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Inhibition of Transforming Growth Factor-β (TGF-β) Signaling by *Scutellaria baicalensis* and *Fritillaria cirrhosa* Extracts in Endometrial Cancer

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ABSTRACT

Transforming growth factor-β (TGF-β), regulates cell proliferation, angiogenesis, metastasis, and is an inducer of epithelial-mesenchymal transition (EMT). Cancer cells exhibit activated TGF-β/SMAD signaling pathway and its inhibition is an attractive strategy for cancer treatment. The Chinese Herbs *Scutellaria baicalensis* (SB) and *Fritillaria cirrhosa* (FC) have been shown to be beneficial to cancer patients, but the mechanisms by which the extracts of two herbs elicit the beneficial effects are unclear. In this study, we have used human endometrial cancer cells to assess the anticancer efficacy of SB and FC on TGF-β signaling pathway components. SB and FC treatment of cancer cells resulted in a significant decrease in expression of TGF-β isoforms, TGF-β receptors, and SMADs. Both herbs effectively inhibited basal and TGF-β1-induced cancer cell proliferation and invasion, which was accompanied with abrogation of Snail, Slug, matrix metalloproteinases (MMPs), αvβ3 integrin, focal adhesion kinase (FAK), and p-FAK expression. An inhibitor of TGF-βRI blocked TGF-β1-induced cell invasion and significantly diminished antitumor effects of SB and FC. These results suggest that SB and FC block endometrial cancer growth by downregulating TGF-β/SMAD signaling pathway. J. Cell. Biochem. 116: 1797–1805, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: proliferation; invasion; EMT; metastasis; natural products

Endometrial cancer (EC) is the most common gynecological malignancy in the United States [Siegel et al., 2014]. EC cases can be classified into two categories based on their clinical and pathological features. Type I ECs are low stage and low grade, and have a better prognosis [Bokhman, 1983]. In contrast, Type II EC cases are advanced stages, aggressive, and have a poor prognosis [Bokhman, 1983]. In the majority of women, endometrial cancer is diagnosed in the early-stages and is curable with primary surgery. Patients with more advanced and/or higher grade disease have a higher risk for recurrence and require multimodality therapy such as surgery, radiation, hormonal therapy, cytotoxic chemotherapy, and therapy with biological agents. Therefore, discovery of new drugs for endometrial cancer treatment is essential.

Chinese herbs have been successfully used in treating different kinds of diseases, including cancer [Wang et al., 2010; Youns et al., 2010]. The extracts of two herbs, *Scutellaria baicalensis* (SB, Chinese name, Huang Qin) and *Fritillaria cirrhosa* (FC, Chinese name, Chuan Bei Mu), have been shown to have antitumor activities in a number of cancers [Kumagai et al., 2007; Ye et al., 2007; Li-Weber, 2009]. Recently, we have studied the mechanism through which SB and FC extracts inhibit endometrial and ovarian cancer cell growth, and demonstrated that both agents caused a dose-dependent cell growth inhibition, which was associated with activation of caspase-3, G0/G1 phase cell cycle arrest, downregulation of cyclins D1 and D3, and induction of p27. Furthermore, our published study also demonstrated that SB and FC attenuated nuclear factor-κB (NFκB) DNA binding, decreased expression of phosphorylated IκBα, abolished activation of NFκB, and culminated in the suppression of NFκB–regulated metastasis–supporting genes in endometrial cancer cells [Kavandi et al, Epub ahead of print].

Many reports suggest that Chinese medicinal herbs efficiently suppress multiple cell signaling pathways to inhibit cell prolifer-

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ation, invasion, metastasis, and angiogenesis [Tan et al., 2011; Lee et al., 2013]. Functional cross-talk between NFκB and transforming growth factor-β (TGF-β) signaling in tumor cells has been demonstrated. TGF-β activation via TGF-β-activated kinase 1 (TAK1) contributes to aberrant NFκB activation in cancer cells [Freudlsperger et al., 2013]. TGF-β inhibits growth in early stage cancer cells and promotes progression and metastasis in advanced stages of cancers. In many cancer cells, profound deregulation of the TGF-β/SMAD signaling pathway leads to cancer growth, invasion, and poor patient prognosis [Massagué, 2012; Principe et al., 2014]. TGF-β1 overexpression in endometrial cancer cells has been reported to be associated with metastasis and poor patient outcome [Gold et al., 1994].

The TGF-β (three isoforms, β1, β2, and β3) is secreted as an inactive latent complex in which the mature growth factor remains associated with its propeptide. To elicit a biological response, TGF-β is released and active TGF-β binds to TGF-β receptors (TGF-βRI, TGF-βRII) and initiates signal transduction. Upon TGF-β stimulation, SMAD-2 and SMAD-3 undergo phosphorylation, triggering an interaction with SMAD-4. The SMAD complex translocates into the nucleus to regulate the target genes and directs cell transactivation. The actions of TGF-β are antagonized by SMAD-7, which interacts with TGF-βRI to prevent phosphorylation and activation of receptor-regulated SMAD 2/3, thereby blocking TGF-β signaling [Massagué 2012 Principe et al., Epub ahead of print].

TGF-β is a potent inducer of epithelial-mesenchymal transition (EMT). In this process, epithelial cells acquire mesenchymal phenotype, resulting in increased motility and invasion potential of cancer cells [Xu et al., 2009; Katsuno et al., 2013]. Several transcription factors, such as the Snail/Slug family and Twist, operate as molecular switches for the EMT program. Overexpression of Snail and Slug in tumor cells negatively regulate E-cadherin expression and are involved in tumor progression and metastasis [Batlle et al., 2000; Bolós et al., 2003].

Adhesion and subsequent invasion of tumor cells through the extracellular matrix (ECM) are regarded as crucial steps in the metastasis cascade. Integrins are considered to be an important modulator of this process since their ligands are components of the ECM. Integrins constitute a large family of cell surface transmembrane molecules that are composed of an α- and a β-subunit. Integrins play an important role in activating TGF-β [Sheppard, 2005; Wipff and Hinz, 2008]. The differential expression and activity of integrins modulate how normal and malignant cells sense and respond to TGF-β in acquiescent and rigid microenvironments. Integrins act as transmembrane scaffolds that connect the ECM to the actin cytoskeletal system; they also function as mediators of mechanotransduction and regulate cell proliferation, migration, invasion, and survival [Desgrosellier and Cheresh, 2010]. Upregulation of β3 integrin in response to TGF-β is critical for initiation of the EMT process [Parvani et al., 2013; Shirakihara et al., 2013].

The MMPs are known to have central roles in degradation of all ECM proteins and basement membrane proteins. Tumor cells, in the course of proliferation-invasion and metastasis, degrade them and escape from original tissues through blood and lymphatic routes, suggesting crucial roles of MMPs in different stages of tumorigenesis. MMPs are upregulated in tumor cells and can regulate their growth by three mechanisms: (1) releasing the cell membrane bound precursors of growth factors, (2) modulating the bioavailability of growth factors that are sequestrated by ECM proteins, or (3) indirectly regulating proliferative signals through integrins [Desgrosellier and Cheresh, 2010].

Focal adhesion kinase (FAK) cohabits with integrin clustering at the site of focal adhesions. FAK signaling plays a pivitol role in TGF-β-induced EMT. High expression of FAK protein is reported for many cancers [Cicchini et al., 2008]. FAK functions as a bridge between integrin signaling pathways and the growth-factor-receptors. The mechanism by which FAK promotes cell adhesion and motility is via conveying ECM signals from integrins to the intracellular compartment [Sieg et al., 2000].

The TGF-β signaling pathway, which regulates cell proliferation, epithelial to mesenchymal transition, invasion, and angiogenesis, is activated in endometrial cancer. Targeting this pathway provides an attractive strategy to treat highly metastatic tumors. Identifying natural products that suppress the TGF-β activated pathway may offer a novel and attractive approach for the treatment of endometrial tumors.

The objectives of this study were to identify TGF signaling proteins that are modulated by extracts of SB or FC in endometrial cancer cells and assess the influence of these herbs on TGF-β-induced pro-tumorigenic activities in endometrial cancer cell lines. We report here that SB and FC inhibit TGF-β induced pro-tumorigenic activities by inhibiting TGF-β isoforms, TGF-β receptors SMADs, EMT promoters, Snail and Slug and attenuating integrins, and MMPs and FAK expression.

MATERIALS AND METHODS

HERBS

Scutellaria baicalensis (Huang Qin) and *Fritillaria cirrhosa* (Chuan Bei Mu) were in the form of powdered concentrates made by hot water extraction and were supplied by Kaiser Pharmaceutical, Co., Ltd. (Tainan, Taiwan).

CULTURE CONDITIONS AND TREATMENT OF ENDOMETRIAL CELL LINES

Human endometrial cancer cell lines Ishikawa and HEC-1B were obtained from Sigma (St. Louis, MO) and the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's Modified Eagle Medium and Eagle's Minimum Essential Medium containing 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY) and 100 U/mL penicillin/streptomycin. The cells were cultured at 37 °C in 5% CO_2. When reached 70–80% confluency, cells were exposed to SB or FC (200 μg/mL), TGF-β1 (10 ng/mL) or their combination for 72 h. The time of treatment and dose of SB or FC was based on our previous studies showing inhibition of cell growth and apoptosis of cancer cells [Kavandi et al, Epub ahead of print]. In a set of experiments, TGF-βR1 blocker (SD-208, 10 μmol/L, Santa Cruz Biotechnology, Dallas, TX) was used to block the action of TGF-β1. Cells were treated with or without TGF-βR1 blocker (10 μmol/L) 2 h prior to treatment with SB, FC, TGF-β1 or their combinations. Cell cultures were treated daily for 72 h and the effects on cell viability and invasion were assessed.

MEASUREMENT OF TGF-β1 CONCENTRATION IN THE CONDITIONED MEDIA

To study the effects of SB and FC on the secretion of TGF-β in culture, endometrial cells were treated for 72 h with SB, FC, or TGF-β1. After culture, cells were removed by centrifugation, and the cell-free media were collected and stored at −80°C until use. After thawing at room temperature, the media were assayed for immunoreactive latent TGF-β by enzyme-linked immunosorbent assay (BioLegend, San Diego, CA) according to the manufacturer's specifications with absorbance at 450 nm in a plate reader (ELX800, Winooski, VT). Experiments were repeated at least three times with different media to minimize the intra- and interassay variation.

CELL VIABILITY ASSAY

Cell viability was evaluated after treatment of cells with SB, FC, and TGF-β1 alone or in combination in the presence or absence of TGF-βR1 blocker using the CellTiter 96 AQueous One Solution cell viability assay (Promega, Madison, WI) according to the instructions of the manufacturer. 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 microtiter plate reader. Relative cell viability was expressed as percentage change of control cells over treated cells.

INVASION ASSAY

The Biocoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA) were used to assess the effects of SB, FC, and TGF-β1 or their combination on the invasive property of endometrial cancer cells. Matrigel chambers were rehydrated at 37°C for 2 h. Treated and vehicle treated cells were detached by trypsin and resuspended in serum-free medium. Medium containing 10% fetal bovine serum medium was applied to the lower chambers of BD BioCoat™ Matrigel Invasion Chambers (BD Biosciences, Bedford, MA) as chemoattractant and then cells were seeded on the upper chambers at a density of 2.5×10^4 cells/well in 100 μl of serum-free medium without SB, FC, or TGF-β1. The chambers were incubated for16–18 h at 37°C. At 18 h after plating, noninvading cells were removed from the upper surface of the membrane by scrubbing. The cells on the lower surface of the membrane were fixed for 2 min in 100% methanol and stained with 1% toluidine blue in 1% sodium borate for 2 min. Cells that invaded through the insert were counted in five random fields per slide. All slides were coded to avoid biased counting. The assay was run in triplicate.

WESTERN BLOT ANALYSIS

The SB, FC, and vehicle treated cells were washed with PBS, and cell lysates were prepared in radioimmune precipitation assay (RIPA) buffer [50 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 0.5% deoxycholate, 1.0% NP40, and 0.1% SDS] supplemented with a protease inhibitor mixture solution (Roche Molecular Biochemicals, Mannheim, Germany). After sonication, cell debris was pelleted by centrifugation, and protein concentration was determined with the BCA Protein Assay Reagent (Pierce, Rockford, IL). Equivalent amounts of proteins were separated onto 10% polyacrylamide gels and then transferred onto methanol-activated PVDF membranes. The membranes were blocked with 5% milk in PBS with 0.05% Tween 20 for 1 h at room temperature, probed with antibodies against TGF-β1, TGF-β2, TGF-β3, TGF-βR1, TGF-βR2, TGF-βR3, pTGF-βR2, pSMAD2/3, SMAD2/3, SMAD-4, Snail, Slug, FAK (Santa Cruz Biotechnology, Dallas, TX), p-FAK, αvβ3, MMP-9 (Cell Signaling Technology, Inc. Danvers, MA), MMP-2, pTGF-βR1 (Abcam Inc. Cambridge, MA), and β-actin (Sigma–Aldrich, St. Louis, MO). The Enhanced Chemiluminescence Detection System (Pierce, Piscataway, NJ), followed by autoradiography, was used for protein visualization. Protein bands were quantified using densitometry software (Bio-Rad, Hercules, CA) and normalized using actin as a loading control. To calculate the relative intensity of each band, individual bands were divided by the corresponding loading control intensity.

STATISTICAL ANALYSIS

Data are presented as the mean of triplicate determinants with SEM. Experiments carried out in triplicate were repeated at least three times. Statistically significant differences were determined between control and treatment groups using two-way ANOVA followed by Tukey post hoc test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

SB AND FC INHIBIT EXPRESSION OF TGF-β ISOFORMS IN ENDOMETRIAL CANCER CELLS AND ATTENUATED TGF-β SECRETION IN THE CULTURE MEDIA

To examine whether SB and FC treatment affects the expression of TGF-β isoforms, endometrial cancer cells were treated with SB or FC for 72 h. Marked decrease in the expression of all the three isoforms was observed with both treatments. However, SB was more potent than FC in suppressing isoforms expression (Fig. 1A). The high levels of TGF-β in tumor tissue, which is primarily released from tumor cells, helps maintain their metastatic nature and exacerbates the creation of a pro-tumor microenvironment. To ascertain if SB and FC regulate secretion of TGF-β, the amount of TGF-β secreted into the culture media was assayed by ELISA. TGF-β secretion by endometrial cancer cells was markedly inhibited by SB and FC treatment compared with vehicle treated cells. Cells exposed to TGF-β1 for 72 h secreted high levels of latent TGF-β compared with vehicle treated cells. Furthermore, SB and FC attenuated TGF-β1 induced secretion of latent TGF-β (Fig. 1B).

SB OR FC ATTENUATE EXPRESSION OF PHOSPHORYLATED TGF-β RECEPTOR 1 AND 2

The first step of TGF signaling is the binding of the TGF-βR2 to TGF-βR1 and phosphorylation of TGF-βR1 [Massagué, 2012]. Expression of TGF-βR1, TGF-βR2, and TGF-βR3 protein was detected in both cell lines (Fig. 2). All the three receptors were expressed in cancer cell lines. Treatment of cells with SB or FC decreased non-phosphorylated TGF-β receptors (Fig. 2A). Interestingly, SB or FC markedly attenuated expression of phosphorylated TGF-βR1 and TGF-βR2 in cancer cells (Fig. 2B).

SB AND FC INHIBIT EXPRESSION OF SMADS

Phosphorylation of SMAD2/3 by the activated TGF-βRI is a key step in the initiation of TGF-β1 signal transduction and in collaboration

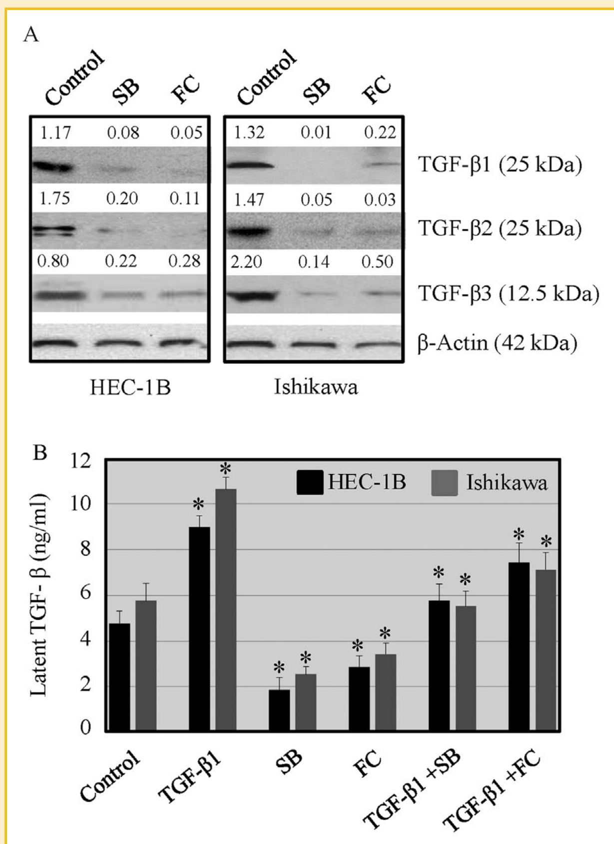

Fig. 1. (A) SB and FC downregulated TGF-β isoforms expression in endometrial cancer cells. The HEC-1B and Ishikawa cells treated with SB and FC (200 μg/mL) for 72 hs were evaluated by Western blot analysis for expression of TGF-β1, TGF-β2, and TGF-β3. β-Actin was used as a loading control. Data shown here are representative of three independent experiments. (B) Concentration of latent TGF-β present in the culture media of endometrial cancer cells treated for 72 h with SB, FC (200 μg/mL), TGF-β1(10 ng/mL), or the combination. The concentration of TGF-β present in the conditioned media of cells was measured by enzyme-linked immunosorbent assay (ELISA), as described in Materials and Methods. Measurement was made at least three times per sample and presented as mean ± SD. *$P < 0.05$ compared to control.

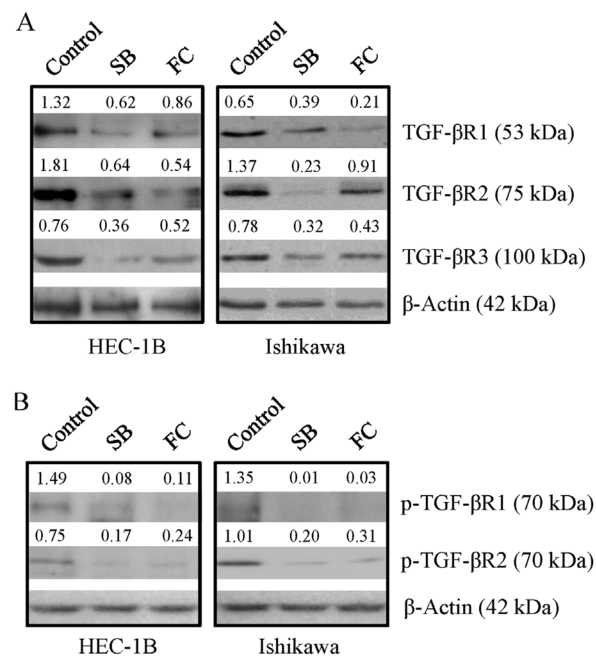

Fig. 2. TGF-β receptors were altered by SB or FC. Endometrial cancer cells (HEC-1B and Ishikawa) treated with SB or FC (200 μg/mL) for 72 h were evaluated by Western blot analysis for expression of non-phosphorylated TGF-βR1, TGF-βR2 and TGF-βR3 (A) and phosphorylated TGF-βR1 and TGF-βR2 (B). β-Actin was used as a loading control. Data shown here are representative of three independent experiments.

with SMAD-4, regulates transcription of TGF-β-induced genes. Untreated cancer cells showed appreciable levels of phosphorylated and total SMAD2/3 proteins compared with attenuated levels of phosphorylated and total SMAD2/3 in SB or FC exposed cells (Fig. 3A). In addition, both herbs significantly reduced SMAD-4 expression (Fig. 3B).

SB AND FC INHIBIT TGF-β1-INDUCED VIABILITY AND INVASION OF ENDOMETRIAL CANCER CELLS

We next examined the effect of SB and FC extracts on TGF-β1-induced cell viability and cell invasion in HEC-1B and Ishikawa cells using MTS and transwell invasion chamber assays. Our results showed that the viability and invasiveness of HEC-1B and Ishikawa cells were increased by TGF-β1 treatment (Fig. 4A and B) compared to vehicle treated cells. On the other hand, SB and FC inhibited basal and TGF-β1-stimulated cell viability and invasiveness (Fig. 4A and B). TGF-βR1 blocker markedly reduced the cell viability and anti-invasive effects of SB and FC (Fig. 4A and B). These results demonstrate that the cytotoxic and anti-invasive effects of the extracts require activation of TGF-βR1.

SB AND FC INHIBIT THE EXPRESSION OF EMT MARKERS IN ENDOMETRIAL CANCER CELLS

Tumor progression is associated with increased expression of EMT-induced transcription factors Snail and Slug. We examined the ability of SB and FC extracts to suppress expression of Snail and Slug using Western blotting. As shown in Figure 5, endometrial cancer cells express appreciable levels of Snail and Slug and their expression significantly increased in the TGF-β treated cells compared with the control cells. SB and FC treatment abrogated the expression of basal and TGF-induced Snail and Slug in both cell lines. These results suggest that SB and FC can down-regulate EMT marker expression, resulting in the reversal of EMT in cancer cells.

SB AND FC ATTENUATE αvβ3 INTEGRINS IN CANCER CELLS

A relationship between metastatic potential of endometrial tumor and changes in the integrin expression has been documented. Increased expression of αvβ3 integrins in endometrial tumors is associated with tumor progression [Lessey et al., 1995]. We found that incubation of endometrial cancer cells with SB and FC extracts reduced both basal and TGF-β-induced αvβ3 expression (Fig. 6). These data suggest that SB and FC reduce the metastasis of

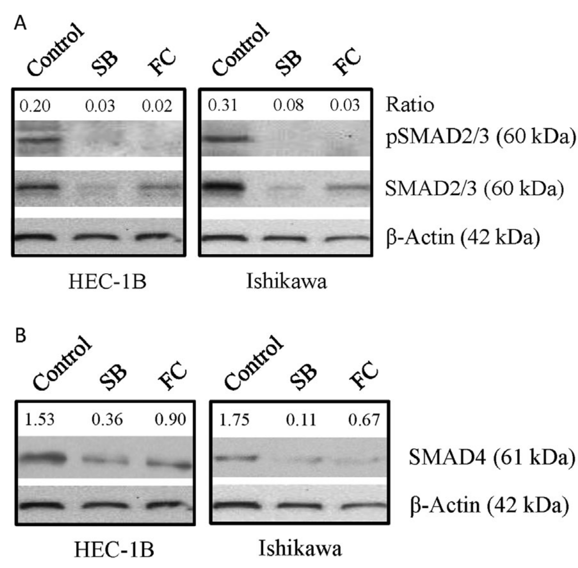

Fig. 3. Downregulation of SMAD expression in endometrial cancer cells by SB and FC. Endometrial cancer cells (HEC-1B and Ishikawa) treated with SB and FC (200 μg/mL) for 72 h were evaluated by Western blot analysis for expression of p-SMAD2/3, SMAD2/3 (A), and SMAD-4 (B). β-Actin was used as a loading control. Data shown here are representative of three independent experiments.

endometrial cancer cells by inhibiting the expression of αvβ3 integrins.

SB AND FC INHIBIT THE EXPRESSION OF TGF-β1-INDUCED MMP-2 AND MMP-9

The MMPs over-expression is associated with tumor invasion and metastasis. Western blotting was performed to investigate the effects of SB and FC on the regulation of MMP-2 and MMP-9 expression in endometrial cancer cells. Compared with the control group, the expression of MMP-2 and MMP-9 was upregulated by TGF-β1, but was inhibited with SB and FC treatment (Fig. 6).

FAK PHOSPHORYLATION IS DECREASED BY SB AND FC IN CANCER CELLS

FAK is an important regulator of cell adhesion and invasion [Cox et al., 2006]. Having shown that SB and FC inhibited endometrial cancer cell invasion, we sought to determine whether FAK activation was altered in these cells. The activation of FAK requires tyrosine phosphorylation at 397 (Y397). Therefore, we examined FAK activation in HEC-1B and Ishikawa cells by Western blotting using phospho-Y397-FAK-specific antibody. As shown in Figure 6, TGF-β1-treated cells exhibited an increased level of FAK phosphorylation compared with vehicle treated cells. SB and FC inhibited basal and TGF-β1 induced FAK phosphorylation.

DISCUSSION

Our recently published study showed that cell viability of immortalized endometrial epithelial and ovarian surface epithelial cells was not affected with the dose (200 μg/mL) of SB and FC used in this study. However, the high dose (500 μg/mL) caused 5–10% decrease in cell viability. Both extracts exhibited 50–60% and 70–90% decrease in cancer cell viability with 200 and 500 μg of herbs, respectively. The SB and FC-induced decrease in cell viability was associated with apoptosis as revealed by activation of caspase 3, cell cycle arrest at G0/G1 phase, attenuation of cyclins D1 and D3, and induction of p27. Furthermore, we also showed that SB and FC inhibited the NFκB pathway, which is activated in tumors [Kavandi et al, Epub ahead of print].

Previous studies have demonstrated cross talk between NFκB and TGF-β signaling pathways [Freudlsperger et al., 2013]. Whether SB and FC affect TGF-β signaling pathway is unknown. The primary objective of this study was to define the molecular basis of SB and FC induced decrease in cell viability by examining changes in TGF-β signaling pathway gene expression in endometrial cancer cells.

TGF-β is a multifunctional secreted polypeptide that signals via receptor serine/threonine kinases and intracellular SMAD effectors. It is expressed at a higher level in tumors and tumor cell lines compared to normal tissues. Several lines of evidence demonstrated that stimulation of TGF-β could induce metastasis and invasion in diversified tumors [Massagué, 2012; Principe et al., Epub ahead of print]. Upon activation of the TGF-β pathway, carcinoma cells exhibit overt and irreversible EMT that lead to more aggressive and metastatic tumors [Hanahan and Weinberg, 2011]. Therefore, inhibition of TGF-β-mediated EMT might be a rational strategy to prevent metastasis. The present study provides compelling evidence that extracts of SB and FC exert modulatory effects on TGF-β signaling and its downstream events, such as cell proliferation and invasion that are the hallmarks of tumorigenesis.

Ligand binding to TGF-β receptors initiates SMAD2/3/4 complex formation. In normal cells, SMADs are primarily concentrated in the cytoplasm. Upon binding of TGF-β to TGF-βR2 and phosphorylation of TGF-βR1, the activated receptor complex phosphorylates SMAD 2/3, which forms a complex with SMAD-4. This complex translocates into the nucleus where it interacts in a cell-specific manner with various transcription factors to regulate the transcription of many genes (Massagué, 2012, Principe et al., Epub ahead of print). In this study, we demonstrated that SB and FC extracts illustrate their anti-proliferative/anti-invasive effects on endometrial cancer cells via inhibition of TGF-β ligands expression in the cells as well as by inhibiting the secretion of TGF-β in culture media. SB and FC treatment decreased expression of phosphorylated TGF-βR1 and TGF-βR2, total and phoshporylated SMAD2/3, and SMAD-4 in cancer cells. These findings suggest that SB and FC suppress TGF-β signaling pathway activation via suppression of TGF-β signaling components.

Results presented herein showed a significant decrease in basal and TGF-β1-induced viability and invasive potential of endometrial cells exposed to SB and FC. Furthermore, the TGF-βR1 blocker almost abolished the inhibitory effects of SB and FC on endometrial cancer cells. These findings strongly suggest that both herbs exert their inhibitory effects on cancer cells primarily via the TGF-β/SMAD signaling pathway. A small but significant decrease in cell viability and invasion seen with SB and FC extracts in TGF-βR1 blocker-treated cells, indicate that beside the TGF-β/SMAD pathway, SB and FC use additional pathways to inhibit malignant

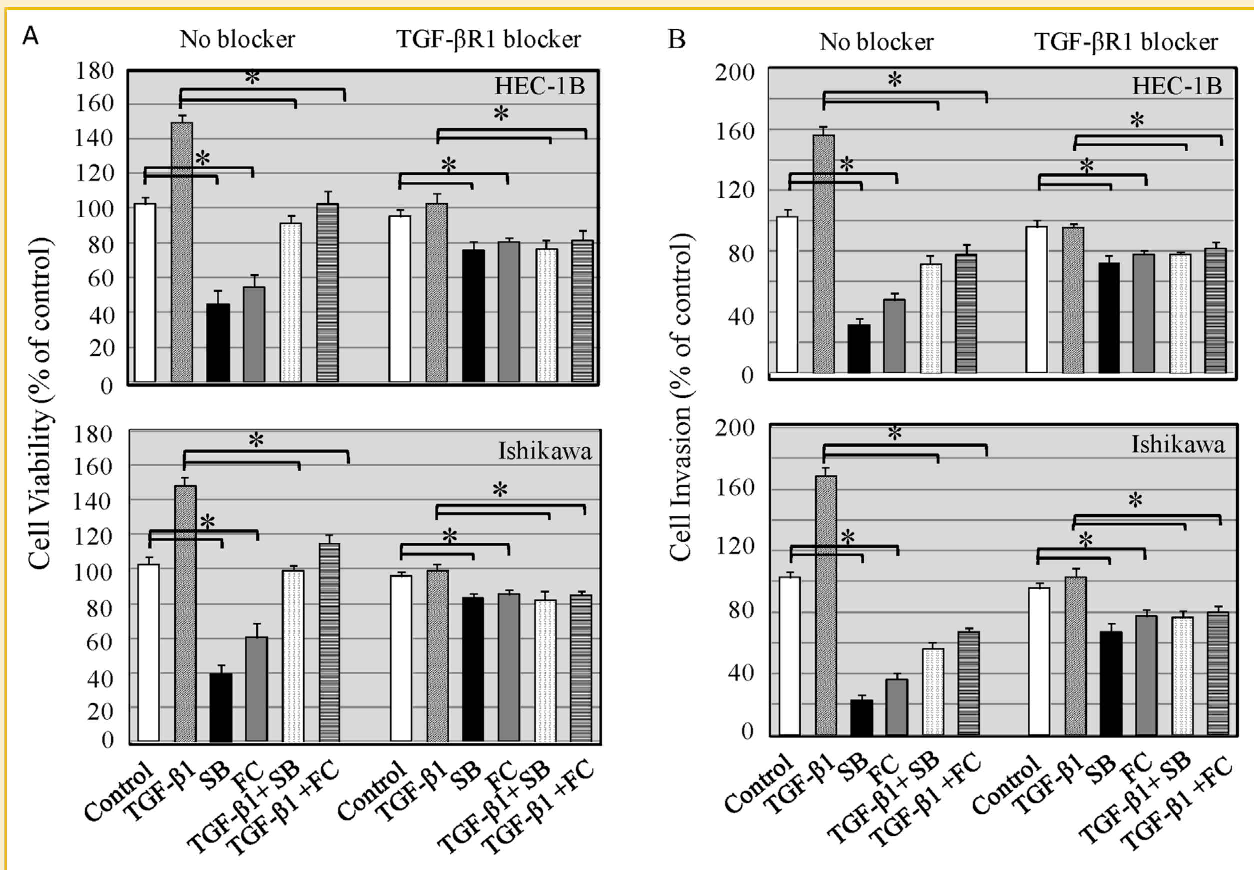

Fig. 4. SB and FC inhibit basal and TGF-β1-induced cell viability and invasion of endometrial cancer cells. HEC-1B and Ishikawa cells were cultured with SB and FC, with or without TGF-β1 (10 ng/mL) for 72 h in the presence or absence of TGF-βR1 (SD 208, 10 μmol/L) blocker, and the effect of treatment on cell viability (A) and invasion (B) was assessed using MTS and cell invasion assays. Data shown are mean ± SEM of values from triplicates. $^*P < 0.05$ (statistically significant) between respective control and the treatment groups. The experiment shown is representative of at least three experiments, all with similar results.

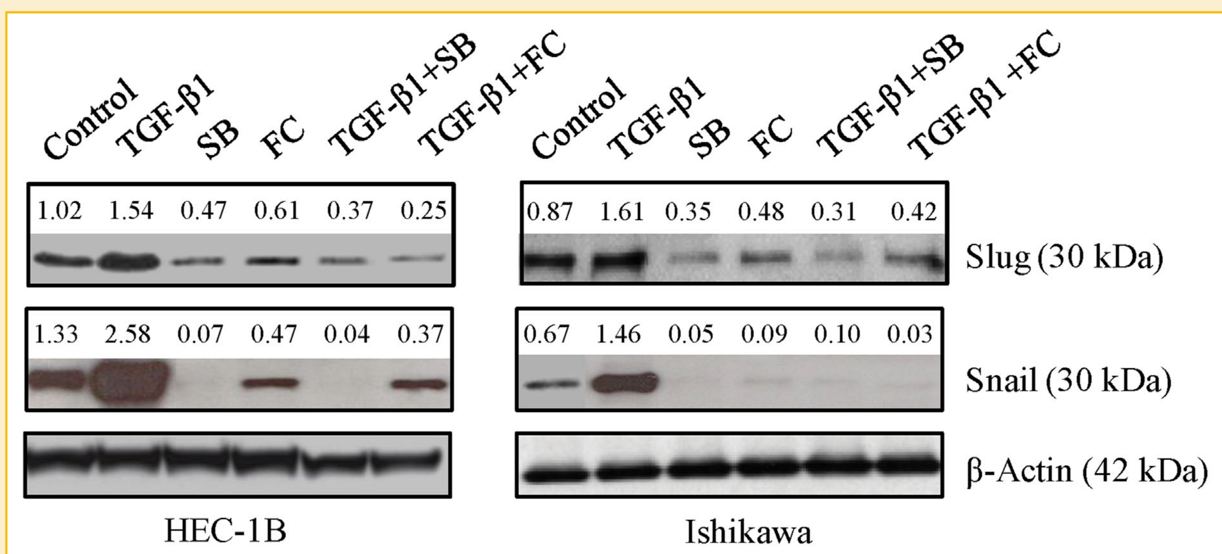

Fig. 5. The EMT-related transcription factors are suppressed by SB and FC. Endometrial cancer cells (HEC-1B and Ishikawa) were cultured with SB and FC, with or without TGF-β1 (10 ng/mL) for 72 h. At the end of incubation period, the expression of Slug and Snail was assessed by Western blot analysis. β-Actin was used as a loading control. Data shown here are representative of three independent experiments.

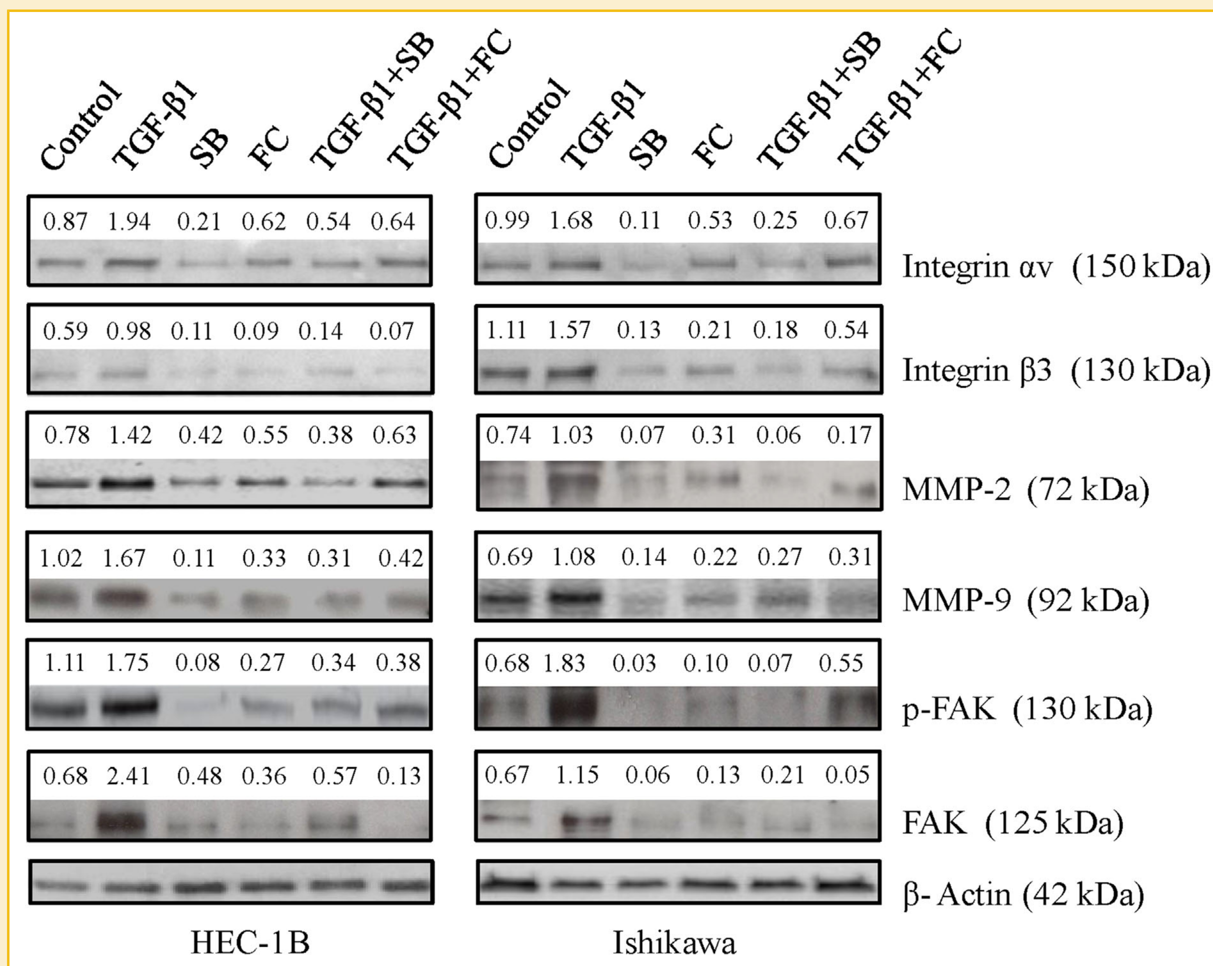

Fig. 6. SB and FC suppressed integrins, MMPs, and FAK in endometrial cancer cells. Endometrial cancer cells (HEC-1B and Ishikawa) were cultured with SB and FC, with or without TGF-β1 (10 ng/mL) for 72 h. At the end of incubation period, the expression αvβ3 integrins, MMP2, MMP9, p-FAK, and FAK was assessed by Western blot analysis. β-Actin was used as a loading control. Data shown here are representative of three independent experiments.

phenotypes. We have previously shown that SB and FC decreased cell viability and invasion by inhibiting the NFκB signaling pathway, which is activated in a number of cancers, including endometrial cancer [Kavandi et al, Epub ahead of print]. These results are consistent with studies indicating inhibition of TGF-β1-induced metastatic potential of colon, breast, and lung cancer cells [Mo et al., 2012; Wang et al., 2013, 2014].

TGF-β1 enhances expression of zinc-finger transcriptional factors Snail and Slug. These transcriptional factors play a central role in EMT. They bind to the promoter Ebox, which represses E-cadherin transcription [Thiery et al., 2009]. Our results demonstrated inhibition of both transcription factors by SB and FC. These findings suggest that SB and FC may inhibit endometrial cancer cell invasion and metastasis by suppressing TGF-β1-induced EMT. These results are in agreement with other studies demonstrating inhibition of TGF-β1-induced EMT with resveratrol, genestin, and berberine in liver and lung cancers [Wang et al., 2013, 2014; Qi et al., 2014].

Although EMT is an organized process involving interaction between different cells and tissue types, aspects of the EMT program can be inappropriately activated in response to microenvironmental alterations and aberrant stimuli and can contribute to cancer progression. EMT could be activated by MMPs [Vandenbroucke et al., 2011]. MMPs play a central role in the degradation of the ECM. The process begins by cleavage of ECM components and unmasking of cryptic sites, resulting in fragments with new biological activities modulating migration, growth, or angiogenesis. The upregulation of MMPs offers clues for tumor metastasis, tumor-induced angiogenesis, tumor invasion and establishment of metastatic foci at the secondary site [Orlichenko and Radisky, 2008; Chetty et al., 2011]. Expression analysis of MMP in endometrial cancer cells demonstrated higher expression of MMP-9 and MMP-2 in cancer cells compared to immortalized endometrial epithelial cells. SB and FC treatment significantly down-regulated basal and TGF-β1-induced MMPs in endometrial cancer cells. These results suggest that SB and FC can prevent extracellular matrix degradation and consequently inhibit tumor metastasis.

The acquisition of metastatic phenotypes in cancers correlates with elevated levels of TGF-β signaling and include essential inputs

derived from integrins [Mamuya and Duncan, 2012]. Specific integrin heterodimers are expressed in different cancer cells, and the growth and migration of the cells depend on the interactions between the integrins and their extracellular ligands. Integrins link the extracellular matrix to intracellular signaling molecules and regulate a number of cellular processes, including adhesion, signaling, motility, survival, gene expression, growth, and differentiation [Deb et al., 2012; Park et al., 2012]. Endometrial cancer cells express high levels of $\alpha v\beta 3$ compared to normal endometrium [Castelbaum et al., 1997] and $\alpha v\beta 3$ has been shown to be involved in tumor metastasis. Here, we showed reduced expression of $\alpha v\beta 3$ integrins in endometrial cancer cells exposed to SB and FC. Inhibition of $\alpha v\beta 3$ integrin by disintegrin or $\alpha v\beta 3$ integrin antibody has been shown to reduce the metastasis of human cancer cells [Boukerche et al., 2008; Liang et al., 2013]. Hence, reducing the expression of $\alpha v\beta 3$ integrins by SB ad FC is a viable treatment option for cancer metastasis. FAK activated by integrins is an important regulator of cell migration and is required for the invasion and metastasis of cancer cells [Mitra et al., 2005; Frame et al., 2010]. FAK is found overexpressed in invadiopodia of tumor cells and FAK silencing inhibits the metastasis of cells [Li and Hua, 2008]. Our results showed a marked inhibition of phospho Y379 FAK in endometrial cancer cells exposed to SB and FC, implicating that these two Chinese herbs may impair invadopodia formation, rendering them less effective for invasion.

In summary, our study suggests that SB and FC inhibit endometrial cancer cell proliferation, invasion, and metastasis by inhibiting TGF-β/SMAD3-mediated EMT and by downregulating TGF-β-activated integrins and FAK expression. Therefore, these herbs, that have a broad spectrum of anticancer activities, could be new promising agents for the treatment of cancer.

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