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# Inhibition of JAK1, 2/STAT3 Signaling Induces Apoptosis, Cell Cycle Arrest, and Reduces Tumor Cell Invasion in Colorectal Cancer Cells[1]

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## Abstract

Abnormalities in the STAT3 pathway are involved in the oncogenesis of several cancers. However, the mechanism by which dysregulated STAT3 signaling contributes to the progression of human colorectal cancer (CRC) has not been elucidated, nor has the role of JAK, the physiological activator of STAT3, been evaluated. To investigate the role of both JAK and STAT3 in CRC progression, we inhibited JAK with AG490 and depleted STAT3 with a SiRNA. Our results demonstrate that STAT3 and both JAK1 and 2 are involved in CRC cell growth, survival, invasion, and migration through regulation of gene expression, such as Bcl-2, $p16^{ink4a}$, $p21^{waf1/cip1}$, $p27^{kip1}$, E-cadherin, VEGF, and MMPs. Importantly, the FAK is not required for STAT3-mediated regulation, but does function downstream of JAK. In addition, our data show that proteasome-mediated proteolysis promotes dephosphorylation of the JAK2, and consequently, negatively regulates STAT3 signaling in CRC. Moreover, immunohistochemical staining reveals that nuclear staining of phospho-STAT3 mostly presents in adenomas and adenocarcinomas, and a positive correlation is found between phospho-JAK2 immunoreactivity and the differentiation of colorectal adenocarcinomas. Therefore, our findings illustrate the biologic significance of JAK1, 2/STAT3 signaling in CRC progression and provide novel evidence that the JAK/STAT3 pathway may be a new potential target for therapy of CRC.

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## Introduction

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays a significant role in various physiological processes, including immune function, cell growth, differentiation, and hematopoiesis [1]. Recently, accumulating evidence indicates that abnormalities in the JAK/STAT pathway are involved in the oncogenesis of several cancers. For example, Lacronique and coworkers [2] reported that constitutive activation of JAK2 was found in childhood T cell acute lymphoblastic leukemia. Constitutive activation of signal transducer and activator of transcription 3 (STAT3) correlates with cell proliferation in breast carcinoma [3] and non–small cell lung cancer [4], and also inhibits apoptosis [5–7]. Conversely, inhibition of JAK/STAT signaling suppresses cancer cell growth and induces apoptosis in various cancers [3,8–12]. Recent studies have also revealed that altered STAT3 activation can contribute to oncogenesis. For example, activation of STAT3 is required for cell transformation by oncogenic Src [13] and by a constitutively active form of Gαo, a heterotrimeric G-protein subunit [14]. These published reports all demonstrate the crucial importance of the JAK/STAT pathway in tumorigenesis and progression.

Colorectal cancer (CRC) is a very common malignancy and one of the leading causes of morbidity and death in the world. Despite our

Abbreviations: CRC, colorectal cancer; JAK, Janus kinase; MMP9, matrix metalloproteinase 9; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor

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growing understanding of oncogenesis and successful identification of protooncogenes and tumor suppressor genes involved in the tumorigenesis of CRC, the biologic and molecular mechanisms in CRC are poorly understood. In general, the molecular mechanisms that control CRC progression are related to the altered expression of different protooncogenes, tumor suppressor genes, cytokines, and their receptors, including Ras, Src, $p27^{kip1}$, $p16^{ink4a}$, interleukin, and epidermal growth factor receptor [15–21]. Notably, these abnormalities involve the JAK/STAT signal transduction pathway. In fact, STAT3 is constitutively activated in various types of human tumors, including colorectal cancer, but very few studies have reported abnormal expression or activation of JAK/STAT in CRC [22]. Ma and coworkers showed that the level of activated phospho-STAT3 (pSTAT3) increased in 45 primary CRC samples compared to adjacent normal mucosae [23]. A significant correlation was also demonstrated between STAT3 and both survivin and Bcl-xl expression in CRC [24]. However, the role of STAT3 in the pathogenesis of CRC has not been examined fully. In addition, the function of JAK, the physiological activator of STAT3, in stimulating STAT3 in CRC cells remains unclear.

To directly assess the biologic significance of JAK/STAT3 signaling in CRC, using AG490, a pharmacological inhibitor of JAK, and small interfering RNA (siRNA) to deplete STAT3 in two human CRC cell lines (SW1116 and HT29), we investigated the changes in cell viability, apoptosis, cell cycle progression, and cell invasive capability. We also evaluated the changes in the expression of several proteins that directly relate to apoptosis (Bcl-2 and survivin), cell cycle regulation ($p16^{ink4a}$, $p21^{waf1/cip1}$, and $p27^{kip1}$), and cell invasion (matrix metalloproteinase 2 [MMP2], 9 [MMP9], vascular endothelial growth factor [VEGF], focal adhesion kinase [FAK], and E-cadherin). In addition, we also examined the expression of STAT3, JAK2, and their active phosphorylated forms in normal colonic epithelium, adenomas, and primary colon adenocarcinomas. Our aim was to determine the role of JAK/STAT3 signaling in CRC progression and test the hypothesis that JAK/STAT3 signaling could serve as therapeutic targets.

## Materials and Methods

### *Cell Culture, Treatment with Pharmacologic Agents, and Transient Transfection of STAT3 siRNA*

Two human colorectal cancer cell lines (SW1116 and HT29) were used in this study, cultured in RPMI 1640 medium (Gibco, Carlsbad, CA) or McCoy's 5A medium (Sigma, St. Louis, MO), respectively, supplemented with 10% fetal bovine serum at 37°C in a humidified 5% $CO_2$ atmosphere.

Both pharmacological inhibitors were purchased from Sigma. AG490, a pharmacological JAK2 inhibitor, was dissolved in ethanol and stored at −20°C until used. It was added at 16 to 24 hours after the cells were seeded at a final concentration of 0, 50, 100, and 150 μM. MG132, a pharmacological proteasome inhibitor, was dissolved in dimethyl sulfoxide and stored −20°C. At the time of the experiment, an aliquot of MG132 was thawed and diluted with tissue culture media to a final concentration of 10 μM.

Commercial STAT3 siRNA was obtained from Dharmacon Inc. (Lafayette, CO) and used to target human STAT3 (GenBank Accession Number: NM_003150). Cells were transfected with siRNA (50 nM) by using the Dharma*FECT* 1 siRNA transfection reagent according to the manufacturer's instructions. Nonspecific siRNA (Dharmacon Inc.) was used as a negative control, and the selective silencing of STAT3 was confirmed by Western blot analysis.

### *Western Blot and Antibodies*

Western blot analysis was performed using standard techniques as described previously [25]. Briefly, the cells were lysed in M-PER Reagent (Pierce, Rockford, IL) containing protease inhibitors. Equal amounts of protein (50-200 μg/lane) from whole-cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After that, proteins were transferred to nitrocellulose (Amersham, Amersham, UK) and were probed with specific primary antibodies, and then with the appropriate HRP-conjugated secondary antibodies (Pierce). Proteins were detected using the enhanced chemiluminescence detection kit (SuperSignal West Femto Substrate; Pierce). For loading control, the membrane was probed with a monoclonal antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Antibodies used in this study were purchased from Cell Signaling Technology Inc., unless otherwise specified: $pJAK3^{Tyr980}$ (Santa Cruz, CA), Bcl-2 (R&D Systems, Minneapolis, MN), and GAPDH (Kangchen, Shanghai, China). All primary antibodies were used at a 1:1000 dilution.

### *Cell Viability Assay*

Cell viability was assessed by a tetrazolium salt (WST-8)–based colorimetric assay in the Cell Counting Kit 8 (CCK-8; Dojindo, Kumamoto, Japan) [26,27]. Briefly, control and treated CRC cells were seeded onto 96-well plates at an initial density of $5 \times 10^3$ cells/well. At specified time points, 10 μl of CCK-8 solution was added to each well of the plate. Then the plate was incubated for 1 hour. Cell viability was determined by scanning with a microplate reader at 450 nm. Data were expressed as the percentage of viable cells as follows: relative viability (%) = $[A_{450}(\text{treated}) - A_{450}(\text{blank})]/[A_{450}(\text{control}) - A_{450}(\text{blank})] \times 100\%$.

### *Cell Cycle Analysis Using Propidium Iodide and Flow Cytometry*

Approximately $1 \times 10^6$ cells were removed at specified time points, washed twice with PBS and fixed in cold ethanol for 30 min, and then incubated with propidium iodide (PI) for 30 minutes. Thereafter, cells were analyzed by a flow cytometer (BD, San Diego, CA).

### *Detection of Apoptosis*

Apoptosis was determined by two methods. For nuclear morphology, cells were fixed and stained with Hoechst 33258 according to the manufacturer's instruction (Beyotime, Beijing, China). Then stained nuclei were observed immediately under a fluorescence microscope (Nikon, Tokyo, Japan). For flow cytometry analysis, an annexin-V fluorescein isothiocyanate/PI double-stain assay was performed in accordance with the manufacturer's protocol (BioVision, Mountain View, CA). Briefly, after treatment, both floating and trypsinized adherent cells ($5 \times 10^5$) were collected and resuspended in 500 μl of binding buffer containing 5 μl of annexin-V fluorescein isothiocyanate and 5 μl of PI, then incubated for 5 minutes in the dark at room temperature. Analysis was immediately performed using a flow cytometer.

### In Vitro *Invasion Assay*

Cell invasion assay was performed as described by Hecht et al. [28]. In brief, chambers with 8-μm-pore polycarbonate membranes,

coated with Matrigel on the upper side, were used (Becton-Dickinson, San Diego, CA). Cells were transfected with STAT3 siRNA (50 nM) for 48 hours. And then $1 \times 10^5$ transfected cells were harvested and seeded in serum-free medium into the upper chamber, whereas medium supplemented with 10% fetal bovine serum was applied to the lower chamber as a chemoattractant to induce invasion. Cells transfected with nonspecific siRNA were used as the negative control. After incubation for 24 hours, nonmigrated cells on the upper chamber of the filter were removed with a cotton swab. Migrated cells on the bottom surface of the filter were fixed, stained and counted. To investigate the inhibitory effects of AG490, the upper chamber was filled with 100 μM AG490 or ethanol at an equivalent volume (as a vehicle control), respectively.

### *ELISA Analysis of VEGF, MMP2, and MMP9 Release*

Cells ($1 \times 10^5$) transfected with STAT3 siRNA (50 nM) were maintained in serum-free medium for 48 hours. The medium was collected, and the concentrations of VEGF, MMP2, and MMP9 in the medium were determined using an ELISA kit (R&D Systems) according to the manufacturer's instruction. To investigate the inhibitory effects of AG490, $1 \times 10^5$ cells were cultured in 24-well culture plates until 90% confluence. Then the spent medium was removed, dishes were washed three times and incubated with serum-free medium containing AG490 or an equivalent volume of ethanol (vehicle control) for 24 hours. Thereafter, the medium was collected and assessed for all three proteins.

### *Tissue Microarray and Immunohistochemical Staining*

All specimens were from patients (38 primary colorectal adenocarcinomas and 46 adenomas) who underwent surgery in Shanghai Renji Hospital from July 2005 to January 2007. The protocol had the approval of the ethics committee of Shanghai Jiao-Tong University School of Medicine Renji Hospital and the research was carried out according to the provisions of the Helsinki Declaration of 1975. None of the patients received preoperative treatments such as radiotherapy or chemotherapy. Each adenocarcinoma was assigned a histologic type according to the World Health Organization classification as follows: well-differentiated adenocarcinoma, moderately differentiated adenocarcinoma, poorly differentiated adenocarcinoma, and mucinous adenocarcinoma. Meanwhile, 15 specimens of normal colonic epithelium, taken from patients without colorectal cancer, were used as a negative control. A tissue microarray (diameter, 1.0 mm; depth, 4 μm) was prepared by Outdo Biotech (Shanghai, China) using standard techniques [29].

The tissue microarray sections were deparaffinized in xylene and rehydrated using a graded series of ethanol. A three-step streptavidin-biotin-horseradish peroxidase method was used, and the expressions of STAT3, pSTAT3$^{Tyr705}$, JAK2, and phospho-JAK2 (pJAK2)$^{Tyr1007/Tyr1008}$ were examined with the primary antibodies (STAT3, dilution 1:100; pSTAT3$^{Tyr705}$, JAK2, and pJAK2$^{Tyr1007/Tyr1008}$, dilution 1:50) using the LSAB+ kit (DakoCytomation, Copenhagen, Denmark) according to the manufacturer's instructions.

The slides were examined independently by two investigators blinded to both clinical and pathologic data. Protein expression was quantified based on the extent of staining (percentage of positive tumor cells) and classified into two categories: negative, 0% to 15% positive tumor cells; positive, more than 15% positive tumor cells.

### *Statistical Analysis*

Results were expressed as the mean ± SD. The data were analyzed for significance by analysis of variance. Analyses comparing the expression of STAT3, JAK2, and their active forms were performed using chi-square analysis, and results were considered significant if the *P* value was less than 0.05.

## Results

### *AG490 and RNAi Induce Downregulation of JAK1, 2/STAT3 Signaling*

Western blot analysis showed a concentration-dependent decrease in the expression of JAK2 and pJAK2 24 hours after treatment with AG490 (Figure 1*A*). pJAK2 was almost undetectable at a concentration of 150 μM AG490. Meanwhile, AG490 exposure also decreased in JAK1 and pJAK1 levels in both CRC cell lines. But both JAK2 and pJAK2 were more markedly downregulated by AG490 treatment than JAK1 and pJAK1. Nevertheless, no significant changes in the JAK3 and pJAK3 levels were seen by AG490 treatment in our study. Therefore, our data suggest that whereas both JAK1 and JAK2 may contribute to abnormalities in JAK/STAT signaling in CRC tumorigenesis and progression, JAK2 might play a more important role. In addition, AG490 decreased the pSTAT3 levels in a concentration-dependent manner in SW1116 and HT29 cells after 24 hours of exposure. However, no detectable changes in the STAT3 level were seen in AG490-treated cells, implying that another pathway is responsible for the activation of STAT3. To selectively reduce the expression of STAT3, we used a siRNA. Western blot analysis revealed that both STAT3 and pSTAT3 (thus activated) were depleted by 59.8% and 80.3% in STAT3 silenced HT29 cells, respectively (Figure 1*B*).

### *The Proteasome Inhibitor, MG132, Inhibits AG490-Induced Downregulation of pJAK2 and pSTAT3*

To test if the proteasome inhibitor, MG132, can inhibit the AG490-induced downregulation of the total JAK1 and JAK2 protein levels, both SW1116 and HT29 cells were incubated in the presence of 100 μM AG490 for 16 hours, treated with MG132 (10 μM) and harvested at 0, 4, or 8 hours later. As seen in Figure 1*C*, although no detectable changes in JAK1, pJAK1, or JAK2 levels were seen, pJAK2 expression, which was low after 16 hours of AG490 treatment, increased in a time-dependent manner. Furthermore, the level of pSTAT3 increased in cells exposed to MG132. These results demonstrate that the proteasome inhibitor MG132 prevents dephosphorylation of the JAK2 kinase, and results in the activation of downstream STAT3 protein.

### *Disruption of JAK1, 2/STAT3 Signaling Is Associated with the Modulation of Some Downstream STAT3 Targets*

We further examined the expression of various apoptosis and cell cycle regulatory proteins known to be downstream targets of the STAT3 pathway. At 24 hours, increased doses of AG490 induced downregulation of Bcl-2 simultaneously with upregulation of $p16^{ink4a}$, $p21^{waf1/cip1}$, and $p27^{kip1}$ in SW1116 cells (Figure 2*A*). However, no change was seen at the protein level for survivin. The overall pattern was similar for HT29 cells, although the upregulation was more marked (Figure 2*A*, *right panel*). In addition, STAT3 downregulation

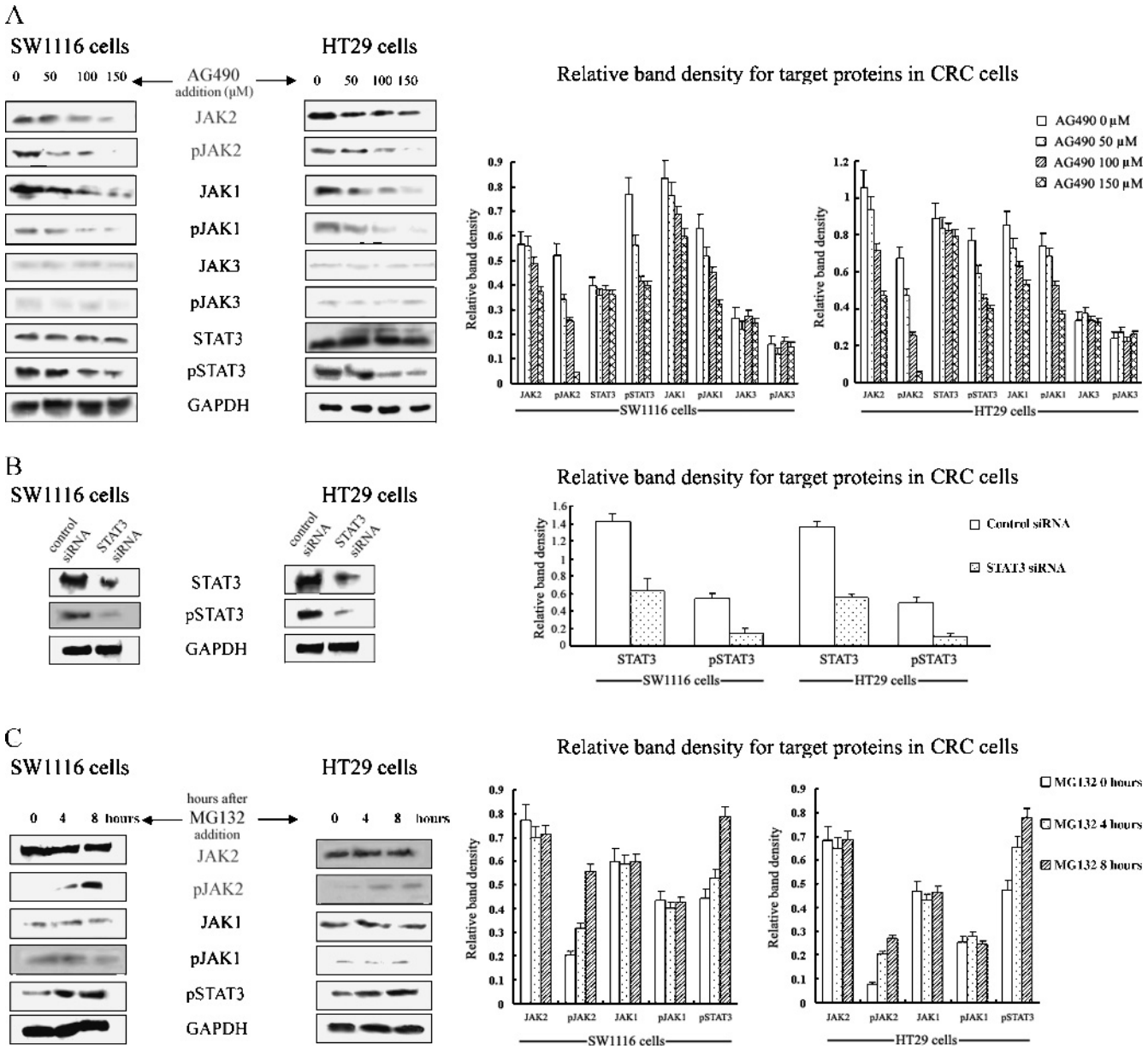


**Figure 1.** AG490 and STAT3 siRNA downregulation of JAK1, 2/STAT3 signaling. (A) Western blot analysis revealed that AG490 induced a concentration-dependent decrease in JAK2 and pJAK2 levels at 24 hours in CRC cells. In the same experiment, decreases in JAK1, pJAK1, and pSTAT3 levels were also identified, although the decrease in JAK3, pJAK3, and STAT3 was not statistically significant. (B) At 72 hours posttransfection, Western blot analysis showed that STAT3 siRNA induced downregulation of STAT3 and pSTAT3 in CRC cells. (C) MG132 blocks downregulation of pJAK2 and pSTAT3, induced by AG490. SW1116 and HT29 cells incubated with AG490 for 16 hours were treated with MG132, a proteasome inhibitor. MG132 induced a time-dependent upregulation of pJAK2 and pSTAT3. The data shown are representative of three separate experiments. GAPDH was used for the loading control. Quantification of the target protein bands relative to GAPDH is shown in the right panel.

by siRNA induced similar effects. At 72 hours posttransfection (Figure 2*B*), including downregulation of Bcl-2 and upregulation of $p16^{ink4a}$, $p21^{waf1/cip1}$, and $p27^{kip1}$.

### *Inhibition of JAK1, 2/STAT3 Signaling by AG490 or siRNA Suppresses CRC Cell Growth*

As detected by the CCK-8 assay (Figure 3*A*), after 24 hours of treatment, AG490 induced a concentration-dependent decrease in the number of viable SW1116 and HT29 cells. Similarly, our results indicated that RNAi-induced STAT3 deficiency inhibited CRC cell growth. This suppression lasted for 72 hours, and the cells recovered 96 hours posttransfection (Figure 3*A*).

### *Inhibition of JAK1, 2/STAT3 Pathway Induces $G_1$ Cell Cycle Arrest and Apoptosis*

To explore the reason for the decrease in cell viability, we examined the effects of JAK1, 2/STAT3 signaling on cell cycle progression and apoptosis. As illustrated in Figure 3*B*, pretreatment of CRC cells with AG490 and STAT3 siRNA blocked the cell cycle in the $G_1$ phase. Furthermore, a dose-dependent $G_1$ cell cycle arrest was also found in

AG490-treated cells (Figure 3*B*). In SW1116 cells, for example, the $G_0/G_1$–phase fraction increased from 38.2% (untreated) to either 52.3%, 63.9%, or 72.3%, at 50, 100, or 150 μM AG490, respectively. These observations are consistent with upregulation of $p16^{ink4a}$, $p21^{waf1/cip1}$, and $p27^{kip1}$ expression, suggesting that the JAK1, 2/STAT3 pathway is involved in cell cycle regulation.

To evaluate whether the decrease in cell viability might have occurred due to apoptotic cell death, we first examined nuclear morphology by staining the cells with Hoechst 33258 after treatment with AG490. SW1116 cells stained with Hoechst 33258 showed typical morphologic features of apoptosis including nuclear condensation and/or fragmentation 24 hours after treatment with 100 μM AG490 (Figure 3*C*).

To quantify apoptotic cell death, we performed flow cytometry analysis (Figure 3*C*). After 24 hours of AG490 treatment, phosphatidylserine extrusion to the outer leaflet of the plasma membrane, as detected by annexin-V binding to the surface of SW1116 cells increased. Our studies demonstrated that there was a 3.33-fold increase in apoptotic SW1116 cells after 24 hours of treatment with 100 μM AG490. Similarly, a 4.8-fold increase in apoptotic HT29 cells was also detected. STAT3 siRNA induced similar effects. At 72 hours after transfection with 50 nM of STAT3 siRNA, the apoptosis of SW1116 and HT29 cells increased 2.55- and 3.50-fold, respectively, when compared with the equivalent transfection with nonspecific siRNA.

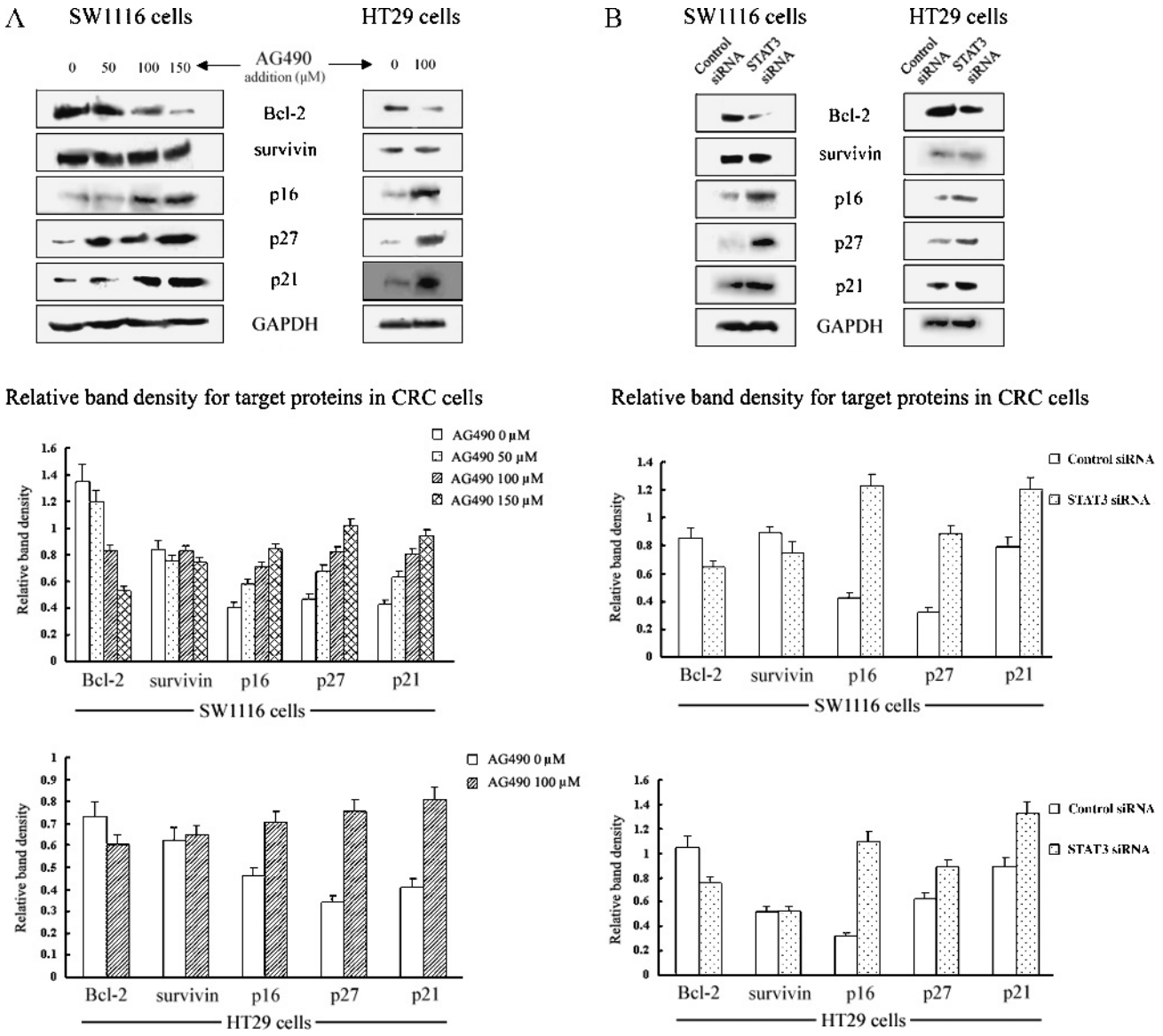


**Figure 2.** Disruption of JAK1, 2/STAT3 signaling is associated with modulation of some STAT3 downstream targets. (A) Western blot analysis showed that AG490 induced concentration-dependent alterations of part, but not all, of the STAT3 downstream targets in SW1116 cells at 24 hours after treatment. Bcl-2 was downregulated, simultaneously associated with the upregulation of $p16^{ink4a}$, $p21^{waf1/cip1}$, and $p27^{kip1}$, whereas survivin showed no detectable change. A similar pattern of changes was identified in HT29 cells. (B) STAT3 siRNA induced similar effects. Indeed, downregulation of Bcl-2, and upregulation of $p16^{ink4a}$, $p21^{waf1/cip1}$, and $p27^{kip1}$ were detected at 72 hours after transfection of the cells. GAPDH was used for the loading control. Quantification of the target protein bands relative to GAPDH is shown in the right panel.

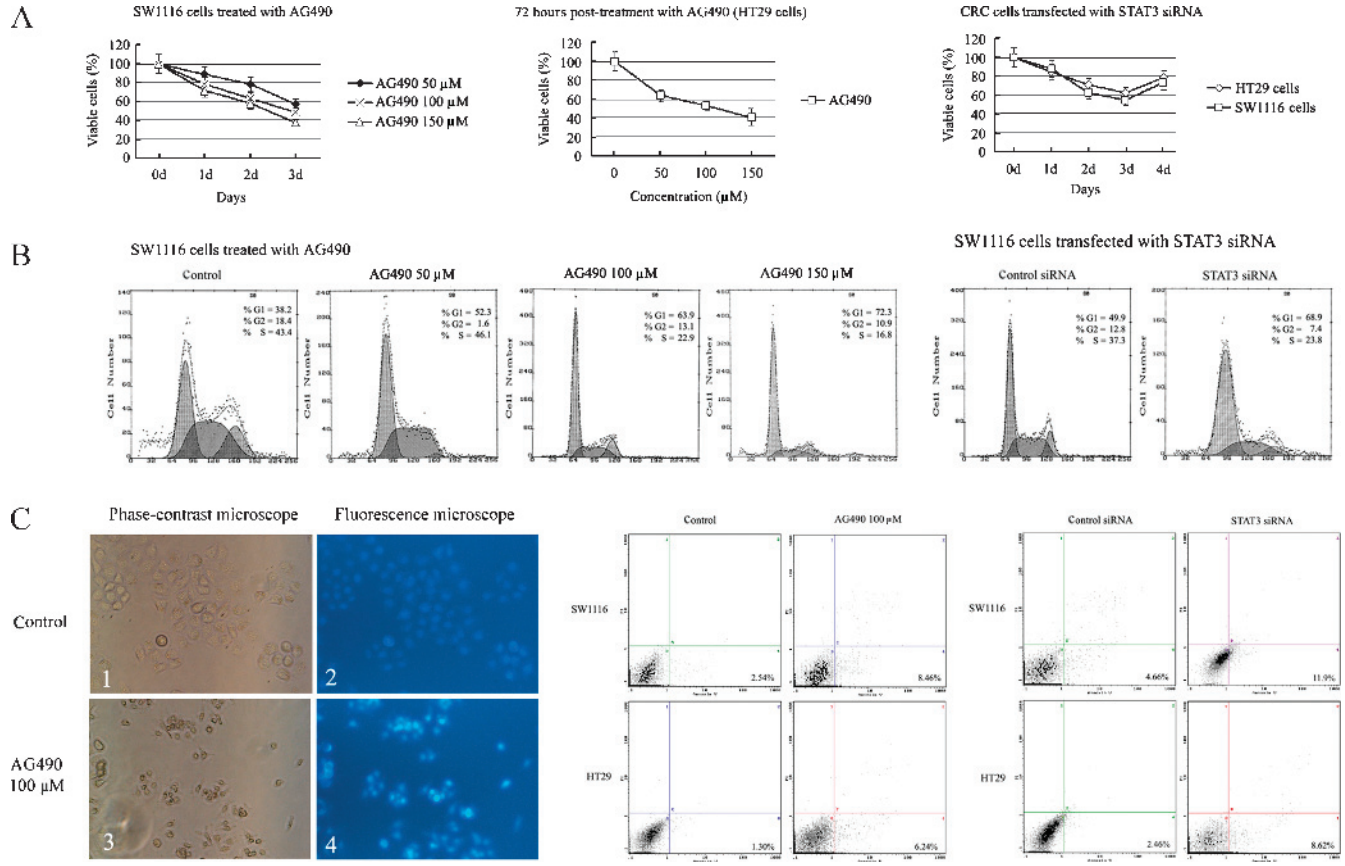


**Figure 3.** The functional role of JAK1, 2/STAT3 signaling on CRC cell growth, cell cycle progression, and apoptosis. (A) Cell viability as determined by the CCK-8 assay of CRC cells treated with AG490 or STAT3 siRNA. SW1116 cells were treated with solvent only (negative controls) or with 50, 100, or 150 μM AG490. The percentage of viable cells was determined as described in the Materials and Methods section. AG490 significantly decreased the number of viable cells. No significant difference was seen in the three dosages of AG490. Treating HT29 cells with AG490 also induced a concentration-dependent decrease in the number of viable cells at 72 hours. In addition, STAT3 siRNA inhibited CRC cell growth. This suppression lasted for 72 hours, and the cell recovered at 96 hours posttransfection. The results represent mean ± SD of three experiments. (B) Cell cycle analysis was performed using SW1116 cells treated with diluent (left) or AG490 (50, 100, or 150 μM) for 24 hours. Compared to the negative control, our results reveal that AG490 induces a concentration-dependent increase in the proportion of cells in the $G_1$ phase. Moreover, treating SW1116 cells with STAT3 siRNA blocked the cell cycle in the $G_1$ phase at 72 hours after transfection. (C) CRC cells was treated with 100 μM AG490 for 24 hours or STAT3 siRNA for 72 hours, and cell apoptosis was detected by morphologic changes and flow cytometric analysis.

### *Disruption of JAK1, 2/STAT3 Signaling Inhibits Colorectal Cancer Cells Invasion*

We next addressed the functional role of JAK1, 2/STAT3 signaling in the invasion of the cells into the surrounding tissue. As shown in Figure 4*A*, AG490 treatment suppressed cell invasion. The number of cells that migrated through the filter decreased to 73.5% in HT29 cells after 24 hours of treatment with 100 μM AG490 when compared to ethanol-treated (vehicle control) cells, demonstrating a potential regulatory role of JAK/STAT signaling on the invasive capability of CRC cells.

To verify if this inhibitory effect was mediated by STAT3, we used siRNA to specifically knockdown STAT3 expression. As illustrated in Figure 4*B*, STAT3 siRNA, but not nonspecific siRNA, effectively suppressed CRC cell invasion. Forty-eight hours after transfection with 50 nM of STAT3 siRNA, the number of SW1116 and HT29 cells that migrated through the filter decreased to 57.4% and 47.5%, respectively, when compared to cells transfected with nonspecific siRNA. These results indicate that STAT3 is a critical mediator involved in JAK/STAT–induced cell invasion.

### *Decreased STAT3 Activation Is Associated with Modulation of E-cadherin, MMP2, and VEGF, But Not FAK and MMP9*

To better understand the mechanisms of JAK1, 2/STAT3 signaling on CRC cell invasion and to reveal downstream events of JAK/STAT3 signaling that are involved in the regulation of cell invasion, we examined the expression of various migration and invasion regulatory proteins by Western blot and ELISA analyses. As shown in Figure 4, *F* and *E*, STAT3 siRNA and increasing doses of AG490 reduced secretion of MMP2 and VEGF, while upregulating E-cadherin expression in CRC cells (Figure 4, *D* and *C*). However, no significant change in MMP9 secretion was seen in cells treated with either STAT3 RNAi or AG490 treatment (Figure 4, *E* and *F*).

Interestingly, although no detectable changes in the expression of FAK were seen by STAT3 siRNA transfection (Figure 4*D*), AG490 induced downregulation of the total FAK protein level in both SW1116 and HT29 cells (Figure 4*C*) in a dose-dependent manner. Our studies imply that: (1) FAK is not required for STAT3-mediated regulation; and that (2) FAK may be a component of the JAK pathway, downstream of JAK.

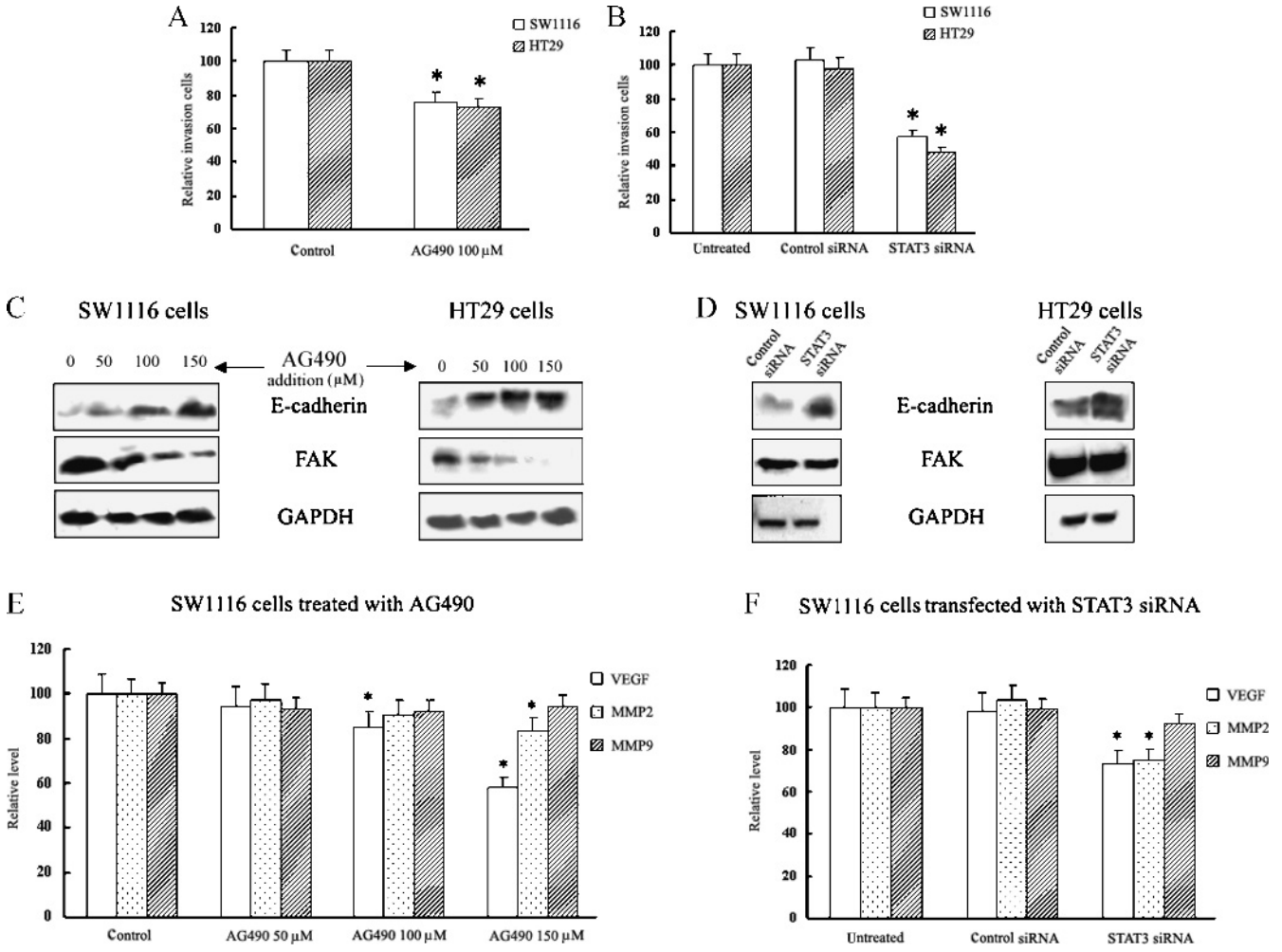


**Figure 4.** The functional role of JAK1, 2/STAT3 signaling on the invasive ability of CRC cell. (A) AG490 suppresses the invasion in CRC cells at 24 hours after treatment. The migrated cell numbers were normalized to that of the negative controls. Our data demonstrated that AG490 significantly inhibits the invasiveness of CRC cells (*$P < .05$). (B) CRC cells transfected with STAT3 siRNA were examined for their invasive capability. At 48 hours posttransfection, the numbers of migrated cells significantly decreased when compared with that of untreated cells (*$P < .05$). (C) Western blot analysis showed that AG490 induced a concentration-dependent upregulation of E-cadherin, simultaneously associated with the downregulation of FAK in CRC cells at 24 hours after treatment. (D) STAT3 siRNA induced an increase in E-cadherin at 72 hours posttransfection of the cells. However, we failed to find the FAK-level changes by STAT3 siRNA transfection. (E) The concentrations of VEGF, MMP2, and MMP9 in AG490-treated SW1116 cells were tested by ELISA. The data are from three individual experiments, and show reduced secretion of MMP2 and VEGF after AG490 treatment (*$P < .05$). (F) Effects of STAT3 siRNA on the secretions of VEGF, MMP2, and MMP9. At 48 hours posttransfection, the concentrations of VEGF and MMP2 were decreased compared to that of untreated cells (*$P < .05$). The experiment was performed three times with consistent findings.

### *Activated STAT3 Is Constitutively Expressed in Colorectal Carcinoma*

Table 1 shows the frequency of expression of STAT3, pSTAT3, JAK2, and pJAK2 by immunohistochemical staining. STAT3 expression was detected in 86.7% of the normal colonic epithelium samples, 89.1% of the adenoma samples, and 100% of the primary colon adenocarcinoma samples. STAT3 staining was detected mainly in the cytoplasm, with occasional nuclear staining. However, pSTAT3 expression, mostly presented in the nucleus, was found in 26.67% of the normal colonic epithelium samples, in 63.0% of the adenoma samples, and in 100% of the primary colon adenocarcinoma samples (Figure 5). Our results suggest that upregulation of activated STAT3 in colon carcinoma could have important implications in colorectal cancer biology.

**Table 1.** Frequency of the Expression of JAK2, STAT3, and Their Activated Forms.

| | *N* | STAT3, *n* (%) | pSTAT3, *n* (%) | JAK2, *n* (%) | pJAK2, *n* (%) |
|---|---|---|---|---|---|
| Normal epithelium | 15 | 13 (86.7%) | 4 (26.7%) | 9 (60%) | 7 (46.7%) |
| Adenoma | 46 | 41 (89.1%) | 29 (63.0%) | 41 (89.1%) | 34 (73.9%) |
| Adenocarcinoma | 38 | 38 (100%) | 38 (100%) | 33 (86.8%) | 31 (81.6%) |
| Differentiation | | | | | |
| Well | 6 | 6 (100%) | 6 (100%) | 5 (83.3%) | 3 (50%) |
| Moderate | 27 | 27 (100%) | 27 (100%) | 23 (85.2%) | 23 (85.2%) |
| Poor | 3 | 3 (100%) | 3 (100%) | 3 (100%) | 3 (100%) |
| Mucinous carcinoma | 2 | 2 (100%) | 2 (100%) | 2 (100%) | 2 (100%) |

pSTAT3 is mostly presented in adenomas and adenocarcinomas, and a significant correlation was found between pJAK2 immunoreactivity and the differentiation of colon adenocarcinomas (*$P < .05$).

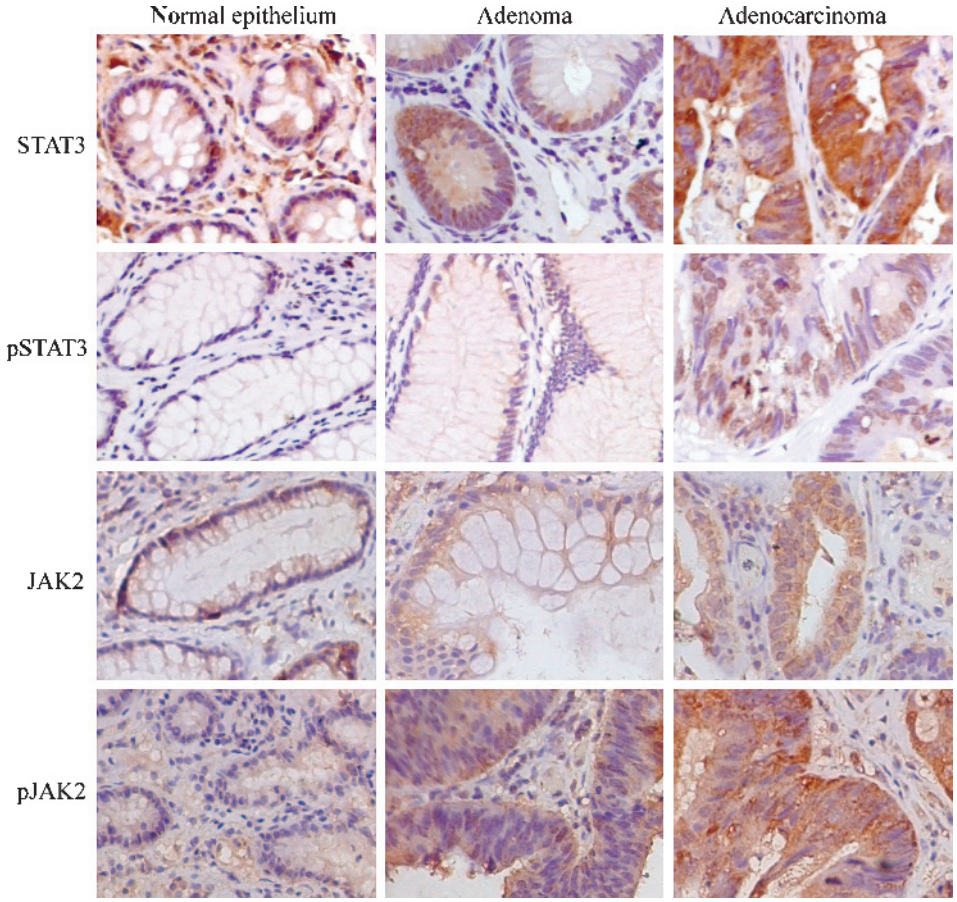


**Figure 5.** Immunohistochemical staining of the tissue microarray. Predominantly, cytoplasmic staining of STAT3 was frequently detected in normal colonic epithelium, adenomas, and primary colon adenocarcinomas. Nevertheless, nuclear staining of pSTAT3 was mostly presented in adenomas and adenocarcinomas. JAK2 and pJAK2 showed predominantly cytoplasm localization. Original magnification, ×200.

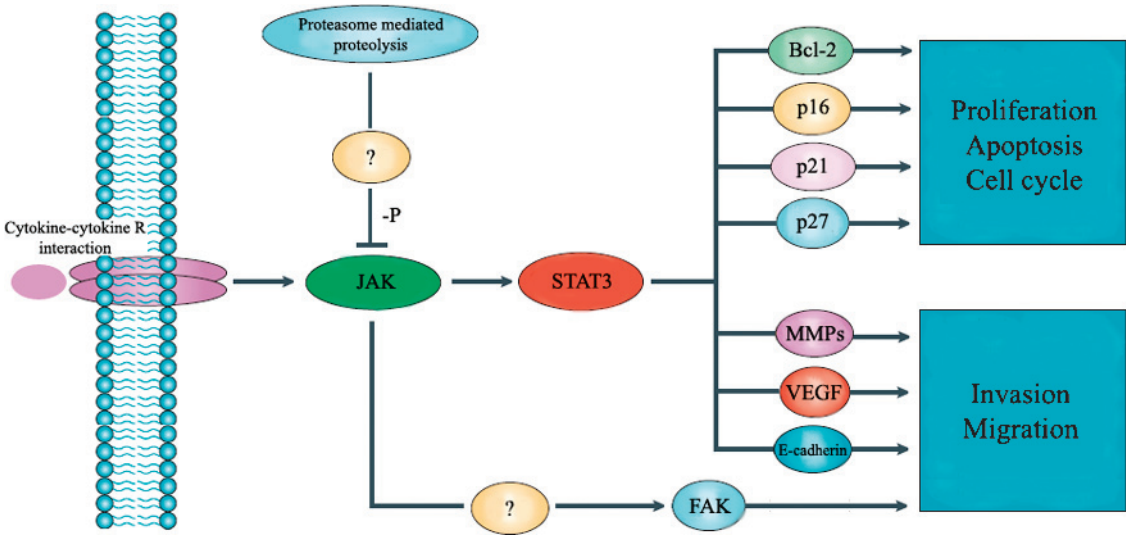


**Figure 6.** Model of the possible contributions of JAK1, 2/STAT3 signaling pathway in CRC development. Proteasome-mediated proteolysis promotes dephosphorylation of the JAK2 kinase and, consequently, negatively regulates JAK/STAT3 signaling in CRC. Other unknown mechanisms may also have been involved in this process. Furthermore, JAK1, 2/STAT3 signaling is implicated in many areas of tumor progression, including cell growth, survival, invasion, and migration by regulation of gene expressions, such as Bcl-2, $p16^{ink4a}$, $p21^{waf1/cip1}$, and $p27^{kip1}$, E-cadherin, VEGF, and MMPs. Additionally, FAK may be a component of the JAK pathway and a downstream of JAK, which may exert its oncogenic effects through interaction with other signal transduction pathways.

### *Activated JAK2 Correlates with the Differentiation of Colon Adenocarcinomas*

JAK2 and pJAK2 showed predominantly cytoplasm localization. Cellular staining with anti-JAK2 antibody occurred in 60%, 89.1%, and 86.8% of the normal colonic epithelium samples, adenoma samples, and colon adenocarcinoma samples, respectively. pJAK2 was observed in 46.7% of the normal tissue samples, in 73.9% of the adenoma samples, and in 81.6% of the adenocarcinoma samples (Figure 5). Thus, adenomas and colon adenocarcinomas showed higher expression of JAK2 and pJAK2 than normal colonic mucosa ($P < .05$). Furthermore, a significant correlation was found between pJAK2 immunoreactivity and the differentiation of colon adenocarcinomas (Table 1).

## Discussion

Despite improvements in determining the molecular mechanisms of CRC tumorigenesis, the specific signal transduction pathways involved have not been fully characterized [30]. Recently, constitutive activation of STAT proteins has been detected frequently in several malignant neoplasms [2,3,5,31–36]. Also, several studies have proposed that STAT3 signaling may be involved in colorectal carcinogenesis [37,38]. However, the precise role of JAK/STAT signaling in human CRC progression has not been fully characterized. In the present study, we provide experimental and mechanistic evidences that abnormalities of JAK1, 2/STAT3 signaling contribute to the progression of CRC.

In this study, we confirmed that AG490 induced a concentration-dependent decrease in the JAK2 protein level in both CRC cell lines, and this change coincided with the downregulation of pJAK2 and pSTAT3. AG490 also decreased the levels of JAK1 and pJAK1, but to a lesser degree than for JAK2 and pJAK2. Conversely, no significant changes in the JAK3 and pJAK3 levels were seen as the result of AG490 treatment. Therefore, our data suggest that whereas both JAK1 and JAK2 may contribute to abnormalities of the JAK/STAT signaling, JAK2 might play a more important role in CRC tumorigenesis and progression. We also found that the proteasome inhibitor MG132 may activate JAK2 kinase and STAT3, as MG132 efficiently blocked the AG490-induced downregulation of pJAK2 and pSTAT3. Therefore, we provide evidence that proteasome-mediated protein degradation promotes dephosphorylation of the JAK2 kinase, and consequently, negatively regulates JAK/STAT3 signaling in CRC. Nevertheless, because MG132 is a nonspecific pharmacologic agent, further studies are needed to substantiate these findings and to delineate how proteasome degradation may regulate JAK/STAT3 signaling in CRC. Our ongoing studies suggested that suppressor of cytokine signaling-1 (an important negative regulator for cellular signaling pathways such as JAK/STAT) may regulate proteasome-mediated downregulation of JAK2 in CRC cells (data not shown).

We further evaluated the biologic significance of JAK/STAT3 activation in the pathogenesis of CRC cells. A pharmacological JAK2 inhibitor, AG490, and STAT3 siRNA were used to selectively block JAK/STAT3 signaling. Our results indicated that downregulation of pJAK1, pJAK2, and pSTAT3 was associated with a gradual decrease in viable cells. Moreover, the decrease in cell viability can be attributed to a significant increase in apoptotic cell death and cell cycle arrest in the $G_1$ phase. The molecular basis for cell apoptosis and cell cycle arrest in CRC was also investigated. Treatment of CRC cells with AG490 or STAT3 siRNA decreased Bcl-2 expression and increased the expression of $p16^{ink4a}$, $p21^{waf1/cip1}$, and $p27^{kip1}$. In addition, although previous studies have shown that STAT3 prevents apoptosis by inducing of survivin [39,40], no change in survivin expression was seen, probably because survivin is regulated by other pathways, such as Akt and NF-κB [41,42]. Therefore, the mechanisms for induction of cell cycle arrest and apoptosis could be attributed at least in part to the altered regulation of these genes.

Invasiveness is a key step that leads to metastasis resulting in poor prognosis [43]. Therefore, it is of great value to study the molecular mechanism of CRC invasiveness. The Matrigel invasion assay showed that an inverse relationship between the invasiveness of CRC cells and inhibition of JAK and STAT3 signaling, demonstrating a potential regulatory effect of JAK1, 2/STAT3 signaling on the invasive capability of CRC cells. Because the downstream events of the JAK/STAT3 pathway are not fully defined, we examined the expression of various migration and invasion regulatory proteins by Western blot and ELISA analyses. Our data suggest that blockade of JAK and STAT3 activity decreases the expression of MMP2 and VEGF, but increases the expression of E-cadherin, suggesting that the JAK/STAT3 pathway may be involved in the regulation of the expression of MMP2, VEGF, and E-cadherin. These results imply that JAK/STAT3 signaling may regulate multiple processes in CRC invasion. First, JAK1, JAK2, and STAT3 activation, by stimulating MMP2 production, could induce degradation of the extracellular matrix. Second, JAK1, 2/STAT3 signaling could regulate CRC invasive capability by affecting angiogenesis. Treatment with AG490 or STAT3 siRNA reduces VEGF secretion by CRC cells, suggesting that the JAK/STAT3 pathway may regulate angiogenesis. Third, the JAK/STAT3 pathway could also function in tumor metastasis and invasion by regulating E-cadherin, a protein belonging to the family of cell-cell adhesion molecules that plays a fundamental role in the maintenance of cell differentiation. Thus, for the first time, we provide mechanistic evidence that the JAK/STAT3 pathway may affect CRC metastasis by multiple mechanisms including proliferation, enzyme-based degradation of the extracellular matrix, angiogenesis, adhesion, and migration. Nevertheless, our findings suggest that FAK is not required for STAT3-mediated regulation, but may be a component of the JAK pathway downstream of JAK. Thus, these findings imply that JAK may exert its oncogenic effects by interacting with other signal transduction pathways, for instance the phosphatidylinositol-3 kinase/protein kinase B pathway, or the activation of other STAT family members [44,45] and not only through the activation of STAT3.

Furthermore, our data on pSTAT3 expression confirmed the results of previous studies [23,38], showing that pSTAT3 expression is markedly increased in colon adenocarcinomas and adenomas (100% and 63%, respectively) compared with expression in normal colonic epithelium (26.7%), and therefore represents a significant correlation between activated STAT3 expression and CRC tumorigenesis. In addition, Kusaba and coworkers reported that activated STAT3 expression is an important factor related to carcinogenesis and CRC invasion [37]. Thus, STAT3 plays a significant role in CRC oncogenesis and could be a potential therapeutic target for CRC treatment. Our study also demonstrated that the expression of pJAK2 increased with the progression of CRC (Table 1), implying a direct relationship between pJAK2 expression and progression of the disease. However, the samples are relatively small and further studies are needed to validate this hypothesis.

In conclusion, this study is the first to have examined in detail the mechanistic role of JAK/STAT3 signaling in CRC tumorigenesis and progression (summarized in Figure 6). Our present findings strongly

suggest that the JAK/STAT3 pathway plays a significant role in CRC progression. We propose that JAK1, JAK2, and STAT3 are novel and worthwhile therapeutic targets for CRC treatment, because they are implicated in many areas of tumor progression, including cell growth, survival, invasion, and migration. Intervention in JAK1, 2/STAT3 signaling may have potential therapeutic value in the treatment of human colorectal cancer.

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