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Pomegranate polyphenolics suppressed azoxymethane-induced colorectal aberrant crypt foci and inflammation: possible role of miR-126/VCAM-1 and miR-126/PI3K/AKT/mTOR

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The antitumorigenic activities of polyphenols such as ellagitannins and anthocyanins in pomegranate (*Punica granatum L.*) have been previously studied where cytotoxic, anti-inflammatory and antioxidant effects were evident in various cancer models. The objective of this study was to investigate the role of miR-126/vascular cell adhesion molecule 1 (VCAM-1) and miR-126/phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) in pomegranate-mediated anti-inflammatory and anticarcinogenic effects *in vivo* and *in vitro*. Sprague-Dawley rats (n = 10 per group) received pomegranate juice (2504.74 mg gallic acid equivalents/l) or a polyphenol-free control beverage *ad libitum* for 10 weeks and were injected with azoxymethane (AOM) subcutaneously (15 mg/kg) at weeks 2 and 3. Consumption of pomegranate juice suppressed the number of aberrant crypt foci (ACF) and dysplastic ACF by 29 and 53.5% (P = 0.05 and 0.04), respectively, and significantly lowered proliferation of mucosa cells. Pomegranate juice significantly downregulated proinflammatory enzymes nitric oxide synthase and cyclooxygenase-2 messenger RNA (mRNA) and protein expression. In addition, it suppressed nuclear factor-κB and VCAM-1 mRNA and protein expression in AOM-treated rats. Pomegranate also inhibited phosphorylation of PI3K/AKT and mTOR expression and increased the expression of miR-126. The specific target and functions of miR-126 were investigated in HT-29 colon cancer cell lines. *In vitro*, the involvement of miR-126 was confirmed using the antagomiR for miR-126, where pomegranate reversed the effects of the antagomiR on the expression of miR-126, VCAM-1 and PI3K p85β. In summary, therapeutic potentials of pomegranate in colon tumorigenesis were due in part to targeting miR-126-regulated pathways, which contributes in the underlying anti-inflammatory mechanisms.

Introduction

Colorectal cancer is the third leading cause of death in the USA (1). According to epidemiological and intervention studies, colon carcinogenesis is significantly influenced by dietary, genetic and environmental factors. These factors may cause disruption of normal cell growth, uncontrolled proliferation and differentiation associated with inflammation and oxidative stress. Owing to the low survival rate of colon cancer, it is crucial to investigate nutritional prevention approaches and their underlying mechanisms of action (2,3).

Epidemiological, preclinical and clinical studies suggested that a diet rich in natural polyphenolics may protect against colonic inflammation and colon cancer (4–6). Clinical and preclinical studies have reported that polyphenolics from pomegranate exert anticarcinogenic, anti-inflammatory and antioxidant activities (7–10). Polyphenols, extracted from pomegranate peels and seed, have shown to induce cytotoxicity in several cancer cell lines such as lung, prostate, breast, and in chemically induced colon cancer in animal studies (7,9,11–15). Predominant polyphenolics in pomegranate include ellagic acid, ellagitannins, punicalagin, flavonoids and 3-glucosides/3,5-diglucosides of the anthocyanins delphinidin, cyanidin and pelargonidin (10,16) and these polyphenolics exhibited antioxidant, anti-inflammatory and anticarcinogenic properties *in vitro* and *in vivo* (7,8,17).

Previous studies have demonstrated the downregulation of a common colon cancer marker, vascular cell adhesion molecule 1 (VCAM-1) (18,19) by polyphenols (20). In addition, pomegranate polyphenols also modulated the expression of phosphatidylinositol-3-kinase (PI3K)/AKT pathways. In the human lung carcinoma cells (A549) and lung tumors in mice, where inflammation was induced by the induction of nuclear factor-κB (NF-κB) and PI3K/AKT signaling pathway, pomegranate polyphenols downregulated inflammation by suppressing PI3K and AKT phosphorylation and decreased the activation of NF-κB (21,22). Insulin-like growth factor (IGF) influenced the regulation of the phosphorylation of PI3K/AKT, which is crucial in the pathogenesis of colon cancer. PI3K/AKT pathway influenced cell survival through phosphorylation of downstream targets such as NF-κB and mammalian target of rapamycin (mTOR). NF-κB regulated inflammatory markers including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (23).

The anti-inflammatory miR-126 has target sites in the promotor regions of VCAM-1 and PI3K p85β subunit, and low expression of miR-126 has been associated with colon cancer (24–26). It was shown previously that red wine polyphenolics increased the expression of miR-126, which was involved in downregulating VCAM-1 (20).

In our preliminary studies in HT-29 colon cancer cells, pomegranate induced apoptosis and increased the expression of miR-126, suppressed VCAM-1, NF-κB, iNOS, COX-2 and decreased the phosphorylation of AKT. Based on these preliminary *in vitro* findings, the objective of this study was to investigate the efficacy and underlying mechanisms of pomegranate in the prevention of AOM-induced aberrant foci in a rat model. The working hypothesis was that miR-126/VCAM-1 and miR-126/PI3K/AKT-mTOR were significantly involved in the anti-inflammatory, cytotoxic and cancer-preventive activities of pomegranate in AOM-treated rats and also *in vitro* in HT-29 colon cancer cells.

Materials and methods

Experimental juice and extract

Rats were divided into two groups: control juice or pomegranate juice. Pomegranate juice was obtained from the Stiebs (Kirkland, WA). For calorie adjustment, 15.7 g sugar and 0.05 g citric acid were added in 100 ml of control juice. Total phenolic content in the pomegranate juice was measured spectrophotometrically by the Folin–Ciocalteu assay against an external standard of gallic acid and expressed as gallic acid equivalents (GAE). The polyphenolic composition of pomegranate juice was determined by high-performance liquid chromatography-mass spectrometry as described previously (10). The extract was also prepared as described in our previous literature (10).

Reagents

Standards for high-performance liquid chromatography-mass spectrometry analysis were obtained from Sigma–Aldrich (St Louis, MI) and Chromadex (Irvine, CA). Antibodies against NF-κB (p65), phosphorylated NF-κB (p65), COX-2, iNOS, IGF and pmTOR were purchased from Cell Signaling

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; COX-2, cyclooxygenase-2; GAE, gallic acid equivalent; IGF, insulin-like growth factor; iNOS, nitric oxide synthase; miRNA, microRNA; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol-3-kinase; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor.

Technology (Beverly, MA). All other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western lighting chemiluminescence reagent was purchased from Perkin-Elmer Life Sciences (Waltham, MA). All primers were purchased from Integrated DNA Technologies (San Diego, CA). mirVana™ extraction kit, reverse transcription and real-time PCR amplification kits were purchased from Applied Biosciences (Foster City, CA). AntagomiR of miR-126 as well as scrambled microRNA (miRNA) were from Dharmacon (Lafayette, CO) (10).

Cell culture

Human colon carcinoma cell lines HT-29, were obtained from American Type Tissue Collection (ATCC) and maintained according to the supplier guidelines (ATCC, Manassas, VA). The cell proliferation was assessed with an electronic cell counter at 48 h (Z2™ Series; Beckman Coulter, Fullerton, CA), as described previously (27) and were showed as net growth.

Animal treatments and tissue collection

Twenty male Sprague-Dawley rats (3-week-old) supplied from Harlan Teklad (Houston, TX) were kept in suspended cages in a room controlled at a temperature of 23 ± 2°C, humidity of 55 ± 5% and 12 h light/dark cycle, and they had free access to regular food pellets and liquids. Animals were randomly distributed by weight into control juice and pomegranate juice groups and fed experimental juices daily instead of water during the entire experiment period. All rats were given intraperitoneal injection of AOM (15 mg/kg body weight; Sigma Chemical Co., St Louis, MO) twice, at 2 and 3 wks, after starting the experimental diets. Rats were killed 6 weeks after the second AOM injection, and colon tissues were collected. One centimeter sections were cut from the distal end of each rat colon, fixed in 4% paraformaldehyde and then embedded in paraffin. Half of the colon was fixed at 70% ethanol for aberrant crypt foci (ACF) determination and the other half was gently scraped for collecting protein and RNA (28,29). The animal use protocol was approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Assessment of ACF

ACF are preneoplastic lesions of adenocarcinoma in the carcinogen-induced colon carcinogenesis. ACF, particularly the dysplastic ACF, are thought to be biomarkers of colon cancer risk and are used as an intermediate endpoint to evaluate nutritional factors (28,29). After 24 h of fixation in 70% ethanol, tissue was stained with 0.5% methylene blue for 1 min, and the total number of ACF was counted using a light microscope at ×40 magnification.

Cell proliferation assay

For Ki-67 immunohistochemistry analysis, sections were treated with primary antibody against Ki-67 (Dilution 1:50; BD Pharmingen, San Jose, CA) and incubated with biotinylated anti-mouse immunoglobulin G, Vectastain ABC Elite kit (Vector Lab, Burlingame, CA). Ki-67-containing nuclei, indicative of proliferating cells, were visible as brown spots within colonic crypt columns. Twenty-five crypt columns per rat were selected for analysis.

Quantitative reverse transcription–PCR

Total RNA from the colon mucosal scraping was isolated using the mirVana™ miRNA Isolation Kit. HT-29 cells were seeded (3×10^5 cells onto a 6-well plate) and incubated for 24 h. Cells were treated with pomegranate extract and messenger RNA (mRNA) was extracted after 24 h (10). Equal amount (1 µg) of mRNAs was converted to complementary DNA using a reverse transcription kit (Invitrogen Corp., Grand Island, NY) (28,29). Real-time PCR reactions were performed using 2 µl of complementry DNA using a Reverse Transcription Kit (Invitrogen). SYBR Green PCR master Mix (Applied Biosystems, Foster City, CA) was used for the quantitative PCR analyses on Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The sequence of primers was designed using Primer3Plus® (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) and were obtained from Integrated DNA Technologies (Coralville, IA). Glyceraldehyde 3-phosphate dehydrogenase was used as the endogenous loading control (10,27).

Western blotting

The mucosal scrapings were homogenized in a protein buffer (500 mM Tris-HCl, 1 M sucrose, 200 mM ethylenediaminetetraacetic acid, 100 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 0.4 M NaF, 10% Triton X-100, 10 mM sodium orthovanadate and protease inhibitor cocktail), were centrifuged at 15 000*g* for 20 min at 4°C, and the supernatant was stored in a 80°C freezer (28,29). HT-29 cells were seeded (1×10^5 onto 6-well plates) and incubated for 24 h to allow cell attachment. They were treated with pomegranate extract (5–25 µg/ml) for 24 h and protein was extracted after 24 h (10). Sixty micrograms of protein was loaded onto 10% electrophoresis gel, followed by electrotransfer onto polyvinylidene difluoride membranes. The blots were probed with the primary antibodies against VCAM-1 (Santa Cruz biotechnology), COX-2 (Cell Signaling Technology, Danvers, MA) and β-actin (Sigma, St Louis, MO) (10,27).

Transfection with antagomiR of miR-126

AntagomiR is a miRNA inhibitor that inhibits a specific miRNA by irreversibly binding the miRNA. Cells seeded (1×10^5 onto 12-well plates) were incubated for 24 h to allow cell attachment. After transfection with 20 nM of antagomiR of miR-126 into cells for 4 h, the transfection mix was replaced with medium containing 25 µg/ml pomegranate extract and incubated for 24 h as described previously (20,27).

Statistical analysis

Quantitative data represent mean values with standard error. Data were analyzed by Student's *t*-test or one-way analysis of variance using Tukey's *post hoc* test ($P < 0.05$) using SAS version 9 (SAS Institute, Cary, NC) (10).

Results

Chemical analysis of pomegranate juice

The polyphenolics profile of pomegranate juice was representative of 100% pomegranate juice (10). The chromatographic profiles showed the ellagitannins such as punicalins (peak 1), punicalagin A (peak 2), punicalagin B (peak 3) and ellagic acid (peak 4) with peak absorption at 280 nm (Figure 1A) (10).

Pomegranate juice intake, food intake and body weight of AOM-treated rats

The concentration of total soluble phenolics of pomegranate juice used for the animal study was 2504.74 mg GAE/l. Rats in pomegranate group (326.557 g body weight) drank an average of 57.211 ml of pomegranate juice everyday (Table I). The dose of total soluble phenolics was 438.95 mg GAE/kg/day for rats. By the calculation of the human equivalent amount of pomegranate juice using the body surface area normalization method (human equivalent dose (mg/kg) =

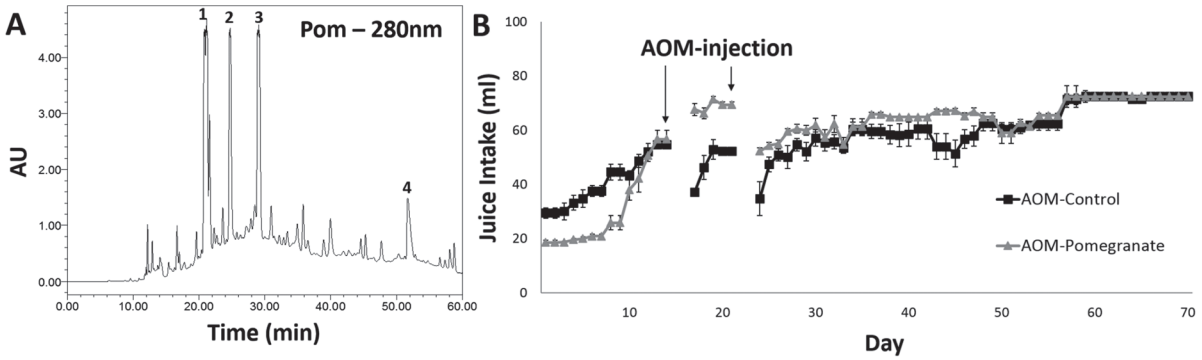

Fig. 1. Representative chromatogram of polyphenolic compounds in pomegranate juice and the daily juice consumption by rats. (**A**) Ellagitannins at 280 nm, tentative peak assignments: 1, punicalins; 2, punicalagin A; 3, punicalagin B; 4, ellagic acid. (**B**) The daily juice consumption by rats was measured every alternative day. Values are mean ± standard error, $n = 20$.

rat dose (mg/kg) × (rat K_m/human K_m(6/37)) (30), the human equivalent dose of pomegranate polyphenolics based on an estimate of the amount consumed in rats was 71.18 mg GAE/kg/day, which equals the intake of 2.13 l juice for an individual of 75 kg body weight. The intake of pomegranate and control beverage was determined every alternative day, and the loss of liquid from bottles was adjusted in the calculation of liquid intake. The intake of liquid was increased compared with other studies with Sprague-Dawley rats within the same weight range (31). The main cause for increased liquid intake in this study was the addition of sugars and citric acid to the control (instead of water) and administering pomegranate juice instead of water. At the same time, the intake of solid food was reduced to about two-thirds of the expected intake for rats within that weight range (31). For the initial 9 days, the intake of pomegranate was slightly but significantly lower compared with the control group by around 10% on average, probably because animals had to get used to the astringency of contained ellagitannins, however, the overall intake of liquids was not significantly different over the entire study duration. For the initial 48 h after each AOM injection (week 2 and 3), the intake of liquids was significantly lower in the control group compared with the pomegranate-treated group, by 3 and 30 ml, respectively. This was potentially due to ameliorating effects of pomegranate on appetite-suppressing effects of AOM (Figure 1B). The intake of solid food was significantly higher in the pomegranate group by 6 g/day compared with the control group. In contrast, the pomegranate group gained less weight compared with the control group, where the final average body weight of the control group was 30 g above the pomegranate group (Table I).

Table I. Effects of pomegranate juice on final body weight, food intake and juice intake

Group	Final body weight (g)	Food intake (g/day)	Juice intake (ml/day)
Control	355.975 ± 30.007	10.460 ± 1.804	53.186 ± 11.31[a]
Pomegranate	326.557 ± 20.034*	16.956 ± 1.430*	57.211 ± 16.75

Each value is a mean ± SD (n = 20). Food intake was measured as the mean (± SD) weight (g) of food intake for 48 h period at 9 weeks after the second AOM injection. Juice intake was calculated as the mean (± SD) volume (ml) of juice consumed for whole study.
[a]ns, not significantly different.
Values are statistically significant at *P < 0.05.

Pomegranate juice inhibited ACF formation in AOM-treated rats

ACF are used as a marker in the assessment of the early stage of carcinogenesis in colons of AOM-treated animals (32). Development of preneoplastic lesions, especially dysplastic ACF (>4 crypts per focus) is found in human and animal colon cancer models and are highly correlated to tumorigenesis (28,29). In this study, the number of ACF and dysplastic ACF was assessed over the entire length of the colon mucosa. The total number of ACF and dysplastic ACF was significantly decreased by the pomegranate treatment by 29 and 53.5%, respectively, (Figure 2A and 2B) compared with the control group (P = 0.05 and 0.04).

Pomegranate juice suppressed cell proliferation

Previous studies showed that pomegranate polyphenolics reduced cell proliferation in colon carcinogenesis (33). Cell proliferation of mucosal tissue harvested from the colon of AOM-induced rats was determined by immunohistochemistry using the Ki-67 antibody as an indicator for the proliferative index (Figure 2C and 2D). The colon mucosa of pomegranate-treated rats showed a significant reduction of Ki-67-positive nuclear staining compared with the control group. In the control group, Ki-67-positive nuclei staining in the colon mucosa were 34.24% of cells, and it was only 23.41% of the cells

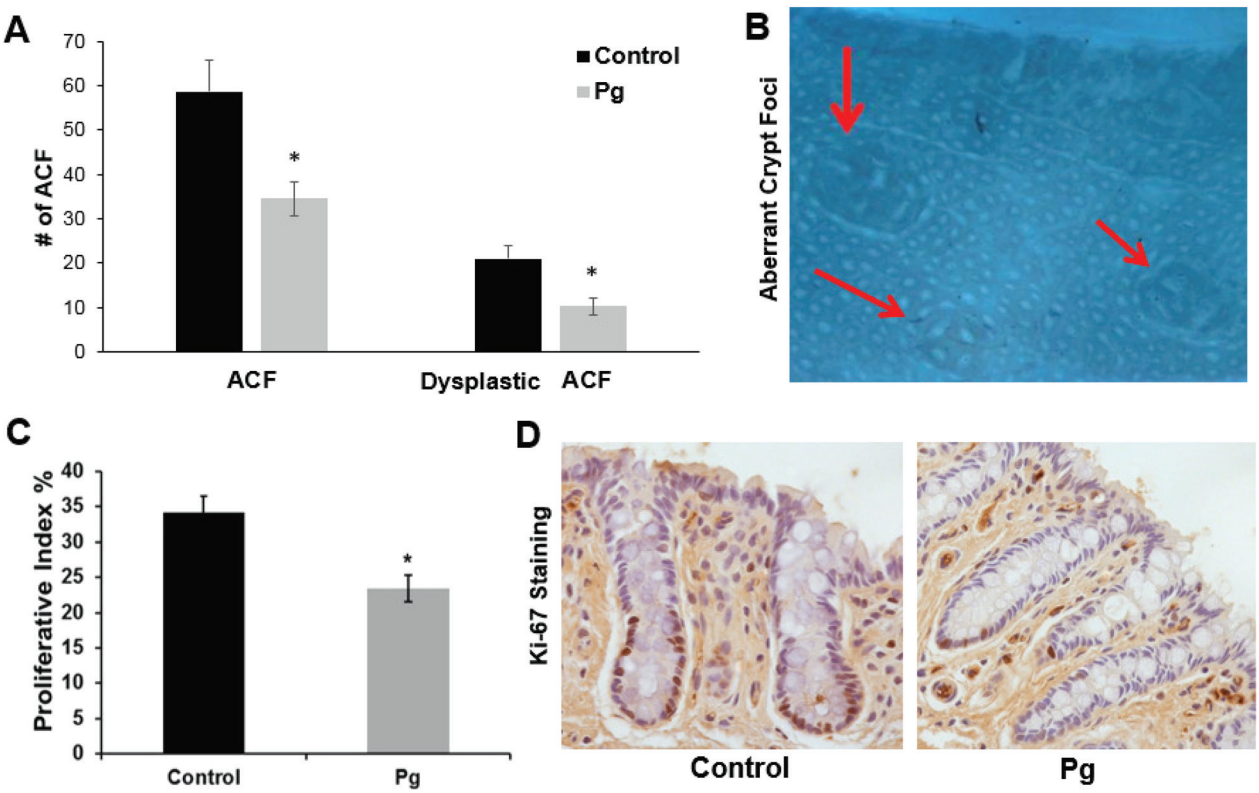

Fig. 2. Effects of pomegranate juice (Pg) on lesion formation and colonocyte proliferation. (**A**) The number of total ACF and dysplastic ACF was significantly decreased by the pomegranate treatment by 29 and 53.5% compared with the control group (P = 0.05 and 0.04), respectively. (**B**) Representative methylene blue staining of ACF in colon tissue. (**C**) Pomegranate juice inhibited cell proliferation compared with the control juice group. Values are mean ± standard error, n = 20. *P < 0.05. (**D**) Immunohistchemistry of Ki-67 in colon mucosa of the rats in control and pomegranate groups.

in pomegranate-treated animals, which represented an 11% reduction compared with the control group ($P = 0.0127$) (Figure 2B).

Pomegranate juice suppressed the expression of inflammatory enzymes COX-2, iNOS, NF-κB (p65) and VCAM-1 in rats treated with AOM

In colon cancer, the overexpression of inflammatory markers plays a significant role. Previously, we demonstrated the anti-inflammatory activities of polyphenols from pomegranate against inflammation in breast cancer (10). In this study, the anti-inflammatory activities of pomegranate juice in rats treated with AOM were investigated. Treatment of AOM-treated rats with pomegranate decreased the expression of COX-2, iNOS and VCAM-1 mRNA and protein (Figure 3A–D). Interactions between pomegranate polyphenolics and NF-κB signaling have been previously reviewed in several studies (21,22,34). In our previous study, pomegranate significantly reduced the expression of constitutive expression and phosphorylation of NF-κB p65 in breast cancer cells BT474 and MDA-MB-231 (10) and revealed the anti-inflammatory properties of pomegranate polyphenolics. Similarly, we have found that pomegranate also suppressed the constitutive expression and phosphorylation of NF-κB p65 in colon cancer cells (Figure 3C and D).

Pomegranate juice modulated the PI3K/AKT/mTOR pathway in AOM-treated rats

The PI3K/AKT signaling pathway plays an important role in carcinogenesis for several types of cancer including colon cancer (24) and influences cell survival pathways through phosphorylation of downstream targets such as NF-κB and mTOR (2,35,36). Previously, we have shown that the anti-inflammatory effects of polyphenols from pomegranate were, in part, mediated through the suppression of the PI3K/AKT pathway in breast cancer cells *in vitro* and *in vivo* (10). IGF regulates the phosphorylation of PI3K/AKT and mTOR. In this study, pomegranate juice decreased the expression of IGF and pPI3K accompanied by decreased expression of pAKT and pmTOR mRNA and protein level (Figure 3E and F).

Pomegranate juice increased the expression of miR-126 in AOM-treated rats

Small non-coding miRNAs are known to influence several biological processes such as cell development, differentiation and maintenance and also to control the process of carcinogenesis (24). In previous studies, it was reported that the expression of miR-126 was significantly reduced in colorectal cancer compared with non-tumor tissue, where miR-126 was involved in suppressing excessive proliferative activity (24,25). miR-126 targets several mRNAs that have a complementary sequence within their 3′-untranslated region such as PI3K/AKT and VCAM-1 (24,26). Previously, we have demonstrated the ability of polyphenolics to increase the expression of miR-126 in lipopolysaccharide-treated human colon-derived CCD-18Co myofibroblast cells (20). Pomegranate extract increased the expression of miR-126 (Figure 3G) and correspondingly, this was accompanied by reduction of VCAM-1, PI3K and AKT mRNA and protein expression in AOM-treated rats (Figure 3C–F).

Pomegranate reduced cell viability and increased markers for apoptosis in HT-29 colon cancer cell lines

It was previously demonstrated that pomegranate polyphenolics suppressed inflammation in HT-29 colon cancer cells (13). However, the specific mechanisms of the chemopreventive effects of pomegranate juice were not elucidated. In order to determine the involvement of miR-126 and its target genes, vascular endothelial growth factor (VEGF) and PI3K p85b in the anti-inflammatory activities of pomegranate, we investigated the further *in vitro* treatment with antagomiR. The cytotoxic activities of pomegranate were shown in HT-29 human colon cancer cells. The result showed a concentration-dependent decrease in cell viability in HT-29 cells after treatment with pomegranate (5–25 μg/ml) after 48 and 72 h (Figure 4A). Reduced cell viability was accompanied by an increase of the activated form of capase-3, a primary apoptosis-executing enzyme, and the cleaved form of its substrate poly (ADP-ribose)-polymerase (Figure 4B).

Pomegranate decreased inflammation and angiogenesis in HT-29 colon cancer cell lines

Previously, we have shown the anti-inflammatory activities of polyphenols from pomegranate in breast cancer (10). In AOM-injected rats, pomegranate inhibited the expression of inflammatory markers, such as VCAM-1. Similarly, in colon cancer cells, pomegranate decreased the expression of inflammatory markers, NF-κB p65, VCAM-1, intercellular adhesion molecule 1, COX-2 and pAKT, mRNA and protein (Figure 4C and D). In addition, pomegranate suppressed the expression of angiogenesis marker VEGF in colon cancer cells, whereas there was no change of VEGF expression by pomegranate during the ACF stage in AOM-injected rats.

Pomegranate increased the expression of miR-126 in HT-29 cells

In HT-29 cells, pomegranate extract increased the expression of miR-126 (Figure 4E) and this was accompanied by reduction of AKT and VCAM-1 mRNA and protein expression (Figure 4C and D). To further understand whether the underlying mechanisms of pomegranate with PI3K and VCAM-1 were based on an increase in miR-126, HT-29 cells were transfected with the 20 nM of miR-126 antagomiR. When transfected cells were treated with 25 μg/ml pomegranate polyphenols extract, this repression of miR-126 by the antagomiR treatment was partially reversed by pomegranate (Figure 5A) and this was accompanied by a decreased expression of VCAM-1 mRNA and protein in transfected cells that were treated with pomegranate (Figure 5B and C). Additionally, when cells were transfected with the miR-126 antagomiR, the mRNA expression of PI3K was increased, and the expression of PI3K mRNA was partially reversed by the treatment of 25 μg/ml pomegranate polyphenols (Figure 5D). Likewise, the transfection of cells with the miR-126 antagomiR partially reversed the effects of pomegranate extract on the expression of miRNA-126 and its target genes VCAM-1 and PI3K. These findings indicated that pomegranate increased miR-126 and was involved in the reduction of VCAM-1 as well as the suppression of PI3K/AKT in HT-29 colon cancer cells.

Discussion

In this study, we demonstrated the cancer-preventive and therapeutic activities of polyphenolics in pomegranate through the suppression of cell proliferation and inflammation. The number of ACF and dysplastic ACF was significantly decreased (Figure 2A) as well as the percentage of proliferative cells (Figure 2B) by pomegranate treatment in AOM-treated rats. In order to confirm *in vivo* findings, the proapoptotic and anti-inflammatory effects of pomegranate polyphenolics were also investigated *in vitro*, in HT-29 colon cancer cells, where pomegranate induced apoptosis and inhibited cell proliferation (Figure 4A). Moreover, similar results were observed in HT-29 colon cancer cell lines where pomegranate upregulated the expressions of capase-3, a primary apoptosis-executing enzyme, and poly (ADP-ribose)-polymerase in HT-29 cells (Figure 4B).

Smaller ACFs may disintegrate and disappear, whereas higher multiplicity (dysplastic) ACF are more likely to develop into neoplastic lesions (32). It has been shown that genetic mutations, epigenetic alterations, genomic instabilities, loss of heterozygosity and defects in mismatch repair systems are present in ACF (37) and therefore, ACF and particularly large or dysplastic ACF affecting four or more crypts, are used as biomarker to assess the risk for colon cancer, and as an intermediate endpoint in the evaluation of nutritional factors (28,29). For this reason, our manuscript distinguishes between ACF and dysplastic ACF.

Polyphenols extracted from pomegranate induced antioxidant, anti-inflammatory and antitumorigenic effects in colon cancer and other cancer models (9,10,12,13,38). In our previous studies, we demonstrated the cytotoxic effect of pomegranate polyphenols in breast

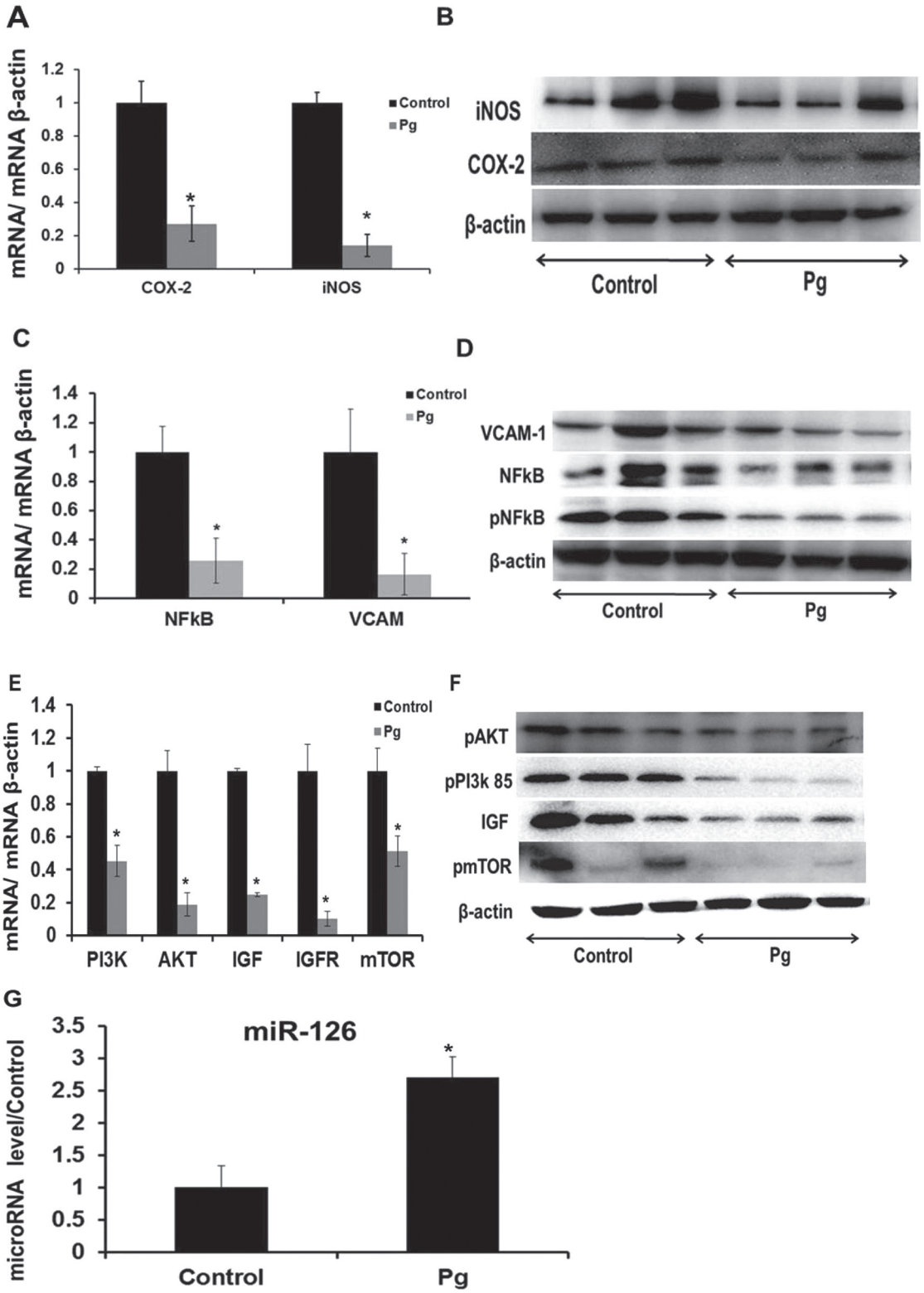

Fig. 3. Effects of pomegranate juice (Pg) on the inflammatory and the mTOR signaling pathways in AOM-treated rats. (**A**) Pomegranate juice decreased the expression of COX-2 and iNOS mRNA and (**B**) protein in AOM-treated rats. (**C**) Pomegranate juice suppressed the expression of VCAM-1 and NF-κB mRNA and (**D**) protein in AOM-treated rats. (**E**) Pomegranate juice decreased in the expression of IGF and pPI3K accompanied by decreased expression of pAKT and pmTOR mRNA and (**F**) protein levels in AOM-treated rats. (**G**) Pomegranate juice increased the expression of miR-126 in AOM-treated rats. All experiments were performed as described in Materials and methods, and results were expressed as mean ± standard error.*Indicates significant changes at $P < 0.05$.

cancer BT474 cells and the inhibition of tumor growth by pomegranate in athymic nude mice bearing BT474 cells as xenografts (10). Previously, pomegranate polyphenols induced apoptosis modulating caspase-3 and targeted multiple pathways in several cancer cell lines including breast, prostate, liver, lung and skin cancer (7,9,10,12,15,22). *In vitro* data in this study demonstrated similar results.

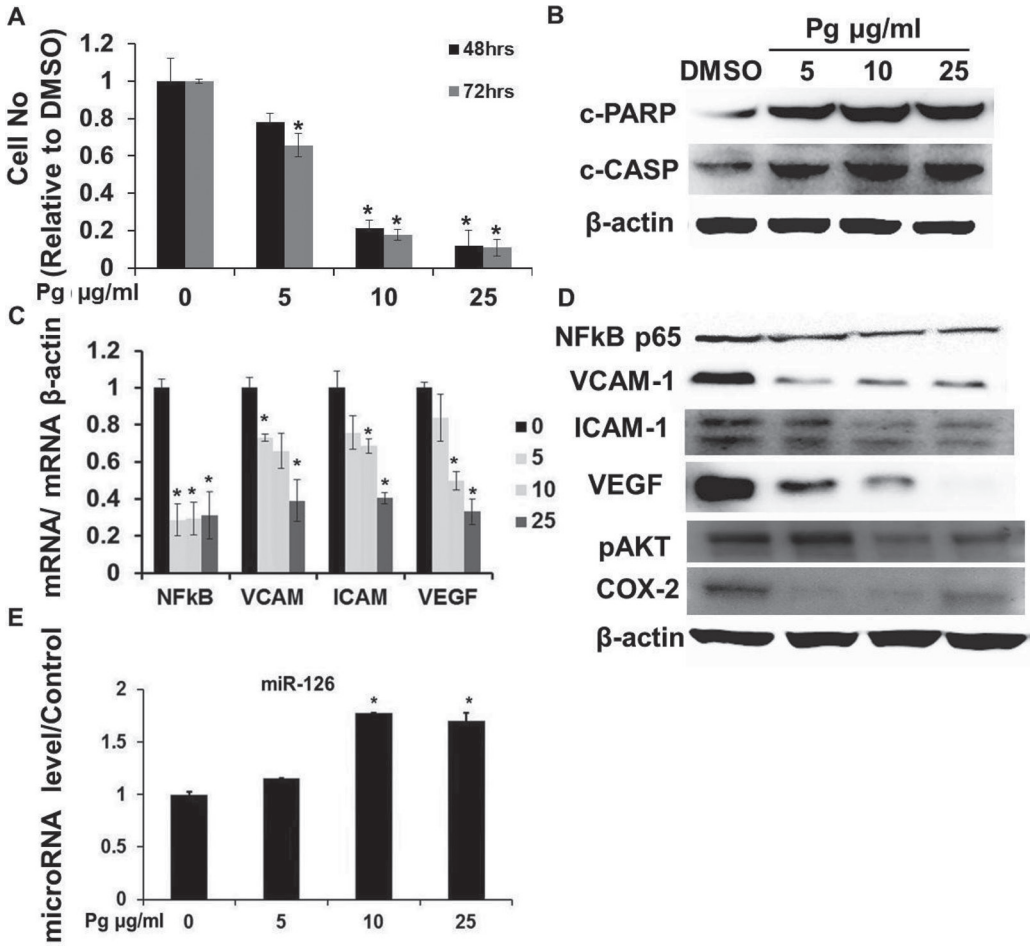

Fig. 4. Effects of pomegranate extract (Pg) on the inflammatory and the mTOR signaling pathways in HT-29 human colon cancer cell lines. (**A**) Pomegranate extract inhibited proliferation in human colon cancer cells. (**B**) Pomegranate extract induced the protein expression of apoptosis-associated proteins (cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase). (**C**) Pomegranate decreased the expression of inflammatory markers NF-κB p65, VCAM-1, intercellular adhesion molecule 1 and angiogenesis marker VEGF mRNA and (**D**) protein in HT-29 human colon cancer cells. (**E**) Pomegranate extract increased the expression of miR-126 in HT-29 cells. Cells were treated with solvent dimethyl sulfoxide (control) or different concentration of pomegranate (5–25 µg/ml) for 24 h. All experiments were performed as described in Materials and methods and were performed at least three times, and results were expressed as mean ± standard error.*Indicates significant changes at $P < 0.05$.

Common colon cancer and inflammatory markers such as VCAM-1 and NF-κB mRNA and protein expression were decreased both *in vitro* and *in vivo* model by pomegranate. Previous studies demonstrated that pomegranate polyphenols decreased inflammation and lung tumor growth in mice by decreasing the activation of NF-κB (21,22). NF-κB plays a central role in inflammatory pathways and controls oncogenes and tumor suppressor genes, as well as adhesion molecules VCAM-1 (39). Cell adhesion molecules such as VCAM-1 disrupt normal cell differentiation, which in turn leads to neoplastic transformation, progression, angiogenesis and metastasis of cancer cells. Thus, increased expression of VCAM-1 possibly influences colon cancer progression (40). In this study, pomegranate suppressed the expression of VCAM-1 in AOM-treated rats (Figure 3C and D). Similar results were observed in HT-29 colon cancer cells (Figure 4C and D), which were accompanied by reducing angiogenesis marker VEGF. Pomegranate suppressed the expression of VEGF in colon cancer cells, whereas there was no change of VEGF expression by pomegranate during the ACF stage in AOM-injected rats. The expression of VEGF may be not changed at the promotion stage by pomegranate but altered at the later tumor stage (41).

In colorectal cancer patients and rodents with chemically induced colon cancer, chronic inflammatory markers such COX-2 and iNOS were upregulated (28,29,42). Excessive NO induced the expression of COX-2, which in turn controlled cell proliferation, inflammation and might inhibit apoptosis in colon cancer cells (42,43). Thus, upregulation of COX-2 could increase cell proliferation (23,44). Previously, it was shown that pomegranate polyphenols suppressed the expression of COX-2 and iNOS and inhibited cell proliferation by inducing cell cycle arrest and apoptosis in HT-29 colon cancer cells (13). In this study, pomegranate decreased mRNA and protein expression of COX-2 and iNOS in AOM-treated rat model (Figure 3A and B) and COX-2 protein level in HT-29 human colon cancer cell (Figure 4D). Pomegranate inhibited cell proliferation in HT-29 colon cancer cells (Figure 4A) and also in AOM rat model showed by Ki-67 staining (Figure 2B) and this was accompanied by the decrease in the number of ACF, a colon cancer biomarker in rats treated with pomegranate juice (Figure 2A).

Previous studies have suggested the potential role of small non-coding miRNAs in the pathogenesis of cancer and its control of diverse biological processes including cell proliferation, development, differentiation, maintenance and carcinogenesis (25). miRNAs are known to function as tumor suppressors or oncogenes. In order to investigate the underlying mechanisms of the downregulation of VCAM-1 and the PI3K/AKT pathway, the potential role of tumor suppressor miR-126, a colonic prognostic marker involved in the anti-inflammatory activity by targeting VCAM-1 (26) was investigated. Pomegranate decreased expression of VCAM-1, which was accompanied by upregulation of miR-126 *in vivo* and *in vitro*. VCAM-1 is regulated

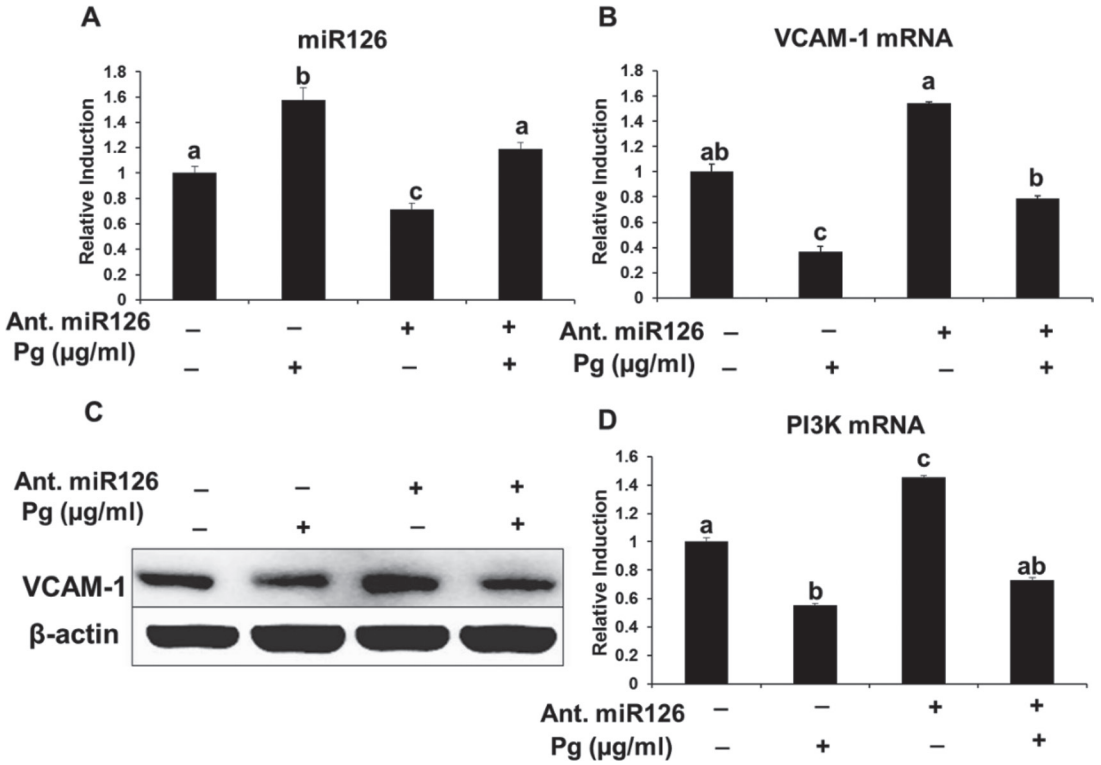

Fig. 5. Effects of pomegranate extract (Pg) on miR-126 levels and its target genes in HT-29 cells transfected with Ant.miR-126. Cells were transfected with 20 nM antagomiR miR-126 and subsequently treated with 25 μg/ml pomegranate extract. (**A**) Pomegranate extract reversed this repression of miR-126 by the antagomiR treatment. (**B**) The reversed expression of miR-126 was accompanied by a decreased expression of VCAM-1 mRNA, (**C**) protein and (**D**) mRNA expression of PI3K in transfected HT-29 colon cancer cells treated with 25 μg/ml pomegranate polyphenols. All experiments were performed as described in Materials and methods and were performed at least three times, and results were expressed as mean ± standard error. *Indicates significant changes at $P < 0.05$. Bars with different letters are significantly different ($P < 0.05$).

by miR-126 via a target-binding site in the 3′-untranslated region of the VCAM-1 mRNA (26). To confirm the involvement of miR-126, a specific antagomiR for miR-126 (Ant-miR-126) was used to decrease miR-126 expression and to increase the expression of VCAM-1, where pomegranate partially but significantly reversed the effects of the antagomiR (Figure 5A–C). Pomegranate was able to increase the levels of miR-126 that had been decreased by the antagomiR and whether as a consequence the levels of VCAM-1 would decrease. The selected concentrations of the antagomiR were low enough to be in part overcome by the effects of the pomegranate extract. For this reason, VCAM-1 was decreased by the pomegranate extract in cells treated with the antagomiR. Likewise, when cells were transfected with the antagomiR of miR-126, the effects of pomegranate extract were partially reversed specifically the expression of miRNA-126 and mRNA and protein expression of its target gene VCAM-1.

Additionally, findings showed that pomegranate downregulated the expression of IGF, PI3K and AKT. Pomegranate reduced the activation of NF-κB and the phosphorylation of PI3K p85β and AKT, and this was consistent with our previous finding where pomegranate extract decreased the expression and phosphorylation of NF-κB and the PI3K/AKT pathways (10). Also, it was shown that pomegranate modulated the expression of IGF and PI3K/AKT, which is crucial in the pathogenesis of colon cancer (Figure 6). The PI3K/AKT pathway controls cell survival mechanism through phosphorylation of downstream targets such as NF-κB and mTOR. The regulatory p85β subunit of PI3K has been shown to be the direct target for miR-126 in colon cancer (24). The decreased expression of PI3K p85β was accompanied by an upregulation of miR-126 (Figure 3E–G) as well as decreased phosphorylation of AKT and reduced phosphorylation of mTOR (Figure 3E and F). In order to confirm the involvement of miR-126 in the regulation of PI3K p85β, a specific antagomiR for miR-126 (ant-miR-126) was used. The antagomiR for miR-126 decreased the expression of miR-126 *in vitro* and increased the expression of the PI3K p85β subunit. Pomegranate reversed the effects of the antagomiR significantly (Figure 5D). As found *in vivo* in rats, CCD-18Co colon myofibroblast cells showed significant reduction of miR-126 and increased expression of PI3K p85β subunit (24).

It has to be noted that animals in this study treated with pomegranate gained less body weight compared with control animals, even though their caloric intake was increased. Previous studies with polyphenolics indicated that the consumption of polyphenolics caused a reduction of weight gain in animals (45,46). It has previously been reported that weight loss had beneficial effects on biomarkers of inflammation in rats (45–47). Therefore, in this study, it was not conclusively clear, whether the observed anti-inflammatory effects of pomegranate polyphenolics were derived from a direct interaction of polyphenolics with inflammatory pathways or whether at least part of the benefits were derived from the anti-inflammatory effects of reduced weight gain in animals treated with pomegranate. Overall, there was no correlation between weight gain and the number of dysplastic ACF ($R^2 = 0.0956$, $P = 0.2439$), for this reason, the effects of reduced weight gain caused by pomegranate might not have significantly contributed to the reduction of dysplastic ACF caused by pomegranate.

In conclusion, results indicated that pomegranate polyphenols exerted cytotoxic and anti-inflammatory effects in AOM-treated rats and colon cancer cells. Interactions of pomegranate with miR-126/VCAM-1 and miR-126/PI3K/AKT/mTOR axes were identified as mechanisms that, at least in part, appeared to be involved in the anti-inflammatory and antiproliferative activities of pomegranate polyphenolics.

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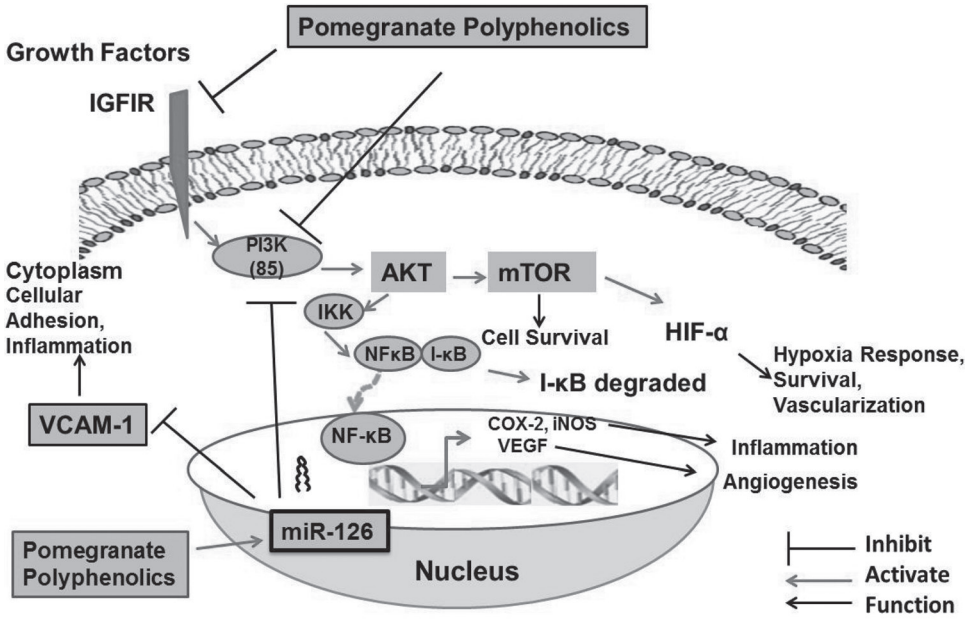

Fig. 6. Schematic representation showing molecular mechanisms involved in colon carcinogenesis treated with pomegranate polyphenols. Pomegranate-targeted miR-126 by modulating both miR-126/VCAM-1 and miR-126/PI3K/AKT/mTOR axes.

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