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Negative regulation of β-catenin/Tcf signaling by naringenin in AGS gastric cancer cell

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Abstract

Functional activation of β-catenin/Tcf signaling plays an important role in early events in carcinogenesis. We examined the effect of naringenin against β-catenin/Tcf signaling in gastric cancer cells. Reporter gene assay showed that naringenin inhibited β-catenin/Tcf signaling efficiently. In addition, the inhibition of β-catenin/Tcf signaling by naringenin in HEK293 cells transiently transfected with constitutively mutant β-catenin gene, whose product is not phosphorylated by GSK3β, indicates that its inhibitory mechanism was related to β-catenin itself or downstream components. To investigate the precise inhibitory mechanism, we performed immunofluorescence, Western blot, and EMSA. As a result, our data revealed that the β-catenin distribution and the levels of nuclear β-catenin and Tcf-4 proteins were unchanged after naringenin treatment. Moreover, the binding activities of Tcf complexes to consensus DNA were not affected by naringenin. Taken together, these data suggest that naringenin inhibits β-catenin/Tcf signaling in gastric cancer with unknown mechanisms.
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Keywords: β-Catenin/Tcf signaling; Naringenin; Nuclear β-catenin; Mechanism

β-Catenin is a multifunctional protein that exerts two important functions in epithelial cells. These diverse functions are realized through interaction with various binding partners. In nonstimulated cells, β-catenin is largely associated with cadherin and connects it, through α-catenin, to the actin cytoskeleton [1]. It is well known that tyrosine modification of β-catenin modulates the formation or disassembly of adherens junctions [2,3]. On the other hand, when not bound to E-cadherin, β-catenin enters the nucleus to act as a transcriptional co-activator, through association with the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) transcription factor. A large number of genes relevant for tumor formation and progression have been identified to be transcriptionally activated by the β-catenin/Tcf complex. Some of them are implicated in growth control and cell cycling (c-Myc, c-Jun, fra-1, cyclin D1, and gastrin), some are relevant for cell survival (Id2, MDR1), and some are implicated in tumor invasion and metastasis (matrilysin, VEGF) [4–10].

In normal cells, most β-catenin protein is present at the cell–cell junctions with very little in cytoplasm or nucleus because of its rapid degradation by the proteasome. In the absence of the secreted factor, Wnt, β-catenin in the cytoplasm is phosphorylated by Axin—adenomatous polyposis coli (APC)—glycogen synthase kinase (GSK) 3β complex. This phosphorylated β-catenin is recognized by β-TrCP, an F-box component of the E3 ubiquitin ligase complex that promotes ubiquitination of β-catenin and increases its susceptibility to degradation by ubiquitin-proteasome system [11]. However, cytosolic β-catenin protein can be stabilized by mutational inactivation of the APC gene, usually leading to a truncated protein product, or by β-catenin

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mutations at regulatory amino-terminal serine residues. In addition, activated Disheveled (Dsh) by Wnt stimulation blocks the ability of GSK3β to phosphorylate β-catenin. This stabilization of β-catenin is detected in different types of cancer [12]. When the failure of this degradation in cells occurs, β-catenin proteins are accumulated in cytoplasm and some of them translocate into the nucleus, leading to activation of the Tcf/Lef transcription factor. Activated β-catenin/Tcf signaling by accumulation of β-catenin in the nucleus has been implicated in human carcinogenesis including colorectal cancer, melanoma, hepatocellular carcinoma, and gastric carcinoma [12–14]. These data strongly suggest that the dysregulation of β-catenin/Tcf signaling may be involved in the development of a broad range of human malignancies. Especially, some studies on the effect of β-catenin/Tcf signaling on gastric cancer were performed recently. Nakatsuru et al. [15] reported APC mutations in 12 of 46 gastric cancers. Clements et al. [16] reported that β-catenin nuclear localization occurs in approximately one-third of gastric tumors and that β-catenin mutations occur in both diffuse- and intestinal-type gastric cancers at a higher rate. These results emphasize the activated β-catenin/Tcf signaling in gastric cancer cells. Therefore, if β-catenin's transcriptional activity can be markedly downregulated, gastric tumor growth will be suppressed.

Flavonoids, polyphenolic compounds distributed widely in plant-based foods, exert diverse biological effects in cultured cells and in vivo. Naringenin (4′,5,7-trihydroxyflavanone), aglycone of naringin, is a predominant flavanone in grapefruits and tomatoes. It has been reported to have several biological effects, such as an anticancer, antimutagenic, and anti-inflammatory activity [17,18]. Based on the antitumor activity of naringenin, we hypothesize that this antitumor activity is mediated by its ability to downregulate the β-catenin/Tcf signaling in gastric cancer cells. To assess the functions of naringenin, we employed AGS human gastric cancer cells. In the present study, we investigated the effect of naringenin against Wnt signaling and its inhibitory mechanism.

Materials and methods

Cell lines and reagents. AGS and HEK293 cell lines were derived from KCLB (Korean Cell Line Bank, Korea). Naringenin was purchased from Sigma. Stock solutions were made in DMSO. TOPflash was provided by Hans Clevers via Bart O. Williams and mutant β-catenin gene (pcDNA S33Y) was provided by Eric R. Fearon.

Isolation of cellular and nuclear extracts. Cells were trypsinized and whole cell proteins were obtained by lysing the cells on ice for 20 min in 700 μl lysis buffer (0.05 M Tris–HCl, pH7.4, 0.15 M NaCl, 1% Nonidet P-40, 0.5 M PMSF 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). The lysates were then sonicated for 20 s and spun at 15,000*g* for 10 min, and the supernatant was saved. Protein determinations were made using the method of Bradford. The nuclear pellets were prepared by resuspending cells in 800 μl lysis buffer (10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, and 0.1 mM EDTA), placing them on ice for 15 min, and then vigorously mixing after the addition of 50 μl of 10% Nonidet P-40. After a 30-s centrifugation (16,000*g*, 4 °C), the pelleted nuclei were resuspended in 120 μl of extraction buffer (50 mM Hepes, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, and 10% glycerol) and incubated on ice for 30 min. Nuclear extracts were stored at −70 °C.

Immunofluorescence. AGS cells were cultured on glass coverslips, and cells treated with naringenin (0, 20, 60, and 80 μM) were washed three times with ice-cold PBS, followed by fixing in cold methanol for 5 min 4°C. After three washes in PBS, the cells were permeabilized by immersion in 0.1% Triton X-100 for 3 min at 4°C. Staining was performed by a 1:100 dilution of mouse anti-β-catenin in PBS plus 2% BSA for 1 h, followed by a 1:100 dilution of FITC-labeled goat anti-mouse Ig with three washes in PBS between antibodies. After an additional three washes in PBS, the cells were mounted in Citifluor and viewed using a Zeiss axioplan microscope equipped with epifluorescence.

Western analysis. Western analysis for the presence of β-catenin, Tcf-4, and GSK3β was performed on the whole extract or nuclear proteins from AGS cells. Whole cell and nuclear extracts were isolated as described above. Protein (20–100 μg) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromophenol blue, and 1.25 M Tris–HCl, pH 6.8) loaded onto a 12% SDS–PAGE gel and run at 120 V for 2 h. Cell proteins were transferred to nitrocellulose (ECL; Amersham, Arlington Heights, IL) for 3 h at 250 mA. Equal loading of the protein groups on the blots was evaluated using the Bradford assay and Ponceau S staining. The nitrocellulose was then blocked with 5% milk in TBST overnight, washed four times, and then incubated with the primary Ab (anti-β-catenin, GSK3β, and Tcf-4 antibody, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. The blots were washed four times with TBST and incubated for 1 h with HRP-conjugated anti-IgG AB (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were developed using a chemiluminescent substrate (WEST-ZOL plus, iNtRON Bio-Technology, Korea). An autoradiograph was obtained with exposure times of 2 min to 12 h.

Electrophoretic mobility shift assay (EMSA). Nuclear extract (3 μg), prepared as described above, was incubated for 30 min at room temperature with ^{32}P-labeled double-stranded oligomer probe (5′-CCCTTTGATCTTACC-3′). To ensure Tcf complexes, unlabeled probes were used. It was analyzed by nondenaturing 4% polyacrylamide gel electrophoresis in 0.5× TBE buffer at 100 V for 40 min. Gels were visualized by autoradiography.

Transfection and luciferase assay. Transient transfection was performed using lipofectamine and plus reagent (Invitrogen, USA). Briefly, 6.5×10^5 cells (AGS, HEK293) were, respectively, seeded in the medium onto 60 mm dish. After 24 h, cells were transfected with 1.5 μg of the luciferase reporter constructs (TOPflash) and 1.5 μg of β-galactosidase gene for normalization. Especially, HEK293 cells were cotransfected with one another gene, pcDNA S33Y gene. After 3 h of post-transfection, inhibitors were added to the medium containing FBS. Cells were incubated for 24 h, lysed in Reporter lysis buffer (Promega, USA), and collected for assays of luciferase and galactosidase activity, respectively.

Results

Naringenin downregulates the β-catenin/Tcf signaling in AGS gastric cancer cell line

As our first objective, we assessed the impact of naringenin, whose structure is shown in Fig. 1, on β-ca-

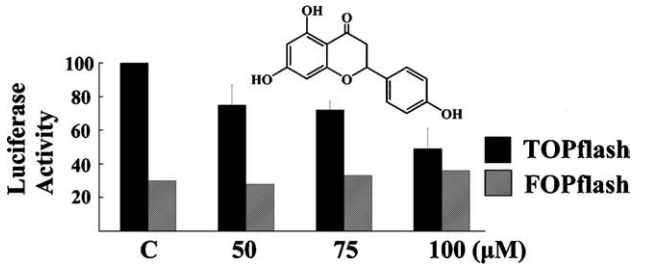

Fig. 1. Naringenin inhibits the transcriptional activity of β-catenin/Tcf in AGS cells. AGS cells were co-transfected with reporter genes harboring Tcf-4-binding sites (TOPflash) or mutant Tcf-binding sites (FOPflash), respectively, and β-galactosidase gene. Three hours post-transfection, increasing amounts of naringenin at the indicated concentration were added to the cells. Luciferase activity was determined 24 h post-transfection, normalized against values for the corresponding β-galactosidase activity. An equivalent volume of DMSO substituted for naringenin was used as a vehicle control. Values represent means ± SEM of four independent experiments.

tenin/Tcf signaling in AGS gastric cancer cell line. AGS cells (missense mutation of glycine to glutamic acid at codon 34 in GSK3β phophorylation sites) have a constitutively active transcriptional activity of β-catenin/Tcf [19]. To score the activation or suppression of Lef/Tcf-dependent transcription, we used reporter gene constructs containing four copies of an either an optimized (TOPflash) or mutant (FOPflash) Tcf-binding element. Cells were cotransfected with TOPflash or FOPflash and pCMV-β-galactosidase to normalize for transfection efficiency and treated with naringenin. Fig. 1 shows that naringenin suppresses the Tcf transcriptional activity in AGS in a concentration-dependent manner. 100 μM naringenin treatment for 24 h reduced β-catenin/Tcf transcriptional activity to 49 ± 12% of that in DMSO-treated cells. The FOPflash activity, contained in mutant Lef/Tcf sites, remained unchanged by naringenin treatment, confirming that the functional binding of β-catenin/Tcf may be important for TOPflash. The results presented in Fig. 1 implicate naringenin as a good candidate for inhibition against β-catenin/Tcf signaling.

Naringenin acts on β-catenin or its downstream elements

We investigated whether naringenin confers its inhibitory effect upstream or downstream of β-catenin/Tcf signaling. HEK293 cells were cotransfected with a constitutively active mutant of β-catenin (pcDNA S33Y), whose product is not phosphorylated by GSK3β–APC–axin complex, together with the reporter constructs. Fig. 2A shows that luciferase activity increased 100-fold by pcDNA S33Y gene transfection into HEK293 cells compared with transfection with pcDNA (mock). One hundred micromolar of naringenin reduced the increased Tcf activity to about 41 ± 3% of that in DMSO-treated cells. This strongly suggests that

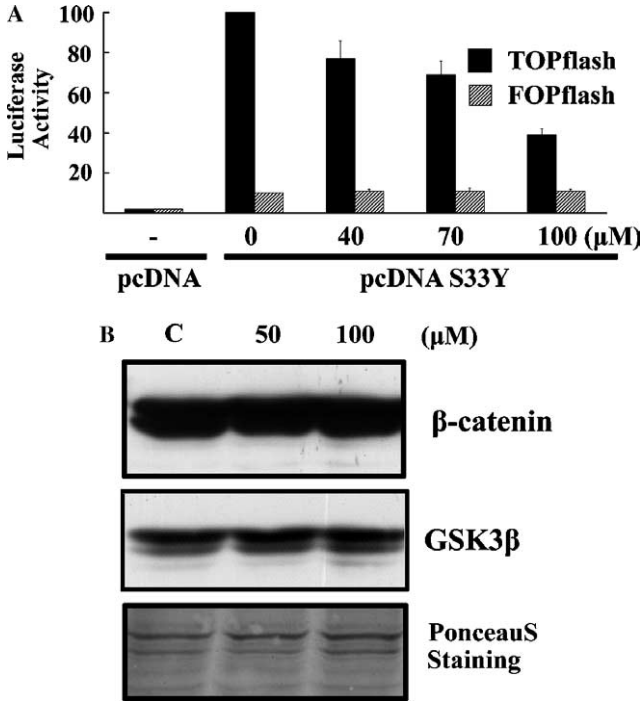

Fig. 2. The inhibition of β-catenin/Tcf signaling by naringenin is not related to the upstream component of the β-catenin pathway. (A) HEK293 cells were co-transfected with TOPflash (or FOPflash), β-galactosidase gene, and pcDNA3.1 (vehicle) or pcDNA S33Y (mutant). Three hours post-transfection, increasing amounts of naringenin at the indicated concentration were added to the cells. Luciferase activity was determined 24 h post-transfection, normalized against values for the corresponding β-galactosidase activity. An equivalent volume of DMSO substituted for naringenin was used as a vehicle control. Values represent means ± SEM of four independent experiments. (B) AGS cells were incubated with naringenin for 24 h and the cellular lysates were isolated for Western blot with GSK3β and β-catenin antibody. Ponceau S staining was used as control.

naringenin exerts its inhibitory effect on β-catenin/Tcf signaling either by acting on β-catenin itself or on downstream components rather than upstream regulators. In addition, Fig. 2B shows that no degradation of cellular β-catenin occurred due to naringenin. This strongly suggests that naringenin does not activate the degradation pathway of cytoplasmic β-catenin. The observations in Fig. 2 indicate that naringenin inhibits β-catenin/Tcf signaling either by acting on β-catenin itself or on downstream components.

Naringenin does not affect the β-catenin distribution in a cell and its nuclear level

We considered the possibility that the naringenin suppressed β-catenin/Tcf signaling by reducing the level of nuclear β-catenin or Tcf-4 proteins. Since it is well known that Tcf signaling activation results from accumulation of nuclear β-catenin [20,21], downregulation of nuclear β-catenin or Tcf-4 proteins may inactivate the transcriptional activity of β-catenin/Tcf. We first

determined the β-catenin distribution upon naringenin treatment for 24 h by indirect immunofluorescence staining in AGS cells. Strong cytoplasmic and a little weaker nuclear staining of β-catenin was visualized in control AGS cells (Fig. 3A). We treated AGS cells with naringenin (20, 60, and 80 μM), but there was no change in the distribution of β-catenin staining (Figs. 3B–D). Next, since β-catenin and Tcf-4 proteins in the nucleus are important in transactivating Tcf signaling, we focused on the levels of nuclear β-catenin and Tcf-4 proteins (Figs. 3E and F). Subconfluent AGS cells were treated with naringenin for 24 h and trypsinized for preparing nuclear extracts for immunoblotting. AGS cells have substantial level of nuclear β-catenin and Tcf-4 products (lane 1 in Fig. 3E). Even in the presence of high concentration (100 μM) of naringenin, however, the amount of nuclear β-catenin and Tcf-4 proteins was unchanged. The same test was done with HEK293 cells, which have low endogenous amounts of β-catenin protein (lane 1 in Fig. 3F). We transiently transfected with constitutively active mutant β-catenin gene into

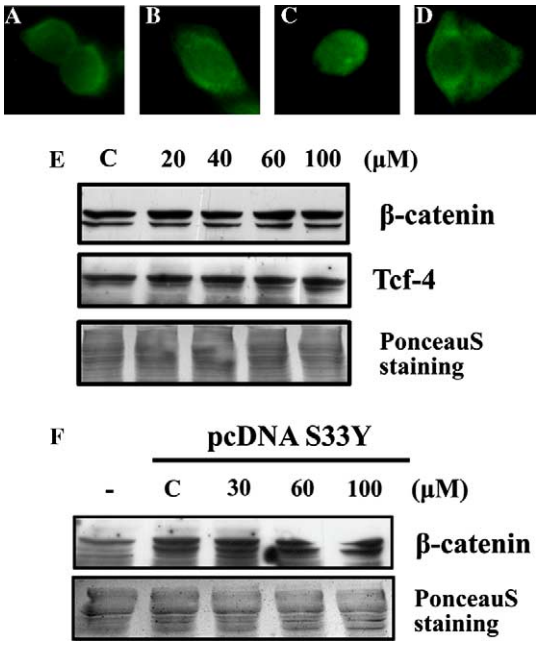

Fig. 3. Subcellular localization of β-catenin and the levels of nuclear β-catenin and Tcf-4 proteins are not affected by naringenin. (A–D) AGS cells were seeded on the slide and were treated with DMSO (A), 20 μM (B), 60 μM (C), and 80 μM (D) naringenin for 24 h. Cells were fixed, permeabilized, and stained with anti-β-catenin monoclonal antibody for indirect immunofluorescence analysis, which is described in detail under Materials and methods. Cells were observed under 400× magnification. (E,F) AGS (E) cells were treated with naringenin for 24 h, while HEK293 cells were transiently transfected with vehicle or constitutively active mutant β-catenin constructs (F) prior to naringenin treatment for 24 h. Lane 1 in (F) is the result of mock transfection (pcDNA). Cells were trypsinized for preparing nuclear extracts, which were used for Western blot with anti-β-catenin antibody or with anti-Tcf-4 antibody. To demonstrate equivalent loading of the lines, Ponceau S staining was used. C, control.

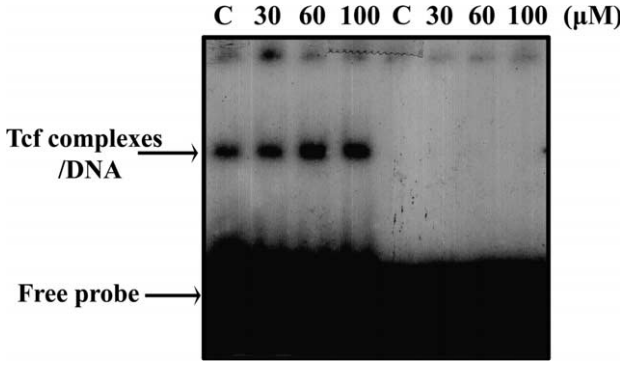

Fig. 4. The binding of Tcf complexes to DNA is not decreased by inhibitor. AGS cells were treated with naringenin for 24 h and nuclear extracts were isolated. Nuclear extracts were isolated from cells treated with DMSO (lanes 1 and 5) or with naringenin at the indicated concentrations (lanes 2–4 and 6–8). EMSA was performed with 3 μg nuclear extracts from treated or untreated cells. Lanes 1–4 are the results of binding to a ^{32}P-labeled optimal Tcf-binding region. Lanes 5–8 show that the 100-fold excess of the unlabeled optimal Tcf-binding region used as a competitor prevents binding of the Tcf complexes to the Tcf-binding region.

HEK293 cells. As a result, lane 2 in Fig. 3F shows that the level of β-catenin products in the nucleus is elevated considerably by gene transfection. Lanes 3–5 shows that naringenin did not affect this elevated level of nuclear β-catenin products. Together, we could conclude that the mechanism of reduced transcriptional activity of β-catenin/Tcf by naringenin is independent of the distribution change of β-catenin and, especially, of the nuclear level of β-catenin and Tcf-4 proteins.

Naringenin does not downregulate the binding of β-catenin/Tcf-4 to its DNA response element

We predicted that the decreased DNA binding by naringenin would be required for repression. Nuclear extracts derived from 24 h naringenin-treated cells were analyzed by EMSA for their ability to associate with ^{32}P-labeled oligonucleotide containing Tcf response elements (TRE) (Fig. 4). AGS cells have substantial binding activity of Tcf to TRE (lane 1). However, binding of the Tcf complexes to their specific-binding element was not decreased by naringenin at any concentration (lanes 2–4). These bindings could be completely eliminated by adding a 100-fold excess of ^{32}P unlabeled probe (lanes 5–8), confirming that the upper bands are specific for Tcf binding. Therefore, the suppressed β-catenin/Tcf signaling matters little to the binding activity of Tcf complexes to TRE.

Discussion

In this report, we have shown that naringenin inhibits β-catenin/Tcf signaling in AGS gastric cancer cell line.

Flavonoids have been demonstrated to inhibit carcinogenesis in vitro and substantial evidence indicates that they can also do so in vivo. Animal studies and investigations using different cellular models suggested that certain flavonoids could inhibit tumor initiation as well as tumor progression [22]. The inhibitory effect of flavonoids against gastric cancer cells was also reviewed extensively in a number of papers. Fukai et al. [23] reported that flavonoids from licorice extract may be useful as chemopreventive agents for peptic ulcer or gastric cancer in *Helicobacter pylori*-infected individuals. Kajomoto et al. [24] reported that sophoranone, a flavonoid compound, inhibited cell growth and induced apoptosis in various lines of cancer cells such as human stomach cancer MKN7 cells. Gastric cancer is the second most common cancer in the world. The etiology of stomach cancer remains unclear, but it has been shown to involve a number of risk factors, such as dietary factors and infection with *H. pylori* [14]. Several lines of evidence implicate the Wnt signaling pathway as a contributor to gastric carcinogenesis [16]. Persons with germ-line mutation of the APC tumor suppressor gene have a 10-fold increased risk of developing gastric cancer as compared with normal persons [25]. Mutations in the APC gene have also been found in sporadic gastric cancers [15]. Recently, β-catenin mutations have also been detected in intestinal type gastric carcinoma tissues and gastric cancer cell lines [26]. Based on these papers, we concluded that β-catenin/Tcf signaling is very important in gastric cancer cells. We hypothesized that the antitumor effects of naringenin are mediated by its ability to downregulate the β-catenin/Tcf signaling. Therefore, this study highlights the potential role of naringenin in the repression of the β-catenin/Tcf signaling and its inhibitory mechanism. Studies on the inhibitory agent against β-catenin/Tcf signaling in cancer cell lines have been performed. Dihlmann et al. [27] and Nath et al. [28] reported that non-steroidal anti-inflammatory drugs (NSAID) and nitric oxide-donating aspirin are good inhibitors of β-catenin/Tcf signaling in colon cancer cell lines, respectively. Dashwood et al. [29] also reported that espigallocatechin-3-gallate (EGCG) inhibits β-catenin/Tcf activity in HEK293 cells transiently transfected with a constitutively active mutant β-catenin gene. In addition, it was revealed by Jaiswal et al. [30] that curcumin inhibits the transcriptional activity of β-catenin/Tcf so as to induce growth arrest and apoptosis in HCT116 cells. Recently, Orner et al. [31] suggested that a combination of tea plus sulindac is highly effective at inhibiting intestinal neoplasia in male Apc^{min} mice via direct or indirect effects on the β-catenin/APC pathway. As the importance of β-catenin as a cause of tumorigenesis increases, many more studies on the β-catenin inhibitor and its inhibitory mechanism are being conducted. Our data of luciferase activity show that β-catenin/Tcf-driven transcription was suppressed strongly by naringenin in AGS gastric cancer cells dose-dependently (Fig. 1). Next, we focused on the inhibitory mechanism. Naringenin also inhibited the constitutively active mutant β-catenin/Tcf signaling in HEK293 cells and the β-catenin proteins in AGS were not degraded by naringenin treatment. Taken together, these results strongly suggest that the inhibitory mechanism of naringenin is not related to the upstream regulators of the β-catenin/Tcf pathway but to β-catenin itself or to the downstream components (Fig. 2). We hypothesized that the β-catenin distribution change caused by naringenin affected β-catenin/Tcf signaling. As we know, β-catenin is ubiquitous and moves freely in a cell. It contributes to the cell–cell adhesion in the membrane and functions as a transcriptional activator in the nucleus. To prove this assumption, we performed immunofluorescence and Western blot. It is well known that the binding of β-catenin to Tcf-4 induces a significant increase in the Tcf transcriptional activity. Dihlmann et al. and Nath et al. reported that subcellular localization of β-catenin protein was not affected by NSAID and NO-donating aspirin, inhibitors against β-catenin/Tcf signaling. That is, the amount of nuclear β-catenin proteins remains unchanged by NSAID and NO-donating aspirin [27,28]. We conducted the same experiments with naringenin (Fig. 3). As with the former inhibitors, the amount of nuclear β-catenin and Tcf-4 proteins was unchanged by naringenin in AGS and HEK293 cells transiently transfected with constitutively active mutant β-catenin gene. Next, we asked whether naringenin affects the DNA-binding properties of the Tcf-4 complexes. To transcript target genes, transcription factors, including β-catenin/Tcf, must bind to consensus DNA. However, Fig. 4 shows that the binding of Tcf complexes to consensus DNA is not blocked either by naringenin in AGS cells.

In summary, we have identified β-catenin/Tcf signaling as a target of naringenin action, with its inhibitory mechanism remaining unrevealed. Our paper helps to reveal the molecular mechanism underlying the anti-tumor effect of naringenin in gastric cancer cells. Given its function in inhibiting β-catenin/Tcf signaling, naringenin may be of interest as a chemotherapeutic agent against tumorigenesis.

Acknowledgments

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