# Elevated Expression of the Oncogene *c-fms* and Its Ligand, the Macrophage Colony-Stimulating Factor-1, in Cervical Cancer and the Role of Transforming Growth Factor-β1 in Inducing *c-fms* Expression

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## Abstract

**Cervical cancer is the third most common gynecologic cancer in the United States. The presence and possible involvement of several cytokines have been studied in cervical cancer; however, very little data, if any, are available on whether cervical tumors are responsive to stimulation by the macrophage colony-stimulating factor-1 (CSF-1). Given the involvement of *c-fms* and its ligand CSF-1 in gynecologic cancers, such as that of the uterus and the ovaries, we have examined the expression of *c-fms* and CSF-1 in cervical tumor ($n$ = 17) and normal cervix ($n$ = 8) samples. The data show that *c-fms* and its ligand are significantly higher in cervical carcinomas compared with normal samples. Immunohistochemistry not only showed that tumor cells expressed significantly higher levels of *c-fms* but also *c-fms* levels were markedly higher in tumor cells than tumor-associated stromal cells. Blocking *c-fms* activity in cervical cancer cells, which express CSF-1 and *c-fms*, resulted in increased apoptosis and decreased motility compared with control, suggesting that CSF-1/*c-fms* signaling may be involved in enhanced survival and possibly invasion by cervical cancer cells via an autocrine mechanism. Combined, the data show for the first time the induction of CSF-1 and *c-fms* in cervical carcinomas and suggest that *c-fms* activation may play a role in cervical carcinogenesis. Additionally, our data suggest that transforming growth factor-β1 may be a factor in inducing the expression of *c-fms* in cervical cancer cells. The data suggest that *c-fms* may be a valuable therapeutic target in cervical cancer.** [Cancer Res 2007;67(5):1918–26]

## Introduction

Cervical cancer is the third most common gynecologic cancer in the United States. It is more prevalent among women of low socioeconomic status and is a major health problem in developing countries. The majority of cervical cancers (85%) are of squamous origin, whereas the rest are adenosquamous or adenocarcinomas. The development of cervical carcinomas seems to be highly associated with human papillomavirus (HPV) infection: ~100% of carcinomas have been shown positive for the presence of HPV DNA (1).

HPV-associated cervical cancer induces inflammatory response due to viral infection, resulting in potential increased macrophage infiltration and induction of cytokines (2, 3). The presence and possible involvement of several chemokines and cytokines have been studied in cervical cancer; however, the molecular mechanisms involved in the disease process are still poorly elucidated (3). One cytokine that has not been examined in much, if any, detail in reference to its involvement in cervical cancer is the macrophage colony-stimulating factor-1 (CSF-1), a cytokine and growth factor that was first identified in macrophages. CSF-1 signals via a membrane tyrosine kinase receptor, *c-fms*, a known proto-oncogene (4). Recent work from several groups suggests that CSF-1 and *c-fms* expression may be involved in the progression of female cancers, such as breast, endometrial, and ovarian cancers (5–7). The concomitant expression of both CSF-1 and *c-fms* has been documented in a variety of cancers, including ovarian and breast cancers, and is usually associated with more aggressive disease (6, 8).

Transforming growth factor-β (TGF-β) is a pleiotropic growth factor that plays contrasting dual roles of initial tumor suppression via inhibition of cellular growth as well as promotion of tumor growth and possibly metastasis during disease progression (9). TGF-β signals through TGF-β receptor complexes (notably, TBRII and TBRI) by binding to TBRII, after which TBRI is recruited to the complex. Activation of TBRI, which is a serine/threonine kinase, results in transmitting the signal into the nucleus via the classic Smads pathway as well as mitogen-activated protein kinase (MAPK; e.g., p38; refs. 9–13). Many tumor cells lose sensitivity to inhibition by TGF-β in the event of loss of TGF-β receptor function or that of intracellular signaling factors, such as Smad3 and/or Smad4 (9, 14). However, there are instances where loss of growth inhibition by TGF-β is not associated with loss of these signaling components (15). This raises the possibility that, in addition to the indirect protumorigenic role of TGF-β by inducing angiogenesis and suppressing antitumor immune response, TGF-β may also positively influence survival of tumor cells directly (16). Previous studies showing that TGF-β1 induced *c-fms* expression in a murine myeloid progenitor cell line and in vascular smooth muscle cells suggested that TGF-β may mediate cellular functions via CSF-1/*c-fms* signaling and prompted us to examine in this study whether TGF-β1 induces *c-fms* expression in cervical cancer cells (17, 18).

Recent studies by our group and others have shown that the serum levels of CSF-1 are increased in cervical cancer patients compared with normal patients (19, 20), suggesting that CSF-1

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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signaling may play an important role in cervical cancer and may be a potential therapeutic target. This study was designed to test whether cervical tumor cells express *c-fms* and its ligand CSF-1 as well as to examine the role of these factors in cervical carcinogenesis. One study has detected equivalent *c-fms* expression in precancerous cervical lesions and normal tissue but did not examine the presence of *c-fms* in cervical carcinomas (21). Whereas several studies have shown the expression of CSF-1 in ovarian and endometrial cancers, no other study, to our knowledge, has examined the expression of this growth factor and its receptor in malignant cervical tumors (6). We report herein increased expression of CSF-1 and its receptor *c-fms* in cervical tumor samples compared with normal cervix. In addition, we have also examined the expression of CSF-1 and *c-fms* in several cervical cancer cell lines and the effects of blocking *c-fms* function on cervical cancer cell survival and motility. We have also shown that TGF-β1 may regulate the expression of *c-fms* in cervical cancer, suggesting a possible mechanism for the induction of CSF-1 signaling in cervical cancer.

## Materials and Methods

**Clinical samples.** Using Institutional Review Board (IRB)-approved protocols at Emory University School of Medicine (Atlanta, GA) and associated hospitals, cervical carcinoma samples ($n$ = 17) and cervical tissue from age-matched normal cases ($n$ = 8) were collected from unselected patients presenting with cervical cancer and women having a hysterectomy for problems unrelated to cervical disease, respectively. Tumors tested were invasive squamous carcinomas (with the exception of two adenocarcinomas), all of which were positive for HPV with International Federation of Gynecology and Obstetrics stage ranging from I to III. Tumor biopsies were carefully taken from nonnecrotic areas of cervical lesions to maximize intact RNA isolation from total tissue. After obtaining informed consent as well as a detailed historical information questionnaire from each patient, the biopsy samples were immediately frozen and stored at −70°C for future analyses.

For immunohistochemistry, archival paraffin blocks of cervical biopsies were obtained from Hospital De Clinicas De Porto Alegre (Porto Alegre, Rio Grande do Sul, Brazil) with local ethical committee approval as well as the University of Texas Health Science Center IRB (San Antonio, TX). Serial 4-μm sections were done, with the first section stained with H&E for pathologic diagnosis and the following sections reserved for immunostaining. All tumors tested were HPV-positive squamous invasive carcinomas.

**Cell lines.** For this study, cervical cancer cell lines CaSki and HeLa were used, both of which were positive for the two highest risk HPV strains (HPV16 and HPV18, respectively), and HT-3 was used representing HPV-negative cervical cancer cells. Furthermore, CaSki cells represent the more common squamous cervical carcinoma, whereas HeLa cells were derived from cervical adenocarcinoma. All cells used in this study were obtained from the American Type Culture Collection (Manassas, VA) and cultured using RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Medium conditions for different experimental treatments are described below.

**siRNA transfection.** SMARTpool-specific siRNA used in the studies below was purchased from Dharmacon (Lafayette, CO). Transfection was done using the highly efficient nucleofection technology as per the manufacturer's protocol (Amaxa, Gaithersburg, MD). Briefly, $1 \times 10^6$ cells per transfection were resuspended in 100 μL of appropriate nucleofection buffer (Amaxa) and mixed with 10 μL of 10 μmol/L SMARTpool siRNA in a cuvette. Transfection was done in the Amaxa Nuclefector II using the appropriate program. For HeLa cells, transfection efficiency using program I-013 was determined at 70% after 24 h. For CaSki cells, transfection of siRNA was done using DharmaFect 1 siRNA transfection kit according to the manufacturer's direction (Dharmacon).

**RNA expression of CSF-1 and *c-fms* by reverse-transcription PCR.** Total RNA was isolated from total normal and cervical cancer tissue samples (nonnecrotic regions were carefully selected by the physician) using TRI Reagent (Sigma, St. Louis, MO) according to the manufacturer's protocol. Total cDNA was synthesized from the RNA by reverse transcription using the murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) and following the protocol provided by the manufacturer. The cDNAs of CSF-1, *c-fms*, and the housekeeping gene *actin* were amplified using sequence-specific primers. The primer set sequences used are as follows: CSF-1, ATGACAGACAGGTGGAACTGCCAG and TCACACAACTTCAGTAGGTTCAGG; *c-fms*, CAGATTGGTATAGTCCCGCTCTCT and TCCAACTACATTGTCAAGGGCAAT; and actin, AAGATGACCCAGATCATGTTTGAGAC and CTGCTGCTGATCCACATCTG. The amplification conditions were carried for 28 cycles using the Platinum Taq Polymerase (Invitrogen, Carlsbad, CA). The amount of starting total RNA (800 ng for CSF-1, 400 ng for *c-fms*, and 200 ng for actin) and cycle number were optimized to reflect the exponential phase of amplification. The PCR products were then visualized by electrophoresis in 1% agarose gels and staining with ethidium bromide. Densitometric analysis was used to quantify the products and normalize to the expression of actin. Real-time PCR was also done using cDNA from reverse transcription reactions using the Platinum Taq Polymerase according to the manufacturer's protocol. To detect amplicon synthesis in the SmartCycler real-time PCR thermal cycler (Cepheid, Sunnyvale, CA), 0.25× Cyber Green dye (Roche, Indianapolis, IN) was added to the reaction mixture. The cycle threshold number ($C_t$) exhibiting the maximum curve growth rate was determined, and the relative gene expression of each sample, normalized to that of actin, was calculated by the formula $2^{Ct\ (\text{actin})\ -\ Ct\ (\text{gene})}$.

**Immunohistochemistry.** Slides were deparaffinized and rehydrated, and antigen retrieval was achieved by boiling the tissues in citrate buffer (pH 6.0). Immunohistochemistry was done with the avidin-biotin complex method using DAKO LSAB+ kit (DAKO, Carpinteria, CA) for CSF-1 and Ultravision kit (Lab Vision, Fremont, CA) for all other antibodies according to the manufacturers' directions.

Endogenous peroxidase and avidin/biotin were blocked before the incubation with primary antibody. The following primary antibodies, dilution, time, and temperature of incubation were used: rabbit polyclonal anti–*c-fms* (Chemicon International, Temecula, CA), 1:50, overnight, 4°C; CSF-1 (R&D Systems, Minneapolis, MN) mouse monoclonal anti-CD68 (marker for monocytes/macrophages; Lab Vision), 1:100, 30 min, room temperature; mouse monoclonal anti–TGF-β1 (Abcam, Cambridge, MA), 1:1,000, 1 h, room temperature; and rabbit polyclonal anti-TBRII (Abcam), 1:100, 30 min, room temperature.

Subsequently, biotinylated secondary antibody, goat anti-mouse or goat anti-rabbit, was applied followed by streptavidin peroxidase. Immunoreactive complexes were detected using 3,3′-diaminobenzidine chromogen exposure for 5 min. Finally, slides were counterstained with methyl green and mounted. Negative controls were obtained omitting primary antibody. Positive controls used were tonsil (for *c-fms*, CSF-1, and CD68), breast carcinoma (for TGF-β1), and placenta (for TBRII).

**Scoring assessment.** Three representative regions of each case were previously chosen after H&E staining from blind pathologic analysis. Sections were analyzed at ×200 magnification according to the H-score (22) using the following formula: H-score = (% cells did not stain × 0) + (% cells stained weak × 1) + (% cells stained moderate × 2) + (% cells stained strong × 3). Therefore, the H-score ranged from 0 (100% of negative cells) to 300 (100% of strong staining). For CD68 staining, cells were individually counted at ×200 magnification with mean area of 0.28 $mm^2$, and values were presented as absolute numbers after correction to the percentage of stroma or epithelium presented.

**Statistics.** Data analysis was done using the Statistical Package for the Social Sciences version 12 (SPPS, Inc., Chicago, IL), and comparisons of variables with normal distributions were analyzed using the Student's $t$ test (e.g., scratch assay), whereas comparison of variables with nonnormal distributions was evaluated using the Mann-Whitney $U$ test (e.g., immunohistochemical H-score). When appropriate, variables are presented as mean ± SD, even the ones with nonnormal distribution. In all tests, $P < 0.05$ was considered statistically significant.

**ELISA.** Cervical cancer cell lines were cultured in RPMI 1640 containing 10% charcoal-stripped FBS, penicillin, and streptomycin at 50% confluence.

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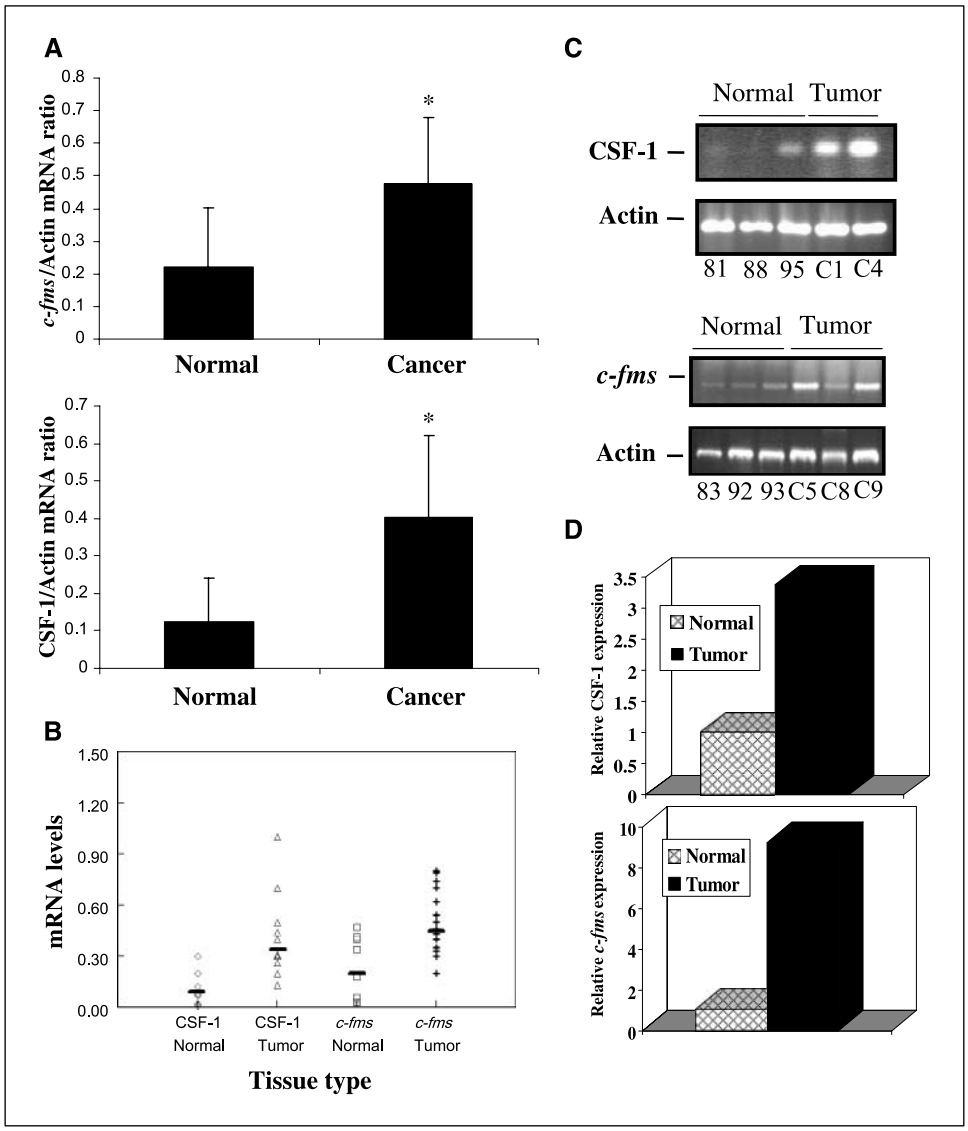


**Figure 1.** Expression of CSF-1 and its receptor *c-fms* in cervical carcinomas. *A,* RNA from individual cervical carcinoma samples ($n$ = 17) and normal cervix tissue ($n$ = 8) was each examined by semiquantitative RT-PCR for the expression of *c-fms* and CSF-1. *Columns,* mean densitometric analysis of *c-fms* and CSF-1 expression normalized to that of actin; *bars,* SD. *, $P$ = 0.008 (for *c-fms* expression) and 0.001 (for CSF-1 expression), Mann-Whitney $U$ (two tailed) test. *B,* distribution of expression [carcinoma samples ($n$ = 17) and normal cervix tissue ($n$ = 8)]. *C,* RT-PCR products of individual representative cases, subjected to agarose gel electrophoresis, showing CSF-1 and *c-fms* expression in cervical tumor and normal tissues. *Bottom,* sample number. *D,* a representative normal sample and a carcinoma sample were subjected to real-time RT-PCR to confirm differential expression of CSF-1 (*top*) and *c-fms* (*bottom*) after normalization to actin.

After 48 h of incubation at 37°C, conditioned media free of cells were collected and centrifuged. CSF-1 levels in supernatants were determined in duplicates using a commercially available sandwich ELISA kit specific to human CSF-1 (Quantikine, R&D Systems) as directed by manufacturer's protocol. CSF-1 concentrations were determined using a standard curve ranging from 0 to 2,000 pg/mL.

**Colony formation assay.** CaSki, HeLa, and HT-3 cells were seeded in six-well plates at 500 per well in phenol red–free RPMI 1640 (containing 10% heat-inactivated charcoal-stripped FBS) with or without 1 μg/mL of neutralizing *c-fms* antibody (2-4A5, Santa Cruz Biotechnology, Santa Cruz, CA). After 10 days of treatment (medium with/without antibody changed every 2 days), cells were fixed and stained with 0.5% crystal violet. Images of colonies were captured using a Nikon SMZ 1500 stereomicroscope with digital camera, ×7.5 magnification (Nikon, Melville, NY), and processed in Corel Photo-Paint X3 (Corel Corp., Eden Prairie, MN) using the Magic Wand Mask Toll to measure in pixels the area of colony formation. Values of treatment group were presented relative to the area measured for controls.

**Apoptosis studies.** After treatment with *c-fms*–specific antibody (2-4A5), CaSki, HeLa, and HT-3 cells were subjected to Annexin V-FITC and propidium iodide double staining using the Apoptosis Detection Kit 1 from BD PharMingen (San Diego, CA) as directed by the manufacturer followed by flow cytometric analysis.

**Cell migration assay.** HeLa and CaSki cells transfected with control siRNA or *c-fms* siRNA ($2 \times 10^6$ per condition) as described above were plated in a six-well plate at confluence per well. The monolayer was wounded using a 10 μL pipette tip, and the cells were incubated for 48 h. Digital images (×400 magnification) were then taken for analysis using a Nikon digital camera mounted on an inverted microscope. The median area was measured using the internet-based software ImageJ.[5]

## Results

**CSF-1 and *c-fms* expression is elevated in cervical carcinomas.** Our previous studies have shown that CSF-1 serum levels are elevated in women with cervical cancer compared with women with normal cervix, suggesting that CSF-1 may play a role in disease progression (19). To investigate whether the expression of CSF-1 and its receptor *c-fms* is elevated in cervical tumors, we examined the expression of these factors in tumor ($n$ = 17) and normal cervix ($n$ = 8) samples by reverse transcription-PCR

[5] http://rsb.info.nih.gov/ij



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(RT-PCR). The data in Fig. 1*A* and *B* show a significant increase in *c-fms* and CSF-1 mRNA levels in cervical carcinoma samples compared with normal samples. Representative data in Fig. 1 show RNA expression by RT-PCR (Fig. 1*C*) and real-time RT-PCR (Fig. 1*D*) in cervical cancer samples compared with normal samples. Given the oncogenic potential of *c-fms*, we further examined its expression at the protein level by immunohistochemistry using antibody specific to *c-fms* (Fig. 2, Table 1). For this purpose, a total of 76 fields from 26 normal cervices and 84 fields from 28 cervical carcinomas was analyzed. Our immunohistochemical analysis showed that *c-fms* is mostly expressed in epithelial cells with low expression in the stromal compartment (Table 1). Importantly, tumor cells exhibited a significantly ($P < 0.001$) higher level of *c-fms* in tumor cells of cervical carcinomas compared with epithelial cells in normal cervical samples (Table 1). Representative tissue sections stained for *c-fms* expression are shown in Fig. 2.

The data suggest that increased *c-fms* levels in tumor cells would make them more responsive to its ligand CSF-1. Consequently, we also examined CSF-1 levels in the tumors using immunohistochemical analysis (Fig. 2). We observed increased CSF-1 expression in cervical tumors compared with normal tissue (Fig. 2). Furthermore, cervical carcinoma samples exhibited higher infiltration of CD68-positve macrophages, which is another source of CSF-1, compared with normal cervical samples, suggesting the availability of increased CSF-1 levels in the tumor microenvironment (Fig. 2; Table 1).

**Blocking CSF-1 signaling induces apoptosis as well as decreases motility and colony formation in cervical cancer cells.** The expression of CSF-1 and *c-fms* by tumor cells raises the possibility of a potential autocrine loop by CSF-1/*c-fms* affecting the growth of the tumor. To examine further whether CSF-1 and *c-fms* are expressed in cervical cancer cells, we tested the expression of these factors in CaSki, HeLa, and HT-3. As shown in Fig. 3*A*, *c-fms* is expressed in HT-3, HeLa, and CaSki cervical cancer cell lines. CSF-1 secretion was also detected by ELISA in these cell lines, with notable higher levels in cultures from HT-3 cells (Fig. 3*A*). The expression of this cytokine and its receptor suggests that CSF-1 may act via an autocrine mechanism in cervical cancer cells. Previous work has shown that CSF-1 can enhance survival and protect against apoptosis in HT-29 colon cancer cell line, and in our previous studies, we have shown that CSF-1 can act as an autocrine survival factor in ovarian and endometrial cells (23–25).

Based on our observations that CaSki, HT-3, and HeLa expressed both CSF-1 and *c-fms*, we examined whether blocking *c-fms* activity using a neutralizing antibody against *c-fms* would affect the growth of these cells. This antibody was used previously to prevent *c-fms* activity and block CSF-1–dependent colony growth of monocytes/macrophages (26). The results in Fig. 3*B* show a drastic increase in the cell death of the antibody-treated cells compared with untreated control. To confirm apoptosis, cells having cytoplasmic and nuclear shrinkage and chromatin condensation were identified morphologically by microscopy (data not shown). Previous work has shown that the CSF-1/*c-fms* signaling pathway is involved in motility of breast cancer cells (27). We examined whether blocking *c-fms* expression by specific siRNA would decrease motility of HeLa and CaSki cells using the scratch test (Fig. 3*C*). Our data show that transfection with *c-fms* siRNA resulted in a 50% reduction in cellular migration compared with cells transfected with control siRNA (Fig. 3*C*).

To determine the effects of autocrine CSF-1 on cervical cancer growth, we did colony formation assay in the presence or absence

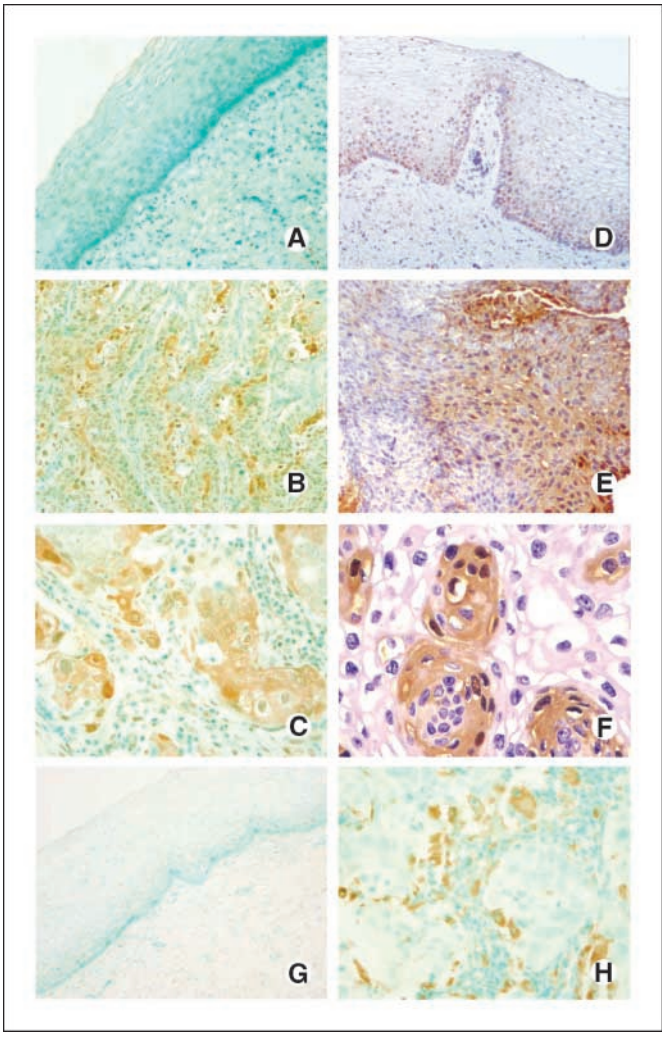


**Figure 2.** Immunostaining for *c-fms*, CSF-1, and CD68 in normal cervix and cervical carcinomas. *A,* normal tissue exhibiting negative cytoplasmic *c-fms* staining. *B,* cervical carcinoma showing intense *c-fms* expression in neoplastic cells. *C,* detail of the cervical carcinoma *c-fms* staining at ×400 magnification. *D,* normal cervical tissue showing negative staining for CSF-1. *E,* strong positive expression of CSF-1 in representative cervical carcinoma. *F,* cervical carcinoma cells exhibiting positive CSF-1 cytoplasmic expression at ×400 magnification. *G,* normal cervix showing the absence of CD68-positive cells. *H,* intense CD68 staining indicates the presence of infiltrating macrophages in the stroma surrounding the neoplastic cells and few in the tumor.

of neutralizing *c-fms* antibody with HeLa, CaSki, and HT-3 cells. The data in Fig. 3*D* show clear reduction in colony formation in the antibody-treated wells, suggesting that CSF-1 signaling is required for growth of cervical cancer cells and that an autocrine mechanism is relevant to the growth of these cells.

**TGF-β1 induces expression of *c-fms* in cervical cancer cells.** As of yet, it is not known how the expression of CSF-1 or *c-fms* is induced in gynecologic cancers. Two early studies have shown induction of *c-fms* by TGF-β1 in a murine myeloid progenitor cell line and in vascular smooth muscle cells (17, 18). Previous reports have also shown that TGF-β1 can induce expression of genes known to be responsive to TGF-β in HT-3, CaSki, and HeLa cells, suggesting that TGF-β1 signaling is intact in these cells (28). Combined, these studies raise the possibility that TGF-β1 may induce the expression of *c-fms* in cervical cancer cells. To examine this, we treated HT-3, HeLa, and CaSki with TGF-β1 and examined the expression of *c-fms*. Figure 4*A* shows about a 4-fold and 2.5-fold

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**Table 1.** Expression of *c-fms, CD68, TGF-β1*, and *TBRII* in normal cervix and cervical carcinoma

| | Epithelium | | | Stroma | | |
|---|---|---|---|---|---|---|
| | Normal cervix | Cervical carcinoma | *P** | Normal cervix | Cervical carcinoma | *P** |
| *c-fms* | 10.79 ± 22.74 | 74.17 ± 59.68 | <0.001 | 3.14 ± 11.69 | 10.48 ± 11.22 | <0.001 |
| CD68 | 2.15 ± 3.88 | 12.85 ± 13.24 | <0.001 | 9.02 ± 7.40 | 25.73 ± 37.76 | <0.001 |
| TGF-β1 † | 77.63 ± 89.79 | 55.89 ± 79.39 | 0.066 | 150 ± 101.40 | 95.18 ± 93.09 | <0.001 |
| TGF-β1 ‡ | 77.63 ± 89.79 | 49.45 ± 63.61 | 0.021 | 150 ± 101.40 | 77.60 ± 78.79 | <0.001 |
| TBRII | 74.18 ± 54.51 | 178.69 ± 84.67 | <0.001 | 145.59 ± 63.21 | 116.07 ± 77.46 | 0.003 |

*Mann-Whitney *U* test.
†Including all cases.
‡Excluding cases of moderate/strong extravascular blood.

increase in the expression of *c-fms* mRNA due to TGF-β1 treatment in HT-3 and HeLa cells, respectively. As *c-fms* is a glucocorticoid-responsive gene, we examined the effects of the synthetic glucocorticoid dexamethasone on *c-fms* expression in these cells as a positive control. The data show a 3-fold and a 6-fold increase in the expression of *c-fms* mRNA due to dexamethasone treatment in HeLa and HT-3 cells, respectively (Fig. 4*A*). To examine the kinetics of TGF-β1 induction of *c-fms* mRNA, we examined *c-fms*

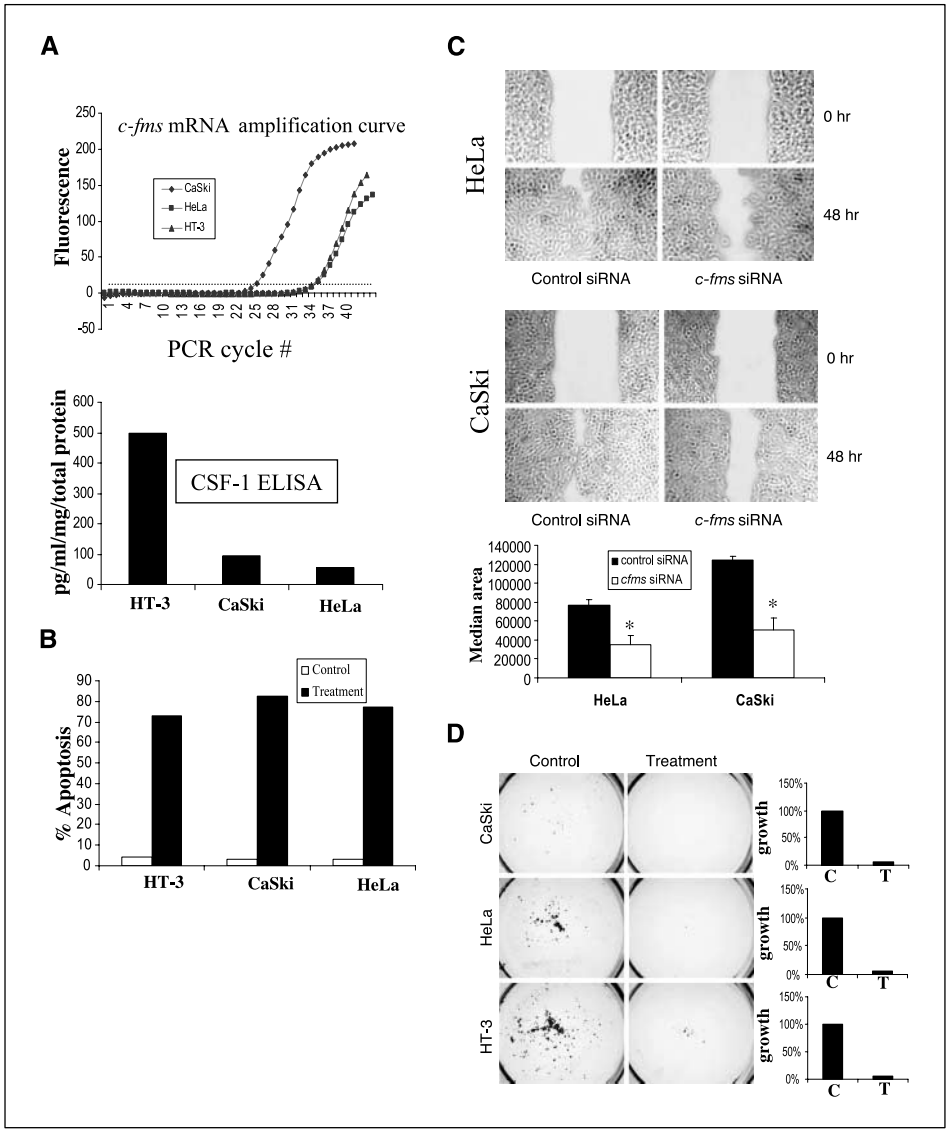


**Figure 3.** Expression of CSF-1 and *c-fms* in cervical cancer cells acts in autocrine fashion to enhance survival and motility. *A,* expression of *c-fms* and CSF-1 in cervical cancer cells was detected by real-time RT-PCR and ELISA, respectively. *Dotted line,* cycle threshold ($C_t$). *B,* effects of blocking CSF-1 signaling on apoptosis were determined by using anti–*c-fms* neutralizing antibody. Flow cytometry was done on Annexin V/propidium iodide double-stained cells as described in Materials and Methods. Apoptotic index. *C,* effects of blocking *c-fms* expression on cell migration were examined using the scratch test. CaSki and HeLa cells transfected with control siRNA or *c-fms* siRNA ($2 \times 10^6$ per condition) were plated in a six-well plate, the monolayer was wounded using a 10 μL pipette tip, and the cells were incubated for 48 h. Representative images of cell migration at 0 and 48 h. Magnification, ×400. The median area was measured using the internet-based software ImageJ (http://rsb.info.gov/ij) and plotted as bar graph. Statistical significance using Student's *t* test ($P < 0.001$). *Bars,* SD. *D,* colony formation assay was done for CaSki, HeLa, and HT-3 cells as described in Materials and Methods. Colonies were visualized by staining cells with crystal violet and digital photography using a Nikon SMZ 1500 stereomicroscope.

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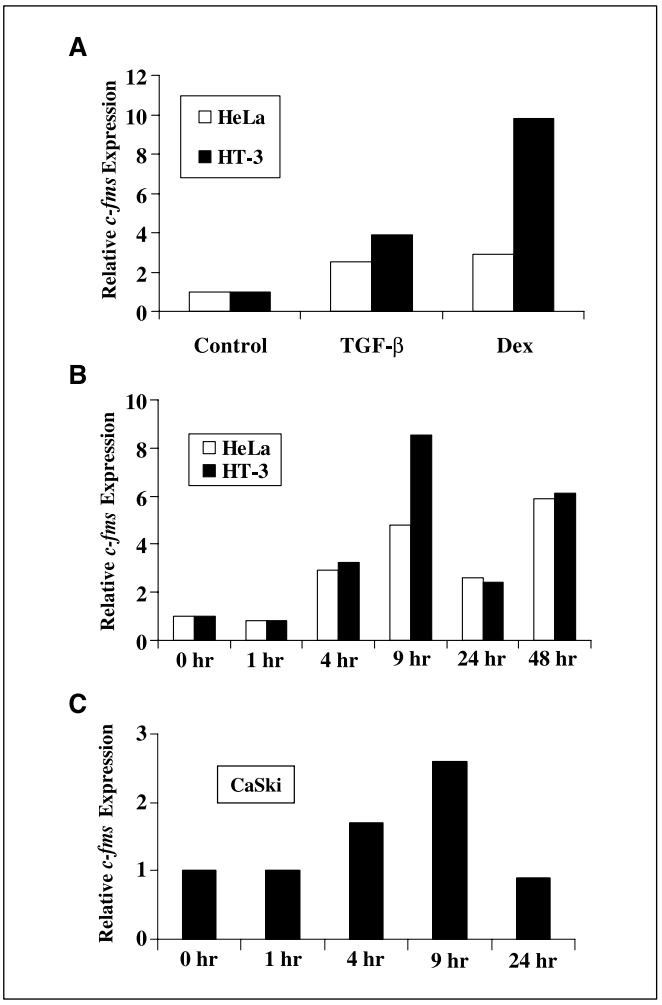


**Figure 4.** TGF-β1 induces the expression of *c-fms*. *A,* expression of *c-fms* is elevated in HeLa and HT-3 cells due to 24-h treatment with TGF-β1 (10 ng/mL) and dexamethasone (*Dex*; 1 μmol/L) as determined by real-time RT-PCR. Data are representative of three independent experiments. *B* and *C,* time course of *c-fms* mRNA expression following TGF-β1 treatment. After 48 h of incubation in phenol red–free RPMI 1640 containing heat-inactivated charcoal-stripped FBS (10%), cells were treated with TGF-β1 (10 ng/mL) at indicated times. RNA was isolated and followed by real-time RT-PCR as described in Materials and Methods.

expression at different time points after TGF-β1 stimulation in HeLa and HT-3 cells. The data show continuous increase in *c-fms* mRNA relative to untreated control, starting at 4 h (about a 3-fold increase) and increasing for 9 to 10 h after treatment to 5- and 8-fold induction in HeLa and HT-3, respectively (Fig. 4*B*). Interestingly, *c-fms* mRNA levels stimulated by TGF-β1 decrease 24 h after treatment but are maintained at about a 2-fold increase over untreated control. At 48 h, there is a distinct increase to ~6-fold over untreated control (Fig. 4*B*). In Fig. 4*C*, TGF-β1 also stimulated *c-fms* expression in CaSki cells (~2.5-fold at 9 h of treatment) but to a lower extent than that observed for HeLa and HT-3 cells (about 5- and 8-fold, respectively, at 9 h of treatment).

To confirm that TGF-β1 induction of *c-fms* in cervical cancer cells is mediated via the TGF-β receptors, we blocked the expression of TBRII using specific siRNA in the presence or absence of TGF-β1 treatment (Fig. 5). The data indicate that induction of *c-fms* by TGF-β1 is diminished when TBRII is down-regulated by siRNA in HeLa cells (Fig. 5*A*). Similarly, transfection of CaSki cells with TBRII siRNA resulted in diminishing the induction of *c-fms* by TGF-β1 (Fig. 5*B*). To further investigate the role of TGF-β1 pathway in the regulation of *c-fms*, we treated cervical cancer cells under TGF-β1 stimulation with SB203580 and SB431542, which are inhibitors of the MAPK p38 and the TGF-β receptor TBRI, respectively. Treatment with these inhibitors at concentrations shown previously (29, 30) to inhibit TGF-β1 action resulted in attenuating *c-fms* induction by TGF-β1 in HT-3 and CaSki cells (Fig. 5*C*). Similar results were obtained with HeLa cells (data not shown). Combined, the results implicate the TGF-β1 pathway in the regulation of *c-fms* expression and suggest that p38 may mediate this activity.

**TGF-β1 levels are significantly higher in normal cervical stroma than cervical tumor stroma, whereas TBRII levels are significantly higher in cervical tumor than in normal epithelium.** The finding that TGF-β1 may be involved in the regulation of *c-fms* prompted us to examine the levels of TGF-β1 and its receptor TBRII in cervical carcinomas. Few studies have examined the levels of TGF-β1 in cervical cancer, yielding conflicting results (31, 32). To examine the role of TGF-β1 in cervical carcinomas in more detail, we determined the levels of TGF-β1 in cervical carcinomas by immunohistochemical analysis. The expression of TGF-β1 seems to be higher in the stroma compared with the epithelial compartment in both cancer and normal cases (Supplementary Figure S1; Table 1). More importantly, TGF-β1 levels were significantly higher in normal cervical stroma compared with stroma in carcinoma samples (Table 1). However, TGF-β1 levels were highly influenced by the presence of extravascular blood, resulting from tumor bleeding, a common occurrence in cervical carcinomas. TGF-β1 expression for carcinoma stroma with moderate/intense extravascular blood was thrice ($P < 0.001$) higher than carcinoma stroma without or with little extravascular blood (Supplementary Table S1; Supplementary Fig. S1). The production of TGF-β1 mainly in the stromal compartment in addition to the supply from intratumoral bleeding suggests that it may act on tumor cells in a paracrine fashion. As an initial step to examine whether TGF-β1 signaling may be active in cervical carcinoma, we did immunohistochemistry to detect TBRII in cervical carcinomas (Supplementary Fig. S1). Our data show that TBRII is expressed in tumor cells and that its levels are significantly higher ($P < 0.001$) in tumor epithelia compared with normal epithelium (Supplementary Fig. S1; Table 1). On the other hand, no difference was detected in the levels of TBRI between carcinoma and normal tissue samples (data not shown).

## Discussion

The increased expression in CSF-1 and its receptor *c-fms* has been observed in several gynecologic cancers, including endometrial and ovarian cancers as well as breast cancer (5, 33). The concomitant expression of both growth factor and receptor by the tumor has been associated with more aggressive and invasive disease, suggesting an autocrine mode of regulation (6, 8). Little information on the involvement of *c-fms* and its ligand CSF-1 in cervical cancer is available. One study found no difference in the expression of *c-fms* in cervical precancerous lesions and normal tissue; however, *c-fms* levels were not examined in cervical carcinomas (21). Several studies, one by our group, showed an increase in serum and peritoneal CSF-1 levels in cervical cancer patients compared with control (3, 19, 20). Combined, these studies raise an important question on whether cervical carcinomas

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express *c-fms* and CSF-1 and whether this expression is higher than in normal tissue. To address this question, we have examined the expression of these factors in cervical carcinomas compared with normal cervical tissue as control. The data show a significant increase in *c-fms* and CSF-1 expression in cervical carcinomas compared with normal cervix, suggesting a possible role for this growth factor signaling pathway in the etiology of cervical cancer, similar to other studies showing the induction of CSF-1 and *c-fms* in other gynecologic cancers, such as those of the uterus and the ovaries (6, 8).

Increased *c-fms* expression revealed by RT-PCR in cervical tumors suggested the possibility that tumor cells may have increased responsiveness to CSF-1 stimulation. Like the RT-PCR data, the immunostaining data clearly show that *c-fms* levels are increased in cervical tumors compared with normal tissue but also show that tumor cells, not necessarily infiltrating monocytes/macrophages or stromal cells, are the main source of this receptor in the carcinoma samples tested. CSF-1 expression by tumor cells and significant macrophage infiltration, another source of CSF-1, in the cervical tumor milieu suggest that autocrine in addition to paracrine CSF-1 production may lead to stimulation of tumor cells possessing increased levels of *c-fms* receptor. Such interactions involving the CSF-1/*c-fms* pathway are involved in disease progression as well as metastasis in other cancers, such as that of the breast (7, 34–36).

Furthermore, our data suggest that autocrine CSF-1 signaling may play a critical role in the survival and motility of cervical cancer cells as evidenced by apoptosis, colony formation, and cell migration assays *in vitro*. Another possibility that we did not test but is the subject of ongoing investigation is that CSF-1 signaling may also be involved in invasive or metastatic capacities of cervical cancer cells.

At this point, it is not clear how *c-fms* or CSF-1 is induced in cervical cancer. Two early studies have shown induction of *c-fms* by TGF-β1 in a murine myeloid progenitor cell line and in vascular smooth muscle cells (17, 18). In addition, a previous study has suggested that TGF-β1 augments CSF-1 activity in bone marrow (37). Our data show that TGF-β1 induces the mRNA expression of

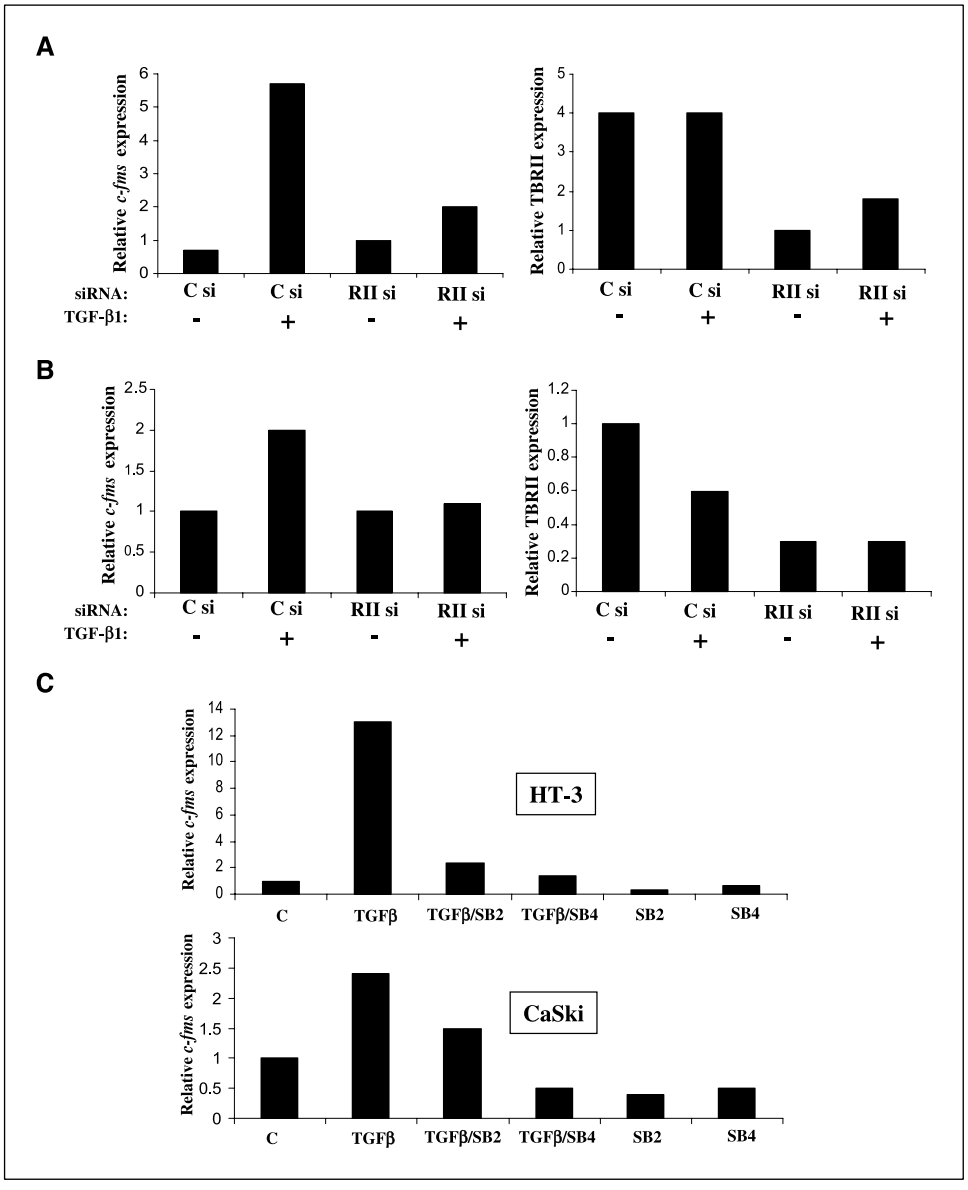


**Figure 5.** Blocking TGF-β pathway results in diminishing the induction of *c-fms* expression by TGF-β1. TBRII siRNA was transfected into HeLa (*A*) and CaSki (*B*) cells using nucleofection technology and DharmaFect kit, respectively, as described in Materials and Methods. Twenty-four hours after transfection, cells were washed with phenol red–free Hanks buffer and RPMI 1640 (with 10% heat-inactivated charcoal-stripped FBS) was added. After 48 h, TGF-β1 (10 ng/mL) was added to appropriate wells. Cells were harvested after 9 h of treatment, total RNA was isolated, and real-time RT-PCR was done with SYBR Green. *C,* in addition, cells were treated with inhibitors of the TGF-β1 pathway [SB203580 (*SB2*) and SB431542 (*SB4*)] in combination with TGF-β1 or alone in HT-3 and CaSki cells. After washing with phenol red–free Hanks buffer, cells were incubated in phenol red–free RPMI 1640 (with 10% heat-inactivated charcoal-stripped FBS) for 48 h. TGF-β1 (10 ng/mL) was added in the presence or absence of SB203580 (10 μmol/L) and SB431542 (5 μmol/L), and cells were incubated for 9 h. Cells were then harvested for RNA isolation. Real-time RT-PCR was done using SYBR Green. Data are representative of at least two independent experiments.

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*c-fms* in cervical cancer cells HeLa, CaSki, and HT-3 and that interruption of TBRII expression by specific siRNA resulted in abolishing this response. We have also shown that treatment with SB431542, which inhibits the activity of the TGF-β receptor TBRI, also attenuates *c-fms* induction by TGF-β1. The data thus implicate TGF-β1 signaling in the regulation of *c-fms*. Furthermore, the p38 inhibitor SB203580 also inhibited TGF-β1 activation of *c-fms*, suggesting that p38 may mediate the regulation of *c-fms*. An active TGF-β signaling pathway was confirmed in these cells by another study, in which the expression of TBRI and TBRII was detected, and treatment with TGF-β resulted in induction of TGF-β–responsive genes (28). Several studies have also shown that p38 is activated on TGF-β1 treatment and that p38 is required for the stimulation of certain gene expression by TGF-β1 (13). An intact TGF-β signaling pathway during tumor progression has been shown to lead to more aggressive disease and metastasis, which is consistent with the proposed role for TGF-β as a protumorigenic factor in cancer progression (14).

The data also showed that *c-fms* response to TGF-β1 in HT-3 (HPV negative) occurred at a somewhat higher extent than HeLa (HPV positive) and that CaSki (HPV positive) exhibited the least response. Whether this difference is due to HPV status or to the functionality of downstream factors of the TGF-β1 pathway is unclear at this time, and further experiment is required to understand the role of HPV in this pathway.

Contradicting studies and the lack of detailed analysis of TGF-β expression in cervical cancer, as well as our observations showing induction of *c-fms* expression by TGF-β1, prompted us to examine levels of TGF-β in cervical carcinomas (31, 32, 38). Data presented here show a decrease in TGF-β1 levels in cervical tumor cells and in associated stromal cells compared with normal tissue. Interestingly, microvascularization and associated extravascular blood (bleeding) seemed to contribute significantly to the levels of TGF-β1 available within the cervical tumor microenvironment and may compensate for the decrease of TGF-β1 in tumor epithelia, which may stimulate tumor cells via a paracrine mechanism.

Furthermore, very few studies have addressed whether TGF-β receptors are expressed in cervical cancer (39, 40). Our immunohistochemical analysis detected the presence of TBRII (as well as TBRI; data not shown) in all cervical carcinomas examined as well as a significant increase in the average expression of TBRII. This suggests that these tumor cells may be responsive to TGF-β1 produced in the stromal compartment, given that TGF-β receptors/signaling components are functional in these cells. Thus, it seems that cervical tumor cells do not necessarily lose the expression of TBRII or TBRI during carcinogenesis, an observation that warrants further studies to examine the functionality of TGF-β receptors in cervical carcinomas.

Although the sample number in this study is too limited to draw a correlation between CSF-1/*c-fms* signaling and cervical carcinoma progression, the data provide strong evidence for the involvement of *c-fms* and its ligand CSF-1 in cervical cancer to warrant the expansion of the study and increase sample number to examine the prognostic value of CSF-1 signaling and to elucidate the possible pathways that CSF-1 signaling may promote tumor growth in cervical cancer. The induction of *c-fms* expression in cervical tumor cells suggests their responsiveness to CSF-1 through potential autocrine or paracrine regulation, suggesting that blocking CSF-1/*c-fms* may be a viable therapeutic strategy in treating cervical cancer. The data also suggest that TGF-β1 may be a factor involved in induction of *c-fms* in cervical tumor cells.

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## References

1. American Cancer Society. Cancer facts and figures 2005. Atlanta (GA): American Cancer Society; 2005.
2. Davidson B, Goldberg I, Kopolovic J. Inflammatory response in cervical intraepithelial neoplasia and squamous cell carcinoma of the uterine cervix. Pathol Res Pract 1997;193:491–5.
3. Punnonen R, Teisala K, Kuoppala T, Bennett B, Punnonen J. Cytokine production profiles in the peritoneal fluids of patients with malignant or benign gynecologic tumors. Cancer 1998;83:788–96.
4. Rettenmier CW, Chen JH, Roussel MF, Sherr CJ. The product of the *c-fms* proto-oncogene: a glycoprotein with associated tyrosine kinase activity. Science 1985; 228:320–2.
5. Baiocchi G, Kavanagh JJ, Talpaz M, Wharton JT, Gutterman JU, Kurzrock R. Expression of the macrophage colony-stimulating factor and its receptor in gynecologic malignancies. Cancer 1991;67:990–6.
6. Kascinski B. Expression of CSF-1 and its receptor CSF-1R in non-hematopoietic neoplasms. Cancer Treat Res 2002;107:285–92.
7. Toy EP, Chambers JT, Kacinski BM, Flick MB, Chambers SK. The activated macrophage colony-stimulating factor (CSF-1) receptor as a predictor of poor outcome in advanced epithelial ovarian carcinoma. Gynecol Oncol 2001;80:194–200.
8. Smith HO, Anderson PS, Kuo DY, et al. The role of colony-stimulating factor 1 and its receptor in the etiopathogenesis of endometrial adenocarcinoma. Clin Cancer Res 1995;1:313–25.
9. Tang B, Vu M, Booker T, et al. TGF-β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. J Clin Invest 2003; 112:1116–24.
10. Chin BY, Petrache I, Choi AM, Choi ME. Transforming growth factor β1 rescues serum deprivation-induced apoptosis via the mitogen-activated protein kinase (MAPK) pathway in macrophages. J Biol Chem 1999;274:11362–8.
11. Arsura M, Panta GR, Bilyeu JD, et al. Transient activation of NF-κB through a TAK1/IKK kinase pathway by TGF-β1 inhibits AP-1/SMAD signaling and apoptosis: implications in liver tumor formation. Oncogene 2003;22:412–25.
12. Kloth JN, Fleuren GJ, Oosting J, et al. Substantial changes in gene expression of Wnt, MAPK, and TNFα pathways induced by TGF-β1 in cervical cancer cell lines. Carcinogenesis 2005;26:1493–502.
13. Hanafusa H, Ninomiya-Tsuji J, Masuyama N, et al. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-β-induced gene expression. J Biol Chem 1999;274:27161–7.
14. Serra R, Crowley MR. Mouse models of transforming growth factor β impact in breast development and cancer. Endocr Relat Cancer 2005;12:749–60.
15. Kang Y, He W, Tulley S, et al. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. Proc Natl Acad Sci U S A 2005;102:13909–14.
16. Kaminska B, Wesolowska A, Danilkiewicz M. TGF β signalling and its role in tumour pathogenesis. Acta Biochim Pol 2005;52:329–37.
17. Inaba T, Ishibashi S, Harada K, et al. Synergistic effects of transforming growth factor-β on the expression of *c-fms*, macrophage colony-stimulating factor receptor gene, in vascular smooth muscle cells. FEBS Lett 1996;399:207–10.
18. Chen AR, Rohrschneider LR. Mechanism of differential inhibition of factor-dependent cell proliferation by transforming growth factor-β1: selective uncoupling of FMS from MYC. Blood 1993;81:2539–46.
19. Adam RA, Horowitz IR, Tekmal RR. Serum levels of macrophage colony-stimulating factor-1 in cervical human papillomavirus infection and intraepithelial neoplasia. Am J Obstet Gynecol 1999;180:28–32.
20. Suzuki M, Ohwada M, Sato I, Nagatomo M. Serum level of macrophage colony-stimulating factor as a marker for gynecologic malignancies. Oncology 1995;52: 128–33.
21. Ngan HY, Liu SS, Yu H, Liu KL, Cheung AN. Proto-oncogenes and p53 protein expression in normal cervical stratified squamous epithelium and cervical intra-epithelial neoplasia. Eur J Cancer 1999;35:1546–50.
22. Detre S, Saclani Jotti G, Dowsett M. A "quickscore" method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. J Clin Pathol 1995;48:876–8.
23. Calatayud S, Warner TD, Breese EJ, Mitchell JA. Modulation by colony stimulating factors of human

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epithelial colon cancer cell apoptosis. Cytokine 2002;20:163–7.
**24.** Gill K, Kirma N, Gunna VS, Santanam N, Parthasarathy S, Tekmal RR. Regulation of colony stimulating factor-1 (CSF-1) in endometrial cells: glucocorticoids and oxidative stress regulate the expression of CSF-1 and its receptor *c-fms* in endometrial cells. Fertil Steril 2001;76:1005–11.
**25.** Keshava N, Gubba S, Tekmal RR. Overexpression of macrophage colony-stimulating factor (CSF-1) and its receptor, *c-fms*, in normal ovarian granulosa cells leads to cell proliferation and tumorigenesis. J Soc Gynecol Investig 1999;6:41–9.
**26.** Dewar AL, Cambareri AC, Zannettino AC, et al. Macrophage colony-stimulating factor receptor *c-fms* is a novel target of imatinib. Blood 2005;105:3127–32.
**27.** Wrobel CN, Debnath J, Lin E, Beausoleil S, Roussel MF, Brugge JS. Autocrine CSF-1R activation promotes Src-dependent disruption of mammary epithelial architecture. J Cell Biol 2004;165:263–73.
**28.** Kang SH, Won K, Chung HW, et al. Genetic integrity of transforming growth factor β (TGF-β) receptors in cervical carcinoma cell lines: loss of growth sensitivity but conserved transcriptional response to TGF-β. Int J Cancer 1998;77:620–5.
**29.** Park JI, Lee MG, Cho K, et al. Transforming growth factor-β1 activates interleukin-6 expression in prostate cancer cells through the synergistic collaboration of the Smad2, p38-NF-κB, JNK, and Ras signaling pathways. Oncogene 2003;22:4314–32.
**30.** Halder SK, Beauchamp RD, Datta PK. A specific inhibitor of TGF-β receptor kinase, SB-431542, as a potent antitumor agent for human cancers. Neoplasia 2005;7:509–21.
**31.** El-Sherif AM, Seth R, Tighe PJ, Jenkins D. Decreased synthesis and expression of TGF-β1, β2, and β3 in epithelium of HPV 16-positive cervical precancer: a study by microdissection, quantitative RT-PCR, and immunocytochemistry. J Pathol 2000;192:494–501.
**32.** Xu Q, Wang S, Xi L, et al. Effects of human papillomavirus type 16 E7 protein on the growth of cervical carcinoma cells and immuno-escape through the TGF-β1 signaling pathway. Gynecol Oncol 2006;101:132–9.
**33.** Kacinski BM, Scata KA, Carter D, et al. FMS (CSF-1 receptor) and CSF-1 transcripts and protein are expressed by human breast carcinomas *in vivo* and *in vitro*. Oncogene 1991;6:941–52.
**34.** Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 2001;193:727–40.
**35.** Heller DS, Hameed M, Cracchiolo B, et al. Presence and quantification of macrophages in squamous cell carcinoma of the cervix. Int J Gynecol Cancer 2003;13:67–70.
**36.** Goswami S, Sahai E, Wyckoff JB, et al. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer Res 2005;65:5278–83.
**37.** Rosenfeld CS. Transforming growth factor-β1 augments macrophage-colony stimulating factor activity on human marrow. Stem Cells 1994;12:527–32.
**38.** Farley J, Gray K, Nycum L, Prentice M, Birrer MJ, Jakowlew SB. Endocervical cancer is associated with an increase in the ligands and receptors for transforming growth factor-β and a contrasting decrease in p27(Kip1). Gynecol Oncol 2000;78:113–22.
**39.** Hazelbag S, Gorter A, Kenter GG, van den Broek L, Fleuren G. Transforming growth factor-β1 induces tumor stroma and reduces tumor infiltrate in cervical cancer. Hum Pathol 2002;33:1193–9.
**40.** Soufla G, Sifakis S, Baritaki S, Zafiropoulos A, Koumantakis E, Spandidos DA. VEGF, FGF2, TGFB1, and TGFBR1 mRNA expression levels correlate with the malignant transformation of the uterine cervix. Cancer Lett 2005;221:105–18.

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