J Cancer Res Clin Oncol
DOI 10.1007/s00432-008-0429-8

ORIGINAL PAPER

Differential IL-4/Stat6 activities correlate with differential expression of regulatory genes *SOCS-1*, *SHP-1*, and *PP2A* in colon cancer cells

Qin Yuan · Pin Dong Li · Ben Hui Li · Xian Zi Yang · Shuang Bing Xu · Xiao Hong Liu · Fu Xiang Zhou · Wen Jie Zhang

Received: 22 December 2007 / Accepted: 23 May 2008

Abstract
Purpose To investigate potential differences in the expression of Stat6 regulatory genes that may influence IL-4/Stat6 activities (phenotypes) in colon cancer cells.
Methods RT-PCR method was employed to examine the constitutive mRNA expression of Stat6 negative regulators *SOCS-1* and *SHP-1*, and positive regulator *PP2A* in colon cancer cell lines HT-29 and Caco-2. Stat6 protein expression and nuclear phosphorylation were detected using Western blotting.
Results Caco-2 cells carrying inactive Stat6^{null} phenotype showed normal constitutive expression of Stat6 but decreased phosphorylation of nuclear Stat6 compared with HT-29 cells carrying active Stat6^{high} phenotype. Stat6^{null} Caco-2 cells expressed increased levels of mRNA and protein of *SOCS-1* and *SHP-1*, and decreased mRNA expression of *PPP2CA* and *PPP2CB,* encoding two critical subunits of PP2A.
Conclusions Constitutively increased expression of Stat6 negative regulators *SOCS-1* and *SHP-1*, together with decreased expression of positive regulator PP2A, may play a role in forming the inactive Stat6^{null} phenotype in colon cancer cells.

Keywords Colon cancer · IL-4 · PP2A · SHP-1 · SOCS-1 · Stat6

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Introduction

Interleukin (IL)-4 is a pleiotropic cytokine involved in a variety of cellular functions including Th2 development, cell growth, and proliferation, the induction of resistance to apoptosis amongst many others (Nelms et al. 1999; Ansel et al. 2006). IL-4 induces its functions primarily through the activation of Stat6 (signal transducer and activator of transcription 6), a molecule with dual function capable of signal transduction and gene transcription. 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. IL-4 addition results in the activation of the signaling pathway via a cascade of interactions including activation of Jak1 and Jak3, tyrosine phosphorylation of IL-4Rα and Stat6, and the dimerization of phosphorylated Stat6 followed by its subsequent translocation to the nucleus (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 to induce various biological functions (Hebenstreit et al. 2006; Zhang et al. 2008).

Functionally active IL-4/Stat6 signaling pathway has important pathophysiologic implications in a variety of cell types, including cancer cells and immune cells (Ansel et al. 2006; Bruns and Kaplan 2006). Activated Stat6 is able to upregulate cell surface molecules such as CD23, CD40, MHC class II, IL-4Rα, and IL-13Rα2 (Hebenstreit et al. 2006), and simultaneously to downregulate proinflammatory cytokines, including IL-12 and tumor necrosis factor-α (TNF-α) (Levings and Schrader 1999). The number of Stat6-regulated genes has been increasing rapidly in the last decade suggesting the pathway's broad importance in physiology and pathophysiology (Hebenstreit et al. 2006).

The Stat6 pathway has been extensively studied in knockout animals. Mice deficient in Stat6 exhibit defective Th2 cell development (Shimoda et al. 1996; Takeda et al. 1996), similar to IL-4Rα-deficient mice (Shirakawa et al. 2000), consistent with the role of the IL-4/Stat6 pathway in T cell differentiation (Nelms et al. 1999). On cancer front of Stat6 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). These findings in animals have been echoed by the observations in humans whose Stat6 signaling is found to be constitutively activated in several cancer types (Bruns and Kaplan 2006). Based on the evidence mentioned above, we favor the hypothesis that a functionally active Stat6 signaling may be beneficial to cancer cells at several stages including carcinogenesis, tumor growth, and metastasis, possibly by promoting an exaggerated Th2 environment, gaining resistance to apoptosis and escaping the host immunosurveillance (Zhang et al. 2004; Ostrand-Rosenberg et al. 2004; Li et al. 2008).

Appropriate regulation of the Stat6 signaling is critical and at least three molecules have been defined to regulate the pathway. Suppressor of cytokine signaling-1 (SOCS-1) is a cytokine-inducible intracellular molecule which has been demonstrated to be a potent inhibitor of the IL-4/Stat6 signaling pathway by suppressing the activation of Jak1, Jak3, and Stat6 in response to IL-4 (Dickensheets et al. 2007; Losman et al. 1999; Hebenstreit et al. 2005). 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 (Dickensheets et al. 2007; Hebenstreit et al. 2005). 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 (Hanson et al. 2003; Rane and Reddy 2002; Haque et al. 1998). On the other hand, protein phosphatase 2A (PP2A), a serine/threonine phosphatase, is able to promote Stat6 activity by dephosphorylating serine of Stat6, as serine phosphorylation can inhibit Stat6 activity (Woetmann et al. 2003). PP2A has a catalytic subunit ($PP2A_C$) whose two isoforms $PP2A_{C\alpha}$ and $PP2A_{C\beta}$ are encoded, respectively by *PPP2CA* and *PPP2CB*. $PP2A_C$ forms the core heterodimer with a 65-kDa structural subunit (PR65/A). The core and a variable regulatory subunit (B) then form the PP2A holoenzyme (Lechward et al. 2001).

Using a semiquantitative electrophoretic mobility shift assay (EMSA), we have previously defined three naturally occurring IL-4-induced Stat6 activational phenotypes, termed as $Stat6^{high}$, $Stat6^{low}$, and $Stat6^{null}$ (Zhang et al. 2003). Attempts to understand molecular mechanisms that have generated the Stat6 phenotypes have failed to correlate them with constitutive Stat6 expression as well as polymorphisms of the *IL4RA* gene (Zhang et al. 2003). In addition, there are no visible differences in constitutive expression of Jak1 and Jak3 among these Stat6 phenotypes (unpublished observations). Other mechanisms must be investigated, of which the regulatory genes of the Stat6 pathway are attractive. Using two well-studied colon cancer cell lines as a model, this study has focused on the regulatory genes of the Stat6 pathway and found that (1) Compared with HT-29 cells carrying active $Stat6^{high}$ phenotype, Caco-2 cells carrying defective $Stat6^{null}$ phenotype show decreased phosphorylation of Stat6 in the nucleus; (2) $Stat6^{null}$ Caco-2 cells exhibit increased mRNA expression of the negative regulators SHP-1 and SOCS-1; and (3) Simultaneously, $Stat6^{null}$ Caco-2 cells express decreased mRNA of *PPP2CA* and *PPP2CB*, two genes encoding critical subunits of PP2A that positively regulates Stat6 activity.

Materials and methods

Cell lines

Human colon cancer cell lines, HT-29 and Caco-2 were from American Type Culture Collection (ATCC). Cell lines were cultured at 37°C with 5% CO_2 in RPMI 1640 medium supplemented with 1% of calf serum (CS), 2.05 mM of L-Glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

Preparation of cytoplasmic and nuclear extracts

HT-29 and Caco-2 cell lines (2×10^6 per cell line) were stimulated with 10 ng/ml or 20 ng/ml recombinant human IL-4 (hIL-4, PeproTech, London, UK) for 30 min at 37°C. Unstimulated cells served as controls. Cell lysates and nuclear extracts were prepared using Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer's instructions (NCPE, Beyotime Institute of Biotechnology,

Haimen, Jiangsu, China). Briefly, cells were washed in ice-cold phosphate-buffered saline (PBS) and resuspended by pipetting up and down ten times in 300 µl of ice-cold cell lysis buffer (10 mM Hepes, pH7.9; 10 mM KCl; 0.1 mM EDTA; 1 mM DTT, 0.4% IGEPAL; protease inhibitors used: Aprotinin 2 µg/ml, 1 mM PMSF, benzamidine 250 µg/ml, leupeptin 2 µg/ml). After sitting on ice for 15 min, cell lysates were spun in a microcentrifuge at 11,400 rpm for 5 min at 4°C and supernatants were aliquoted and stored at −70°C for Western blot analysis when needed. Nuclear pellets were then washed in 500 µl of ice-cold cell lysis buffer and resuspended in 150 µl of nuclear extraction buffer (0.4 M NaCl; 20 mM Hepes pH 7.9; 1 mM EDTA; 1 mM DTT; 1 mM PMSF). After vigorously shaking at 4°C for 30 min, nuclear extracts were aliquoted and stored at −70°C until use. Cell lysates and nuclear extracts were quantified using a BCA Protein Assay kit (BPA, Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). EMSA for phenotyping Stat6 activities was described previously (Li et al. 2008).

Preparation of total cellular protein extracts

Total cellular protein extracts of HT-29 and Caco-2 cell lines (1×10^6 per cell line) were prepared using Whole Cell Lysis/Extraction Buffer (WCLB, Applygen, Beijing, China) according to the manufacturer's instructions. The whole cell lysates were quantified using a BCA Protein Assay kit (BPAK, Beyotime institute of Biotechnology, HaiMen, JiangSu, China), aliquoted and stored at −70°C until use.

Determination of Stat6 activational phenotypes

Phenotyping of IL-4-activated Stat6 was performed using an EMSA-based semiquantitative method we developed earlier (Zhang et al. 2003), and HT-29 and Caco-2 cell lines were phenotyped for IL-4/Stat6 activity in a previous report (Li et al. 2008, Fig. 1).

Reverse transcriptional-PCR (RT-PCR)

Cells were cultured in standard culture flask at a concentration of 1×10^6 cells/flask and allowed for 4 days of spontaneous growth. On the fourth day prior to harvest, cells were stimulated or unstimulated with 10 ng/ml rhIL-4 (hIL4, Sigma, St Louis, MO, USA) for 6 h. These stimulated and unstimulated cells (5×10^6 per cell line) were served as IL-4 (+) group and IL-4 (−) group, respectively. Total RNA was isolated using TRIzol reagent (Fermentas Co., USA) according to the manufacturer's instructions. The quantity of isolated RNA was determined by an ultraviolet spectrophotometer at 260/280 nm. First-strand cDNA synthesis

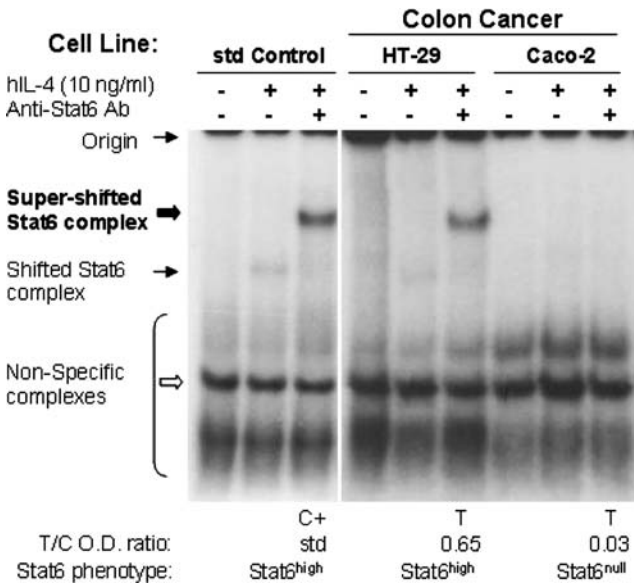

Fig. 1 Colon cancer cell lines HT-29 and Caco-2 show distinct IL-4/Stat6 EMSA profiles corresponding to differential Stat6 phenotypes. A previously phenotyped EBV cell line was used as standard control (Control) with $Stat6^{high}$ phenotype (Zhang et al. 2003). Nuclear proteins were unstimulated (−) or stimulated (+) with human IL-4 (hIL-4) and, with (+) or without (−) addition of specific anti-Stat6 antibody (anti-Stat6 Ab) before separation on 5% PAGE gels. Optical density (OD) readings over anti-Stat6 Ab super-shifted bands (indicated by *solid thick arrow*) were obtained for HT-29 (T) and Caco-2 (T), and compared with that of C (*Lane 3*), respectively. The results were expressed as a ratio of test sample OD versus control OD (*T/C* OD ratio; reproduced from Li et al. 2008 with permission)

was accomplished with an Oligo-$(dT)_{18}$ primer (0.5 µg/µl) used with RevertAid™ First Strand cDNA Synthesis Kit (TOYOBO, Tokyo, Japan). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a control in all tests. Primer sequences and PCR parameters used in RT-PCR were shown in Table 1. PCR was performed in a final volume of 50 µl containing 3 µg cDNA, 10 pmol/µl of each oligonucleotide primer, 10× *Taq* buffer with KCl, 25 mmol/l magnesium chloride, 0.2 mmol/l of each dNTP, DEPC treated water, and 1.25 U *Taq* DNA polymerase (MBI Fermentas, USA) in prealiquoted tubes. Following PCR, 10 µl of each amplified product was electrophoresed on an ethidium bromide (EB)-stained 2% agarose gels, then all bands were visualized and analysed under ultraviolet transillumination by gel imaging scanning system (GeneGenius from Syngene, England). Individual RT-PCR tests were repeated on at least six independent occasions.

Western blot analyses

Whole cell protein extracts (35 µg/sample), cytoplasmic and nuclear protein extracts (25 µg/sample) were heated in SDS-PAGE protein loading buffer (Beyotime institute of Biotechnology, Haimen, Jiangsu, China) at 95°C for

Table 1 Sequences of primers used in RT-PCR

Genes	Sequences	PCR conditions[a]	Size (bp)
STAT6	F: 5′-CAATCCACTCCTTCCTTTCTAT -3′	50 (30)	273
	R: 5′-TGTCTGTATGTTCCTGCCTATC -3′		
STAT4	F: 5′- AGCCATCTCGGAATAACTT-3′	58 (30)	378
	R: 5′-TCTCTCAACACCGCATACACAC -3′		
SHP-1	F: 5′-GTCGGAGTACGGGAACATCACC-3′	61 (30)	397
	R:5′-CCCAGGGCTTTATTTACAAGAGGAG-3′		
SOCS-1	F : 5′-GGAGCGGATGGGTGTAGGGG -3′	64 (30)	178
	R: 5′- GAGGTAGGAGGTGCGAGTTCAG -3′		
SOCS-7	F: 5′- CAATGTCAAATCCCTCCAGCACC-3′	62 (30)	325
	R: 5′- CCCAACCTTCCTGTTCCCTCACT-3′		
CISH	F: 5′- ACAGGCAGGACCTTGTCTCACC-3′	58 (30)	401
	R: 5′- CTTGGTTCCAGCAAGATGTCTCA-3′		
PPP2CA	F: 5′- TCGTCGTACCCCAGACTACTTC -3′	55 (30)	422
	R: 5′- TGATTGTTTGCTGCTATCCTTAT -3′		
PPP2CB	F: 5′- GGAAATCACGAAAGCCGACA -3′	55 (30)	290
	R: 5′- GAAATACCCCATCCACCACG -3′		
GAPDH	F: 5′-CATGAGAAGTATGACAACAGCCT-3′	56 (30)	113
	R: 5′-AGTCCTTCCACGATACCAAAGT-3′		

F forward primer, *R* reverse primer

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

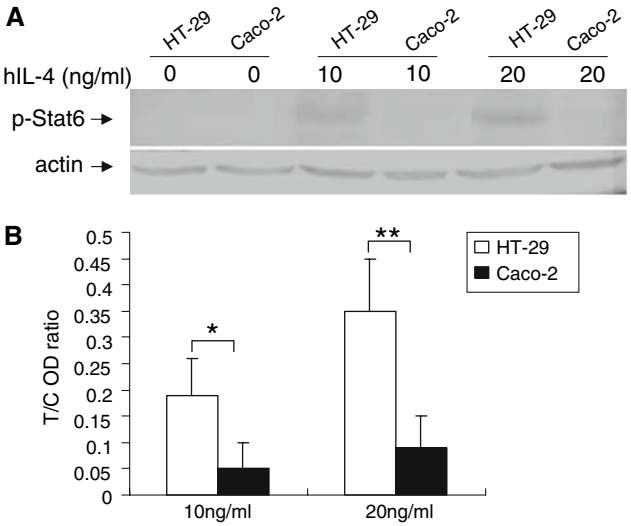

Fig. 2 Western blot analyses of nuclear extracts reveal little or no visible phosphorylated Stat6 (p-Stat6) in Stat6^{null}-carrying Caco-2 cells. **a** HT-29 and Caco-2 cells were either unstimulated (0) or stimulated with hIL-4 (10 or 20 ng/ml) for 30 min and extracted nuclear proteins (25 μg) were separated on 10% SDS-PAGE gels, blotted and then incubated with specific antibodies separately as indicated. **b** Three independent tests show little or no detectable p-Stat6 in Caco-2 cells. As shown, Caco-2 cells appear to have very weak OD reading as compared with HT-29 cells. *T* represents the OD reading of the target p-Stat6, and *C* is the OD reading of internal control protein actin. The results are expressed as an OD ratio of *T* versus *C* within the same lane. * $P < 0.05$; ** $P < 0.01$

10 min and separated on 10% SDS-PAGE (Stat6, SHP-1, phospho-Stat6) and 15% SDS-PAGE (SOCS1) Gel (Beyotime institute of Biotechnology, HaiMen, JiangSu, China,). After electrophoresis, separated proteins were transferred onto a nitrocellulose membrane overnight at 4°C and blocked in 5% nonfat milk in TBS-Tween for 3 h at RT. Then the membrane was incubated overnight at 4°C with the primary antibodies against Stat6 (Poly6252, rabbit IgG), SHP-1 (rabbit monoclonal IgG), SOCS-1 (rabbit polyclonal IgG), phospho-Stat6 (rabbit polyclonal IgG) or *β*-actin (rabbit polyclonal IgG, catalog: sc-1616-R, Santa Cruz Biotechnology, CA, USA), respectively. Following the addition of horseradish peroxidase (HRP)-labeled secondary antibodies, the blots were visualized using a 3,3′-diaminobenzidine tetrahydrochloride substrate kit (ZLI-9032, ZSGB, Beijing, China). Densitometric analyses were performed to quantitate protein band densities using a computer-based image analysis system (GeneGenius from Syngene, England).

Statistical analysis

Statistical analysis was performed using independent sample *t* test provided in a statistical software package SPSS (Version 11.5). Statistical significance was defined as a *P* value less than 0.05 for all analyses and the data were presented as mean ± standard deviation (M ± SD).

Results

IL-4-induced Stat6 activities in HT-29 and Caco-2 colon cancer cells

Using a semiquantitative EMSA and an established Stat6^{high} phenotype as standard control (Zhang et al. 2003); we were able to assign Stat6 activational phenotypes (Stat6

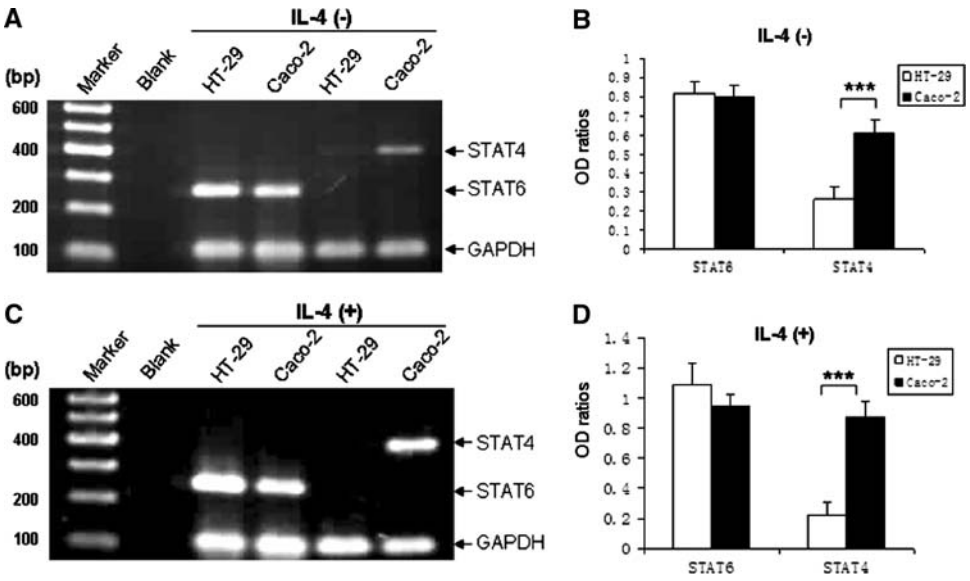

Fig. 3 Defective Stat6^{null} phenotype carried by Caco-2 is not attributable to constitutive expression of *STAT6* gene. Cells were cultured in spontaneous media for 4 days before mRNA extraction and each RT-PCR test was repeated on four independent occasions starting from cell culture. **a** Cells were untreated with IL-4 (−). There is no difference in *STAT6* mRNA expression by RT-PCR assay between HT-29 and Caco-2 cells. However, a clear and dense band of *STAT4* mRNA expression is seen for Caco-2 but barely visible for HT-29. **b** Semiquantitative measuring of four independent tests reproduces the findings in (**a**). T/C OD ratios of target gene (T) versus control gene *GAPDH* (C) are: *STAT6*, 0.82 ± 0.06 for HT-29 and 0.80 ± 0.06 for Caco-2, respectively; *STAT4*, 0.26 ± 0.07 for HT-29 and 0.61 ± 0.07 for Caco-2, respectively. **c** Cells were treated with IL-4 (+). As shown, there is no apparent difference in *STAT6* mRNA expression between HT-29 and Caco-2 cells although the bands for both cells appear to be denser than those of untreated with IL-4 in (**a**). The *STAT4* mRNA expression may be higher for Caco-2 as compared with that in (**a**) but no band can be seen for HT-29, similar to what has been seen in (**a**). **d** Semiquantitative measuring of four independent tests reassures the findings in (**c**). *T/C* OD ratios are obtained as in (**b**): *STAT6*, 1.09 ± 0.14 for HT-29 and 0.95 ± 0.07 for Caco-2, respectively; *STAT4*, 0.22 ± 0.09 for HT-29 and 0.88 ± 0.10 for Caco-2, respectively. *** $P < 0.001$ by statistical analyses

DNA-binding activities) for HT-29 and Caco-2 colon cancer cell lines (Fig. 1). HT-29 was assigned as active Stat6^{high} phenotype (high DNA-binding activity) and Caco-2 was assigned as defective Stat6^{null} phenotype (no discernable DNA-binding activity) as reported earlier by us (Li et al. 2008).

IL-4/Stat6 phenotypes correlate with phosphorylated Stat6 but not constitutive Stat6 levels

To confirm the null activation of Stat6 in Caco-2 cells, we tested nuclear phosphorylation status of Stat6 in the cell line. As shown in Fig. 2, Western blot analyses revealed IL-4-induced phosphorylation of Stat6 in Stat6^{high}-carrying HT-29 cells but little or no visible phosphorylated Stat6 in Stat6^{null}-carrying Caco-2 cells. This was in contrast to constitutive expression of Stat6 at both the mRNA (Fig. 3a, b) and protein (Fig. 7a, d) levels, in keeping with our previous findings in EBV-B cell lines carrying differential Stat6 phenotypes (Zhang et al. 2003). However, it was interesting to note that Stat6^{null} Caco-2 cells appeared to have significant constitutive mRNA expression of *STAT4*, which was not seen in Stat6^{high} HT-29 cells (Fig. 3). The expression of Stat4 is inducible by IL-12 and IL-23, and involved in pro-inflammatory response (Watford et al. 2004; Neurath 2007; Pang et al. 2007). The constitutive *STAT4* expression in Stat6^{null} Caco-2 cells may be relevant to our previous findings that Stat6^{null} cells exhibited increased spontaneously apoptosis and constitutive expression of IL-12 (Galka et al. 2004; Zhang et al. 2004).

Defective Stat6^{null} phenotype correlates with increased expression of negative regulator genes SHP-1 and SOCS-1, and decreased expression of positive regulator genes PPP2CA and PPP2CB

As Stat6^{null} phenotype was not due to constitutive Stat6 expression but a defect in Stat6 phosphorylation (Figs. 2, 3a, b, 7a, d), we next tested regulatory genes of the Stat6 pathway. SHP-1 and SOCS-1 are known to negatively regulate Stat6 activation and PP2A, known to positively regulate Stat6 activation (Hebenstreit et al. 2006). As shown in Fig. 4a, b, mRNA of both *SHP-1* and *SOCS-1* were highly transcribed in Stat6^{null} Caco-2 cells but no or little transcribed mRNA could be seen in Stat6^{high} HT-29 cells. However, reciprocal profiles were observed for *PPP2CA* and *PPP2CB* which were underexpressed in Stat6^{null} Caco-2 cells as compared with Stat6^{high} HT-29 cells (Fig. 6a, b).

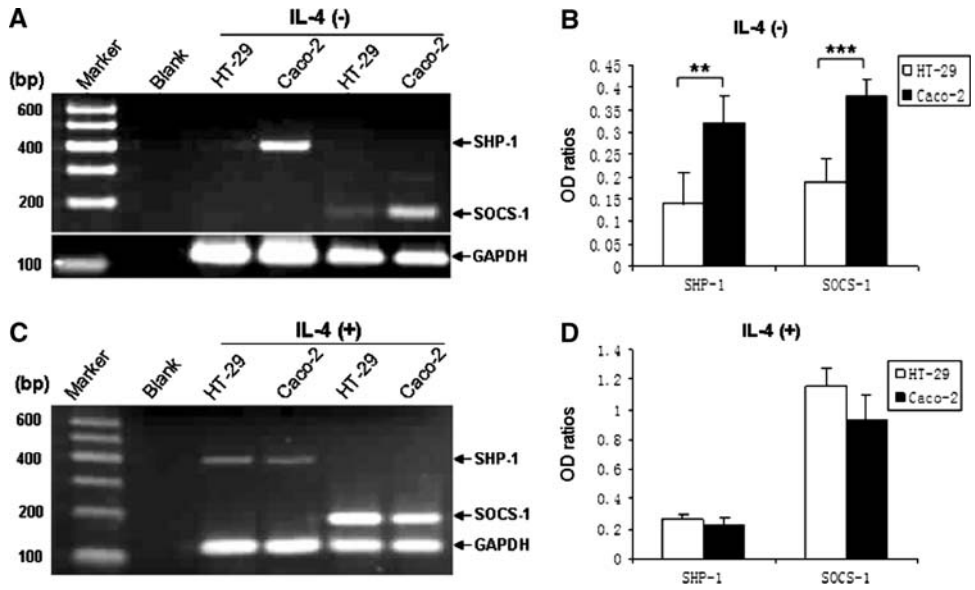

Fig. 4 Stat6^{null}-carrying Caco-2 cells overexpress *SHP-1* and *SOCS-1*, both of which are negative regulator genes of the Stat6 signaling. As in Fig. 3, cells were cultured in spontaneous media for 4 days before mRNA extraction and each RT-PCR test was repeated on four independent occasions starting from cell culture. **a** Cells were untreated with IL-4 (−). Stat6^{null}-carrying Caco-2 cells show a dense band of *SHP-1* which is not seen in Stat6^{high}-carrying HT-29 cells. In addition, Caco-2 also expresses more *SOCS-1* mRNA than HT-29. **b** Semiquantitative measuring of 4 independent tests reassures the findings in **a** and *T/C* OD ratios obtained are: *SHP-1*, 0.14 ± 0.07 for HT-29 and 0.32 ± 0.06 for Caco-2, respectively; *SOCS-1*, 0.19 ± 0.05 for HT-29 and 0.38 ± 0.04 for Caco-2, respectively. **c** It appears that, after IL-4 stimulation (+), the differences in *SHP-1* and *SOCS-1* expression as shown in (**a**) and (**b**) have disappeared between the two cells. **d** The findings in (**c**) are reproducible from 4 independent tests after IL-4 addition (+) and T/C OD ratios are: *SHP-1*, 0.26 ± 0.04 for HT-29 and 0.23 ± 0.04 for Caco-2, respectively; *SOCS-1*, 1.16 ± 0.12 for HT-29 and 0.93 ± 0.17 for Caco-2, respectively. ** $P < 0.01$; *** $P < 0.001$

On the other hand, it was interesting to note that, in comparison with Stat6^{high} HT-29 cells, Stat6^{null} Caco-2 cells expressed higher mRNA levels of *SOCS-7* and *CISH* (Fig. 5a, b), of which *SOCS-7* is known to negatively regulate Stat3 and Stat5 signaling pathways (Martens et al. 2005). Using Western blot analyses and antibodies available to us, the increased expression of SHP-1 protein in Stat6^{null} Caco-2 cells was confirmed while SOCS-1 protein in Caco-2 cells appeared to be more than that in HT-29 cells although not statistically significant (Fig. 7b, c, d).

Stat6^{high} HT-29 cells actively respond to IL-4 and upregulate the regulator genes of the Stat6 pathway

To examine the functional fitness of Stat6 phenotypes, IL-4 responsiveness was tested for Stat6^{high}- and Stat6^{null}-carrying colon cancer cells. As shown in Fig. 4c, d Stat6^{high} HT-29 cells responded to IL-4 more vigorously than Stat6^{null} Caco-2 cells and upregulated mRNA levels of *SHP-1* and *SOCS-1*, reversing the differences between the two as shown in Fig. 4a, b. It was also noted that, Caco-2 cells appeared to have expressed more mRNA of *SOCS-1* after IL-4, although to a lesser degree than HT-29 (Fig. 4), suggesting a diminished but not abolished function of Stat6 in Caco-2 cells, in keeping with our previous findings in breast cancer cells (Zhang et al. 2008). On the other hand, upon IL-4 addition, very similar expression profiles were also observed for regulator genes *SOCS-7* and *CISH* between HT-29 and Caco-2 (Fig. 5), again demonstrating an impaired Stat6 function in Caco-2 cells. However, the situation was reciprocal for *PPP2CA* and *PPP2CB* in these two cell lines (Fig. 6a, b). In addition, IL-4 stimulation appeared to have upregulated mRNA expression of the *PPP2CA* and *PPP2CB* in Stat6^{null} Caco-2 cells (Fig. 6c, d). All together, these observations suggested that Stat6^{null} was likely a defective phenotype with residual functionality in terms of IL-4 responsiveness.

Discussion

Functionally active Stat6 plays an important role in cancer cells, which may determine the fate of cancer cell's differentiation, proliferation, and survivability. Supporting such a hypothesis are the observations of constitutively activated Stat6 in many human malignancies (Bruns and Kaplan 2006), spontaneous rejection of implanted tumors and resistance to metastatic disease in Stat6$^{-/-}$ (Stat6 null phenotype) mice (Kacha et al. 2000; Ostrand-Rosenberg et al. 2000, 2004). It is very interesting to note that defective IL-4/Stat6^{null} phenotype, which is naturally occurring, appears to be present in at least three cell types with different tissue

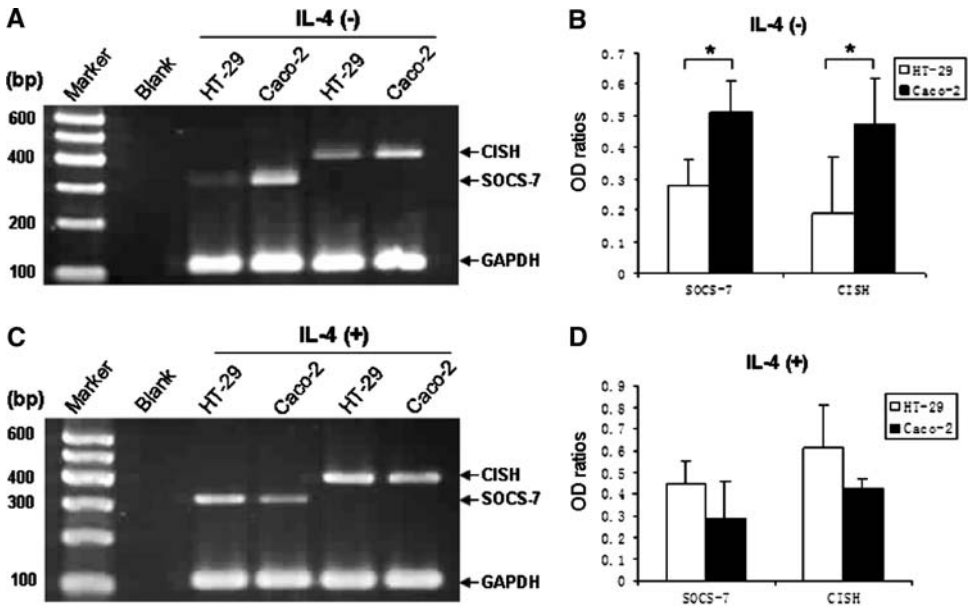

Fig. 5 Stat6^{null}-carrying Caco-2 cells overexpress the regulatory genes *SOCS-7* and *CISH*. Cells were spontaneously cultured for 4 days before mRNA extraction and each RT-PCR assay was independently tested on four occasions starting from cell culture. **a** Cells were untreated with IL-4 (−). Stat6^{null}-carrying Caco-2 shows a dense band of *SOCS-7* while Stat6^{high}-carrying HT-29 shows a faint band of the gene product. On the other hand, Caco-2 also expresses more *CISH* mRNA than HT-29. **b** The observations in (**a**) are reproducible from four independent RT-PCR tests and *T/C* OD ratios are obtained as in Fig. 4: *SOCS-7*, 0.28 ± 0.08 for HT-29 and 0.51 ± 0.10 for Caco-2, respectively; *CISH*, 0.19 ± 0.18 for HT-29 and 0.47 ± 0.15 for Caco-2, respectively. **c** IL-4 stimulation (+) induces HT-29 cells to express more mRNA of *SOCS-7* and *CISH* resulting in the disappearance of the differences as seen in (**a**). **d** As shown again, increased mRNA levels of both genes are reproducible when cells are independently tested for four times with IL-4 stimulation. The corresponding *T/C* OD ratios (M ± SD) are: *SOCS-7*, 0.45 ± 0.10 for HT-29 and 0.29 ± 0.17 for Caco-2, respectively; *CISH*, 0.61 ± 0.20 for HT-29 and 0.43 ± 0.04 for Caco-2, respectively. * $P < 0.05$

origins including EBV-B cells (Zhang et al. 2003), breast cancer cells (Zhang et al. 2008), and colon cancer cells (Fig. 1; Li et al. 2008).

Another interesting phenomenon of the Stat6^{null} phenotype is the carrier cell's susceptibility to apoptosis in spontaneous culture. Thus far, we have found such susceptibility to apoptosis in three different cell types mentioned above that carry the Stat6^{null} phenotype including EBV-B cells (Galka et al. 2004), breast cancer cells (Zhang et al. 2008), and colon cancer cells (Li et al. 2008). The involvement of Stat6 signaling in regulating apoptosis (Nelms et al. 1999) is supported by the evidence that knocking down the expression of *STAT6* in active Stat6^{high} HT-29 cells (Fig. 1) using RNAi technology produces a less active Stat6 status, similar to Stat6^{null} phenotype, and renders the otherwise apoptosis-resistant HT-29 cells less proliferative and susceptible to apoptosis (Zhang et al. 2006). Taken together, these findings suggest that IL-4-induced Stat6 activities or phenotypes as shown here may serve as a biomarker helpful in predicting the apoptotic fate of cancer cells.

On the other hand, constitutively overexpressed Stat4 in Stat6^{null}-carrying Caco-2 cells (Fig. 3) may further deteriorate the Caco-2 cells' susceptibility to apoptosis as Stat4 pathway is activated by IL-12 and IL-23 and involved in Th1/Th17 proinflammatory and proapoptotic responses in nature (Watford et al. 2004; Wurster et al. 2000; Thierfelder et al. 1996). In this context, it may be relevant that proinflammatory cytokines IL-12, INF-γ and TNF-α are constitutively elevated in Stat6^{null} EBV-B cells (Zhang et al. 2004) and Stat6^{null} colon cancer cells (Li et al. 2008). The current finding that Stat4 is constitutively overexpressed in Stat6 inactive cells may suggest an imbalanced Stat4 versus Stat6 signaling relevant to imbalanced Th1/Th17 versus Th2 responses, which may significantly alter the biology in host cells.

To understand the underlying mechanism(s) that generate naturally occurring Stat6 activational 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 IL-4 receptor α polymorphisms, such as I50V, S503P and Q576R variants (Mitsuyasu et al. 1998), which all fail to correlate with Stat6 phenotypes (Zhang et al. 2003). In addition, no differences have been observed in constitutive expression of Jak1 and Jak3 kinases among different Stat6 phenotypes (Xu et al. submitted). Here in colon cancer cells studied, Stat6 phenotypes do not correlate with constitutive expression of Stat6 (Fig. 7a), in keeping with the previous

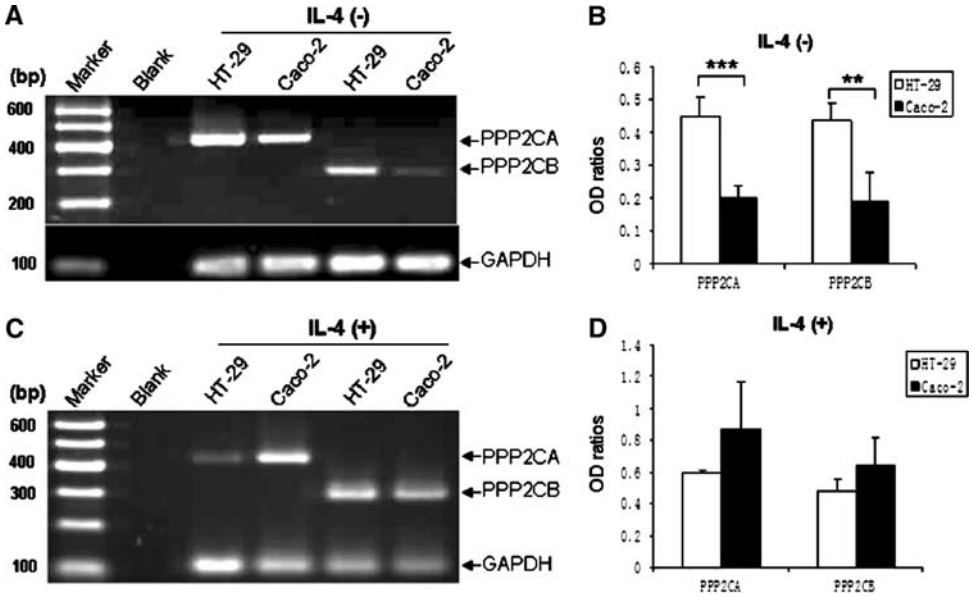

Fig. 6 Stat6^{null}-carrying Caco-2 cells show underexpressed levels of *PPP2CA* and *PPP2CB*, the genes coding for catalytic subunits $PP2_{C\alpha}$ and $PP2_{C\beta}$ of the positive regulator PP2A. Cells were spontaneously cultured for 4 days before mRNA extraction and each RT-PCR assay was independently tested on four occasions starting from cell culture. **a** Cells were untreated with IL-4 (−). Stat6^{null}-carrying Caco-2 exhibits much reduced mRNA levels of *PPP2CA* and *PPP2CB* as compared with Stat6^{high}-carrying HT-29. **b** The findings in (**a**) are reproducible from four independent RT-PCR tests and *T/C* OD ratios are obtained as in Fig. 4: *PPP2CA*, 0.45 ± 0.06 for HT-29 and 0.20 ± 0.04 for Caco-2, respectively; *PPP2CB*, 0.44 ± 0.05 for HT-29 and 0.19 ± 0.09 for Caco-2, respectively. (C) Cells were treated with IL-4 (+). As shown, IL-4 stimulation upregulates the mRNA levels of both *PPP2CA* and *PPP2CB*, especially in the Caco-2 cells. **d** *T/C* OD ratios obtained from four independent tests show the reproducibility of the findings in (**c**): *PPP2CA*, 0.60 ± 0.01 for HT-29 and 0.87 ± 0.30 for Caco-2, respectively; *PPP2CB*, 0.48 ± 0.07 for HT-29 and 0.64 ± 0.18 for Caco-2, respectively. ** $P < 0.01$ and *** $P < 0.001$, respectively

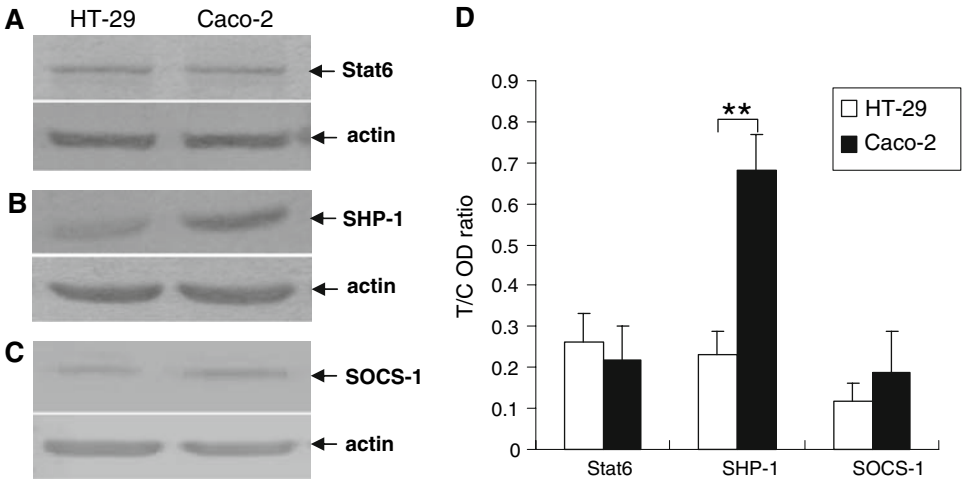

Fig. 7 Stat6^{null}-carrying Caco-2 cells show increased constitutive expression of SHP-1 at protein level. Whole cell extracts were analysed using Western blotting assay for Stat6 (**a**), SHP-1 (**b**) and SOCS-1 (**c**). In (**d**), T/C OD ratios of three independent analyses were obtained from comparisons of Target protein OD readings (*T*) versus Control protein actin OD readings (*C*), a method similar to that as shown in Figs. 1, 2, and 4. It is clear that there is no detectable difference in Stat6 levels between the two cells. As can be seen, the expression of SHP-1 in Caco-2 cells is much higher than that in HT-29 cells. Although not statistically significant, the level of SOCS-1 appears to be higher in Caco-2 cells than in HT-29 cells from three independent tests. ** $P < 0.01$

observations in EBV-B cells (Zhang et al. 2003). However, the mechanism(s) that generate the observed IL-4/Stat6 phenotypes are complex (Zhang et al. 2003) and it will be necessary to investigate the cell surface display of IL-4 receptor in several aspects such as its constitutive expression and/or density, expression kinetics before and after IL-4

treatment at different time intervals, which will certainly offer additional information for the differences in IL-4-induced activation of Stat6 and gene transcription within these cells.

The discovery that Stat6 is apparently underphosphorylated in Stat6^{null} cells (Fig. 2), has led us to examine whether regulator genes, such as *SHP1* and *SOCS1*, may be involved in phenotypic differences of Stat6. Interestingly as shown in Fig. 4, the constitutively expressed levels of *SOCS1* mRNA and *SHP1* mRNA in Stat6^{null} Caco-2 cells are significantly increased as compared with those in Stat6^{high} HT-29 cells. In addition, IL-4 is shown to induce the expression of both *SHP1* and *SOCS1* in Stat6 active HT-29 cells as expected, but much less so in Stat6 inactive Caco-2 cells (Fig. 4c, d). Furthermore, such differences in mRNA expression are also visible at protein levels at least for SHP-1 (Fig. 7b, c). These findings may favor a simple hypothesis that overexpressed negative regulators SHP-1 and SOCS-1 could be able to significantly weaken the activation of Stat6. However, such a simple hypothesis may not be sufficient to fully explain the formation of the constitutive Stat6^{null} phenotype. Therefore, investigating other negative regulators of various Stat pathways should provide additional information as to understand the observed characteristics of cancer cells carrying Stat6^{null} phenotype.

For example, SOCS-7 has been reported to inhibit the phosphorylation of Stat3 and Stat5, and attenuate their nuclear translocation (Martens et al. 2005). *CISH*, the first *CIS/SOCS* gene identified, has been shown to be a negative feedback regulator of the Stat5 pathway (Matsumoto et al. 1999; Ram and Waxman 1999; Yoshimura 2005). As shown in Fig. 5, we have observed significantly increased mRNA expression of *SOCS7* and *CISH* in Stat6^{null}-carrying Caco-2 cells, similar to that of *SHP1* and *SOCS1*. Thus far, it is not known whether *SOCS7* and *CISH* are IL-4 responsive. Here, we show for the first time that IL-4 induces the expression of these two regulator genes (Fig. 5c, d), implying a possibility that *SOCS7* and *CISH* are likely regulator genes of the Stat6 pathway (Haque et al. 2000) warranting further investigations. It may not be coincident that, in a recent study in breast cancer cell lines, *CISH* is upregulated after IL-4 stimulation (Zhang et al. 2008). On the other hand, both Stat3 and Stat5 have also been reported to be constitutively activated in a variety of cancer types, including colon cancer (Bromberg 2002). In this context, the heightened expression of negative regulator genes *SHP1* and *SOCS1*, together with *SOCS7* and *CISH*, in the Stat6^{null}-carrying Caco-2 colon cancer cells may suggest a collective effect of an exaggerated negative regulation on several Stat pathways, including Stat6, that render cancer cells prone to apoptosis and less invasive (Li et al. 2008).

Having examined the negative regulators, we next investigated genes involved in positive regulation of the Stat6 pathway, which may be equally important in terms of varying Stat6 activational phenotypes. As mentioned previously, *PPP2CA* and *PPP2CB* encode two key isoforms, $PP2A_{C\alpha}$ and $PP2A_{C\beta}$, respectively, forming the catalytic subunit $PP2A_C$ of the PP2A enzyme (Lechward et al. 2001). PP2A is able to promote Stat6 activation (Woetmann et al. 2003) by which positively regulates the Stat6 signaling. As shown in Fig. 6, the mRNA expression of both *PPP2CA* and *PPP2CB* is remarkably lower in the Stat6^{null}-carrying Caco-2 cells than in the Stat6^{high}-carrying HT-29 cells, with reciprocal profiles to those of the negative regulator genes, *SHP-1* and *SOCS1* (Fig. 4) as well as to those of the *SOCS7* and *CISH* (Fig. 5), respectively. Interestingly, IL-4 appears to be able to upregulate mRNA levels of *PPP2CA* and *PPP2CB* in Stat6^{null} Caco-2 cells, which has not been reported thus far. These observations suggest that positive regulator genes *PPP2CA* and *PPP2CB* are likely involved in the formation of the defective Stat6^{null} phenotype.

In summary, we have shown that IL-4-induced Stat6 activity is defective in Caco-2 colon cancer cells and such a Stat6^{null} activity or phenotype correlates with impaired phosphorylation of Stat6. The increased expression of negative regulator genes *SHP-1* and *SOCS-1* and the decreased expression of positive regulator genes *PPP2CA* and *PPP2CB* may explain in part, if not all, the formation of the defective Stat6^{null} phenotype. On the other hand, the elevated expression of the negative regulator genes *SOCS7* and *CISH* of the Stat5 and Stat3 pathways may provide added biological influence in Stat6^{null} cells. Constitutively overexpressed Stat4 in Stat6^{null} cells may suggest imbalanced Stat4 versus Stat6 signalings relevant to imbalanced Th1/Th17 versus Th2 responses that ultimately determine the biological fate of the host cell.

Acknowledgments This work was supported by a grant from the National Natural Science Foundation of China (NSFC No. 30470981) awarded to W.J. Zhang. We thank S.Q. Liu and S.P. Liu for excellent technical assistance in cell culture and flowcytometry analyses.

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