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Direct evidence for the critical role of NFAT3 in benzo[*a*]pyrene diol-epoxide-induced cell transformation through mediation of inflammatory cytokine TNF induction in mouse epidermal Cl41 cells

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Nuclear factor of activated T cell (NFAT)-3 is a member of the transcription factor NFAT family, which has been demonstrated to be responsible for the up-regulation of the pro-inflammatory cytokine tumor necrosis factor (TNF) in the immune system. Our most recent studies have also shown that TNF is able to induce cell transformation in mouse epidermal Cl41 cells by induction of cyclooxygenase-2 (COX-2) expression. To provide direct evidence for NFAT3 in the environmental carcinogen-caused carcinogenic effect, (±)-benzo[*a*]pyrene-7,8-diol-9,10-epoxide (B[*a*]PDE), an ultimate environmental carcinogen metabolized from benzo[*a*]pyrene, was utilized. We found that exposure of Cl41 cells to B[*a*]PDE was able to induce cell transformation in Cl41 cells, while specific knock-down of NFAT3 resulted in the dramatic inhibition of this cell transformation. The tumorigenicity of B[*a*]PDE-caused transformed cells was confirmed in nude mice, whereas the tumor formation of B[*a*]PDE-treated NFAT3 small interference RNA (siRNA) knock-down cells was significantly reduced. Further studies showed that the role of NFAT3 in B[*a*]PDE-caused cell transformation was mediated by up-regulation of its downstream targeted gene TNF. This conclusion was based on the findings that inhibition of NFAT3 activation by either FK506 or NFAT3 siRNA dramatically down-regulated the TNF induction upon B[*a*]PDE exposure, and that knock-down of TNF by its specific siRNA also led to abrogation of B[*a*]PDE-induced cell transformation in Cl41 cells and their tumorigenicity in nude mice. Collectively, these results provide direct evidence for the important role of NFAT3 activation in B[*a*]PDE-induced cell transformation by up-regulation of TNF expression in mouse epidermal Cl41 cells, further suggesting that B[*a*]PDE may exert its tumor promotion effect on skin carcinogenesis, at least partially, by inducing TNF expression.

Introduction

Polycyclic aromatic hydrocarbons are ubiquitous environmental contaminants which are produced mainly from cigarette smoke, incomplete combustion of fossil fuel in transportation and residential heating and power generation (1). Benzo[*a*]pyrene (B[*a*]P), having been used as a prototype carcinogenic polycyclic aromatic hydrocarbon since its isolation from coal tar in the 1930s, is a well-characterized human complete carcinogen (2,3). (±)-benzo[*a*]pyrene-7,8-diol-9,10-epoxide (B[*a*]PDE) is an ultimate metabolite of B[*a*]P by cytochrome P450 enzymes and is actively involved in the development of smoking-associated cancers, including skin and lung cancers (4,5). The carcinogenic activity of B[*a*]P and its metabolite, B[*a*]PDE, is supported by epidemiologic studies and convincingly proven previously by both *in vitro* and *in vivo* studies (2,3,6).

The mouse skin model of multistage carcinogenesis has demonstrated that cancer development results from a synergism between genotoxic and non-genotoxic factors (7,8). The former induces irreversible genetic alterations (tumor initiation), whereas the latter promotes tumor development by favoring the clonal outgrowth of the genetically altered cells (tumor promotion). After absorption, B[*a*]P can be metabolized to some metabolites containing vicinal diol epoxides, which can form covalent adducts with DNA (9,10). It is generally believed that the resultant mutations in DNA are the essential initiating steps in carcinogenesis (10,11). In addition to its DNA-damaging or tumor-initiating activity, B[*a*]PDE may also contain tumor-promoting activity since topical application of low doses of B[*a*]PDE alone twice a week for a long-time exposure is able to cause tumor development (12). Whereas the mutagenic effects of B[*a*]P and B[*a*]PDE, which are mainly associated with the initiation stage of carcinogenesis, were extensively explored, the effects of B[*a*]P and B[*a*]PDE on tumor promotion remain largely unexplored. Although the exact molecular mechanisms of tumor promotion are elusive, it is believed that alterations in the events regulating cell apoptosis, inflammation and cell proliferation play important roles in promoting cancer development.

Chronic inflammation is one of the most important factors associated with cancer development. It has been estimated that the chronic inflammation accounts for ~20% of human cancers (13). The tumor promotion in mouse skin is characterized by keratinocyte hyperproliferation and inflammation at an early stage. The pro-inflammatory cytokine tumor necrosis factor (TNF) is a major mediator of inflammation (14). In malignant disease, TNF has originally been considered as an anticancer agent because it induces the destruction of blood vessels and mediates the killing of certain tumors (15). However, recent studies have provided substantial evidence showing that TNF acts as an endogenous tumor promoter in both cell culture and animal models (16). TNF knockout mice (TNF−/−) are more resistant to exposure of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) than that in wild-type mice (17,18). TNF receptor I knockout mice (TNFR−/−) could decrease the chance and size of the liver metastasis of intrasplenic administration of the colonic adenocarcinoma cell line (19). Our previous work also showed that TNF had effects similar to TPA in the induction of the anchorage-independent growth of mouse epidermal Cl41 cells (20).

Nuclear factor of activated T cell (NFAT) is an important transcription factor originally identified in T cells (21,22). NFAT3 is one of five members in the NFAT family, including NFAT1/c2 and NFAT2/c1, NFAT3/c4, NFAT4/c3 and NFAT5 (23). The NFAT activation is initiated by dephosphorylation of the NFAT regulation domain located in the N-terminal of the DNA-binding domain. Dephosphorylated NFAT translocates into the nucleus, binds to consensus DNA sites and controls the expression of its target genes, such as TNF and COX-2 (24–27). Our recent studies have demonstrated that mouse epidermal Cl41 cells only express NFAT3 and NFAT4, while other members are not detectable (28), and NFAT3 is critical for TNF-caused cell transformation in Cl41 cells (29). However, to the best of our knowledge, there is no direct evidence showing the implication of NFAT in real environmental carcinogen-caused carcinogenic effects. Therefore, current studies address this question by using the

Abbreviations: B[*a*]P, benzo[*a*]pyrene; B[*a*]PDE, (±)-anti-benzo[*a*]pyrene-7,8-diol-9,10-epoxide; FBS, fetal bovine serum; MEM, Eagle's minimal essential medium; NFAT, nuclear factor of activated T cell; siRNA, small interference RNA; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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environmental carcinogen, B[*a*]PDE, in mouse epidermal JB6 Cl41 cells, which has been widely used as a model for tumor promotion study (30–32).

Materials and methods

Plasmids and reagents

CMV-neo and NFAT-luciferase reporter plasmids were constructed as described in our previous reports (33,34). TNF-luciferase reporter plasmid containing the human TNF promoter region (−1260/+60) was a generous gift from Dr Peter Johnson at National Cancer Institute–Frederick as described in previous studies (35). Fetal bovine serum (FBS) and Eagle's minimal essential medium (MEM) were purchased from BioWhittaker (Walkersville, MD). The substrate for the luciferase assay was purchased from Promega (Madison, WI,). Lipofectamine was obtained from Gibco BRL (Rockville, MD). Benzo[*a*]pyrene (B[*a*]P) was purchased from Sigma; B[*a*]PDE was purchased from Eagle-Picher Industries, Chemsyn Science Laboratories (Lenexa, KS); both B[*a*]P and B[*a*]PDE were dissolved in dimethyl sulfoxide at stock concentration of 2 mM. FK506 was from Alexis Biochemicals (San Diego, CA).

Cell culture

The JB6 P^+ mouse epidermal Cl41 cell line was cultured in monolayers at 37°C, 5% CO_2 using 5% FBS MEM containing 2 mM L-glutamine and 25 μg gentamicin per ml as described previously (36). The cultures were dissociated with trypsin and transferred to new 75 cm^2 culture flasks (Fisher, Pittsburgh, PA) twice a week.

Construction of siRNAs expression vector

The specific small interference RNAs (siRNAs) for mouse TNF were designed by siRNA converter on the Web site of Ambion (Austin, TX) according to the gene sequence in GenBank, synthesized by Invitrogen (San Diego, CA). The target sequences was 5′-AATTCGAGTGACAAGCCTGTA-3′ (bases 412–432 of NM 013693, mouse TNF mRNA). The siRNA sequences were controlled via BLAST search and did not show any homology to other known mouse genes. The siRNAs were inserted into pSuppressor vector (Imgenex Co, San Diego, CA) and verified by DNA sequencing. The target sequences for mouse NFAT3/NFATc4 was 5′-GCCATTGACTCTGCAGATG-3′ (bases 1409–1427 of NM023699, mouse NFAT3 mRNA) and constructed as in our previous published studies (28,29). The recombinant siRNA vectors were designated as siNFAT3 and siTNF, respectively. Constructs containing the reversed target sequences were used to make control siRNA constructs.

*Generation of stable transfectant*s

Cl41 cells were cultured in a six-well plate until they reached 85–90% confluence. Five microgram of NFAT luciferase, 4 μg of TNF-luciferase reporter plasmid DNA in combination with 1 μg of siNFAT3 or mock pSuppressor plasmid, 5 μg of plasmid siTNF and 5 μg of plasmid mock pSuppressor plasmid were mixed with 10 μl of Lipofectamine reagent (Gibco BRL), respectively, and used to transfect each well in the absence of serum. After 6 h, the medium was replaced with 5% FBS MEM without penicillin/streptomycin. Approximately 36–48 h after the beginning of the transfection, the medium was replaced with 5% FBS MEM containing 500 μg/ml G418 (Gibco BRL). After selection for 28–45 days with G418, the stable transfectants were identified by measuring the basal level of luciferase activity and examining the inhibitory effect of siRNAs on the expression of their targets. Stable transfectants, Cl41-NFAT-Luc mass3, Cl41-TNF-Luc mass1, Cl41-siNFAT3/TNF-Luc mass1, Cl41-siNFAT3/TNF-Luc mass2, Cl41-siTNF mass1, Cl41-siTNF mass2 and Cl41-vector mass1, were established and cultured in G418-free 5% FBS MEM for at least 2 weeks before each experiment.

Gene reporter assay

Confluent monolayers of stable Cl41 transfectants were trypsinized, and 8×10^3 viable cells suspended in 100 μl of MEM supplemented with 5% FBS were seeded into each well of 96-well plates. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. After the cell density reached 80–90%, the cells were exposed to B[*a*]PDE at final concentrations as indicated in the figure legends for NFAT transactivation or TNF induction. The cells were extracted with lysis buffer at different time points after B[*a*]PDE treatment, and their luciferase activities were determined by the luciferase assay using a luminometer (Wallac 1420 Victor 2 multilabel counter system) after the addition of 50 μl of lysis buffer for 30 min at 4°C. The results are expressed as NFAT activity or TNF induction relative to medium control containing the same concentration (0.1%) of dimethyl sulfoxide only (relative NFAT activity or relative TNF induction).

Reverse transcriptase–polymerase chain reaction

Cl41 transfectants (2×10^5) were cultured in each well of six-well plates to 80% confluence. After exposure to B[*a*]PDE for 12 h, the cells were extracted with Trizol reagent (Invitrogen), and the total RNA was isolated following the manufacturer's instructions. One microgram of total RNA was reverse transcribed (Superscript II, Invitrogen), and the cDNA was subjected to polymerase chain reaction amplifications using primer pairs specific for mouse TNF (forward: 5′-CCA GAC CCT CAC ACT CAG AT-3′; backward: 5′-AAC ACC CAT TCC CTT CAC AG-3′) and β-actin (forward: 5′-CAT CCG TAA AGA CCT CTA TGC C-3′; backward: 5′-ACG CAG CTC AGT AAC AGT CC-3′) which served as the internal control. The polymerase chain reaction cycling conditions were 25 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 45 s.

Western blot

Cl41 transfectants (2×10^5) were cultured in each well of six-well plates to 70–80% confluence with normal culture medium. The cell culture medium was replaced with 0.1% FBS MEM with 2 mM L-glutamine and 25 μg of gentamicin and cultured for 33 h. The cells were exposed to B[*a*]PDE for various time periods, and then extracted with sodium dodecyl sulfate sample buffer. The cell extracts were separated on polyacrylamide-sodium dodecyl sulfate gels, transferred and probed with primary antibodies, including antibodies specific against NFAT3, Protein kinase C alpha (PKCα) and c-Jun. The protein bands specifically bound to primary antibodies were detected using an AP-linked secondary antibody and by an enhanced chemifluorescence (ECF) western blotting system (37).

Anchorage-independent growth

Cl41 transfectants (2×10^4) were cultured in each well of six-well plates to 30–40% confluence with normal culture medium. The cells were treated with 1 μM B[*a*]PDE or 8 μM B[*a*]P for 1 day and then recovered in fresh medium for 2 days. After the repeated treatment with B[*a*]PDE and B[*a*]P for 8 weeks, the cells were used for anchorage-independent growth assay which was performed as described previously (20). Briefly, 2.5 ml of 0.5% agar in BME supplemented with 10% FBS was laid onto each well of six-well tissue culture plates. One milliliter of Cl41 cells were mixed with 2 ml of 0.5% agar BME and layered on top of the 0.5% agar layer. The plates were incubated at 37°C in 5% CO_2 for 3 weeks. The colonies were then counted under microscopy and ones with >16 cells were scored.

Tumorigenicity assays

Six 5-week-old female nude mice were randomly divided into two experimental groups, i.e. Cl41-TNF-Luc medium control group and Cl41-TNF-Luc B[*a*]PDE-treated group. Each nude mouse was injected subcutaneously in two spots with 2×10^6 of Cl41 transfectants in 100 μl of growth medium for each spot. The mice were killed by CO_2 asphyxiation 2 weeks after the inoculation, and tumor dimensions were measured using calipers and tumor volume (mm^3) was calculated using the following formula: 0.5236 (L × W × H) as described in previous studies (38,39), where L is tumor length, W is width and H is height.

Statistical analysis

The significance of the difference between different groups was determined with the Student's *t*-test. The results are expressed as mean ± SD. The differences were considered significant at a $P < 0.05$.

Results

NFAT3 activation plays an essential role in B[a]PDE-induced cell transformation in Cl41 cells

It has been demonstrated that topical application of low doses of B[*a*]PDE alone twice a week for long-time exposure is able to cause tumor development, implicating that B[*a*]P not only has tumor-initiating activity but also possesses tumor-promoting activity (12). To test the potential involvement of NFAT in B[*a*]PDE-induced cell transformation, we observed the effect of B[*a*]PDE treatment on NFAT transactivation in Cl41 cells by using the NFAT-luciferase gene reporter assay. The results showed that B[*a*]PDE could significantly induce NFAT transactivation in a time- and dose-dependent manner in Cl41 cells (Figure 1A and B), while B[*a*]P exposure only showed a marginal effect on NFAT activation (Figure 1A and B). These data suggest that NFAT activation is involved in Cl41 cell response to B[*a*]PDE exposure. We further evaluated the potential transformation capability of B[*a*]PDE on Cl41 cells. We found that long-term repeated exposure of Cl41 cells to B[*a*]PDE was able to induce marked

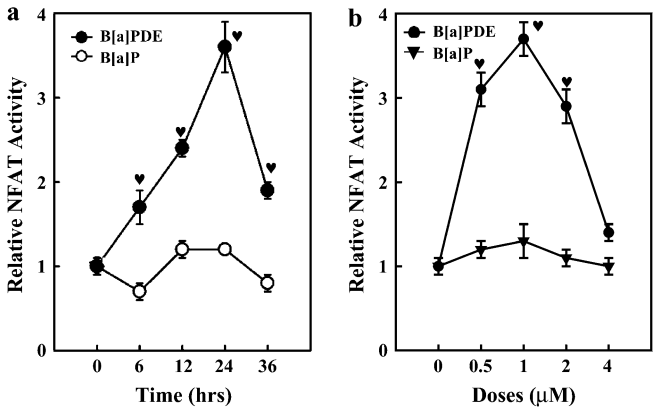

Fig. 1. Induction of NFAT activation upon B[*a*]PDE exposure in Cl41 cells. C141-NFAT-Luc mass3 cells (8×10^3) were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were exposed to (**A**) 2 μM of B[*a*]PDE or B[*a*]P or (**B**) various concentrations of B[*a*]PDE and B[*a*]P as indicated for 12 h. The treated cells were extracted with lysis buffer, and the luciferase activity was measured using the Promega luciferase assay. The results were then presented as NFAT transcription activity relative to the control (relative NFAT activity). Each bar indicates the mean and SD of triplicate assay wells. The symbol (heart shaped) indicates a significant increase compared with that of medium control ($P < 0.01$).

anchorage-independent cell growth in the soft agar assay, whereas B[*a*]P did not show an observed effect (Figure 2A and B), which is consistent with its effects on NFAT activation. The failure of B[*a*]P to induce NFAT activation and cell transformation may be explained by the low activity of cytochrome P450 in Cl41 cells, which has also been found to account for much lower activity of B[*a*]P in triggering activator protein-1 (AP-1) signal pathways activation as compared with B[*a*]PDE in Cl41 cells (40). It is well recognized that B[*a*]P exerts its function mainly through its intermediate metabolite B[*a*]PDE, so our following studies have focused on B[*a*]PDE.

Our most recent published studies have demonstrated that NFAT3 and NFAT4 are two members that can be detected in Cl41 cells (28,29), and NFAT3 is critical for TNF-mediated cell transformation (29). Our published studies have also shown that introduction of NFAT3 siRNA construct is able to specifically knock-down the NFAT3 protein expression, while it did not have any inhibitory affect on the protein expression of NFAT4, signal transducer and activator of transcription 3 (Stat3), nuclear factor kappa B (NF-κB) p65 and p50 subunits in Cl41 cells expression (28). To determine whether NFAT3 is involved in the B[*a*]PDE-induced cell transformation, we employed this well-characterized specific NFAT3 siRNA construct to establish the Cl41 cells stably co-transfected with the TNF-luciferase reporter. The knock-down of the siRNA on NFAT3 expression was analyzed by western blot assay. Consistent with our previous reports, the NFAT3 protein level was markedly knocked-down in siNFAT3 transfectants

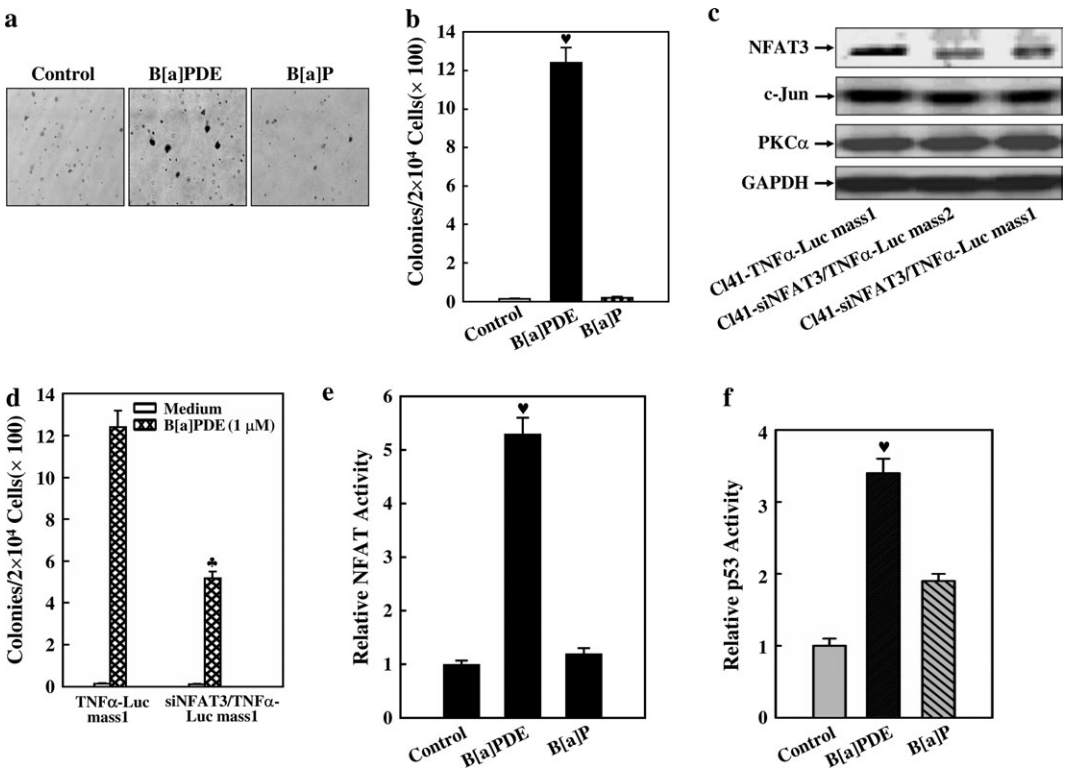

Fig. 2. Knock-down of NFAT by siNFAT3 resulted in the inhibition of B[*a*]PDE-induced cell transformation in Cl41 cells. (**A**, **B** and **D**) Cl41 cells, Cl41 TNF-Luc mass1 and Cl41 siNFAT/TNF-luc mass1 were repeatedly treated with 1 μM of B[*a*]PDE or 8 μM of B[*a*]P for 8 weeks and then subjected to anchorage-independent growth assay as described in Materials and Methods. After being cultured in a 37°C, 5% CO_2 incubator for 3 weeks, the cell colonies were counted under microscopy, and colonies with >16 cells were scored. Schematic diagrams outlining the anchorage-independent cell growth. Each bar indicates the mean and SD from triplicate assays. The symbol (heart shaped) indicates a significant increase as compared with that from medium control ($P < 0.01$). The symbol (club shaped) indicates a significant decrease as compared with that from Cl41 transfected with control siRNA ($P < 0.01$). (**C**) Cl41-TNF-Luc mass1 (2×10^5), Cl41-siNFAT3/TNF-Luc mass1 and Cl41-siNFAT3/TNF-Luc mass2 cells were extracted with sodium dodecyl sulfate sample buffer. The cell extracts were separated on polyacrylamide-sodium dodecyl sulfate gels, transferred and probed with specific antibodies against NFAT3, c-Jun, PKCα and GAPDH. The protein band specifically bound with the primary antibody was detected by using anti-rabbit IgG-AP-linked secondary antibody and an ECF western blotting system. GAPDH was used as a control for protein loading. (**E** and **F**) The long-term treated Cl41 cells were transiently transfected with (**E**) NFAT-Luc or (**F**) p53-Luc and treated with B[*a*]PDE or B[*a*]P for 12 h. The treated cells were extracted with lysis buffer, and the luciferase activity was measured using the Promega luciferase assay. The results were presented as (**E**) NFAT transcription activity or (**F**) p53 activity relative to the control. Each bar indicates the mean and SD of triplicate assay wells. The symbol (heart shaped) indicates a significant increase compared with that in medium control ($P < 0.01$).

as compared with that in the control siRNA transfectant (Figure 2C), whereas the protein of c-Jun, an important component of another transcription factor AP-1, and PKCα, a protein kinase involved in many stress responses, did not show any changes (Figure 2C). These results indicate that the NFAT3 siRNA does specifically knock-down NFAT3 protein expression. Consistent with the knock-down of NFAT3 protein expression, B[*a*]PDE-induced cell transformation was also dramatically inhibited in siNFAT transfectants as compared with that in control siRNA transfectants (Figure 2D), demonstrating that NFAT3 plays an important role in B[*a*]PDE-induced cell transformation. To exclude any intrinsic alteration on the NFAT activation during the long-term treatment of B[*a*]PDE, we determined the B[*a*]PDE-induced NFAT transactivation in the long-term treated cells (Figure 2E), similar to that observed in normal cultured Cl41 cells (Figure 1). Meanwhile, in view of the important role of p53 mutation during carcinogenesis under some context, we examined the potential change on p53 function in the long-term treated cells. As shown in Figure 2F, exposure of long-term treated Cl41 cells to B[*a*]PDE can induce p53 activation, suggesting an intact p53 in this cell, and p53 mutation may not be required for B[*a*]PDE-induced transformation of Cl41 cells.

NFAT3 is required for the tumor formation of B[a]PDE-transformed cells in nude mice

Previous studies demonstrated that the capacity of anchorage-independent growth of Cl41 cells in soft agar indicates the tumorigenicity of the cells in nude mice (30–32). To evaluate the tumorigenicity of B[*a*]PDE-treated cells, the nude mice were injected subcutaneously in two spots with 2×10^6 of B[*a*]PDE-treated Cl41 cells to observe the formation and size of tumors. The results showed that long-term repeated exposure of Cl41 cells to B[*a*]PDE dramatically increased the tumorigenicity in nude mice (Figure 3A and B, $P < 0.01$, $n = 6$), while cells without B[*a*]PDE treatment did not show any tumor formation (Figure 3A). The introduction of siNFAT did reduce the tumor size dramatically (Figure 3A and B, $P < 0.01$, $n = 6$). The comparison of tumor formation in nude mice between control siRNA transfectant and siNFAT transfectant suggests that NFAT3 is required for tumor-promoting activity caused by B[*a*]PDE exposure.

B[a]PDE exposure induced TNF expression in Cl41 cells

TNF up-regulation has been demonstrated to be induced in 9,10-dimethyl-1,2-benzanthracene (DMBA)/TPA multistages of the mouse skin carcinogenesis model and seems to play an essential role in the tumor development by favoring the tumor promotion stage (17,41). In view of our previous study showing that TNF can induce the anchorage-independent growth of mouse epidermal Cl41 cells to the similar extent of TPA, a well-characterized tumor promoter (20), we proposed that TNF might contribute to the transformation of Cl41 cells induced by B[*a*]PDE exposure. To test this hypothesis, we investigated whether TNF expression can be induced by B[*a*]P and its metabolite of B[*a*]PDE in mouse epidermal Cl41 cells. The Cl41 cells were stably transfected with luciferase reporter plasmid containing the human TNF promoter region, and then exposed to B[*a*]P or B[*a*]PDE. Consistent with NFAT activation and cell transformation activity, exposure of Cl41 cells to B[*a*]PDE caused significant TNF induction, while B[*a*]P exposure only showed a marginal effect (Figure 4A). The induction of TNF by B[*a*]PDE in Cl41 cells was further confirmed by time course and dosage studies (Figure 4B and C). The maximum induction of TNF occurred between 12 and 24 h after the exposure to B[*a*]PDE (Figure 4B). These data indicate that B[*a*]PDE exposure is able to induce TNF expression, suggesting that TNF may be a mediator of B[*a*]PDE-induced cell transformation.

NFAT3 is required for TNF induction upon B[a]PDE exposure in Cl41 cells

In T cells, the expression of TNF is mainly regulated by the NFAT pathway (42). To test the role of NFAT activation in up-regulation of TNF upon B[*a*]PDE, FK506, a chemical inhibitor for the calcineurin/NFAT pathway, was employed. As shown in Figure 5A, pretreatment of cells with FK506 resulted in the inhibition of NFAT transactivation induced by B[*a*]PDE (Figure 5A). Moreover, B[*a*]PDE-induced TNF induction was also inhibited by FK506 pretreatment (Figure 5B), suggesting that the NFAT pathway may be one of the mediators for TNF induction upon B[*a*]PDE exposure. Considering that FK506 is a chemical inhibitor, which may cause some non-specific effects, co-transfectants of siNFAT with TNF luciferase was utilized. The results showed that TNF induction by B[*a*]PDE was dramatically inhibited in siNFAT3 transfectants as compared with those in the control siRNA transfectants in all time and doses observed (Figure 5C and D). It should be noted that the inhibition of NFAT activation by FK506 seems to be greater than its inhibition on TNF transcription (Figure 5A and B), while NFAT3 siRNA can block the TNF transcription upon B[*a*]PDE exposure. Those discrepancies may be caused by relative non-specific effects of FK506, which may not only affect NFAT activation but also affect some other signaling pathways involved in the regulation of TNF transcription. Collectively, these results indicate that NFAT3 is required for TNF induction by B[*a*]PDE in Cl41 cells. It should also be noted that knock-down of NFAT3 expression with NFAT3 siRNA decreased TNF transcription by >95%, while it only showed 60% inhibition of cell transformation (Figure 2D). The

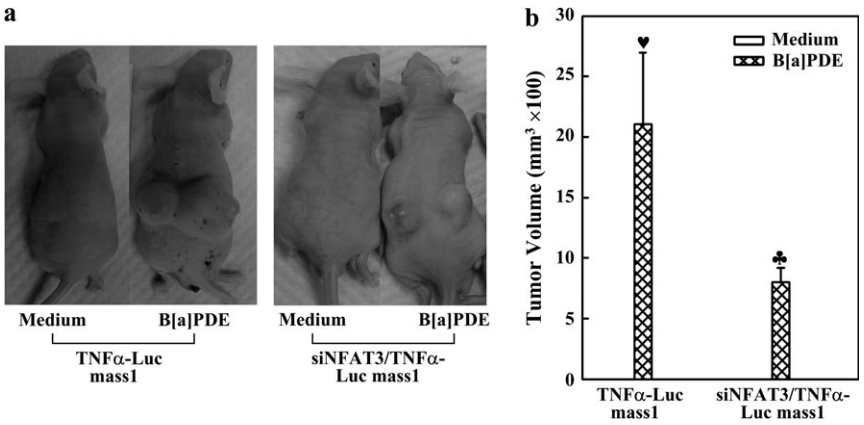

Fig. 3. Knock-down of NFAT3 by siNFAT3 reduced the tumor formation in the nude mice. Cl41-TNF-luc mass1 and Cl41 siNFAT/TNF-luc mass1 were repeatedly treated with 1 μM of B[*a*]PDE or 8 μM of B[*a*]P for 8 weeks as described in Materials and Methods. In all, 2×10^6 of these two kinds of cells were injected subcutaneously into each spot of 5-week-old female nude mice. Two weeks after the inoculation (**A**) the tumors were taken pictures and (**B**) the tumor dimensions were measured using calipers and tumor volume (mm^3) was calculated using the formula of 0.5236 (L × W × H). The symbol (heart shaped) indicates a significant increase as compared with that from Cl41-TNF-luc mass1 cells without B[*a*]PDE treatment ($P < 0.01$, $n = 6$). The symbol (club shaped) indicates a significant decrease as compared with that from Cl41 transfected with control siRNA transfectant, Cl41-TNF-luc mass1 ($P < 0.05$).

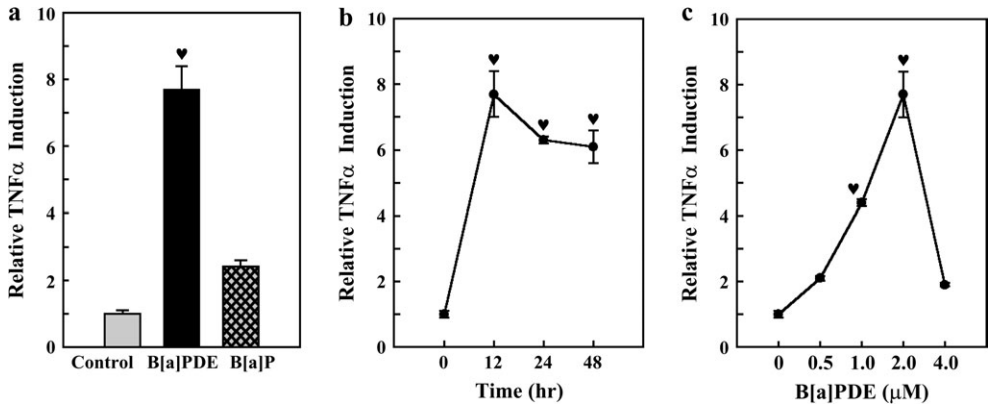

Fig. 4. Induction of TNF by B[*a*]PDE in mouse epidermal Cl41 cells. C141-TNF-Luc mass1 cells (8×10^3) were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were exposed to (**A**) 2 μM of B[*a*]PDE or B[*a*]P for 12 h (**B**) or various time periods as indicated (**C**) or with various concentrations of B[*a*]PDE as indicated for 12 h. The cells were then extracted with lysis buffer, and the luciferase activity was measured using the Promega luciferase assay kit. The results were presented as TNF luciferase activity relative to medium control (relative TNF induction). Each bar indicates the mean and SD of triplicate assay wells. The symbol (heart shaped) indicates a significant increase from medium control ($P < 0.01$).

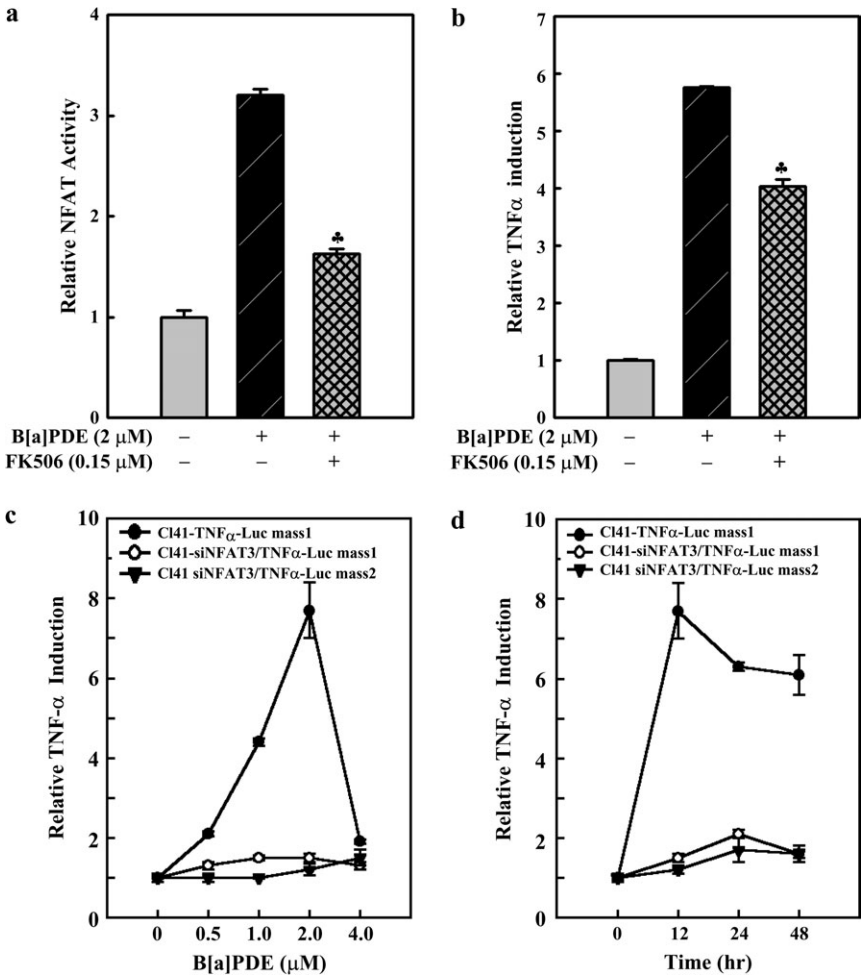

Fig. 5. Requirement of NFAT3 activation for B[*a*]PDE-induced TNF expression in mouse epidermal Cl41 cells. (**A**) C141-NFAT-Luc mass3 and (**B**) C141-TNF-Luc mass1 (8×10^3) were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were pretreated with 0.15 μM of FK506 for 1 h and then exposed to 2 μM of B[*a*]PDE for 12 h. (**C**–**D**) Cl41-TNF-Luc mass1, Cl41-siNFAT3/TNF-Luc mass1 or Cl41-siNFAT3/TNF-Luc mass2 cells were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were exposed to (**C**) various concentrations of B[*a*]PDE for 12 h or (**D**) 2 μM of B[*a*]PDE for various time points as indicated. The treated cells were extracted with lysis buffer, and the luciferase activity was measured using the Promega luciferase assay kit. The results were then presented as NFAT transcription activity relative to the control (relative NFAT activity) or TNF luciferase activity relative to medium control (relative TNF induction). Each bar indicates the mean and SD of triplicate assay wells.

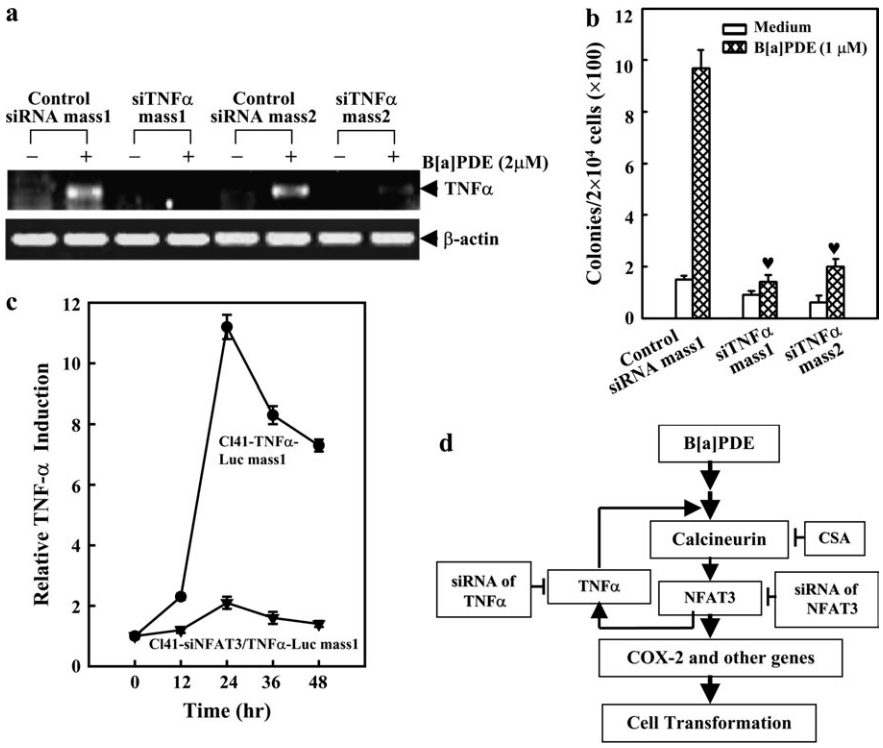

Fig. 6. Essential role of TNF induction in B[*a*]PDE-induced cell transformation. (**A**) Cl41 control siRNA mass1 (2×10^5) and Cl41 control siRNA mass2, Cl41-siTNF mass1, Cl41-siTNF mass2, were seeded into each well of six-well plates. After being cultured at 37°C overnight, the cells were exposed to 2 μM of B[*a*]PDE for 12 h, and then extracted with Trizol reagent for the total RNA isolation. TNF was amplified with their specific primers by reverse transcriptase–polymerase chain reaction for 30 cycles. β-actin was used as an internal control. (**B**) Cl41 control siRNA mass1, Cl41-siTNF mass1, Cl41-siTNF mass2 were repeatedly treated with 1 μM of B[*a*]PDE for 8 weeks as described in Materials and Methods, and then subjected to anchorage-independent growth assay as described above. Each bar indicates the mean and SD from triplicate assays. The symbol (heart shaped) indicates a significant decrease as compared with that from Cl41 transfected with vector control ($P < 0.01$). (**C**) The long-term treated Cl41-TNF-Luc mass1 and Cl41-siNFAT3/TNF-Luc mass1 were exposed to 2 μM B[*a*]PDE for indicated times, and then extracted with lysis buffer. The luciferase activity was measured using the Promega luciferase assay, and the results were presented as TNF induction relative to the control (relative TNF induction). Each bar indicates the mean and SD of triplicate assay wells. (**D**) Proposed model for outlining the mechanism involved in B[*a*]PDE-induced cell transformation.

explanation for this may be due to the complexity of cell transformation, which may need activation of multiple signaling pathways, such as AP-1 and NF-κB. This notion is supported by our previous finding that B[*a*]PDE exposure is able to activate AP-1 and NF-κB (43).

TNF is a major downstream mediator for B[a]PDE-induced cell transformation in Cl41 cells

To provide direct evidence for the identification of TNF required in B[*a*]PDE-induced cell transformation, we established the stable Cl41 cells transfected with siTNF or control siRNA vector, and verified the inhibitory effect of siTNF on the TNF transcriptional level by reverse transcriptase–polymerase chain reaction. As shown in Figure 6A, B[*a*]PDE-induced TNF expression was significantly inhibited by the transfection of siTNF. The Cl41-siTNF cells were then exposed to 1 μM of B[*a*]PDE for 8 weeks and then subjected to soft agar assay. The results showed that knock-down of TNF by its siRNA dramatically inhibited the anchorage-independent cell growth induced by the long-term repeated exposure of B[*a*]PDE (Figure 6B). Moreover, the induction of TNF by B[*a*]PDE in Cl41-TNF-Luc and Cl41-siNFAT/TNF-Luc cells was not affected by the long-term treatment of B[*a*]PDE (Figures 5D and 6C).

It should be noted that the inhibition of the anchorage-independent cell growth by siNFAT3 was not as efficient as siTNF, although siNFAT3 almost totally inhibited TNF induction by B[*a*]PDE. This may be due to certain cell transformation inhibitory signals that are also regulated by the NFAT pathway. This notion was supported by our most recent finding that inducible nitric oxide synthase (iNOS) is up-regulated via the NFAT3-dependent pathway, and provides inhibitory signaling for TPA-induced cell transformation (28). In fact, Lee *et al.* (44) has reported that NFATc3 plays a critical role in suppressing the development of mammary gland tumors in female mice. Together these results provide convincing direct evidence that the induction of TNF through NFAT3 plays an essential role in the cell transformation induced by B[*a*]PDE exposure.

Discussion

NFAT is a transcription factor that has been well studied in the regulation of cytokine expression in lymphocytes. Our most recent studies show that it is also involved in cell response to environmental carcinogen exposure; however, its real function in cell response to environmental carcinogen exposure has not yet been investigated. In this study, we used B[*a*]P and its metabolite, B[*a*]PDE as an environmental carcinogen to evaluate the role of NFAT3 in cell transformation in the cell culture model and their tumorigenicity in nude mice. We demonstrated that long-term repeated exposure of B[*a*]PDE is able to induce the transformation of mouse epidermal Cl41 cells, and that NFAT3 plays a critical role in this cell transformation as well as tumorigenicity in nude mice. Moreover, we provide the first direct evidence for the requirement of TNF induction through the NFAT3-dependent pathway for B[*a*]PDE-induced cell transformation.

NFAT has been suspected to be involved in environmental carcinogenic responses (45). For instance, nickel compounds are linked with human lung and nasal cancer in epidemiological studies, and are also found to cause carcinogenic effects in cultured cells and animal models (46). In mouse embryo fibroblasts, nickel sulfide

and nickel chloride induce the transcriptional activity of NFAT through the generation of reactive oxygen species (45). In the same cell model, asbestos, another environmental agent associated with lung cancer, also increases the activity of NFAT to transactivate its target gene through similar mechanisms of nickel compounds (47). Ultraviolet radiation is another major carcinogen and induces NFAT-dependent transcription through a calcium-dependent pathway in both JB6 mouse epidermal cell lines and in the skin of NFAT-luciferase reporter transgenic mice (33). NFAT1 nuclear localization is also involved in the cellular response of human keratinocytes to Ultraviolet B (48). NFAT activation is involved in the production of certain cytokines and other genes that are associated with cancer development (28,29,46). In human lung bronchial epithelial cells, arsenite induces COX-2 expression through the transactivation of NFAT3 (49). It is found that in both colon carcinoma cells and mouse skin epidermal cells, the NFAT pathway was involved in the expression of COX-2, which has extensive functions in both inflammation and cancer development (29,50,51). Thus, the NFAT pathway may be a missing link between chronic inflammation and cancer (52). Current studies have provided more direct evidence for the important role of NFAT3 in carcinogenesis. We demonstrate that the exposure of cells to B[*a*]PDE results in an increase in TNF expression in Cl41 cells, and that this process is dependent on NFAT3. Furthermore, B[*a*]PDE also induces cell transformation in the same cell model dependent on both TNF and NFAT3, since either TNF or NFAT3 siRNA is able to block the cell transformation induced by B[*a*]PDE treatment. More direct evidence comes from the tumorigenecity assay using nude mice in which the knock-down of NFAT3 significantly decreases the volumes of tumors. Consistently with this study, exogenous TNF treatment also induces anchorage-independent growth through the NFAT3/COX-2-dependent pathway in the same cells (29). Our results indicate that NFAT3 functions as both the upstream and downstream of TNF, which lead us to propose the model of NFAT3 in B[*a*]PDE-induced cell transformation and tumorigenesis (Figure 6C).

As a central mediator of inflammation, the pro-inflammatory cytokine TNF was recently identified as a potential endogenous tumor promoter (14,16,17,41). *In vitro* studies indicated that TNF can induce cell transformation of mouse epidermal JB6 P^+ cells (20). The direct evidence came from the studies showing that TNF knockout (TNF−/−) mice had 10-fold fewer skin tumors than wild-type mice after exposure to TPA (17); neutralizing antibodies to TNF is sufficient to inhibit TPA-induced skin tumor formation (18); TNF receptor I knockout mice (TNFR−/−) developed less liver metastasis with smaller size tumors compared with wild-type mice after intrasplenic administration of the colonic adenocarcinoma cell line (19).

TNF plays a critical role in the inflammation by initiating a so-called inflammatory cascade consisting of inflammatory cytokines, chemokines, growth factors and endothelial adhesion factors, recruiting a variety of activated cells at the site of tissue damage (14,53). Therefore, TNF has been an important inflammatory cytokine targeted in the therapies of inflammation-related diseases, including Crohn's disease (54,55) and rheumatoid arthritis (55,56). A recombinant anti-TNF antibody shows astonishing therapeutic effects in these diseases (55,56). In recent years, the role of inflammation in cancer development has attracted more and more attention (57). It has been proposed that inflammation may act as a promoter to accumulate more mutation in cells, drive those mutant cells to proliferation and give pre-neoplastic and neoplastic cells a growth advantage (58). Moreover, the chronic inflammatory conditions may lead to constitutive NF-κB activation, which in turn leads to the expression of pro-survival genes and prevention of damaged cells from apoptosis (59), and causes neoplastic growth (60). This notion was supported by the findings that TNF induction by okadaic acid could promote the initiated cells or various tissues surrounding the initiated lesion to produce clonal expansion and transformation (17). It has also been demonstrated that the inflammatory process triggers NF-κB activation through up-regulation of TNF in adjacent endothelial and inflammatory hepatocytes (61). In this study, we demonstrated that TNF exerts an important tumor promotion effect on the Cl41 cells repeatedly exposed to B[*a*]PDE over a long period of time. TNF may mediate this effect by affecting some signal pathways including PKCα, c-jun N-terminal kinases, AP-1 and NF-κB that have been suggested to be required for cell transformation (20,31,62,63). TNF may also affect the expressions of some important genes involved in regulating cell proliferation, apoptosis and tumorigenesis. Our recent study showed that TNF was able to markedly increase the expression of COX-2, and this COX-2 expression is required for TNF-induced cell transformation (29). In addition, TNF has been reported to regulate epithelial expression of matrix metalloproteinase-9 (MMP-9) and integrin during tumor promotion (64).

Recent studies have shown that the TNF promoter region contains the binding sites for the NFAT, activating transcription factor-2 (ATF-2)/Jun, Ets/Elk and specificity protein 1 (Sp 1) proteins and the CREB-binding protein (CBP)/p300 family of coactivator proteins (65–67); and our most recent studies have demonstrated that point mutation of the NFAT binding site in the TNF promoter region results in impairment of the NFAT-dependent TNF generation upon Ultraviolet B radiation (68). The mechanisms of transcriptional activation involved in TNF gene expression are inducer and cell type specific. In the current study, we demonstrated that exposure of mouse epidermal Cl41 cells to B[*a*]PDE induced significant NFAT activation, which is responsible for B[*a*]PDE-induced TNF expression because inhibition of the NFAT pathway by either FK506 or NFAT3 siRNA markedly blocked the induction of TNF by B[*a*]PDE. Moreover, NFAT3 siRNA transfection significantly inhibited the cell transformation induced by long-term repeated exposure of B[*a*]PDE, which is consistent with the result that knock-down of TNF by its siRNA significantly inhibited B[*a*]PDE-induced cell transformation. These results are also consistent with previous reports that expression of a constitutively active NFAT mutant in preadipocytes 3T3-L1 promotes both anchorage-independent cell growth and the formation of tumors in athymic nude mice (69), and transfection of dominant-negative NFAT in integrin β_4^+ breast carcinoma cells inhibited carcinoma invasion (70). Given the essential role of NFAT in TNF induction, we consider TNF to be one of the most important mediators of the carcinogenic effect of the NFAT pathway.

In the assay using the multistage carcinogenesis model in mouse skin, the induction of skin tumors in mice by B[*a*]PDE is accomplished by a multistage process involving initiation and promotion (71). The initiation effect of B[*a*]PDE is observed in mouse skin by the topical application of a single dose of B[*a*]PDE, followed with twice weekly applications of a tumor promoter, such as TPA, for 24 weeks (71), and the tumor promotion activity of B[*a*]PDE is demonstrated by repeated topical application of B[*a*]PDE which causes cancer development (12,72,73). In the present study, we treated Cl41 cells with B[*a*]PDE repeatedly for 8 weeks and found that Cl41 cells were transformed as indicated by both anchorage-independent cell growth and tumor formation in nude mice. In addition, TNF induction through NFAT3 played an essential role in the transformation of the mouse epidermal cells induced by long-term repeated B[*a*]PDE exposure. Investigation of skin cancer development in TNF-deficient mice induced by the repeated application of B[*a*]P or B[*a*]PDE may provide more direct evidence to support the notion proposed in this study; such studies are in progress.

It needs to be mentioned here that the roles of NFAT3 in the regulation of its target genes are various upon stimuli, which contribute to the discrepancy of its effects on cell transformation. For example, in the current study, we found that NFAT3 positively regulates B[*a*]PDE-induced TNF transcription, which is responsible for transformation upon B[*a*]PDE treatment. However, in terms of same Cl41 cells' response to tumor promoter TPA, NFAT3 enhances the transcription of iNOS, which has been proved to be a negative regulator for cell transformation (28). So the outcome of NFAT3 in Cl41 cells response to TPA is inhibition of cell transformation (28). In the case of epidermal growth factor-induced cell transformation, NFAT3 is not required for iNOS transcription (74). Those differential roles of NFAT3 upon various carcinogen exposures in the regulation of the same downstream target gene, such as iNOS, could be explained by multiple transcriptional

factor binding sites in the promoter region of the same target gene. The final outcome of the target gene expression is dependent on the crosstalks and/or interactions of those transcription factors that have been activated by carcinogens at the same time. The roles of NFAT3 in cell transformation induced by different carcinogens are dependent on multiple genes that are regulated by multiple signaling pathways in response to the carcinogens. It has been clearly demonstrated that different carcinogens activate the different signaling pathways even though some pathways are overlap activated. Our published studies demonstrate that NFAT3 positively regulated COX-2, by which mediates cell transformation in Cl41 cells' response to TNF (29), and B[*a*]PDE treatment resulted in TNF induction, by which causes the cell transformation. On the other hand, NFAT3 is not critical for cell transformation induced by TPA and epidermal growth factor (28, 74). This discrepancy of role of NFAT3 in the regulation of cell transformation in response to various carcinogens could be explained by differential multiple signaling pathways initiated by those carcinogens although all of them activate the same NFAT3 pathway because NFAT3 pathway might crosstalk to other signaling pathways that are activated at the same time. Those crosstalks and the balance of the positive and negative pathways will coordinately determine the transformation potential of these stimuli. Based on those studies, we conclude that the exact role of NFAT in the modulation of gene expression and cell transformation *in vitro* have to be explored based on various carcinogens.

In summary, the present study indicated that B[*a*]PDE is a potent form of B[*a*]P metabolites eliciting TNF expression through the NFAT3-dependent pathway in Cl41 cells, and the induction of TNF through the NFAT3 pathway is critical for the cell transformation induced by long-term repeated exposure of B[*a*]PDE. This study provides the first direct evidence that NFAT3-dependent TNF induction is an important mediator in B[*a*]PDE-induced tumor promotion. These findings may not only shed light on unraveling the mechanisms of B[*a*]PDE-induced carcinogenesis but also suggest that both the NFAT pathway and TNF may serve as the targets for cancer chemoprevention.

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