RESEARCH ARTICLE

2′-Hydroxyflavanone induces apoptosis through Egr-1 involving expression of Bax, p21, and NAG-1 in colon cancer cells

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Scope: Natural flavanones exhibit cancer preventive and/or therapeutic effects. The objective of this study was to investigate the molecular mechanism underlying the action of the antitumor activity of hydroxyflavanone using the HCT116 colon cancer cell line.
Methods and results: We investigated the effect of hydroxyflavanones on antitumor activity. We found that 2′-hydroxyflavanone (2′-HF) potently inhibited the clonogenicity of HCT116 cells. 2′-HF triggered apoptosis in both wild-type and p53-null HCT116 cells, as revealed by DNA fragmentation and caspase activation. 2′-HF upregulated nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1) expression through induction of Egr-1. Silencing of NAG-1 or Egr-1 using small interfering RNA (siRNA) could attenuate 2′-HF-induced apoptosis. Egr-1 also upregulated the proapoptotic gene Bax and the cell cycle inhibitor p21.
Conclusion: Dietary 2′-HF may possess antitumor activity against human colon cancer.

Keywords:
Apoptosis / Colon cancer / Egr-1 / Hydroxyflavanone / NAG-1

1 Introduction

Colorectal cancer continues to be one of the predominant cancers in both sexes globally. Certain risk factors such as heredity, age, smoking, inflammatory bowel disease, and diet exert a strong influence on the development of colorectal cancer. Ethnological studies have reported that a diet high in red meat and low in fresh fruit, vegetables, and fish increases the risk of colorectal cancer [1, 2]. It is generally recognized that dietary bioactive polyphenols have chemopreventive or therapeutic effects in colon cancer cells [3]. Flavonoids are one of the major classes of natural phenols that are widely distributed in fruits and vegetables. They can be divided into several classes including chalcones, flavonols, flavones, flavanones, anthocyanidins, and isoflavonoids. Flavonoids generally display a broad spectrum of pharmacological activities, including antioxidative and anti-inflammatory activities [4, 5]. Also, dietary flavonoids can reduce the risk of some malignant neoplasms and suppress tumor cell growth [3, 6, 7].

Among flavonoids, flavanones are present in most plants and are concentrated in seeds and fruit skin, and certain flavanones in food also have cancer-preventative effects. Naringenin, a trihydroxyflavanone, is abundant in grapefruit and other citrus fruits [8] and displays antitumor activity through induction of apoptosis in various cancer cells [9–11]. Hesperidin, a flavanone glycoside from orange juice, is known to have cancer-preventative effects [12]. Structure–activity studies have demonstrated that flavonoids with more hydroxyl groups exhibit increased antioxidant and anti-inflammatory activities [13]. Moreover, polyhydroxyflavonoids such as genistein (5,7,4′-trihydroxyisoflavone), apigenin (5,7,4′-trihydroxyflavone), and quercetin (3,3′,4′,5,7-pentahydroxyflavone) are effective inducers of apoptosis in diverse cancer cells [3,14,15]. However, flavanones containing no or a monohydroxyl group are even more potent inhibitors of colon cancer cell proliferation than are polyhydroxy-

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Abbreviations: HF, hydroxyflavanone; **NAG-1**, nonsteroidal anti-inflammatory drug-activated gene 1; **PARP**, poly(ADP-ribose) polymerase; **PI**, propidium iodide

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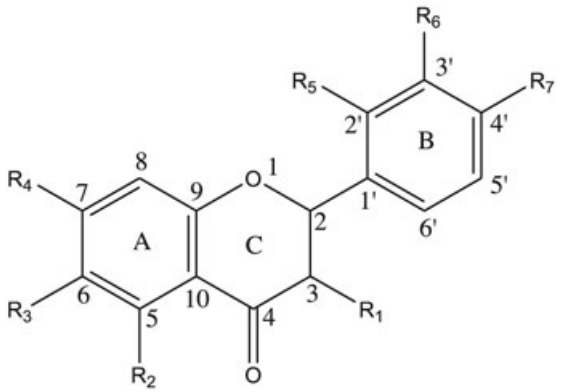

common name*	R_1	R_2	R_3	R_4	R_5	R_6	R_7
2'-Hydroxyflavanone	H	H	H	H	OH	H	H
3'-Hydroxyflavanone	H	H	H	H	H	OH	H
4'-Hydroxyflavanone	H	H	H	H	H	H	OH
6-Hydroxyflavanone	H	H	OH	H	H	H	H
7-Hydroxyflavanone	H	H	H	OH	H	H	H
4',5,7-trihydroxyflavanone (Naringenin)	H	OH	H	OH	H	H	OH
(2R,3R)-3,3',4',5,7-pentahydroxyflavanone (Taxifolin)	OH	OH	H	OH	H	OH	OH

* The stereochemistry of all derivatives at C-2 is Rectus.

Figure 1. Chemical structures and nomenclature of flavanone derivatives.

flavanones [16, 17]. But the detailed molecular mechanisms underlying the effects of hydroxyflavanones remain unclear.

In the present study, we investigated the effect of hydroxyflavanones on the induction of apoptosis using the HCT116 colon cancer cell line. Our data demonstrated that 2′-hydroxyflavanone (2′-HF) potently inhibited the clonogenicity of HCT116 cells, probably due to induction of caspase-mediated apoptosis through upregulation of the proapoptotic transcription factor Egr-1 and its downstream targets Bax, p21, and nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1).

2 Materials and methods

2.1 Materials

HCT116 and SW620 human colon cancer cells, MCF7 human breast cancer cells, and FHC human fetal normal colonic cells (CRL-1831) were obtained from the American Type Culture Collection (Rockville, MD). p53-null HCT116 cells (HCT116/p53$^{-/-}$) and their parent cells (HCT116/p53$^{+/+}$) were kindly provided by Dr. Bert Vogelstein (John Hopkins University School of Medicine, Baltimore, MD). Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT). All flavanone derivatives (Fig. 1) were purchased from Indofine Chemical Co. (Hillsborough, NJ). Rabbit polyclonal antibodies against p21, Egr-1, and glyceraldehyde phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cleaved-caspase-3, cleaved-caspase-7 (Asp198), poly(ADP-ribose) polymerase (PARP), and p53 were obtained from Cell Signaling Technology (Beverly, MA). The Alexa Fluor 555-conjugated secondary antibody was obtained from Invitrogen (Carlsbad, CA). The Dual-Glo® Luciferase Assay System for firefly and *Renilla* luciferase activities was purchased from Promega (Madison, WI).

2.2 Colony formation assay

For long-term survival assay, mid-log phase cultured HCT116 cells were plated onto 24-well tissue culture plates (1×10^3 cells/well) and treated with different concentrations (5, 10, 15, and 20 μM) of various HF compounds for 7 days. The cells were then fixed with 6% glutaraldehyde and stained as described previously [18].

2.3 Quantification of apoptosis by flow cytometry

For quantification of apoptosis, cellular DNA content was analyzed by flow cytometry as described previously [18]. Briefly, HCT116 cells (1×10^5 cells/sample) were treated with 20 μM 2′-HF for 24 or 48 h, fixed in 70% ethanol, washed twice with PBS, and then stained with 50 μg/mL propidium iodide (PI) solution containing 0.1% Triton X-100, 0.1 mM ethylenediaminetetraacetic acid, and 50 μg/mL RNase A. The cellular DNA content was analyzed, and the population in the sub-G0/G1 phase of the cell cycle was measured by flow cytometry in a FACSCalibur (Becton Dickinson Immunocytometry Systems; San Jose, CA).

2.4 Quantification of cell viability

HCT116 cells were seeded onto 96-well plates (1×10^3 cells/well) and treated with either vehicle (DMSO) or various concentrations of flavanones (10–40 μM) for 48 h. Cell viability was assessed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) with the water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] as a substrate, according to the manufacturer's instructions. For vital staining, HCT116 cells (1×10^5 cells/sample) were treated with 40 μM 2′-HF for 24 h, followed by incubation with 2.5 μg/mL PI for 5 min at room temperature. After detachment, PI-stained dead cells were analyzed by flow cytometry in a FACSCalibur.

2.5 Western blotting

Cells were lysed in a buffer containing 20 mM HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/mL leupeptin, and 1 mM PMSF. Western blotting was performed as described previously [19]. Signals were developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Inc.; Piscataway, NJ).

2.6 In situ detection of activated caspase-7

HCT116 cells plated on coverslips were treated with either DMSO (vehicle) or 40 μM 2′-HF for 24 h. They were then fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100, as described previously [18]. Cleaved caspase-7 was stained by incubation with anti-cleaved caspase-7 (Asp198) antibody for 90 min and then with Alexa-Fluor 555-conjugated secondary antibody for 30 min. Nuclear DNA was stained with Hoechst 33258 (Sigma-Aldrich). Labeled cells were examined under an EVOSf1® fluorescence microscope.

2.7 Annexin V staining

After treatment with 2′-HF, cells were fixed and incubated with fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Pharmingen; San Diego, CA) according to the manufacturer's instructions. Fluorescence-positive cells were analyzed by flow cytometry in a FACSCalibur instrument.

2.8 Generation of stable cell lines expressing siRNA

Generation of expression plasmid for shRNA targeting to Egr-1 mRNA (pSilencer/siEgr1#2) has been described elsewhere [20]. To generate siRNA targeting to NAG-1 mRNA, we used siRNA Target Finder, a web-based algorithm maintained by Ambion (http://www.ambion.com/techlib/misc/siRNA_design.html). The oligonucleotide sequences corresponding to nucleotides +785 to +805 (5′-AAACATGCACGCGCAGATCAA-3′) downstream of the transcription start site of NAG-1 were selected and used to design hairpin siRNA template oligonucleotides using a web-based tool (http://www.ambion.com/techlib/misc/psilencer_converter.html). Two complementary oligonucleotides were synthesized, annealed, and ligated into the pSilencer 2.1-U6 siRNA expression vector (Ambion) as per the manufacturer's instructions. For the generation of stable cells expressing Egr-1 siRNA or NAG-1 siRNA, HCT116 cells were transfected with pSilencer/siCont, pSilencer/siEgr1#2, or pSilencer/siNAG-1 using Nucleofactor (AMAXA Inc., Gaithersburg, MD). Stable transfectants were selected using 400 μg/mL G418 2 days after transfection. After 2 weeks, silencing of Egr-1 or NAG-1 expression was determined by Western blotting.

2.9 Construction and site-directed mutagenesis of the human NAG-1 promoter

A human NAG-1 promoter fragment spanning −1000 to +21 was synthesized by PCR from human genomic DNA (Promega) using the following primers: 5′-TAGAGA CAGGGT TTCTCCATG-3′ (forward primer; −1000/−980) and 5′-TTGCGGCTCTGAGCTGGGACT-3′ (reverse primer; +1/+21). The amplified PCR products were cloned into a pT&A vector (Real Biotech Corporation, Taipei, Taiwan) and digested with *Kpn*I and *Bgl*II sites. The fragments were ligated into the *Kpn*I and *Bgl*II sites of pGL4.17 Luc2/neo (Promega), designated pNAG1-Luc(−1000/+21). A series of deletion constructs of NAG promoter fragments were synthesized by PCR. The primer sequences were: 5′-CCACCTCTCCAGTGAGAGTCT-3′ (forward, −500/−480), 5′-TGTGGTCATTGGAGTGTTTAC-3′ (forward, −110/−90), and 5′-TTGCGGCTCTGAGCTGGGACT-3′ (common reverse, +1/+21). The PCR products were ligated into a pT&A vector, followed by ligation into the pGL3-basic vector using *Kpn*I and *Bgl*II sites. The resulting constructs

were named pNAG1-Luc(−500/+21), pNAG1-Luc(−110/+21), and pNAG1-Luc(−46/+21). The primers used to generate point mutations in the Egr-1/SP-1 overlapping core motif were forward 5′-AGGAtttCGGGACTGAGCAttCGG-3′ (−74/−51, lower-case letters are mutated bases). The resulting construct was named pNAG1-Luc(−507/+21) mtEgr1/SP1. The specific identities of all plasmid constructs were verified by DNA sequencing.

2.10 NAG-1 promoter-reporter assay

HCT116 cells were seeded onto 12-well plates and transfected with 0.2 μg NAG-1 promoter constructs using LipofectAMINE 2000 (Invitrogen Life Technologies, San Diego, CA). To monitor the transfection efficiency, a pRL-null plasmid (50 ng) encoding *Renilla* luciferase was included in all transfections. Where indicated, a mammalian expression vector encoding Egr-1 or SP-1 was also included. At 24 h post-transfection, the cells were treated with 20 μM 2′-HF. After 8 h, the levels of firefly and *Renilla* luciferase activity were measured sequentially from a single sample using the Dual-Glo® Luciferase Assay System. Luminescence was measured with a luminometer (Centro LB960; Berthold Tech, Bad Wildbad, Germany).

2.11 Chromatin immunoprecipitation assay

The cross-linking of protein to DNA and chromatin immunoprecipitation was performed as described previously [21]. The following promoter-specific primers were used to PCR amplify *NAG-1* gene promoter sequences: 5′-ACTGAGGC CCAGAAATGTGC-3′ (target region forward primer, −199/−180), 5′-GACCAGATGCTGCCGGACC-3′, (target region reverse primer, −19/−1), 5′-AATCCCAGTA CTTTGGGAGGC-3′ (off-target region forward primer, −1982/−1962), 5′-GCCTCCTGAGTAGCTGGGACT-3′ (off-target region reverse primer, −1845/−1825).

2.12 Statistical analysis

Each experiment was repeated at least three times. Data are presented as the mean ± SD. Statistical analysis were performed by the Student's *t*-test using Microsoft Excel® 2007. A p-value of <0.05 was considered statistically significant.

3 Results

3.1 Effect of HFs on the clonogenicity of HCT116 colon cancer cells

To evaluate the effectiveness of structure-related HFs on antitumor activity, various HFs were tested for their capacity to inhibit tumor cell growth by long-term clonogenic survival of HCT116 colon cancer cells. Exponentially growing HCT116 cells were exposed to different concentrations of flavanones for 7 days. Colony-formation assay showed that mono-HFs, with the exception of 7-HF, displayed inhibitory effects on the clonogenicity of HCT116 cells (Fig. 2). Of these, 2′-HF was the most potent. In contrast, poly-HFs, such as naringenin (4′,5,7-trihydroxyflavanone) and taxifolin (dihydroquercetin; 3,3′,4′,5,7-pentahydroxyflavanone), showed no obvious inhibitory effects. These data suggest that mono-HFs possess potential antitumor activity. Further study focused on characterizing the antitumor activity of 2′-HF.

3.2 2′-HF induces apoptosis in HCT116 cells

To address whether inhibition of clonogenicity of HCT116 cells by 2′-HF is associated with cytotoxicity of 2′-HF, we performed a cell viability assay. Exponentially growing HCT116 cells were treated with various concentrations of flavanoids for different periods of time, and cell viability was determined. Similar to the long-term clonogenic assay, 2′-HF exhibited the most prominent inhibition of cell viability (Fig. 3A). Apoptosis is one of the important mechanisms by which anticancer agents inhibit the growth of cancer cells. To understand the mechanism by which 2′-HF inhibits the viability of HCT116 cells, exponentially growing cells were exposed to 20 μM 2′-HF for 24 or 48 h, and the population of sub-G1 phase cells, a hallmark of apoptosis, was determined by a flow cytometry. Accumulation of G2/M phase cells was observed in cells treated with 2′-HF at 24 h, after which their numbers declined, whereas the population of sub-G1 phase cells persistently increased from 24 h following 2′-HF treatment (Fig. 3B), suggesting that 2′-HF triggers apoptosis. To determine whether 2′-HF induces cell death, HCT116 cells were treated with 2′-HF for 24 h and then stained with propidium iodide (PI), which is an intercalating DNA-binding agent that is membrane impermeant and generally excluded from viable cells; thus it can be used for identifying dead cells in a population. Flow cytometric analysis showed that 2′-HF substantially increased the number of dead cells (Fig. 3C). Although we could not rule out the involvement of necrotic cell death, these data suggested that 2′-HF exhibits antitumor activity through apoptosis in HCT116 colon cancer cells. PI is an intercalating DNA-binding agent that is membrane impermeant and generally excluded from viable cells; thus it can be used for identifying dead cells in a population.

Activation of caspase frequently plays a key role in apoptosis induced by chemotherapeutic agents. To determine whether caspase was involved in 2′-HF-induced apoptosis, Western blot analysis was performed using antibodies recognizing the cleaved forms of caspase. Treatment of HCT116 cells with 2′-HF gradually increased the cleavage of both caspase-3 and caspase-7 in a dose- and time-dependent manner (Fig. 4A). The poly(ADP-ribose) polymerase (PARP), a chromatin-associated enzyme that plays an important role

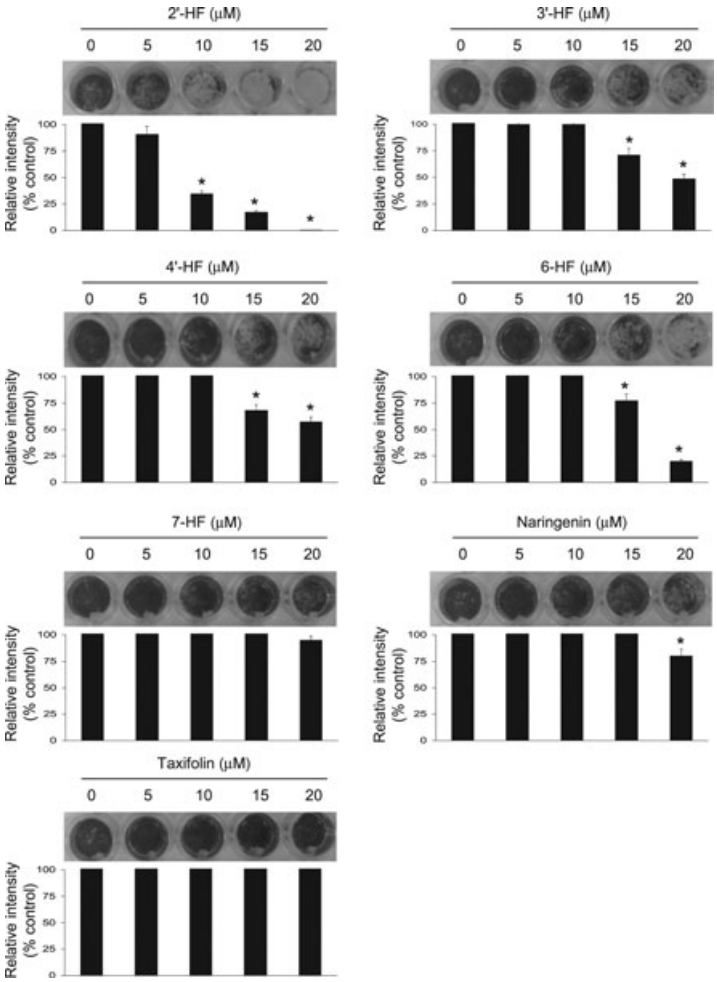

Figure 2. Inhibitory effects of monohydroxylated flavanones on the clonogenicity of HCT116 cells. HCT116 cells (5×10^3 cells/well) were seeded and cultured in the absence or presence of hydroxyflavanones (0, 5, 10, 15, and 20 μM). After 7 days of treatment, colonies were stained with crystal violet (top) and quantified by densitometry (bottom). Data represent the mean ± SD of three independent experiments normalized to the vehicle control (set 100%). Statistical significance of comparisons was analyzed by Student's *t*-test. *, $p < 0.05$ compared with vehicle-treated control.

in DNA repair and the recovery of cells from DNA damage, is cleaved by caspase-3 or caspase-7 [22]. The amount of cleaved PARP was also increased after 2′-HF treatment as a function of dose and time. Activation of caspase was further supported by immunofluorescence microscopy using an antibody specific for the cleaved form of caspase-7 (Fig. 4B). We also observed that 2′-HF-treated cells were characterized by shrunken and fragmented nuclei with condensed chromatin, whereas the nuclei of control cells appeared to be normal with dispersed chromatin. Collectively, these results indicate that the induction of apoptotic cell death by 2′-HF probably occurs through a caspase-dependent pathway after approximately 24 h of 2′-HF exposure.

To test the generality of the effect of 2′-HF on the induction of apoptosis, we examined its effect in different cancer cell lines. Treatment with 2′-HF strongly induced the cleavage of PARP in SW620 colon cancer (Fig. 5A) and MCF7 breast cancer (Fig. 5B) cells. In contrast, 2′-HF had no effect on normal FHC colon cells (Fig. 5C) and HEK293 human embryonic kidney cells (Fig. 5D). These data suggest that 2′-HF induces apoptosis in diverse cancer cells but not in normal cells.

3.3 p53 is not critical for 2′-hydroxyflavanone-induced apoptosis

The tumor-suppressor protein p53 is responsible for many forms of genotoxic agent-induced apoptosis. In an attempt to find the molecular mechanism underlying 2′-HF-induced apoptosis, we investigated the role of p53 using wild-type (HCT116/$p53^{+/+}$) and p53-null HCT116 cells (HCT116/$p53^{-/-}$) [23]. Western blot analysis showed that 2′-HF dose dependently increased levels of p53 and its downstream target p21 in wild-type HCT116 cells but not in p53-null HCT116 cells (Fig. 6A). Unexpectedly, the levels of cleaved caspase-7 and PARP were higher in p53-null HCT116 cells as compared with wild-type cells following 2′-HF exposure (Fig. 6A).

One of the earliest cellular changes during apoptosis is translocation of phosphatidylserine (PS), a membrane phospholipid, from the inner to the outer membrane. To determine the appearance of PS in the outer membrane following 2′-HF treatment, annexin V-FITC staining was performed. The population of annexin V-stained cells was more strongly increased in p53-null HCT116 cells than in

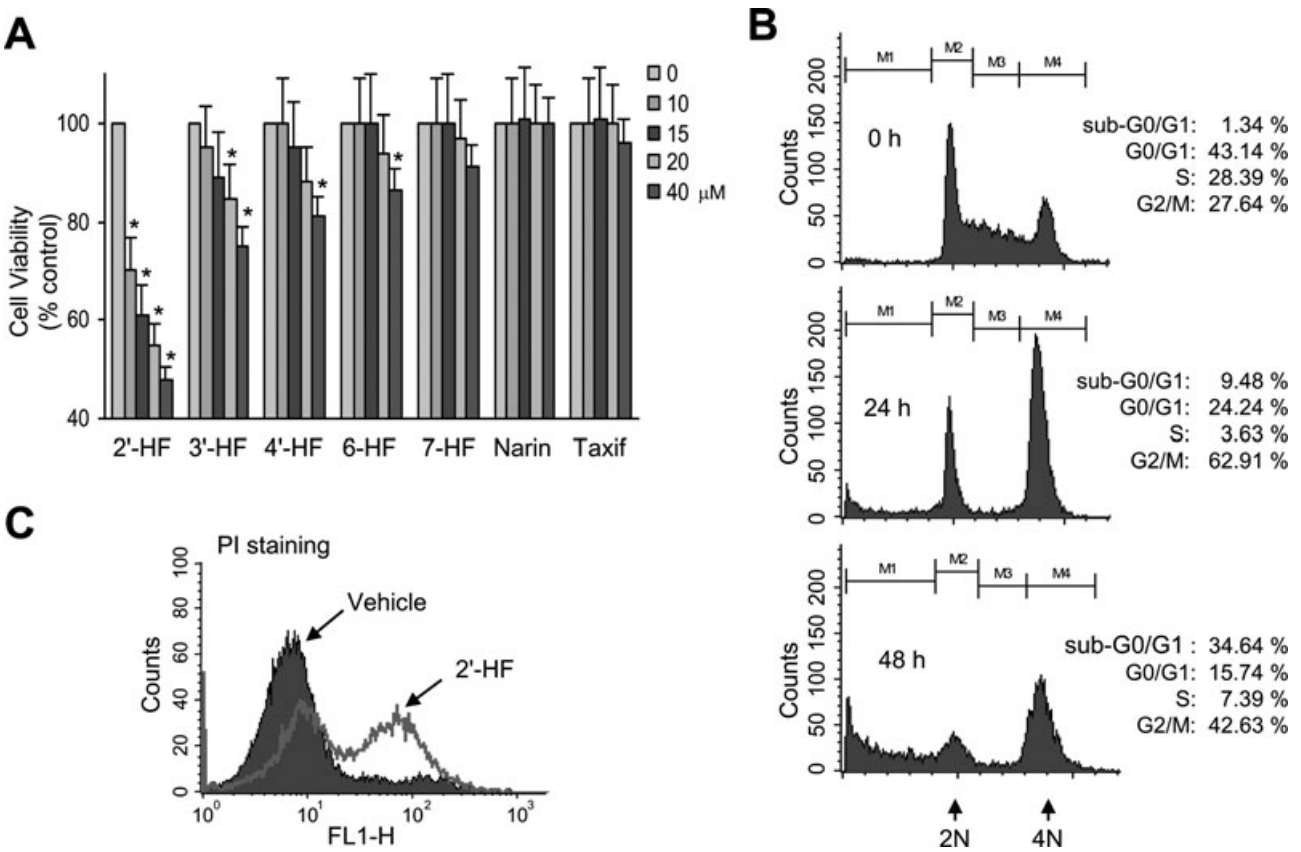

Figure 3. Effect of 2′-HF on the cytotoxicity in HCT116 cells. (A) HCT116 cells (1×10^3 cells/sample) were treated with different concentrations (0, 10, 15, 20, and 40 μM) of flavanones for 48 h, and cell viability was measured using a Cell Counting Kit-8. Data represent the mean ± SD for two independent experiments performed in triplicate. Statistical significance of comparisons was analyzed by Student's *t*-test. A *p*-value of <0.05 was considered significant. *, $p < 0.05$ compared with vehicle-treated control. (B) FACS analysis. HCT116 cells (1×10^5 cells/sample) were treated with 20 μM 2′-HF for 24 or 48 h. Cells were harvested, fixed with ethanol, and stained with propidium iodide. The cellular DNA content was then determined by flow cytometry. 2N, diploid; 4N, tetraploid; M1, sub-G0/G1 phase; M2, G0/G1 phase; M3. S phase; M4, G2/M phase. (C) Detection of dead cells. HCT116 cells (1×10^5 cells/sample) were treated with either DMSO (vehicle) or 40 μM 2′-HF for 24 h and then incubated with propidium iodide (PI). The population of PI-stained cells was determined by flow cytometry.

wild-type HCT116 cells (Fig. 6B), suggesting that a p53-independent mechanism is also involved in 2′-HF-induced apoptosis. Many human cancers contain a p53 mutation, which can mediate resistance to chemotherapy-induced apoptosis. Thus, it seems likely that 2′-HF may trigger apoptosis of cancer cells, irrespective of p53 status.

3.4 NAG-1 is involved in 2′-HF-induced apoptosis

NAG-1 is a member of the TGF-β super-family. NAG-1 is known to induce apoptosis in several tumor cells, including breast, osteosarcoma, and prostate cancer cells [24–28]. To investigate the possible involvement of NAG-1 in 2′-HF-induced apoptosis, we established cell lines stably expressing NAG-1 siRNA (HCT116/siNAG1) or scrambled siRNA (HCT116/siCont). We found that 2′-HF accumulated NAG-1 protein in a dose-dependent manner in control HCT116/siCont cells (Fig. 7A). Stable knockdown of NAG-1 by siRNA was evaluated by Western blotting. Knockdown of endogenous NAG-1 substantially attenuated the ability of 2′-HF to cleave caspase-7 and its downstream substrate PARP, two markers of apoptosis (Fig. 7A). We also observed that HCT116/siNAG1 cells displayed resistance to the 2′-HF-induced appearance of PS in the outer membrane of cells, as revealed by Annexin V-FITC staining (Fig. 7B). These data suggest that NAG-1 plays an important role in 2′-HF-induced apoptosis.

3.5 A proximal *cis*-acting element is necessary for 2′-HF-induced NAG-1 promoter activation

To determine whether 2′-HF upregulates NAG-1 expression at the transcriptional level, we constructed a human NAG-1 promoter reporter, pNAG1-Luc(−1000/+21), and measured NAG-1 promoter activity following 2′-HF

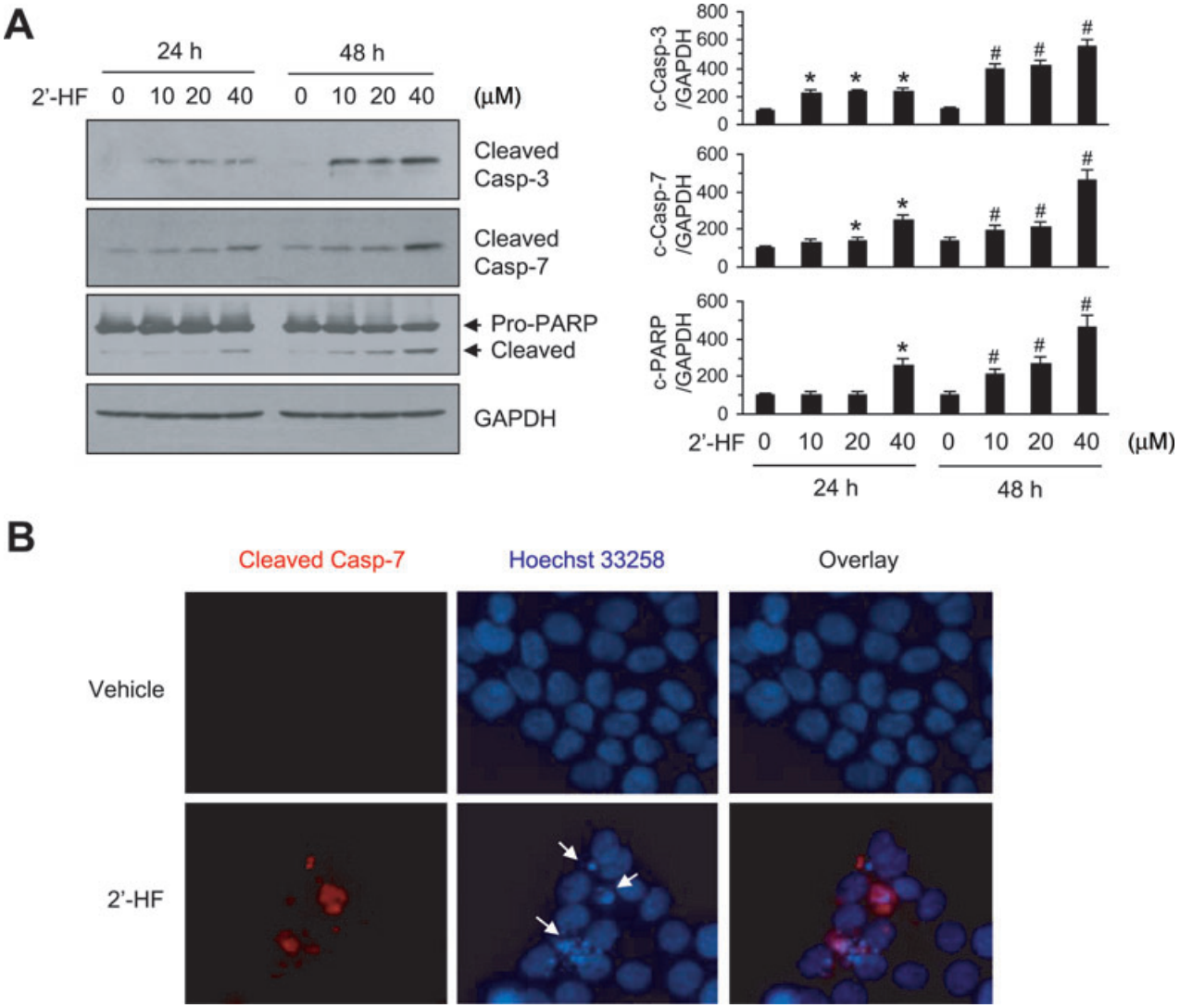

Figure 4. Effect of 2′-HF on the activation of caspases. (A) HCT116 cells were treated with different concentrations of 2′-HF for 24 or 48 h, and then whole-cell lysates were prepared and subjected to Western blotting using antibodies against the indicated proteins. GAPDH antibody was used as an internal control to show equal protein loading. Quantitative analysis of band intensities was performed by densitometry. Data represent the mean ± SD of three independent experiments normalized to the vehicle control (set to 100%). Statistical significance of comparisons was analyzed by Student's *t*-test. A *p*-value of <0.05 was considered significant. *, $p < 0.05$ compared with vehicle-treated control (24 h); #, $p < 0.05$ compared with vehicle-treated control (48 h). (B) HCT116 cells were either treated with DMSO (vehicle) or 40 μM 2′-HF for 24 h. Cleaved caspase-7 was stained with anti-cleaved caspase-7 (Asp198) antibody for 90 min and then with Alexa-Fluor 555-conjugated secondary antibody for 30 min. Chromosomal DNA (blue) was stained with Hoechst 33258. Arrows indicate shrunken or fragmented nuclei. Fluorescence staining was analyzed by EVOSf1® fluorescence microscope.

treatment. As shown in Fig. 8A, 2′-HF dose-dependently increased NAG-1 promoter-reporter activity.

Several putative transcription-factor binding sites are localized within the 5′-regulatory region of the *NAG-1* gene (Fig. 8B). To identify the *cis*-acting regulatory element in the *NAG-1* gene, we made progressively deleted NAG-1 promoter–reporter constructs. Even when −1000 to −110 were deleted, 2′-HF-induced reporter activity remained high; however, further deletion to −46 resulted in complete loss of reporter activity (Fig. 8B). These data suggest that a proximal region between −110 and −46 of the NAG-1 promoter contains a *cis*-acting regulatory element responsive to 2′-HF.

3.6 Egr-1 *trans*-activates the NAG-1 promoter through its binding sites

A previous study demonstrated that quercetin induces NAG-1 expression through Egr-1 [29]. Sequence analysis using the web-based program MatInspector (Genomatix) showed

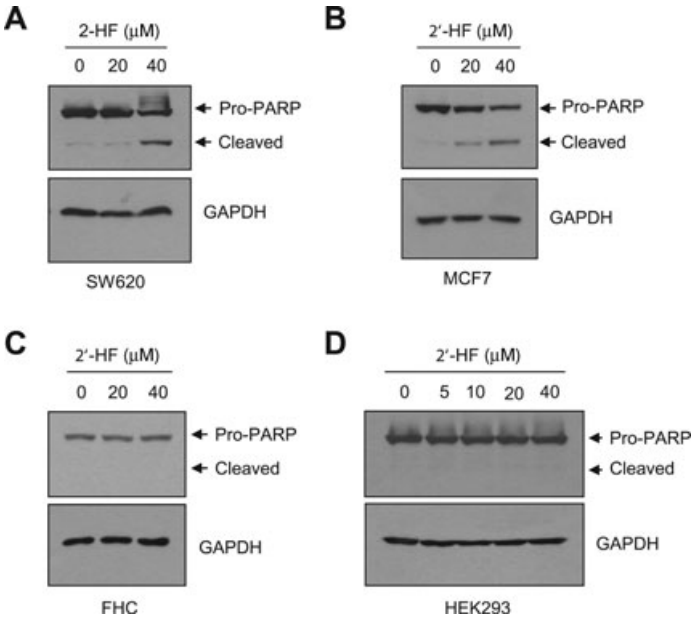

Figure 5. Effect of 2′-HF on the induction of apoptosis in diverse cell types. SW620 (A), MCF7 (B), FHC (C), and HEK293 (D) cells were treated with various concentrations of 2′-HF for 48 h, and then whole-cell lysates were prepared and subjected to Western blotting using antibodies against PARP. GAPDH antibody was used as an internal control to show equal protein loading. Similar results were obtained from two additional independent experiments.

that two consensus Egr-1-binding motifs overlap with SP-1 between nucleotides −80 and −40 within the NAG-1 promoter. To evaluate the role of Egr-1/SP-1-binding elements located between −110 and −46 in 2′-HF-induced NAG-1 transcription, we introduced double mutations (GGGC → TTTC and CAGG → CATT) into the core Egr-1/SP-1-binding motif of the pNAG1-Luc(−110/+21) plasmid, yielding pNAG1-Luc(−110/+21)mtEgr1/SP1. These mutations completely eliminated the response to 2′-HF (Fig. 9A). Thus, the putative Egr-1 regulatory elements within the promoter region are critical for transcriptional activation of the *NAG-1* gene in response to 2′-HF in HCT116 cells.

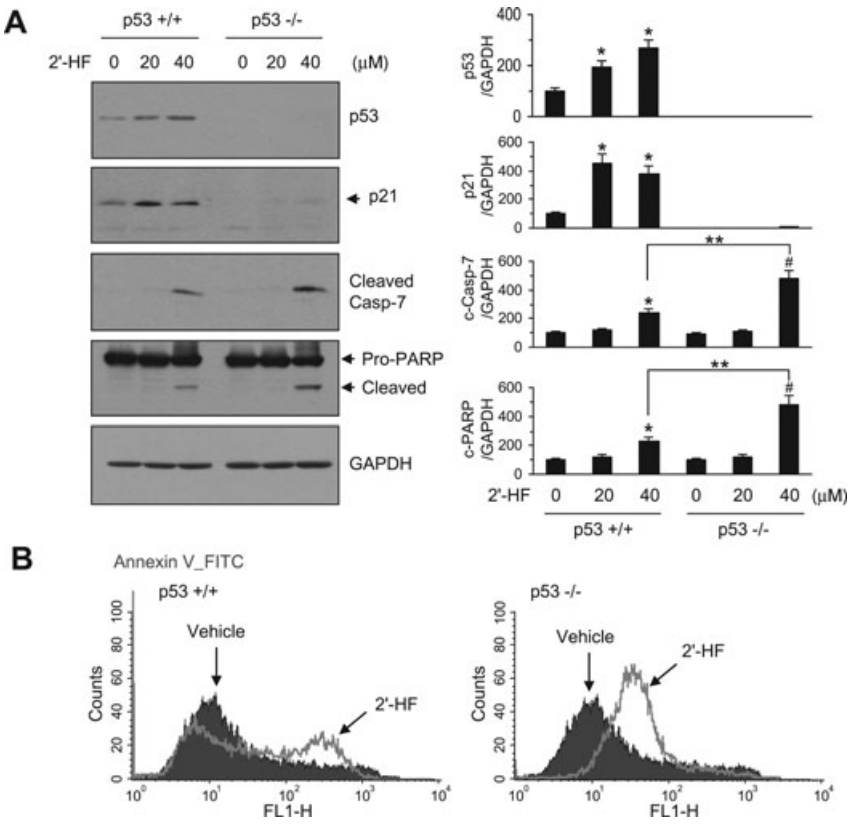

Figure 6. Effect of p53 depletion on 2′-HF-induced apoptosis. (A) Wild-type ($p53^{+/+}$) or p53-null ($p53^{-/-}$) HCT116 cells were treated with various concentrations of 2′-HF for 24 h, and then whole-cell lysates were prepared and subjected to Western blotting using antibodies against the indicated proteins. GAPDH antibody was used as an internal control to show equal protein loading. Quantitative analysis of band intensities was performed by densitometry. Data represent the mean ± SD of three independent experiments normalized to the vehicle control (set to 100%). Statistical significance of comparisons was analyzed by Student's *t*-test. A *p*-value of <0.05 was considered significant. *, $p < 0.05$ compared with vehicle-treated control ($p53^{+/+}$); #, $p < 0.05$ compared with vehicle-treated control ($p53^{-/-}$); **, $p < 0.05$ compared with indicated group. (B) Detection of apoptosis. Wild-type ($p53^{+/+}$) or p53-null ($p53^{-/-}$) HCT116 cells were treated with either DMSO (vehicle) or 40 μM 2′-HF for 12 h and then incubated with annexin V-FITC. The percentage of annexin V-stained cells was determined by flow cytometry.

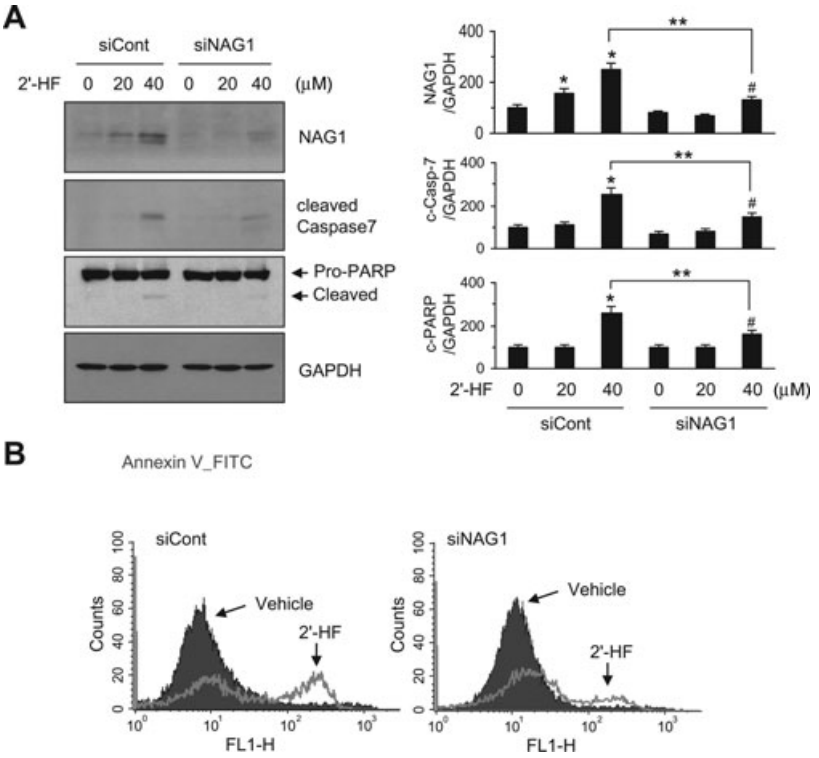

Figure 7. Role of NAG-1 in 2′-HF-induced apoptosis. (A) HCT116/siCont or HCT116/siNAG1 cells were treated with 2′-HF (20 or 40 μM) for 24 h. Whole-cell lysates were prepared and subjected to Western blotting using antibodies against the indicated proteins. GAPDH antibody was used as an internal control to show equal protein loading. Quantitative analysis of band intensities was performed by densitometry. Data represent the mean ± SD of three independent experiments normalized to the vehicle control (set to 100%). Statistical significance of comparisons was analyzed by Student's *t*-test. A *p*-value of <0.05 was considered significant. *, $p < 0.05$ compared with vehicle-treated control (siCont); #, $p < 0.05$ compared with vehicle-treated control (siNAG1); **, $p < 0.05$ compared with indicated group. (B) Detection of apoptosis. HCT116/siCont or HCT116/siNAG1 cells were treated with either DMSO (vehicle) or 40 μM 2′-HF for 12 h and then incubated with annexin V-FITC. The percentage of annexin V-stained cells was determined by flow cytometry.

As Egr-1 and SP-1 core motifs between −110 and −46 overlap, we determined whether Egr-1 or SP-1 alone could trans-activate the NAG-1 promoter. HCT116 cells were cotransfected with the pNAG1-Luc(−110/+21) reporter and expression plasmid for Egr-1 or SP-1, and luciferase activity was measured. We found that Egr-1, but not SP1, strongly enhanced both basal and 2′-HF-induced reporter activity (Fig. 9B), suggesting that the *NAG-1* gene is transcriptionally regulated by Egr-1 in response to 2′-HF.

To verify whether Egr-1 directly binds to the NAG-1 promoter at the chromatin level, we cross-linked DNA and its bound proteins in 2′-HF-treated HCT116 cells using formaldehyde. Cross-linked DNA–protein complexes were subjected to chromatin immunoprecipitation using a rabbit anti-Egr-1 or anti-SP-1 antibody. Normal rabbit IgG was used as a negative control. The resulting immunoprecipitated DNA was amplified by PCR using primers designed to the promoter region (−199 to −1) of the *NAG-1* gene. Input genomic DNA was used as a positive control. As shown in Fig. 9C, a noticeable increase in the amount of protein-bound DNA in 2′-HF-treated cells was detected using the anti-Egr-1 antibody, but not the anti-SP-1 antibody or normal rabbit IgG. To determine the specificity of Egr-1 binding to the promoter, the same ChIP DNA was amplified using off-target primers. The off-target region (−1982 to −1825) was not amplified, although positive results were obtained from input genomic DNA. These data indicate that Egr-1 physically interacts with the *NAG-1* gene promoter. Collectively, Egr-1 trans-activates the NAG-1 gene promoter through direct binding to the promoter in response to 2′-HF in HCT116 cells.

3.7 Egr-1 is important for 2′-HF-induced apoptosis

To evaluate the upregulation of Egr-1 by 2′-HF treatment, serum-starved HCT116 cells were treated with 40 μM 2′-HF, and the amount of Egr-1 was measured by Western blotting. A time-course study showed that the level of Egr-1 increased within 1 h, with a maximum increase at approximately 2 h, after which the level had dropped considerably by 4 h but remained high as compared to the basal level (Fig. 10A).

To corroborate the role of Egr-1 in 2′-HF-induced NAG-1 expression, stable cell lines expressing Egr-1 siRNA (HCT116/siEgr-1) or scrambled siRNA (HCT116/siCont) were established. Knockdown of Egr-1 expression was evaluated by Western blotting (Fig. 10B). Silencing of endogenous Egr-1 substantially attenuated 2′-HF-induced NAG-1 expression (Fig. 10C). Interestingly, Bax and p21 expression was also reduced in HCT116/siEgr-1 cells, although p53 expression in HCT116/siEgr-1 cells was similar to that in HCT116/siCont cells, suggesting that Bax and p21 are also regulated by Egr-1 irrespective of p53.

To determine whether silencing of Egr-1 affects the induction of apoptosis, we analyzed the cleavage of caspase-7 and PARP. As shown in Fig. 10D, 2′-HF-induced

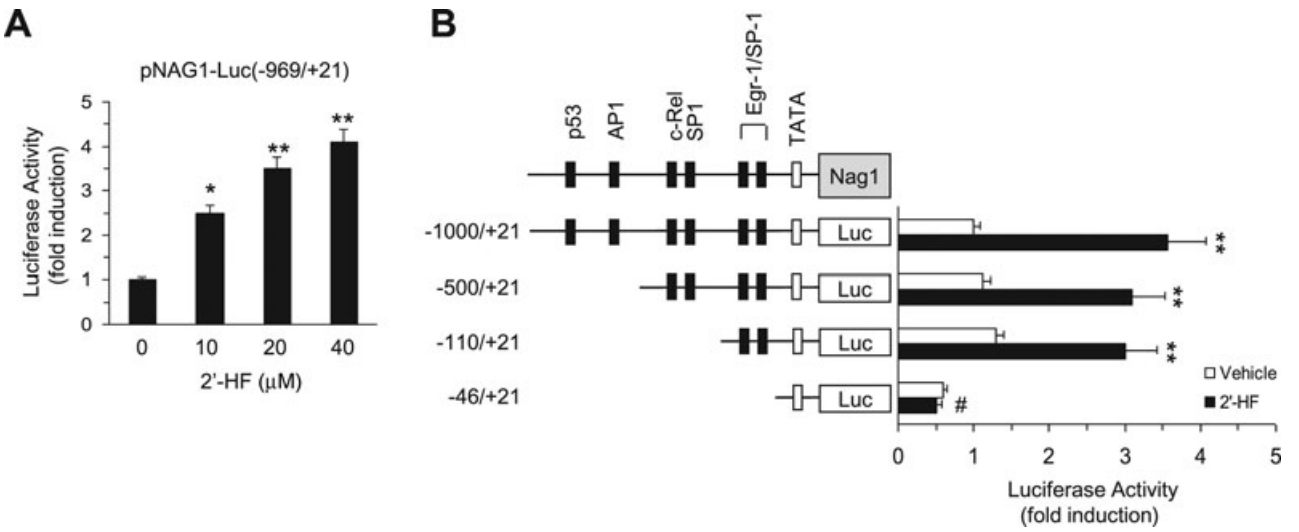

Figure 8. Effect of 2′-HF on the activation of the NAG-1 promoter. (A) HCT116 cells were transfected with 0.2 μg of the human NAG-1 promoter construct pNAG1-Luc(−1000/+21) and 50 ng pRL-null vector. After 48 h, cells were treated with different concentrations of 2′-HF for 8 h, and their luciferase activities were measured. Values for firefly luciferase were normalized to those for Renilla luciferase. Data represent the mean ± SD of three independent experiments, each performed in triplicate. *, $p < 0.05$; **, $p < 0.01$ compared with vehicle-treated cells. (B) HCT116 cells were transfected with 50 ng pRL-null vector and 0.2 μg of NAG-1 promoter construct [pNAG1-Luc(−1000/+21), pNAG1-Luc(−500/+21), pNAG1-Luc(−110/+21), or pNAG1-Luc(−46/+21)], as indicated. Then, 48 h post-transfection, cells were treated with 20 μM 2′-HF for 8 h, and their luciferase activities were determined. Values for firefly luciferase were normalized to those for Renilla luciferase. Data represent the mean ± SD of three independent experiments, performed in triplicate. **, $p < 0.01$; #, not significant; each compared with vehicle-treated cells.

cleavage of caspase-7 and PARP was strongly attenuated in HCT116/siEgr-1 cells as compared with HCT116/siCont cells. Furthermore, the population of annexin V-FITC-stained cells following 2′-HF treatment was also reduced in HCT116/siEgr-1 cells as compared with HCT116/siCont cells (Fig. 10E). These results identify Egr-1 as an important transcription factor responsible for 2′-HF-induced apoptosis. Collectively, Egr-1 plays a key role in 2′-HF-induced apoptosis through upregulation of proapoptotic genes, including Bax and NAG-1.

4 Discussion

It is now well recognized that bioactive polyphenols present in edible plants can interfere with cancer initiation, promotion, and progression, acting as chemopreventive agents [3]. Certain flavanones, such as naringenin and hesperidine, are known to have antitumor activity through induction of apoptosis in various cancer cells. However, the detailed mode of action of flavanones is still not completely understood. Previously, we analyzed the relationship between the structural properties of flavanone derivatives and the induction of Egr-1 using comparative molecular field analysis (COMFA) and comparative molecular similarity indices analysis (CoMSIA). We found that the hydrophilic region near the C-2′ position of the B-ring of the flavanones is important for Egr-1 expression [30]. In the present study, we investigated the effect of various HFs on antitumor activity using the HCT116 colon cancer cell line system. We found that flavanone hydroxylated at C-2′ (2′-HF) showed the most potent inhibitory activity against the clonogenicity of HCT116 cells, while the C-7 hydroxylation (7-HF) showed relatively weak activity compared with other flavanones, suggesting that structure-related hydroxylation might be more important for antitumor activity than the number of hydroxyls added. Our results are consistent with previous findings that C-2′ hydroxylation is critical for the antitumor activity of flavanones in caspase-3-dependent apoptosis in colon cancer cells [17]. The results emphasize the potential benefits of dietary hydroxyflavanones for prevention or therapy of human colon cancer.

Many anticancer agents are associated with the activation of apoptotic pathways. Apoptosis is mediated by the caspase cascade, leading to cellular shrinkage with chromatin condensation. During apoptosis, the processing of native 113-kDa PARP to its 89- and 24-kDa forms is catalyzed by effector caspases (caspase-3 and -7), which is a hallmark of caspase activation. In this study, we showed that 2′-HF increased the cleavages of caspase-3, caspase-7 and PARP in colon cancer cells but had no effect on PARP cleavage in FHC human colon epithelial cells and HEK293 human embryonic kidney cells. These data suggest that 2′-HF stimulates caspase-mediated apoptosis in colon cancer cells, but not in normal cells. We thus sought to identify the mechanism of 2′-HF induction of apoptosis in colon cancer cells. Here, we show for the first time that Egr-1 plays an important role in 2′-HF-induced apoptosis through upregulation of Bax, p21, and NAG-1 irrespective of p53 status.

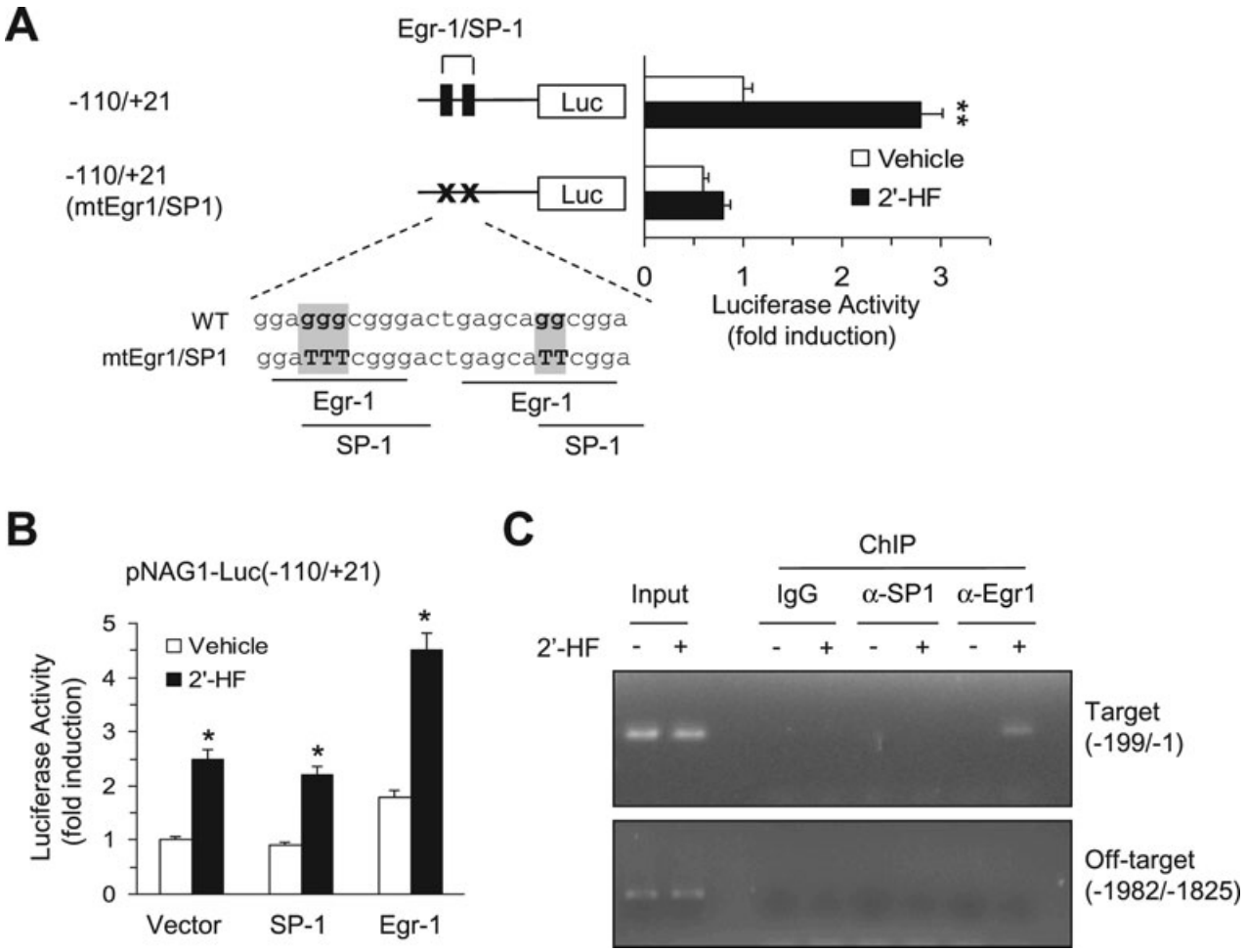

Figure 9. Association of Egr-1 to the NAG-1 promoter. (A) HCT116 cells were transiently transfected with 50 ng pRL-null vector and 0.2 μg wild-type pNAG1-Luc(−110/+21) or pNAG1-Luc(−110/+21)mtEgr1/SP1 construct carrying both mutations in the Egr-1/SP-1-binding sites. Then, 48 h after transfection, cells were either left untreated or treated with 20 μM 2′-HF for 8 h and analyzed for luciferase activity. Mutated nucleotides are highlighted by a gray box. Values for firefly luciferase were normalized to those for Renilla luciferase. Data represent the mean ± SD of three independent experiments, each performed in triplicate. **, $p < 0.01$ compared with vehicle-treated cells. (B) HCT116 cells were cotransfected with 50 ng pRL-null vector and 0.2 μg wild-type pNAG1-Luc(−110/+21) and an expression plasmid encoding SP-1 or Egr-1. Forty-eight hours post-transfection, cells were treated with 20 μM 2′-HF for 8 h and analyzed for luciferase activity. Values for firefly luciferase were normalized to those for Renilla luciferase. *, $p < 0.05$ compared with vehicle-treated cells. (C) ChIP assay. HCT116 cells treated with 20 μM 2′-HF for 2 h were cross-linked, lysed, and immunoprecipitated with normal rabbit IgG (negative control), anti-Egr-1, or anti-SP-1 antibody. Precipitated DNA was analyzed by standard PCR using primers specific for the target region (−199 to −180) or off-target region (−19 to −1). One aliquot of input DNA was used as a positive control.

p53 is a well-characterized transcription factor that functions as a tumor suppressor, inhibiting cell growth and inducing apoptosis in response to DNA damage, primarily through induction of p21 and Bax. p21, also known as CDKN1A, WAF1, or CIP1, regulates cell cycle progression at G1 or G2/M phase through inhibition of cyclin E-cyclin-dependent protein kinase (Cdk) 2 or the cyclin B/Cdk1 complex [31, 32]. Bax promotes the release of cytochrome c from mitochondria, resulting in activation of caspase-9, which initiates the apoptotic caspase cascade. Thus, p53 plays an important role in the regulation by chemotherapeutic agents of cell growth arrest and apoptosis in diverse cell types. Given that 2′-HF caused an accumulation of p53 in dose- and time-dependent manners in HCT116 cells, we examined its involvement in 2′-HF-induced apoptosis. Unexpectedly, we found that 2′-HF induced apoptosis in p53-null HCT116 cells, suggesting that 2′-HF may trigger apoptosis of tumor cells through both p53-dependent and -independent mechanisms. Indeed, 2′-HF-induced apoptosis is independent of p53 in HT29 and COLO205 colon cancer cells [17]. Thus, p53 may be involved in the inhibition of cell cycle progression through upregulation of p21 but does not appear to be critical for 2′-HF-induced apoptosis in HCT116 cells.

NAG-1, also known as macrophage inhibitory cytokine-1 (MIC-1), growth differentiation factor-15 (GDF-15), prostate differentiation factor (PDF), or placental bone morphogenetic

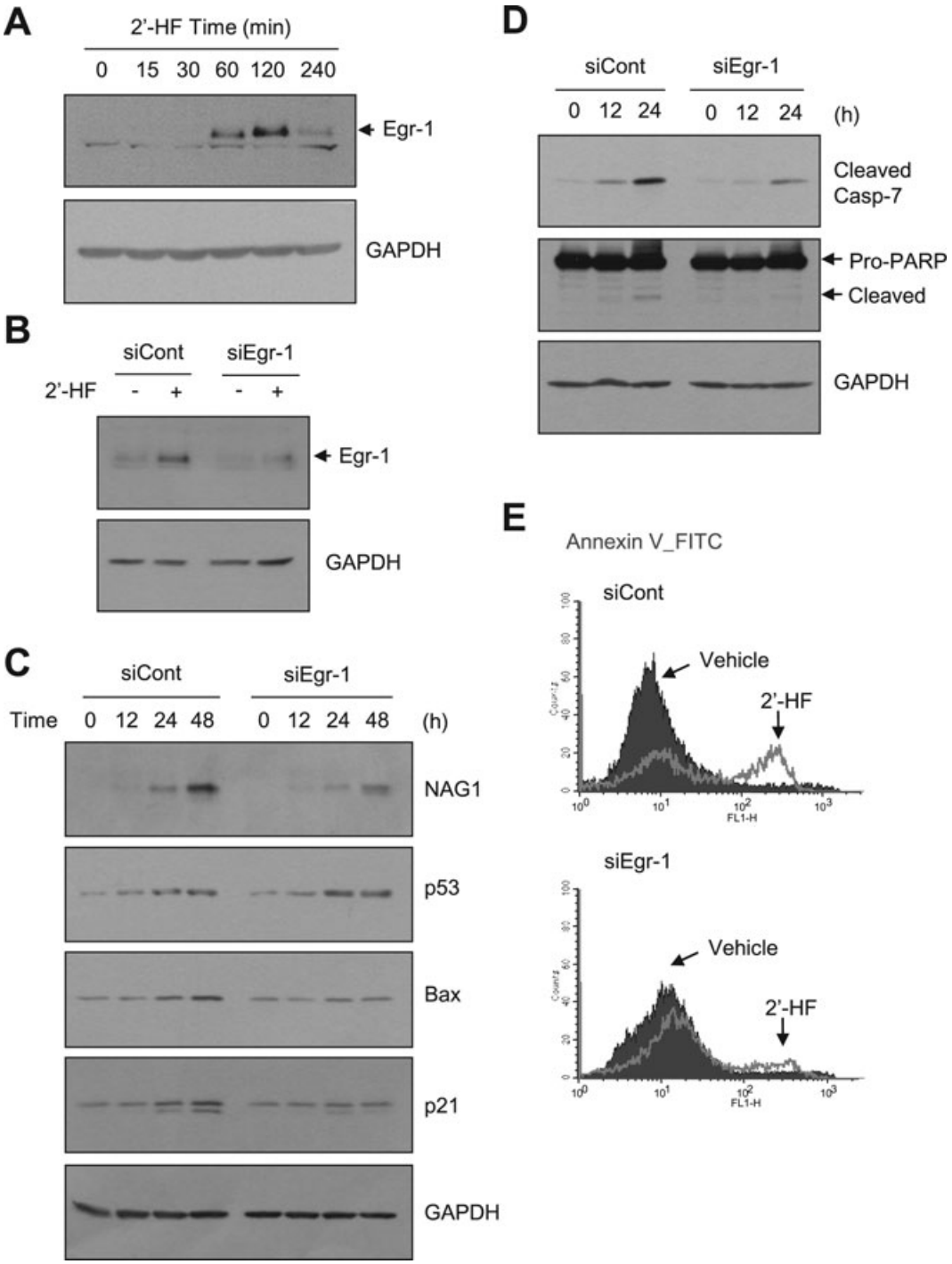

Figure 10. Functional role of Egr-1 in 2′-HF-induced apoptosis. (A) HCT116 cells were serum-starved with 0.5% serum for 24 h, followed by treatment with 40 μM 2′-HF for different periods of time. Whole-cell lysates were prepared and subjected to Western blotting using anti-Egr-1 antibody. GAPDH antibody was used as an internal control to show equal protein loading. (B) Serum-starved HCT116 cells expressing scrambled control (HCT116/siCont) or Egr-1 siRNA (HCT116/siEgr-1) were treated with 40 μM 2′-HF for 2 h. Silencing of Egr-1 expression was verified by Western blotting using anti-Egr-1 antibody. GAPDH antibody was used as an internal control to show equal protein loading. (C) and (D) HCT116/siCont or HCT116/siEgr-1 cells were treated with 40 μM 2′-HF for various periods of time. Whole-cell lysates were then prepared and subjected to Western blotting using antibodies against the indicated proteins. GAPDH antibody was used as an internal control to show equal protein loading. (E) Detection of apoptosis. HCT116/siCont or HCT116/siEgr-1 cells were treated with either DMSO (vehicle) or 40 μM 2′-HF for 12 h and then incubated with annexin V-FITC. The percentage of annexin V-stained cells was determined by flow cytometry.

protein (PLAB), is a member of the TGF-β superfamily. Several studies have shown that forced expression of NAG-1 in HCT116 colon cancer cells results in reduced tumor growth [24], whereas knockdown of NAG-1 by siRNA was shown to block 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced apoptosis in LNCaP prostate cancer cells [28]. NAG-1 upregulation induced apoptosis in DNA damaging agent-treated MCF7 breast cancer cells [25] and in quercetin-treated HCT116 colon cancer cells [29]. These results imply that NAG-1 induces growth arrest or apoptosis in many cancer cells. Transcriptional activation of the *NAG-1* gene is mediated by various *cis*-acting regulatory elements within the promoter, involving NF-κB and Egr-1 [28, 29, 33]. We found that expression of both Egr-1 and NAG-1 was upregulated in 2′-HF-treated HCT116 cells.

Egr-1 is a Cys2/His2-type zinc-finger transcription factor that regulates cell growth, differentiation, development, and death [34]. Egr-1 can directly *trans*-activate the proapoptotic *Bax* gene independently of p53 [35]. Indeed, sodium arsenite stimulates Egr-1 expression and promotes apoptosis through Egr-1-dependent induction of Bax in p53-mutated HaCaT keratinocytes [36]. The functional role of Egr-1 in 2′-HF-induced apoptosis is based on several lines of evidence: (i) Egr-1 was rapidly induced in response to 2′-HF treatment [8]; (ii) knockdown of endogenous Egr-1 expression using siRNA almost completely abolished 2′-HF-induced apoptosis; (iii) knockdown of Egr-1 substantially reduced expression of proapoptotic Bax and NAG-1 as well as cell cycle inhibitor p21 by 2′-HF; and (iv) silencing of NAG-1 by siRNA abrogated 2′-HF-induced apoptosis.

In conclusion, 2′-HF has an antitumor effect by inducing Egr-1-mediated p21, Bax, and NAG-1. As mutations within the *p53* gene locus frequently occur in diverse human tumors, 2′-HF might be useful for prevention or therapy of p53-mutated cancer.

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