RESEARCH ARTICLE

The inhibitory effects of 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone on human colon cancer cells

Peiju Qiu[1,2], Huashi Guan[2], Ping Dong[1], Shanshan Guo[1], Jinkai Zheng[1], Shiming Li[3], Yun Chen[4], Chi-Tang Ho[3], Min-Hsiung Pan[5], David Julian McClements[1] and Hang Xiao[1]

[1] Department of Food Science, University of Massachusetts, Amherst, MA, USA
[2] Marine Drug and Food Institute, Ocean University of China, Qingdao, Shandong, P. R. China
[3] Department of Food Science, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA
[4] Center for Pharmacogenomics, Washington University, St. Louis, MO, USA
[5] Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan

Received: January 30, 2011
Revised: March 16, 2011
Accepted: March 24, 2011

Scope: Previously, we reported that 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5HHMF), a polymethoxyflavone found in citrus peels, potently inhibited the growth of multiple human colon cancer cells. Herein, we further investigated the anti-cancer mechanisms of 5HHMF in human colon cancer cells.

Methods and results: Colony formation assay revealed that 5HHMF dose dependently inhibited colony formation of multiple colon cancer cells. Western blot analysis demonstrated 5HHMF decreased nuclear β-catenin levels and increased the E-cadherin level in a dose-dependent manner. 5HHMF also modified plasma membrane-associated proteins, such as K-Ras, EGFR, and their downstream effectors, such as Akt. Moreover, treatments with 5HHMF inhibited nuclear translocation of NF-κB, which may contribute to its anti-cancer effects. Add-back study showed that the inhibitory effect of 5HHMF was not associated with the production of reactive oxygen species (ROS). In addition, 5HHMF treatment inhibited the capillary tube formation of human umbilical vein endothelial cells (HUVECs) on matrigel, suggesting a potential anti-tumor angiogenesis effect.

Conclusion: Our results demonstrated that 5HHMF suppressed multiple oncogenic molecular events in colon cancer cells.

Keywords:
Angiogenesis / β-Catenin / EGFR / 5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone / K-Ras

1 Introduction

Despite the increasing efforts on research and development, cancer is continuing to be a leading cause of human mortality around the world, especially in the developed countries. Colon cancer is the second leading cause of cancer mortality in the United States [1]. As a complex disease, colon carcinogenesis involves multiple genetic and epigenetic events in cell proliferation, cell survival, inflammation, and tumor angiogenesis [2]. K-Ras mutations and epidermal growth factor receptor (EGFR) overexpression are two of the most common oncogenic events in colon cancer [3–9]. Compelling evidence has suggested that mutations in K-Ras can lead to resistance to anti-EGFR treatments because K-Ras acts downstream of EGFR [10–17]. Aberrant Wnt/β-catenin/Tcf4 signaling is frequently observed during colon carcinogenesis, which leads to activation of genes such as cyclin D1, myc, and VEGF [18]. NF-κB activation also contributes to colon carcinogenesis in part by causing inflammatory reactions in both colon tumor cells and their surrounding cellular micro-environment [19–20].

Natural products have contributed to the development of important therapeutics to combat various diseases including

Correspondence: Professor Hang Xiao, Department of Food Science, University of Massachusetts, Amherst, MA, USA
E-mail: hangxiao@foodsci.umass.edu
Fax: +1-413-545-1262

Abbreviations: EGFR, epidermal growth factor receptor; **5HHMF,** 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone; **HUVECs,** human umbilical vein endothelial cells; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NAC,** *N*-acetyl-cysteine; **ROS,** reactive oxygen species

cancer. However, this development has been greatly hampered by a lack of mechanistic understanding of molecular actions of natural products [21]. Certain fruits, vegetables, and herbs with diversified pharmacological properties have been shown to be rich sources of phytochemicals with the anti-carcinogenic potential [22, 23]. Recently, we and others have identified a unique class a flavonoids, hydroxylated polymentoxyflavones from citrus peels, as potential anti-cancer agents [24–28]. These hydroxylated polymethoxyflavones were found to extensively inhibit the growth of multiple human lung [26], colon [24], leukemia [27], and breast cancer cells [28, 29]. Among these flavonoid compounds, we studied three major ones, namely 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5HHMF, Fig. 1), 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5HPMF), and 5-hydroxy-6,7,8,4′-tetramethoxyflavone (5HTMF), and found that they potently induced apoptosis and cell-cycle arrest in multiple human colon and lung cancer cells [24, 26]. Our results also indicated that these anti-carcinogenic effects were dependent on p53, Bax, and p21 proteins [25]. Herein, we further investigated the mode of actions of 5HHMF by examining its effects on important oncogenic signaling pathways such as Wnt/β-catenin, EGFR/K-Ras/Akt, and NF-κB.

2 Materials and methods

2.1 Cell culture and treatments with 5HHMF

Human colorectal cancer cell lines (HCT116, HT29, and SW620) and human umbilical vein endothelial cells (HUVECs) were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA). HCT116 and HT29 cells were maintained in McCoy's 5A media (ATCC) supplemented with 5% heat-inactivated FBS (Mediatech, Herndon, VA, USA), 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin (Sigma-Aldrich) at 37% with 5% CO_2 and 95% air. SW620 cells were maintained in DMEM media supplemented with 5% heat-inactivated FBS, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin at 37% with 5% CO_2 and 95% air. HUVECs were cultured in endothelial growth medium 2 (EGM-2) as reported previously [30]. Cells were kept sub-confluent and media were changed every other day. All cells used were between 3 and 30 passages. 5HHMF was isolated as previously described [26, 31, 32]. Dimethylsulfoxide (DMSO) was used as the vehicle to deliver 5HHMF, and the final concentration of DMSO in all experiments was 0.1% in culture media.

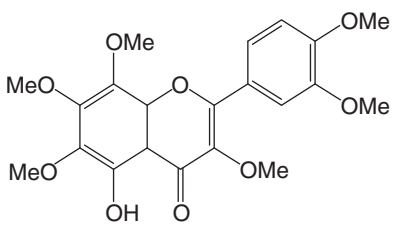

Figure 1. Chemical structure of 5HHMF.

2.2 Colony formation assay

HCT116 (1000 cells/well), HT29 (1000 cells/well), and SW620 (1000 cells/well) cells were seeded in six-well plates. After 24 h, cells were treated with various concentrations of 5HHMF in 2 mL of serum complete media. After 10 days of culture, media were removed and colonies were washed with PBS. Colonies were then stained with 0.2% crystal violet for 10 min. After thorough rinse with PBS, the colonies were counted and scanned with a high-resolution scanner (HP).

2.3 MTT assay for cellular viability

HCT116 (1500 cells/well), HT29 (2000 cells/well), and SW620 (4000 cells/well) cells were seeded in 96-well plates. After 24 h, the cells were treated with 5HHMF, *N*-acetylcysteine (NAC), or 5HHMF plus NAC at different concentrations in 200 μL of serum complete media. After 48 h treatments, cells were subject to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as we previously described [24]. Briefly, culture media were replaced by 100 μL fresh media containing 0.5 mg/mL of MTT (Sigma-Aldrich). After 1-h incubation at 37°C, MTT-containing media were removed and the reduced formazan dye was solubilized by adding 100 μL of DMSO to each well. After gentle mixing, the absorbance was monitored at 570 nm using a plate reader (Elx800TM absorbance microplate reader, BioTek Instrument, Vermont, USA).

2.4 Membrane preparation

Cell culture media were collected and spun down to recover any floating cells. Adherent cells were washed with ice-cold PBS and then collected with cell scrapers in the presence of buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, and protease inhibitor cocktail (Sigma-Aldrich) at 1–100 dilution) [33]. After combining with floating cells, cells were sonicated on ice four times at 5 s each, and then centrifuged at 100 000 × *g* for 1 h. The pellets were then solubilized with lysis buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) (1:100) on ice for 30 min after thorough mixing. Membrane fraction was collected as the supernatant after centrifugation at 10 000 × *g* for 10 min.

2.5 Preparation of nuclear fractions

The nuclear fractions were extracted using the NE-PER® Nuclear and Cytoplasmic Extraction kit. After treatment of 5HHMF for 48 h, the medium was aspirated and the cells were washed twice with ice-cold PBS (10 mM, pH 7.4). The cells were incubated in 0.2 mL ice-cold lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF (pH 7.9)) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem) for 10 min, after which 12.5 μL of 10% NP-40 was added and the contents were mixed on a vortex and then centrifuged for 1 min (14 000 × *g*) at 4°C. The supernatant was saved as cytosolic lysate and stored at −80°C. The nuclear pellet was resuspended in 50 μL of ice-cold nuclear extraction buffer (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF (pH 7.9)) with freshly added protease inhibitor cocktail for 30 min with intermittent mixing. The samples were centrifuged for 5 min (14 000 × *g*) at 4°C, and the supernatant (nuclear extract) was obtained and stored at −80°C. The protein concentration was determined by the BCA protein assay kit (Thermo Fisher).

2.6 Capillary tube formation assay

Capillary tube formation assays were carried out as described in [34, 35]. In brief, 2×10^5 HUVECS cells were plated in six-well plates coated with Low Growth Factor Matrigel (BD Biosciences, San Jose, CA, USA) and incubated overnight in the presence of serum and 10 ng/mL VEGF165 (R&D Systems, Minneapolis, MN, USA). The number of tubes, defined as projections that connect two-cell bodies per low-powered field (50 ×) for each well, was determined for at least five fields by an observer blinded to experimental groups according to protocols described in Refs. [35, 36].

2.7 Immunoblot analysis

For immunoblot analysis, equal amount of proteins (20–50 μg, depending on the proteins of interest) was resolved over 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes containing the transferred protein were blocked in blocking buffer (5% nonfat dry milk, 1% Tween-20 in 20 mM Tris-buffered saline, pH 7.6) for 2 h at room temperature, and then incubated with appropriate monoclonal primary antibody in blocking buffer overnight at 4°C. After incubation with appropriate secondary antibodies, the membranes were washed three times with Tris buffer with Tween-20 and then visualized using enhanced chemiluminescence (Boston Bioproducts, Ashland, MA, USA). Antibodies against β-catenin, E-cadherin, NF-κB, EGFR, phospho-EGFR (Tyr1068), Akt, phosphor-Akt (Ser 473), histone H3, and β-tubulin were from Cell Signaling Technology. Antibody against K-Ras was from Santa Cruz Biotechnology. Antibody against β-actin was from Sigma-Aldrich.

2.8 Statistical analyses

All data were presented as mean ± SD. Student's *t*-test was used to test the mean difference between two groups. Analyses of variance (ANOVA) model was used for the comparison of the differences among more than two groups. A 1% significant level was used for all the tests.

3 Results

3.1 5HHMF suppresses colony formation of human colon cancer cells

As shown in Fig. 2, different human colon cancer cells showed different capabilities in forming colonies. The number of colonies formed followed the order of SW620 > HCT116 > HT29. In all three cells, 5HHMF showed dose-dependent inhibitory effects on the colony formation. In HT29 cells, 5HHMF at 1, 3, 4, and 5 μM decreased the number of colonies by 15, 22, 70, and 91%, respectively (Fig. 2A). In HCT116 cells, 5HHMF at 1, 2, 3, and 4 μM decreased the number of colonies formed by 30, 35, 70, and 94%, respectively (Fig. 2B). Compared with HCT116 and HT29 cells, SW620 cells were more sensitive to 5HHMF treatment (Fig. 2C). For example, 5HHMF at 3 μM caused an inhibition of 91% on the colony formation in SW620 cells, while the same concentration of 5HHMF caused 22% and 70% inhibition in HT29 and HCT116 cells, respectively.

3.2 5HHMF inhibits β-catenin signaling

Aberrant β-catenin signaling is one of the important molecular events in the development of colon cancer. We examined the effect of 5HHMF on β-catenin signaling. By Western blot analyses, we measured nuclear levels of β-catenin protein in HCT116 colon cancer cells. Based on our previous results [24, 25], we chose to use 5HHMF at concentrations of 3, 6, and 9 μM. At 6 μM (EC50), 5HHMF can cause about 50% growth inhibition in HCT116 cells after 48 h treatment. As shown in Fig. 3A, 5HHMF dose dependently decreased the nuclear levels of β-catenin, and at 9 μM 5HHMF caused a 70% reduction of β-catenin level in the nucleus. One potential event in the suppression of β-catenin signaling is the up-regulation of E-cadherin protein [37]. Next, we examined the effects of 5HHMF on E-cadherin and found that 5HHMF dose dependently

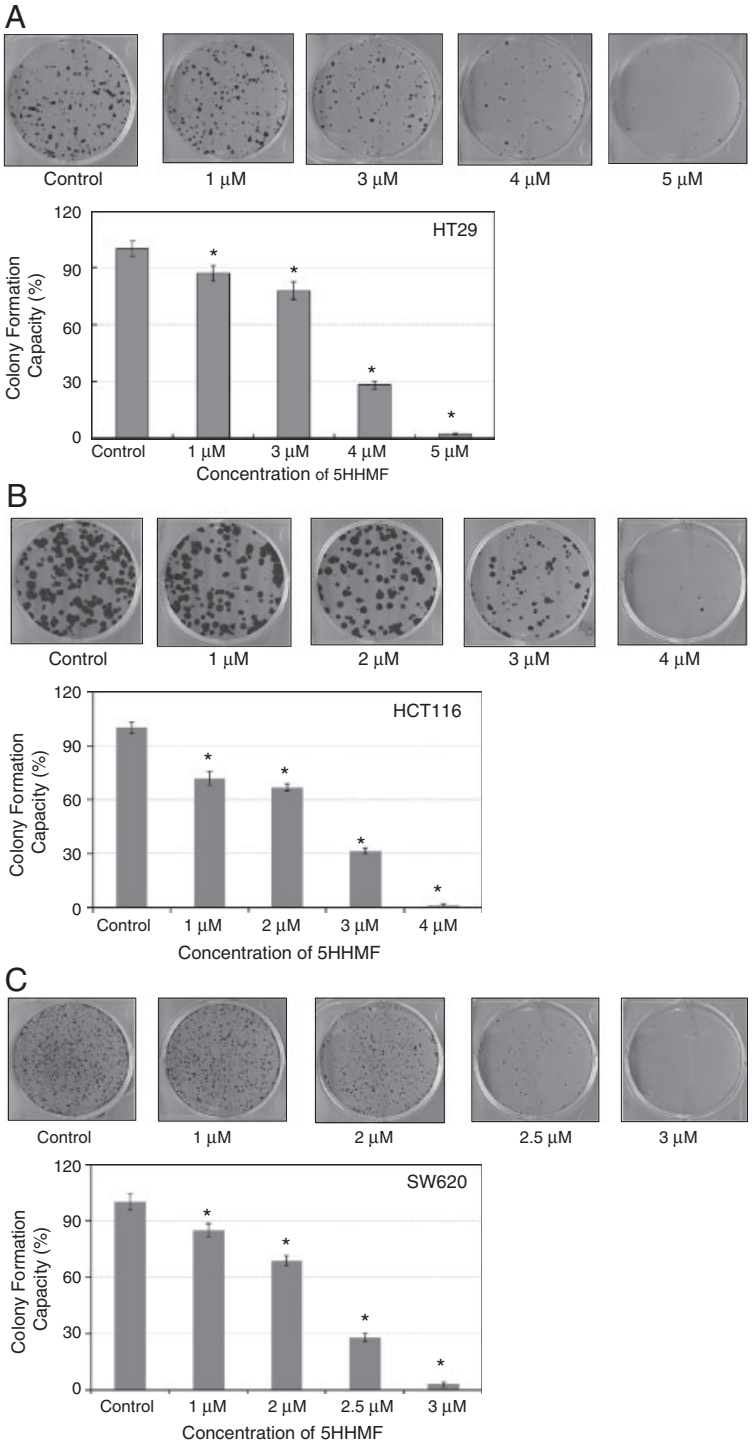

Figure 2. Inhibitory effect of 5HHMF on colony formation of different human colon cancer cells. HT29 (A), HCT116 (B), and SW620 (C) cells were seeded in six-well plates. After 24 h of incubation, cells were treated with serial concentrations of 5HHMF as indicated in the figure. After 10 days of treatments, the number of colonies formed was counted as described in Methods. Data represent mean $\pm$ SD ($n = 3$), and all the treated groups showed significant difference in comparison with the controls ($^{*}p < 0.01$).

increased the level of E-cadherin in HCT116 cells (Fig. 3B). For example, 5HHMF at 9 μM increased the level of E-cadherin by 110%. Moreover, we calculated the ratio of β-catenin/E-cadherin and found that 5HHMF at the concentrations of 3, 6, or 9 μM decreased the ratio of β-catenin/E-cadherin by 10, 40, or 90%, respectively (Fig. 3C).

3.3 5HHMF inhibits EGFR/K-Ras signaling

We further examined the effect of 5HHMF on membrane protein EGFR, K-Ras, and their downstream signaling proteins, i.e. Akt and NF-κB. As shown in Fig. 4, after 48 h treatment, 5HHMF did not significantly decrease the levels

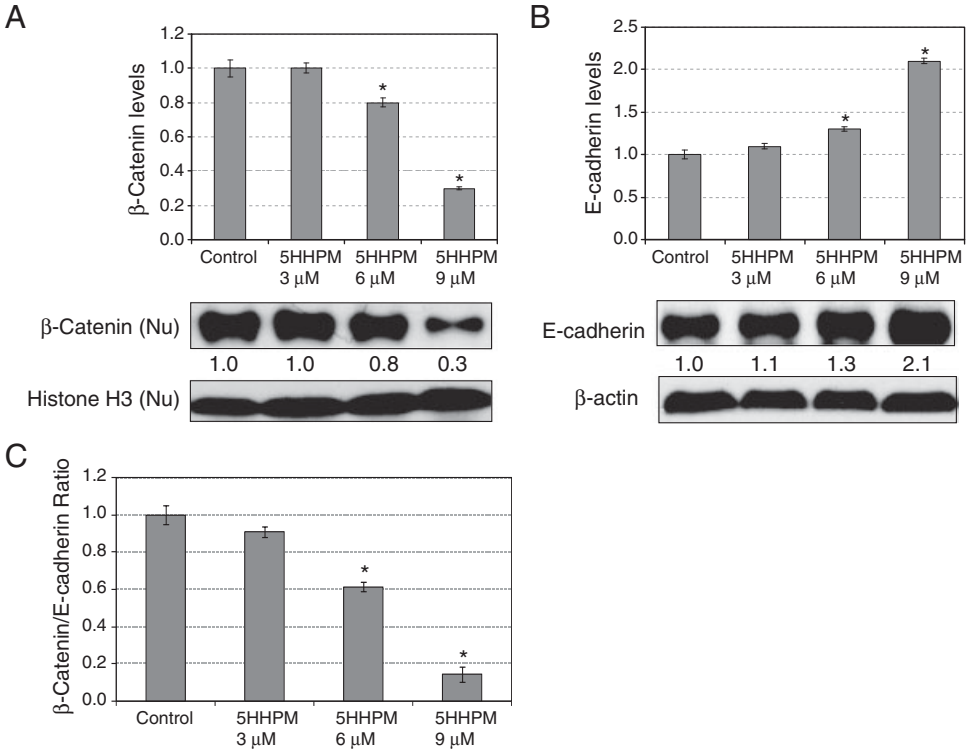

Figure 3. Effects of 5HHMF on β-catenin and E-cadherin in HCT116 human colon cancer cells. HCT116 cells were seeded in 15-cm dishes for 24 h, and then cells were treated with serial concentrations of 5HHMF. After 48 h of incubation, cells were harvested for Western immunoblotting as described in Methods. The numbers underneath the blots represent band intensities (normalized to the loading controls, mean of three independent experiments) measured by the Image J software. The standard deviations (all within ± 15% of the means) were not shown. The experiments were repeated for three times. β-Actin and histone H3 were used as equal loading controls. 'Nu' indicates the nuclear fraction. Data in C represent mean ± SD ($n = 3$), and '*' indicated that the treated groups showed significant difference in comparison with the controls ($p < 0.01$).

of EGFR in cell membrane or whole cell lysate (Fig. 4A and B). However, 5HHMF at 6 and 9 μM caused substantial decrease in the phosphorylation (Tyr 1068) by 50–60% (Fig. 4A). Treatment with 5HHMF dose-dependently decreased the levels of K-Ras in whole cell lysate. Similarly, 5HHMF treatment caused significant decrease in K-Ras levels in membrane fraction. For example, 5HHMF at 3, 6, and 9 μM decreased membrane-associated K-Ras levels by 30, 60, and 90%, respectively. In contrast, the K-Ras levels in cytosol fraction were not significantly affected by 5HHMF treatments. Akt is an important downstream effector of EGFR/K-Ras signaling. Our results showed that 5HHMF at 3, 6, and 9 μM decreased the level of Akt phosphorylation (Ser 473) by 40, 50, and 90%, respectively, whereas the total Akt levels were not changed by 5HHMF treatments. We further measured the impact of 5HHMF on NF-κB (Fig. 4C) and found that treatment of cells with 5HHMF inhibited nuclear translocation of NF-κB/p65 in a dose-dependent manner. For example, 5HHMF at 3, 6, and 9 μM significantly decreased the levels of NF-κB/p65 in nucleus by 20, 50, and 90%.

3.4 Growth inhibition by 5HHMF is not associated with ROS

A previous study has shown that 5HHMF increased the levels of cellular reactive oxygen species (ROS), which was associated with its inhibitory effects on HL-60 leukemia cells [27]. Herein, we tested whether the growth inhibition caused by 5HHMF in colon cancer cells was associated with potential production of ROS. First, we optimized the concentrations of the antioxidant NAC that acts as a cellular ROS scavenger [27]. Based on cell viability assay, the NAC concentration ranges used in HT29, HCT116, and SW620 cells were 10–200, 10–50, and 10–50 μM, respectively (Fig. 5). At these concentrations, the colon cancer cell viability was not significantly affected (< 10%) by NAC. We used 5HHMF at 8, 4, and 6 μM as positive controls in HT29, HCT116, and SW620 cells to cause considerable growth inhibition (about 40–70%). However, the addition of NAC (at different concentrations) to 5HHMF-treated cells did not affect the growth inhibition caused by 5HHMF in all three cancer cells. These results indicated that ROS may not be associated with growth inhibition caused by 5HHMF.

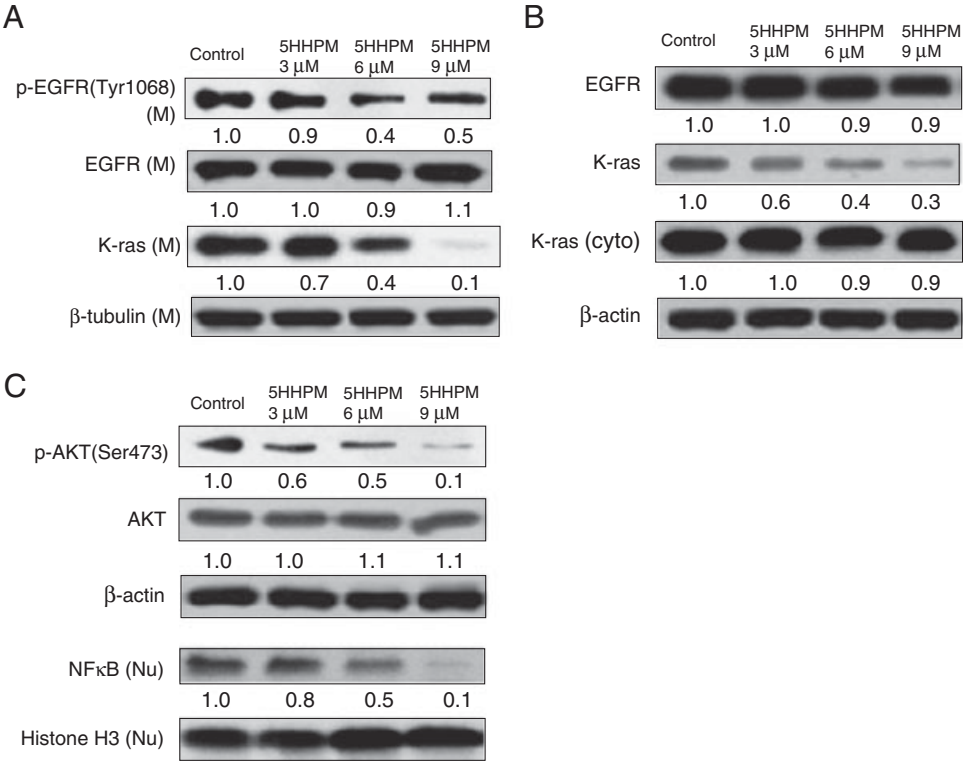

Figure 4. Effects of 5HHMF on EGFR, K-Ras, Akt, and NF κB in HCT116 human colon cancer cells. HCT-116 cells were seeded in 15-cm dishes for 24 h, and then cells were treated with serial concentrations of 5HHMF. After 48 h of incubation, cells were harvested for Western immunoblotting as described in Methods. The numbers underneath the blots represent band intensities (normalized to the loading controls, means of three independent experiments) measured by the Image J software. The standard deviations (all within ± 15% of the means) were not shown. The experiments were repeated for three times. β-Actin, β-tubulin, and histone H3 were used as equal loading controls. 'M', 'cyto', and 'Nu' indicate the membrane, cytosol, and nuclear fractions, respectively.

3.5 5HHMF inhibits angiogenesis

Angiogenesis plays an important role in tumorigenesis and metastasis. We next examined the effects of 5HHMF on the capillary tube formation of HUVECs. As shown in Fig. 6, HUVECs formed extensive capillary tubes after they were seeded on the surface of matrigel layer for 16 h. Treatments with 5HHMF caused a dose-dependent reduction on the number of tubes formed by HUVECS. Specifically, 5HHMF at 6 and 9 μM decreased the number of tubes formed by 20 and 70%, respectively.

4 Discussion

Majority of colon cancer harbor activating mutations of the Wnt signaling pathway, and these mutations can ultimately lead to the stabilization and accumulation of β-catenin in the nucleus of cancer cells where β-catenin can bind to the transcription factors Lef and Tcf, leading to the activation of gene expression and promotion of cellular proliferation [38]. Our results demonstrated that 5HHMF treatments dose-dependently suppressed nuclear translocation of β-catenin in colon cancer cells, which may contribute to the suppressive effects of 5HHMF on colon cancer cell growth. The transmembrane cell adhesion protein E-cadherin has been well-recognized as a tumor suppressor, and it plays a crucial suppressive role in the transition from adenoma to carcinoma in multiple epithelial cancers, including colon cancer [39]. The cytoplasmic domain of E-cadherin can interact with β-catenin to form an adherent junction complex, which can prevent β-catenin nuclear translocalization and β-catenin/LEF-1-mediated transactivation [40, 41]. We observed that 5HHMF increased the cellular levels of E-cadherin in colon cancer cells, which is concomitant with the decrease of nuclear levels of β-catenin. As a consequence, the ratio of β-catenin to E-cadherin was significantly reduced by up to seven-fold in colon cancer cells. These results suggested that 5HHMF upregulates E-cadherin that anchors β-catenin in plasma membrane and cytoplasm, which in turn leads to decreased levels of β-catenin in the nucleus of colon cancer cells. The lowered nuclear β-catenin level may contribute the decreased ability to form colonies by colon cancer cells.

EGFR expression is detected in 80% of human colon cancers [8, 9]. Tumors with high EGFR expression usually have poor prognosis [42], which have led to the clinical usage of EGFR inhibitors to treat colon cancer. Our results demonstrated that treatments of 5HHMF did not affect the levels of EGFR in colon cancer cells, but deactivated EGFR by decreasing the phosphorylation level of EGFR at Tyr 1068. As a key downstream effector of EGFR signaling, K-Ras plays critical roles in colon carcinogenesis, and 30–50% human colon cancers harbor K-Ras mutations [3–7]. Compelling evidence has indicated that patients with tumors positive for the K-Ras mutations do not benefit from

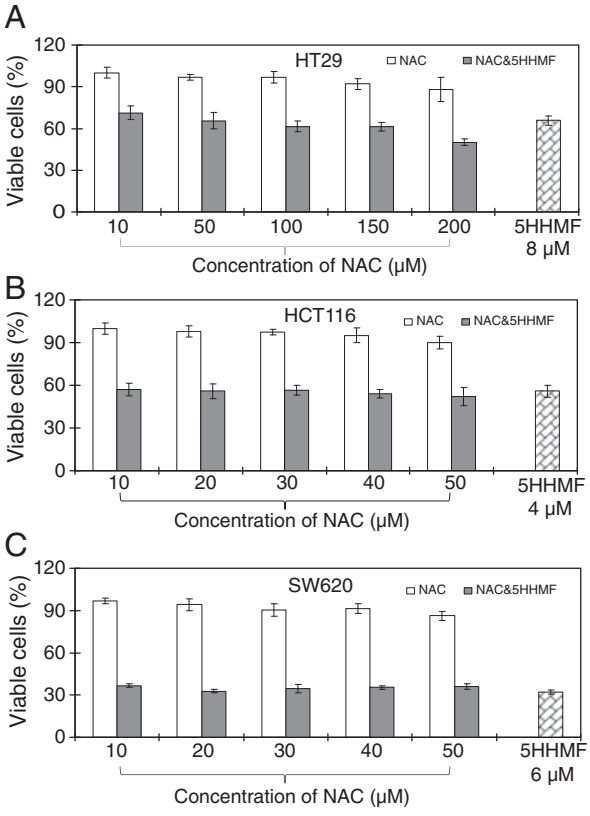

Figure 5. The effects of NAC on 5HHMF-induced growth inhibition of human colon cancer cells. HT29 (A), HCT116 (B), and SW620 (C) cells were seeded in 96-well plates. After 24 h incubation, cells were treated with 5HHMF alone (8, 4, and 6 μM for HT29, HCT116, and SW620, respectively), NAC alone (10–200, 10–50, and 10–50 μM for HT29, HCT116, and SW620, respectively), or 5HHMF+NAC. After 48 h of incubation, the cell viability was measured by the MTT method as described in Section 2. Data represent mean ± SD ($n = 6$).

anti-EGFR treatments [11], This is due to that oncogenic mutations in K-Ras circumvent the EGFR blockade and continue to activate downstream oncogenic signaling pathways, such as PI3K/Akt pathway. K-Ras is a small G-protein and its membrane association plays a key role in K-Ras activation. Our results showed that 5HHMF treatments significantly decreased the levels of membrane-bound K-Ras in colon cancer cells. Consistent with the aforementioned inhibitory effects of 5HHMF on EGFR/K-Ras, we observed that 5HHMF treatments further inhibited activation of Akt, a key downstream effector of EGFR/K-Ras signaling. These results suggested that 5HHMF can be used to inhibit aberrant K-Ras activation including those caused by K-Ras mutations. The fact that 5HHMF can inhibit EGFR activation and concomitantly decrease membrane-bound K-Ras level provide a rationale to utilize 5HHMF to treat colon cancer that harbors both EGFR activation and K-Ras mutations. In the meantime, 5HHMF can also be used in combination with other anti-EGFR treatments to enhance inhibitory effects against colon cancer resistant to anti-EGFR treatments due to K-Ras mutations.

NF-κB mediates molecular events closely associated with colon carcinogenesis [19, 20]. NF-κB consists of a Rel-homology domain for DNA binding, dimerization and interaction with its inhibitors IκBs [43]. Upon receipt of upstream signals, IκBs are phosphorylated and proteolytically removed from the complex with NF-κB, which releases NF-κB and leads to nuclear translocation of NF-κB that acts as a transcription factor to regulate genes involved in cell growth and survival [44]. Our results showed that 5HHMF treatments dose dependently inhibited nuclear translocation of NF-κB in colon cancer cells, and 5HHMF at 9 μM almost abolished the nuclear level of NF-κB. NF-κB can promote cell proliferation and growth by activating phosphoinositide 3-kinase (PI3-K)-AKT-mammalian target of rapamycin (mTOR) signaling pathway [43]; therefore, the inhibitory effects of 5HHMF on NF-κB may also contribute to the suppressive activity of 5HHMF on Akt activation observed in colon cancer cells (Fig. 4). Since NF-κB activation plays important roles in carcinogenesis of multiple organ sites, our results suggested that 5HHMF may be used as an inhibitor of NF-κB signaling in different cancer types.

Induction of apoptosis in cancer cells is an effective method to control cancer cell growth and expansion. Hydroxylated polymethoxyflavones have been shown to induce apoptosis in different cancer cells. Hydroxylation of polymethoxyflavones was found to be critical for enhancing their pro-apoptotic effects in breast [28, 29] and colon cancer cells [24]. In breast cancer cells, the pro-apoptotic effects of hydroxylated polymethoxyflavones were associated with a Ca^{2+}-mediated pathway [28, 29]. ROS have been recognized as important factors in the inhibition of cancer cell growth by inducing apoptosis. It has been reported that 5HHMF caused ROS generation in HL-60 human promyelocytic leukemia cells, which led to irreparable DNA damage and apoptosis [27]. Herein, we tested the extent to which ROS played a role in the growth inhibition caused by 5HHMF in three human colon cancer cells (HT29, HCT116, and SW620). NAC was used to scavenge potential ROS generated by 5HHMF in these colon cancer cells. Our results showed that the addition of NAC to 5HHMF-treated colon cancer cells did not rescue the cells from 5HHMF caused growth inhibition (Fig. 5). This suggested that ROS were not associated with growth inhibition by 5HHMF in three colon cancer cells tested. The possible explanation on this discrepancy between colon cancer cells and HL-60 leukemia cells in terms of their response to ROS generated by 5HHMF is that (i) colon cancer cells may be less sensitive to ROS in comparison with HL-60 cells and (ii) amount of ROS generated by 5HHMF in colon cancer cells might be less than that in HL-60 cells. Taken together, our results demonstrated that different cancer cells may respond differently to the potential ROS production caused by 5HHMF, and it is important to monitor the particular role of 5HHMF-induced ROS production in different cancer cells.

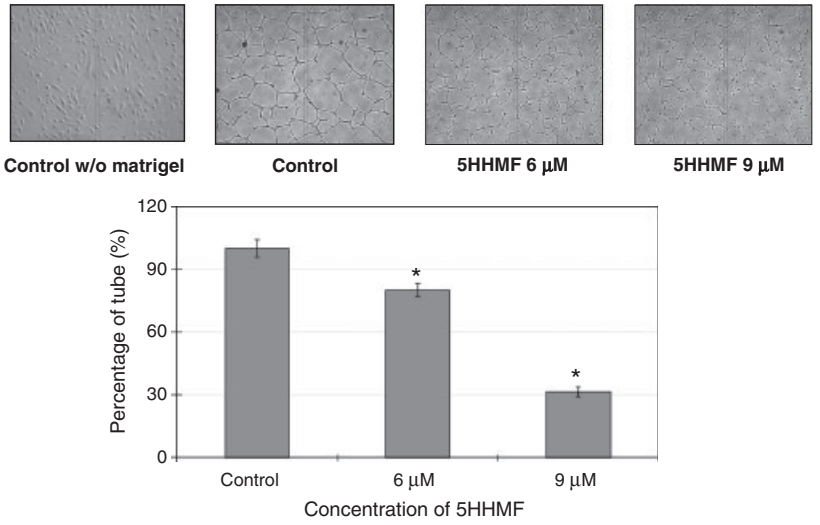

Figure 6. Effects of 5HHMF on tube formation in HUVECs cells. HUVECs cells were seeded in Matrigel-coated six-well for 24 h, and then cells were treated with serial concentrations of 5HHMF. After 16 h of incubation, the number of capillary tubes formed was counted under a microscope. Data represent mean ± SD ($n = 6$). '*' indicates the statistical significance ($p < 0.01$).

It has been well recognized that tumor invasion and metastasis are the major causes of cancer treatment failure. Tumor can form new blood vessel by a process called angiogenesis that is a key factor for tumor invasion and metastasis. During angiogenesis, tumor utilizes existing endothelium and endothelial precursor cells to generate new blood vessel, which involves complex actions, such as extracellular matrix degradation, proliferation, and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes [45–47]. Herein, we used HUVECs to mimic tube formation process during angiogenesis, and to determine the extent to which 5HHMF may inhibit angiogenesis. We observed that 5HHMF treatments caused a significant decrease in the number of tubes formed in a dose-dependent manner. Our results suggested that 5HHMF is not only capable of inhibiting tumor cells growth but also can suppress new tumor blood vessel formation. This is of importance because decreased tumor angiogenesis may greatly limit the expansion of tumor mass and decrease the possibility of tumor invasion and metastasis.

In summary, the present study demonstrated potent inhibitory effects of 5HHMF on colony-forming ability of colon cancer cells. These activities were associated with the suppressive effects of 5HHMF on multiple oncogenic signaling pathways, such as EGFR/K-Ras signaling, Wnt/β-catenin signaling, and NF-κB signaling. Moreover, we also demonstrated that 5HHMF inhibited angiogenesis, which may play an important role in inhibiting tumor invasion and metastasis. Taken together, our results provided new information on the mode of actions of 5HHMF as a cancer inhibitory agent, which warrants further in vivo investigation on the anti-cancer efficacy of 5HHMF.

This work was supported in part by an NIH grant CA139174, a grant from American Institute for Cancer Research, a Hatch grant from the University of Massachusetts Amherst, and by a Pilot Grant from the Center for Excellence in Apoptosis Research at the Pioneer Valley Life Sciences Institute.

The authors have declared no conflict of interest.

5 References

[1] Jemal, A., Siegel, R., Ward, E., Murray, T. et al., Cancer statistics, 2007. *CA – Cancer J. Clin.* 2007, *57*, 43–66.

[2] Janakiram, N. B., Rao, C. V., Molecular markers and targets for colorectal cancer prevention. *Acta Pharmacol. Sin.* 2008, *29*, 1–20.

[3] Smith, G., Carey, F. A., Beattie, J., Wilkie, M. J. et al., Mutations in APC, Kirsten-ras, and p53 – alternative genetic pathways to colorectal cancer. *Proc. Natl. Acad. Sci. USA* 2002, *99*, 9433–9438.

[4] Giaretti, W., Venesio, T., Sciutto, A., Prevosto, C., et al., Near-diploid and near-triploid human sporadic colorectal adenocarcinomas differ for KRAS2 and TP53 mutational status. *Genes Chromosomes Cancer* 2003, *37*, 207–213.

[5] Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., et al., Prevalence of ras gene mutations in human colorectal cancers. *Nature* 1987, *327*, 293–297.

[6] Forrester, K., Almoguera, C., Han, K., Grizzle, W. E., Perucho, M., Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. *Nature* 1987, *327*, 298–303.

[7] Andreyev, H. J., Norman, A. R., Cunningham, D., Oates, J. R., Clarke, P. A., Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. *J. Natl. Cancer Inst.* 1998, *90*, 675–684.

[8] Messa, C., Russo, F., Caruso, M. G., Di Leo, A., EGF, TGF-alpha, and EGF-R in human colorectal adenocarcinoma. *Acta Oncol.* 1998, *37*, 285–289.

[9] Porebska, I., Harlozinska, A., Bojarowski, T., Expression of the tyrosine kinase activity growth factor receptors (EGFR,

ERB B2, ERB B3) in colorectal adenocarcinomas and adenomas. *Tumour Biol.* 2000, *21*, 105–115.

[10] Wheeler, D. L., Dunn, E. F., Harari, P. M., Understanding resistance to EGFR inhibitors-impact on future treatment strategies. *Nat. Rev. Clin. Oncol.* 2010, *7*, 493–507.

[11] Jiang, Y., Kimchi, E. T., Staveley-O'Carroll, K. F., Cheng, H., Ajani, J. A., Assessment of K-ras mutation: a step toward personalized medicine for patients with colorectal cancer. *Cancer* 2009, *115*, 3609–3617.

[12] Van Cutsem, E., Kohne, C. H., Hitre, E., Zaluski, J. et al., Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N. Engl. J. Med.* 2009, *360*, 1408–1417.

[13] Lievre, A., Bachet, J. B., Le Corre, D., Boige, V. et al., KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res.* 2006, *66*, 3992–3995.

[14] Di Fiore, F., Blanchard, F., Charbonnier, F., Le Pessot, F., et al., Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. *Br. J. Cancer* 2007, *96*, 1166–1169.

[15] De Roock, W., Piessevaux, H., De Schutter, J., Janssens, M. et al., KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. *Ann. Oncol.* 2008, *19*, 508–515.

[16] Amado, R. G., Wolf, M., Peeters, M., Van Cutsem, E. et al., Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J. Clin. Oncol.* 2008, *26*, 1626–1634.

[17] Karapetis, C. S., Khambata-Ford, S., Jonker, D. J., O'Callaghan, C. J. et al., K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N. Engl. J. Med.* 2008, *359*, 1757–1765.

[18] Segditsas, S., Tomlinson, I., Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* 2006, *25*, 7531–7537.

[19] Sakamoto, K., Maeda, S., Targeting NF-kappaB for colorectal cancer. *Exp. Opin. Ther. Targets* 2010, *14*, 593–601.

[20] Vaiopoulos, A. G., Papachroni, K. K., Papavassiliou, A. G., Colon carcinogenesis: learning from NF-kappaB and AP-1. *Int. J. Biochem. Cell Biol.* 2010, *42*, 1061–1065.

[21] Cheng, K. W., Wong, C. C., Wang, M., He, Q. Y., Chen, F., Identification and characterization of molecular targets of natural products by mass spectrometry. *Mass Spectrom. Rev.* 2010, *29*, 126–155.

[22] Hong, W. K., Sporn, M. B., Recent advances in chemoprevention of cancer. *Science* 1997, *278*, 1073–1077.

[23] Rudolf, E., Andelova, H., Cervinka, M., Polyphenolic compounds in chemoprevention of colon cancer – targets and signaling pathways. *Anticancer Agents Med. Chem.* 2007, *7*, 559–575.

[24] Qiu, P., Dong, P., Guan, H., Li, S. et al., Inhibitory effects of 5-hydroxy polymethoxyflavones on colon cancer cells. *Mol. Nutr. Food Res.* 2010, *54*, S244–S252.

[25] Qiu, P., Guan, H., Dong, P., Li, S. et al., The p53-, Bax- and p21-dependent inhibition of colon cancer cell growth by 5-hydroxy polymethoxyflavones. *Mol. Nutr. Food Res.* 2011, *55*, 613–622.

[26] Xiao, H., Yang, C. S., Li, S., Jin, H. et al., Monodemethylated polymethoxyflavones from sweet orange (*Citrus sinensis*) peel inhibit growth of human lung cancer cells by apoptosis. *Mol. Nutr. Food Res.* 2009, *53*, 398–406.

[27] Pan, M. H., Lai, Y. S., Lai, C. S., Wang, Y. J. et al., 5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone induces apoptosis through reactive oxygen species production, growth arrest and DNA damage-inducible gene 153 expression, and caspase activation in human leukemia cells. *J. Agric. Food Chem.* 2007, *55*, 5081–5091.

[28] Sergeev, I. N., Li, S., Colby, J., Ho, C. T., Dushenkov, S., Polymethoxylated flavones induce Ca(2+)-mediated apoptosis in breast cancer cells. *Life Sci.* 2006, *80*, 245–253.

[29] Sergeev, I. N., Ho, C. T., Li, S., Colby, J., Dushenkov, S., Apoptosis-inducing activity of hydroxylated polymethoxyflavones and polymethoxyflavones from orange peel in human breast cancer cells. *Mol. Nutr. Food Res.* 2007, *51*, 1478–1484.

[30] Chen, Y., Leal, A. D., Patel, S., Gorski, D. H., The homeobox gene GAX activates p21WAF1/CIP1 expression in vascular endothelial cells through direct interaction with upstream AT-rich sequences. *J. Biol. Chem.* 2007, *282*, 507–517.

[31] Li, S., Lo, C. Y., Ho, C. T., Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel. *J. Agric. Food Chem.* 2006, *54*, 4176–4185.

[32] Wang, Z., Li, S., Jonca, M., Lambros, T. et al., Comparison of supercritical fluid chromatography and liquid chromatography for the separation of urinary metabolites of nobiletin with chiral and non-chiral stationary phases. *Biomed. Chromatogr.* 2006, *20*, 1206–1215.

[33] Park, H. J., Kong, D., Iruela-Arispe, L., Begley, U. et al., 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors interfere with angiogenesis by inhibiting the geranylgeranylation of RhoA. *Circ. Res.* 2002, *91*, 143–150.

[34] Chen, Y., Gorski, D. H., Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood* 2008, *111*, 1217–1226.

[35] Gorski, D. H., Leal, A. J., Inhibition of endothelial cell activation by the homeobox gene Gax. *J. Surg. Res.* 2003, *111*, 91–99.

[36] Patel, S., Leal, A. D., Gorski, D. H., The homeobox gene Gax inhibits angiogenesis through inhibition of nuclear factor-kappaB-dependent endothelial cell gene expression. *Cancer Res.* 2005, *65*, 1414–1424.

[37] Conacci-Sorrell, M., Zhurinsky, J., Ben-Ze'ev, A., The cadherin–catenin adhesion system in signaling and cancer. *J. Clin. Invest.* 2002, *109*, 987–991.

[38] Kongkanuntn, R., Bubb, V. J., Sansom, O. J., Wyllie, A. H., Harrison, D. J., Clarke, A. R., Dysregulated expression of beta-catenin marks early neoplastic change in Apc mutant mice, but not all lesions arising in Msh2 deficient mice. *Oncogene* 1999, *18*, 7219–7225.

[39] Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H., Christofori, G., A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* 1998, *392*, 190–193.

[40] Aberle, H., Schwartz, H., Kemler, R., Cadherin–catenin complex: protein interactions and their implications for cadherin function. *J. Cell. Biochem.* 1996, *61*, 514–523.

[41] Orsulic, S., Huber, O., Aberle, H., Arnold, S., Kemler, R., E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. *J. Cell. Sci.* 1999, *112*, 1237–1245.

[42] Mayer, A., Takimoto, M., Fritz, E., Schellander, G. et al., The prognostic significance of proliferating cell nuclear antigen, epidermal growth factor receptor, and mdr gene expression in colorectal cancer. *Cancer* 1993, *71*, 2454–2460.

[43] Shen, H. M., Tergaonkar, V., NFkappaB signaling in carcinogenesis and as a potential molecular target for cancer therapy. *Apoptosis* 2009, *14*, 348–363.

[44] Basseres, D. S., Baldwin, A. S., Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. *Oncogene* 2006, *25*, 6817–6830.

[45] Cavallaro, U., Christofori, G., Molecular mechanisms of tumor angiogenesis and tumor progression. *J. Neurooncol.* 2000, *50*, 63–70.

[46] Folkman, J., Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1995, *1*, 27–31.

[47] Haas, T. L., Milkiewicz, M., Davis, S. J., Zhou, A. L. et al., Matrix metalloproteinase activity is required for activity-induced angiogenesis in rat skeletal muscle. *Am. J. Physiol. Heart Circ. Physiol.* 2000, *279*, H1540–H1547.

