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PROSPECTS

Knockdown of NFAT3 Blocked TPA-Induced COX-2 and iNOS Expression, and Enhanced Cell Transformation in Cl41 Cells

Jingxia Li,[1] Lun Song,[1] Dongyun Zhang,[1] Lixin Wei,[2] and Chuanshu Huang[1]*

[1]Nelson Institute of Environmental Medicine, New York University School of Medicine, Tuxedo, New York 10987

[2]Tumor Immunology & Gene Therapy Center, Eastern Hospital of Hepatobiliary Surgery, Second Military Medical University, Shanghai 200438, China

Abstract The nuclear factor of activated-T-cells (NFAT) family is a ubiquitous transcription factor that mediates regulation on various gene expressions. Recent studies indicate that NFAT may implicate in cancer process, mainly through its direct regulation on the cyclooxygenase-2 (COX-2) gene expression. There is also evidence suggesting another aspect of NFAT in tumor suppression. However, the according mechanism remains unknown. In this study, we used a small interfering RNA (siRNA) expression construct to study the role of NFAT3 in the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced cell transformation with the tumor promotion-sensitive mouse epidermal Cl41 cells. Our results showed that TPA was able to induce NFAT3 activation in Cl41 cells. Stable transfection of NFAT3 siRNA specifically reduced endogenous NFAT3 expression. At the same time, TPA-induced expression of both COX-2 and inducible nitric oxide synthase (iNOS) were blocked. However, anchorage-independent transformation in response to TPA was significantly enhanced in NFAT3 siRNA stable transfectants as compared with vector transfectants. Moreover, treatment with the iNOS specific inhibitor aminoguanidine (AG) also enhanced Cl41 cells transformation induced by TPA. As COX-2 expression is proved to be required for cell transformation in Cl41 cells in our recent studies, our results demonstrate that the inducible NFAT3-mediated iNOS upregulation represents a novel potent tumor-suppressing pathway and may contribute to the tumor suppressor functions of NFAT protein. J. Cell. Biochem. 99: 1010–1020, 2006.

Key words: NFAT3; iNOS; COX-2; TPA; cell transformation

The nuclear factor of activated-T-cells (NFAT) is a family of transcription factors composed of five proteins (NFAT1–NFAT5), originally identified as Ca^{2+}-dependent transcriptional factors responsible for mediating expression of a series of cytokine genes in activated T-cells [Horsley and Pavlath, 2002; Hogan et al., 2003; Viola et al., 2005]. Later studies revealed that the expression of NFAT proteins is widly present in a variety of tissue cell types and their roles are far beyond the immune system [Santini et al., 2001; Baksh et al., 2002; Caetano et al., 2002; Chuvpilo et al., 2002; Graef et al., 2003; Neal and Clipstone, 2003; Pu et al., 2003; Jauliac et al., 2002; Zaichuk et al., 2004; Jayanthi et al., 2005]. It has been demonstrated that the NFAT signaling pathway plays important roles in the regulation of genes that control cell cycle progression, cell proliferation, differentiation, and apoptosis in different tissues, revealing a broader role of these proteins in normal cell physiology [Santini et al., 2001; Baksh et al., 2002; Caetano et al., 2002; Chuvpilo et al., 2002; Graef et al., 2003; Pu et al., 2003; Jayanthi et al., 2005]. In addition, several reports also have

Abbreviations used: AG, aminoguanidine; COX-2, cyclooxygenase-2; FBS, fetal bovine serum; iNOS, inducible nitric oxide synthase; NFAT, nuclear factor of activated-T-cells; NO, nitric oxide; NFκB, nuclear factor-κB; siRNA, small interfering RNA; TNF-α, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Jingxia Li and Lun Song contributed equally to this work.

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*Correspondence to: Dr. Chuanshu Huang, Nelson Institute of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, NY 10987. E-mail: chuanshu@env.med.nyu.edu

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addressed the involvement of NFATs in some aspects of malignant cell transformation and tumorigenic processes [Neal and Clipstone, 2003; Jauliac et al., 2002; Zaichuk et al., 2004]. However, the subset of genes regulated by NFAT proteins and their implication in different stage of tumor development has not been fully clarified.

Cyclooxygenase-2 (COX-2) and the inducible nitric oxide synthase (iNOS) have drawn great attentions in the field of cancer research during the past several years [Prescott and Fitzpatrick, 2000; Hinz and Brune, 2002; Xu et al., 2002; Crowell et al., 2003]. Upregulation of COX-2 has been documented in a variety of human cancer cells and attributes to tumor development via promoting neoangiogenesis and increasing cancer cells resistance to apoptosis [Sheng et al., 1998, 2001; Mann et al., 2001; Kuwano et al., 2004]. Elevated expression of iNOS is also observed in some carcinogen-induced tumorigenesis and responsible for the overproduction of nitric oxide (NO), a signaling molecule eliciting numerous important cellular responses in tumor cells [Ohshima and Bartsch, 1994; Cobbs et al., 1995; Chen and Stoner, 2004]. However, other reports have addressed the opposite effect of iNOS in cell transformation [Dhar et al., 2003; Liu et al., 2003]. Therefore, the roles of iNOS in tumorigenesis are still obscure. In many circumstances, induction of COX-2 and iNOS is concomitantly occurred [Di Rosa et al., 1996], suggesting an intricate link between these two pathways in vivo. *COX-2* gene has been reported to be a direct downstream target of NFAT and can be transcriptional inducible by NFAT [Iniguez et al., 2000; Yan et al., 2006], whereas the role of NFAT in the regulation of iNOS has not been investigated.

In the present study, we provide evidence indicating that NFAT3, a member of the NFAT transcription factor family mainly expressed in tissue other than lymphoid cells, involves in mediating the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced iNOS and COX-2 expression in the mouse Cl41 epidermal cell line. We found that suppressing NFAT3 expression with small interfering RNA (siRNA) in Cl41 cells concomitantly blocks the induction of both COX-2 and iNOS induced by TPA exposure. Moreover, we observed that abolishing endogenous NFAT3 expression or inhibition of iNOS activity by its specific inhibitor enhances the sensitivity of Cl41 cells to TPA-induced transformation. We, thus, for the first time demonstrate that NFAT3 plays a role in the regulation of iNOS and propose that this special signaling pathway might confer suppressing effects on cellular transformation, suggesting a tumor suppressor activity of this NFAT family member.

MATERIALS AND METHODS

Cell Culture and Construction of siRNA Expression Vector

JB6 P^+ mouse epidermal cell line, Cl41, was cultured in Eagle's Minimal Essential Medium (MEM, Calbiochem San Diego, CA) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies, Inc., Rockville, MD). The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in air. The siRNA expression plasmid was constructed using Gene Suppressor™ System (Imgenex Co., San Diego, CA). An inverted repeat DNA oligonucleotide designed with the aid of siRNA Target Finder published on the website http://www.ambion.com/techlib/misc/siRNA_finder.html (Ambion, Inc., Austin, TX) was synthesized (Invitrogen, Carlsbad, CA), annealed, and inserted into the *Sal*I and *Xba*I sites of the vector. The sequence of the oligonucleotide was 5′-tcgaGCCATTGACTCTGCAGATGgagtactgCATCTGCAGAGTCAATGGCttttt-3′ (forward) and 5′-ctagtttttGCCATTGACTCTGCAGAT GcagtactcCATCTGCAGAGTC AATGGC-3′ (reverse), where capital letters show the target sequence of mouse NFAT3, lowercase show the 8 nt spacer, 5 A nt terminal signal, and *Xho*I and *Xba*I compatible restriction sites overhang. The construct was named as siNFAT3/pSuppressor.

Generation of Stable Cotransfectants

The COX-2-luciferase reporter plasmid contains upstream 5′-flanking region of human *COX-2* gene promoter linked to the luciferase reporter was described in previous studies [Subbaramaiah et al., 2002; Yan et al., 2006]. The iNOS-luciferase reporter plasmid was kindly provided by Dr. Geller (University of Pittsburgh) [De Vera et al., 1996]. The NFAT-luciferase reporter plasmid and the TNFα-luciferase reporter plasmid were described in previous studies, respectively [Huang et al., 2000, 2001; Zhang et al., 2001]. Cl41 cells were cultured in a 6-well plate until they reached

85–90% confluence. COX-2 (1 μg), iNOS, or TNFα-luciferase reporter plasmid DNA, 5 μg of siNFAT3/pSuppressor or empty pSuppressor vector, or 1 μg of NFAT-luciferase reporter plasmid DNA and 5 μg of pSuppressor, and 20 μl of LipofectAMINE reagent (Gibco BRL, Rockville, MD) were used in each well transfection in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM without penicillin/streptomycin. Approximately 36–48 h after transfection, the stable transfectants were selected with 500 μg/ml of G418 (Gibco BRL). The following stable transfectants, Cl41 NFAT mass1, Cl41 siNFAT COX-2 mass1, Cl41 siNFAT iNOS mass1 and mass2, Cl41 COX-2 mass1, Cl41 iNOS mass1, and Cl41 siNFAT TNFα mass1 were established and cultured in G418-free 5% FBS MEM for at least two passages before each experiment.

Assays for Induction of NFAT or COX-2 and iNOS Promotor Activities

Confluent monolayer cells of the transfectants were trypsinized, and 8×10^3 viable cells in 100 μl of 5% FBS MEM were added to each well of 96-well plates. 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 10 ng/ml of TPA (Promega, Madison, WI) in 100 μl of 0.1% FBS MEM. The luciferase activity was determined by the luciferase assay at different time points using a luminometer (Wallac 1420 Victor 2 multilable counter system) after the addition of 50 μl of lysis buffer for 30 min at 4°C. The results were expressed as NFAT, COX-2, or iNOS transcriptional induction relative to medium control (Relative NFAT, COX-2, or iNOS induction). The Student's *t*-test was used to determine the significance of the differences of COX-2 and iNOS induction among various transfectants. The differences were considered significant at a $P \leq 0.05$.

Western Blot Analysis

Cl41 cells (2×10^5) or its stable transfectants were seeded in each well of 6-well plates. For NFAT expression level determination, each stable transfectant was harvested when cells were 80–90% confluence. For COX-2 induction analysis, Cl41 cells or its stable transfectants were starved in 0.1% FBS MEM for 12 h and then exposed to TPA (10 ng/ml) for various time periods. The whole cell extracts were prepared with boiling buffer (1% SDS, 1 mM Na_3VO_4, 10 mM Tris-HCl, pH 7.4) and then the protein concentrations were determined using Bio-Rad Protein Assay Kit. The cell extracts were separated on polyacrylamide–SDS gels, transferred, and probed with antibodies against NFAT1, 2, 3, 4 (Santa Cruz Biotechnology, CA), STAT1, 3 (Cell Signal), NF-κB p65, NF-κB p50 (Santa Cruz Biotechnology), COX-2 (Cayman Chemical, MI) or β-actin (Sigma, St. Louis, MO). The protein band was detected using the ECF Western blotting system.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis of iNOS mRNA

The iNOS mRNA levels in Cl41 cells with or without TPA exposure were measured by ThermoScript™ RT-PCR system (Invitrogen). Cl41 cells or its stable transfectants were treated same as for COX-2 induction assay described above, total RNA was isolated with Trizol reagent (Gibco BRL) and then RT-PCR was performed as suggested by the manufacturer. The following primers are used: mouse iNOS sense: 5′-CCTTGTTCAGCTACGCCTTC-3′; mouse iNOS antisense: 5′-CTGAGGGCTCTGTTG-AGGTC-3′; mouse β-actin sense: 5′-CATCCG-TAAAGACCTC TATGCC-3′; mouse β-actin antisense: 5′-ACGCAGCTCAGTAACA-GTCC-3′. PCR amplification program for mouse iNOS mRNA: denaturation at 94°C for 30 s; annealing at 53°C for 90 s, and extension at 72°C for 60 s for 25 cycles. Housekeeping gene β-actin was amplified as a control.

Anchorage-Independent Colony Assays

Soft agar colony formation assays were performed as described previously [Huang et al., 1997]. Briefly, 2.5 ml of 0.5% agar in BMEM supplemented with 10% fetal bovine serum and 10 ng/ml of TPA was layered onto each well of 6-well tissue culture plates. Cl41 (1 ml) cells or its stable transfectants (1×10^3) were mixed with 2 ml of 0.5% agar BMEM supplemented with 10% FBS and 10 ng/ml TPA and layered on top of the 0.5% agar layer. For the analysis of the effect of aminoguanidine (AG) (Alexis Biochemicals) on TPA-induced transformation response, both layers of agar were supplemented with TPA (10 ng/ml) or different doses of AG either alone or as a combination of AG and TPA. Plates were incubated at 37°C in 5% CO_2 for 2 weeks, and the number of colonies was scored as described previously [Huang et al., 1997].

RESULTS

TPA Induced NFAT Transactivation in Cl41 Cells

The activation of NFAT proteins has been shown to be an essential pathway not only in normal cell physiology, but also in some tumorigenic process [Horsley and Pavlath, 2002; Hogan et al., 2003; Viola et al., 2005]. However, the mechanism of different NFAT transcription factor family members in tumorigenesis is much less investigated [Viola et al., 2005]. Due to its role in blood vessels formation and angiogenesis, NFAT3 has been indicated to might be oncogenic potential [Horsley and Pavlath, 2002; Viola et al., 2005]. Therefore, to investigate whether NFAT3 is involved in TPA-induced cell transformation in tumor promotion sensitive mouse epitheral Cl41 cells, NFAT luciferase reporter gene was introduced into the mouse epidermal Cl41 cells, and the stable transfactants were selected with G418 and then TPA-induced NFAT reporter activity was measured. As shown in Figure 1, TPA can induce significant NFAT transactivation in Cl41 cells. The peak induction of NFAT activity is about 2.5-fold of the untreated control with 48 h of TPA exposure. This result indicated that NFAT is involved in TPA-induced response in Cl41 cells.

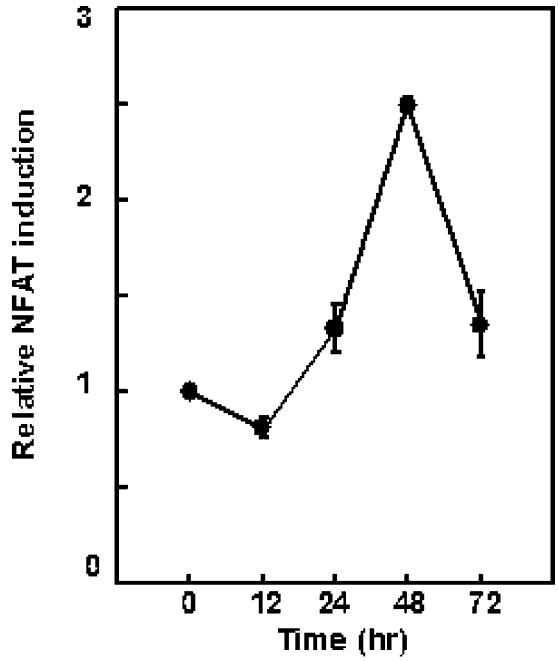


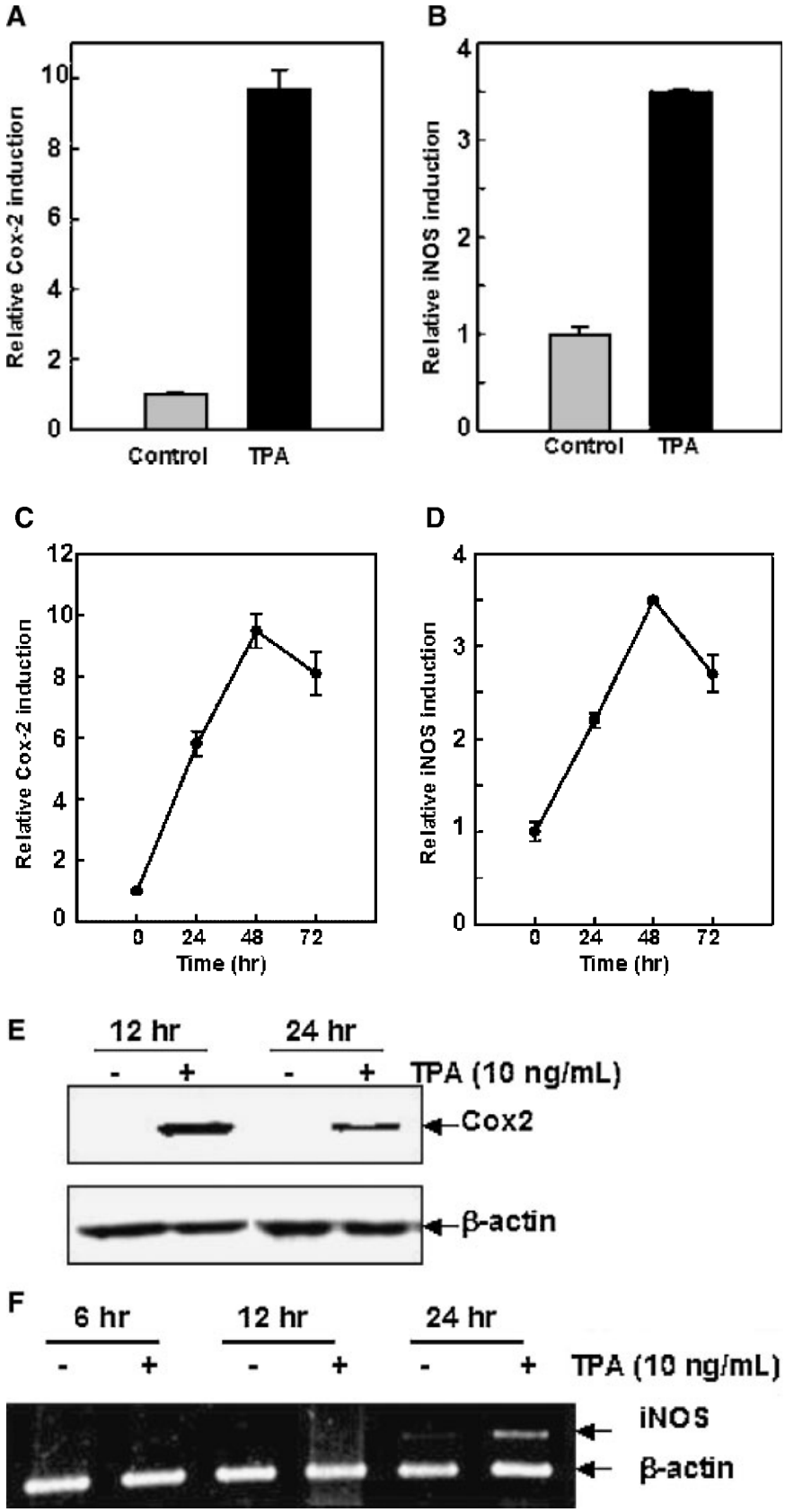
Fig. 1. TPA induced NFAT transactivation in Cl41 cells. NFAT luciferase reporter gene was introduced into the mouse epidermal Cl41 cells and the stable transfactants were selected with G418. Cl41 NFAT mass (8×10^3) were seeded into each well of 96-well plates. After reaching to 80–90% confluence, the cells were exposed to TPA (10 ng/ml) for different time periods and the luciferase activity was measured using the luminometer as described in the Materials and Methods. The results were presented as the induction of NFAT relative to medium control (relative NFAT activity). Each bar indicates the mean and standard deviation of triplicate wells.

TPA Induced Both COX-2 and iNOS Expression in Cl41 Cell

Upregulation of COX-2 and iNOS has been found associated with cellular transformation induced by numerous carcinogens [Ohshima and Bartsch, 1994; Sheng et al., 1998; Prescott and Fitzpatrick, 2000; Xu et al., 2002; Chen and Stoner, 2004]. Consistently, the COX-2 and iNOS promoter-driven luciferase reporters were separately transfected into Cl41 cells, and the stable transfectants were established with G418 selection. The results showed that the luciferase expression driven by either one of the two promoters was significantly increased after exposures of cells to TPA. The TPA-induced luciferase activities increased up to nearly 10-fold in Cl41 COX-2 mass1 (Fig. 2A) and to more than 3.5-fold in Cl41 iNOS mass1 (Fig. 2B). This induction appeared to be in a time-dependent manner (Fig. 2C,D). Consistent with the luciferase reporter gene assay, strong induction of both COX-2 protein and iNOS mRNA expression was also observed in Cl41 cells treated with TPA (Fig. 2E,F). These data indicated that TPA exposure is able to induce the expression of both COX-2 and iNOS in Cl41 cells, which is consistent with the findings from our laboratory in mouse embryo fibroblasts (MEFs) (data not shown) and others in mouse skin cells [Chun et al., 2004], and further excluding the possibility that this phenomenon is cell type dependent.

Knockdown of Endogenous NFAT3 Resulted in Dramatic Decrease of COX-2 Induction in Response to TPA in Cl41 Cell

NFAT is one of the transcription factors that have been reported to mediate COX-2 transcription [Iniguez et al., 2000]. To investigate whether the TPA-induced COX-2 upregulation is through the NFAT pathway in Cl41 cells, a siRNA expression construct specific for NFAT3, a member of the NFAT transcriptional factor family mainly expressed in non-lymphoid cells, was cotransfected with COX-2- luciferase repor-


A
Relative Cox-2 induction
0
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Control
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TPA (10 ng/mL)
Cox2
β-actin
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6 hr
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24 hr
- + - + - +
TPA (10 ng/mL)
iNOS
β-actin

(Fig. 2)

ter into Cl41 cells. As shown in Figure 3A, NFAT3 siRNA transfection dramatically suppressed NFAT3 expression by more than 80%, while without affecting the levels of other NFAT family members (NFA1, 2, 4), suggesting that the effect of the siRNA molecule is specifically targeted to NFAT3. After stable transfection, the Cl41 siNFAT3 COX-2 mass1 was treated with TPA and the induction of the luciferase activity was compared with that in Cl41 COX-2 mass1. The results showed that stable cotransfection with the NFAT3 siRNA construct almost completely blocked the TPA-induced COX-2 promoter transactivation. The induction of the COX-2 promoter-driven luciferase activity in response to TPA exposure was declined from ~9.6-fold in the Cl41 COX-2 mass1 to ~1.6-fold in Cl41 siNFAT3 COX-2 mass1 (Fig. 3B). And the inhibitory effects of NFAT3 siRNA on the TPA-induced activation of COX-2 promoter appeared in a time-dependent manner (Fig. 3D). Consistent with results of the luciferase reporter gene assay, immunoblotting analysis also confirmed that the induction of COX-2 protein expression was blocked in siNFAT3 stable transfectant (Fig. 3F). We further showed that siNFAT3 transfection did not affect the expressions of NF-κB p65, NF-κB p50, STAT1, and STAT3 (Fig. 3A), all of which are also involved in the transcriptional regulation of COX-2 or iNOS [Yamamoto et al., 1998; Kleinert et al., 2004]. Thus we conclude that the event of TPA-induced COX-2 is dependent of the NFAT3 pathway.

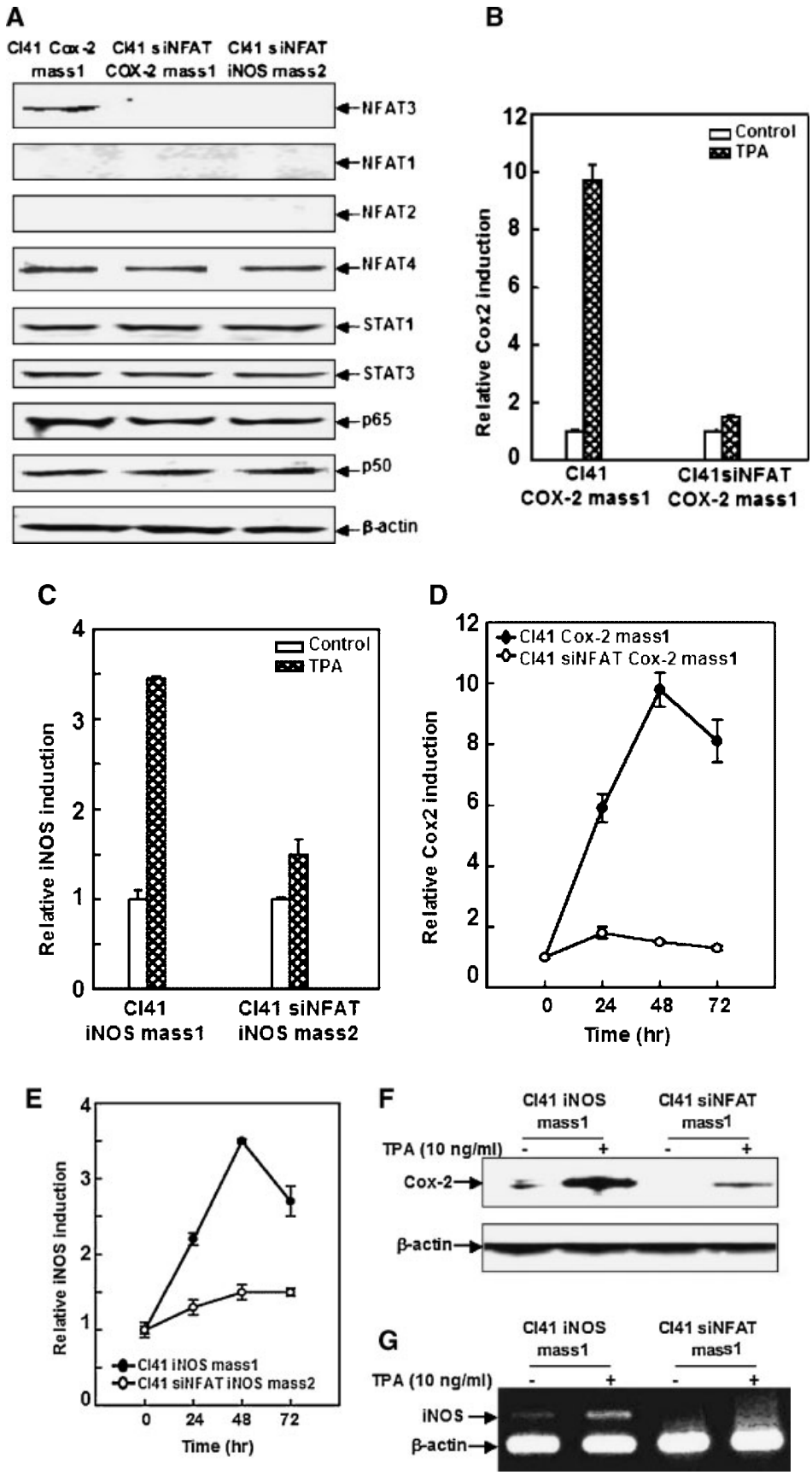
Reduction of NFAT3 Expression by NFAT3 siRNA Blocked TPA-Induced iNOS Expression in Cl41 Cells

iNOS is an enzyme responsible for the generation of NO, a signaling molecule eliciting numerous important cellular responses in tumor cells [Ohshima and Bartsch, 1994; Cobbs et al., 1995; Xu et al., 2002; Chen and Stoner, 2004]. However, potential role of NFAT in regulation of iNOS expression has not been investigated yet to the best of our knowledge. To investigate whether the TPA-induced iNOS upregulation is through the NFAT pathway, the NFAT3 siRNA construct was cotransfected with iNOS-luciferase reporter into Cl41 cells. Cl41siNFAT3 iNOS mass1 was treated with TPA and the induction of iNOS transcription was measured by determination of iNOS-promoter driven luciferase activity as compared with that in Cl41 iNOS mass2. The results showed that stable cotransfection with the NFAT3 siRNA construct significantly reduced the response of the iNOS promoter-driven luciferase induction in response to TPA stimulation (Fig. 3C). The TPA-induced luciferase activities only had a 1.4-fold increase in the Cl41 siNFAT iNOS mass2 as compared to 3.9-fold increase in Cl41 iNOS mass1 ($P < 0.001$). This inhibitory effect was observed in all time points studied (Fig. 3E). In addition, RT-PCR analysis further confirmed that both basal level and TPA-induced iNOS mRNA expression were totally blocked with siNFAT3 transfection (Fig. 3G). Thus, those results together demonstrated that that NFAT3 plays a critical role in TPA-induced iNOS induction in Cl41 cells.

Knockdown of NFAT3 Enhanced TPA-Induced Cl41 Cell Transformation

The NFAT proteins can act as different modulations according to different physiologic and pathophysiologic conditions, consequently regulating a different set of genes expression profile and conferring different biological consequences [Horsley and Pavlath, 2002; Hogan et al., 2003; Viola et al., 2005]. In a recent study, we demonstrated that suppressing endogenous NFAT3 inhibits TNF-α-induced Cl41 cell transformation [Yan et al., 2006], suggesting a potential oncogenic role of NFAT3 under this condition. However, suppressing NFAT3 expression only blocks the COX-2, and not the iNOS transcription induced by TNF-α [Yan et al., 2006], contrasting to the aforementioned phenomenon with TPA stimulation. We proposed that TPA might elicit a special functional

Fig. 2. Effects of TPA on the induction of COX-2 and iNOS expression in Cl41 cells. **A–D**: Cl41 COX-2 mass1 and Cl41 iNOS mass2 (8×10^3) were seeded into each well of 96-well plates, and cultured in 5% FBS MEM at 37°C. The cells were exposed to TPA (10 ng/ml) diluted in 0.1% FBS MEM after the cell density reached 80–90%. Cells were harvested 48 h later (A and B) or at various time points as indicated (C and D). The luciferase activities of COX-2 and iNOS were measured and the results were presented as the induction of COX-2 or iNOS relative to medium control (relative COX-2 induction or iNOS induction). **E**, **F**: Cl41 cells (2×10^5) were seeded into 6-well plate and stimulated with TPA (10 ng/ml) for various time periods after starved in 0.1% FBS MEM for 12 h. Whole cell extracts (E) and total RNA (F) were prepared and the expression of COX-2 protein was detected by Western blot and iNOS mRNA was determined by RT-PCR as described in Materials and Methods. β-actin was detected as loading control.


A
Cl41 Cox-2 mass1
Cl41 siNFAT COX-2 mass1
Cl41 siNFAT iNOS mass2
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p50
β-actin
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Cl41 siNFAT mass1
TPA (10 ng/ml)
Cox-2
β-actin
G
Cl41 iNOS mass1
Cl41 siNFAT mass1
TPA (10 ng/ml)
iNOS
β-actin

(Fig. 3)

modulation on the NFAT3 that might not occur in the case of TNF-α treatment. To further delineate the significance of this distinct NFAT3 pathway in carcinogenesis, Cl41 cells with NFAT3 siRNA stable transfection were used to test their sensitivity to the TPA-induced anchorage-independent growth, a well-characterized assay for cellular transformation [Huang et al., 1997]. Compared with the control vector transfectant (Cl41 COX-2 mass1), we consistently found that knockdown of NFAT3 expression with its specific siRNA greatly increased the anchorage-independent growth capacity of Cl41 COX-2 cell mass cultures induced by TPA. Similar observations were also found when we used other Cl41 cell stable transfectants for this experiments (Fig. 4), excluding the possibility that the effect of siNFAT3 on TPA-induced cell transformation is caused by clone selections. Based on these results, we anticipate that NFAT3 also possesses a tumor suppressing effect that might link with its roles in iNOS regulation.

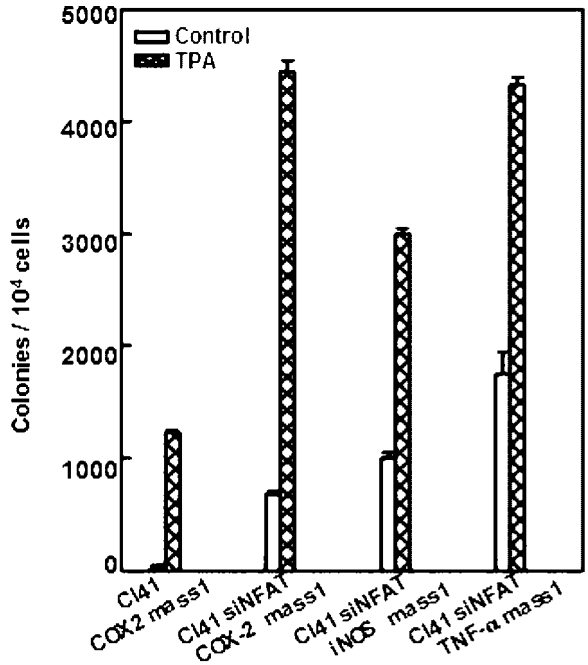

Fig. 4. Effects of NFAT3 siRNA on TPA-induced cell transformation. Independent Cl41 siNFAT stable transfectants with the expression of COX-2, iNOS, or TNFα luciferase reporter genes and Cl41 COX-2 mass1 (1×10^4) were exposed to TPA (10 ng/ml) in 1 ml of 0.33% MEM agar containing 10% FBS over 2.5 ml of 0.5% MEM agar containing 10% FBS in each well of 6-well plates. The cultures were maintained in a 37°C, 5% CO_2 incubator for 2 weeks, and the cell colonies were scored as described in our previous studies [Huang et al., 1997]. The experiments were repeated at least three times. Each bar indicates the mean ± SD from triplicate wells.

iNOS Specific Inhibitor Enhanced TPA-Induced Transformation Response in Cl41 Cells

In order to further confirm that NFAT3-induced iNOS upregulation in response to TPA represents a tumor suppressing pathway, we examined cell transformation induced by TPA in the absence or presence of a well-described specific iNOS inhibitor, AG [Islas-Carbajal et al., 2005]. As shown in Figure 5, treatment with AG alone did not induce cell transformation in Cl41 cells. However, AG produced a dose-dependent enhancement of TPA-induced transformation response. TPA can induce colony formation to sixfold over the control, and AG increased the response to 1.5–2.2-fold at the concentration of 0.25–2 mM over TPA level. This result indicates that inhibition of iNOS make Cl41 cells more sensitive to TPA-induced cell transformation and supports our notion that iNOS is responsible for suppressing the tumor phenotype induced by NFAT3.

DISSCUSSION

The NFAT family transcription factors are ubiquitous regulators for the expression of a various number of gene expression and play important roles in the control of cellular response to the change of growth environments [Santini et al., 2001; Baksh et al., 2002; Caetano et al.,

Fig. 3. Specific knockdown of NFAT3 expression resulted in dramatic reduction of COX-2 and iNOS induction in response to TPA in Cl41 cells. **A**: 2×10^5 of Cl41 COX-2 mass1, Cl41 siNFAT COX-2 mass1, or Cl41 siNFAT iNOS mass2 were cultured in each well of 6-well plates to 95–100% confluence. The cell extracts were prepared with SDS-sample buffer and the expression levels of NFAT3 and other transcriptional factors were detected with specific antibodies. β-actin was used as protein loading control. **B–E**: Cl41 COX-2 mass1, Cl41 siNFAT COX-2 mass1, Cl41 iNOS mass1, and Cl41 siNFAT iNOS mass2 (8×10^3) cells were seeded into each well of 96-well plates. The cells were treated with TPA for 48 h (B and C) or various time points as indicated (D and E). The luciferase activities were determined, and the results were presented as relative COX-2 induction (B and D) or relative iNOS induction (C and E). Each bar indicates the mean and standard deviation of triplicate wells. **F**, **G**: Cl41 iNOS mass1 and Cl41 siNFAT mass1 (2×10^5) were seeded into 6-well plate and stimulated with TPA (10 ng/ml) for 12 h (F) or 24 h (G) after starved in 0.1% FBS MEM. The expressions of COX-2 protein and iNOS mRNA were detected. β-actin was used as a loading control.

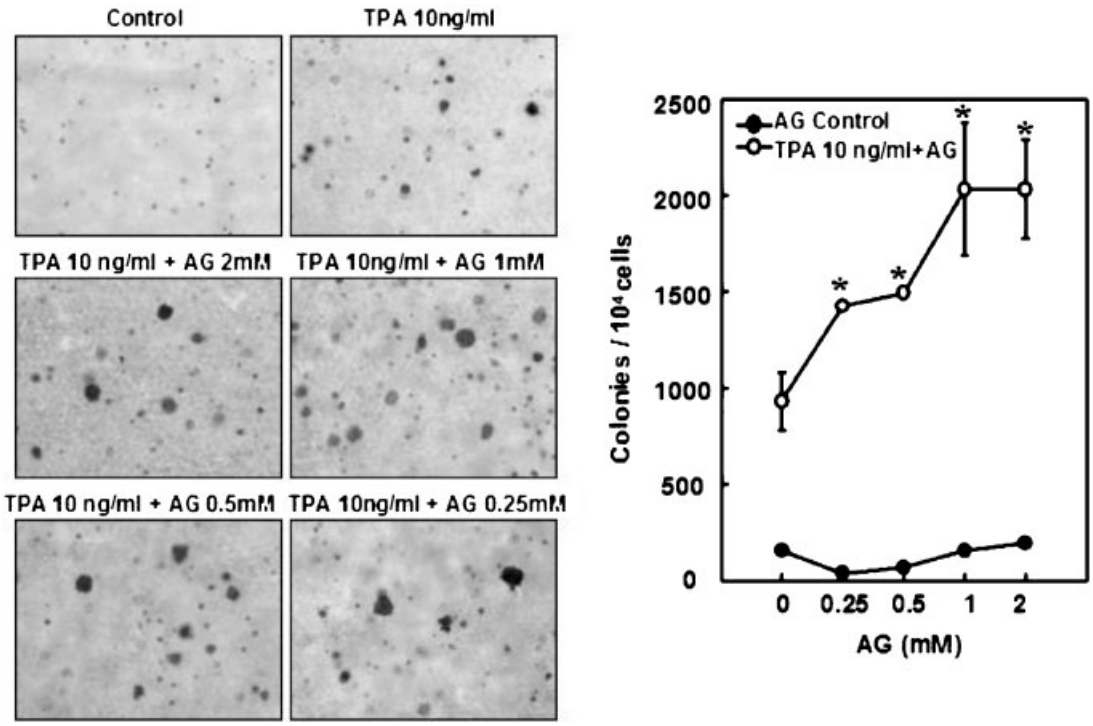

Fig. 5. iNOS-specific inhibitor enhanced TPA-induced transformation response in Cl41 cells. Cl41 cells were exposed to different doses of AG in the absence or presence of TPA (10 ng/ml). Cell transformation was performed and the results were presented as described in Figure 4.

2002; Horsley and Pavlath, 2002; Chuvpilo et al., 2002; Graef et al., 2003; Hogan et al., 2003; Pu et al., 2003; Jayanthi et al., 2005; Viola et al., 2005]. Recently, there have been evidences indicating that the NFAT proteins contribute to multiple aspects of carcinogenesis [Neal and Clipstone, 2003; Jauliac et al., 2002; Zaichuk et al., 2004], and its direct transcriptional activation of COX-2 expression is one of the mechanisms of NFAT activity in tumorogenesis [Duque et al., 2005]. Consistently, our previous work demonstrated that diminishing endogenous NFAT3, a member of the NFAT family, inhibits COX-2 induction and blocks the transformation-sensitive Cl41 cells transformation under the treatment of TNF-α [Yan et al., 2006]. However, there were also evidences suggesting another property of the NFAT proteins in the cancer process [Ranger et al., 2000]. Glimcher and colleagues found that deletion of NFATc2, another member of NFAT family, leads to the uncontrolled proliferation of abnormal extraarticular cartilage cells in NFATc2-null mice and these NFATc2-deficient cartilage cells exhibit cancer phenotypes both in vitro culture and in vivo [Ranger et al., 2000], suggesting a potential tumor suppressor role of NFATc2 in this special cell lineage. These controversial results about the significance of the NFAT pathway in cancer reflect the fact of the versatility of NFAT proteins in cell fate determination that is dependent on their functional modulations under the certain condition [Hogan et al., 2003; Viola et al., 2005]. Dissecting the distinct aspects of NFAT in the course of cellular transformation is thus of importance.

In the present study, we describe a novel action of NFAT3 on inhibition of cell transformation through a pathway on iNOS regulation, a key inducible enzyme for the production of the signaling molecule NO. By stable transfection of a NFAT3-specific siRNA construct into Cl41 cells, we found that diminishing endogenous NFAT3 expression significantly enhances the sensitivity of Cl41 cells to TPA-induced transformation, in contrast to our previous finding that knockdown of endogenous NFAT3 blocks the Cl41 cells transformation induced by TNF-α [Yan et al., 2006]. The different behaviors of NFAT3 in cellular transformation might attribute to the discrepancy of its effects on iNOS regulation under these two conditions. In

the present study, we showed that suppression of NFAT3 expression concomitantly inhibited both COX-2 and iNOS induction in response to the TPA stimulation, suggesting that the TPA-induced iNOS upregulation depends on the activity of NFAT3. However, suppressing NFAT3 only affects COX-2, but not iNOS induction under TNFα treatment [Yan et al., 2006], indicating that TNFα can induce iNOS upregulation through other pathways. We further showed that iNOS specific inhibitor was able to enhance TPA-induced cell transformation response. Also, overproduction of NO generated by the NO donors was reported to inhibit transformation and tumor phenotype, regardless of the COX-2 expression levels [Dhar et al., 2003; Liu et al., 2003]. Thus, we anticipate that induction of iNOS via a special activity of NFAT3 represents a putative tumor suppressing mechanism. Ablation of NFAT3 by the specific siRNA results in Cl41 cells more sensitive to the TPA-induced transformation while delivering an opposite effect in the case of TNFα.

The molecular basis for mediating NFAT3 on iNOS regulation is still unknown. It seems that iNOS is not a direct target for NFAT because there is no NFAT binding site found in the iNOS promoter element [De Vera et al., 1996; De Gregorio et al., 2001]. Also, our previous work demonstrated that iNOS induction by the TNF-α is not dependent on the NFAT pathway [Yan et al., 2006]. We propose that the activity of NFAT3 on iNOS transcription is either conditionally coregulated with certain other pathway or regulated indirectly. Because deregulation of both NFAT and iNOS has been implicated in cancers, further elucidating the mechanisms that link these two signaling in vivo and their corresponding biologic significance is warranted.

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