NRF2 AND CHEMOPREVENTION: SIGNALING, EPIGENETICS AND ROLE IN INTESTINAL CARCINOGENESIS

by

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Prevention is better than cure. The carcinogenesis could take as long as 20 to 30 years to develop from initiated cells to malignant tumor, therefore providing us various opportunities to prevent the appearance of tumors with the use of chemopreventive compounds in the early stage. Chemoprevention becomes an increasing important concept and has led to the intense research about the mechanisms of actions of various chemopreventive compounds. They can be generally classified into blocking agents and suppressing agents. The chemopreventive compounds usually prevent or slow progression of cancer by maintaining a low oxidative stress and inflammatory environment in cells. This is brought about by the activation of Nrf2, the key protein being investigated in our lab.
In this dissertation, I will be discussing the use of compounds as suppressing agents and blocking agents, how compounds activates Nrf2 signaling, how novel Nrf2 interaction partner IQGAP1 mediates Nrf2-Keap1 signaling axis, how expression level of Nrf2 could be regulated epigenetically, apart from the well-known post-translational control by Keap1-Ubiquitinase-Protesome axis and finally how loss of Nrf2 could enhance intestinal tumorigenesis in Apc(min/+ ) mice.
Acknowledgement and/or Dedication

I would like to thank the past and current lab members for their enormous help. Particularly, I would like to thank Dr. Kong for his patience and continuous support of my studies. I want to dedicate this dissertation to my family.
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1. INTRODUCTION

Carcinogenesis is a long term process which could take as long as 20-30 years to develop from initiated cells to advanced and metastatic cancer. It is a multi-step event comprising three different stages, including initiation, promotion and progression (1-3). Initiation occurs when cellular insults disturb the normal genetic and epigenetic events. Promotion follows when cellular signals stimulate the expansion of the initiated cells. Finally, progression is when the clonal group of cells continues to expand into malignant tumors (1-3). Increasing evidence from epidemiological and pathological studies suggest that cancer could be prevented or their progression could be stopped (4). In this context, the prevention of cancer by the use of natural or synthetic compounds has been described as chemoprevention (5). Chemopreventive compounds could target the initiation stage of carcinogenesis or by stopping the promotion and progression of cancer or their combinations. Prevention of cancer initiation can be achieved by limiting the exposure of cells to carcinogenic substances via either by inhibiting their activation or by increasing their detoxification and subsequent removal (6). On the other hand, chemopreventive compounds can also suppress progression and promotion of carcinogenesis by interfering with various signaling pathways involving oxidative stress (7), inflammation (8) and cellular proliferation. Finally, these compounds can also induce
cell cycle arrest and apoptosis and therefore they could target all different stages of carcinogenesis, including initiation, promotion and progression.

Numerous studies have shown that many natural dietary compounds can potently modulate various molecular targets, leading to prevention of cancer initiation, promotion and progression. Dietary fruits and vegetables are regarded as rich sources of chemopreventive compounds and are widely investigated due to their low toxicity but significant chemopreventive efficacies (9). Two classes of the most abundant naturally occurring dietary chemopreventive compounds are polyphenolic and the isothiocyanate (ITC)-containing compounds with totally different chemical structures. Polyphenolic compounds are characterized by their phenolic functional groups while the ITC-containing compounds are characterized by sulphur-containing -N=C=S functional group (Fig 1). Curcumin, dimethylbenzoylmethane (DBM) and green tea polyphenols are some of the commonly studied polyphenolic compounds. Ally isothiocyanate (AITC) from cabbage, mustard and horseradish, benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) from watercress, garden cress and sulforaphane (SFN) from broccoli, cauliflower, brassicas and kale are commonly used ITC-containing compounds (Table 1).
Many of the chemopreventive effects of cruciferous vegetables are attributed to the ITCs, rather than their parent moiety, the glucosinolates. The general structures of glucosinolates consist of β-D-thioglucose group, a sulfonated oxime group and a side chain derived from methionine, phenylalanine, tryptophane or

**Fig 1.** Chemical structure of PEITC and SFN. Both PEITC and SFN are isothiocyanates, which are characterized by sulfur containing N=C=S functional group

**Table 1.** Sources of Isothiocyanates (ITCs) from Dietary Vegetables

<table>
<thead>
<tr>
<th>Structure</th>
<th>Chemical name</th>
<th>Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃-S-CH₂-CH₂-CH₂-N=C=S</td>
<td>3-Methylthiopropyl</td>
<td>Cabbages</td>
</tr>
<tr>
<td>CH₃-S-CH₂-CH₂-CH₂-CH₂-N=C=S</td>
<td>4-Methylthiobutyl</td>
<td>Arugula</td>
</tr>
<tr>
<td>CH₃-S-CH₂-CH₂-CH₂-CH₂-N=C=S</td>
<td>3-Methylsulfinylpropyl (iberin)</td>
<td>Broccoli, Brussels sprouts, cabbages</td>
</tr>
<tr>
<td>CH₃-S-CH₂-CH₂-CH₂-CH₂-N=C=S</td>
<td>4-Methylsulfinylbutyl (SFN)</td>
<td>Broccoli, Cauliflower</td>
</tr>
<tr>
<td>CH₂=CH₂-N=C=S</td>
<td>2-Propanyl (AITC)</td>
<td>Mustards, cabbages, Brussels sprouts, Cauliflower</td>
</tr>
<tr>
<td>CH₂=CH₂-N=C=S</td>
<td>Benzyl (BITC)</td>
<td>Lepidium cress</td>
</tr>
<tr>
<td>CH₂=CH₂-N=C=S</td>
<td>2-Phenethyl (PEITC)</td>
<td>Watercress, radishes, turnips</td>
</tr>
</tbody>
</table>
branch-chained amino acids (10). The glucosinolates are not bioactive and appear to have no chemopreventive effects unless they are converted to ITCs and indole-3 carbinols (I3C) by hydrolysis catalysed by myrosinase. Disruption of plant cells during harvesting, processing or chewing, releases myrosinase which comes into contact with glucosinolates and hydrolyses them to different ITCs (11). It is estimated that in humans, intake of SFN or PEITC can range from 50 to 200 μmol after ingesting 100 g (wet weight) of broccoli and watercress (12, 13). In rats, after an oral dose of 50 μmol of SFN, the plasma concentration of SFN can peak at 20 μM at 4 hours and decline with a half-life of about 2.2 hours. This increase in plasma concentration of SFN is accompanied by an induction of genes that are important in cellular defense mechanisms and cell cycle regulation (14). In mice fed with diets supplemented with 300 (~5 μmol/day) and 600 ppm (~10 μmol/day) of SFN for 3 weeks, the steady state levels of SFN in plasma and intestine reached 124-254 nM and 3-13 nmol/g of tissue (roughly equivalent to about 3-10 μM of total SFN). These concentrations of SFN had been shown to be effective in prevention of adenoma formation in Apc(min/+)+/− mice (15). On the other hand, plasma concentration of PEITC in rats could reach 9.2 to 42.1 μM after oral dose of 10 and 100 μmol/kg of PEITC (16). These in vivo pharmacokinetic data show that μM concentrations of PEITC and SFN are achievable in vivo. In animal and cell culture models, μmol doses or μM concentrations of PEITC
and SFN have been shown to prevent cancer through different mechanisms both in vivo and in vitro (9, 11).

In this dissertation, we will discuss the diverse cellular and molecular targets regulated by phytochemicals, how phytochemicals could act as cancer blocking agents and suppressing agents, how phytochemicals prevent cancer through activation of Nrf2 signaling, how novel Nrf2 interaction partner IQGAP1 mediates Nrf2 signaling, how Nrf2 expression level could be control epigenetically and finally, how loss of Nrf2 could enhance intestinal tumorigenesis in Apc(min/+ ) mice.
2. PEITC as a cancer suppressing agent: cell cycle arrest effect

\textit{in-vitro}

2.1 INTRODUCTION

Colon cancer is one of the leading causes of cancer death in the US. Epidemiological studies have demonstrated an inverse association of colon cancer with intake of cruciferous vegetables (17). Dietary isothiocyanates are present in large quantities in cruciferous vegetables including watercress, broccoli and cabbage (18). Many isothiocyanates (ITCs) such as sulphoraphane (SFN), phenethyl isothiocyanate (PEITC) and ally isothiocyanate (AITC) are highly effective in chemoprevention and have anti-tumor activities \textit{in-vitro} and \textit{in-vivo}. Apart from the colorectum, isothiocyanates inhibit cancer formation in various tissues such as lung, esophagus, mammary gland, liver, small intestine and bladder (19).

While the exact mechanisms by which PEITC exerts its anti-tumor activity are still unclear, MAPK and AP-1 pathways are believed to be involved. Using PC-3 cells as a model, it was shown that PEITC can activate JNK and ERK signaling and mediate the transcriptional activity of AP-1, which in turn regulate the cell death (20). Apart from transcriptional control, PEITC may inhibit cap-dependent translation by regulating the level and phosphorylation of 4E-BP1 which may be an important mechanism in PEITC-induced apoptosis (21).
A lot of studies have been focused on PEITC induction of apoptosis in cancer cell lines. Previously, our lab demonstrated PEITC can induce apoptosis in HT-29 cells in a time- and dose-dependent manner via the mitochondria caspase cascade, and the activation of JNK is critical for the initiation of the apoptotic processes (22). In the present study, we demonstrated that in addition to activating apoptosis, MAPKs, p38 in particular have an important role in regulating cell cycle of HT-29 cells.

2.2 MATERIALS AND METHODS

Cell culture and reagents

Human colorectal cancer cell lines HT-29 was obtained from American Type Culture Collection (ATCC). The cells were maintained in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), 2.2g/l sodium bicarbonate, 100 U/ml penicillin and 100ug streptomycin. Before treatment, the medium was removed and the cells were starved with 0.5% serum MEM overnight. Phenethyl isothiocyanate (PEITC) was obtained from Sigma (St Louis, MO). Cyclin A, D and E were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). pRb, phospho-cdc2-Tyr15, p-JNK, p-ERK, pp38 were purchased from Cell Signalling Technology (Beverly, MA). Specific MAPKs inhibitors SP600125, PD98059 and SB203580 were purchased from Calbiochem Technology (San Diego, CA).
**Flow cytometry analysis of cell cycle distribution**

HT-29 cells were seeded in 60-mm petri dishes in complete MEM overnight. The cells were then serum-starved for 24 h in serum free MEM medium. At treatment, the medium was replaced with complete MEM containing either 0.1% DMSO (as a negative control) or a varying dose of PEITC (5, 10 and 25uM). Following PEITC treatment, the cells were collected by trypsinization. Cells were collected by centrifugation at 1000rpm for 5 min. The cell pellet was then re-suspended in 500ul PBS, and the resulting cell suspension was passed through a 26.5 gauge needle 3 times. The cells were then fixed by adding 500ul ethanol. The fixed cells were then stained by 100ug/ml RNase A and 10ug/ml propidium iodide (PI) at 4°C in the dark for 30 min. Cell cycle distribution was then analyzed by flow cytometry using FACS analysis core facility of University of Medicine and Dentistry of New Jersey.

**Western blotting analysis**

After treatment, HT-29 cells in six-well plates were washed with ice-cold PBS and lysed with 200 ul of whole cell lyses buffer (10 mM Tris–HCl, pH 7.9, 250 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.5% Triton X-100, 10% glycerol, 1 mM proteinase inhibitor mixture, 1 mM phenylmethylsulfonyl
fluoride, 100 mM Na₃VO₄, 5 mM ZnCl₂, 2 mM indole acetic acid). The cell lysates were centrifuged at 12,000 g for 10 min at 4°C. The protein concentrations of the whole cell lysate supernatants were determined using a Bio-Rad protein assay kit. An equal amount of protein (20 µg) was then resolved on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane using semi-dry transfer system. The membrane was blocked in 5% non-fat milk for 1 h at room temperature, then incubated overnight at 4°C with a primary antibody specifically recognizing cyclin D1 (sc-718), cyclin A (sc-596), cyclin E (sc-247) or actin (sc-1616), p21Cip1 (sc-6246) (Santa Cruz Biotechnology) and p-JNK, pERK, pp38, pRb, phospho-cdc2-Tyr15 (Cell Signalling Biotechnology). After incubation with the primary antibody, the membrane was washed with TBST (20 mM Tris–HCl, 8 g/l NaCl, 0.1% Tween 20, pH 7.6) three times, then incubated in horseradish peroxidase-conjugated secondary antibody (1:5000 dilution) for 45 min at room temperature followed by an additional three washes with TBST. Detection was performed using ECL reagents (Bio-Rad).

2.3 RESULTS

*PEITC induced G1 cell cycle arrest in HT-29 cells*

HT-29 cells were treated with various concentrations (0, 5, 10, 25 µM) of PEITC for 24 h. Flow cytometry was performed and the results showed a significant
increase in the percentage of G1 phase cells at 25 µM (figure 1). In agreement with our previous study, flow cytometry showed that apoptotic cells increase from 5% to about 11% at high concentrations after PEITC treatment. On the other hand, the percentage increase in G1 cells increase from 53% at concentration 0 µM to 71% at 25 µM. Together, these results suggest that apart from apoptosis, PEITC may exert its effect through cell cycle arrest.

**PEITC decreased expression of cyclins**

To determine the effect of PEITC on the expression level of various common proteins involved in cell cycle control, western blotting was performed. Cyclin D belongs to the G1 cyclins which promote passage through restriction point in late G1. Cyclin D forms complex with CDK4 which hyperphosphorylates Rb, leading to activation of E2F and enhanced transcription of cyclins A and E. As shown in figure 2, PEITC decreased the expression of cyclin A, D and E in a dose-dependent manner. However, PEITC has no effect on p21, p27 and p53 (data not shown). Instead, pRb maybe the target where PEITC exerts its effect on HT-29, since increase in PEITC leads to a reduction in pRb (figure 2).

**PEITC decrease expression of cyclins through a β-catenin independent pathway**
Aberrant activation of Wnt signaling pathway is known to be essential in colorectal carcinogenesis. To elucidate if β-catenin is involved in PEITC depression of cyclins, the expression of β-catenin and E-cadherin was examined. As shown in figure 3a, the total β-catenin and E-cadherin level remains unchanged after PEITC treatment. As shown in figure 3b, the nuclear β-catenin level do not change even PEITC increases, suggesting that β-catenin pathway may not be involved in the PEITC effects on HT-29 cells.

**PEITC induced pp38 activity, leading to G1 cell cycle arrest**

To investigate the role of different MAPKs on cell cycle arrest induced by PEITC, specific inhibitors of different MAPKs were used. JNK and ERK inhibitors do not significantly suppress G1 cell cycle arrest induced by PEITC (data not shown). However, by inhibiting the pp38 activity using specific inhibitor SB 203580, we showed that the cell cycle arrest was attenuated. As shown in figure 4, the percentage of cells in G1 phase was 46.64% and 70.58% without and with treatment of 25 µM PEITC respectively. By inhibiting the action of p38, the percentage of cells in G1 phase after treatment of 25 µM PEITC drops from originally 70.58% to 50.62% (p38 activity being inhibited). Therefore, we conclude that pp38 is involved in PEITC-mediated cell cycle arrest.
**PEITC mediated cyclin level through pp38 activity**

As shown in figure 5b, cyclin A and D expression, but not cyclin E (data not shown) decreased at the PEITC 25 µM while inhibitor of pp38 partially restore the expression. This suggests pp38 activity may play a role in regulating the expression of cyclins, probably regulation on cdc2 and pRb. pRb expression decreases upon PEITC treatment while inhibition of pp38 partially inhibits the depression. In contrary, phospho-cdc2-Tyr15 expression increases upon PEITC treatment and is restored by inhibition of pp38 (figure 5b).

**2.4 DISCUSSION**

Previous studies supported that isothiocyanates like PEITC and sulforaphane have anti-colon tumor effect. SFN regulates genes involved in apoptosis, cell growth and inflammation in the small intestinal polyps of Apc(min/+) mice (23). It has been shown that the sulforaphane exert its anti-cancer effect through differential regulation of cell cycle and subsequent events lead to cell death (24). Little studies have been done on the anti-proliferative effect of PEITC on HT-29 cells. In this study, however, we showed for the first time that in addition to apoptosis, PEITC inhibits cancer cell growth through cell cycle arrest.
Cyclin A, D and E were found to be down-regulated by PEITC in HT-29 cells. The results are consistent with flow cytometry results which show an increase in G1 cell cycle arrest. Several upstream signaling events have been suggested as responsible for up-regulations of cyclin A, D and E. For example, β-catenin can translocate into nucleus to up-regulate cyclin D through the Wnt signaling pathway (25). IKKα stabilize β-catenin and induce the cyclins (26), leading to their proliferative effect. However, our results showed that PEITC do not suppress cyclin D through the β-catenin pathway.

At the heart of the cell-cycle control system is a family of protein kinases known as cyclin-dependent kinases (Cdks). Cdk activity is controlled by a complex array of enzymes and proteins, one of which is known as cyclins. Cyclin D belongs to the G1 cyclins which promote passage through restriction point in late G1, cyclin E belongs to the G1/S phase cyclins and cyclin A belongs to S phase cyclins. Our study showed that PEITC arrests HT-29 cells in G1 phase through suppression of cyclins in a dose dependent manner. It is known that DNA damage leads to the activation of p53, which in turn increase the expression of p21 that binds to G1/S Cdk and inhibits their activity, thus arresting the cells in G1 phase. However, our results show that PEITC do not regulate the Cdk activity through p53 and p21.

We are then interested to know what is the upstream signaling for the
PEITC-induced decrease in cyclin A, D and E levels. We have found that β-catenin pathway is not involved since the basal level of β-catenin did not increase after PEITC treatment, nor did its nuclear localization. Specific inhibitor for JNK and ERK attenuates the G1 cell cycle arrest but is not significant (data not shown). However, we showed that abrogation of p38 attenuates the G1 cell cycle arrest induced by PEITC. This suggests that MAPK could be an upstream signaling controlling cyclins expression mediated by PEITC. Particularly, we provide a plausible mechanism explaining how pp38 can mediate the effect of PEITC on HT-29 cells. Combining all the evidence, we suggest that activation of pp38 is responsible for the G1 cell cycle arrest. Studies have shown that p38MAPK pathway is activated in response to the commonly used DNA-damaging agents cisplatin, doxorubicin, and camptothecin and that it has cell-cycle checkpoint function (27). It has been shown that pp38 can phosphorylate cdc25A and phosphorylation of cdc2 at tyr15 leads to cell cycle arrest (28). Our results showed that PEITC induce pp38 expression, leading to an increase in phospho-cdc2 at tyr15. The increase in inhibitory phosphorylation of this cyclin dependent kinase may results in a decrease in phosphorylation of Rb. And with more Rb binding to E2F, the cyclin expression is suppressed.
**Fig 1.** The effect of PEITC on cell cycle of HT-29 cells. HT-29 cells were treated with PEITC (25 μM) for 24 h. PEITC significantly increased G1 cells from 51% at PEITC 0 μM to 73% at PEITC 25 μM. Student’s t-test was used to compare the means between treated (25 μM) groups vs control (0 μM) groups *p=0.01

**Fig 2.** (A) The effect of PEITC on cell cycle arrest markers in HT-29 cells. HT-29 cells were treated with an increasing dose of PEITC (0, 5, 10, 25 μM) for 24 h and cell cycle arrest markers were blotted. PEITC significantly suppressed the expression of cyclin A, D, E and pRb dose dependently. (B) Densitometry data are representative of 3 independent experiments.
Fig 3. (A) The effect of PEITC on β-catenin expression. The expression level of β-catenin and E-cadherin in total cell lysate were not altered by administration of PEITC. (B) Densitometry data are representative of two independent experiments. (C) The effects of PEITC on β-catenin nuclear localization. The nuclear localization of β-catenin was not altered by administration of PEITC. (D) Densitometry data are representative of two independent experiments.
Fig. 4. The effect of p38 inhibitor on G1 phase arrest of HT-29 cells induced by PEITC. PEITC (25 μM) significantly induced G1 cell cycle arrest while treatment of p38 inhibitor significantly attenuated this cell cycle arrest. Student’s t-test was used to compare the means between the groups *p=0.01
Fig. 5. (A) The effect of PEITC on expression of p38MAPK. PEITC induce the expression of phosphorylated p38 at PEITC 25 µM. The data are representative of three independent experiments. (B) The effect of p38 inhibitor on G1 phase arrest markers of HT-29 cells induced by PEITC. PEITC suppressed cyclin A, D and pRb, induced phosphor-cdc2 at tyr15 while treatment of p38 inhibitor significantly attenuated this. The data are representative of three independent experiments.
3. DBM as a cancer blocking agent: Nrf2/phase II gene induction *in-vivo*

3.1. INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer of men and women and the third leading cause of cancer mortality with an estimated new cases and deaths from colon and rectal cancer in the United States in 2009: New cases: 106,100 (colon); 40,870 (rectal); Deaths: 49,920 (colon and rectal combined). The etiology of colon cancer is multifactorial, including familial, environmental, dietary and other unknown factors. Familial adenomatous polyposis (FAP) is an autosomal dominant inherited condition in humans that is characterized by the progressive development of hundreds to thousands of adenomatous colon polyps. The gene associated with FAP (APC) appears to be an early mutational event found to be responsible in 50-80% of the colorectal cancer cases and Apc(min/+ ) mice have been used extensively for gastrointestinal carcinogenesis research (29). However, a single gene mutation alone such as Apc might not be sufficient to drive colon carcinogenesis. Therefore, chemical induced-carcinogenesis models are also widely utilized. Aberrant crypt foci (ACF) are early morphological changes observed in rodents after administration of colon-specific carcinogen such as azoxymethane AOM. ACF are considered to be putative preneoplastic lesions (30, 31) and are widely used as a surrogate biomarker.
to rapidly evaluate chemopreventive potential of compounds. ACF share many morphological and biochemical characteristics with tumors and ACF found on the surface of cancer-predisposed colons of rodents have been regarded as early-appearing pre-neoplastic lesions. However, it is not clear if such lesions are truly pre-cancerous lesions for colorectal cancers in rodents.

Inflammatory bowel diseases (IBD), such as ulcerative colitis (UC; chronic inflammation of colon mucosa) and Crohn’s disease (CD; chronic inflammation often involves small intestine along with colon and other organs) have been strongly linked to an increase risk of colorectal cancer (32). Indeed, IBD ranks among the top three high-risk conditions for CRC, together with hereditary syndromes of FAP and hereditary nonpolyposis colorectal cancer (HNPCC) (32). In this context, the azoxymethane (AOM)-initiated and promoted by the inflammatory dextran sulfate sodium (DSS) would appear to simulate colon tumor development in human (33, 34). AOM causes oxidative and DNA damage, which is then promoted by inflammatory agent DSS. DSS induces colitis and promotes AOM-induced colon cancer in mice (33). Although the AOM alone model recapitulates many features of human CRC, AOM/DSS appears to represent a potentially more physiologically relevant model since human CRC is often caused by promoting factor (eg. inflammation) in addition to initiation factors (eg. somatic mutation caused by carcinogens and or inheritance).
Phenethyl isothiocyanates (PEITC) is a metabolite of glucosinolate which are found abundantly in cruciferous vegetables such as watercress. The chemopreventive efficacy of PEITC has been widely investigated in vitro and in vivo. It has been shown that PEITC induces cell cycle arrest and apoptosis in colon (35), prostate (36) and lung cancer cells (37). Recent studies suggested that other potential mechanism of actions of PEITC include ROS-mediated cell killing (38), Nrf2 induction (39) and protein binding-mediated apoptosis and cell cycle arrest (36, 37, 40). The efficacy of PEITC as a chemopreventive agent has also been shown in various animal cancer models including rat esophagus (41), prostate (42) and lung (43). However, in terms of PEITC’s efficacy in the colon, two previous studies appear to show some contradictory results. One earlier study showed that PEITC was effective in chemoprevention of colonic ACF in Fischer rats (44) while a recent report showed that there was no significant difference in the number of ACF found between the PEITC-treated group and the control group (45). Furthermore, as discussed above, ACF lesions might not be true indicators of pre-cancerous lesions for CRC and hence in this study we investigated PEITC’s efficacy in AOM/DSS induced mouse CRC model, as well as examined PEITC’s potential in vivo mechanisms.

Dibenzoylmethane (DBM) is a beta-diketone structurally-related to curcumin, which is a minor constituent of licorice that possesses anticarcinogenic properties in
several animal models (46, 47). Early study shows that the efficacy of DBM in the prevention of rat mammary DNA adducts and tumors induced by 7,12-dimethylbenz[a]anthracene (DMBA) (47). Our previous study showed the chemopreventive potential of DBM using Apc(min/+) mouse model and that the combination of DBM and another isothiocyanate sulforaphane (SFN) appears to enhance the effectiveness of inhibition of carcinogenesis in the Apc(min/+) mice (46). The mechanism of actions of DBM remained unclear. In vitro, it was reported that DBM induced cell cycle deregulation in human prostate cancer LNCap cells (48). The anti-inflammatory effect of DBM has been shown to be related to the modulation of arachidonic acid metabolism (49). In vivo, DBM was shown to activate Nrf-2 detoxification pathway and inhibit benzo(a)pyrene induced DNA adducts in lungs (50). Considering the results of these studies, we thereby investigated the chemopreventive efficacy and the potential in vivo mechanism of actions of DBM in colon cancer using the AOM/DSS model.

3.2. MATERIALS AND METHODS

Animals, chemicals and diets

Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). 15-20 mice were used for each treatment group. AOM was purchased from the
Sigma-Aldrich (St Louis, MO) and DSS was purchased from ICN Biochemicals. Both PEITC and DBM were purchased from Sigma and were sent to Dyets, Inc. (Bethlehem, PA) where they were mixed into 0.05% PEITC and 1% DBM with AIN-76 diet.

**Experimental procedure**

The experimental protocol for the present study is shown in Figure 1. Animals were maintained at 12 h light/12 h dark cycles with free access to water and food (AIN-76A powder diet from Dyets, Inc.). After 1 week of acclimatization, animals were randomly divided into 6 groups of between 15-20 animals in each group and fed with pellet-diets according to the protocol in Figure 1. After 20 weeks, the mice were sacrificed, and their colons were evaluated for polyps and other markers as described below.

**Histopathological analysis**

At the end of the experiment, all the mice were sacrificed by CO\textsubscript{2}. At autopsy, the large bowel was flushed with saline and excised. It was cut open longitudinally along the main axis and washed with saline. The large bowel was then carefully inspected for the presence of polyps, the size and the number of polyps was carefully measured. The polyps and the apparently normal mucosa were excised and fixed in 10% buffered formalin for at least 24 h. Paraffin-embedded sections of the large
bowel were then made by routine procedures. Any histopathological alterations in the colon were examined on hematoxylin and eosin (H&E)-stained sections.

**Preparation of tissue homogenates and Western blotting**

Colonic normal mucosa and polyps were excised from individual animal and pooled together based on the treatment groups. To prepare total cell lysates, about 3 to 5 polyps were incubated with 400 μl lysis buffer in an ice-cold French Douncer for 15 min before homogenization of 40 strokes. The homogenates were cleared by centrifugation at 14,000 x g for 10 min at 4°C. The supernatants were diluted and the protein concentrations were measured by bicinchoninic acid kit (Pierce). A total of 30 μg of proteins were resolved on precasted SDS-PAGE gels (Bio-Rad), and Western blotting was performed. The primary antibodies used include actin, Nrf2, GSTM1, HO-1, NQO-1, p21, p27 (Santa Cruz), cleaved-caspase-3, cleaved-caspase-7 and Bim (Cell Signaling).

**Statistical analysis**

The differences between the groups in terms of the multiplicity and the average size of tumors per mouse were determined by Student's t test, whereas the difference in the percentage of mice with tumors was tested by Fisher's exact test.

3.3. RESULTS
**Dietary administration of PEITC or DBM for 20 weeks significantly reduced AOM/DSS-induced polyps without affecting body weight.**

Figure 2 shows that neither the body weight nor any noticeable signs of toxicity were observed in the treatment groups as compared with the control group during the PEITC and DBM treatments. Mice in the control group (AOM/DSS) had a 70% tumor incidence, an average polyp number of 1.6 and an average polyp size of 5 mm (Figure 3). Dietary supplementation of 0.05% PEITC or 1% DBM before initiation (AOM) reduced the polyps incidence to 37.5% (p<0.05) and 36.4% (p<0.05) (Figure 3A); average polyps number to 0.375 (p<0.05) and 1.09 (Figure 3B); average polyps size to 1.5 mm (p<0.05) and 3.36 mm (Figure 3C). On the other hand, dietary consumption of 0.05% PEITC after initiation (AOM) reduced the polyps incidence to 40% (p<0.05) (Figure 3A); average polyps number to 0.6 (p<0.05) (Figure 3B); average polyps size to 2.6 mm (p<0.05) (Figure 3C), while 1% DBM after initiation (AOM) showed no apparent inhibitory effect at all (polyps incidence of 77.8%, average polyps number of 1.89, average polyps size of 5.78 mm) (Figures 3A-3C).

**General histological observation of AOM/DSS-induced polyps and normal mucosa in C57/B6 mice treated with PEITC or DBM.**
AOM/DSS-treated mice resulted in histological alterations of colonic mucosa, including infiltration of inflammatory cells into the lamina propria and loss of crypts (Figure 4B). PEITC or DBM treatment decreased the loss of crypts in the colonic mucosa (Figure 4C, 4D). The polyps developed in AOM/DSS-treated group were mostly adenocarcinomas, treatments with PEITC or DBM did not reverse the severity of the adenocarcinomas developed in the AOM/DSS-treated mice (Figure 4E, 4F).

**Dietary administration of PEITC or DBM altered proteins involved in cell cycle regulation, apoptosis and Nrf2-regulated detoxification enzymes.**

DBM greatly induced Nrf2 and its downstream detoxifying enzymes in normal mucosa. Administration of DBM but not PEITC before AOM initiation induced Nrf2 protein (Figure 5A, lane 3). However, DBM administration after initiation had no effect on Nrf2 induction (Figure 5A, lane 5). The Nrf2-mediated downstream detoxifying/antioxidant enzymes GSTM1, HO-1 and NQO-1 were also greatly induced by DBM treatment before AOM initiation. PEITC induced p21 and cleaved-caspase-3 and -7 in the polyps. The expression level of cell cycle arrest protein p21 was low in polyps (Figure 5B, lane 1). PEITC administration induced p21 in the polyps, before as well as after AOM initiation (Figure 5B, lane 2 and 4). Administration of PEITC or DBM had no effect on p27 induction. Cleaved-caspase-3 and cleaved-caspase-7 were more
robustly induced by PEITC before AOM than after AOM initiation, and Bim was 
induced by PEITC and DBM before AOM initiation (Figure 5B, lane 2 and 4).

3.4. DISCUSSION

The purpose of the present study was to investigate the efficacy of PEITC and 
DBM in the cancer chemoprevention of colon cancer. In addition, we aimed to 
elucidate the potentially in vivo mechanisms of actions of PEITC and DBM that would 
contribute to their cancer inhibitory effects. Previously, the use of the AOM–ACF 
model to determine the chemopreventive efficacy of PEITC was somewhat 
contradictory (44, 45). One paper reported that PEITC inhibited the formation of ACF 
while another paper did not. The efficacy of DBM has been demonstrated in the lung, 
in the small intestine, but not in the colon. In this study, we showed that both PEITC 
and DBM are effective chemopreventive compounds in colon carcinogenesis induced 
by AOM/DSS model (although DBM is only effective before AOM initiation); their in 
vivo molecular targets and mechanisms appear to be somewhat different.

PEITC appears to be a more potent compound than DBM due to the 
significantly lower average tumor numbers and sizes. Importantly, if DBM was 
administrated after AOM initiation, the inhibitory effect of DBM in tumor incidence, 
number and size was completely attenuated as compared to DBM administered
before AOM initiation. Interestingly, the major mechanism of action of DBM in chemoprevention appears to be targeting the molecular events before AOM initiation. This could be through increasing the Nrf2-mediated detoxifying/antioxidant genes for carcinogen detoxification leading to decreased carcinogen-induced oxidative stress, DNA and protein damages. The tumor incidence, number and size remained unchanged for PEITC, whether administrated before or after AOM initiation. These indicate that in contrast to DBM, PEITC’s effect may not depend only on the prevention of AOM initiation but also after AOM initiation. Indeed, the inhibitory effect could be due to induction of cell cycle arrest and apoptosis during the progression of carcinogenesis. Interesting, our recent study using the genetic TRAMP prostate mouse tumor model shows that DBM is more effective when given before the prostatic intraepithelial neoplasia (PIN) development at 8 weeks old, whereas it is somewhat less effective when DBM is given at 12 weeks when PIN apparently have developed (51). It will be interesting whether PEITC given at a later time point 12 weeks or older TRAMP mice will be as effective as when it was given at 8 weeks old (42).

In this study, we have also examined the normal mucosa and the polyps sections after PEITC and DBM treatments. The AOM/DSS treatment promoted the formation of polyps and most of them were adenocarcinomas. The normal mucosa
from AOM/DSS-treated group was exemplified with the loss of colonic crypts and the infiltration of inflammatory cells into the lamina propria. The polyps showed no signs of decreased severity for those polyps obtained from mice treated with PEITC or DBM as compared to the controls. However, we did observe less or no crypt-loss in the mucosa for the PEITC- and DBM-treated groups. These general observations from the histological analysis suggest that PEITC and DBM have no apparent effect in reversing the tumor grade of already formed malignant polyps.

Comparisons of some of the potential anti-carcinogenesis markers in the normal mucosa versus the polyps between the different groups using Western blotting shed some lights on the potential different in vivo mechanisms elicited by PEITC and DBM. DBM appears to induce a higher Nrf2 expression level, resulting in a more robust induction of phase II detoxifying and antioxidant genes, particularly GSTM1, HO-1 and NQO1, which would play an important role in detoxifying carcinogenic reactive intermediates and reactive oxygen/nitrogen species (RONS). Several studies have shown that DBM is effective in reducing DNA-carcinogen adducts in the lungs (50) and mammary glands (52). In this context, the same mechanism could be applied in the colon. Induction of Nrf2 and its downstream target defense enzymes by DBM before AOM initiation would lead to AOM detoxification and potentially less DNA damage. In contrast, when cell cycle and
apoptosis markers were compared, PEITC appears to have greater ability to induce cell cycle arrest and apoptosis than DBM, and these results are quite analogous to our previous study with SFN in the Apc(min/+)) mouse model (46). PEITC appears to be more potent in inducing p21, Bim and cleaved-caspase-3 and -7 than DBM. This might possibly explain why the tumor incidence, number and size were not affected with PEITC was given either before or after AOM initiation. Our result suggests that AOM detoxification might not be the major mechanism for PEITC’s chemopreventive effects, rather PEITC’s ability to induce apoptosis and cell cycle arrest and possibly keeps the transformed malignant colon cells in check would be the mechanism. However, future studies involving proteomic and or metabolomics would be needed to further elucidate the potential in vivo differential mechanisms.

In summary, using the AOM/DSS-induced colon cancer model, we showed that the major in vivo mechanism for PEITC appears to be induction of cell cycle arrest and apoptosis while DBM’s in vivo mechanism of action could be through AOM and or RONS detoxification by Nrf2-regulated detoxifying/antioxidant enzymes. This appears to be consistent with our observation that PEITC is effective in reducing tumor incidence, numbers and size whereas DBM is only effective towards inhibiting tumor incidence when used before AOM initiation. These differential in vivo mechanisms between PEITC and DBM could potentially offer new insights into the in
vivo anti-carcinogenesis mechanisms by different dietary cancer chemopreventive compounds and would help to advance future clinical human colorectal chemoprevention trials.

![Experimental protocol for chemoprevention study](image)

**Fig. 1.** Experimental protocol for chemoprevention study with PEITC and DBM in AOM/DSS mice. Ten-week-old mice were used for each group and fed with AIN-76 diet or diet supplemented with 0.05% PEITC or 1% DBM before AOM injection (pre-initiation) or after AOM injection (after initiation). Twenty weeks later, the mice were killed for analysis.
Fig. 2. No observable weight loss of mice. The mice were weighed every week from the start of the treatment for 20 weeks. The administration of compounds did not cause significant weight loss over time.
Fig 3. PEITC and DBM decreased tumor incidence, average tumor number and size. (A) 0.05% PEITC and 1% DBM before initiation (AOM) reduced the polyps incidence from 70% to 37.5% and 36.4% and 0.05% PEITC after initiation (AOM) reduced the polyps incidence to 40%. (B) 0.05% PEITC and 1% DBM before initiation (AOM) reduced average polyps number from 1.6 to 0.375 and 1.09 and 0.05% PEITC after initiation (AOM) reduced average polyps number to 0.6; (C) 0.05% PEITC and 1% DBM before initiation (AOM) reduced the average polyps size from 5 mm to 1.5 mm and 3.36 mm and 0.05% PEITC after initiation (AOM) reduced the average polyps size to 2.6 mm, 1% DBM after initiation (AOM) showed no inhibitory effect at all (polyps incidence of 77.8%, average polyps number of 1.89, average polyps size of 5.78 mm).
Figure 4. General histological observation of AOM-DSS polyps and normal mucosa in C57/B6 mice treated with PEITC or DBM. (A) No loss of crypts was observed in mice without AOM/DSS treatment. (B) AOM/DSS treatment resulted in observable crypts loss in normal mucosa. (C) PEITC or (D) DBM treatment decreased loss of crypts in normal mucosa and a better crypt organization was observed. (E) Treatment with PEITC or (F) DBM did not reverse the severity of the adenocarcinomas developed in the AOM/DSS-treated mice.
Figure 5. PEITC or DBM altered proteins involved in cell cycle regulation, apoptosis and Nrf2-regulated detoxification enzymes. (A) DBM greatly induced Nrf2 and its downstream detoxifying enzymes. DBM but not PEITC before AOM initiation induced Nrf2 protein in the normal mucosa. The Nrf2 regulated downstream detoxifying enzymes GSTM1, HO-1 and NQO-1 were also greatly induced by DBM treatment. (B) PEITC greatly induced p21 and cleaved-caspase 3 and 7. PEITC administration showed a substantial p21 induction in the polyps, before or after initiation. Induction of another cell cycle arrest marker p27 was less substantial. Cleaved-caspase-3 and 7 (C-casp3 and 7) was substantially induced by PEITC administrated both before and after AOM initiation. Induction of pro-apoptotic protein Bim was also higher by PEITC than by DBM.
4. Nrf2 novel interaction partner IQGAP1 and its signaling

4.1. INTRODUCTION

The Nrf2 pathway, which is activated in response to different extracellular stimuli (39, 53-55), modulates oxidative stress in cells (56, 57). Nrf2, a transcription factor belongs to the Cap “n” Collar (CNC) family has been shown to regulate the induction of phase II/antioxidant genes including glutathione S-transferase (GST), NAD(P)H: quinone oxidoreductase 1 (NQO1), UDP-glucuronosyl transferase (UGT), γ-glutamylcystein synthetase (γGCS) and heme oxygenase-1 (HO-1) (58). In unstimulated conditions, Nrf2 levels are generally low since it is sequestrated by Kelch-like ECH-associated protein 1 (Keap1) and is targeted by ubiquitinase for ubiquitination. The ubiquitinated Nrf2 are then degraded by proteosomes (59). However, in response to oxidative stress, Nrf2 dissociates from Keap1 and translocates into nucleus, where Nrf2 binds to a cis-acting element called anti-oxidant responsive element/electrophile response element [ARE/EpRE, 5’-(A/G)TGACNNNGC(A/G)-3] at 5’-flanking promoter regions of the phase II/antioxidant genes (60).

Nrf2 activation has been shown to be regulated by different kinases, among
them mitogen-activated protein kinases (MAPKs) (61, 62) and protein kinase C (PKC) (63). MAPK cascades are activated in response to a variety of extracellular stimuli.

The most widely studied MAPK pathway is the ERK cascade. ERK has been shown to be activated and is required for activation of Nrf2 induced by different stimuli, including dietary compounds such as PEITC (39) or inducers of oxidative stress. In vitro kinase assays showed that PEITC could induce the phosphorylation of ERK, which in turn phosphorylates Nrf2 and activates it (39).

We identified IQGAP1 as a novel binding partner of Nrf2. IQGAP1, like other IQGAP family such as IQGAP2 and IQGAP3, share conserved protein structure including calmodulin-binding IQ motifs and the GTPase activating protein (GAP) homology domain (64). IQGAP1 was first identified as a Rac and Cdc 42 binding protein coupled with actin. It is widely accepted that IQGAP1 inhibits its GTPase activity by recruiting Cdc42 and Rac1 (65, 66). IQGAP protein interacts with numerous cellular signaling molecules and coordinates various cellular events in actin and tubulin cytoskeletons through four major domains (67): CHD, calponin homology domain for actin binding (68); WW, WW domain for ERK2 binding (69); IQ, IQ motifs for calmodulin (70), myosin light chain (71) and S100B binding (72); GRD, GAP-related domain for CDC42 and Rac1 binding (65, 73); dCT, distal C-terminal domain for E-cadherin, β-catenin (66) and CLIP-170 binding (66, 74).
Previous reports indicate IQGAP1 acts as a scaffold protein and modulates MEK/ERK activity (75). On the other hand, our preliminary results show that IQGAP1 binds Nrf2. Taken these results together, we hypothesized that IQGAP1 acts as a scaffold for Nrf2 and MEK/ERK machinery and modulate Nrf2 activity in response to MAPK activator PEITC. We present evidence that IQGAP1 and Nrf2 associate both in vitro and in cells. The knockdown of IQGAP1 by shRNA attenuate PEITC or MEK-induced activation of MEK/ERK pathway, interaction of IQGAP1 and Nrf2, Nrf2 nuclear translocation, and phase II gene expression. These data suggest that IQGAP1 plays an important role in MEK-ERK-Nrf2 signaling.

4.2. Materials and Methods

Cell Culture

HEK, MEF and HeLa cells were maintained at 37 °C in a humidified atmosphere of 5% CO2/95% air in MEM medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and 50 U/ml of penicillin/streptomycin mixture (Gibco BRL, Grand Island, NY). Cells were grown to 60–80% confluence and trypsinized with 0.05% trypsin containing 2 mM EDTA.

Construction of Plasmid for IQGAP1 silencing
siIQGAP DNA duplex was inserted into pRNAT-CMV3.2/neo/cGFP vector (Genscript, NJ, USA). The sequence for pRNAT-silQ was 5'-GAT CCT TCT ACG GTC AAT AGC TTC ATT TGA TAT CCG ATG CTA TTG ACC GTA GAA CGC-3'. The duplexes were synthesized, and purified by high pressure liquid chromatography (Integrated DNA Technologies). Synthesized DNA were annealed to form double strand and cut with BamHI and XhoI restriction enzyme sites and ligated into pRNAT-CMV3.2/neo/cGFP vector.

**Transient Transfection**

HEK cells were transfected using the GeneJuice (Novagen) or FUGENE6 (Roche) according to the manufacturers’ instructions. For each well, 500 ng of each plasmid or in combination were added into 100 µl of OptiMEM with 3 µl GeneJuice. The various amounts of plasmids were also applied for certain experiments as indicated in the figure legend. pcDNA3.1 was added to make total amount of plasmids equal. The transfection mixture were mixed vigorously and incubated at room temperature for 15 mins. After that, the cells were incubated with transfection mixtures for 20-24 h at 37°C incubator. The cells were then subjected to further treatments.

**Reporter Gene Activity Assays**
Cells transfected with ARE-luciferase plasmid were washed once with PBS, scraped, and lysed in 200 μl of reporter lysis buffer (Promega) on ice for 10 min. After centrifugation at 12,000 rpm for 5 min at 4°C, 10 μl of lysate was mixed with luciferase substrate (Promega), and the ARE-luciferase activity was measured using a Sirius luminometer (Berthold Detection System). Luciferase activity was normalized by measuring the conventional β-gal activity by the transfection with pCDNA3.1-/hisB/lacZ plasmid coding galactosidase.

**Western Blot Analysis**

HEK or HeLa cells were plated in 6-well culture dishes at ~4.0 x 10^5 cells/well for 16 h prior to plasmid transfection. After transfection, cells were scraped and lysed with RIPA lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl, pH 7.4, 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na3VO4, with protease inhibitor cocktail tablet) for 30 min on ice followed by centrifugation at 14,800 x g for 15 min. The protein concentration of the supernatant was measured by using the BCA reagent. Protein (20 μg) was loaded on 4-15% criterion Tris-HCl gel (Biorad) and electrotransferred onto polyvinylidene difluoride (PVDF) membrane in Tris–glycine buffer (pH 8.4) containing 20% methanol. The membrane was then blocked in 5% fat-free dry milk in phosphate-buffered saline with 0.1% Tween-20 (PBS-T) for 1 h.
Then membranes were probed with primary antibodies and horseradish peroxidase-conjugated secondary antibody by standard Western blotting procedures. The proteins were visualized with the femto signal chemiluminescent substrate (Pierce) under the image analyzer (Bio-rad).

**Immunoprecipitation (IP)**

HEK or HeLa cells were plated on 100 mm dishes and cultured until over 95% confluence. Next, the treated cells were harvested and lysed with M-PER (Pierce) buffer for cytosolic extract. Four hundred micrograms of cytosolic samples were subjected to immunoprecipitation (IP) assay using anti-IQGAP1 (H-109) antibodies. In this experiment, Dynabead G beads (Invitrogein) were used for IP. The procedures were followed by the manufacture’s protocol. Briefly, 10 μg of antibodies were incubated with Dynabead protein G for 10 min to make antibody-bead complex first. Then, this complex was incubated with cytosolic lysates for 10 min at room temperature. After washing the IP complexes, IP samples were suspended with 200 μl of 2xSDS sample buffer and 20 μl of aliquots were subjected to Western blotting using anti-Nrf2 (C-20) antibody or IQGAP1 (H-109).

**In vitro binding assays and in vitro kinase assays**
IQGAP1 fragments bound to Dynabead G beads were incubated with His-Nrf2 in 500μl of buffer for 3 hours at 4°C. After centrifugation, samples were washed and resolved by SDS-PAGE. Alternatively, His-Nrf2 bound to His-beads was incubated with different IQGAP1 fragments. Proteins that bound to Nrf2 were washed and resolved by SDS-PAGE. In vitro kinase assays were performed as described previously.

**Quantitative RT-PCR**

RNA extraction and qRT-PCR analysis were performed as described in SYBR Green Quantitative PCR Protocol. Primer sequences of UGT, HO1, NQO1, GST were described previously.

**4.3. RESULTS**

*IQGAP1 binds Nrf2 in HEK cells and PEITC further stimulates the binding.*

We have identified that IQGAP1 and Nrf2 interact in-vitro. To ascertain whether IQGAP1 and Nrf2 interact in a normal cellular environment, immunoprecipitation was performed. We observed that endogenous Nrf2 binds to IQGAP1 in HEK cells. Nrf2 binding to IQGAP1 was increased when we blocked the proteosomal degradation of Nrf2 using MG132 (10μM) (Figure 1A). Little Nrf2 was detected in samples immunoprecipitated with NIRS (rabbit serum), confirming that IQGAP1 and
Nrf2 specifically interact in HEK cells. Upon PEITC stimulation, the binding between IQGAP1 and Nrf2 increased (Figure 1B).

**IQGAP1 mediates PEITC-induced expression of phase II/antioxidant genes.**

Transfection of HEK cells with shIQGAP1 in FUGENE transfection reagent resulted in more than 50% knockdown of IQGAP1 protein (Figure 2A). We have previously documented PEITC activates MAPK cascade (MEK/ERK), enhancing Nrf2 activity and inducing phase II/antioxidant genes. In stimulated conditions, PEITC induce activation of Nrf2 and expression of phase II/antioxidant genes, including HO-1, NQO1 and UGT mRNA. The phase II/antioxidant mRNAs were induced 3-5 folds in HEK cells by PEITC but were attenuated in IQGAP1-knockdown HEK cells (Figure 2B). Specifically, western blot of HO-1 revealed a similar result. HO-1 protein was induced ~3.5 fold in HEK cells by PEITC but was attenuated in IQGAP1-knockdown HEK cells (Figure 2C). Taken these results together, IQGAP1 is necessary for PEITC-induced expression of phase II/antioxidant genes.

**IQGAP1 mediates PEITC-induced nuclear expression of Nrf2**

PEITC time-dependently induced HO-1 protein, with a maximal expression from 6 to 24 hours. However, the induction of HO-1 protein was substantially attenuated
in IQGAP1-knockdown HEK cells (Figure 3A). To determine if IQGAP1 modulates nuclear expression of Nrf2 induced by PEITC, the amount of Nrf2 in nucleus after PEITC treatment was determined in IQGAP1-knockdown HEK cells. Maximal Nrf2 nuclear translocation occurred in HEK cells from 2 to 6 hours after PEITC treatment, but the amount of Nrf2 nuclear translocation was comparatively lower in IQGAP1-knockdown HEK cells during that period (Figure 3B). These results indicate that IQGAP1 is required for PEITC-induced nuclear translocation of Nrf2.

**Nrf2 binds to the IQ domain of IQGAP1**

The Nrf2 binding domain of IQGAP1 was investigated using IQGAP1 constructs (Figure 4A) obtained from Dr. David Sacks, Harvard. The IQGAP1 proteins were generated according to the protocol of TNT® Quick Coupled Transcription /Translation Systems and were mixed with purified His-Nrf2. The mixtures were then subjected to immunoprecipitation using anti-myc antibody and were pulled down by DynaG beads. It was revealed that the N-terminal half (amino acid residues 1 to 863) of IQGAP1 binded to Nrf2; further examination revealed the specific binding of Nrf2 to IQ domain of IQGAP1 (Figure 4B). Immunoprecipitation using Ni-NTA beads to pull down His-Nrf2 together with the binding IQGAP1 fragments showed that Nrf2 specifically binds to the IQ domain (amino acid residues 699 to 905) of IQGAP1.
IQGAP1 mediates MEK/ERK-induced ARE expression

Our previous studies have shown that activation of MEK/ERK pathway was required for PEITC-induced phase II/antioxidant gene expression. We showed that PEITC-induced MEK/ERK activation was dependent on IQGAP1, with an increase in pMEK and pERK 30 mins after PEITC treatment on HEK cells but was attenuated in IQGAP1-knockdown HEK cells (Figure 5A). To test if phase II/antioxidant gene induced by overexpression of MEK was dependent on IQGAP1, we transfected the cells with ARE-luciferase plasmid together with HA-ERK2 or/and DNEE-MEK. Overexpression of ERK did not turn on the MEK/ERK pathway, since increase of pERK and pMEK was not observed. On the other hand, overexpression of MEK activated the MEK/ERK pathway, indicated by increase of pMEK and pERK. IQGAP1 is necessary in activation of MEK/ERK since the amounts of pMEK and pERK were attenuated in IQGAP1-knockdown HEK cells (Figure 5B). Activation of MEK/ERK concomitantly induced ARE-luciferase to ~4.5 fold but was attenuated in IQGAP1-knockdown HEK cells (Figure 5C), indicating activation of MEK/ERK pathway is required for Nrf2-activation and this MEK/ERK/Nrf2 pathway is IQGAP1 dependent.
**Nrf2 is Phosphorylated by MEK1/ERK2 In Vitro and In Vivo and IQGAP1 mediates the ERK/Nrf2 interaction.**

The possible functional significance of increase of MEK/ERK/Nrf2 interaction was investigated. HeLa cells were transfected with ERK2 and DNEE-MEK1 for 24 hrs, then the cells were harvested and the HA-ERK2 was immunoprecipitated and blotted against HA and p-ERK1/2. We showed that the overexpressed ERK2 were mostly phosphorylated by MEK1 (Figure 6A). *In vitro* experiments showed Nrf2 was phosphorylated by MEK1/ERK2. MBP, GST, or GST-Nrf2 proteins were incubated with immunoprecipitated HA-ERK2 prepared from the HeLa cells with/without MEK stimulation, in the presence of [γ-32P]ATP in kinase assay buffer at 30°C for 30 min, then resolved by SDS-PAGE and visualized by autoradiography. We showed that low level of phosphorylated ERK resulted in small amount of Nrf2 phosphorylation *in vitro*, while MEK/ERK overexpression significantly phosphorylated ERK, leading to a large amount of phosphorylated Nrf2 *in vitro* (Figure 6B). To show that Nrf2 was phosphorylated by MEK/ERK *in vivo*, MEFs were co-transfected with GFP-Nrf2, HA-ERK2, and DNEE-MEK1. GFP-Nrf2 was immunoprecipitated and the binding proteins were analyzed. We showed that overexpression of MEK and ERK significantly increase the proportion of phosphorylation of overexpressed Nrf2 and an increase binding of ERK2 to the phosphorylated Nrf2, which was not observed when only ERK
was overexpressed (Figure 6C). Next, the interaction between ERK and Nrf2 was investigated. We showed that activation of MEK is required to increase interaction between ERK and endogenous Nrf2 or overexpressed Nrf2 and knockdown of IQGAP1 attenuates the interaction between ERK and Nrf2 even after MEK activation (Figure 6D).

**MEK/ERK activation stabilized Nrf2 by interfering with Keap1-dependent ubiquitination of Nrf2 and was IQGAP1 dependent**

MEK/ERK activation stabilized the ectopically expressed GFP-Nrf2 protein and IQGAP1 appeared to attenuate the stabilization. Cells were treated with MG132 to stabilize the ubiquitinated-Nrf2 for easy detection. Our results showed that activation of MEK/ERK decreased ubiquitination of Nrf2, suggesting interference with Nrf2/Keap1 interaction. In contrary, the decreased ubiquitination of Nrf2 was not observed in IQGAP1-knockdown cells. In fact, there was a higher basal level of ubiquitination of Nrf2 in IQGAP1-knockdown cells, indicating IQGAP1 played a role in MEK/ERK stabilization of basal of Nrf2 level (Figure 7A). Stabilization of Nrf2 by MEK/ERK activation was IQGAP1-dependent. After cycloheximide (CHX) treatment, the level of GFP-Nrf2 in HEK cells was compared with IQGAP1-knockdown cells. We showed that MEK/ERK activation stabilized Nrf2 protein, but Nrf2 protein degraded
with a half life of approximately 30 mins in IQGAP1-knockdown cells (Figure 7B).

*Phosphorylated Nrf2 increases binding with small Mafs, potentiates ARE-mediated gene expression and was IQGAP1 dependent*

Again, GST-Nrf2 was phosphorylated by ERK2 in vitro (Figure 8A). The in vitro phosphorylated GST-Nrf2 was incubated with whole cell lysate from MEFs. The GST-Nrf2 was then pulled down by GSH-resin and the pull-down products were blotted against Nrf2 or MafF/G/K. We showed that phosphorylated Nrf2 increased binding with different small Mafs (Figure 8B). We also showed that phosphorylated Nrf2 increases binding with small Mafs in MEF cells (Figure 8C). Furthermore, MafG and MEK1/ERK2 synergistically upregulate Nrf2-induced ARE-iediated gene expression (Figure 8D), indicating the functional significance of increased binding between phosphorylated Nrf2 and MafG. MEK/ERK appeared to stabilize the ectopically expressed Nrf2 and MafG and increase their binding but the same effects were not observed in IQGAP1-knockdown HEK cells (Figure 8E), indicating IQGAP1 is required for MEK/ERK-mediated Nrf2/MafG interaction and stabilization.

*IQGAP1 modulates PEITC-stimulated activation of Nrf2 in a concentration-independent manner*
Because Nrf2 activation is dependent on IQGAP1, we assessed whether IQGAP1 modulated Nrf2 activities in a dose-dependent manner. To obtain different expression levels, IQGAP1 was transiently overexpressed or knocked down. Serum starved cells were stimulated by PEITC, nuclear fractions were collected at 2 hours after treatment and cytoplasmic fractions were collected 6 hours after treatment and were analyzed by western blotting. We observed PEITC induced Nrf2 translocation and HO-1 protein only within a close range of IQGAP1 concentration close to endogenous levels in HEK cells. Interestingly, both increasing and decreasing intracellular IQGAP1 reduced PEITC-induced nuclear translocation of Nrf2 2 hours after treatment, leading to decreased HO-1 expression 6 hours after PEITC treatment (Figure 9).

**IQGAP1 knockout reduced NQO1 expression induced by a variety of compounds**

We used MEF-WT and MEF-IQGAP1-KO cells to compare the ARE activation induced by different compounds. PEITC, SFN, BHA, tBHQ and Curcumin were shown to induce the NQO1 expression to various degrees in MEF-WT cells but the induction by various treatments was consistently decreased in MET-KO cells, indicating the importance of IQGAP1 in ARE activation (Figure 10).
4.4. DISCUSSION

Previously, we have isolated and identified several possible Nrf2 signaling-partner proteins using One-strep tag purification system. Among the newly identified Nrf2 partners, IQGAP1, a scaffolding protein was further investigated on the Nrf2 signaling. Here, we found that IQGAP1 modulates Nrf2 activation by PEITC. In that regard, we postulate that IQGAP1 protein may scaffold Nrf2 and MEK/ERK signaling, which leads to activation and nuclear translocation of Nrf2 to induce phase II/antioxidant genes.

Nrf2 has been considered as a key transcriptional factor for the regulation of ARE-mediated gene expression in response to oxidative and electrophilic insults. MAPK signaling has been shown to be activated by various Nrf2-inducer chemicals such as PEITC (39), BHA (76) and 3H-1,2-dithiole-3-thione (D3T) (77). Interestingly, ERK2 activation has been shown to be necessary for induction of Nrf2-regulated genes induced by these chemicals. We have identified the MEK-ERK-Nrf2 sequential phosphorylation as the major pathway of Nrf2 activation. But the mechanism of how do these proteins come together to form a functional module remains unclear.

Using One-strep tag purification system, we observed that Nrf2 binds IQGAP. GST-IQGAP1 co-immunoprecipitates with Nrf2 confirm their direct binding. Co-immunoprecipitation of IQGAP and Nrf2 in HEK and HeLa cells under stimulated or
un-stimulated conditions (PEITC/SFN treatments) further confirm their binding in a physiological environment. IQGAP1 binds an array of proteins including calmodulin, Cdc42, Rac1, actin, β-catenin, E-cadherin, and CLIP-170. Recently, evidence has been provided that IQGAP acts a scaffold protein and modulates the Raf-MEK-ERK pathway. It has been shown that IQGAP1 binds Raf, MEK and ERK directly and modulates their functions and activation (78). Changing IQGAP1 concentration impairs the ability of EGF to activate MEK and ERK. Given the experimental evidence suggesting that IQGAP1 binds Nrf2, integrates MEK-ERK signaling pathway, we hypothesized that IQGAP1 may be an unrecognized scaffold for the MEK-ERK-Nrf2 signaling pathway. The evidence presented here supports this hypothesis.

Previous reports suggest that activation of MEK-ERK phosphorylates Nrf2 and induce its subsequent translocation into the nucleus. It was shown that PEITC induced Nrf2-dependent ARE activity and HO-1 expression were attenuated by inhibiting the ERK signaling. On the other hand, activation of the ERK signaling by transfection of ERK also resulted in elevation of ARE activity and HO-1 expression. *In-vitro* kinase assay showed ERK could directly phosphorylates GST-Nrf2. These data suggest a model that in PC-3 cells, PEITC activates ERK, which in turn phosphorylates Nrf2 leading to its nuclear translocation (39). Further experiments comparing this signaling pathway in HEK cells and IQGAP1 knockdown HEK cells suggest that
knockdown of IQGAP1 severely impairs PEITC-induced activation of ERK-Nrf2 pathway.

Decreasing intracellular IQGAP1 concentration leads to marked impairment of basal Nrf2 activity. IQGAP1 knockdown HEK cells expressed less ARE compared with normal HEK cells, implicating IQGAP1 modulates basal Nrf2 activity. The inducible Nrf2 activity is also modulated by IQGAP1, supported by data showing that PEITC-induced HO-1, UGT, NQO1 and ARE expression were all abrogated in IQGAP1 knockdown HEK cells. The decrease in phase II/antioxidant gene IQGAP1 knockdown HEK cells is due a decrease in nuclear expression of Nrf2. PEITC induced a maximal nuclear translocation of Nrf2 2 hours after treatment, but the amount was significantly attenuated in IQGAP1 knockdown HEK cells.

On the other hand, upon PEITC stimulation or when Nrf2 is in excess, an increased binding of Nrf2 to IQGAP1 is observed. The binding of Nrf2 to IQGAP1 has functional significance. Our data showed that under stimulated conditions (PEITC or activation of MEK-ERK), increased Nrf2 binds to IQGAP1, and this interaction is abolished if IQGAP1 is knockdown. The decreased IQGAP1-Nrf2 interaction in IQGAP1 knockdown HEK cells could be due to decreased MEK-ERK signaling as observed by the decrease in pMEK and pERK in IQGAP1 knockdown HEK cells. Activating the MEK-ERK axis by transfection of ERK or constitutive active MEK alone
provides us with more insights. Transfection of constitutive MEK increases pMEK together with pERK, correlating with increase of IQGAP1-Nrf2 interaction. On the other hand, overexpression of ERK alone has no effect on MEK-ERK activation and interaction of IQGAP1-Nrf2 was not observed. Increased phosphorylation of Nrf2 by MEK/ERK is important in binding with sMafs. In particular, MafG, Nrf2, MEK and ERK synergistically upregulate ARE-mediated gene expression, indicating the importance of this signaling cascade.

Another line of evidence also support a functional module of MEK-ERK-Nrf2 could take place on IQGAP1. Other groups have shown that both MEK and ERK bind to the N-terminal (1-600aa) of IQGAP1. Further studies showed MEK binds to IQ domain and ERK binds to WW domain of IQGAP1 respectively (75). Interestingly, our data also show Nrf2 binds directly to IQ-domain of IQGAP1, implicating Nrf2 and MEK-ERK complex are in close proximity and regulated in the same fashion. Maximal activation of Nrf2 by PEITC was observed only when all client proteins (MEK, ERK and Nrf2) are assembled on IQGAP1. The components have to be present in an appropriate stoichiometric ratio. It was said that the concentration of IQGAP1, MEK2 and ERK2 in MCF-7 cells were 0.8uM, 6.9 uM and 15.5uM (75). Thus, changing the amount of IQGAP1 will result in large change in the stoichimtries of IQGAP1 to MEK, ERK and Nrf2. The Nrf2 activity induced by PEITC (measured by ARE and HO-1) in HEK,
shIQ-HEK, dsIQ-HEK cells support this data. Activation of Nrf2 by PEITC was observed only when cellular concentration was close to normal levels. However, the stiochiometry required for optimal activation could vary from cell to cell and would need further study.

A model of IQGAP1 functioning as a scaffold in the MAPK kinase pathway has been proposed. When the concentration of IQGAP1 is too low, the functional complexes are unable to form, whereas when concentration of IQGAP1 is in excess, IQGAP1 could harbor only one or two components of the cascades, which cannot function optimally. In either cases, suboptimal activation of MEK/ERK/Nrf2 signaling leads to a decrease in phosphorylated Nrf2 and decrease sMafs binding. Heterodimerization of Nrf2 with sMafs in the nucleus are known to be important in transactivation of genes regulated through ARE (79). This could be due to the enhancement of nuclear retention of Nrf2 via masking NESzip motif after Nrf2 heterodimerizes with sMafs (80).

In summary, we have identified IQGAP1 as a novel binding partner of Nrf2. We provide evidence that IQGAP1 participates and modulates activation of Nrf2. Furthermore, our data suggest that IQGAP1 modulates Nrf2 activation through regulation of MEK-ERK signaling. Our study provides a novel hypothesis that IQGAP1 links the MAPK and Nrf2 signaling together. Our data suggest that a change in
intracellular IQGAP1 concentration could severely impair Nrf2 activation ability, thus diminishes our cellular defense against oxidative damages or carcinogens. IQGAP1 has been shown to be involved in organizing ROS-dependent VEGF signaling and promotes endothelial cell migration and proliferation (81), ROS-dependent tyrosine phosphorylation of VE-cadherin and loss of cell-cell contacts (82) and hyperoxia-induced p47 phox translocation and ROS species generation in lung endothelial cells (83). Knowing the interplay between IQGAP1, Nrf2 and oxidative stress will provide us with more insights how IQGAP1 mediates our cellular defense against oxidative damages or carcinogens, helping us to explain disease mechanisms such as oxidative-stress induced carcinogenesis such as hepatotumorigenesis (84). In addition, the fact that IQGAP1 mediates PEITC-induced phase II gene expression imply that the chemopreventive effects of a wide range of compounds could also be IQGAP1-dependent. In fact, we also showed that IQGAP1-knockdown cells have decreased phase II gene expression (NQO1) in response to compounds, demonstrating the importance of IQGAP1-Nrf2 interplay in chemoprevention.
**Fig 1.** IQGAP1 and Nrf2 interact in a normal cellular environment. (A) We immunoprecipitated HEK cells lysates with IQGAP1 antibody and NIRS (rabbit serum). The HEK cells were treated with or without MG132 (a proteosomal inhibitor) for 6 hrs. (B) HEK cells were treated with PEITC for 20 mins and immunoprecipitation was performed.
Fig 2. IQGAP1 mediates PEITC-induced expression of phase II/antioxidant genes. (A) HEK cells were transfected with plasmid pcDNA3.1 and shIQGAP1 for 24 hrs. The downregulation of IQGAP1 protein was measured. (B) HEK cells transfected with plasmid shIQGAP1 or pcDNA3.1 were treated with PEITC for 6 hrs. mRNA expression of NQO1, UGT1A1 and HO1 were determined. (C) HEK cells transfected with plasmid shIQGAP1 or pcDNA3.1 were treated with PEITC for 6 hrs. Protein expression of HO1 was determined.
**Fig 3.** IQGAP1 mediates PEITC-induced nuclear accumulation of Nrf2. HEK cells transfected with pcDNA3.1 or shIQGAP1 were treated with PEITC. Cytoplasmic fraction and nuclear fraction were collected 0, 0.5, 2, 6 and 24 hrs after treatment. (A) Protein expression of HO1 was determined (B) Nuclear accumulation of Nrf2 was determined.
Fig 4. Nrf2 binds to IQ domain of IQGAP1. The IQGAP1 proteins were generated according to TNT protocol. They were then mixed with purified His-Nrf2. (A) Schematic diagram of the IQGAP1 constructs. (B) DynaG beads and c-myc antibody were used to pull down proteins that form complexes with myc-tagged IQGAP1 proteins. (C) Ni-NTA beads were used to pull down proteins that formed complexes with His-Nrf2.
**Fig 5.** IQGAP1 mediates MEK/ERK-induced ARE expression. (A) HEK cells transfected with pcDNA3.1 or shIQGAP1 were treated with PEITC for 10 mins. MEK and ERK phosphorylation were determined. (B) HEK cells were transfected with combination of plasmids for 24 hrs. MEK and ERK phosphorylation were determined. (C) HEK cells were transfected with combination of plasmids for 24 hrs. ARE induction was determined.
**Fig 6.** Nrf2 is phosphorylated by MEK1/ERK2 In-vitro and In-vivo and IQGAP1 mediates the ERK2/Nrf2 interaction. (A) HeLa cells were transfected with HA-ERK2 and DNEE-MEK1 for 24 hrs, then the cells were harvested and the HA-ERK2 was immunoprecipitated and blotted against HA and p-ERK1/2. (B) MBP, GST, or GST-Nrf2 proteins were incubated with immunoprecipitated HA-ERK2 prepared from the HeLa cells with/without MEK stimulation, in the presence of [γ-32P]ATP in kinase assay buffer at 30°C for 30 min, then resolved by SDS-PAGE and visualized by autoradiography. (C) MEFs were co-transfected with GFP-Nrf2, HA-ERK2, and DNEE-MEK1. GFP-Nrf2 was immunoprecipitated and blotted against Nrf2, phosphorylated Nrf2 and HA-ERK2. (D) HEK cells and IQGAP1 deficient HEK cells were transfected with HA-ERK2 and DNEE-MEK1. HA-ERK2 was immunoprecipitated and blotted against Nrf2.
Fig 7. MEK/ERK activation stabilized Nrf2 by interfering with Keap1-dependent ubiquitination of Nrf2 and was IQGAP1 dependent. (A) HEK cells were transfected and GFP-Nrf2 were immunoprecipitated and blotted with ubiquitin antibody. (B) HEK cells were transfected as indicated for 24 hrs and treated with CHX for 0, 0.5 and 1 hr. The cells were harvested and GFP-Nrf2 expression was blotted.
Fig 8. Phosphorylated Nrf2 increases binding with small Mafs, potentiates ARE-mediated gene expression and was IQGAP1 dependent. (A) GST-Nrf2 was phosphorylated by ERK2 in vitro. (B) The in vitro phosphorylated GST-Nrf2 was incubated with whole cell lysate from MEFs. The GST-NrF2 was then pulled down by GSH-resin and the pull-down products were blotted against Nrf2 or MafG/F/G/K. (C) Phosphorylated Nrf2 increases binding with small Maf proteins in MEF cells. (D) MafG and MEK1/ERK2 synergistically upregulate Nrf2-induced ARE-mediated gene expression (E) MEK/ERK appeared to stabilize the ectopically expressed Nrf2 and MafG and increase their binding but the same effects were not observed in IQGAP1-knockdown HEK cells.

Fig 9. IQGAP1 modulates PEITC-stimulated activation of Nrf2 in a concentration-independent manner. IQGAP1 was transiently overexpressed and knocked down by transfecting HEK cells with Ds-red-IQGAP1 (0.5 μg and 1 μg) and shIQGAP1 (0.5 μg and 1 μg) respectively. The cells were then stimulated by PEITC. (A) Cytoplasmic fractions were collected at 6 hours after PEITC treatment and HO-1 protein level was determined. (B) Nuclear fractions were collected at 2 hours after PEITC treatment and Nrf2 protein level was determined.
**Fig 10.** IQGAP1 knockout reduced NQO1 expression induced by a variety of compounds. IQGAP1-WT and IQGAP1-KO MEF cells were treated with different compounds (PEITC, SFN, BHA, tBHQ and CUR) for 6 hrs and NQO1 expression was blotted.
5. Nrf2 epigenetic regulation

5.1 INTRODUCTION

Prostate cancer, as well as many other age-related cancers, is characterized by increased intracellular oxidative stress (85, 86). Chronic oxidative stress and its associated pathological conditions including inflammation and metabolic disorders have been postulated to drive somatic mutations and neoplastic transformation, thus could play an important role in the development and progression of prostate cancer (87). Increased oxidative stress or reactive oxygen species (ROS) levels could be a consequence of increased ROS generation and/or decreased antioxidant capacities and or ROS detoxification. Recently the impaired antioxidant defense system in carcinogenesis of prostate cancer has been gaining increased attentions. The cellular antioxidant defense system comprises a battery of antioxidant/detoxifying enzymes and proteins such as superoxide dismutase (SOD), catalase, hemeoxgenase (HO), UDP-glucuronosyltransferases (UGT), glutathione peroxidase (GPx), glutathione-S-transferases (GST), and NAD(P)H:quinone oxidoreductase (NQO) (88). Down-regulation of GST by DNA methylation appears to be quite common in human prostate cancer that it has been developed as a diagnostic marker (89). The expression and the activities of SOD, catalase and GPx
have been reported to be decreased in prostate carcinoma tissues as well as in plasma and erythrocytes (89-92). Recent studies from our laboratory and others have found that the expression levels of SOD, UGT1A1, NQO1 and several GST family genes were significantly suppressed in prostate tumors in Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) mice (93-95). Although the down-regulation of GST enzymes in human prostate cancer have been linked to the promoter hypermethylation of GST genes (89, 96); promoter DNA hypermethylation does not appear to cause GST gene repression in TRAMP tumors (95). Instead, down-regulation of nuclear factor-erythroid 2p45 (NF-E2)-related factor 2 (Nrf2), a key regulator of cellular antioxidant enzymes, may be responsible for the transcriptional suppression of GSTs and other phase II detoxifying enzyme genes (94). Nrf2 is a helix–loop–helix basic leucine zipper transcription factor that regulates the expression of many phase II detoxifying and antioxidant enzymes via its binding to the antioxidant response element (ARE) in the promoter region (88). Knockout of Nrf2 in mice substantially abrogated the inducible expression of ARE-mediated detoxifying and antioxidant enzymes, and rendered these mice highly susceptible to carcinogens and/or oxidative damages (97, 98). In this context, previously we have found that the protein expression levels of Nrf2 and Nrf2-target gene heme-oxygenase-1 (HO-1) were attenuated in the skin tumors of a mouse skin
carcinogenesis model (99). Similarly, the expression of Nrf2 as well as its downstream target genes such as UGT1A1, GSTM1 and NQO1 were found to be gradually downregulated in prostate tumors with the progression of prostate tumorigenesis in TRAMP mice (93). Frolich et al. recently reported the expression of Nrf2 and GST mu family genes were significantly decreased in TRAMP prostate tumor, and linked this phenomenon to increased oxidative stress and DNA damage in prostate cancer. Meta-analysis of tissue expression profiling data from Oncomine database (www.oncomine.org) suggested that the expressions of Nrf2 and several GST mu genes are also gradually down-regulated in human prostate cancers (94). The currently accepted paradigm of Nrf2 regulation appears to be mainly achieved via post-translational mechanisms. As such, Nrf2 is functionally suppressed by the Kelch-like Erythroid-cell-derived protein with CNC homology (ECH)-Associated Protein 1 (Keap1), which binds to and sequesters Nrf2 in the cytoplasm leading to the degradation of Nrf2, and thus prevents Nrf2 nuclear translocation and transactivation of its target genes (100). Upon challenges by oxidative or electrophilic stresses that could involve potential modification of critical cysteine residues in Keap1 and or Nrf2 itself coupled with phosphorylation by kinases, Nrf2 is released from Keap-1, translocates into the nucleus, dimerizes with small Maf proteins, binds to ARE and transcriptionally activates Nrf2-ARE target genes (88). To
date, it is not clear as to how the expression of Nrf2 in human prostate cancer or in TRAMP mouse tumor is suppressed. Epigenetic or epigenomic mechanisms, particularly DNA methylation, have been frequently implicated in the alterations of gene expression in prostate cancer (101, 102). Coordinated hypermethylation of APC and GSTP1 in early carcinogenesis has been utilized as potential diagnostic markers to detect prostate cancer (103). In addition, alteration of DNA methylation profiles has been linked with cancer progression (104). DNA methylation, coupled with histone modifications, would affect the interactions of the promoters of critical genes with transcriptional corepressors and coactivators leading to changes in gene expressions, which could be one of the driving forces for prostate carcinogenesis. Silencing of multiple genes by DNA methylation has been reported in TRAMP prostate tumors and cell lines derived from TRAMP prostate tumors (104-106). Inhibition of DNA methyltransferase activity by 5-aza-29-deoxycytidine (5-aza) has been shown to prevent prostate tumorigenesis in TRAMP mice (107). In addition, the expression of Keap1 has been reported to be regulated by DNA methylation in lung cancer (108). In the present study, we first identified a CpG island in the upstream 59-flanking region of the murine Nrf2 gene followed by interrogation of the DNA methylation status of the whole CpG island via bisulfate genomic sequencing. We found that certain CpG sites in the distal region of the CpG island were
hypermethylated in the TRAMP tumor tissues as well as in tumorigenic TRAMP-C1 and -C2 cells, but not in normal prostatic tissue and non-tumorigenic TRAMPC3 cells. Utilizing methylated reporter assay, chromatin immunoprecipitation (ChIP) assay and treatments with trichostatin A (TSA)/5-aza, we provided compelling evidence that the expression of Nrf2 is epigenetically regulated during the development of prostate tumors in TRAMP mice. Nrf2 expression was suppressed by methylation of certain CpG sites and this was accompanied by the recruitment of MBD2 and trimethyl-histone H3 (Lys9) to the Nrf2 gene promoter in prostate tumor of TRAMP mice.

5.2. MATERIALS AND METHODS

Reagents and Cell Culture

All the enzymes used in the present study were obtained from New England Biolabs Inc. (Ipswich, MA) unless specified. Human recombinant insulin was purchased from Invitrogen. Dual luciferase assay system, luciferase reporter vectors pGL 4.75 with CMV promoter and pGL 4.15 were obtained from Promega (Madison, WI). All other chemicals were purchased from Sigma (St Louis, MO, USA). TRAMP C1 and C3 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 5 mg/ml human recombinant insulin, 1028 mol/L 5-androstan-
17b-ol-3-one, and antibiotics. The cells were grown at 37°C in a humidified 5% CO2 atmosphere.

**Animals**

Female hemizygous C57BL/TGN TRAMP mice, line PB Tag 8247NG, and male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were bred on same genetic background and maintained in the Animal Care Facility of Rutgers University. Housing and care of the animals were in accordance with the guidelines established by the University’s Animal Research Committee consistent with the NIH Guidelines for the Care and Use of Laboratory Animals. Transgenic males used in the current study were routinely obtained as [TRAMP 6 C57BL/6] F1 or as [TRAMP 6 C57BL/6] F2 offspring. Identities of transgenic mice were confirmed by the PCR-based genotyping. Throughout the experiment the animals were housed in cages with wood chip bedding in a temperature-controlled room (68–72°F) with a 12-h light dark cycle, at a relative humidity of 45–55%, and fed with irradiated AIN-76A diet (DYETS Inc, Bethlehem, PA).

**Bisulfite Genomic Sequencing (BGS)**

Genomic DNA was isolated from the palpable prostate tumors of 24 weeks old
TRAMP mice (n=12), apparently normal prostate tissue of 24 weeks old C57BL/6J mice (n=10), TRAMP-C1 and C3 cells using the DNeasy tissue kit (Qiagen, Valencia, CA). The bisulfite conversion was carried out using 500 ng of genomic DNA with the use of EZ DNA Methylation Gold Kits following manufacturer's instructions (Zymo Research Corp., Orange, CA). The converted DNA was amplified by PCR using Platinum Blue PCR SuperMix (Invitrogen, Grand Island, NY) with specific primer sets, with the translation initiation site (TIS) defined as position 1. The PCR products were purified by gel extraction using the Qiaquick™ gel extraction kit (Qiagen, Valencia, CA), then cloned into pCR4 TOPO vector using a TOPOTM TA Cloning kit (Invitrogen, Grand Island, NY). Plasmids DNA from at least 10 colonies per each group were prepared using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and sequenced (DNA Core Facility, Rutgers/UMDNJ, Piscataway, NJ).

**Plasmids**

The genomic sequence of murine Nrf2 containing the promoter region was retrieved from NCBI mouse genome data base. Two murine Nrf2 promoter segments, 21065–1 and 21367–1 with the translation initiation site (TSS) referred to as position 1, were amplified from mouse genomic DNA isolated from normal mouse prostate using the following primers: 1065 forward,
5’-GGTACCTAAGTACGTGAAAGGAACCCTGAGA-3’; 1367 forward, 5’-GGTACCAACAGTCACTACCACCACCA-3’; and a common reverse primer, 5’-CTCGAGGCTGAGGGCGGACGCTG-3’. The PCR products were cloned into pCR4 TOPO vector using a TOPO TA Cloning kit (Invitrogen, Grand Island, NY) then digested with KpnI and XhoI and inserted into pGL4.15 luc2P/Hygro vector. All the sequences of recombinant plasmids were verified by sequencing (DNA Core Facility, Rutgers/UMDNJ, Piscataway, NJ). The CpG-methylated reporters were generated by treating the reporter plasmids with methyl-transferase M. SssI according to the instruction provided by manufacturer. Briefly, 5 mg reporter constructs were incubated with 5 units of M. SssI for 1 hr in NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM MgCl2, and 1 mM dithiothreitol) supplemented with 160 mM Sadenosylmethionine at 37°C. Methylated plasmids were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and the concentrations of all plasmids were determined by agarose gel electrophoresis. The efficiency of methylation reactions was confirmed by digestion using the methylation-dependent Hhal and Hpall restriction endonucleases.

Transfection and Luciferase Reporter Assay

TRAMP C1 cells were plated in 24-well plates for 24 hrs, then transfected with
100 ng of the indicated reporter plasmids by using GeneJuice (Novagen, Madison, WI) according to the manufacturer’s instructions. 25 ng of pGL 4.75, which contains a Renilla reniformis luciferase gene driven by CMV promoter, was cotransfected as internal control. 24 hrs after transfection, the cells were lysed in dual luciferase lysis buffer, and 10 ml aliquots of the cell lysate were assayed using a dual luciferase assay kit with a Sirius luminometer (Berthold Technologies, Pforzheim, Germany). The transcriptional activities of each constructs were calculated by normalizing the firefly luciferase activities with corresponding Renilla luciferase activities, and were reported as folds of induction compared with the activity of empty pGL 4.15 vector. The values are mean±SD of four separate samples.

**Cell Treatments and Western Blotting**

Cells were plated in 6-well or 12-well plates for 24 hrs, then treated with 2 mM 5-aza, 200 nM TSA, or 1 mM 5-aza plus 100 nM TSA in media containing 0.5% FBS for 60 hrs, or 48 hrs followed by incubation in the presence of 5 mM tert-butylhydroquinone (tBHQ) for additional 12 hrs. After treatments, the cells were harvested in radioimmunoprecipitation assay (RIPA) buffer (Sigma, St Louis, MO). The protein concentrations of the cleared lysates were determined by using the bicinchoninic acid method (Pierce, Rockford, IL), and aliquots each containing 20 mg
of total protein were resolved by 4%–15% SDS-polyacrylamide gel electrophoresis (Bio-rad, Hercules, CA). After electrophoresis, the proteins were electro-transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The PVDF membrane was blocked with 5% fat-free milk in phosphate-buffered saline-0.1% Tween 20 (PBST), then sequentially incubated with specified primary antibodies and HRP-conjugated secondary antibodies. The blots were visualized by SuperSignal enhanced chemiluminiscence (ECL) detection system and documented using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA).

**RNA Isolation and Reverse Transcription-PCR**

Total RNA was extracted from the treated cells using the Trizol (Invitrogen, Carlsbad, CA). Steady-state mRNA levels of Nrf2 and NQO1 were determined by semi-quantitative reverse transcription-PCR (RT-PCR). First-strand cDNA was synthesized from 2 mg of total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. The cDNA was used as the template for PCR reactions performed using Platinum Blue PCR SuperMix (Invitrogen, Grand Island, NY). The PCR products were isolated by agarose gel electrophoresis and visualized by EB staining using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA). Primers to specifically amplify
the genes involved were shown in Table 1.

**Chromatin Immunoprecipitation (ChIP) Assay**

Chromatin immunoprecipitation (ChIP) assay was carried out using Millipore’s Magna-ChIP A kit (Millipore, Lake Placid, NY) following manufacturer’s protocol. In brief, freshly prepared 18.5% formaldehyde was added to the cells at a final concentration of 1%. Cells were incubated at 37°C for 10 min, then excess formaldehyde was quenched by addition of 5M glycine. After washing twice, the cells were scraped into 2 ml cold PBS containing protease inhibitor cocktail. The cells were pelleted and then resuspended in Cell Lysis Buffer containing protease inhibitor cocktail. Nuclei were isolated after Dounce homogenization and resuspended in Nuclear Lysis Buffer containing protease inhibitor cocktail. The samples were sonicated on ice using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ) to shear the cross-linked DNA to an average length of 200–1000 bp and centrifuged at 12,000 rpm to remove insoluble material. The chromatin solutions were diluted 10-folds using dilution buffer, and 10 ml of each was reserved as total input control. Diluted chromatin solutions were precleared with salmon sperm DNAprotein A magnetic beads for 1 hr, and then incubated with protein A magnetic beads and antibodies specific for MBD2, Pol II, H3Ac and H3K9me3 (Millipore, Lake Placid, NY) or
nonspecific IgG overnight at 4°C. The immunoprecipitated complex-magnetic beads were collected using magnetic separator and washed according to manufacturer’s instruction. The pellets were then incubated with proteinase K in ChIP Elution buffer for 2 hrs at 62°C with shaking to elute immunocomplex and reverse cross-link. The samples were incubated at 95°C for 10 min and DNA was purified according to manufacturer’s instruction. 1 ml of each of the purified DNA was used as template for 30 cycles of PCR amplification using designated primers (Table 1). The PCR products were then analyzed by agarose gel electrophoresis and visualized using EB staining. Primer set was designed to cover the DNA sequence from position 21190 to 21092 (mNrf2P1) in which the first 5 CpGs locate. To determine the association of RNA polymerase complex II to the Nrf2 promoter, another primer set was designed to cover the sequence closer to the transcription start site (TSS, 262 to +20, mNrf2P2). Primers covering β-actin promoter region was used as a control to verify the efficacy of ChIP assays.

5.3. RESULTS

**Hypermethylation of Specific CpG Sites in the CpG Island in Murine Nrf2 Gene**

The genomic sequence of Nrf2 gene (NC_000068.6: 75544698- 75513576 Mus musculus chromosome 2, reference assembly (C57BL/6J), including 2 kb of its
59-upstream sequence) was analyzed for CpG island using CpG island Finder (http://people.usd.edu/sye/cpgisland/CpGIF.htm). Since several mRNA variants with different transcription start sites (TSS) have been reported in the literature (109-111), therefore in the present study we define the translation initiation site (TIS) as position 1 to avoid any possible confusion. A CpG island was identified between -1175 and +1240, with a GC content of 61.53%, CpG observed/expected ratio of 0.66 and a total of 150 CpGs. The CpG islands include the murine Nrf2 promoter, the first exon and part of the first intron (Figure 1A). Similar results were obtained when using other criteria and algorithm (http://www.uscnorris.com/cpgislands2/cpg.aspx, data not shown). 10 sets of bisulfite genomic sequencing (BGS) primers were designed using the BiSearch web server (http://biseum.enzim.hu/, (112)) to cover the 59-flanking region spanning from 21226 to +844 of the murine Nrf2 gene. Bisulfite-converted genomic DNA derived from 12 palpable prostate tumors of 24 weeks old TRAMP mice and 10 apparently normal prostate tissues of C57BL/6J mice was used as templates. As shown in Figure 1B, although the majority of the CpG island is barely methylated, the first 5 CpG sites were found to be hypermethylated (96%) in prostate tumors compared to apparently normal prostate tissues (4%). The following 10 CpGs that are separated by two CpG free gaps (21131 to 21060 and 2886 to 2798) also displayed differential methylation status in tumors (34%)
compared to normal tissues (2%). In addition, another region located in the first intron appears to be sparsely CpG-methylated in prostate tumor (4.5%). TRAMP C1, C2 and C3 cell lines were originated from a heterogeneous tumor of 32-week TRAMP mouse (113). While TRAMP C1 and C2 cells are tumorigenic when grafted into syngenic C57BL/6J hosts, TRAMP C3 cells are not. Genomic DNA from C1 and C3 cells was bisulfite-sequenced as above to detect the methylation status of the CpG island in Nrf2 gene. Surprisingly, the methylated CpG “hot spots” as found above in the TRAMP prostate tumor were also methylated in tumorigenic C1 cells but not in non-tumorigenic TRAMP C3 cells (Figure 1C).

**Methylation of the First 5 CpGs Significantly Suppressed the Transcriptional Activity of Mouse Nrf2 Promoter**

To investigate the functional role of methylation of specific CpG sites, particularly the first 5 CpGs in the CpG island, luciferase reporters driven by the Nrf2 promoter with or without the first 5 CpGs (designated as 21367 and 21065, respectively) were constructed as described in Materials and Methods (Figure 2A). The plasmids were methylated using M. sssI CpG methyltransferase in vitro and the methylation status was confirmed by Hpal/HhaII digestion. As shown in Fig. 2B, the 21065 Nrf2 promoter, which had been reported previously (109, 114), substantially
increased the luciferase activity to about 200 folds. In contrast, the 21367 Nrf2 promoter showed a less potent transcriptional activity (67 folds). Importantly, in vitro CpG methylation of the reporter construct resulted in a dramatic decrease of the transcriptional activity of the Nrf2 promoter (84% decrease); while, the activity of the promoter was decreased by only 23.4% by CpG-methylation.

**Hypermethylated CpG Island was associated with Methyl-CpG-Binding Domain (MBD2) and histone modifications, which is reversible by 5-aza/TSA treatment.**

The repression of gene expression by CpG methylation is generally achieved by MBD proteins that bind to methylated DNA leading to recruitment of chromatin remodeling and transcriptional repressor complexes (115). In the current study, ChIP assays were employed to examine the proteins that could be potentially associated with Nrf2 promoter in TRAMP C1 and C3 cells. Based on the methylation status of Nrf2 promoter, primer sets were designed to cover the first 5 CpGs and the TSS to detect the association of specified DNA-binding proteins as well as RNA polymerase II (Pol II). The specificity of ChIP assays were verified by nonspecific IgG which did not pull down anything. As a positive control, β-actin promoter is equally associated with Pol II in C1 and C3 (Figure 3A). The binding of Pol II to the Nrf2 gene TSS was significantly decreased in C1 cells as compared to in C3 cells, indicating a suppressed
transcription of Nrf2 in C1 cells (Figure 3A & B). In agreement with this observation, the binding of MBD2 and tri-methylated histone 3-lys9, (H3K9me3) to the methylated CpGs in Nrf2 promoter was higher in C1 cells than in C3 cells, whereas the association of acetylated histone 3 (H3Ac) displayed a reversed pattern (Figure 3A & B). Methyl CpG binding protein 2 (MeCP2) and mammalian Sin3A (mSin3A) were also tested for their binding with this Nrf2 promoter; however no detectable binding was observed (data not shown). The methylation status of DNA is regulated by DNA methyltransferases (DNMTs) and 5-aza is a DNMT inhibitor, is often used to examine the effects of DNA methylation (116). As shown in Figure 3C & D, 5-aza treatment of C1 cells decreases the binding of MBD2 and H3K9me3 to the Nrf2 promoter, while the H3Ac exhibits no observable effect. However, combined treatment of C1 cells with 5-aza and a histone deacetylase (HDAC) inhibitor, TSA, substantially increases the binding of H3Ac to the Nrf2 promoter, and further decreases the bindings of MBD2 and H3K9me3 to the Nrf2 promoter. Consistent with the above results, the recruitment of Pol II to the Nrf2 promoter also increases correspondingly, whereas Pol II’s binding to β-actin promoter was not changed (Figure 3C).

The Transcriptional Induction of Nrf2 and NQO-1 in TRAMP Cell Lines Was
Correlated with CpG Islands Methylation Status and Could Be Restored by 5-aza/TSA Treatment

We and others have previously reported that the expression of Nrf2 and its target genes is suppressed in TRAMP prostate tumors (93, 94). Marvis et al., had reported that the expression of GST family genes was suppressed in TRAMP C2 cells and 5-aza and TSA treatment could restore the expression (95). Here we examined the expression of Nrf2 and one of its downstream target genes NQO1 in TRAMP C1 and C3 cells. As shown in Figure 4A, the mRNA level of Nrf2 is significantly lower in C1 cells than in C3 cells. The expression of NQO1 was readily induced by tBHQ, a classic Nrf2 agonist, in C3 cells, while such induction was blunted in C1 cells (Figure 4A & C). Treatment of C1 cells with 5-aza and TSA modestly restored Nrf2 expression and significantly enhanced the induction of NQO1 by tBHQ. However, the treatment of C3 cells under the same conditions exhibited no effect on the expression of Nrf2 or NQO1 (Figure 4A, B & C). Western blotting was performed to examine the protein expression levels of Nrf2, NQO1, MBD2, H3Ac and H3K9me3 (Figure 4D). The protein levels of Nrf2 and NQO1 were lower in C1 cells than in C3 cells, and 5-aza and TSA treatment enhanced the induction of NQO1 protein by tBHQ. Although 5-aza and TSA treatment did not increase the basal level of Nrf2 protein the treatment significantly augmented tBHQ-induced Nrf2 protein expression. TSA treatment
strongly increased acetylated histone 3 protein while had no effect on histone 3 methylation status. Interestingly, although the protein level of MBD2 was not affected by 5-aza and TSA treatment, it was much higher in C1 cells than in C3 cells (Figure 4D).

5.4. DISCUSSION

Previous findings from several laboratories including our laboratory have demonstrated that Nrf2 plays an essential role in the development of various cancers (117). Nrf2 regulates the expression of antioxidant and phase II detoxifying enzymes including NQO1, HO-1 and GST (117). Therefore, the control of transcriptional activation of Nrf2 and Nrf2-target genes would appear to be an important homeostatic mechanism that protect cellular injuries or damages resulted from oxidative stress (117). Nrf2 deficiency could lead to defect in the cellular defense system against oxidative stress, potentially resulting in cancer initiation, promotion and progression (118). The repressed expression of antioxidant and detoxifying enzymes such as GSTP1 in prostate cancer has extensively been studied (85, 89). However the role of Nrf2 in prostate cancer have not received enough attention until recently (93, 94). Frolich et al. reported that the down-regulation of Nrf2 appears to be responsible for the reduced GST expression, elevated oxidative
stress and DNA damage in prostate tumorigenesis in TRAMP mice (94). We have recently found that the expression of Nrf2 as well as Nrf2-target genes is gradually down-regulated during the progression of prostate cancer in TRAMP mice (93). Previous analysis of the online human prostate gene expression data sets demonstrated that the expression of Nrf2 and GST (94) as well as NQO1 was gradually decrease during human prostate carcinogenesis. Furthermore, it has been reported that several GST genes are down-regulated in primary but not in metastatic TRAMP tumors (95). In current study, we found that the expression of Nrf2 and the induction of NQO1 were compromised in tumorigenic TRAMP C1 cells but not in nontumorigenic TRAMP C3 cells (Figure 4A). This suppression of Nrf2 expression and Nrf2-target gene NQO1 in both of these TRAMP cell lines would exclude the possibility that Nrf2 expression would be affected by the SV40 transgene, since these TRAMP cell lines do not express the SV40 transgene (113). DNA methylation has been implicated in the silencing of the GSTP1 gene in human prostate cancer, and similarly DNA methylation silencing of several other genes are also implicated in TRAMP prostate tumor (89, 105, 106). However, interestingly MassARRAY Quantitative DNA Methylation Analyses (MAQMA) analysis of the 5’9 region of several GST genes displayed no significant differences between normal prostatic epithelial cells and prostate tumor from the TRAMP mice (95). Recently, several
reports show that in both TRAMP and Rb2/2 prostate tumors, an Rb/ E2F-dependent increase of DNMT1 expression and methylation activity (107, 119). Hypermethylation Nrf2 promoter was ruled out using MSP and that 5-aza treatment had no effect on Nrf2 expression (with data not shown) (94). We analyzed the 5' flanking region of Nrf2 gene and identified a CpG island that extends to position -1175 (Figure 1A). Using bisulfite sequencing, which would be more specific in identifying CpG methylation and would reveal more details about DNA methylation than MSP, we found that the first 5 CpGs in the CpG island are hypermethylated in TRAMP prostate tumors and in the tumorigenic TRAMP C1 cells but not in normal prostate tissues and non-tumorigenic TRAMP C3 cells (Figure 1B). Remarkably, these 5 CpGs are located adjacent to the previously reported Nrf2 promoter (114). Thus, the methylation status of these specific CpGs appears to be correlated with the tumorigenicity as well as Nrf2 expression and NQO1 induction (Figures 1 and 4). Notably, similar pattern of specific methylation of the distal CpG island has also been observed in the Keap1 gene in lung cancer cells (108). It is important to note that some of our current findings appear to be somewhat contradictory with the results reported previously (94), however previous findings of extensive down-regulation of Nrf2 and GST during prostate tumor progression in TRAMP mice (94), are consistent with our current results. To determine the functional role of methylation of these 5
CpGs in the suppression of Nrf2 expression, luciferase reporters of the f2 promoter with or without these 5 CpGs were constructed (Figure 2A). The Nrf2 promoter possesses very GC-rich noncanonical promoter which contains neither TATA box nor a CCAAT box (109), however, this Nrf2-promoter potently activated the transcription of luciferase reporter gene (Figure 2B). Interestingly, the addition of sequences from 21065 to 21367 appears to be repressive to the transcriptional activity of the Nrf2 promoter (Figure 2B). Such repressive sequence could function by recruiting specific repressing factors (120), but the exact mechanism accounting for this repressive function of this sequence even in the absence of methylation would require further investigation. Nevertheless, when the reporters were methylated in vitro by CpG methyltransferase, the luciferase reporter activity of the Nrf2 promoter with the additional sequence containing the 5 CpGs (pGL-1367) was reduced by about 84%. In contrast, methylation of the reporter without the additional sequence resulted in about 23% reduction (Figure 2B). This is probably due to the heavy methylation of the whole construct including the luciferase gene (but further study would be needed to prove this). Altogether, these results suggest that the extra 5 CpGs (21065 to 21367) could play a critical role in methylationdependent suppression of Nrf2 promoter activity. The role of CpG methylation in suppressing Nrf2 expression and activation was tested by treatment with 5-aza in TRAMP cells. 5-Aza has previously
been shown to be able to prevent early disease progression, delay androgen-independent disease and improve survival of TRAMP mice (107, 121). In addition, stage and phenotype-specific CpG island methylation and DNA methyltransferase expression have been well documented during prostate cancer progression in TRAMP mice (122, 123). These published findings suggest the relevance of using this TRAMP system to interrogate the possible role of epigenetic alterations in prostate carcinogenesis. 5-aza treatment of TRAMP C1 cells modestly increased the mRNA level of Nrf2, while combined treatments of 5-aza and TSA induced a more prominent increase of Nrf2 (Figure 4A). This result is consistent with the report by Mavis et al., in which combined 5-aza and TSA treatments significantly enhanced the expression of GST genes (95). The protein level of Nrf2 in TRAMP C1 cells remained unaffected by either 5-aza or 5-aza/TSA treatments, however, addition of a potent Nrf2-activator tBHQ would enhance the accumulation of Nrf2 protein (Figure 4B). As expected, the induction of NQO1 mRNA and protein levels displayed a similar trend with that of the Nrf2 protein. It is highly likely that since Nrf2 signaling is primarily regulated via post-translational mechanisms, without challenges with Nrf2-activators such as tBHQ, Nrf2 protein would be rapidly turned over by proteosome-dependent degradation (100). The precise reason as to why tBHQ is needed for NQO1 induction would require further study. To further
delineate the molecular mechanism by which the specific CpG-methylation suppresses Nrf2 expression, ChIP assays were performed. The result reveals that the binding of MBD2 and H3K9m3 to the specific CpGs was substantially higher in TRAMP C1 cells than in TRAMP C3 cells correlating with the fact that the CpGs were minimally methylated in TRAMP C3 cells (Figure 3A). In contrast, AcH3 displayed an opposite binding pattern to the same CpGs sequence in TRAMP C1 cells as compared to TRAMP C3 cells, and similarly the binding of Pol II to the transcription start site showed similar pattern as AcH3 (Figure 3A). These methylation-dependent associations of corepressors could be modulated by 5-aza and TSA treatments and our results (Figure 3B) correlated very well with the transcription level of Nrf2 (mRNA level) in these two cell lines (Figure 4A). MBD2 has been reported to mediate epigenetic silencing of 14-3-3s in TRAMP C1 cells and human LNCaP prostate cells (105), and has been shown to be involved in the transcriptional repression of GSTP1 in MCF-7 breast cancer cells (124). MBD2 and other MBD proteins bind to methylated CpGs and recruit corepressor complexes which contain HDACs, chromatin remodeling proteins as well as other proteins leading to the repression of the expression of hypermethylated genes (115). Interestingly, the protein level of MBD2 was much higher in TRAMP C1 cells than in TRAMP C3 cells (Figure 4A), suggesting a possible common MBD2-mediated epigenetic suppression of Nrf2 in TRAMP C1 cells.
In contrast, the protein level of AcH3 was apparently lower in TRAMP C1 cells than in TRAMP C3 cells but was increased tremendously by TSA treatment (Figure 4D). Since the expression of MBD2 would be dependent on HDAC activities, our above results would potentially explain the requirement of HDAC inhibitors in order to effectively modulate corepressors binding and to maximally restore the reexpression of Nrf2 as well as induction of NQO1 in TRAMP C1 cells. In addition, when this suppressive sequence containing the extra 5 CpGs was further analyzed using the Transcriptional Elements Search System (TESS, http://www.cbil.upenn.edu/tess) based on the TRANSFAC V6.0 database, several transcription factor binding sites were identified, including the binding sites of E2F1-p107 and NF-E2 (data not shown). The exact binding proteins and their functions leading to the suppression of Nrf2 expression would require further investigation. In summary, our present results clearly demonstrate that the expression of Nrf2 is suppressed by promoter CpG methylation in TRAMP prostate tumors. The existence and possible biological consequences of such epigenetic mechanism in the regulation of Nrf2 expression in human prostate cancer is currently under investigation in our laboratory. To the best of our knowledge, this is the first report revealing the epigenetic regulation of Nrf2 in prostate tumorigenesis. These findings would certainly open the door for further study on the role of Nrf2 as a plausible target for cancer chemoprevention and a
possible diagnostic marker for detection of human prostate cancer.
Fig 1. Hypermethylation of Nrf2 promoter in TRAMP prostate was correlated with tumorigenesis. (A) A CpG island was identified in the 5'-flanking region of mouse Nrf2 gene, spanning from position -1175 to +1240 with the translation initiation site set as position 1. The sequences covered by bisulfite genomic sequencing (-1226 to +844) and contain methylated CpGs are schematically presented with CpG sites indicated by vertical lines. (B) The methylation patterns and extents of CpG sites in the promoter of Nrf2 gene in TRAMP prostate tumor and apparently normal prostate were determined by bisulfite genomic sequencing as described in Material & Methods. Black dots indicate methylated CpGs and open circles indicate non-methylated CpGs. (C) The methylation patterns and extents of CpG sites in the promoter of Nrf2 gene in TRAMP C1 and C3 cells were determined. The CpGs in the sequence between +296 to +594 are not displayed because methylation is insignificant.
Fig 2. Methylation of the first 5 CpGs inhibited the transcriptional activity of Nrf2 promoter. (A) The construction of luciferase reporters is schematically presented. Nrf2 promoters with (-1367-1) or without (-1065-1) the extra sequence containing the first 5 CpGs were amplified from mouse genomic DNA and inserted into pGL4.15 vector. The resulted reporters were designated as pGL-1367 and pGL-1065, respectively. (B) pGL-1367 or pGL-1065 reporters, either methylated by CpG methyltransferase or not, were co-transfected with pGL4.75 vector which contains a Renilla reniformis luciferase gene driven by CMV promoter into TRAMP C1 cells, and the luciferase activities were measured after 24 hrs. The transcriptional activities of each constructs were calculated by normalizing the firefly luciferase activities with corresponding Renilla luciferase activities, and are represented as folds of induction compared with the activity of empty pGL4.15 vector. The values are mean ±SD of four separate samples.
Fig 3. Hypermethylated CpG island was associated with MBD2 binding and histone modifications and 5-aza/TSA treatment reversed the association. (A) ChIP assay was performed to detect the binding of indicated proteins to specific regions of Nrf2 gene cross-linked and immunoprecipitated from TRAMP C1 and C3 cells. The results from 3 independent experiments were qualified by densitometry as shown in (B). (C) TRAMP C1 cells were treated with vehicle, 5-aza or 5-aza+TSA as described, then the cells were subjected to ChIP assay. The results from 2 independent experiments were quantified by densitometry as shown in (D). ChIP assays were performed as described in Material & Methods using antibodies against Pol II, MBD2, H3K9m3 and AcH3. 3 sets of ChIP primers were used, with Nrf2P1 covers the first CpGs (-1190 to -1092) in the CpG island and Nrf2P2 covers a region close to TSS (-62 to +20). Nonspecific IgG was employed as a negative control and binding of Pol II to β-actin promoter was used to verify the efficiency of ChIP assay. The experiments were repeated at least twice with similar results.
Fig 4. The expression of Nrf2 and NQO-1 in TRAMP cell lines was correlated with CpG islands methylation status and could be restored by 5-aza/TSA treatment. TRAMP C1 and C3 cells were treated with 2 µM 5-aza, 200 nM TSA, or 1 µM 5-aza plus 100 nM TSA for 60 hrs or 48 hrs followed by incubation in the presence of 5 µM tBHQ for further 12 hrs. After treatments, the cells were harvested for total protein or RNA extractions. (A) the mRNA levels of Nrf2 and NQO1 were determined by RT-PCR, with GAPDH serving as internal control, and the results from 3 independent experiments were quantified by densitometry and the results are shown in (B) and (C). (D) the protein levels of Nrf2, NQO1, MBD2, H3K9me3 and H3Ac were determined by Western blotting, with actin as a loading control. Each experiment was repeated at least twice with similar results.

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Table 1. Primers used for RT-PCR and ChIP assay
6. Nrf2 knockout enhanced intestinal tumorigenesis in Apc mice

6.1 INTRODUCTION

More than 80% of colorectal cancer patients harbor a mutation in Apc gene (125). Apc is a suppressor gene that is present in the normal intestinal mucosa, with an increasing expression in mature epithelial cells in the upper crypt region (126). Apc antagonize the pro-proliferative Wnt pathway by inhibition of nuclear accumulation of β-catenin, controlling the expression of downstream target genes such as cyclin D1 and c-MYC which promote cell proliferation (127-130). The Apc(min/+)] mice is an excellent model for studying intestinal tumorigenesis since it resembles familial adenomatous polyposis. However, the role of Nrf2 in this genetic model has never before been investigated.

The nuclear transcription factor Nrf2 plays a central role in regulation of oxidative stress and inflammation. Previously, we have demonstrated that chronic inflammation in Nrf2−/− mice resulted in higher expression inflammatory markers and cytokine, together with a higher inflammatory damage to the colonic crypt cells, as compared with Nrf2+/+ mice (97). Induction of mutation in the colon by administration of AOM prior to inflammation promotion resulted in higher tumor incidence and numbers in Nrf2−/− mice (98). These results indicate that
Nrf2-dependent inhibition of inflammation appears to be important in inhibition of mutation-initiated colorectal carcinogenesis.

Inflammation promotes intestinal tumorigenesis in Apc(min/+) mice. Compounds that are known to be anti-inflammatory such as curcumin and DBM were shown to be effective in inhibition of intestinal tumorigenesis in Apc(min/+) mice (46, 131). These compounds were also known to be potent Nrf2-inducer, which exerts their anti-cancer effect through inhibition of excess oxidative damages and inflammatory damages. In the current study, we showed that loss of Nrf2 dose-dependently promoted intestinal tumorigenesis, induced inflammatory markers, and contributed to a higher proliferative potential in the intestinal crypts.

6.2 MATERIAL AND METHOD

Mice.

C57BL/6J male mice heterozygous for the Apc allele (Apc(min/+)) were purchased from the Jackson Laboratory. C57BL/6J female mice homozygous for the Nrf2 allele (Nrf2-/-) were previously described. Apc(min/+)Nrf2+/-(F1) mice were established by mating Apc(min/+)+ males with Nrf2-/- females, and subsequent mating between the F1 mice yielded colony of different genotypes, including Apc(min/+), F1 and Apc(min/+)+Nrf2-/- (F2). Allele-specific PCR assays were used for
Tissue harvesting and tumor assessment.

At 20 weeks of age, Apc(min/+), F1 and F2 mice were sacrificed by CO₂ asphyxiation. The entire small intestine was dissected and washed in PBS and examined for the presence of adenomas, and the number and size of adenomas were recorded.

Protein extraction and Western Blotting.

Adenomas and normal appearing intestinal tissues from Apc(min/+), F1 and F2 mice were retrieved. Adenomas of size >2 mm were pooled within each mice. The tissues were incubated with PBST inhibitor cocktail and were grinded with douppler for 50 times. The lysate were than centrifuged and the debris removed. Protein concentration was determined and 20μg of total protein from each sample were compared.

Immunohistochemistry.

The intestines were placed in 10% phosphate-buffered formalin for 24 hours. The samples were then dehydrated in ascending concentration of ethanol (80, 95
and 100%), cleared in xylene and embedded in paraffin. 4μm section were cut from paraffin-embedded blocks and mounted on glass slides. The immunohistochemistry of PCNA was performed according to manufacturer’s instruction.

**LTB₄ assays.**

Snap-freeze tissues were incubated with 500μL homogenization buffer (0.1M phosphate buffer, pH 7.4, containing 1mM EDTA and 10μM indomethacin). The tissues were homogenized with the Biodisruptor using the following setting: 10 sec ON/20 second OFF per cycle for 5 mins. The tissue homogenates were then centrifuged at 10,000xg for 10 mins. The supernatants were collected and the assays were performed following the protocols provided by the manufacturer. The protein concentrations for each sample were also determined and were used for normalization.

**6.3 RESULTS**

*Higher polyps numbers and larger polyps in Apc(min/+)Nrf2KO mice.*

We compared the number of the adenomas that developed in Apc(min/+) F1 and F2 mice 20 weeks of age. Apc(min/+) mice (n=18, 10 male, 8 female) developed, on average, 36.3±8.1 polyps per mouse in the small intestine. The average number of
polyps developed was 43.2±11.7 in the small intestine of F1 mice (n=30, 17 male, 13 female), and 57.8±15.8 in the small intestine of F2 mice (n=10, 7 male, 3 female) (Figure 1). The number of adenomas developed in F1 and F2 mice were significantly higher than Apc(min/+), (p<0.05 and p<0.001). We further compared the number of polyps developed in the three parts (proximal, middle and distal) of the small intestine across each genotype. The average number of polyps developed was 3.7±3.2, 3.9±2.5 and 5.8±2 in the proximal part of small intestine; 15.3±5.2, 17.4±8 and 24.7±10.6 in the middle part of the small intestine and 16.9±7, 22.4±6.8 and 28±11.3 in the distal part of small intestine in Apc(min/+), F1 and F2 mice, respectively (Figure 2). There was a significant increase in polyps numbers in the middle part of the intestine in F2 mice compared to Apc(min/+), (p<0.01). In the distal part, there was also significant increase in polyps number in F1 and F2 mice compared to Apc(min/+), (p<0.01 and p<0.01). We also classified the polyps into small (</=2mm) and large (>2mm) polyps and compared the difference between Apc(min/+), and F2 mice (Figure 3). The distribution of sizes of polyps along the intestine was noteworthy. Most of the polyps developed were small polyps (</=2mm). In the Apc(min/+), small polyps contributed to 58.57% of the polyps developed in proximal part, 77.64% in middle part and 81.59% in the distal part. In F2 mice, large polyps contributed to 54.29% of the polyps developed in proximal
part, 24.32% in middle part and 26.19% in distal part, which are 12.86%, 1.96% and 7.78% higher when compared to Apc(min+)/mice.

**Lower expression of phase II marker NQO1 and Higher expression of inflammatory markers: western blotting**

We compared the expression of NQO1 across the three genotypes. While there was not too much difference in expression of NQO1 between Apc and F1, expression of NQO1 was shown to be substantially lower in the F2 group (Figure 4). It is also noteworthy that although Nrf2 is knockout in the F2 group, there were still expression of NQO1, indicating Nrf2 is not the sole regulator of its expression *in-vivo*.

Lower expression of phase II genes have been shown to correlate with a higher expression of inflammatory markers. To investigate if the inflammatory signals could possibly contributes to the enhanced intestinal adenoma formation in Nrf2 knockout genotype (F2 mice), we examined the levels of iNOS, COX2, cPLA and 5LOX proteins. We showed that inflammatory signals were augmented in Nrf2 knockout genotype. There were generally higher expression of iNOS, cPLA and 5LOX in normal-appearing tissue and higher expression of cPLA and COX2 in polyps in F1 and F2 mice compared to Apc(min+)/mice (Figure 4).
**Higher levels of LTB₄ in Apc(min/+)$\times$Nrf2KO mice**

Leukotrienes (LTB₄) are important mediators of the arachidonic acid metabolism by 5LOX, and have been shown to contribute to intestinal tumorigenesis in Apc(min/+) mice. LTB₄ level in normal-appearing tissue appeared to be higher throughout the intestine in the F1 and F2 mice, and the increase was statistically significant in the distal part of the intestine (Figure 5A). LTB₄ level in polyps in distal part of intestine was 569±203 pg/mg protein in the F2 mice, significantly higher than 187±56 pg/mg protein in Apc(min/+)$\times$Nrf2KO mice ($p<0.001$) and 361±141 pg/mg protein in F1 mice ($p<0.001$) (Figure 5B).

**PCNA staining: Higher proliferation of intestinal crypts in Apc(min/+)$\times$Nrf2KO mice**

To investigate if crypt proliferation could be affected by the enhanced inflammatory and higher oxidative stress environment in the Nrf2-knockout genotype, we examined the level of PCNA by immunohistochemistry in the normal-appearing intestinal tissues. PCNA was primarily expressed in the intestinal crypts (Figure 5). We observed a generally much higher number of crypts expressing PCNA along the middle and distal part of the small intestine in F2 mice compared to Apc(min/+) mice (Figure 5), indicating an environment with higher oxidative stress and inflammation possibly contributes to enhanced number of proliferative crypts.
6.4 DISCUSSION

We have previously documented the effect of Nrf2 knockout in animal models. Nrf2-knockout mice are more susceptible to DSS-induced inflammatory damages in the colon (97), and could lead to higher tumor incidence in the colon when pre-treated with mutation inducing agent AOM (98). These data indicates the importance of constitutive expression of Nrf2 in the inhibition of inflammation which slows the progression of carcinogenesis. On the other hand, a more direct consequence of Nrf2-knockout is probably a higher oxidative stress, which has also been commonly seen in many tumorigenesis models.

In this study, we have demonstrated that Nrf2 has a role in suppression of intestinal tumor formation in Apc(min/+) mice. We have shown that loss of Nrf2 led to an increase in number and size of polyps developed in the intestine in Apc(min/+) mice. Apc(min/+) mice have been a great model since most of human familial adenomatous polyposis patients have Apc mutation and since 80-90% spontaneous polyposis also has a mutation in Apc gene. Of particular importance are the inflammatory markers including the iNOS, COX2, 5LOX and cPLA2. Separate studies have pointed out loss of iNOS, COX2 and cPLA could ameliorate the intestinal tumorigenesis in Apc(min/+) model. For example, knockout of iNOS or administration
of iNOS inhibitor (132, 133) significantly decreased intestinal adenoma development.

Deletion or mutation of the cPLA$_2$ gene also suppresses Apc(min+) mice tumorigenesis (134, 135). In this study, we have shown that loss of Nrf2 led to an overexpression of these inflammatory markers. These data supports our hypothesis that Nrf2 expression suppresses Apc(min+) tumorigenesis through inhibition of inflammation, as indicated by the increased expression of iNOS, COX2, cPLA proteins in F2 mice.

The expression of these markers in the intestinal cell types in the Apc(min+) mice has been previously described. Briefly, there was consistent evidence that iNOS was expressed both in normal-tissue and polyps, and iNOS usually localize in the epithelial cell of the villus but not in the crypt cells. COX2, on the other hand, were more specifically expressed in polyps (136), particularly the myofibroblasts in the connective tissue, and the stromal cells in the musculris mucosa providing support to the epithelial cells of the villus.

The role of inflammation on tumor growth has been demonstrated, but how inflammation initiates tumor has been less well-defined. The initiation of polyps in Apc(min+) model has been vigorously discussed before. It has been described that conditional knockout of Apc gene resulted in the increases in apoptotic body and crypt compartment enlargement, together with more c-myc and cyclin D staining in
the crypt cells. The complete knockout of Apc had no apparent effect on morphology on epithelial cells, implicating that the differentiated cells are more resistance to Apc gene mutation (137). The loss of heterozygosity of Apc (LOH) has known to be a common phenomenon in polyps. There were reports showing that Apc(+-) genotype is common in FAP patients, but all polyps (100%) were Apc(-/-) genotype, indicating LOH as the initiation event of polyps appearance. We do not know if knockout of Nrf2 could increase the number of incidence of LOH and therefore increases tumor number formation, but this is very possible and worth investigating. On the other hand, Nrf2 probably also plays a central role in the regulation of the growth of the initiated LOH sites with the already aberrant Wnt/β-catenin pathway. We have shown that the number of crypt cells stained with PCNA was also a lot higher in F2 mice compared to Apc(min/+) mice, suggesting the higher number of tumors in F2 mice could be due to more hyperproliferative crypts. The hypothesis that Nrf2-regulation of oxidative stress specifically controls the proliferative activity of intestinal stem cells (ISCs) has recently been tested in Drosophila (138). Repression of Nrf2 by its negative regulator Keap1 is required for ISCs proliferation (138), collaborating with our results that a higher oxidative stress due to loss of Nrf2 could lead to higher potential of crypt proliferation.
An environment enhanced of inflammatory signals and oxidative stress leading to enhanced damages to the cells could promote the development of the tumor. There was one study describing the suppressor function of cytokine signaling 3 (SOCS3) that limits the damage-induced crypt-hyperproliferation and inflammation-associated tumorigenesis in the colon (139), which could lend us insights on how inflammation in the epithelial cells would signals a hyperproliferative crypts. Our data suggest that there was probably an increase in nitric oxide due to increased iNOS expression and an increase in oxidative stress due to loss of Nrf2 and decreased NQO1 expression. A combination of nitric oxide and superoxide favors the production of peroxynitrite anion (ONOO-) which could result in tissue injury in inflammation. One important interaction of peroxynitrite occurs with nucleic acids, which produces 8-hydroxydeoxyguanosine which indicates DNA damage and possible strand breaks (140). However, we did not observed significant difference of 8-hydroxydeoxyguanosine in both normal-appearing intestinal tissues and polyps between Apc(+-), F1 and F2 mice (data not shown), indicating DNA-damage induced by enhanced oxidative stress might not be a major pathway driving the enhanced intestinal tumorigenesis in F2 mice. On the other hand, nitric oxide also damages cellular proteins by nitrosylation which could lead to apoptosis resistance and carcinogenesis (141). Lipid peroxidation by free radicals could further complicate the
problem (142). Also, we observed a higher expression of cPLA protein in F2 polyps than Apc(min/+) mice, which could exacerbate the size expansion of polyps. This result agrees with a previous study that there was a marked increased cPLA mRNA in the polyps which correlated with polyps size (135). An increased expression of cPLA protein could also lead to higher tumor numbers, showed by an 83% reduction in tumor number in the small intestine comparing cPLA2 wild-type and heterozygous genotypes (134).

Apc loss could up-regulate expression of COX2. It has been suggested that APC plays a role in either directly or indirectly in the translational regulation of COX2 (143), which could partly due to stabilization of COX2 mRNA through interaction of its AU-rich elements of 3’-UTR with beta-catenin (144). We observed Nrf2 played a role in the regulation of COX2 expression. Apart from COX2, 5LOX was also consistently over-expressed in F2 mice. 5LOX plays an important role in the synthesis of leukotriene B4 (LTB4) from arachidonic acid and has long been recognized as a potent mediator of inflammation. We showed that the 5LOX-LTB4 axis was consistently up-regulated by knockout of Nrf2. Taken the data together; the most consistent pathway affected by the loss of Nrf2 is an upregulation of cPLA2 expression which catalyzes an increase release of arachidonic acid from membrane phospholipids, the increase of COX-2 and 5LOX expression further augments the
production of inflammatory mediators such as PGE\(_2\) and LTB\(_4\), leading to an enhanced inflammatory environment.

Taken together, our data showed that Nrf2 knockout leads to an enhanced oxidative and inflammatory environment which possibly contributes to an increased level of free radicals, PGE\(_2\), LKTB\(_4\) and NO accumulation in cells, possibly leading to hyperproliferation of crypts and size expansion of polyps, which correlates with an increase in number and size in polyps developed in F2 mice compared to Apc(min/+) mice.
Fig 1. Higher polyps numbers in Apc(min+)/Nrf2KO mice. Total number of polyps that developed in the intestine of Apc(min+)/, Apc(min+)/Nrf2(+/−) (F1) and Apc(min+)/Nrf2(−/−) F2 mice 20 weeks of age were compared. *F1 vs Apc(min+)/, p<0.05 ** F2 vs Apc(min+)/, p<0.001.

Fig 2. Higher number of polyps in the distal part of the intestine in Apc(min+)/Nrf2KO mice. Number of polyps developed in the proximal, middle and distal part of intestine were compared across the genotypes. *F1 vs Apc(min+)/, p<0.01 ** F2 vs Apc(min+)/, p<0.01.
**Fig 3.** Larger polyps in Apc(min+)/Nrf2KO mice. We classified the polyps into small ($<2\text{mm}$) and large ($>2\text{mm}$) polyps and compared the difference between Apc(min+)/+ and F2 mice in the proximal, middle and distal part of intestine.

**Fig 4.** Lower expression of NQO1 and higher expression of inflammatory markers. The normal-appearing intestinal tissues and polyps were lysed and blotted against actin, NQO1, iNOS, cPLA, COX2 and 5LOX. The expression of these markers were compared across genotypes.
Fig 5. Higher level of LTB₄ in Apc(min/+)Nrf2KO mice. LTB₄ ELISA assays were performed in (a) normal-appearing intestinal tissues and (b) polyps across genotypes.
Fig 6. More intense PCNA staining in Apc(min/+)Nrf2KO mice. PCNA Immunohistochemistry was performed and the staining was compared.
7. Summary

In summary, we showed that phytochemicals prevent cancer through two mechanisms: blocking cancer initiation and suppressing cancer progression. These were illustrated with the use of PEITC and DBM in both in-vitro and in-vivo models. PEITC suppressed cancer through induction of G1 cell cycle arrest which was p38 dependent. The same tumor suppressing effect of PEITC was shown in AOM/DSS model with an increased protein expression of p21 and cleaved caspases. On the other hand, DBM was shown to prevent cancer initiation in the same model through activation of Nrf2 and its induction of antioxidant/detoxifying genes.

The regulation of Nrf2 signaling has come to our attention. The importance of MEK/ERK activation of Nrf2 induced by phytochemicals has been well-studied. We added a new perspective in this signaling pathway and show that IQGAP1 mediates phytochemicals-induced MEK/ERK/Nrf2 activation cascades. We showed that in IQGAP1-deficient cells, PEITC-induced MEK/ERK activation, Nrf2 translocation, Nrf2/MafG binding and induction of antioxidant/detoxifying genes were substantially attenuated. We also showed that IQGAP1 was required for bringing MEK, ERK and Nrf2 to form a complex in response to PEITC.

Finally, we also showed that expression of Nrf2 could be epigenetically regulated. 5 CpG sites were shown to be consistently methylated in tumors in TRAMP
mice and tumorigenic TRAMP C1 cells, in correlation to low Nrf2 expression. The methylated CpG sites were shown to associate with more MBD2, H3K9me3, less H3-Ac and Pol-II binding. In addition, the combined treatment of 5-aza and TSA could restore Nrf2 expression through demethylation of Nrf2 promoter and inhibition of histone deacetylase.

In conclusion, we have discussed the diverse cellular and molecular targets regulated by phytochemicals, how phytochemicals could act as cancer blocking agents and suppressing agents, how phytochemicals prevent cancer through activation of Nrf2 signaling, how novel Nrf2 interaction partner IQGAP1 mediates Nrf2 signaling, and finally, how Nrf2 expression level could be control epigenetically. This understanding of Nrf2 would allow us to design better chemopreventive compounds in the future.
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