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Indole-3-carbinol Inhibits Sp1-Induced Matrix Metalloproteinase-2 Expression To Attenuate Migration and Invasion of Breast Cancer Cells

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Indole-3-carbinol (I3C), a major indole metabolite in cruciferous vegetables, has been shown to inhibit invasion of breast cancer cells. This study addressed the effect of I3C on the expression of matrix metalloproteinases (MMPs) and clarified the underlying mechanism. Migration, invasion, and MMP-2 activity of MCF-7 breast cancer cells were dose-dependently inhibited by I3C. In addition, the MMP-2 mRNA level was also reduced by I3C. Promoter deletion and mutation analysis suggested that I3C inhibited MMP-2 gene transcription via the −85/−7 bp promoter region and the Sp1 transcription factor binding site located within the −72/−64 bp promoter region was important for the inhibition. Chromatin immunoprecipitation assay confirmed that Sp1 proteins constitutively bound to this consensus sequence in vivo and that the binding was attenuated by I3C. In addition, I3C inhibited the extracellular signal-regulated kinase (ERK) signaling pathway in MCF-7 cells. The results suggest that I3C inhibits MMP-2 expression by blocking the ERK/Sp1-mediated gene transcription to attenuate migration and invasion of breast cancer cells.

KEYWORDS: Indole-3-carbinol; matrix metalloproteinase; Sp1; extracellular signal-regulated kinase

INTRODUCTION

Indole-3-carbinol (I3C) is the major bioactive food component in cruciferous vegetables and has been shown to exhibit anticancer activity on various cancer cell lines (*1, 2*). In addition, I3C exerts a chemopreventive effect on carcinogen-induced tumor development in experimental animals (*3, 4*) and inhibits tumor growth in vivo (*5*). Several mechanisms have been suggested to be involved in the inhibition of tumorigenesis by I3C. First, I3C may up-regulate cell cycle inhibitors including p21 and p27 to repress proliferation of cancer cells (*6*). Second, I3C may up-regulate pro-apoptotic Bax protein and increase the Bax/Bcl-2 ratio to promote apoptosis of cancer cells (*7*). Third, I3C may attenuate carcinogen bioactivation in vivo by modulating the expression of phase I and II metabolic enzymes (*8*). Fourth, I3C may reduce DNA damage caused by various carcinogens by inducing DNA repair enzymes to block DNA strand breakage (*9*). Fifth, I3C may inhibit angiogenesis by suppressing proliferation of blood endothelial cells (*10*).

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Inhibition of migration, invasion, and possibly metastasis by I3C was originally demonstrated by Fan's group (*11, 12*). The authors showed that I3C up-regulated the tumor suppressor genes PTEN and BRCA1 and the cell−cell adhesion protein E-cadherin to repress invasion and migration of breast cancer cells. However, it is possible that some other mediators may also be involved in I3C-induced inhibition of tumor invasion.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that selectively degrade components of the extracellular matrix (*13*). The MMP family consists of at least 20 enzymes and may be subgrouped into different types based on sequence characteristic and substrate specificity. MMPs are synthesized as inactive precursors and activated by proteolytic cleavage. Among human MMPs reported, MMP-2 (also named gelatinase-A) and MMP-9 (gelatinase-B) show substrate specificity toward type IV collagen, the major component of basement membrane, and their expressions are strongly linked with tumor metastasis in various types of human cancer. The

Table 1. Inhibition of Migration of MCF-7 Cells by I3C

time (h)	nonrecovered area (%)		
	C	50 μM	100 μM
0	100	100	100
48	63.6 ± 2.0	74.9 ± 2.1*[a]	84.8 ± 1.6*
96	33.0 ± 1.4	52.6 ± 4.4*	65.4 ± 2.9*

[a] *, $P < 0.05$ when compared to the control group.

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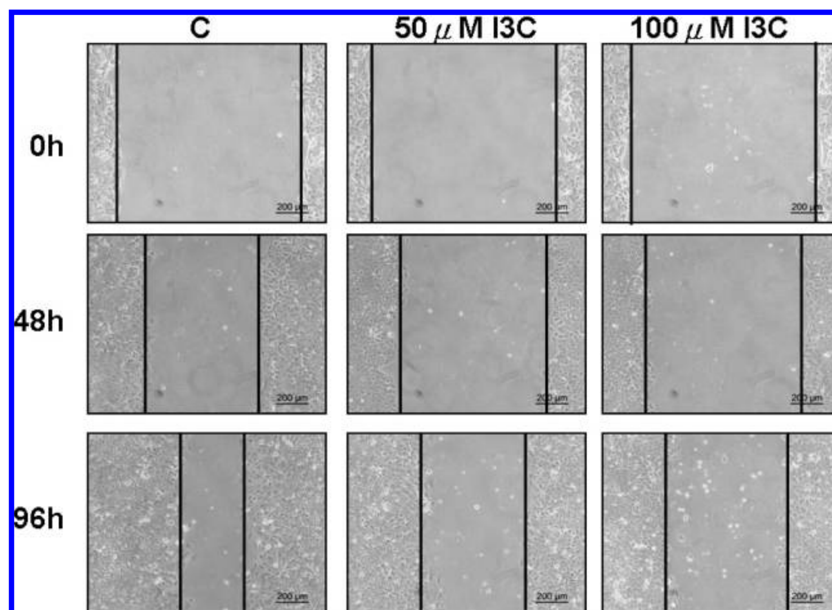

Figure 1. Effect of I3C on the migration of MCF-7 cells. Cells were treated without (C) or with various concentrations of I3C. Lines were marked along the wounded area at each plate, and pictures were taken at 0, 48, and 96 h to observe the nonrecovered areas.

expressions of MMP-2 and MMP-9 are regulated by Sp1 transcription factor. For example, Sp1 has been shown to play a critical role in the induction of MMP-2 and the control of cell invasion in astroglioma cells and gastric cancer cells (*14, 15*). In addition, epidemiological study demonstrated a strong and positive correction in allele-specific transcriptional regulation of MMP-2 and Sp1 (*16*). Similarly, Sp1 is also involved in the control of MMP-9 expression in neuroblastoma cells (*17*).

One of the main upstream regulators of Sp1 are the extracellular signal-regulated kinases (ERKs). This signaling pathway also plays an important role in the enhancement of cell migration and invasion. Overexpression of constitutively active mitogen-activated protein kinase MEK1 stimulated ERK activation and induced an invasive phenotype in transfected epithelial cells (*18*). Moreover, fibroblast growth factor-2 and chemical carcinogen 12-*O*-tetradecanoylphorbol-13-acetate (TPA) also triggered the ERK signaling pathway to promote MMP-9 secretion and to enhance cell invasion (*19*). In this study, we test whether MMPs are molecular targets of I3C and address the underlying mechanism by which I3C regulates MMPs.

MATERIALS AND METHODS

Cell Culture and Reagents. MCF-7 breast cancer cells were obtained from the cell bank of the National Health Research Institute (Miaoli, Taiwan). Cells were cultured in DMEM/F12 medium containing 10% charcoal-stripped fetal calf serum (FCS) and antibiotics. I3C was obtained from Sigma Chemical (St. Louis, MO). Antibodies against phospho-AKT (no. 9271), AKT (no. 9272), phospho-ERK (no. 9101), and ERK (no. 9102) were obtained from New England Biolabs (Ipswich, MA). LipofecTAMINE reagent was obtained from Life Technologist (Rockville, MA). The luciferase assay system was from Promega (Madison, WI). The EnzChek MMP assay kit was purchased from Molecule Probes (Invitrogen) (Eugene, OR).

Plasmids. Full-length human MMP-2 promoter-luciferase construct and a series of deletion or mutant constructs were created as described previously (*14*) and were kindly provided by Dr. E. N. Benveniste (University of Alabama at Birmingham). CMV-Sp1 was kindly provided by Dr. S. T. Smale (University of California). The constitutively active MEK1 expression vector CMV-MEK1 was kindly provided by Dr. M. Z. Lai (Academic Sinica, Taiwan).

In Vitro Scratch Assay. Cells were grown to confluence, and a wound was created in the center of the cell monolayer by gentle removal of the attached cells with a sterile plastic pipet tip. The ability of the cells to migrate into the wound area was assessed after 48 and 96 h. The wound areas of control or I3C-treated groups were photographed, and lines were marked along the wounded area at each plate. The measure of the wounded area was calculated by the following formula: area = length × width. The percentage of nonrecovered wound area was calculated by dividing the nonrecovered area after 48 and 96 h by the initial wound area at zero time.

In Vitro Invasion Assay. In vitro invasion assay was performed by using 24-well transwell units with Matrigel (Becton Dickinson Labware, Bedford, MA) coated on the upper side of polycarbonate filters (pore size = 8 μm) as described previously (*20*). MCF-7 cells treated without or with various concentrations of I3C for 24 h were collected. Three thousand cells resuspended in 100 μL of serum-free medium were placed in the upper part of the transwell unit and allowed to invade for 24 h. The lower part of the transwell unit was filled with 10% FCS medium. After incubation, noninvaded cells on the upper part of the membrane were removed with a cotton swab. The total number of cells that invaded the lower chamber (which were localized on the bottom surface of the polycarbonate filters) were fixed in formaldehyde, stained with Giemsa solution, and counted under a microscope. Each experiment was done in triplicate, and results from three independent experiments were expressed as mean ± SE.

MMP Activity Assay. Conditioned medium of MCF-7 cells treated without or with I3C was harvested, and the MMP activity was studied by the EnzChek MMP assay kit using fluorescein-conjugated gelatin (DQ gelatin) as substrate and purified type IV collagenase as positive control enzyme. Experiments were carried out according to the procedures of the manufacturer, and the output signal (excitation wavelength, 495 nm; emission wavelength, 520 nm) was recorded for 2 min at 37 °C by using a fluorescent reader (BMG LABTECH). Results of three independent experiments were expressed as mean ± SE.

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from MCF-7 cells treated without or with I3C, and MMP-2 expression was investigated by using the OneStep RT-PCR kit as described previously (*24*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to check the efficiency of cDNA synthesis and PCR amplification. cDNA synthesis was performed at 50 °C for 30 min, and the condition for PCR was 22 cycles of denaturation (94 °C/1 min), annealing (60 °C/1min), extension (72 °C/1 min), and 1 cycle of final extension (72 °C/10 min). The primers used were as follows: MMP-2-forward, 5′-CATGTCGCCCCTAAAACAGA-3′; MMP-2-reverse, 5′-CCATCAA-ACGGGTATCCATC-3′; GAPDH-forward, 5′-CCCATCACCATCT-

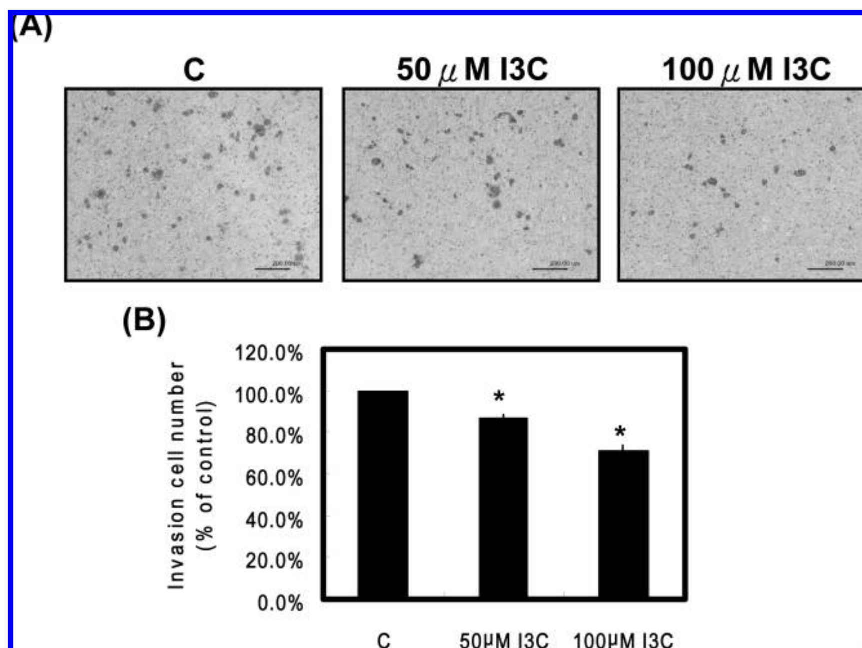

Figure 2. Effect of I3C on the invasion of MCF-7 cells: (**A**) Cells were treated without (C) or with various concentrations of I3C and subjected to transwell assays (typical pictures were taken at 24 h after seeding of cells into upper wells); (**B**) invaded cell numbers were calculated, and results of three independent experiments are expressed as mean ± SE [∗, $P < 0.05$ when compared with the control (C) group].

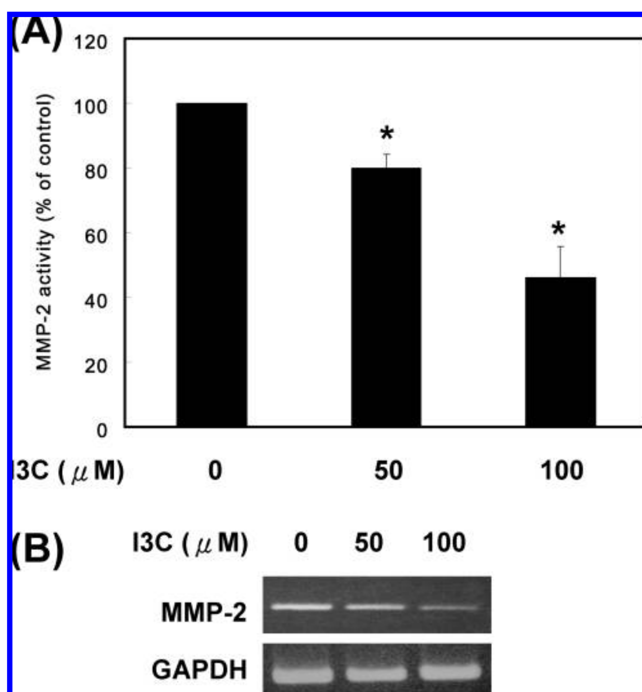

Figure 3. Inhibition of MMP-2 activity and expression by I3C: (**A**) Conditioned medium of MCF-7 cells treated without or with various concentrations of I3C was harvested, and the MMP-2 activity was studied by the EnzChek MMP assay kit. The MMP-2 activity of the control group was defined as 100%. Results of three independent experiments are expressed as mean ± SE. ∗, $P < 0.05$ when compared with the control group. (**B**) Cells were also harvested, and the mRNA level of MMP-2 was detected by RT-PCR.

TCCAG-3′; and GAPDH-reverse, 5′-CAGTCTTCTGGGTGGCAGT-3′. After reaction, PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Promoter Activity Assays. Activity of full-length or mutant MMP-2 promoter constructs was investigated as described previously (*20*). In brief, cells were plated onto six-well plates at a density of 100000 cells/well and grown overnight. Cells were cotransfected with 2 μg of MMP-2 promoter constructs for 5 h by the LipofecTAMINE method. In some experiments, the CMV-Sp1 expression vector was also cotransfected with MMP-2 promoter constructs. After transfection, cells were cultured in 10% FCS medium for 48 h. Luciferase activities were assayed by using the assay systems according to the procedures of the manufacturer and were normalized for cellular protein concentrations. Results from three independent assays were expressed as mean ± SE.

Chromatin Immunoprecipitation (ChIP) Assay. Cells treated without or with 100 μM I3C were fixed with 1% formaldehyde at 37 °C for 10 min. ChIP assays were performed as described previously (*21*). The primers (sense, 5′-GTCCTGGCAATCCCTTTGTA-3′; antisense, 5′-GGGGAAAAGAGGTGGAGAAA-3′) were used for PCR amplification of the MMP-2 promoter, which contains the Sp1 site at the −72/−64 bp region.

Immunoblotting. Drug-treated or plasmid-transfected cells were washed with phosphate-buffered saline (PBS) and harvested as described previously (*21*). Protein concentration was measured by using a BCA protein assay kit (Pierce, Rockford, IL), and an equal amount of proteins was subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes, and proteins were detected by probing the membranes with different primary antibodies. Enhanced chemiluminescence reagents were used to detect the phosphorylation status of AKT and ERK or the expression of Sp1.

Statistical Analysis. The associations between control and I3C-treated groups were assessed using Student's *t* test. Statistical significance was defined as $P < 0.05$.

RESULTS

Inhibition of Migration and Invasion of MCF-7 Cells by I3C. The migration ability of MCF-7 cells was assessed by in vitro scratch assay. As shown in **Figure 1**, I3C repressed the migration of MCF-7 cells in a dose-dependent manner. Quantitative analysis indicated that nonrecovered areas of the control group were 63.6 and 33.0% at 48 and 96 h, respectively (**Table 1**). The nonrecovered areas were 74.9 and 52.6% in the 50 μM I3C-treated group, and a more dramatic reduction of migration was found in the 100 μM I3C-treated group (**Figure 1**). Similar results were also observed in the cell invasion assay (**Figure 2A**). I3C at concentrations of 50 and 100 μM inhibited cell

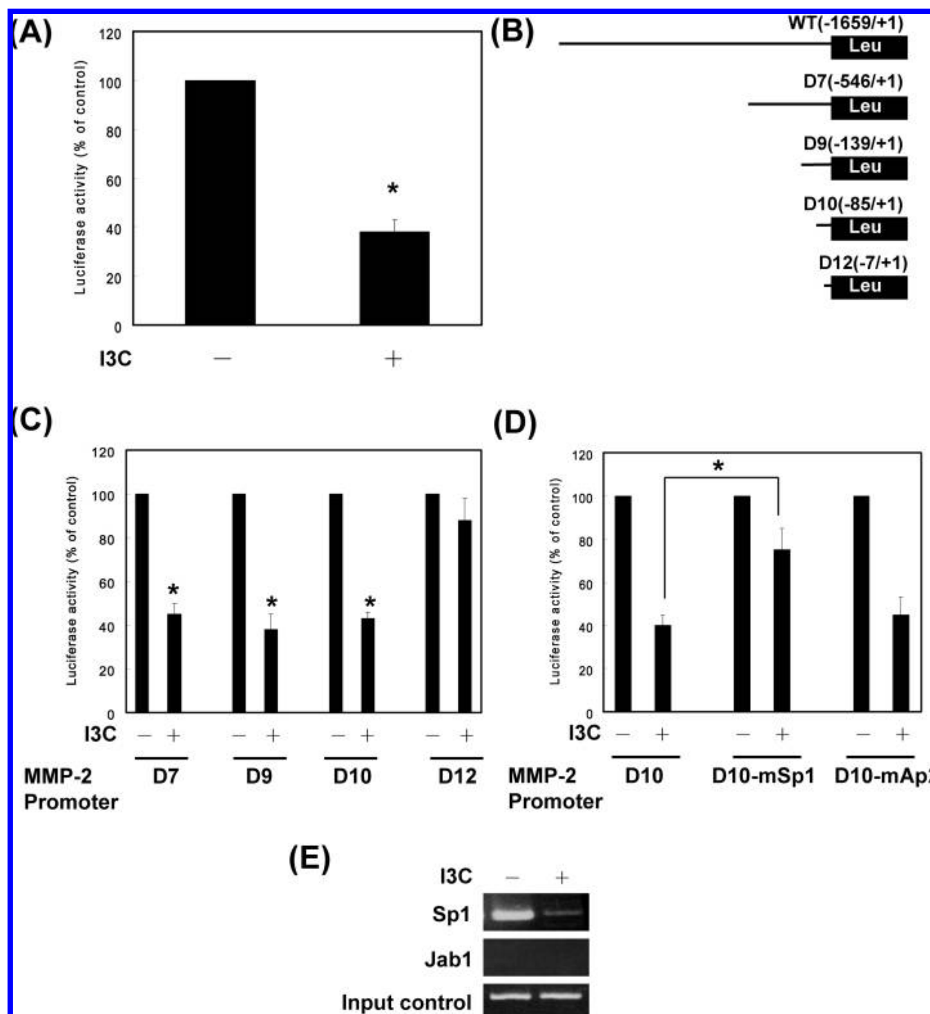

Figure 4. Effect of I3C on the binding of Sp1 to MMP-2 promoter. (**A**) Cells were transfected with wild-type MMP-2 (WT, −1659/+1) promoter construct and treated without (–) or with 100 μM I3C for 24 h. Results of three independent experiments are expressed as mean ± SE, and the luciferase activity of the control group was defined as 100%. ∗, $P < 0.05$ when compared with the control group. (**B**) MMP-2 promoter deletion constructs used in this study. (**C**) Cells were transfected with various MMP-2 promoter deletion constructs and treated without (–) or with 100 μM I3C for 24 h. Results of three independent experiments are expressed as mean ± SE, and the luciferase activity of the control group was defined as 100%. ∗, $P < 0.05$ when compared with the control group. (**D**) Cells were transfected with D10, D10-mSp1 (D10 promoter in which the Sp1 site located at −72/-64 region was mutated), or D10-mAP2 (D10 promoter in which the Ap2 site at the −61/−53 region was mutated and treated without (–) or with 100 μM I3C for 24 h. Results of three independent experiments are expressed as mean ± SE, and the luciferase activity of the control group was defined as 100%. ∗, $P <$ 0.05 when the mutation significantly reversed the I3C-induced inhibition of promoter activity. (**E**) Cells were treated without (–) or with 100 μM I3C for 24 h. ChIP assay was performed as described under Materials and Methods. Anti-Jab1 antibody against Jab1, the coactivator of Ap1 transcription factor, was used as a negative control to verify the specific binding of Sp1 to MMP-2 promoter.

invasion by 13 and 30%, respectively (**Figure 2B**). These data indicate that I3C is effective in the suppression of migration and invasion of breast cancer cells.

Down-regulation of Enzymatic Activity and Expression of MMP-2 by I3C. In the cell invasion assay, cells needed to degrade the matrigel coated on the membrane to invade the lower chamber. Because MMPs have been found to be involved in matrix degradation, we tested whether enzymatic activity and expression of MMPs were affected by I3C. Our preliminary result indicated that MCF-7 cells expressed a high level of MMP-2 but little MMP-9 (data not shown). We found that I3C inhibited MMP-2 activity in a dose-dependent manner (**Figure 3A**), which was accompanied by down-regulation of MMP-2 mRNA level (**Figure 3B**).

Inhibition of Sp1-Mediated MMP-2 Promoter Activity by I3C. To clarify whether I3C directly repressed MMP-2 gene transcription, we performed a promoter activity assay. Indeed, MMP-2 promoter activity was inhibited by 100 μM I3C (**Figure 4A**). Various promoter deletion constructs (**Figure 4B**) were then used for study, and our data showed that the I3C responsive region was mapped to the −85/−7 region of the MMP-2 promoter (D10 promoter construct) (**Figure 4C**). Previous studies including ours have demonstrated that Sp1 and Ap-2 transcription factor binding sites existed within this region and that these two consensus binding sequences are important for the regulation of MMP-2 expression (*14, 20*); therefore, we used Sp1 (D10-mSp1) and Ap-2 (D10-mAp-2) mutation constructs to address the importance of these binding sites. Mutation of the Sp1 site partly reversed the inhibitory effect of I3C, whereas mutation of Ap-2 had no effect (**Figure 4D**). These data indicate that Sp1 was involved in the inhibition of MMP-2 by I3C. However, it should be noted that Sp1 could not completely counteract the effect of I3C, suggesting that some other transcription factors may also participate in this process. The ChIP assay indeed demonstrated that Sp1 was bound to the MMP-2 promoter and that this binding was attenuated by I3C

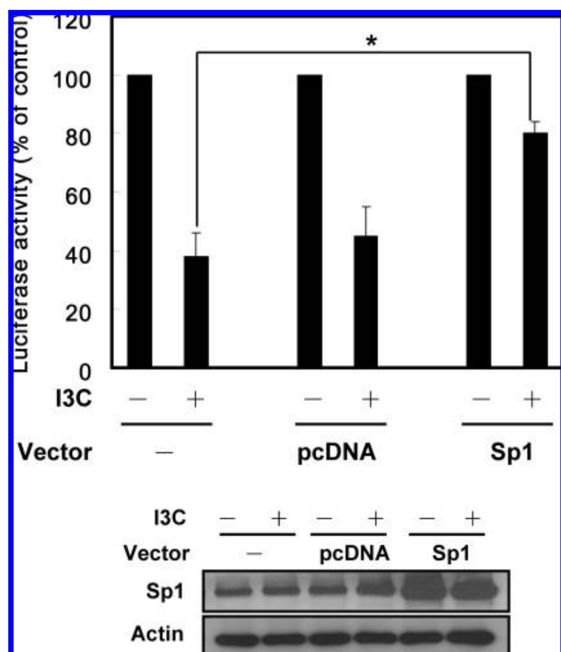

Figure 5. Ectopic expression of Sp1 on I3C-induced down-regulation of MMP-2. Cells were cotransfected with MMP-2 D10 promoter construct and pcDNA or Sp1 expression vector and treated without (–) or with (+) I3C (100 μM) for 24 h. Luciferase activity was determined, and the protein level of Sp1 was investigated by Western blot analysis. $*$, $P < 0.05$ when compared to the control group without vector transfection.

(**Figure 4E**). The binding of Sp1 to the MMP-2 promoter was specific because an antibody against Jab1, an unrelated nuclear protein that could not bind to DNA, could not pull down the MMP-2 promoter under the same experimental condition. More importantly, ectopic expression of Sp1 significantly reversed I3C-induced inhibition of MMP-2 promoter activity (**Figure 5**).

Inhibition of ERK Signaling Pathway by I3C. We also addressed the underlying signaling by which I3C inhibited MMP-2. Our data indicated that I3C suppressed ERK, but not AKT, kinase activity (**Figure 6A**) in MCF-7 cells. ERK activity is known to be controlled by the upstream regulator MEK1, and mutation of serine 218 and 222 to aspartic acid leads to constitutive activation of MEK1 (*22*). In addition, expression of this mutant MEK1 increases ERK activity dramatically in cells (*22*). Indeed, ectopic expression of MEK1 reversed the inhibition of MMP-2 promoter activity by I3C (**Figure 6B**). Collectively, our results suggest that I3C inhibits MMP-2 expression via repression of the ERK/Sp1 signaling pathway. which leads to inhibition of migration and invasion.

DISCUSSION

In this study, we test the effect of I3C on cell invasion and MMP expression and find that I3C suppresses MMP-2 expression in human breast cancer cells. Moreover, we elucidate that I3C inhibits MMP-2 expression by suppressing the ERK/Sp1-mediated MMP-2 gene transcription. Previous studies have demonstrated that Sp1 is a critical regulator which controls both basal and extracellular signal-stimulated MMP-2 expression (*14, 20*). Because I3C has been reported to disrupt the interaction of Sp1 with a DNA-binding site within the CDK6 promoter to suppress CDK6 expression (*23*), it seems not surprising that I3C may inhibit MMP-2 via repression of Sp1-mediated MMP-2 transcription. We provide clear evidence that the Sp1 site located within the −72/−64 region of the MMP-2 promoter is important for I3C-induced inhibition and that ectopic expression of Sp1 may partly counteract the inhibition by I3C. In addition, our ChIP assay further verifies the binding of Sp1 to the MMP-2 promoter, which can be reduced by I3C. We also address the upstream regulator that controls the Sp1 transcription activity by I3C. A recent study demonstrated that ERK may directly phosphorylate Sp1 at Thr453 and Thr739 to enhance its DNA binding activity and transcriptional activity (*24*). In addition, Li et al. demonstrate that I3C inhibits the expression of MAP2K3, MAP2K4, MAP4K3, and MAPK3, upstream activating kinases for ERK (*25*). These results lead us to hypothesize that I3C inhibits the ERK kinase signaling pathway, which in turn reduces Sp1 phosphorylation and attenuates Sp1-induced MMP-2 expression. This hypothesis is strongly supported by the observation that ectopic expression of MEK1 effectively reverses the inhibition of MMP-2 by I3C. Another transcription factor, Ap-2, has been shown to play a role in the control of MMP-2 expression. However, Ap-2 is not involved in the inhibition of MMP-2 by I3C because mutation of the Ap-2 binding site does not affect I3C-mediated repression.

An interesting finding of our study is that I3C does not significantly affect AKT activity in MCF-7 cells. The reason is currently unclear. However, the inhibitory effect of I3C on AKT activation may be dependent on cell type. For example, inhibition of AKT activity by I3C has been clearly demonstrated

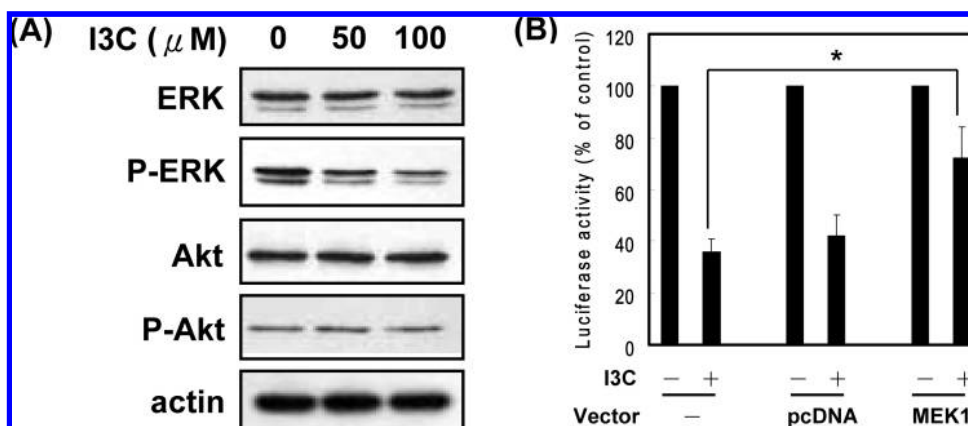

Figure 6. Involvement of the ERK signaling pathway in I3C-induced inhibition of MMP-2: (**A**) Cells were not treated or treated with various concentrations of I3C for 24 h. ERK and AKT kinase activities were studied by detecting the phosphorylation status of these two kinases. (**B**) Cells were cotransfected with MMP-2 D10 promoter construct, pcDNA, or MEK1 expression vector. Luciferase activity was determined 24 h after transfection, and results of three independent experiments are expressed as mean ± SE. $*$, $P < 0.05$ when compared to the control group without vector transfection.

in prostate cancer cells (*26*). I3C potently suppresses AKT activation and induces significant apoptosis in different human prostate cancer cell lines such as PC-3. Conversely, the effect of I3C on AKT in breast cancer cells has been little reported. A search of the association between I3C and AKT in breast cancer in the PUBMED yielded only four publications. Two of them studied the effect of 3,3′-diindolylmethane, but not I3C, on AKT activity. Howel et al. found that I3C inhibited AKT and induced apoptosis in MDA-MB-468 but not in the nontumorigenic HBL100 cells (*27*). Rahman et al. also reported the inactivation of AKT and induction of apoptosis in two breast cancer cell lines, MCF10DCIS.com and MCF10CA1a, which were derived from the spontaneously immortalized human breast epithelial cell line (*28*). Therefore, to the best of our knowledge, the effect of I3C on AKT activity has never been addressed in MCF-7 cells. Whether the difference in the genetic background of these different breast cancer cell lines may affect their response to I3C needs further investigations. Taken together, we conclude that I3C may repress Sp1-mediated MMP-2 transcription to attenuate MMP-2 expression and invasion in breast cancer cells.

However, MMP-2 is not the only target for I3C to inhibit invasion and metastasis of breast cancer. A recent study shows that MMP-9 is another target suppressed by I3C in MDA-MB-231 breast cancer cells (*29*). These authors also demonstrate that I3C suppresses MMP-9 expression via inhibition of the NF-κB signaling pathway. Importantly, these authors find that I3C also inhibits the expression of chemokine receptor CXCR4. The involvement of CXCR4 in the metastasis of breast cancer was originally suggested by Muller et al. (*30*). They demonstrated that CXCR4 is highly expressed in breast cancer cells, whereas its ligand CXCL12 (or SDF-1) exhibits peak levels of expression in organs representing the first destinations of breast cancer metastasis. Therefore, breast cancer cells with high expression of CXCR4 are attracted by a chemical gradient to the organs that express large amounts of CXCL12. Because I3C inhibits CXCR4 expression, it seems possible that I3C may reduce bone metastasis of breast cancer cells. By using the SCID-human mouse model, Rahman et al. indeed confirmed this hypothesis (*29*). Both MMP-9 and CXCR4 are NF-κB-regulated genes. Therefore, I3C is also a negative regulator of the NF-κB signaling pathway.

Although the antiproliferative and apoptosis-inducing actions (and their underlying mechanisms) have been characterized in the past cascade, the antimetastatic effect of I3C is relatively unclear. Rahman's and our studies have demonstrated that MMPs (MMP-2 and MMP-9) and chemokine receptors (CXCR4) are key targets for I3C to repress tumor invasion and metastasis. These results also elucidate that I3C inhibits the upstream signaling pathways including ERK and IKK to attenuate Sp1 and NF-κB-mediated induction of these metastasis-promoting molecules. Taken together, I3C may be useful for the treatment and prevention of metastasis of breast cancer.

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