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Damnacanthal, a noni component, exhibits antitumorigenic activity in human colorectal cancer cells☆,☆☆

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Abstract

Damnacanthal, an anthraquinone compound, is isolated from the roots of *Morinda citrifolia* L. (noni), which has been used for traditional therapy in several chronic diseases including cancer. Although noni has been consumed for a long time in Asian and Polynesian countries, the molecular mechanisms by which it exerts several benefits are starting to emerge. In this report, we examined systematic approaches on the cancer-suppressing capability of damnacanthal in colorectal tumorigenesis. Damnacanthal exhibits cell growth arrest as well as caspase activity induction in colorectal cancer cells. We also examined several potential target proteins and found that the proapoptotic protein nonsteroidal anti-inflammatory activated gene-1 (NAG-1) is highly induced. Subsequently, we have found that damnacanthal also enhances transcription factor CCAAT/enhancer binding protein β (C/EBPβ), which controls NAG-1 transcriptional activity. Blocking of C/EBPβ by shRNA results in the reduction of NAG-1 expression as well as caspase activity in the presence of damnacanthal. Taken together, these results indicate that damnacanthal increases antitumorigenic activity in human colorectal cancer cells and that C/EBPβ plays a role in damnacanthal-induced NAG-1 expression.

Keywords: Damnacanthal; Noni; NAG-1; GDF15; C/EBPβ; Colorectal cancer

1. Introduction

Cancer is a major public health problem in the United States with an estimated 1.5 million new cancer cases expected in 2010 [1]. It seems that there is still no satisfactory improvement in the success of cancer treatment, although anticancer agents are constantly being introduced. Prevention research is a promising approach to reduce cancer incidence. In particular, chemoprevention using herbal medicine has been emerging due to its anticancer activity. Several medicinal plants and herbs, and their various phytochemical constituents including phenolics, flavonoids, carotenoids, and alkaloids, confer protective effects against a wide range of cancers [2].

Morinda citrifolia L. (Rubiaceae), commonly called noni, is one of the traditional folk medicinal plants that has a long history of use in Polynesia. Noni has been reported to have a broad range of health benefits for cancer, infection, diabetes, asthma, cough, hypertension, pain, ulcers, wounds, hemorrhoids and rheumatoid arthritis [3,4]. Several mechanisms have been proposed to understand how noni manifests its anticancer effect, such as the inhibition of angiogenesis [5], prevention of carcinogen–DNA adduct formation [6] or activation of immune responses [7,8]. Subsequent studies using various animal models showed that noni has antitumorigenic activity *in vivo* [6,9,10].

Damnacanthal (Dam), an anthraquinone compound, was isolated from the roots of *M. citrifolia* L. and identified as a potent inhibitor of $p56^{lck}$ tyrosine kinase activity through high-volume screening of natural product extracts [11]. Dam also exhibits cytotoxic activity against breast cancer cell lines as well as small cell lung cancer cell lines [12]. In addition to its anticancer activity, Dam exhibits antifungal activity against *Candida albicans* and antituberculosis activity against *Mycobacterium tuberculosis* [12]. However, the molecular targets by which Dam acts against several pathophysiological diseases have not been elucidated.

Abbreviations: DMSO, dimethyl sulfoxide; C/EBPβ, CCAAT/enhancer binding protein β; ChIP, chromatin immunoprecipitation; NAG-1, nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1; RARα, retinoic acid receptor α; ERK, extracellular-signal-regulated kinase; dnERK, dominant-negative ERK.

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Nonsteroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1, also known as GDF15) is a member of the transforming growth factor β superfamily, which has proapoptotic and antitumorigenic activities [13]. It is believed that NAG-1 may serve as a molecular target of many phytochemicals [14]. Transgenic mice (*NAG-1*$^{Tg+/BL6}$, C57/BL6 background) expressing human NAG-1 have been developed by our group [15], and we have reported that *NAG-1*$^{Tg+/BL6}$ mice are resistant to chemically and genetically induced intestinal polyp formation [15] as well as lung tumors and inflammation [16]. These data indicate that NAG-1 plays a pivotal role in colon and lung tumorigenesis *in vivo*. It also has been reported that several transcription factors contribute to NAG-1 expression at the transcription level, including EGR-1 [17], p53 [18], CCAAT/enhancer binding protein β (C/EBPβ) [19] and ATF3 [20].

In the present study, we have shown that Dam exhibits antitumorigenic activity as assessed by several *in vitro* assays. We report a novel finding of C/EBPβ induction by Dam, followed by NAG-1 induction in human colorectal cancer cells. This sequential pathway seems to be mediated by the extracellular-signal-regulated kinase (ERK) pathway.

2. Materials and methods

2.1. Materials

Human colorectal cancer cell lines (HCT-116, SW480 and LoVo) were purchased from American Type Culture Collection (Manassas, VA, USA). The cell culture media were purchased from Hyclone (Logan, UT, USA) and Mediatech (Herndon, VA, USA). NAG-1 antibody was previously described [13], and cyclin D1, p53, C/EBPβ and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The p21 antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). All chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA), unless otherwise specified.

2.2. Damnacanthal

Damnacanthal was extracted from the root of *M. citrifolia* L. in Thailand, as previously reported [21]. The isolated pure compound was verified by various methods: (a) comparison of R_f value with reference standard checked by thin-layer chromatography, (b) measuring the melting point of samples compared to the previous report and (c) investigating mass spectrum data by the mass spectroscopy with LC system coupled to a MicrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) and determining ^{1}H NMR and ^{13}C NMR spectra determination by an INOVA-500 nuclear magnetic resonance spectroscopy.

2.3. Cell culture

HCT-116 cells were cultured in McCoy's 5A medium. SW480 was maintained in RPMI 1640 medium, and LoVo cells were maintained in Ham's F-12 medium. All culture media were supplemented with 10% fetal bovine serum (Hyclone), 50 U/ml penicillin and 50 μg/ml streptomycin.

2.4. Human NAG-1 promoters and expression vectors

The *NAG-1* promoters and internal deletion constructs were described previously [19,22]. The *C/EBPβ*, *C/EBPα*, *C/EBPδ*, *RARα* expression vectors were also described previously [19], as were the wild-type and dominant-negative constructs of *ERK2* [23].

2.5. Cell proliferation analysis

The effect of Dam on cell proliferation in HCT-116 and SW480 cells was investigated using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Cells were seeded at the concentration of 1000 cells/well for HCT-116 and 2000 cells/well for SW480 in 96-well tissue culture plates in four replicates. The cells were then treated with 100 nM and with 1, 10 and 100 μM of Dam in the presence of serum. At 1, 2 and 4 days after treatment, 20 μl of CellTiter 96 Aqueous One Solution was added to each well, and the plate was incubated for 1 h at 37°C. Absorbance at 490 nm was recorded in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.6. Cell cycle analysis

Cells were plated at 4×10^5 cells/well in six-well plates, incubated for 24 h and then treated with 1, 10 and 50 μM of Dam in the presence of serum for 72 h. The cells (attached and floating cells) were then harvested, washed with phosphate-buffered saline (PBS), fixed by the slow addition of 1 ml cold 70% ethanol and stored at −80°C. The fixed cells were pelleted, washed with 50% and 20% ethanol, followed by PBS, and stained with 0.5 ml of 70 μM propidium iodide containing 1 mg/ml RNase in PBS for 15 min at room temperature. Twenty thousand cells were examined by flow cytometry, using a Becton Dickinson fluorescence-activated cell sorter equipped with CellQuest software, by gating on an area vs. width dot plot to exclude cell debris and cell aggregates.

2.7. Caspase 3/7 enzyme activity

Enzyme activity of caspase 3/7 was analyzed using the Caspase-Glo 3/7 Assay kit (Promega) according to the manufacturer's protocol. The cells were harvested with radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors, and the same volume of Caspase-Glo 3/7 reagent was added to the cell lysates (30 μg protein) in 96-well plates and incubated at room temperature in the dark for 1 h. The luminescence was measured using a FLX800 microplate reader (BioTek).

2.8. Clonogenic assay

The effect of Dam on HCT-116 cell colony formation was investigated using clonogenic assay. HCT-116 cells were plated (in triplicates) in 12-well plates at 300 cells per well. After 24 h, cells were then treated with dimethyl sulfoxide (DMSO) or with 1, 10 and 50 μM of Dam in the presence of serum. Plates were incubated for 2 weeks at 37°C. Cell colonies were fixed with 0.4 ml of 100% methanol for 5 min and stained with 0.4 ml of Giemsa solution (Acros Organics, Fair Lawn, NJ, USA) for 5 min. The colonies were counted by using Colony Version 1.1 program (Fuji Photo Film Co., Stamford, CT, USA).

2.9. Soft agar cloning assay

Soft agar assays were performed to compare the clonogenic potential of HCT-116 cells in semisolid medium. HCT-116 cells at the concentration of 3000 cells per well were resuspended in 2 ml of 0.4% agar in McCoy's 5A medium in the presence of DMSO or 1, 10 or 50 μM of Dam and plated on top of 2 ml of 0.8% agar in six-well plates. Plates were incubated for 2 to 3 weeks at 37°C. Cell colonies were visualized by staining with 0.5 ml of *p*-iodonitrotetrazolium violet (Sigma, Germany). The colonies were counted by using Colony Version 1.1 program (Fuji Photo Film Co.).

2.10. Cell migration assay

HCT-116 cells were plated in 12-well plates at a density of approximately 2.5×10^5 cells/well and grown to confluency. Cell monolayer was scratched with a sterile pipette tip, and the cells were treated with DMSO or 1, 10 and 50 μM of Dam. Wound size was monitored at various time points by observation under microscope, and the degree of cell migration was quantified by the ratio of gap distance at 48 h to that at 0 h.

2.11. Western blot analysis

Cells were grown to 60%–80% confluence in 6-cm plates followed by 24-h treatment of various concentrations of Dam or DMSO as vehicle control in the absence of serum. Total cell lysates were isolated using RIPA buffer (1×PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin and 5 μg/ml leupeptin) and phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF). The protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard. Thirty or fifty micrograms of protein was separated on sodium dodecyl sulfate–polyacrylamide gel and transferred for 90 min onto a nitrocellulose membrane (Pall Life Sciences, Pensacola, FL, USA). The blots were blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T) and probed with a specific primary antiserum in TBS-T and 5% nonfat dry milk at 4°C overnight. After three washes with TBS-T, the blots were incubated with horseradish-peroxidase-conjugated secondary antibody for 1 h and washed several times. Proteins were detected by the enhanced chemiluminescence system (Thermo Scientific, Rockford, IL, USA).

2.12. Plasmid transient transfections

Transient transfections were performed using PolyJet (Signagen Laboratories, Ijamsville, MD, USA) according to the manufacturer's protocol. Briefly, HCT-116 cells were plated in 12-well plates at the concentration of 2×10^5 cells per well. The next day, plasmid mixtures containing 0.75 μg of the reporter gene and 0.075 μg of *pRL-null* vector were co-transfected for 24 h. For the co-transfection experiment, 0.375 μg of *NAG-1* promoter and 0.375 μg of expression vectors were co-transfected with 0.075 μg of *pRL-null* vector. The transfected cells were then incubated with DMSO or 10 μM of Dam for 24 h. For the ERK kinase inhibitor experiment, 0.75 μg of *NAG-1* promoter and 0.075 μg of *pRL-null* vector were co-transfected for 24 h, and the transfected cells were pretreated with 1 or 5 μM of U0126 (ERK inhibitor) for 30 min. The pretreated cells were then incubated with DMSO or 20 μM of Dam for 24 h. Cells were harvested in 1×

luciferase lysis buffer (Promega), and luciferase activity was measured and normalized to the *pRL-null* luciferase activity using a dual luciferase assay kit (Promega).

2.13. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as described previously [19].

2.14. RNA interference

Interference for C/EBPβ was performed as described previously [19]. Briefly, 2.5 μg of control shRNA or *C/EBPβ* shRNA was transfected using PolyJet (Signagen Laboratories) for 48 h. The transfected cells were treated with either DMSO or 20 μM Dam in serum-free media for 24 h, and the cells were then harvested using RIPA buffer.

2.15. Statistical analysis

Statistical analysis was performed with the Student's *t* test, with statistical significance set at **P*<.05, ***P*<.01 and ****P*<.001.

3. Results

3.1. Dam inhibits cell proliferation and induces caspase activity in colorectal cancer cell

We first investigated the antiproliferative effects of Dam on human colorectal carcinoma, HCT-116 and SW480 cells. Cells were treated with 100 nM or with 1, 10 or 100 μM Dam for 1, 2 and 4 days. Treating HCT-116 cells with Dam resulted in a significant reduction of cell proliferation at 1 μM (*P*<.05), 10 μM (*P*<.01) and 100 μM (*P*<.01) after 4 days of treatment compared to DMSO-treated cells. SW480 cells also showed a significant reduction of cell proliferation at 10 μM (*P*<.05) and 100 μM (*P*<.001) after 4 days of treatment with Dam (Fig. 1A). These data suggest that Dam has a cytotoxic effect on colorectal cancer cells, both in p53 wild-type (HCT-116) and p53-mutated (SW480) cells. Based on preliminary data in which we observed a strong growth inhibitory effect of Dam in human colorectal cancer cells, we selected doses of 1, 10 and 50 μM to determine the possible inhibitory effect of Dam on cell cycle progression and apoptosis. As shown in Fig. 1B, treatment of HCT-116 cells with Dam resulted in a significant enrichment in the number of cells in the S/G_1 and G_2/G_1 phases at concentration of 50 μM compared to DMSO-treated cells. To observe whether Dam affects apoptosis in human colorectal carcinoma cells, we treated HCT-116 cells with DMSO or 10 μM Dam, and caspase 3/7 activity was measured. As shown in Fig. 1C, 10-μM Dam treatment significantly increased caspase 3/7 activity (3.2-fold, *P*<.05) compared to that of DMSO. These results support that Dam induces cell cycle arrest and caspase activity in HCT-116 cells.

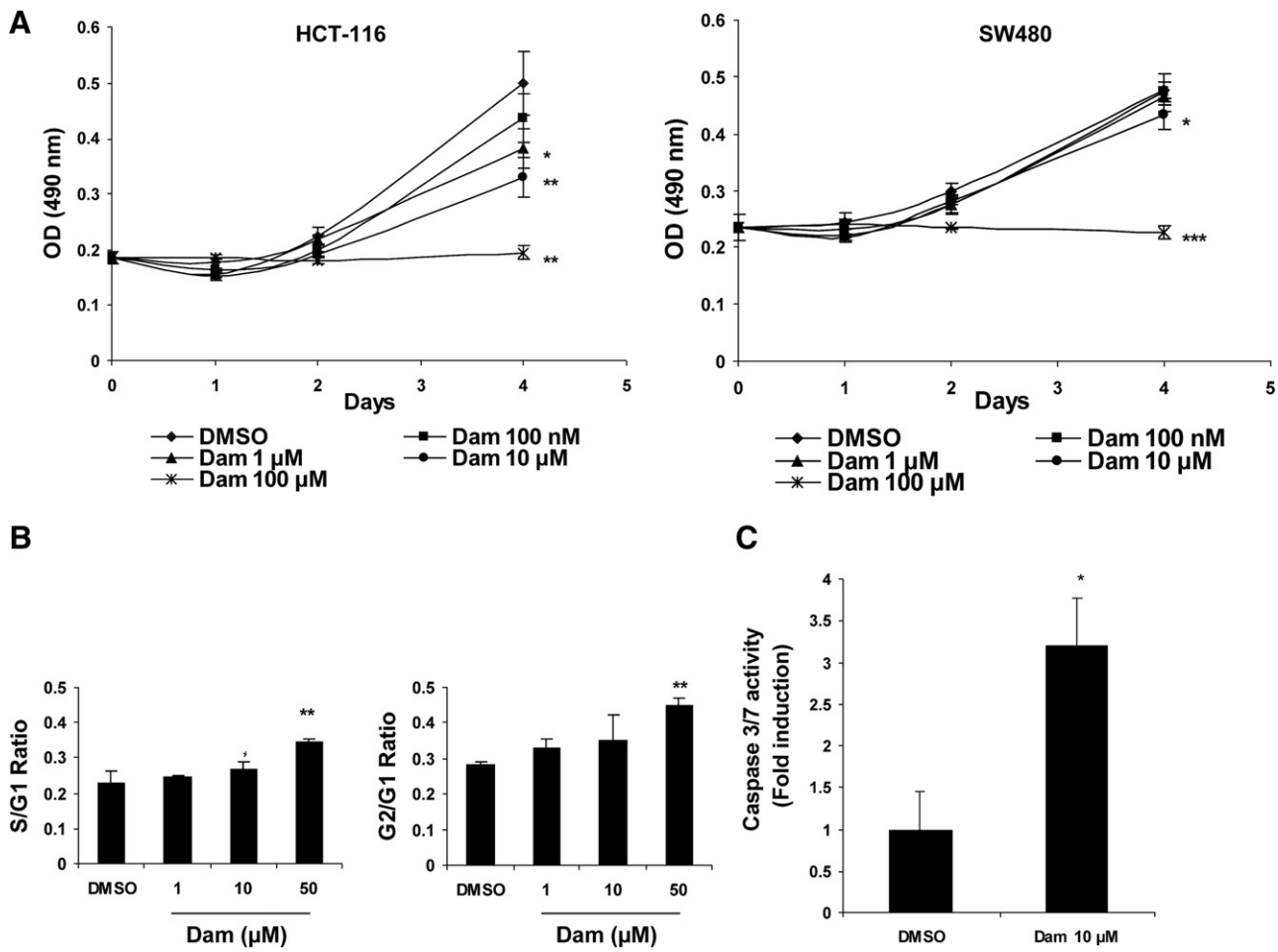

Fig. 1. Damnacanthal inhibits cell proliferation and induces caspase activity in colorectal cancer cells. (A) HCT-116 (left panel) and SW480 (right panel) cells were treated with DMSO or various concentrations of Dam for 4 days. Cell growth was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Values are expressed as mean±S.D. of four replicates. **P*<.05, ***P*<.01, ****P*<.001 vs. DMSO-treated cells. (B) Flow cytometric analysis of DMSO- or Dam-treated HCT-116 cells. Cell were treated with DMSO or 1, 10 and 50 μM of Dam for 72 h and analyzed for cell cycle progression as described in Materials and Methods. S/G_1 and G_2/G_1 ratios are expressed as mean±S.D. of three replicates. **P*<.05, ***P*<.01 vs. DMSO-treated cells. (C) Caspase 3/7 enzymatic activity was measured as described in Materials and Methods. HCT-116 cells were treated with DMSO or 10 μM of Dam for 24 h. The *y*-axis represents fold induction over DMSO-treated cells. Values are expressed as mean±S.D. of three replicates. **P*<.05 vs. DMSO-treated cells.

3.2. Antitumorigenic activity of Dam

Antitumorigenic activity of Dam was investigated using several assays including cloning efficiency, soft agar and cell migration assays. As shown in Fig. 2A, Dam treatment for 2 weeks showed significant decreasing colony number in HCT-116 cells in a concentration-dependent manner, at concentrations of 10 μM (18 colonies, $P<.05$) and 50 μM (five colonies, $P<.01$), compared to that of DMSO (43.33 colonies). We also investigated anchorage-independent cell growth by the soft agar cloning assay because the ability to form colonies in soft agar is reflective of tumorigenicity. Dam-treated cells showed a dramatic inhibition of clonogenic capacity at 10 μM (286.33 colonies, $P<.01$) and 50 μM (105.67 colonies, $P<.001$) compared to DMSO treatment (576 colonies) (Fig. 2B). Furthermore, cell migration was investigated. HCT-116 cells were plated in a 12-well plate, grown to confluency and scratched and treated with DMSO or with 1, 10 and 50 μM of Dam as described in Materials and Methods. The migration ratio was documented by photography at 0 and 48 h. As shown in Fig. 2C, Dam-treated cells significantly inhibited the migration of HCT-116 cells at 1 μM (0.41, $P<.01$), 10 μM (0.46, $P<.001$) and 50 μM (0.78, $P<.001$) compared to DMSO treatment (0.26). These results indicate that Dam exhibits antitumorigenic activity as assessed by several *in vitro* assays.

3.3. Dam induces NAG-1 expression in HCT-116 cells

To elucidate the molecular mechanism by which Dam affects antitumorigenesis in colorectal cancer cells, HCT-116 and SW480 cells were treated with 0.1, 1 or 10 μM of Dam for 24 h. As shown in Figs. 3A and B, NAG-1 was induced in HCT-116 cells in a dose- and time-dependent manner. Cyclin D1 expression was reduced at 10 μM of Dam, whereas p21 and p53 did not alter their expression in the presence of Dam. PARP cleavage was seen at 10-μM Dam treatment only in HCT-116 cells, where NAG-1 is induced. In contrast, NAG-1 was not increased in SW480 cells, nor was PARP cleavage increased. Dam treatment also increased NAG-1 expression in a time-dependent manner, wherein we were beginning to see NAG-1 induction at 2 h and saw it highly increased at 24 h (Fig. 2B). NAG-1 induction was not only in HCT-116 cells, but also in LoVo (p53 wild type) cells (Fig. 3C).

3.4. C/EBPβ mediates Dam-induced NAG-1 expression

Because phytochemicals increase several transcriptional factors that play an important role in NAG-1 expression [19,24,25], we next investigated whether Dam activated NAG-1 at the transcriptional level. We first performed a deletion assay using the four serial

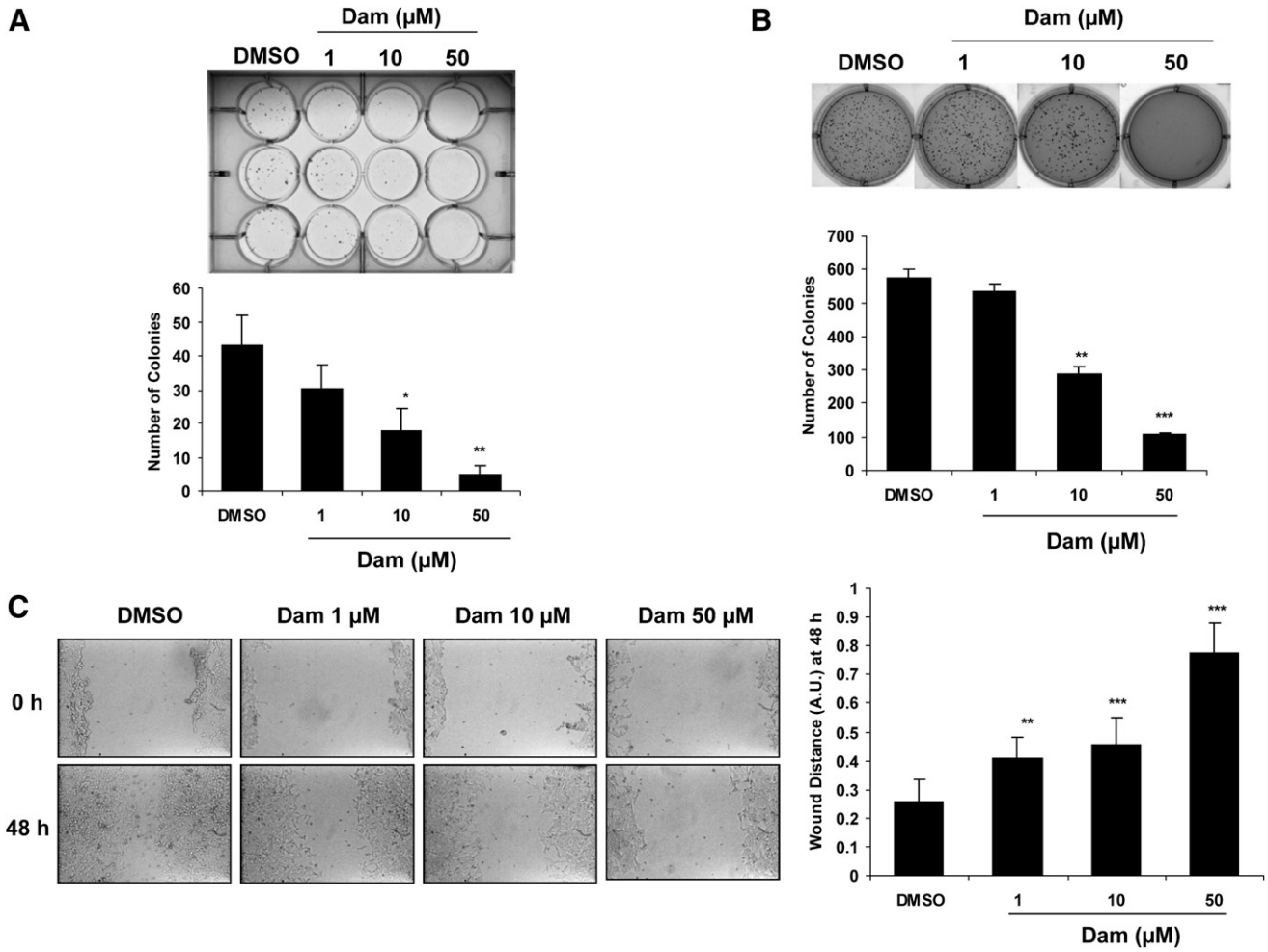

Fig. 2. Antitumorigenic activity of Dam. (A) Clonogenic assay. HCT-116 cells were treated with DMSO or 1, 10 and 50 μM of Dam for 2 weeks, and cell colonies were stained and counted. The data represent mean±S.D. of three replicates. *$P<.05$, **$P<.01$ vs. DMSO-treated cells. (B) Soft agar cloning assay. HCT-116 cells were grown in 0.4% soft agar in the presence of DMSO or 1, 10 or 50 μM of Dam for 2 weeks, and then cell colonies were stained and counted. Values are expressed as mean±S.D. of three replicates. **$P<.01$, ***$P<.001$ vs. DMSO-treated cells. (C) Cell migration assay. HCT-116 cells were plated, scratched and treated with DMSO or 1, 10 and 50 μM of Dam. Wound size was monitored at 0 and 48 h by observation under an optical microscope (left panel) at 100× magnification. The degree of cell migration was quantified by the ratio of gap distance at 48 h to that at 0 h (right panel). Values are expressed as mean±S.D. of three replicates. **$P<.01$, ***$P<.001$ vs. DMSO-treated cells.

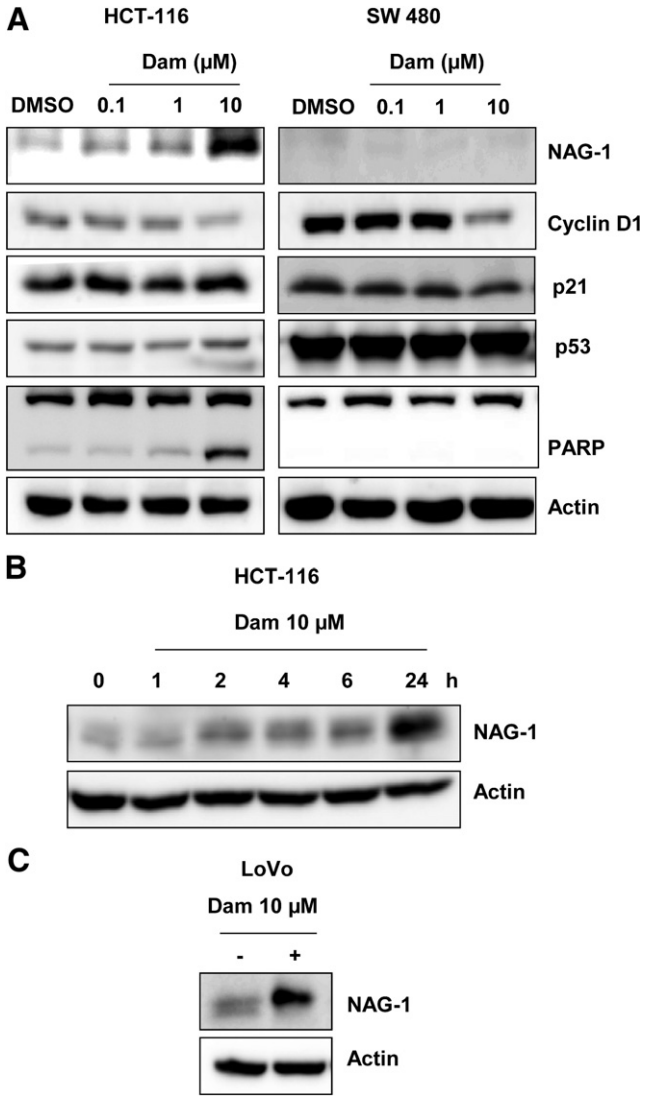

Fig. 3. Damnacanthal induces NAG-1 expression in HCT-116 cells. (A) HCT-116 (left panel) and SW480 (right panel) cells were treated with DMSO or with 0.1, 1 and 10 μM of Dam for 24 h, and Western blot was performed for NAG-1, cyclin D1, p21, p53, PARP and actin. (B) HCT-116 cells were treated with 10 μM of Dam. At the indicated times, the cell lysates were harvested to perform Western blot analysis for NAG-1 and actin. (C) LoVo cells were treated with DMSO or 10 μM of Dam for 48 h, and Western blot was performed for NAG-1 and actin.

deletion constructs of human *NAG-1* promoter (*pNAG-1*-3500/+41, *pNAG-1*-1086/+41, *pNAG-1*-474/+41 and *pNAG-1*-133/+41) to observe whether Dam affects transcriptional regulation of the *NAG-1* gene. *NAG-1* promoters were transfected into HCT-116 cells and treated with 10 μM of Dam for 24 h. As shown in Fig. 4A, Dam treatment resulted in the stimulation of *NAG-1* promoter activity in all the constructs tested, implying that the −133 and +41 region of the promoter is responsible for Dam's effect.

It has been reported that there are several *cis*-acting elements in this region, including C/EBPs, RARα, XF1 and FKMR [22]. To confirm the responsible site for the transactivation of *NAG-1* gene by Dam, we utilized internal deletion clones for C/EBPs, RARα, XF1 and FKMR. HCT-116 cells were transfected with wild-type or internal-deletion constructs, and treated with 10 μM of Dam for 24 h. As shown in Fig. 4B, none of the internal deletion clones, including C/EBP, FKMR, RARα and XF1, showed significant differences in Dam-induced promoter activity compared to wild-type promoter. Interestingly, transfection with double deletion clone lacking two potential C/EBP binding sites (CEBP1 and CEBP2) abolished the basal and Dam-induced promoter activity. These data suggest that these two regions on the C/EBP binding site are important for *NAG-1* promoter activation by Dam treatment.

To find the *trans*-acting elements responsible for Dam-induced *NAG-1* transactivation, we tested whether overexpression of different types of C/EBP or RARα affects *NAG-1* transactivation. We co-transfected expression vectors for *C/EBPβ1*, *C/EBPβ2*, *C/EBPα1*, *C/EBPδ* and *RARα* with the *NAG-1* promoter constructs (*pNAG-1*-133/+41), and promoter activity was determined. As shown in Fig 4C, C/EBPβ1, C/EBPβ2 and RARα expression showed a significant increase of Dam-induced promoter activity, whereas C/EBPα and C/EBPδ expression slightly decreased Dam-induced promoter activity (Fig. 4C). To confirm the binding of C/EBPβ to this site, the ChIP assay was performed using C/EBPβ antibody. As shown in Fig. 4D, C/EBPβ binds to the −133/+41 region of the *NAG-1* promoter, whereas rabbit control IgG did not pull down any protein to this region. Taken together, these results indicate that two C/EBPβ binding sites (CEBP1 and CEBP2) are pivotal *cis*-acting elements to confer Dam-induced NAG-1 expression.

3.5. Effect of kinase inhibitors on NAG-1 expression

Transcriptional activity of C/EBPβ is regulated by its translation or posttranslational modulation [26,27]. To examine whether Dam increases C/EBPβ expression, HCT-116 cells were treated with DMSO or with 0.1, 1, 10 or 20 μM of Dam for 24 h, and Western blot was performed using C/EBPβ specific antibody. As shown in Fig. 5A, C/EBPβ protein increased in HCT-116 cells in a dose-dependent manner. To explore the upstream molecular mechanism involved in Dam-induced NAG-1 and C/EBPβ expression, we examined several signaling pathways that are affected by Dam. We screened several kinase-specific inhibitors at a concentration that does not deviate from their selectivity. HCT-116 cells were pretreated with kinase inhibitors for 30 min, prior to incubation with 20 μM of Dam. As shown in Fig. 5B, the Dam-induced NAG-1 expression was strongly suppressed in the presence of U0126 (ERK inhibitor) and RO318220 (PKC inhibitor). In addition, we found that C/EBPβ was also suppressed by U0126 and RO318220. These results suggest roles of the ERK and PKC protein kinase pathways in Dam-induced NAG-1 and C/EBPβ expression. The construct *pNAG-1*-133/+41 was further transfected into HCT-116 cells and treated with 1 or 5 μM of U0126 (ERK inhibitor) for 30 min prior to incubation with 20 μM of Dam. As shown in Fig. 5C, *NAG-1* promoter activity is diminished in the presence of U0126 compound in a dose-dependent manner. This result demonstrates that Dam affects ERK activity, followed by the induction of NAG-1 expression at the transcriptional level.

To confirm the possible regulatory effect of ERK on Dam-induced NAG-1 expression, HCT-116 cells were transfected with empty vector, *ERK2* wild type, or dominant-negative ERK2 and treated with 10 μM of Dam for 24 h. As shown in Fig. 5D, transfection with dominant-negative ERK suppressed Dam-induced NAG-1 expression compared to control empty vector and *ERK2* wild-type transfected cells with Dam treatment. Similar results were obtained in C/EBPβ expression. These results indicated that Dam affects ERK2 activity, followed by the induction of C/EBPβ and NAG-1 expression in HCT-116 cells.

3.6. C/EBPβ and NAG-1 are involved in Dam-induced apoptosis

To explore whether C/EBPβ expression contributes to Dam-induced apoptosis, we performed knockdown of the endogenous C/EBPβ gene by RNA interference and analyzed apoptosis-related caspase 3/7 activity. HCT-116 cells were transfected with shRNA for *C/EBPβ*. As shown in Fig. 6A, transfection with *C/EBPβ* shRNA clearly suppressed Dam-induced C/EBPβ. Moreover, we also explored NAG-1

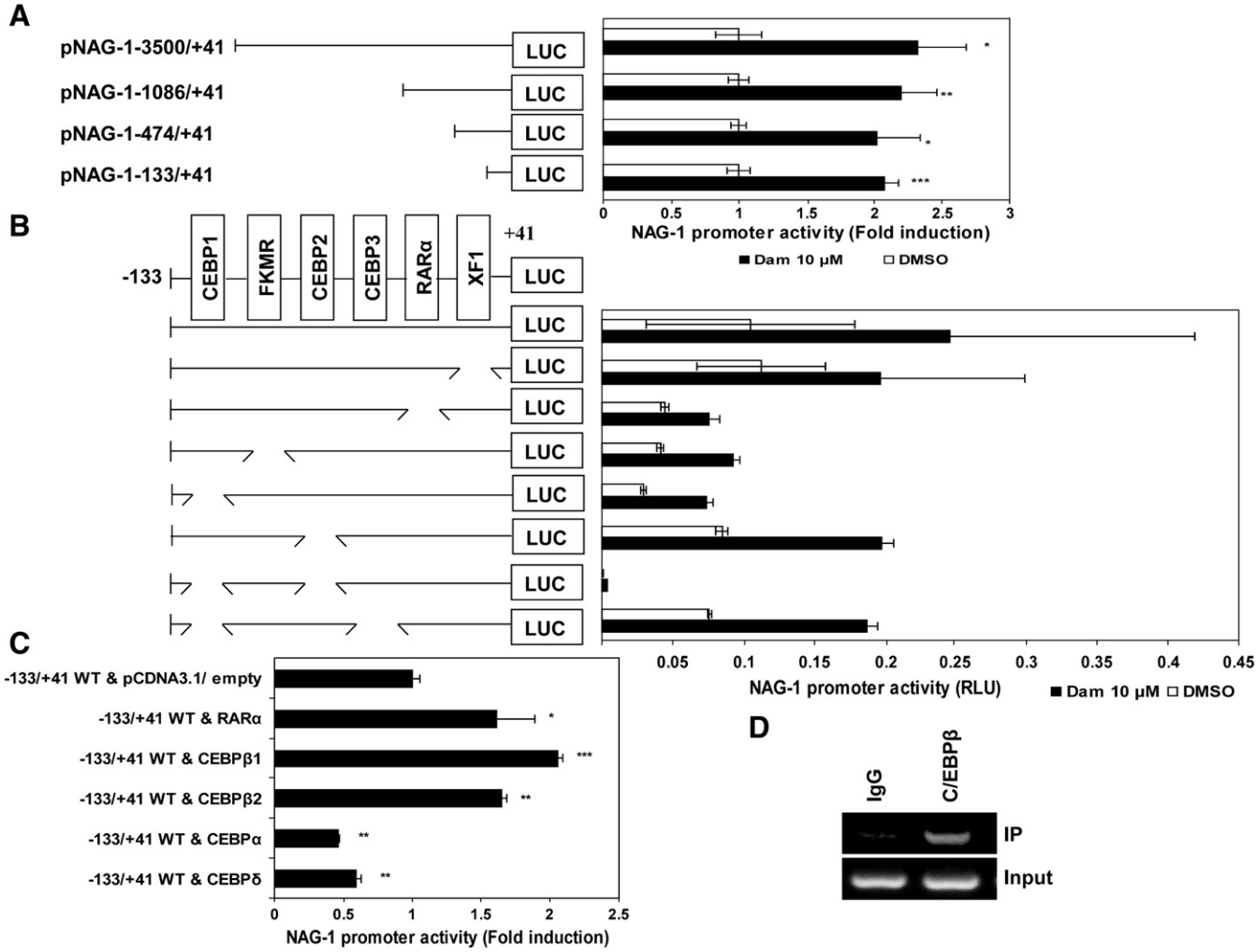

Fig. 4. Identification of the promoter region responsible for Dam-induced *NAG-1* transactivation. (A) Each indicated construct of the *NAG-1* promoter and *pRL-null* vector was transiently transfected into HCT-116 cells and then treated with DMSO or 10 μM of Dam for 24 h. The promoter activity was measured as a ratio of firefly luciferase signal/renilla luciferase signal. Fold induction refers to ratio of luciferase (LUC) activity of Dam-treated cells compared to DMSO-treated cells of each construct. The results are the mean±S.D. of three replicates. **P*<.05, ***P*<.01, ****P*<.001 vs. DMSO-treated cells. (B) HCT-116 cells were transfected with each internal deletion construct of the *NAG-1* promoter (pNAG-1-133/+41) and then treated with 10 μM of Dam for 24 h. The *x*-axis shows relative luciferase unit of each construct. RLU, relative luciferase unit. (C) The *NAG-1* promoter (pNAG-1-133/+41) was co-transfected with indicated expression vector as described in Materials and Methods and then treated with 10 μM of Dam for 24 h. The *x*-axis shows fold induction over empty vector. **P*<.05, ***P*<.01, ****P*<.001 vs. control vector-treated cells. (D) Chromatin immunoprecipitation assay was performed using a DNA–protein complex treated with 20 μM of Dam for 24 h as described in Materials and Methods. The sequence of the *NAG-1* promoter region (−131/+137) was amplified by PCR primer pairs as described previously [19]. The input represents PCR products obtained from 1% aliquots of chromatin pellets escaping immunoprecipitation.

expression in *C/EBPβ* shRNA- transfected cells. In the presence of 20 μM of Dam, *NAG-1* expression was decreased in *C/EBPβ* shRNA-transfected cells compared to control shRNA transfected cells with Dam treatment. Treatment of *C/EBPβ* shRNA-transfected cells with Dam decreased caspase 3/7 activity (1.5-fold, *P*<.001) compared to control shRNA-transfected cells with Dam treatment (3.3-fold) (Fig. 6B), indicating an important role of C/EBPβ in Dam-induced caspase activity.

4. Discussion

Cancer is second only to heart disease as the leading cause of death in the United States, and colorectal cancer is one of the most prevalent causes of cancer-related mortality in the Western world [28]. Further development of therapeutic and preventive means of controlling this disease is clearly needed. Epidemiological studies have suggested that phytochemicals found in dietary plant products, including fruits, vegetables, beverages, herbs and spices, play an important role in carcinogenesis, and a number of these compounds have been found to inhibit tumorigenesis in experimental animals and/or exhibit potent biological properties [29]. One such phytochemical is Dam, an anthraquinone and a naturally occurring component of noni root. In this study, the cell proliferation assay showed that Dam treatment results in the inhibition of cell growth compared to vehicle-treated cells in two different colorectal cancer cells (Fig. 1). Cell growth retardation properties of Dam are more evident by the soft agar cloning, clonogenic and cell migration assays (Fig. 2). All these results show that Dam increased antitumorigenic activity by increased expression of C/EBPβ, followed by NAG-1 expression.

NAG-1 is a proapoptotic protein that exhibits antitumorigenic activity in culture cells as well as in animal models [15,22]. NAG-1 transgenic mice (overexpression) formed fewer tumors in carcinogenic- and genetic-induced animal models [15]. Interestingly, NAG-1 is induced by many phytochemicals, having antitumorigenic and anti-inflammatory activities [14]. Therefore, NAG-1 may be an important protein that controls compound-induced antitumorigenesis as well as anti-inflammation. Indeed, not only phytochemicals but also drugs that show an anti-inflammation property increase NAG-1 expression

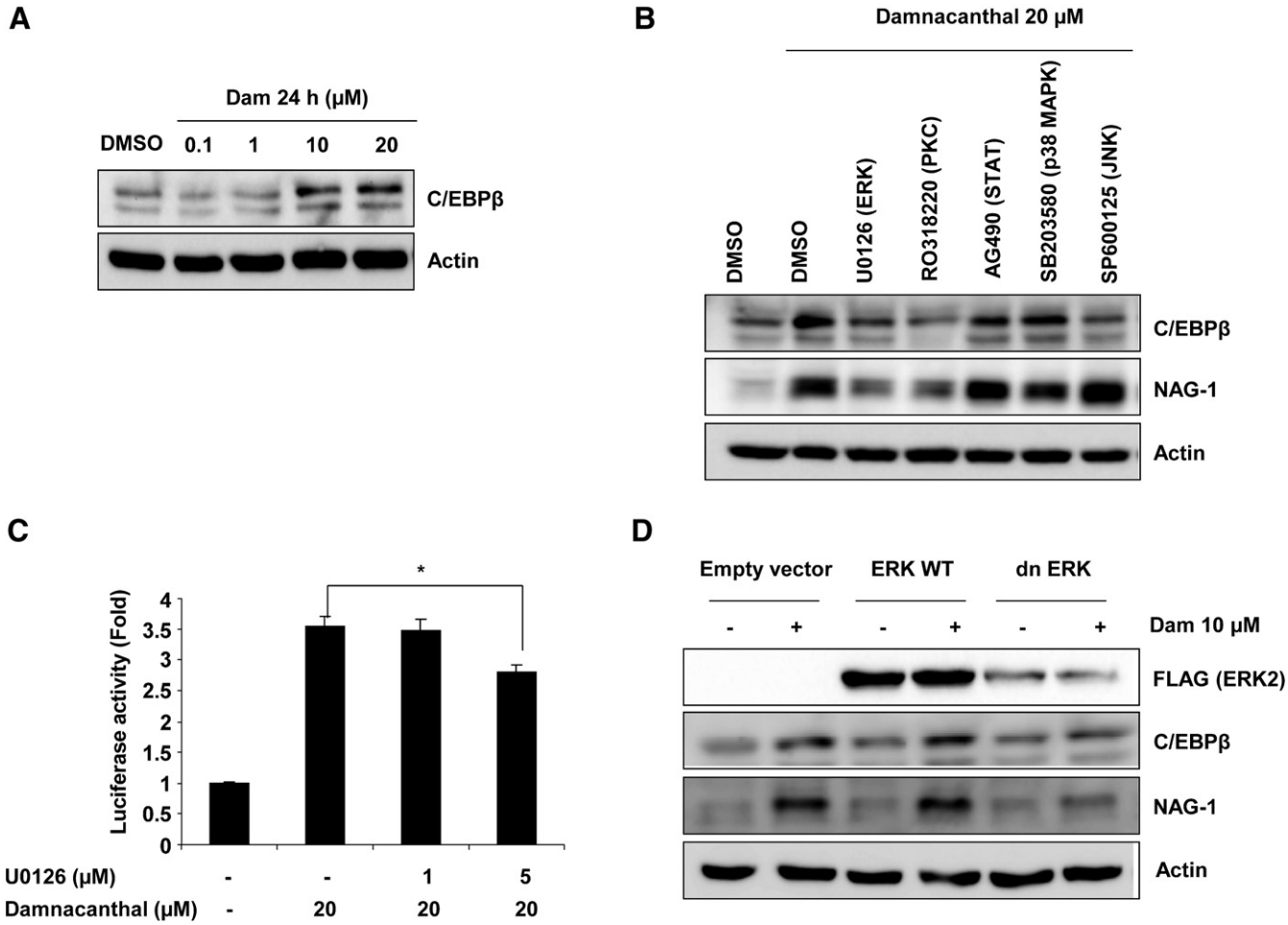

Fig. 5. ERK and PKC mediate C/EBPβ and Dam-induced NAG-1 expression. (A) HCT-116 cells were treated with DMSO or 0.1, 1, 10 and 20 μM of Dam for 24 h, and Western blot was performed for C/EBPβ and actin as described in Materials and Methods. (B) HCT-116 cells were pretreated with different kinase inhibitors (U0126, 5 μM; RO318220, 2.5 μM; AG490, 50 μM; SB203580, 15 μM; SP 600125, 30 μM) for 30 min and treated with 20 μM of Dam for 24 h. Western blot was performed for C/EBPβ, NAG-1 and actin. (C) The *NAG-1* promoter (pNAG-1-133/+41) and *pRL-null* vector were transfected for 24 h as described in Materials and Methods, pretreated with U0126 (1 or 5 μM) and then treated with DMSO or 20 μM of Dam for 24 h. The *y*-axis shows fold induction over DMSO-treated cells. *P<.05 vs. Dam 20-μM-treated cells. (D) HCT-116 cells were transfected with pcDNA 3.1 empty vector, *ERK2* wild type or a dominant-negative ERK2 as described in Materials and Methods, and treated with DMSO or 10 μM of Dam for 24 h. Western blot was performed for FLAG, C/EBPβ, NAG-1 and actin.

[30]. Thus, NAG-1 may be a molecular target protein for these compounds that control cell growth and apoptosis.

Many transcription factors have been identified that control NAG-1 transcriptional regulation. These include EGR-1, p53, ATF3 and PPARγ [14].

We observed transcriptional up-regulation of the *NAG-1* by Dam and identified three putative C/EBP binding sites (CEBP1, CEBP2 and CEBP3) in the *NAG-1* promoter (between −133 and +41). No single deletion of each C/EBP binding site changed Dam-mediated *NAG-1* transactivation. Interestingly, double deletion of C/EBP binding sites (CEBP1 and CEBP2) completely inhibited basal as well as Dam-induced promoter activity, indicating the simultaneous significant role of these two sites. In addition, co-transfection with expression vectors and ChIP assay indicates that C/EBPβ mediates *NAG-1* transactivation by direct binding to the *NAG-1* promoter. Recently, we have also shown that C/EBPβ also plays an important role in capsaicin-induced NAG-1 expression with ATF3, C/EBPβ and GSK-3β complexes [19]. These complexes are facilitated by capsaicin via PKCδ activation. Although we have shown that Dam affects NAG-1 expression through a C/EBPβ-dependent mechanism, the mechanism by which Dam up-regulates NAG-1 is different from that of capsaicin. First, Dam increases C/EBPβ expression, while capsaicin did not increase C/EBPβ expression. Rather, capsaicin induces C/EBPβ activity by phosphorylation [19]. Since the activity of C/EBPβ is also regulated by expression, overexpression of C/EBP by Dam may modulate cell growth and apoptosis by activating their target genes such as *NAG-1*. Our data support that NAG-1 is a Dam-regulating C/EBPβ target gene involved in apoptosis of human colorectal cancer cells. Secondly, Dam utilizes the ERK pathway to increase NAG-1 as well as C/EBPβ expression, while the effect of MEK inhibitor on capsaicin-induced NAG-1 expression is marginal [19]. Indeed, it has been shown that Dam increases ERK activity by phosphorylation [31] and that ERK plays an important role in C/EBPβ expression in colorectal cancer cells [32]. Thus, we propose that Dam affects ERK activity, followed by C/EBPβ expression. These sequential pathways may ultimately affect NAG-1 induction (Fig. 6C). ERK also mediates NAG-1 expression by different anticancer compounds [33,34]. Hiramatsu et al. screened tropical plant extracts for substances that induce normal morphology in K-ras^{ts}-NRK cells [21]. As a result, they isolated Dam from the chloroform extract of the root of noni and found that Dam was an inhibitor of Ras function. Taken together with our data, it is suggested that Dam alters several kinase pathways, leading to antitumorigenesis in cancer cells.

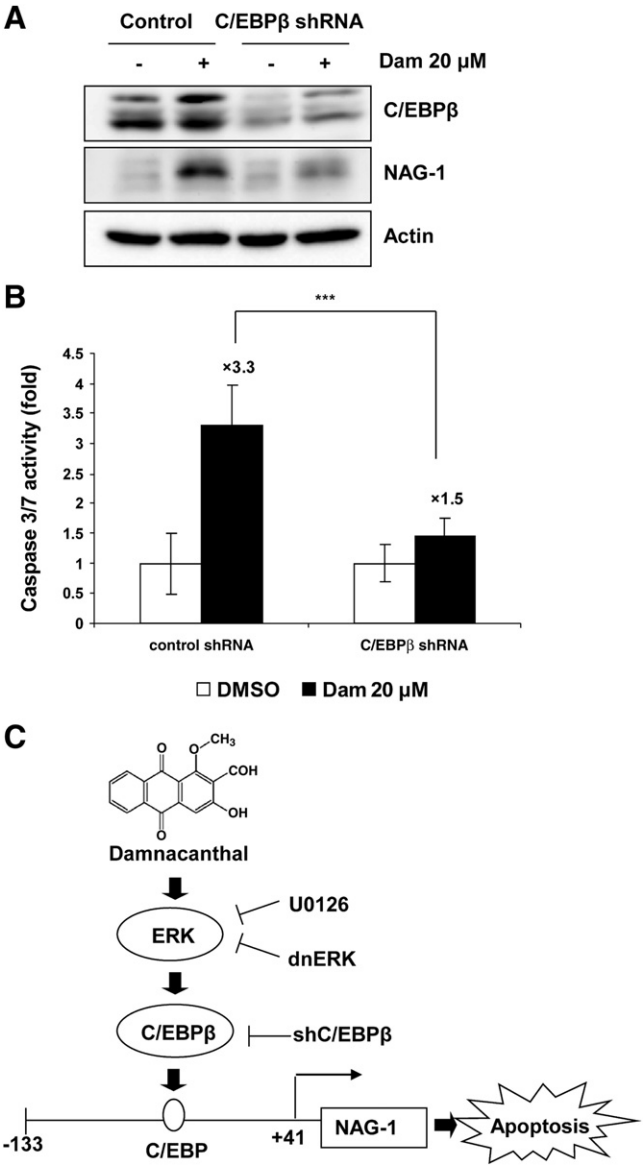

Fig. 6. Knockdown of C/EBPβ alters Dam-induced NAG-1 expression and apoptosis. (A) HCT-116 cells were transfected with control or *C/EBPβ* shRNA for 48 h and then treated with DMSO or 20 μM of Dam for 24 h. Western analysis was performed for C/EBPβ, NAG-1, and actin. (B) HCT-116 cells were transfected with control or *C/EBPβ* shRNA for 48 h and then treated with DMSO or 20 μM of Dam for 24 h. Caspase 3/7 enzymatic activity was measured as described in Materials and Methods. Values are expressed as mean±S.D. of three replicates. ***$P<.001$ vs. control shRNA-transfected Dam treatment cells. (C) Proposed mechanism by which Dam induces NAG-1 transcription in human colorectal cancer cells. Dam activates C/EBPβ through the ERK kinase pathway. Enhanced expression of C/EBPβ increases the binding affinity of C/EBPβ onto the *NAG-1* promoter and activates transcription of the *NAG-1* gene. Up-regulation of the *NAG-1* gene results in an increase of apoptosis in colorectal cancer cells.

It has been known that various types of transcription factors, including those in the GATA family and ATF/CREB family, directly bind to the C/EBP site, regulating target genes [19,35–38]. Although double deletion of a C/EBP binding sites or knockdown of C/EBPβ completely suppressed NAG-1 transactivation and protein expression (Fig. 4B, Fig. 6A), we do not exclude the possibility that other proteins modified by Dam may involve NAG-1 regulation by changing binding affinity to the C/EBP binding site. With respect to the biological activity of C/EBPβ in colorectal cancer, it has been known that C/EBPβ is a positive regulator of COX-2 [39,40]. However, a recent study showed a direct association between strong suppression of colon tumor growth and C/EBPβ overexpression in nude mice bearing CW-2 human colon cancer tumors [41]. Thus, detailed studies are required for pro- or antitumorigenic activity of C/EBPβ in colorectal carcinoma.

In conclusion, Dam, a bioactive compound from noni roots, activates ERK pathway and enhanced expression of C/EBPβ. Overexpression of C/EBPβ directly activates transcription and expression of NAG-1 and subsequently increases apoptosis in human colorectal cancer. These results will provide potential benefits of Dam for further preclinical or clinical practice, and Dam may be a useful cancer prevention/therapeutic agent in human colorectal carcinoma.

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