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Curcumin Suppresses Constitutive Activation of STAT-3 by Up-Regulating Protein Inhibitor of Activated STAT-3 (PIAS-3) in Ovarian and Endometrial Cancer Cells

Manush Saydmohammed, Doina Joseph, and Viqar Syed*

Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814-4799

ABSTRACT

Signal transducer and activator of transcription-3 (STAT-3) is constitutively activated in ovarian and endometrial cancers and is implicated in uncontrolled cell growth. Thus, its disruption could be an effective approach to control tumorigenesis. Curcumin is a dihydroxyphenolic compound, with proven anti-cancer efficacy in various cancer models. We examined the anti-tumor mechanism of curcumin on STAT-3 and on the negative regulators of STAT-3, including suppressors of cytokine signaling proteins (SOCS-1 and SOCS-3), protein inhibitors of activated STAT (PIAS-1 and PIAS-3), and SH2 domain-containing phosphatases (SHP-1 and SHP-2) in ovarian and endometrial cancer cell lines. Treatment of cancer cells with curcumin induced a dose- and time-dependent decrease of constitutive IL-6 expression and of constitutive and IL-6-induced STAT-3 phosphorylation, which is associated with decreased cell viability and increased cleavage of caspase-3. The inhibition of STAT-3 activation by curcumin was reversible, and phosphorylated STAT-3 levels returned to control levels 24 h after curcumin removal. Compared to normal cells baseline expression of SOCS-3 was high in cancer cells and a marked decrease in SOCS-3 expression was seen following curcumin treatment. Overexpression of SOCS-3 in curcumin-treated cells increased expression of phosphorylated STAT-3 and resulted in increased cell viability. Normal ovarian and endometrial cells exhibited high expression of PIAS-3 protein, whereas in cancer cells the expression was greatly reduced. Curcumin increased PIAS-3 expression in cancer cells. Of significance, siRNA-mediated knockdown of PIAS-3 overcomes the inhibitory effect of curcumin on STAT-3 phosphorylation and cell viability. In conclusion, curcumin suppresses JAK-STAT signaling via activation of PIAS-3, thus attenuating STAT-3 phosphorylation and tumor cell growth. J. Cell. Biochem. 110: 447–456, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: STAT3; JAKs; ENDOMETRIAL CANCER; OVARIAN CANCER; STAT3 INHIBITORS

Ovarian cancer is the most lethal gynecological cancer in the United States and a high mortality rate makes this disease a major health concern for women, while endometrial carcinoma is a worsening public health problem in the United States. At a time when the overall incidence and mortality from many cancers is falling, the number of new cases and deaths related to endometrial cancer is rising. Thus, there is a need to develop effective methods to prevent these two cancers.

Signal transducer and activator of transcription-3 (STAT-3) belongs to the STAT family of proteins, which are both signal transducers and transcription factors. STAT-3 integrates signals from a variety of extracellular stimuli and kinase pathways and regulates genes involved in many key cellular processes. Constitutive activation of STAT-3 is found in an astounding number of cancers, including ovarian and endometrial cancer [Bowman et al., 2000; Bromberg 2000; Syed et al., 2002]. Constitutive STAT-3 signaling participates in oncogenesis by stimulating cell proliferation, promoting angiogenesis, mediating immune evasion, and conferring resistance to apoptosis induced by conventional therapies [Levy and Darnell, 2002; Niu et al., 2002; Real et al., 2002]. The inappropriate activation of STAT-3 in cancer not only contributes directly to malignant tumor behavior but also provides an opportunity for therapeutic intervention.

In normal tissues, STAT-3 is widely expressed as inactive monomers in the cytoplasm that become activated upon tyrosine phosphorylation. This phosphorylation allows the STAT-3 molecules

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*Correspondence to: Dr. Viqar Syed, PhD, Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Room# A-3080, 4301 Jones Bridge Road, Bethesda, MD 20814-4799.
E-mail: viqar.syed@usuhs.mil, syedv2@mail.nih.gov

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to form homodimers or heterodimers. The dimer can then enter the nucleus and activate the transcription of various genes [Levy and Darnell, 2002]. In normal cells, STAT-3 is transiently activated and then inactivated by a group of signaling proteins, such as protein inhibitors of activated STATs (PIAS), suppressors of cytokine signaling proteins (SOCS), and SH2-containing tyrosine phosphotase (SHP1 and SHP2) cascades.

The PIAS family of proteins (PIAS1, PIAS3, PIASx, and PIASy) regulate STAT activity [Shuai and Liu 2005]. Upon cytokine stimulation, PIAS-1 and PIAS-3 bind activated STAT-1 and STAT-3, respectively, and prevent their ability to bind to DNA [Shuai and Liu, 2005]. Increased PIAS-3 expression was detected in human cancers, including lung, breast, and brain tumors [Wang and Banerjee, 2004], and associated with apoptosis in prostate cancer cells [Wible et al., 2002] and growth suppression in human lung cancer cells [Ogata et al., 2002].

Another group of proteins, SOCS, have been suggested to be part of the negative feedback regulation of JAK-STAT signaling [Starr and Hilton, 1999; Krebs and Hilton, 2001]. These proteins are reported to inactivate JAKs and block the access of STATs to receptor binding sites. SOCS inactivate JAKs; cytokine-induced STAT inhibitor binds to the sites of STAT on the activated receptor and blocks STAT-receptor binding. Protein tyrosine phosphatases (PTPases) including Src homology 2 (SH2) domain-containing PTPases SHP-1 and SHP-2, are other important regulators of cytokine signaling. SHP-1 and SHP-2 have two SH2 domains at the N terminus and a PTPase catalytic domain at the C terminus, which catalyze the tyrosine dephosphorylation of JAKs, and other cellular proteins, and thus play critical roles in the control of cytokine signaling [Jiao et al., 1996; You et al., 1999].

Currently several natural agents are under study for their assessment as preventive agents against cancer. Curcumin or diferuloylmethane, commonly known as turmeric [Shishodia et al., 2005], is widely used in Asian cuisine. It is widely recognized for its anti-inflammatory, anti-microbial, and wound healing activities [Aggarwal et al., 2006]. Because of its relatively low- or non-cytotoxicity [Aggarwal et al., 2006], curcumin has recently emerged as one of the most promising and powerful chemopreventive and chemotherapeutic agents [3] capable of inhibiting proliferation of a variety of tumor cells in vitro and suppressing in vivo tumor formation in animal models [Limtrakul et al., 1997; Karunagaran et al., 2005].

Although the molecular mechanism and targets of curcumin action are well known and several publications [Bharti et al., 2003; Chakravarti et al., 2006; Rajasingh et al., 2006] have shown that curcumin suppresses STAT-3, this study is the first to explore the effects of curcumin on the negative regulators of activated STAT-3 such as PIAS, SOCS, SHP-1, and SHP-2 in ovarian and endometrial cancer cells.

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

The endometrial cancer cell lines RL95-2 and Ishikawa were obtained from the American Type Culture Collection (Manassas, VA). Normal epithelial endometrial cells were established and characterized by Kyo et al. [2003]. We obtained the cells from Dr. Satoru Kyo (Kanazawa University, School of Medicine, Kanazawa, Japan). Human ovarian surface epithelial, HOSE 642, and ovarian cancer cell lines OVCA 420 and OVCA 429 were provided by Dr. S.C. Mok (M.D. Anderson Cancer Center, Houston, TX). The HOSE 642 cell line was derived from normal ovaries obtained from a 47-year-old normal woman with non-cancerous gynecologic indications. Primary cultures were established from surface scrapings of normal ovaries and immortalized with human papillomavirus E6 and E7 genes. All primary cultures had an epithelial cell phenotype, that is, cobblestone morphology, epithelial cytokeratin staining, responsiveness to transforming growth factor-β, no detectable CA125 production, and lack of in vivo tumorigenicity [Tsao et al., 1995]. OVCA cell lines OVCA 420 and OVCA 429 were established cell lines derived from patients with late-stage serous ovarian adenocarcinomas, as described by Bast et al. [1981]. The endometrial and ovarian cancer cell lines were grown in Eagle MEM (Invitrogen Corporation, Carlsbad, CA), and in a 1:1 mixture of medium 199/MCDB 105 (Sigma, St. Louis, MO), respectively; supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen Corporation), 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

TREATMENT OF OVARIAN AND ENDOMETRIAL CANCER CELL LINES

To study the effect of curcumin on cell proliferation, ovarian and endometrial cancer cells were cultured in 96-well plates at 1,000 cells per well in medium containing FBS. Twenty-four hours later, the medium was replaced with the same medium containing various doses of curcumin (10–50 μM) for 72 h. Curcumin stock solution was 20 mM in DMSO. Control cells were exposed to DMSO alone. After treatment, cell viability was measured using a Cell Proliferation Kit (Promega, Madison, WI). To evaluate p-STAT-3 expression, 2×10^5 ovarian and endometrial cancer cells were seeded in a T-25 flasks (Falcon, Becton Dickinson Labware, Bedford, MA) and treated either with various doses of curcumin (3.12–50 μM) for 1 h or with 50 μM for various time points (15–240 min). At the end of the treatment period, the cells were harvested for protein extraction. To study the effect of curcumin on IL-6-induced p-STAT-3, cells were cultured as described above except that cells were pretreated with IL-6 (10 ng/ml) for 1 h prior to treatment with curcumin (50 μM) for 1 h. The cells were washed, the curcumin was removed, and fresh medium was added. Cells were harvested for protein extraction after 1, 3, 6, 24, 48, and 72 h of curcumin removal. To determine whether the curcumin-induced apoptosis in OVCA and endometrial cancer cell lines was mediated by intracellular caspases activation, cell cultures were pretreated with the caspase inhibitor DEVD (10 μM) for 60 min before exposure to curcumin (50 μM) for 24 h.

CELL VIABILITY ASSAY

One thousand cells per well were plated in 96-well plates. Cytotoxicity was determined using the CellTiter96 Aqueous One Solution cell viability assay (Promega) according to the manufacturer's instructions. Cells were cultured for 3 days in the presence of various doses of curcumin. On the fourth day, the CellTiter 96 Aqueous One Solution Reagent (20 μl) was added into each well of

the 96-well assay plate containing the samples in 100 µl of culture medium. Absorbance was measured at 490 nm using a ELX800 Plate Reader. Absorbance of untreated cells was set as 100% viability, and absorbance of cell-free wells containing medium was set as zero.

WESTERN BLOTTING

To obtain cellular extracts, cells were lysed in lysis buffer (50 mM Tris [pH 8.0], 250 mM NaCl, 0.5% Nonidet P-40) and proteins were quantified with a protein determination kit (Pierce, Rockford, IL). Proteins (20 µg) from OVCA 420, OVCA 429, RL-95, and Ishikawa cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 10% gels and transferred to polyvinylidene difluoride (PVDF) membranes. Blots were blocked with 5% bovine serum albumin in Tris-buffered saline/0.1% Tween-20 for 1 h. Blots were probed with anti-STAT3, anti-phospho-STAT3, cleaved caspase-3, anti-phospho-JAK-1, and JAK-2 antibodies (1:1,000; Cell Signaling, Beverly, MA), with anti-phospho-SHP-1 and SHP-2 antibodies (1:1,000 dilution; Abcam, Cambridge, MA, and Cell Signaling, respectively), with anti-SOCS-3 (1:200, Santa Cruz Biotechnology, Inc.), with anti-SOCS-1 (1:100, Santa Cruz Biotechnology, Inc.), with anti-PIAS1 (1:200 Santa Cruz Biotechnology, Inc.), with anti-PIAS3 (1:200, Santa Cruz Biotechnology, Inc.), with anti-IL-6 antibody (1:600 dilution, Santa Cruz Biotechnology, Inc.), or with β-actin antibody (1:25,000 dilution; Sigma), overnight at 4°C and then incubated with corresponding secondary antibodies (1:2,000 dilution) in 5% bovine serum albumin in Tris-buffered saline/0.1% Tween-20 for 1 h at room temperature. After washing, bound antibodies were detected by using an enhanced chemiluminescence (ECL) detection system (Pierce).

TRANSFECTION OF PIAS3 siRNA OR SOCS3 PLASMID

SOCS-3 gene was excised from pORF vector (InvivoGen, San Diego, CA) by using *Nco* I and *Nhe* I enzymes and subsequently cloned into pcDNA3.1 expression vector (Invitrogen Corporation). OVCA 429 and Ishikawa cells were cultured (each at 1×10^5 cells per well) in six-well plates for 24 h. The cultures were treated with or without curcumin (50 µM) for 3 days. After 3 days, cells were transfected with either siRNA for PIAS3 (5.0 µM, Santa Cruz Biotechnology, Inc.) or SOCS3 plasmid (1 µg/ml) or scrambled control siRNA (5.0 µM, Santa Cruz Biotechnology, Inc.), or control plasmid by the use of LipofectAMINE 2000 (Life Technologies, Inc.) in serum-free medium for 3 h at 37°C. Cultures were incubated with complete medium for 72 h. Cell viability was analyzed by MTS assay and cellular extracts were prepared for the Western blotting of p-STAT3, total STAT-3, PIAS-3, and SOCS-3. The overall transfection efficiency for cells assessed by X-Gal staining assay against pSV-βgalactosidase vector transfected cells was 68–71%.

STATISTICAL ANALYSIS OF THE DATA

Results are expressed as the mean ± SEM of at least three independent experiments. Data were analyzed by ANOVA, followed by Tukey's post hoc test. Statistical significance was inferred at ($P < 0.05$).

RESULTS

CURCUMIN INDUCES GROWTH INHIBITION OF OVARIAN AND ENDOMETRIAL CANCER CELLS BY INDUCING APOPTOSIS

The effects of curcumin on cell proliferation in two ovarian (OVCA 420 and OVCA 429) and two endometrial (Ishikawa and RL95-2) cancer lines were investigated over a wide concentration range. Cells were treated with curcumin (10–50 µM) for 72 h and cell viability was assessed by MTS assay. Results revealed that treatment with curcumin (40–50 µM) decreased cell viability by about 70–80% in ovarian cancer cells whereas a 60–80% decrease in cell viability was observed in endometrial cancer cells (Fig. 1A). In most cancer cells, caspases are present in the pro-forms (inactive) and require site-specific cleavage of the protein to become active and participate in the process of apoptosis. Data presented in Figure 1B showed significant cleavage of active caspase-3 in curcumin-treated cells. Since DEVD-FMK inhibits caspase activation, we then studied whether DEVD-FMK could block curcumin-mediated apoptosis. Compared with curcumin treatment, preincubation of OVCA and endometrial cancer cells with DEVD-FMK (10 µM) for 1 h prior to curcumin treatment resulted in the inhibition of caspase cleavage as observed by immunoblot analysis (Fig. 1B).

CURCUMIN INHIBITS CONSTITUTIVE IL-6 AND STAT-3 PHOSPHORYLATION IN OVARIAN AND ENDOMETRIAL CANCER CELLS

To investigate the anti-tumorigenic actions of curcumin and its mechanism of action in ovarian and endometrial cancer cells, we first examined the effects of curcumin on the expression of constitutively activated IL-6 and Tyr^{705} or Ser^{727} phosphorylated STAT-3 that are implicated in the pathogenesis of many cancers such as ovarian and endometrial. Ovarian and endometrial cancer cells were treated with concentrations of curcumin ranging from 0 to 50 µM for 1 h and then examined for the expression of IL-6, and Tyr^{705} or Ser^{727} phosphorylated STAT-3 by Western blot analysis. Curcumin decreased levels of IL-6 protein in ovarian and endometrial cancer cells in a dose-dependent manner (Fig. 2A). A marked decrease in Tyr^{705}-pSTAT-3 expression in OVCA cell lines treated with 25 µM curcumin and the complete inhibition of Tyr^{705}-pSTAT-3 expression were noted in cells exposed to 50 µM curcumin (Fig. 2A). The endometrial cells appear to be less sensitive to curcumin. Expression of Tyr^{705}-pSTAT-3 was moderately decreased in Ishikawa cells treated with 25 µM curcumin. A marked decrease in Tyr^{705}-pSTAT-3 expression was apparent in endometrial cancer cells treated with 50 µM curcumin (Fig. 2A). Curcumin failed to inhibit the phosphorylation of STAT-3 at Ser^{727} in endometrial and ovarian cancer cells (Fig. 2A).

We then examined the optimum time required for the down-regulation of Tyr^{705}-pSTAT-3 in ovarian and endometrial cancer cells. Curcumin inhibited the constitutively active STAT-3 in the OVCA cells. The inhibition appeared as early as 30 min in OVCA 420 and 60 min in OVCA 429 cells. Curcumin at 50 µM for 240 min completely inhibited STAT-3 phosphorylation in OVCA cells. One-hour incubation of Ishikawa cells with curcumin (50 µM) resulted in a marked decrease in Tyr^{705}-pSTAT-3 expression; however, noticeable inhibition of Tyr^{705}-pSTAT-3 was apparent in

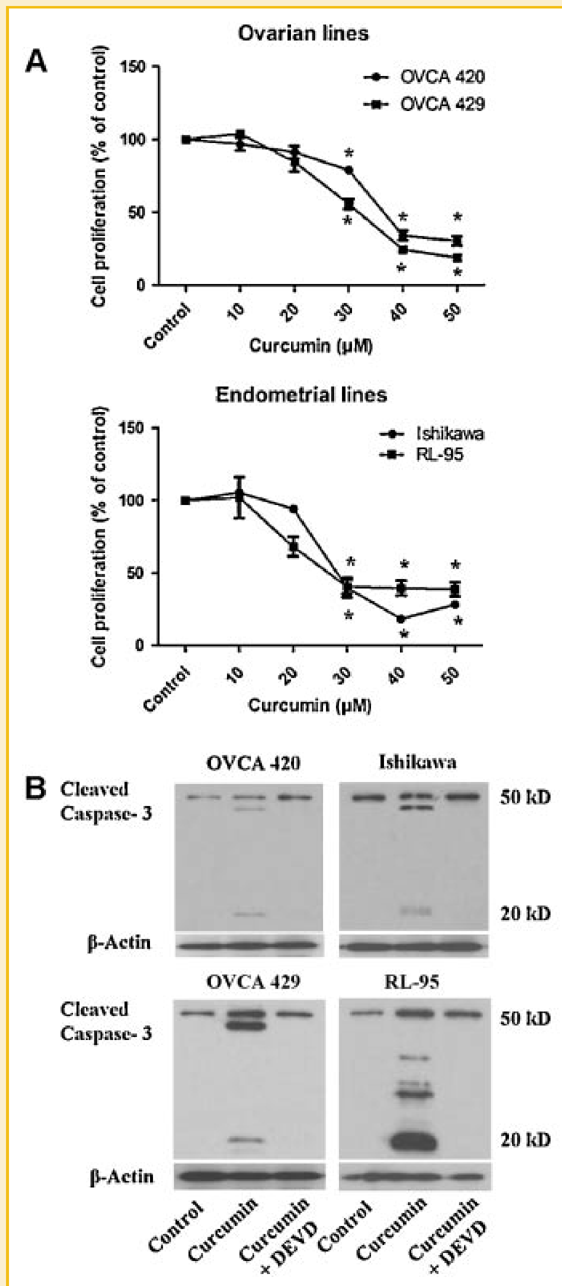

Fig. 1. Curcumin inhibits cell viability by inducing apoptosis of ovarian cancer (OVCA 420 and OVCA 429) and endometrial cancer (Ishikawa and RL-95) cells. A: To investigate a dose-dependent effect of curcumin on cell viability, cells were plated in 96-well plates as described in the Materials and Methods Section and treated with indicated concentrations of either curcumin or DMSO for 72 h. Cell viability was measured by CellTiter96 Aqueous One solution assay by determining the absorbance at 490 nm using ELX800 Plate Reader (BioTek, Winooski, VT). The data shown here are an average ± SEM of four replicate wells and are representative of three independent experiments. B: Ovarian cancer (OVCA 420 and OVCA 429) and endometrial cancer (Ishikawa and RL-95) cells were treated with curcumin (50 µM) for 24 h with or without pretreatment with DEVD-fmk (10 µM) for 1 h. At the end of treatments, cell lysates were prepared and analyzed by Western blotting for cleaved caspase-3, and protein loading was checked by stripping and re-probing the membranes for β-actin.

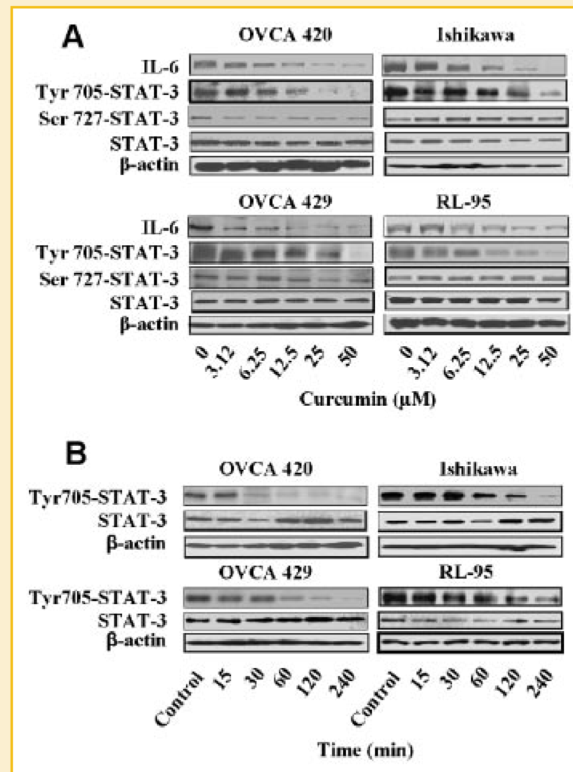

Fig. 2. Curcumin attenuates Tyr^{705}p-STAT-3 in ovarian and endometrial cancer cells. A: To study the dose-dependent effect the ovarian cancer (OVCA 420 and OVCA 429) and endometrial cancer (Ishikawa and RL-95) cells were treated with either DMSO (control, C) or different concentrations of curcumin for 1 h. B: To study the time-dependent effect, cells were treated with curcumin (50 µM) for 15, 30, 60, 120, and 240 min. Cells were harvested and 20 µg of protein from the whole-cell extract was loaded in each lane. The blots were probed with anti-human IL-6, Tyr^{705}p-STAT-3, Ser^{727}p-STAT-3, or total STAT-3 antibodies. β-Actin was used as a loading control.

RL-95 cells after 120 h of cell exposure to curcumin (Fig. 2B). Levels of total STAT-3 were not affected by curcumin treatment in all the cell lines tested (Fig. 2B).

CURCUMIN-INDUCED INHIBITION OF STAT-3 PHOSPHORYLATION IS REVERSIBLE IN OVARIAN AND ENDOMETRIAL CANCER CELLS

We next determined whether curcumin-induced inhibition of STAT-3 phosphorylation was reversible. The ovarian and endometrial cancer cells were treated for 60 min with curcumin (50 µM), and then washed twice with PBS to remove the curcumin. The cells were then cultured in fresh medium for various lengths of time and the levels of Tyr^{705}p-STAT-3 were determined by Western blotting. In ovarian and endometrial cancer cells, curcumin induced the suppression of STAT-3 phosphorylation, while the removal of curcumin resulted in a gradual increase in phosphorylated STAT-3 after 24 h in both cell types. The levels of Tyr^{705}p-STAT-3 returned to control levels after 72 h of curcumin removal from the media of endometrial cancer cells. However, in ovarian cancer cells (OVCA 420 and OVCA 429) partial reversal was seen at 72 h. No significant changes were observed in total STAT-3 levels in ovarian and endometrial cancer cells (Fig. 3).

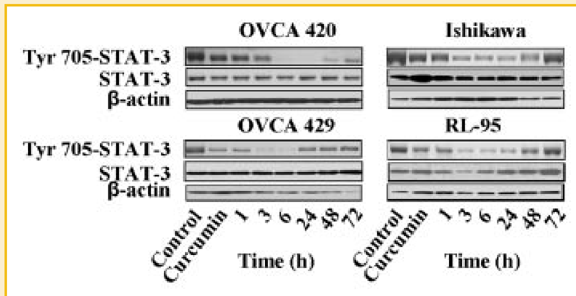

Fig. 3. Curcumin-induced inhibition of STAT phosphorylation is reversible. Ovarian cancer (OVCA 420 and OVCA 429) and endometrial cancer (Ishikawa and RL-95) cells were treated with curcumin (50 μM) for 1 h and washed twice with PBS to remove curcumin before addition of fresh medium. Cells were removed at indicated times and lysed to prepare the whole-cell extract. Twenty micrograms of whole cell extracts were resolved on 10% SDS–PAGE, transferred to a PVDF membrane, probed for Tyr^{705}p-STAT3 and stripped and reprobed for total STAT-3. β-Actin was used as a loading control.

CURCUMIN INHIBITS IL-6-INDUCIBLE STAT-3 PHOSPHORYLATION IN OVARIAN AND ENDOMETRIAL CANCER CELLS

Given that IL-6-induced signals are mediated through STAT-3 phosphorylation, it is logical to then investigate the effect of IL-6 and curcumin on STAT-3 phosphorylation in ovarian and endometrial cancer cells. Although these cells express high levels of Tyr^{705}p-STAT-3 (Fig. 1A), Figure 4 shows that IL-6-induced STAT-3 phosphorylation in ovarian and endometrial cancer cell lines was reduced by curcumin treatment. Exposure of cells to curcumin for 1 h markedly suppressed IL-6-induced STAT-3 phosphorylation in all cell lines. In both ovarian and endometrial cancer cell lines the levels of Tyr^{705}p-STAT-3 returned to levels comparable to respective control cells after 24 and 48 h of treatment (Fig. 4).

CURCUMIN INHIBITS JAK IN OVARIAN AND ENDOMETRIAL CANCER CELLS

Phosphorylation of STATs depends on the activation of JAKs. Members of the JAK family of protein tyrosine kinases phosphorylate and activate cytoplasmic STAT proteins. JAK-2 is a recognized activator of STAT-3 [Darnell, 1997]. We investigated whether the inhibitory effects of curcumin on the activation of STAT were due to the suppression of JAK expression. First we examined the expression of constitutively active JAK-1 and JAK-2 in normal and malignant ovarian and endometrial cell lines. As shown in Figure 5A, two ovarian cancer cell lines (OVCA 420 and OVCA 429) constitutively express phosphorylated JAK-1 and JAK-2 compared to normal HOSE cells. JAK-2, but not JAK-1, expression was high in the normal endometrial cell line (NEE) compared to endometrial cancer cell lines (Ishikawa and RL-95). Curcumin inhibited p-JAK-1 and p-JAK-2 expression in ovarian and endometrial cancer cell lines (Fig. 5B). Expression of total JAK-1 and JAK-2 was not altered by curcumin treatment (results not shown).

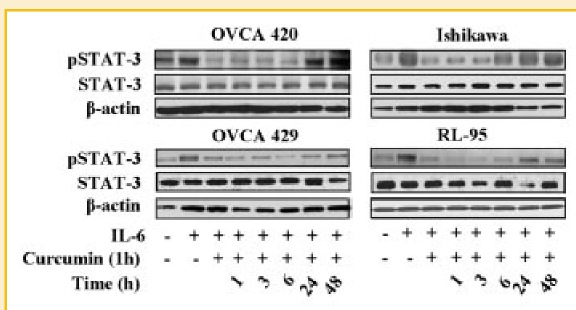

Fig. 4. Curcumin inhibits IL-6-induced Tyr^{705}p-STAT-3 in human ovarian and endometrial cancer cells. Ovarian (OVCA 420 and OVCA 429) and endometrial (Ishikawa and RL-95) cancer cells were treated with IL-6 (10 ng/ml) for 1 h and then incubated with curcumin (50 μM) for 1 h. The cells were washed and resuspended in fresh medium. Cells were removed at indicated times and lysed to prepare the whole-cell extract. The extracts were resolved on 10% SDS–PAGE, electrotransferred to a PVDF membrane, probed for the Tyr^{705}p-STAT-3 (upper panel), and stripped and reprobed for total STAT-3 (lower panel). β-Actin was used as a loading control.

SUPPRESSION OF SHP-2 EXPRESSION BY CURCUMIN IN OVARIAN AND ENDOMETRIAL CANCER CELLS

Three families of proteins, SHP, PIAS, and SOCS, inhibit specific and distinct aspects of JAK/STAT signal transduction. We next addressed the question of how curcumin affects these proteins. Two members of the SHP family—SHP-1 and SHP-2—are implicated in the negative regulation of STAT signaling. Expression of SHP-1 and SHP-2 was evaluated in normal and malignant ovarian and endometrial cells. Both SH2-containing phosphatases were overexpressed in the ovarian cancer cell line compared to normal ovarian cells. On the contrary, high levels of SHP-2 were found in normal endometrial cells (NEE), whereas SHP-2 was down-regulated in endometrial cancer cells. SHP-1 levels were unaltered in normal and malignant endometrial cells. Marked decrease in SHP-2 expression was seen in ovarian cancer cells following curcumin treatment (Fig. 5B).

PIAS-3 EXPRESSION IS DIMINISHED IN OVARIAN AND ENDOMETRIAL CANCER CELLS AND UP-REGULATED FOLLOWING CURCUMIN TREATMENT

To understand the mechanism by which curcumin inhibits STAT-3 phosphorylation, we examined the effect of curcumin on the negative regulators of STAT-3. PIAS-3 functions as a negative regulator of STAT-3 signaling by interfering with its ability to bind DNA [Shuai and Liu, 2005]. PIAS-1 and PIAS-3 protein levels were examined by Western blot analyses in both normal and malignant ovarian and endometrial cells lines. Normal endometrial cells and HOSE 642 cells expressed high levels of PIAS-3 compared to endometrial and ovarian cancer cell lines. Treatment of cancer cells with curcumin for 24 h up-regulated the expression of PIAS-3 protein. However, the expression of PIAS-1 was not affected by curcumin (Fig. 5C). These data implicate that persistent down-regulation of PIAS-3 as a basis for pSTAT-3 activation and enhanced cell growth in ovarian and endometrial cancer cells.

CURCUMIN ATTENUATES SOCS-3 EXPRESSION IN CANCER CELLS

We next examined the effect of curcumin on the SOCS proteins. Generally, SOCS proteins are present in cells at very low levels but are rapidly transcribed after the exposure of cells to cytokine stimulation. SOCS proteins act in a negative feedback loop to inhibit JAK activation. SOCS-3 is not only an endogenous inhibitor of

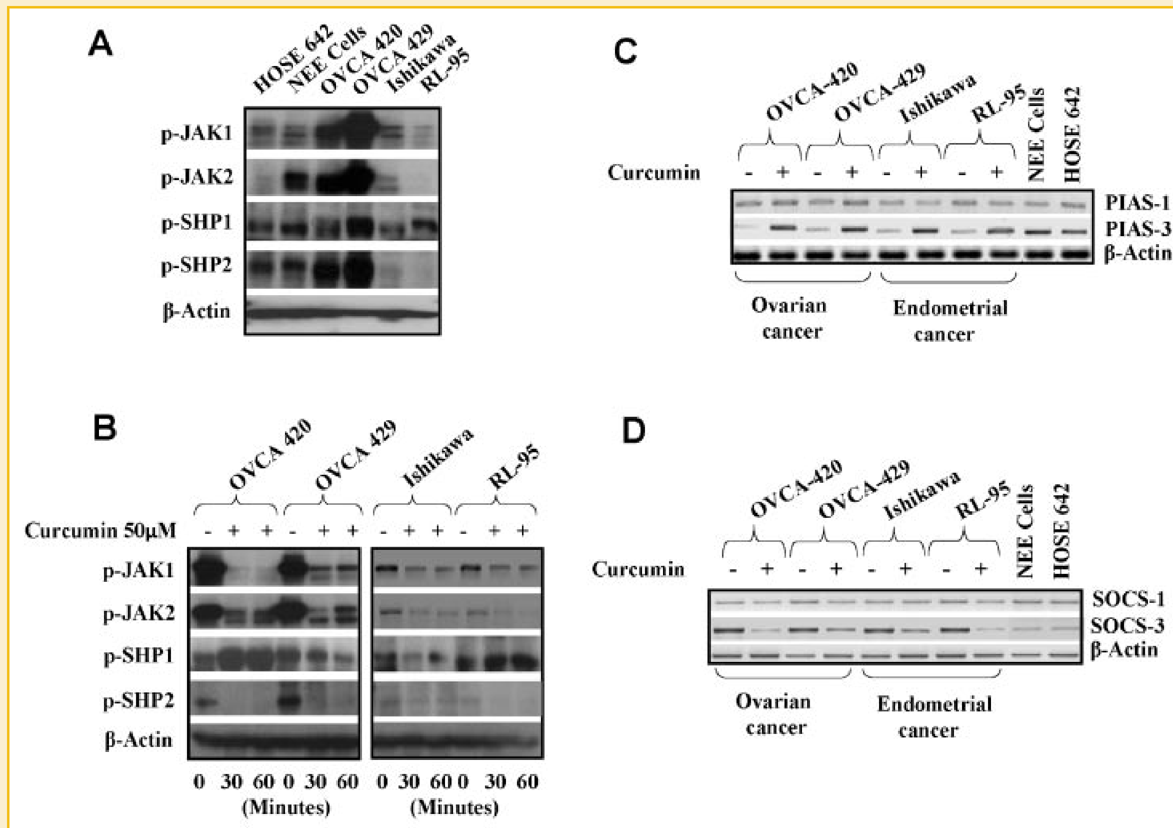

Fig. 5. Curcumin up-regulates PIAS-3 expression in human ovarian and endometrial cancer cells. Ovarian (OVCA 420 and OVCA 429) and endometrial (Ishikawa and RL-95) cancer cells were treated with curcumin (50 μM) for 24 h. Cell lysates were analyzed for (A) basal (B) curcumin-regulated expression of JAK-1 and JAK-2, SHP-1, and SHP-2, (C) PIAS-1, and PIAS-3 and (D) SOCS-1 and SOCS-3 expression. β-Actin was used as a loading control. Extracts from normal ovarian surface epithelial cells (HOSE-642) and normal endometrial epithelial (NEE) cells were used as controls. Data shown here are representative of three independent experiments.

STAT-3 but also a STAT-3 transcriptional target. Thus, we tested the effects of curcumin on the expression of SOCS-1 and SOCS-3 in ovarian and endometrial cancer cells. The expression of SOCS-3 was elevated in cancer cells compared to normal endometrial and ovarian surface epithelial cells. Treatment of cancer cells with curcumin significantly inhibited the expression of SOCS-3. SOCS-1 expression in cancer cells was not affected following curcumin treatment (Fig. 5D).

SILENCING OF PIAS-3 OR OVEREXPRESSION OF SOCS-3 IN CURCUMIN-TREATED CELLS REVERSE THE EFFECT OF CURCUMIN ON ACTIVATED STAT-3 AND CELL VIABILITY

Our results demonstrate increased expression of PIAS-3 and decreased SOCS-3 expression following curcumin treatment, which is associated with decreased Tyr^{705}p-STAT-3 expression and decreased cell viability of OVCA and endometrial cells. To explore if silencing PIAS-3 and over expressing of SOCS-3 would reverse the effect of curcumin on STAT-3 activation, ovarian and endometrial cancer cells were treated with or without curcumin (50 μM) for 3 days and transfected with siRNA against PIAS-3 or with SOCS-3 plasmid to inhibit PIAS-3 and up-regulate SOCS-3 expression, respectively. Cell viability was assessed by MTS assay. In all the cell lines tested, siRNA against PIAS-3 inhibited PIAS-3 expression in curcumin-treated and non-treated transfected cells compared to scrambled siRNA-transfected cells (Fig. 6A). Transfection of SOCS-3 plasmid resulted in the up-regulation of SOCS-3 expression in curcumin-treated and non-treated transfected cells compared to control plasmid-transfected cells (Fig. 6B). No change was noted in Tyr^{705}p-STAT-3 and total STAT-3 expression in non-curcumin-treated OVCA and Ishikawa cells. However, a marked decrease of Tyr^{705}p-STAT-3 was observed in curcumin-treated cells (Fig. 6A,B). Interestingly, a marked increase of Tyr^{705}p-STAT-3 was seen in curcumin-treated cancer cells transfected with siRNA of PIAS-3 or SOCS-3 plasmid. Transfection of curcumin-treated cells with siRNA of PIAS-3 or SOCS-3 plasmid abrogated curcumin-induced cell growth inhibition (Fig. 6A,B), whereas a significant increase in cell viability was observed when non-curcumin-treated cells were transfected with siRNA of PIAS-3 or SOCS-3 plasmid (Fig. 6A,B).

DISCUSSION

It is widely known that aberrant activation of STAT-3 occurs in many human tumors [Bowman et al., 2000; Turkson and Jove, 2000]. Several studies employing genetic and pharmacological approaches to modulate constitutive STAT-3 activity have substantiated the critical role of aberrant STAT-3 activity in malignant transformation and tumor progression, and hence

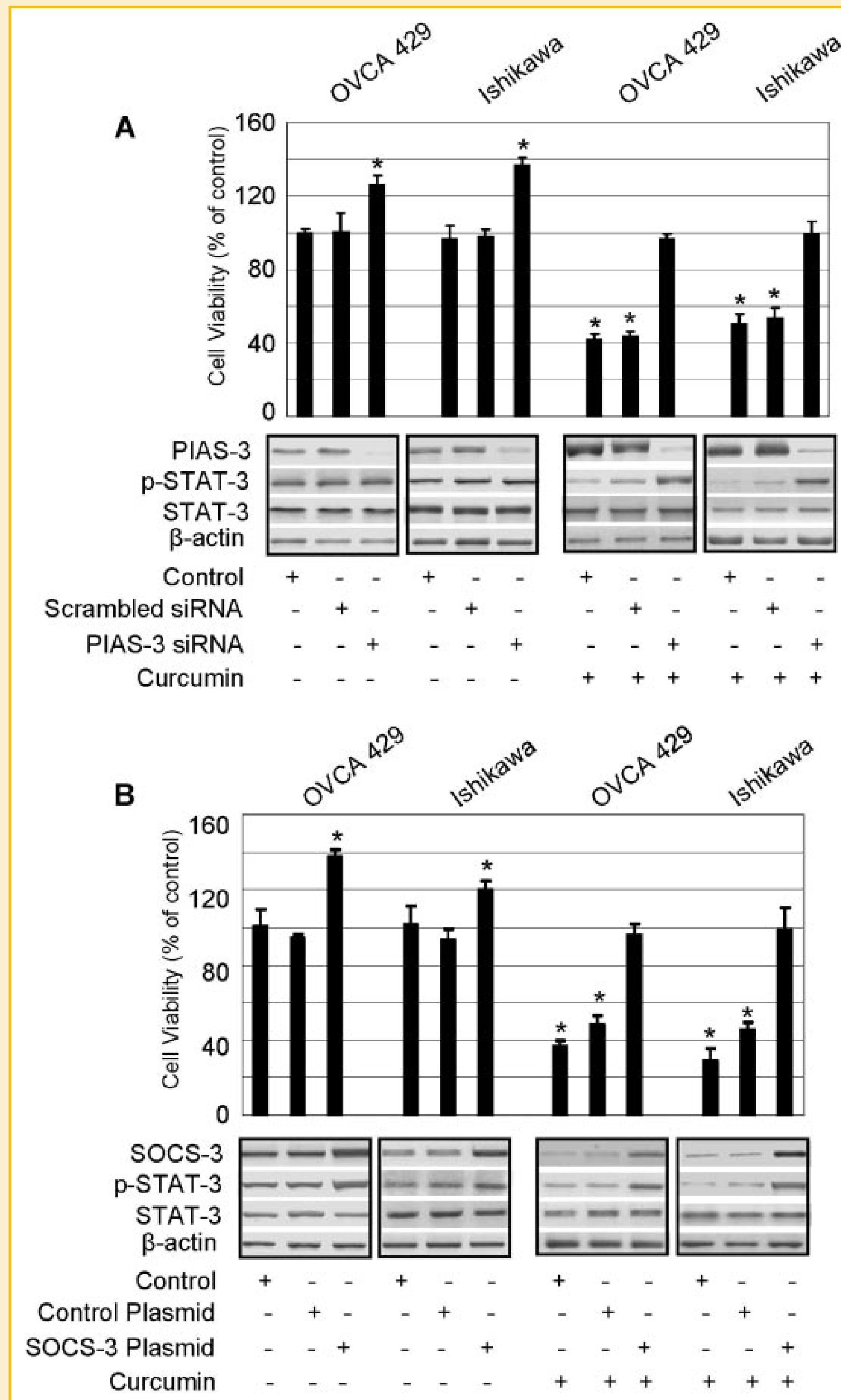

Fig. 6. Silencing of PIAS-3 and ectopic expression of SOCS-3 in curcumin-treated cells reverse the effect of curcumin on STAT-3 activation and cell viability. Ovarian (OVCA 429) and endometrial (Ishikawa) cancer cell lines were treated with curcumin (50 μM) for 3 days. Treated and non-treated cells were transfected with siRNA against PIAS-3 (A) or SOCS-3 plasmid (B). Three days later, cell lysates were analyzed for protein levels of PIAS-3, SOCS-3, Tyr^{705}p-STAT-3, total STAT-3 and cell viability was measured by MTS assay. Data are the means of two experiments with triplicate samples. Bars are mean ± SEM and asterisk (*) indicates a statistically significant difference ($P < 0.05$).

endorse STAT-3 as a novel cancer drug target. Thus, a pharmacologically safe and effective agent that can block constitutive or inducible activation of STAT-3 has potential for use in cancer treatment.

Curcumin has recently emerged as one of the most promising and powerful chemopreventive and chemotherapeutic agents because of its relatively low- or non-cytotoxicity [Campbell and Collett, 2005; Aggarwal et al., 2006]. Curcumin has been shown to interact with a

wide variety of proteins, including transcription factors (e.g., STAT-3), and modify their expression and activity. Although curcumin has been shown to suppress proliferation of a wide variety of cancer cells via inhibition of phosphorylated STAT-3, the mechanism is not fully understood. To date, there is no data delineating the effects of curcumin on proteins regulating STAT-3 signaling in ovarian and endometrial cancers. The goal of this study was to determine whether curcumin exerts its anti-proliferative effects through modulation of the negative regulators of STAT-3 signaling pathway in gynecological cancers. We specifically focused on STAT-3 because we have previously shown the relationship between STAT-3 activation and ovarian cancer cell proliferation [Syed et al., 2002]. To the best of our knowledge, the present study is the first report showing the effect of curcumin on the three families of STAT-3 inhibitor proteins, PIAS, SOCS, SHP-1, and SHP-2, that inhibit specific and distinct aspects of STAT signal transduction in ovarian and endometrial cancer cells.

In this study, we have identified several alterations in the JAK-STAT signaling cascade in ovarian and endometrial cancer cells compared to their respective normal cells. (1) Ovarian and endometrial cancer cells exhibit high levels of IL-6 and curcumin inhibits IL-6 expression in these cells. (2) High levels of Tyr^{705}p-STAT-3 were detected in OVCA and endometrial cancer cells in the absence of IL-6. (3) In ovarian cancer cells the expression of phosphorylated JAK-1 and JAK-2 was higher than in HOSE cells, where as low expression of JAK-2 was seen in endometrial cancer cells compared to normal endometrial epithelial cells. (4) Curcumin inhibited phosphorylated JAK-1 and JAK-2 in ovarian and endometrial cancer cells. (5) PIAS-3 was lower in ovarian and endometrial cancer than in normal cells, while curcumin up-regulated the expression of PIAS-3 in cancer cells. (6) SOCS-3 was elevated in cancer cells compared to normal cells and SOCS-3 expression was inhibited following curcumin treatment. SOCS-1 expression was similar in normal and malignant cells and was unaffected by curcumin. (7) Significantly higher SHP-1 and SHP-2 expression was seen in ovarian cancer cells compared to HOSE cells. (8) SHP-1 expression levels were unaltered in normal versus endometrial cancer cells; however, SHP-2 levels were down-regulated in endometrial cancer cells compared to normal cells. (9) Curcumin attenuated the expression of SHP-2 in ovarian cancer cells. (10) Curcumin had no effect on SHP-1 expression. To our knowledge, these findings present the first evidence of JAK/STAT signaling proteins modulated by curcumin in cancer cells.

Our data show that curcumin in a dose- and time-dependent manner inhibited the viability of ovarian and endometrial tumor cell lines as determined with the MTS assay. The growth-inhibitory effects of curcumin were apparent within 24 h of treatment. In both OVCA cell lines, about 80% of cell inhibition was achieved after 3 days of exposure. The concentration of curcumin used in this study is comparable to that used in other in vitro studies in which curcumin inhibited cell proliferation and induced apoptosis in human basal cell carcinomas, bladder cancer cells, human colon cancer cells, hepatoblastomas, myeloid leukemic cells, head and neck squamous cell carcinoma cells, T cell leukemia, and Hodgkin's lymphoma cells [Jee et al., 1998; Bharti et al., 2003; Collett and Campbell, 2004; Uddin et al., 2005; Chakravarti et al., 2006; Rajasingh et al., 2006; Mackenzie et al., 2008].

Ovarian and endometrial cancer patients are shown to have high levels of IL-6 in their serum compared to age matched disease free females [Tempfer et al., 1997; Bellone et al., 2005]. The IL-6 secreted by tumor cells via autocrine/paracrine mechanisms contributes to constitutively activated STAT-3 and plays a significant role in cell proliferation [Syed et al., 2002]. In this study, we demonstrated a dose-dependent decrease of IL-6 expression in cancer cells following curcumin treatment. Our results also showed that in vitro treatment with curcumin blocked the constitutive phosphorylation of STAT-3 in ovarian and endometrial cancer cells. The inhibition of phosphorylated STAT-3 was apparent after 1 h exposure of cells to a high dose of curcumin. Our results are consistent with earlier reports showing the inhibition of phosphorylated STAT-3 with curcumin in head and neck squamous cell carcinoma cells and multiple myeloma cells [Bharti et al., 2003; Chakravarti et al., 2006; Rajasingh et al., 2006]. In fact the curcumin-induced decrease in IL-6 production in cancer cells essentially mirrors the corresponding decrease in STAT-3 phosphorylation. These results suggest that the effects of curcumin on IL-6 expression may in part explain the inhibitory effect of curcumin on STAT-3 phosphorylation in ovarian and endometrial cancer cells.

To investigate the mechanism of curcumin-induced STAT-3 inhibitory effects in ovarian and endometrial cancer cells, we analyzed proteins upstream of STAT-3. The roles of JAK-1 and JAK-2 have been implicated in STAT-3 activation. As shown in Figure 5B, phosphorylation of JAK-1 and JAK-2 was suppressed by treatment with curcumin in ovarian and endometrial cancer cells. It is likely that the inhibition of Tyr^{705} STAT-3 is due to the inhibition of JAK-2 because the inhibition of JAK-2 phosphorylation occurred within 30 min after the treatment, whereas STAT-3 phosphorylation was inhibited within 60 min after the treatment (Fig. 2B). This suggests that curcumin could manifest its effect on STAT-3 activation through the inhibition of JAKs. In addition to Tyr^{705} STAT-3 phosphorylation, STAT-3 Ser^{727} phosphorylation has been proposed to participate in the regulation of STAT-3 phosphorylation. Nevertheless, we found that curcumin selectively inhibits JAK-STAT-3 tyrosine phosphorylation but does not affect STAT-3 Ser^{727} phosphorylation. This finding indicates that curcumin inhibition of JAK-STAT signaling is independent of serine phosphorylation.

All cells harbor constitutive suppressors of activated STAT, among them are SOCS, PIAS, and SHPs. These inhibitors inhibit different parts of the JAK-STAT pathway. SOCSs are cytokine-inducible endogenous inhibitors of STAT-3 that work in a classical negative feedback loop. SOCS proteins bind through their SH2 domain to phosphotyrosine residues in either cytokine receptors or JAK and thus can suppress STAT-3 signaling. SOCS-1 and SOCS-3 are known to inhibit JAK phosphorylation. Our results showed an overexpression of SOCS-3 in cancer cells compared to normal cells. Elevated SOCS-3 expression was reported in human breast cancer and melanoma tissues as well as in primary lymphoma cells [Takeuchi et al., 2005; Fojtova et al., 2007]. Thus, the parallel elevated expression of both activated STAT-3 and SOCS-3 proteins in ovarian and endometrial cancer cells is plausible and may contribute to the anti-apoptotic phenotype of these cells. SOCS-3 is

a negative regulator of the STAT-3 signaling pathway, and a transcriptional target. Marked decrease in SOCS-3 expression following curcumin treatment of cancer cells is attributed to decreased STAT-3 expression endorsing that SOCS-3 is a STAT-3-induced protein.

We observed a loss a constitutive PIAS-3 in ovarian and endometrial cancer cells. It has been proposed that PIAS-1 and PIAS-3 function by blocking the DNA binding activity of STAT-1 and STAT-3, respectively [Chung et al., 1997; Liu et al., 2001]. Low expression of PIAS-3 in ovarian and endometrial cancer cells may be a contributing factor to the STAT-3 activation observed in these cancers. These results are in accordance with studies by Brantley et al. [2008] demonstrating elevated levels of PIAS-3 in control tissue compared to reduced levels of PIAS-3 expression in glioblastoma multiforme (GBM) tissue. There are reports describing deficient PIAS-3 expression in various human cancers [Zhang et al., 2002]. Results of the present study suggest that the loss of PIAS-3 in ovarian and endometrial cancer cells contributes to enhanced STAT-3 and subsequent cell proliferation and curcumin inhibits STAT-3 phosphorylation by up-regulating PIAS-3 in cancer cells.

SHP-1 and SHP-2 are present constitutively in cells and play a role in STAT deactivation by dephosphorylating JAK, and are implicated in negative regulation of JAK/STAT signaling pathways. SHP-1 is mainly expressed in epithelial cells [Banville et al., 1995], as opposed to SHP-2, which is ubiquitously expressed. SHP-1 is altered in most cancer cells resulting in hyperphosphorylation of JAK-1 and JAK-2. Our results showing overexpression of SHP-1 in ovarian cancer cells are consistent with studies demonstrating overexpression of SHP-1 in solid tumors such as pancreatic and breast cancers [Yip et al., 2000; Wu et al., 2003a,b]. Mok et al. [1995] found a two- to fourfold increase in the expression of SHP-1 in 7 of the 8 ovarian cancer cell lines tested, including ovarian cancer cell lines used in this study, and a two- to threefold increase in the expression of SHP-1 in 10 of 11 invasive ovarian epithelial cancer tissues.

In summary, prior to this report, there was no information on the status of JAK/STAT signaling proteins in ovarian and endometrial cancer cells and their regulation by curcumin. The significance of this study is that we have identified a perturbance in a plethora of JAK-STAT signaling proteins in cancer cells. Our data demonstrated to our knowledge for the first time that PIAS-3 is down-regulated and SOCS-3 is up-regulated in ovarian and endometrial cancer cells, while curcumin up-regulated PIAS-3 and decreased SOCS-3 expression, which inhibited STAT-3 phosphorylation and tumor cell viability.

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