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Indole-3-carbinol and 3,3′-diindolylmethane induce expression of NAG-1 in a p53-independent manner☆

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Abstract

Indole-3-carbinol (I3C), present in cruciferous vegetables, and its major in vivo product 3,3′-diindolylmethane (DIM), have been reported to suppress cancer development. However, the responsible molecular mechanisms are not fully understood. Nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) is a TGF-β superfamily gene associated with pro-apoptotic and anti-tumorigenic activities. The present study was performed to investigate whether I3C and DIM influence NAG-1 expression and to provide the potential molecular mechanism of their effects on anti-tumorigenesis. The I3C repressed cell proliferation and induced NAG-1 expression in a concentration-dependent manner. In addition, DIM increased the expression of NAG-1 as well as activating transcription factor 3 (ATF3), and the induction of ATF3 was earlier than that of NAG-1. The DIM treatment increased luciferase activity of NAG-1 in HCT-116 cells transfected with NAG-1 promoter construct. The results suggest that I3C represses cell proliferation through up-regulation of NAG-1 and that ATF3 may play a pivotal role in DIM-induced NAG-1 expression in human colorectal cancer cells. Furthermore, the mixture of I3C with resveratrol enhances NAG-1 expression, suggesting the synergistic effect of these two unrelated compounds on NAG-1 expression.

Keywords: I3C; GDF-15; NAG-1; ATF3; p53; DIM; Apoptosis

Colorectal cancer is one of the most prevalent cancers in the world. In the United States, colon cancer is the second most prevalent cause of cancer death and the third most common cancer among adults, accounting for approximately 15% of all cancers diagnosed annually [1]. Recently, chemoprevention by dietary compounds has received considerable attention as an effective approach for cancer prevention since epidemiologic studies demonstrated that the consumption of phytochemical-rich plant foods, including whole grains, vegetables, and fruits, is highly associated with a reduced risk for cancer incidence [2]. One such phytochemical is indole-3-carbinol (I3C), an autolysis product of glucobrassicin, a naturally occurring component of *Brassica* vegetables, such as cabbage, broccoli, and *Brussels sprouts* [3]. It has been reported that high doses of I3C in the diet of rodents reduce the incidence of cancer in the colon [4], liver [5], endometrium [6], and epidermis [7]. Furthermore, in vitro studies have shown

☆ *Abbreviations:* I3C, indole-3-carbinol; DIM, 3,3′-diindolylmethane; NSAIDs, nonsteroidal anti-inflammatory drugs; NAG-1, NSAID-activated gene-1; ATF3, activating transcription factor 3.

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that I3C inhibits the growth of human colon [8], prostate [9], and breast [10,11] cancer cells.

I3C is converted to numerous condensation products with distinctive biological activities. The 3,3′-diindolylmethane (DIM) is a major acid condensation product of I3C [12]. Subsequently, DIM has been found to suppress colon and mammary tumor growth in rats [13], as well as in breast [14] and prostate cancer cells [15] in vitro. DIM is readily detected in the livers and feces of rodents fed with I3C, whereas the parent I3C compound could not be detected in tissues of I3C-treated rodents, suggesting that DIM, not I3C, may mediate the observed physiological effects of dietary I3C in vivo. In in vitro studies, both I3C and DIM mediate the antiproliferative effect by inducing a G1 cell cycle arrest through disruptions in the expression and activity of specific G1-acting cell cycle components [16–18]. Although several studies have investigated the apoptotic effects of high concentrations of I3C in several carcinoma cells [15,19], relatively little is known about the molecular mechanisms that selectively control the anti-proliferative and anti-tumorigenic responses of these indole compounds.

Originally discovered by our group, NAG-1 (NSAID-activated gene) is an important gene regulated by a number of cyclooxygenase (COX) inhibitors and chemopreventive chemicals [20]. NAG-1, a member of the transforming growth factor-β (TGF-β) superfamily, is also known as macrophage inhibitory cytokine-1 (MIC-1) [21], placental transformation growth factor-β (PTGFB) [22], prostate derived factor (PDF) [23], growth differentiation factor 15 (GDF-15) [24], and placental bone morphogenetic protein (PLAB) [25]. The expression of NAG-1 is regulated by NSAIDs and a large number of other related chemicals that are chemopreventive. For example, genistein and resveratrol act through p53-dependent mechanisms to regulate induction of NAG-1 [26,27]. The chemopreventive agent troglitazone regulates NAG-1 expression via the tumor suppressor gene EGR-1, while prostaglandin J_2, a PPARγ agonist, upregulates the expression of NAG-1 by a PPARγ-dependent mechanism [28]. Furthermore, we have reported that NAG-1 is a downstream of PI3K/AKT/GSK-3β signaling pathway [29]. Thus, transcriptional regulation of NAG-1 is complex and the promoter sequence contains many different *cis*- and *trans*-acting promoter elements [30]. In addition, NAG-1 expression is also regulated by post-transcriptional mechanisms as reported for the chemopreventive agent, AHPN [31]. We were prompted to examine NAG-1 expression by indole compounds because NAG-1 has an anti-tumorigenic activity as assessed by in vivo and in vitro assay [20,32,33], and is regulated by many anti-tumorigenic compounds. Therefore, the current study was conducted to investigate the potential effects of I3C and DIM on the proliferation of colon carcinogenesis, and to characterize the possible molecular mechanisms involved in these effects. We report, for the first time, that ATF3 and NAG-1 mediate the anti-tumorigenic activity of I3C and DIM. Furthermore, adding another phytochemical, resveratrol, to I3C augments the expression of NAG-1 induction.

Materials and methods

Components and materials. I3C and DIM were purchased from Sigma (St. Louis, MO) and LKT Laboratories (St. Paul, MN). Resveratrol and I3C/resveratrol capsules were obtained from Dr. Terri Mitchell (Life Extension Foundation, NY). All chemicals were purchased from Fisher Scientific, unless otherwise specified. Human colorectal carcinoma HCT-116 cells were purchased from American Type Culture Collection (Manassas, VA), whereas HCT-116 p53−/− cells were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Antibodies for ATF3 and actin were purchased from Santa Cruz (Santa Cruz, CA). NAG-1 antibody was described previously [20]. Cell culture media and serum were purchased from Invitrogen (Carlsbad, CA).

Cell culture and measurement of cell proliferation. HCT-116 cells were maintained in McCoy's 5A medium with 10% fetal bovine serum (FBS) and 10 μg/ml gentamycin. The cells between 6 and 15 passages were used in these studies. The effect of the I3C on cell proliferation was investigated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Briefly, cells were seeded at the concentration of 1000–2000 cells/well in 96-well tissue culture plates in eight replicates and maintained overnight. The cells were treated with 0, 10, 50 or 100 μM I3C. At 1, 2, 3, and 4 day after treatment, 20 μl MTS solution was added to each well and the plate was incubated for 1 h at 37 °C, and absorbance at 490 nm was recorded in an ELISA plate reader (Bio-Tek Instruments, Winooski, VT).

Transient transfections. Transient transfections were performed using the Lipofectamine procedure according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). HCT-116 cells were plated in 12-well plates at the concentration of 10^5 cells/well. After growth for 18 h, plasmid mixtures containing 0.5 μg NAG-1 promoter linked to luciferase and 0.05 μg pRL-null vector were transfected for 5 h according to the manufacturer's protocols. The transfected cells were cultured in the absence or presence of DIM for 24 h. The cells were harvested in 1× luciferase lysis buffer, and luciferase activity was determined and normalized to the pRL-null luciferase activity with a dual luciferase assay kit (Promega, WI).

Western analysis. Cells were grown to 60–80% confluency in 60-mm plates, followed by 24 h treatment in the presence of indicated I3C or DIM. Cells were washed with PBS, and cell lysates were isolated in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) and centrifuged at 12,000 rpm for 5 min at 4 °C. Protein concentration was determined with BCA protein assay (Pierce, Rockford, IL) using BSA as standard. The proteins (30 μg) were separated on 12% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were incubated with a specific anti-serum against human NAG-1 [20], ATF3 (Santa Cruz, CA) or actin in TBS +0.05% Tween 20 (TSB-T) containing 5% nonfat dried milk at 4 °C overnight. After three washes with TBS-T, the blots were incubated with peroxidase-conjugated anti-rabbit or anti-goat IgG for 1 h at room temperature, visualized using ECL (Amersham Biosciences), and quantified by Scion Image Software (Scion, Frederick, MD).

Results

I3C inhibits cell proliferation and induces NAG-1 expression in HCT-116 cells

To investigate the effects of I3C on the growth of colorectal cancer cells in culture, HCT-116 cells were incubated with different concentrations of I3C for 4 days, and cell proliferation was measured. As shown in Fig. 1A, treatment with 10 μM I3C did not influence the cell growth rate. However, HCT-116 cells treated with 50 μM significantly reduced the cell growth rate by 26% and 24% at 3 and 4 days, while treatment with 100 μM reduced the cell growth rate by 36%, 68%, and 77% at 2, 3, and 4 days, respectively. NAG-1 is linked to cell growth arrest and apoptosis, and is responsible for the chemopreventive effects of several dietary compounds in a variety of carcinoma models [20,22,26–28,32,34]. To investigate whether I3C-induced growth arrest is associated with NAG-1 expression, HCT-116 cells were grown and treated with different concentrations of I3C and NAG-1 expression was measured by Western analysis. As shown in Fig. 1B, NAG-1 expression increased by approximately 3.5- and 4.8-fold in HCT-116 cells treated with 50 and 100 μM I3C, respectively. This result is consistent with I3C-induced cell growth arrest shown in Fig. 1A, suggesting that I3C inhibits cell proliferation in human colon cancer cells, probably by the induction of NAG-1 expression.

NAG-1 induction by I3C is independent of p53

Previous studies show that NAG-1 expression is regulated by p53 proteins at the transcriptional level by some compounds [22,26,27,35]. I3C effects on anti-tumorigenesis are known to be mediated in a p53-independent manner [15,36,37]. To investigate whether NAG-1 expression induced by I3C is mediated by p53, we used p53 null HCT-116 cells (HCT-116 p53−/−) and examined NAG-1 expression in the presence of I3C. Results showed increases of NAG-1 expression by 100 μM I3C in both HCT-116 (p53+/+) and HCT-116 (p53−/−) cell lines, indicating that NAG-1 is up-regulated by I3C through p53-independent mechanisms in human colon cancer cells (Fig. 2A). I3C is chemically unstable in acidic environments and is rapidly converted in the stomach to 3,3′-diindolylmethane (DIM), a major condensation product [12,38]. Therefore, we also treated HCT-116 cells with 25 μM DIM and measured NAG-1 expression. As shown in Fig. 2B, DIM also induces NAG-1 expression in both p53+/+ and p53−/− HCT-116 cells. These data indicate that both I3C and DIM induce NAG-1 expression in a p53-independent manner.

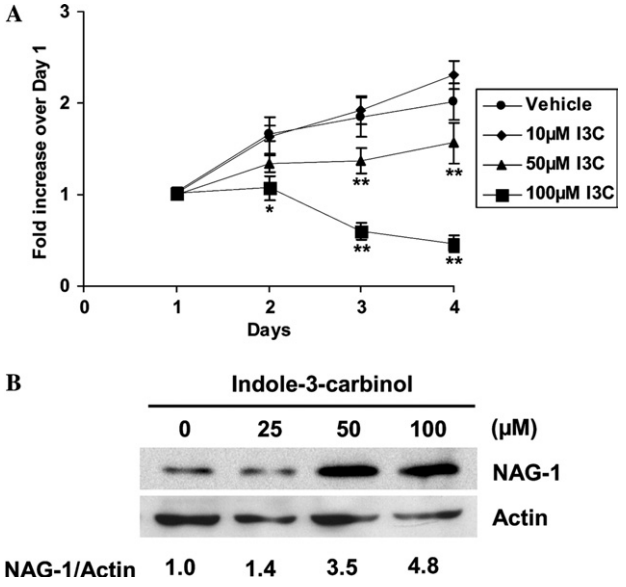

Fig. 1. Growth arrest and dose response of NAG-1 protein in I3C-treated HCT-116 cells. (A) HCT-116 cells were treated with vehicle or different concentrations of I3C for 4 days. Cell growth was measured using CellTiter96 Aqueous One Solution Cell Proliferation Assay Kit (Promega, WI). Values are expressed as means ± SD of eight replicates. $*P < 0.01$, $**P < 0.001$ versus vehicle. (B) HCT-116 cells were treated with vehicle or different concentrations of I3C for 24 h. Subsequently, 30 μg of total cell lysates was subjected to 14% SDS–PAGE. NAG-1 and actin antibodies were probed. The blots are representative of two independent experiments. The relative NAG-1 levels normalized by actin are shown on the bottom.

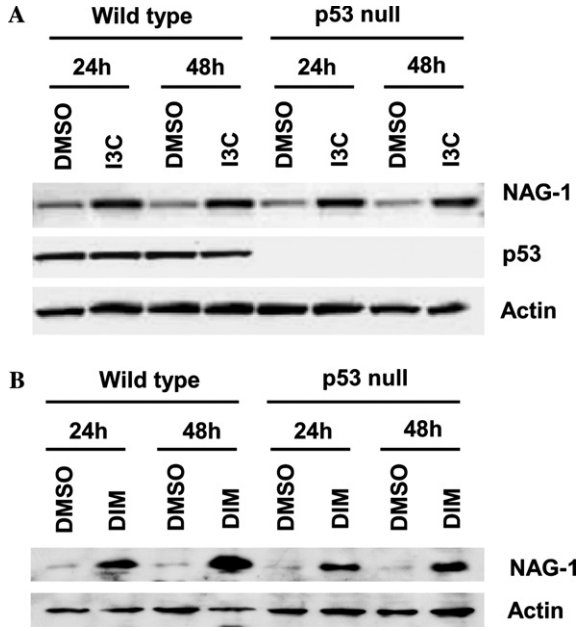

Fig. 2. I3C-induced NAG-1 expression in p53 wild-type and p53 null HCT-116 cells. The p53 wild-type and p53 null HCT-116 cells were treated with vehicle or 100 μM I3C (A), and vehicle or 12.5 μM DIM (B), for 24 or 48 h and Western analysis was performed as described in Fig. 1. NAG-1, p53 or actin antibodies were probed.

DIM induces ATF3 expression earlier than NAG-1 expression

Because our previous data demonstrated that NAG-1 was regulated by EGR-1, ATF3, p53, and Sp1 transcriptional factors [27,28,30,34,39], we conducted this study to examine whether DIM influences the expression of transcription factors. Expression of EGR-1 was not influenced by DIM treatment (data not shown). We found, however, that ATF3 expression was increased in a dose-dependent manner in HCT-116 cells treated with 12.5 and 25 μM DIM for 24 h (Fig. 3A). In addition, NAG-1 expression is also induced by 4.0- and 5.2-fold after addition of 12.5 and 25 μM DIM, respectively. However, NAG-1 and ATF3 protein expression decreased in HCT-116 cells treated with 50 μM DIM. To address whether ATF3 expression is possibly linked to NAG-1 expression, time course experiments of both ATF3 and NAG-1 protein were performed in HCT-116 cells treated with 25 μM DIM. NAG-1 expression began to increase at 4 h while ATF3 expression increased as early as 2 h (Fig. 3B), suggesting the possibility that the induction of ATF3 plays a pivotal role for the expression of NAG-1 in the presence of DIM. Indeed, it has been reported that ATF3 is one of the transcriptional factors that possibly regulate NAG-1 expression at the transcriptional level [34].

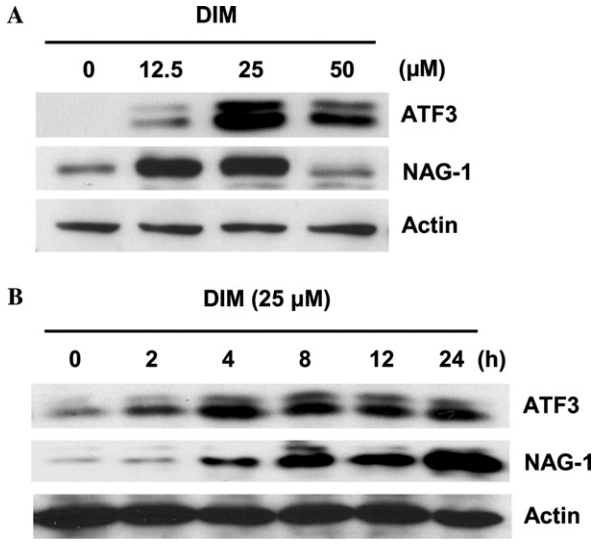

Fig. 3. Dose- and time-dependent induction of ATF3 and NAG-1 by DIM. (A) HCT-116 cells were treated with vehicle or different concentrations of DIM for 24 h. (B) HCT116 cells were treated with 25 μM DIM at different time points. Western analysis was performed using NAG-1, ATF3, and actin antibodies as described in Fig. 1. The blots are representative of two independent experiments.

DIM induces NAG-1 promoter activity

To test whether DIM affects transcriptional regulation of NAG-1, we measured promoter activity using luciferase constructs (pNAG3500/41 and pNAG1086/41) containing NAG-1 promoter region [30]. Constructs were transfected into HCT-116 cells and treated with 25 μM DIM for 24 h. As shown in Fig. 4, DIM treatment resulted in the 3.5- and 3.4-fold induction of luciferase activity in pNAG3500/41 and pNAG1086/41, respectively. As a control, pGLBasic promoterless vector was transfected and no increase of luciferase activity was found in the presence of DIM. It is notable that there is no p53 binding site in these constructs, indicating that DIM-induced NAG-1 promoter activity is p53-independent.

The mixture of I3C and resveratrol augments NAG-1 expression

An important aspect of dietary chemoprevention is that it may be combined with other phytochemicals to potentially enhance the therapeutic effects. Unlike I3C, resveratrol induces NAG-1 expression in a p53-dependent manner [27]. I3C may work synergistically with resveratrol to induce cell growth arrest because I3C stops cell growth at G1 phase in the cell cycle [9,36,40], while resveratrol stops it at G2/M phase [27]. Resveratrol and I3C/resveratrol mixture were treated into HCT-116 cells. A resveratrol capsule contains 20 mg resveratrol and 120 mg quercetin, whereas I3C/resveratrol capsule contains 7 mg resveratrol, 40 mg quercetin, and

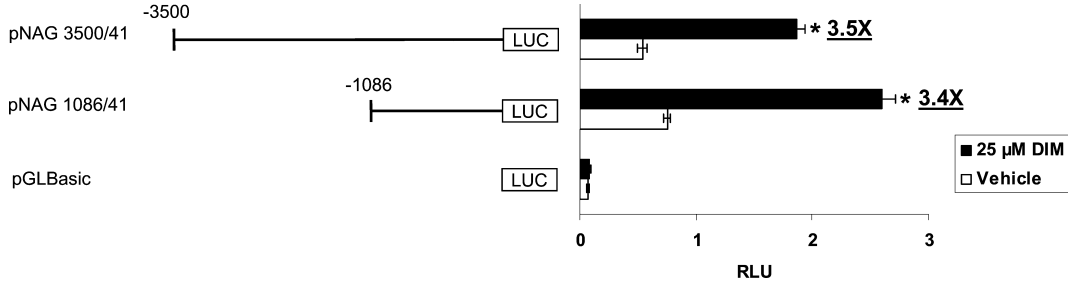

Fig. 4. NAG-1 promoter assay in DIM-treated HCT-116 cells. The pNAG3500/41 and 1086/41 constructs were linked to luciferase and transiently transfected with pRL-null vector in HCT-116 cells. After transfection, the cells were treated with vehicle or 25 μM DIM for 24 h and luciferase activity was measured and expressed as relative luciferase unit (firefly luciferase signal/*Renilla* luciferase signal). The results show means ± SD of three separate transfections. $^{*}P < 0.001$ versus vehicle.

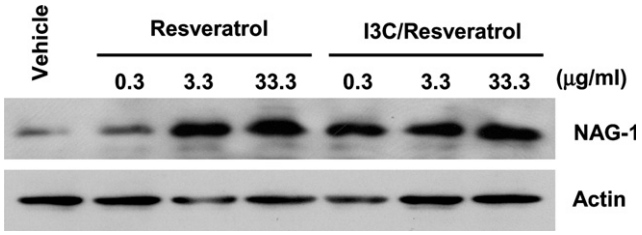

Fig. 5. NAG-1 induction by resveratrol and mixture of I3C/resveratrol in HCT-116 cells. HCT-116 cells were treated with vehicle, different concentrations of resveratrol or mixture of I3C/resveratrol for 24 h. Western analyses of NAG-1 and actin were performed. 0.3 μg/ml resveratrol capsule represents 0.05 μM resveratrol, whereas 0.3 μg/ml I3C/resveratrol capsule represents 0.025 μM of resveratrol and 1 μM I3C.

200 mg I3C. As shown in Fig. 5, the mixture of I3C/resveratrol capsule enhances NAG-1 expression at 0.3 μg/ml concentration, compared to resveratrol capsule itself. However, the treatment of resveratrol or I3C/resveratrol at 3.3 and 33.3 μg/ml concentrations resulted in the similar induction of NAG-1 expression. This result suggests that at a lower concentration, I3C and resveratrol may work synergistically to induce NAG-1 expression, and also provide knowledge of the better effects of combinatory chemoprevention in human colorectal cancer.

Discussion

The development of targeted pharmaceutical agents from dietary sources to inhibit cancers depends on precise information concerning the mechanisms of control and the functions of regulated genes that can potentially control tumor cell proliferation. In the present study, we demonstrate that both I3C and its major metabolite, DIM, stimulate NAG-1 expression, which might be the hallmark of an anti-proliferative pathway by indole compounds in human colon cancer cells.

NAG-1 is a novel TGF-β superfamily gene, which mediates the biological functions of different types of chemopreventive compounds including NSAIDs, PPARγ ligands, and many dietary compounds. Regulation of NAG-1 appears to be complex, involving both transcriptional and post-transcriptional mechanisms [31,39]. NAG-1 is regulated via a number of different *cis*- and *trans*-acting elements present in the promoter sequence, and most of the chemicals and drugs we have used increase NAG-1 by transcriptional mechanisms. The transcriptional regulation of NAG-1 by chemopreventive compounds is mediated by several mechanisms [27,28,39]. One of the genes involved in the regulation of NAG-1 expression is the p53 tumor suppressor gene. In fact, some dietary compounds, including resveratrol and genistein, induce NAG-1 expression through the p53 tumor suppressor protein [26,27]. We have investigated the involvement of p53 in I3C-induced NAG-1 expression and found that I3C and DIM induce NAG-1 expression in a p53-independent manner (Fig. 2). Similarly, it has been reported that DIM induces apoptosis in human breast and prostate cancer cells through p53-independent pathways [15,41]. Thus, unlike other phytochemicals, indole compounds may induce their anti-tumorigenic activity through a p53-independent manner. In this report, we have further found that the induction of ATF3 transcriptional factor may be an important target protein to regulate NAG-1 expression by indole compounds. Indeed, we have also shown that epicatechin gallate (a component of green tea) enhances NAG-1 expression by ATF3 transcription factor, not by p53 [34]. Taken together with previous reports, this finding illustrates the complexity of the regulation of NAG-1 expression. Further studies are required to better understand these regulatory mechanisms.

It has been reported that I3C is unstable and is converted into mainly DIM. Thus, DIM, rather than I3C, is probably the major compound initially available to cells after ingestion of I3C, although both I3C and DIM are active to induce apoptosis in MCF-7 breast carcinoma cells growing in culture [37,41]. In our studies, NAG-1 induction by DIM was greater than that by I3C in colon cancer cells because treatment with 50 μM I3C and 12.5 μM DIM showed a similar induction of NAG-1 (Figs. 1B and 3A). This is in agreement with observations that DIM is a more effective compound in suppressing the growth of human prostate cancer cells [15].

Our promoter assay showed that −1086 bp region of the NAG-1 promoter is necessary for DIM-induced NAG-1 expression. The NAG-1 promoter contains several binding sites for transcription factors previously identified as important for its regulation [30]. In this report, we suggest that ATF3 may be a potential activator of NAG-1 promoter (Fig. 3). Recently, ATF3 has been postulated as a tumor suppressor gene because ATF3 coordinates the expression of genes which may be linked to cancer [42,43]. Interestingly, it has been reported that both ATF3 and NAG-1 were co-induced by the treatment of 5F-203 anti-tumorigenic compound [44] as well as sulindac [45]. In the current study, we found that ATF3 expression is induced by DIM treatment and ATF3 is expressed earlier than NAG-1, supporting that ATF3 expression plays an important role for the induction of NAG-1 in the presence of DIM (Fig. 3).

The current study provides information on anti-tumorigenic activity by I3C and DIM. The molecular mechanisms by which I3C and DIM possess chemopreventive effects on colon cancer are largely unknown; however, NAG-1 and ATF3 likely play important roles as mediators of anti-tumorigenic effects. Furthermore, the current study demonstrates the coordinated gene responses to I3C or DIM and its combination with other phytochemicals expand our understanding of the nutrient regulation of gene transcription in chemoprevention.

A better understanding of the anti-tumorigenic properties of I3C and its metabolite in colon cancer could extend a novel nutritional management and therapy of cancer.

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