

# NFAT3 is specifically required for TNF- $\alpha$ -induced cyclooxygenase-2 (COX-2) expression and transformation of C141 cells

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## Summary

NFAT family is recognized as a transcription factor for inflammation regulation by inducing the expression of pro-inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), the key mediator of inflammation, which was reported to induce cell transformation in mouse epidermal C141 cells. In this study, we demonstrated that TNF- $\alpha$  was able to induce NFAT activation, as well as the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The induction of COX-2 by TNF- $\alpha$  was abolished by knockdown of NFAT3 with its siRNA, while the induction of iNOS was not effected. Moreover, TNF- $\alpha$ -induced anchorage-independent cell growth was significantly inhibited by NFAT3 siRNA and cyclosporine A, a chemical inhibitor for the calcineurin/NFAT pathway, which suggests the importance of NFAT3 in regulating

TNF- $\alpha$ -induced anchorage-independent cell growth. Consequently, impairment of COX-2 by its siRNA or selective inhibitor also inhibited TNF- $\alpha$ -induced anchorage-independent cell growth. Taken together, our results indicate that NFAT3 plays an important role in the regulation of TNF- $\alpha$ -induced anchorage-independent cell growth, at least partially, by inducing COX-2 expression in C141 cells. These findings suggest that NFAT3/cyclooxygenase-2 act as a link between inflammation and carcinogenesis by being involved in the tumor promotion stage.

Key words: TNF- $\alpha$ , Cyclooxygenase-2, NFAT3, Cell transformation, Carcinogenesis

## Introduction

It has been thought that carcinogen exposure and chronic inflammation are two important associated factors involved in cancer development. It has been estimated that chronic inflammation accounts for approximately 20% of human cancer (Coussens and Werb, 2002). Whereas the causal relationship between carcinogen exposure and cancer development has been intensely investigated, the molecular and cellular mechanisms linking chronic inflammation to tumor development remain largely uninvestigated. The proinflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a major mediator of inflammation (Locksley et al., 2001). In malignant disease, TNF- $\alpha$  has originally been considered as an anticancer agent, because it induces the destruction of blood vessels and mediates the killing of certain tumors (Malik and Balkwill, 1988). However, recent studies have provided substantial evidence showing that TNF- $\alpha$  acts as an endogenous tumor promoter in both cell culture and animal models (Szlosarek and Balkwill, 2003). TNF- $\alpha$ -knockout mice (TNF- $\alpha^{-/-}$ ) are more resistant to exposure to the chemical carcinogen 12-O-tetradecanoylphorbol-13-acetate (TPA) than are wild-type mice (Moore et al., 1999; Scott et al., 2003). TNF- $\alpha$  receptor I (TNFRI)-knockout mice (TNFR<sup>I</sup> $^{-/-}$ ) have a reduced incidence of and developed smaller liver metastases

following intrasplenic administration of a colonic adenocarcinoma cell line (Kitakata et al., 2002). Our previous work also showed that TNF- $\alpha$  had similar effects to TPA in the induction of the anchorage-independent growth of mouse epidermal C141 cells (Huang et al., 1999).

TNF- $\alpha$  can trigger multiple signal pathways that lead to activation of transcription factors such as AP-1, NF- $\kappa$ B and NFAT (Gaur and Aggarwal, 2003; Schaft et al., 2003). Arnott et al. demonstrated that AP-1 activation is involved in TNF-mediated tumor promotion (Arnott et al., 2002). Most recently, Pikarsky et al. reported that hepatocyte NF- $\kappa$ B activation, triggered by inflammatory processes through upregulation of TNF- $\alpha$  in adjacent endothelial and inflammatory cells, plays a key role in the development of cholestatic hepatitis, and followed hepatocellular carcinoma in Mdr2-knockout mouse (Pikarsky et al., 2004). Anti-TNF- $\alpha$  treatment or induction of I $\kappa$ B-superrepressor in later stages of tumor development resulted in apoptosis of transformed hepatocytes and failure to progress to hepatocellular carcinoma (Pikarsky et al., 2004). Nonetheless, it still remains to be intensively investigated whether other pathways are also involved in the regulation of TNF- $\alpha$ -promoted inflammation-associated cancer development, and what the target genes of the transcription factors that mediate the promotion of cancer development are.

NFAT is another important transcription factor that can be activated by TNF- $\alpha$  (Schaft et al., 2003). It has originally been identified in T cells to regulate the IL-2 promoter (Chow et al., 1999; Jain et al., 1995). Up to now, at least five members of NFAT have been identified: NFAT1/c2 and NFAT2/c1 are the major NFATs involved in T-cell activation, NFAT3/c4 is expressed primarily in nonlymphoid tissues, NFAT4/c3 is expressed mainly in thymus, and NFAT5 is a transcription factor crucial for cellular response to hypertonic stress (Rao et al., 1997). NFAT is expressed in cytoplasm, and its activation is regulated by Ca<sup>2+</sup> and the Ca<sup>2+</sup>/calmodulin-dependent serine phosphatase calcineurin (Rao et al., 1997). NFAT activation is initiated by dephosphorylation of the NFAT regulation domain located N-terminal to the DNA-binding domain. Dephosphorylated NFAT translocates into the nucleus, binds to consensus DNA sites and controls the expression of its target genes including IL-3, GM-CSF, TNF- $\alpha$  and COX-2 (Cockerill et al., 1993; Iniguez et al., 2000; McCaffrey et al., 1994; Shannon et al., 1997). Recent studies indicate that the NFAT signaling pathway is involved in the regulation of cell growth and development in a wide variety of different tissues and cell types (Hogan et al., 2003; Horsley and Pavlath, 2002).

It has been reported that TNF- $\alpha$  promotes cancer growth, invasion and metastasis through induction of multiple inflammatory mediators, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Surh et al., 2001; Xu et al., 2002). Cyclooxygenase is the rate-limiting enzyme in the conversion of arachidonic acid to prostanoids (Sheng et al., 2001; Smith et al., 1996). Two COX isoforms have been cloned (Feng et al., 1993), of which COX-1 is constitutively expressed, whereas the expression of COX-2 is low or nondetectable in most tissues, but can be readily induced in response to cell activation by cytokines, growth factors and tumour promoters. Thus, COX-1 is considered a housekeeping gene and thought to be responsible for the synthesis of prostanoids involved in cytoprotection of the stomach and for the production of the pro-aggregatory prostanoid thromboxane by the platelets. By contrast, COX-2 is an inducible immediate-early gene, and its role has been related to inflammation, reproduction and carcinogenesis (Kirschenbaum et al., 2001; Liu et al., 1998; Tsujii and DuBois, 1995; Tsujii et al., 1997; Tsujii et al., 1998). Expression of COX-2 is elevated in a variety of human malignancies and in their precursor lesions. Furthermore, genetic deletion or pharmacological inhibition of COX-2 suppresses tumour growth in several animal models of carcinogenesis. In humans, elevated COX-2 expression is associated with poor prognosis in adenocarcinomas of the digestive tract and the breast, and a selective inhibitor of COX-2 reduced polyp burden in patients who suffer from familial adenomatous polyposis. iNOS is one of the three isoforms of nitric oxide synthase (NOS), which catalyzes the oxidative deamination of L-arginine to produce nitric oxide (NO). NO promotes DNA damage and inhibits DNA repair (Jaiswal et al., 2000), and is thought to play an important role in the tumor promotion and induction of neoplastic cell transformation (Mordan et al., 1993; Robertson et al., 1996).

In the current study, we employed mouse epidermal JB6 P<sup>+</sup> Cl41 cells to investigate the role of NFAT3 in the regulation of COX-2 and iNOS induction by TNF- $\alpha$ , and the contribution of NFAT3 and its downstream target gene to TNF- $\alpha$ -induced

anchorage-independent growth of Cl41 cells. The JB6 P<sup>+</sup> Cl41 cell is a transformation-susceptible cell line, and has been widely used as a model for anchorage-independent cell growth to indicate cell transformation based on the fact that cells growing in soft agar can form tumors in nude mice (Colburn et al., 1988; Huang et al., 1998; Li et al., 1997; Yu et al., 2002). We demonstrated that induction of COX-2, but not iNOS by TNF- $\alpha$  was abolished by knockdown of NFAT3 expression by its specific siRNA, and TNF- $\alpha$ -induced anchorage-independent growth was greatly inhibited by knockdown of NFAT3 or COX-2. These results suggest that COX-2 induction through NFAT3 plays an important role in regulation of TNF- $\alpha$ -induced anchorage-independent cell growth.

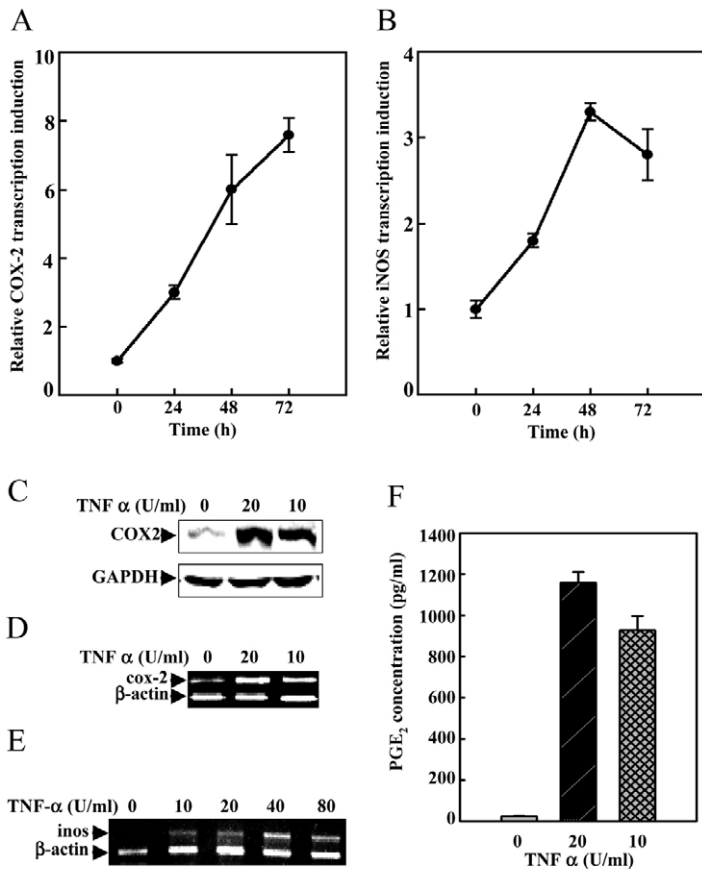
## Results

### Induction of COX-2 and iNOS promoter activity by TNF- $\alpha$ in mouse epidermal Cl41 cells

Previous studies indicate that COX-2 induction appears to be in a highly cell type-specific and stimulus-specific manner (Fournier et al., 1997; Itoh et al., 2003; Lin et al., 2004; Pang and Knox, 1997). It has been reported that COX-2 could be induced by pro-inflammatory cytokines, such as TNF- $\alpha$ , in a variety of cells (Fournier et al., 1997; Itoh et al., 2003; Lin et al., 2004; Pang and Knox, 1997). TNF- $\alpha$  was also reported to induce iNOS expression in certain cell types when it was combined with LPS or other cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ) and IL-1 $\beta$  (Chan et al., 1999; Dhar et al., 2003; Lee et al., 2003; Robbins et al., 1997). However, the effect of TNF- $\alpha$  alone on iNOS induction remains unclear. Here we demonstrated that treatment of Cl41 with 20 U/ml TNF- $\alpha$  resulted in significant induction of COX-2 and iNOS at transcription level in Cl41 cells in a time-dependent manner (Fig. 1A,B). The inductions of COX-2 and iNOS by TNF- $\alpha$  were further confirmed by western blot and/or RT-PCR, respectively (Fig. 1C-E). As a consequence of COX-2 induction, the production of PGE<sub>2</sub> also dramatically increased after TNF- $\alpha$  treatment (Fig. 1F). These results demonstrated that TNF- $\alpha$  could induce both COX-2 and iNOS expression in Cl41 cells.

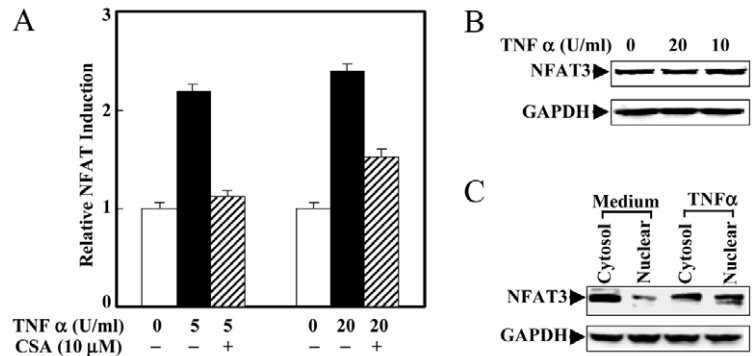
### Requirement of NFAT3 for TNF- $\alpha$ -induced expression of COX-2 but not iNOS

The expression of COX-2 and iNOS is regulated primarily at the transcriptional level via distinct pathways in different cell types (Gorgoni et al., 2001; Hogan et al., 2003). For example, in the Jurkat human leukemic T cells, AP-1 and NFAT are required for the induction of COX-2 expression, while CREB is not essential (Iniguez et al., 2000). The knockout of C/EBP $\beta$  in macrophages blocks COX-2 expression but has no effect on COX-2 expression in fibroblasts (Gorgoni et al., 2001). To unravel the potential role of NFAT3 in the induction of COX-2 and iNOS by TNF- $\alpha$  in Cl41 cells, we first determined whether TNF- $\alpha$  is able to activate NFAT in Cl41 cells. As shown in Fig. 2A, TNF- $\alpha$  exposure led to significant NFAT transactivation in Cl41 cells in the NFAT luciferase reporter assay, which can be obviously inhibited by CSA, a chemical inhibitor of NFAT pathway. In addition, the NFAT translocation assay showed that TNF- $\alpha$  exposure led to a reduction of NFAT3 protein in cytosol but an increase in NFAT3 protein in the nucleus (Fig. 2C), which suggests that NFAT3 protein translocates from the cytosol to the nucleus. By



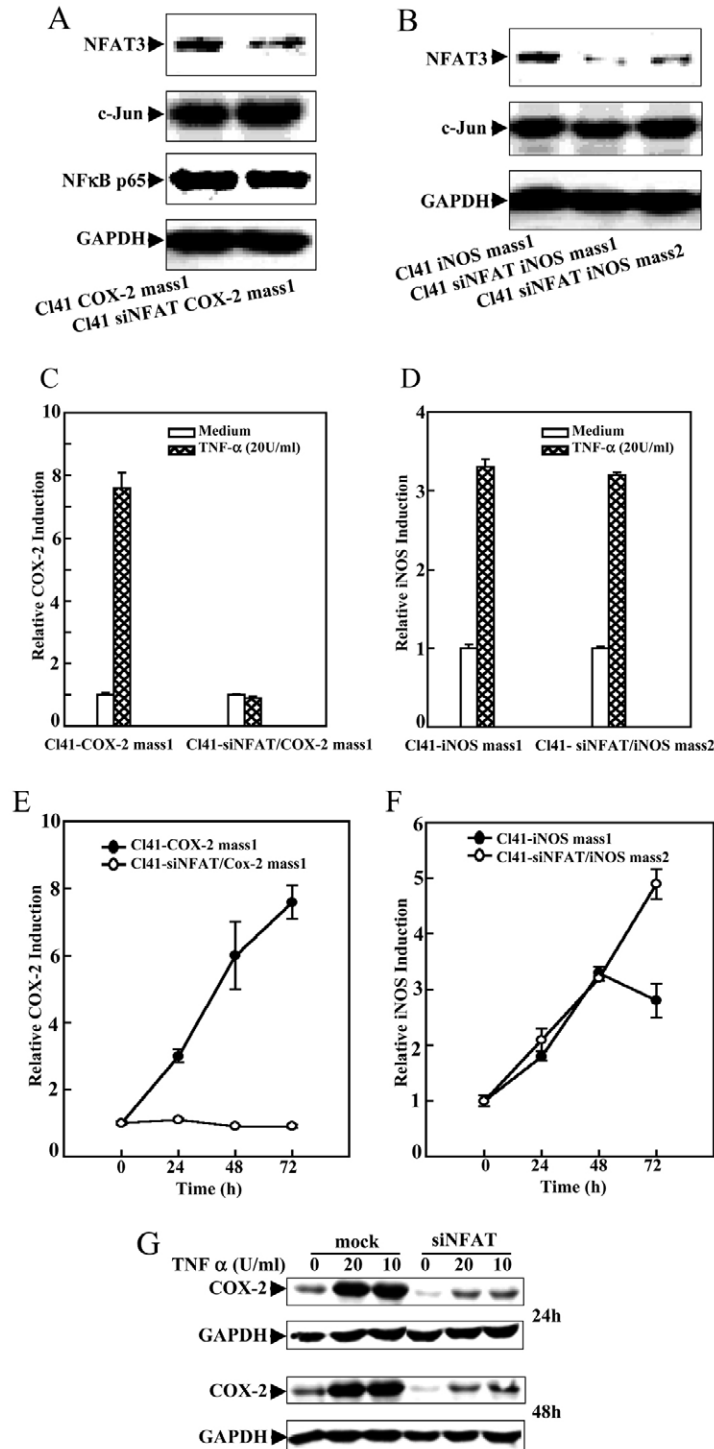
**Fig. 1.** Induction of COX-2 and iNOS by TNF- $\alpha$  in CI41 cells. (A,B)  $8 \times 10^3$  CI41-COX-2 mass1 (A), and CI41-iNOS mass1 (B) cells were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, the cells were treated with 20 U/ml TNF- $\alpha$  for various time periods as indicated. The cells were then extracted with lysis buffer, and the luciferase activity was measured as described in the Materials and Methods. The results were expressed as COX-2 or iNOS relative luciferase activity (RLU). Each bar indicates the mean and standard deviation of triplicate wells. (C) CI41 cells ( $2 \times 10^5$ ) were seeded into each well of a 6-well plate, and cultured in 5% FBS MEM at 37°C. When the cell density reached 70–80%, the culture medium was replaced with 0.1% FBS MEM. After being cultured for 24 hours, the cells were exposed to various concentrations of TNF- $\alpha$  as indicated. The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The western blot was carried out with specific antibodies against COX-2 and GAPDH, respectively, as described in Materials and Methods. GAPDH was used as the protein loading control. (D,E) After being exposed to various concentrations of TNF- $\alpha$  for 24 h, CI41 cells were extracted with Trizol reagent for the total RNA isolation. COX-2 (D) and iNOS (E) were amplified with their specific primers by RT-PCR for 30 cycles.  $\beta$ -actin was used as an internal control. (F)  $8 \times 10^3$  of CI41 cells were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, the cells were treated with 10 and 20 U/ml of TNF- $\alpha$  for 48 hours. The PGE2 levels in the 48 hour culture medium were determined by ELISA according to the manufacturer's instructions. The results were expressed as the mean  $\pm$  s.d. of triplicate samples.

contrast, TNF- $\alpha$  treatment failed to show any obvious effect on the expression level of NFAT3 (Fig. 2B). These results strongly indicate that TNF- $\alpha$  is able to induce NFAT activation in CI41 cells. We further established stable CI41 cells transfected with NFAT3-specific siRNA and verified the silencing effect of the siRNA on NFAT3 expression by western blot. As shown in Fig. 3A,B, transfection of siNFAT3 resulted in a decrease in NFAT3 protein levels of more than 75% in both CI41-siNFAT3/COX-2 mass1 and CI41-siNFAT3/iNOS mass1 cells when compared with CI41 cells transfected with mock vector, whereas the protein levels of c-Jun, a key component of transcription factor AP-1, and NF $\kappa$ B p65 subunit were not affected by siNFAT3. In addition, NFAT3 siRNA has been demonstrated to have no effect on the expression of other members of NFAT in our previous study (Li et al., 2006). Then we observed the effect of siNFAT3 on the induction of COX-2 and iNOS by TNF- $\alpha$ . As shown in Fig. 3C,D, knockdown of NFAT3 expression blocked COX-2 induction in CI41 cells, but did not show any inhibitory effect on TNF- $\alpha$ -induced iNOS expression. These findings were further confirmed by time course studies (Fig. 3E,F). Moreover, the blockage of both base level and TNF- $\alpha$ -induced COX-2 expression by siNFAT3 was found at the protein level by western blot (Fig. 3G). These results indicated that the NFAT pathway plays an important role in regulating TNF- $\alpha$ -induced COX-2, but not TNF- $\alpha$ -induced iNOS,



**Fig. 2.** Activation of NFAT3 by TNF- $\alpha$  in CI41 cells. (A)  $8 \times 10^3$  CI41-NFAT Luc cells were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, cells were treated with 5 or 20 U/ml of TNF- $\alpha$  in combination with or without 10  $\mu$ M CsA for 24 hours. The luciferase activity was measured as indicated in Fig. 1A. The results were expressed as the relative NFAT activity. Each bar indicates the mean and standard deviation of triplicate wells. (B) CI41 cells ( $2 \times 10^5$ ) were seeded into each well of a 6-well plate, and cultured in 5% FBS MEM at 37°C. When the cell density reached 70–80%, the cells were exposed to various concentrations of TNF- $\alpha$  for 24 hours, and then washed once with ice-cold PBS and extracted with SDS-sample buffer. The NFAT3 protein levels in the extracts were detected by western blot. (C) CI41 cells were treated with 20 U/ml TNF- $\alpha$  for 12 hours, and then extracted, as described in Materials and Methods. The NFAT3 protein levels in cytoplasm (C) and nuclear extracts (N) were detected by western blot.





**Fig. 3.** Requirement of NFAT3 for COX-2 but not iNOS induction, by TNF- $\alpha$  in CI41 cells. (A,B)  $2 \times 10^5$  cells of CI41 transfectants were seeded into each well of a 6-well plate, and the cells were extracted with Tris-Glycine SDS sample buffer after the cell density reached 90-100%. Western blotting was carried out with specific antibodies as indicated. NF- $\kappa$ B and c-Jun were used as specific controls for NFAT3 siRNA, and GAPDH was used as the protein loading control. (C-F) CI41-COX-2 mass1, CI41-siNFAT3/COX-2 mass1, CI41-iNOS mass1 cells and CI41-siNFAT3/iNOS mass2 ( $8 \times 10^3$ ), were seeded into each well of a 96-well plate. The cells were treated with TNF- $\alpha$  for 72 hours and the luciferase activities were determined. The results were expressed as COX-2 (C,E) and iNOS (D,F) transcriptional induction relative to medium control (relative COX-2 induction or relative iNOS induction). Each bar indicates the mean and standard deviation of triplicate wells. (G) CI41 COX-2 mass1 and CI41 siNFAT3/COX-2 mass1 ( $2 \times 10^5$ ) were seeded into each well of a 6-well plate, and cultured in 5% FBS MEM at 37°C. When the cell density reached 70-80%, the cells were exposed to various concentrations of TNF- $\alpha$  as indicated for 24 hours and 48 hours. The induction of COX-2 at protein level was detected by western blot.

explanation for this may be due to the fact that COX-2 induction and anchorage-independent cell growth could require a certain level of NFAT in the cells. Thus, the low level of NFAT in the cells may not be able to initiate any COX-2 transcription and anchorage-independent cell growth. COX-2 transcription is regulated by NFAT, AP-1 and NF $\kappa$ B. Our data showed that NFAT siRNA specifically reduced NFAT protein expression by more than 80%, and did not show any inhibitory effect on AP-1 c-Jun, NF $\kappa$ B p65 and iNOS expression. We therefore concluded that the effects of NFAT siRNA on COX-2 expression are specific.

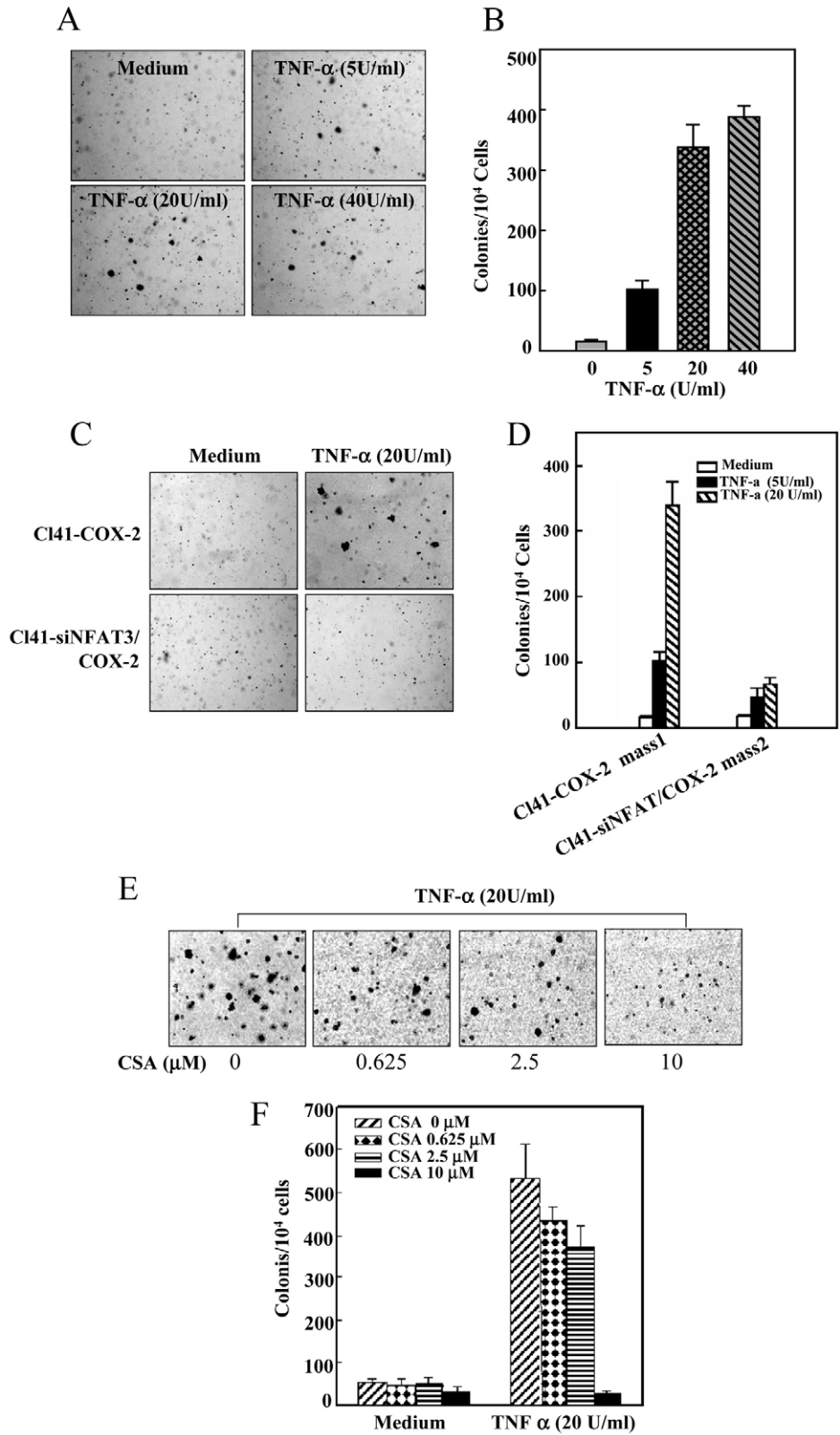
#### Essential role of NFAT3 in TNF- $\alpha$ -induced anchorage-independent growth of CI41 cells

Overexpression of the active mutant NFAT has been reported to induce 3T3-L1 cell transformation (Jauliac et al., 2002; Neal and Clipstone, 2003), and the NFAT family has also been shown to be involved in tumor cell invasion (Jauliac et al., 2002). To reveal the potential contributions of NFAT3 in TNF- $\alpha$ -induced anchorage-independent cell growth, the stable CI41 transfectants of siNFAT3 were exposed to 5, 10, 20 and 40 U/ml of TNF- $\alpha$  in soft agar for 3 weeks. Consistent with our previous findings, incubation of CI41 cells with TNF- $\alpha$  led to the anchorage-independent growth of CI41 cells in a dose-dependent manner (Fig. 4A,B). The maximal induction of anchorage-independent cell growth appeared to be at a concentration of TNF- $\alpha$  at 20-40 U/ml (Fig. 4A,B).

TNF- $\alpha$ -induced anchorage-independent cell growth was dramatically inhibited in the CI41 cells stably transfected with siNFAT3 (Fig. 4C,D), suggesting that NFAT3 plays a crucial role in TNF-induced anchorage-independent cell growth. The requirement of NFAT3 for TNF-induced anchorage-independent cell growth was also confirmed by the findings that cyclosporine A, a chemical inhibitor of the calcineurin/NFAT pathway, was able to inhibit TNF- $\alpha$ -induced anchorage-independent cell growth of CI41 cells (Fig. 4E,F).

which is consistent with previous reports that there is no NFAT binding site in the 5'-flanking region of the iNOS promoter, and that there are NFAT regulatory elements in the 5'-flanking promoter region of human COX-2 (de Gregorio et al., 2001; Iniguez et al., 2000). It may be noted that the knockdown level of the expression of the endogenous NFAT3 protein by NFAT3 siRNA is more than 75%, whereas the effects of the NFAT3 siRNA on the COX-2 promoter activity and TNF- $\alpha$ -induced anchorage-independent cell growth are around 90-100%. The

**Fig. 4.** Essential role of NFAT3 in TNF- $\alpha$ -induced anchorage-independent growth of CI41 cells. (A,C) CI41-COX-2 mass1, CI41-siNFAT3/COX-2 mass2 ( $1 \times 10^4$ ) were exposed to indicated concentrations of TNF- $\alpha$  in 1 ml of 0.33% MEM agar containing 5% FBS over 2.5 ml of 0.5% MEM agar containing 5% FBS in each well of a 6-well plate. After being cultured in a 37°C, 5% CO<sub>2</sub> incubator for 3 weeks, the cell colonies were counted under microscopy, and colonies with more than 16 cells were scored. Each bar indicates the mean and s.d. from triplicate assays. (B,D) Schematic diagrams outlining the anchorage-independent cell growth in Fig. 4A,C. (E) The soft agar assay was performed as indicated in Fig. 3A,  $2 \times 10^4$  cells were exposed to 20 U/ml of TNF- $\alpha$  in combination with various concentrations of CsA as indicated for 3 weeks. (F) Schematic diagram outlining the anchorage-independent cell growth in Fig. 4E.



### Involvement of COX-2 in NFAT3-mediated anchorage-independent growth of CI41 cells induced by TNF- $\alpha$

Previous studies have demonstrated that NFAT can activate expression of a number of genes, including IL-2, IL-3, GM-CSF, TNF- $\alpha$  and COX-2 (Duque et al., 2005; Rao et al., 1997). In light of the important role of COX-2 in regulating cell proliferation, cell survival, and tumorigenesis, it is of great interest to investigate its potential contribution to TNF- $\alpha$ -induced anchorage-independent cell growth. Thus, we constructed the COX-2-specific siRNA vector, and established stable CI41-siCOX-2 cells. As shown in Fig. 5A, siCOX-2 was able to inhibit TNF- $\alpha$ -induced COX-2 expression. More importantly, TNF- $\alpha$ -induced anchorage-independent cell growth was also dramatically inhibited by either siCOX-2 (Fig. 5B,C) or NS398, a selective COX-2 chemical inhibitor (Fig. 5D,E). These results indicate that COX-2 induction, as a downstream event of NFAT3 pathway activation, at least partially accounts for TNF- $\alpha$ -induced anchorage-independent cell growth.

### Discussion

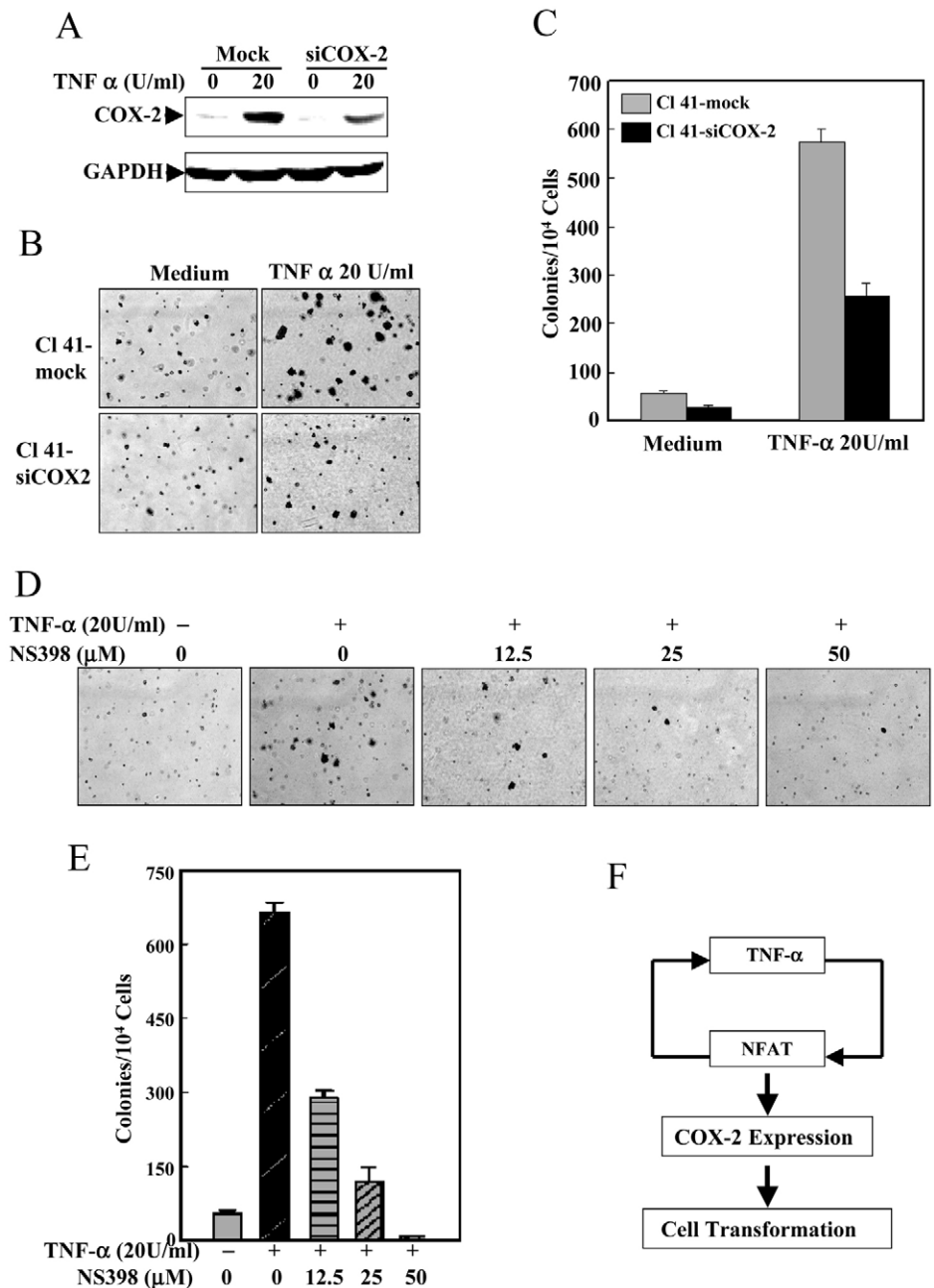
The importance of the tumor microenvironment and inflammation is gaining more and more attention in cancer development (Coussens and Werb, 2002). As a central

mediator of inflammation, the pro-inflammatory cytokine TNF- $\alpha$  was originally considered as an anticancer agent; however, recent studies provided substantial evidence showing that TNF- $\alpha$  may act as an endogenous tumor promoter

(Locksley et al., 2001; Szlosarek and Balkwill, 2003). In vitro studies indicated that TNF- $\alpha$  can induce anchorage-independent growth of mouse epidermal JB6 Cl41 cells (Huang et al., 1999). In animal models, TNF- $\alpha$  knockout (TNF- $\alpha^{-/-}$ ) mice had tenfold fewer skin tumors than wild-type mice after exposure to TPA (Moore et al., 1999). Neutralizing antibodies to TNF- $\alpha$  are sufficient to inhibit TPA-induced skin tumor formation (Scott et al., 2003); TNF- $\alpha$  receptor I (TNFR1)-knockout mice (TNFR1 $^{-/-}$ ) developed less liver metastasis with smaller tumors compared with wild-type mice after intrasplenic administration of a colonic adenocarcinoma cell line (Kitakata et al., 2002). But nonetheless, the underlying molecular mechanisms remain to be extensively resolved. In this study, we demonstrated that induction of COX-2 through NFAT3 plays an important role in regulation of TNF- $\alpha$ -induced anchorage-independent cell growth of Cl41 cells.

TNF- $\alpha$  could induce the activation of multiple signaling pathways and transcription factors, which are believed to be crucial for the promotion of cell transformation. In previous studies, other laboratories and our laboratory demonstrated that activation of JNKs, AP-1 and NF- $\kappa$ B is required for TNF- $\alpha$ -induced cell transformation in JB6 P $^{+}$  cell model (Huang et al., 1999; Li et al., 1997). Arnott et al. compared the activation of PKC $\alpha$  and AP-1 in wild-type and TNF- $\alpha^{-/-}$  mice and revealed that TNF- $\alpha$  was able to mediate tumor

promotion via a PKC $\alpha$ - and AP-1-dependent pathway (Arnott et al., 2002). Very recently, Pikarsky et al. reported that NF- $\kappa$ B activation in hepatocytes, triggered by the inflammatory process through upregulation of TNF- $\alpha$  in adjacent endothelial and inflammatory cells, plays a key role in the development of cholestatic hepatitis and followed hepatocellular carcinoma in Mdr2-knockout mouse (Arnott et al., 2002). In the present study, we demonstrated that NFAT3, the NFAT transcription factor family member that is expressed in nonlymphoid cells, is another important transcription factor involved in regulating TNF- $\alpha$ -mediated cell transformation. Knockdown of NFAT3 expression by its specific siRNA or inhibition of its activity by CsA significantly impaired TNF- $\alpha$ -induced anchorage-independent growth of Cl41 cells. Whereas the NFAT signaling pathway is best known for its role in the regulation of the





immune response, it has become increasingly apparent that this pathway may also play a role in the regulation of a wide variety of cellular responses, such as cell growth and differentiation (Hogan et al., 2003; Horsley and Pavlath, 2002). Previous studies have shown that antagonists of the NFAT family of transcription factors, by using the calcineurin inhibitor CsA, exhibit strong anti-tumor-promoting activity (Mosieniak et al., 1997). CsA was reported to inhibit the growth of C6 glioma cells and induce apoptotic cell death (Mosieniak et al., 1997). Pretreatment of mouse skin with FK506 before each TPA treatment almost completely inhibited tumor formation (Jiang et al., 1993). Since both FK506 and CsA can have calcineurin-independent effects, it is necessary to address the role of NFAT in carcinogenesis with molecular approaches. Neal and Clipstone reported that expression of a constitutively active NFAT2/c1 mutant in 3T3-L1 preadipocytes promotes both anchorage-independent cell growth and the formation of tumors in athymic nude mice (Neal and Clipstone, 2003). Jauliac et al. showed that transfection of dominant negative NFAT1/c2 or NFAT5 in integrin  $\beta_4^+$  breast carcinoma cells inhibited carcinoma invasion, while the expression of a constitutively active calcineurin was able to increase carcinoma invasion significantly (Jauliac et al., 2002). In light of the fact that TNF- $\alpha$  can activate NFAT, which in turn induces the expression of TNF- $\alpha$ , we anticipate that there may be a positive feedback loop between TNF- $\alpha$  and NFAT that is able to augment the inflammation cascade and thereby facilitate tumor promotion (Fig. 5F).

Through activation of signal pathways and transcription factors, TNF- $\alpha$  induces expression of numerous genes including inflammatory mediators, such as COX-2 and/or iNOS, in a variety of cells (Chan et al., 1999; Dhar et al., 2003; Fournier et al., 1997; Itoh et al., 2003; Lee et al., 2003; Lin et al., 2004; Pang and Knox, 1997; Robbins et al., 1997). However, the contribution of these target genes to TNF- $\alpha$ -mediated tumor promotion remain obscure. COX-2 and iNOS are important enzymes that mediate the inflammatory process. They are thought to play an important role in carcinogenesis. Overexpression of COX-2 stimulates cancer proliferation (Sheng et al., 2001; Smith et al., 1996), promotes angiogenesis (Tsujii et al., 1998), inhibits apoptosis (Tsujii and DuBois, 1995), and increases metastatic potential (Tsujii et al., 1997). COX-2 inhibitors are shown to inhibit tumor cell growth (Bae et al., 2001; Liu et al., 1998; Mann et al., 2001) and cell transformation (Chinery et al., 1998; Wong et al., 2004). In animal models, overexpression of human COX-2 in transgenic mice is sufficient to induce mammary gland tumorigenesis (Liu et al., 2001), and a null mutation for COX-2 in APC $\Delta^{716}$ -knockout mice markedly reduces the number and size of intestinal tumors (Oshima et al., 1996). Recently, Krysan et al. reported that PGE2 can activate MAPK/Erk pathway signaling and cell proliferation in non-small-cell lung cancer cells in an epidermal growth factor receptor-independent manner (Krysan et al., 2005; Wang et al., 2005); Wang et al. reported that COX-2-derived PGE2 regulates COX-2 expression by activation of a Ras-mitogen-activated protein kinase signaling cascade, and the COX-2-PGE2-COX-2 self amplifying loop mimics the effects of constitutively active Ras that allows for a distinct growth advantage of intestinal adenoma (Krysan et al., 2005; Wang et al., 2005). Here, we demonstrated that induction of COX-2, but not iNOS via activation of NFAT3, is involved in

TNF- $\alpha$ -induced anchorage-independent cell growth. Impairment of COX-2 either by siRNA or its selective inhibitor markedly inhibited TNF- $\alpha$ -induced anchorage-independent cell growth (Fig. 5). Given that the capacity of anchorage-independent growth of C141 cells in soft agar indicates the tumorigenicity of the cells in nude mouse (Colburn et al., 1988; Huang et al., 1998; Li et al., 1997; Yu et al., 2002), COX-2 induction at least partially contributed to TNF- $\alpha$ -induced transformation of C141 cells. In addition, Dhar et al. reported that specific and non-specific iNOS inhibitors significantly reduced TNF- $\alpha$ -induced NO production in C141 cells, whereas both of them enhanced the TNF- $\alpha$ -induced cell transformation (Dhar et al., 2003), which suggests that iNOS may not play an essential role in TNF- $\alpha$ -induced cell transformation in C141 cells (Dhar et al., 2003). In this study, we found that even though TNF- $\alpha$  was able to induce iNOS expression, this induction was through NFAT3-independent pathways, revealing that NFAT3 siRNA blocked the TNF- $\alpha$ -induced anchorage-independent cell growth via iNOS-independent pathways.

The promoter region of human COX-2 contains a canonical TATA-box and multiple regulatory elements, transcription factors such as nuclear factor  $\kappa$ B (NF- $\kappa$ B), activator protein-1 (AP-1), NFAT, nuclear factor interleukin-6 (NF-IL-6)/CCAAT/enhancer-binding protein (C/EBP), and cAMP-response element-binding protein (CREB) (de Gregorio et al., 2001; Iniguez et al., 2000), which are involved in the regulation of COX-2 expression in cell type and stimuli-dependent manner (Chun and Surh, 2004). AP-1 has been demonstrated to mediate activation of the COX-2 promoter by HER-2/neu, v-Src, platelet-derived growth factor or TPA (Subbaramaiah et al., 2002). NF- $\kappa$ B has been shown to be a positive regulator of COX-2 expression in murine macrophages (D'Acquisto et al., 1997) and human colon adenocarcinoma (Kojima et al., 2000) cell lines exposed to LPS. In mesangial cells, activated NFAT2 by endothelin-1 upregulated COX-2 gene expression (Sugimoto et al., 2001). Duque et al. revealed that NFAT is required for COX-2 induction by TPA plus the calcium ionophore A23187 in human colon carcinoma cell lines (Duque et al., 2005). In this study, we demonstrated that knockdown of NFAT3 by its specific siRNA caused the decrease in COX-2 expression induced by TNF- $\alpha$ , indicating that NFAT3 is important for the induction of COX-2. So we provided the first direct evidence that NFAT3 plays an essential role in the regulation of COX-2 expression in nonlymphoid mouse epidermal cells.

In addition, cell transformation induced by TNF- $\alpha$  is a very complicated process, which results from the coordination of many factors, including the activation of multiple signal pathways and the induction of many molecules. Either of these factors may be required but not enough for this process, thus inhibition of either of these factors may inhibit anchorage-independent cell growth. Our previous study demonstrated that JNK also plays an essential role in this process. Inhibition of JNK dramatically blocked TNF- $\alpha$ -induced anchorage-independent cell growth (Huang et al., 1999). However, it remains to be investigated whether there is any crosstalk between the NFAT and JNK pathways, or whether there is some integration of signaling through these two pathways, or whether there are additional pathways that are also essential for TNF- $\alpha$ -induced anchorage-independent cell growth. These

issues are currently being investigated in our laboratory. In addition, molecules other than COX-2 may also contribute to TNF- $\alpha$ -induced cell transformation through NFAT3 pathway. In fact, it has been demonstrated that expression of a constitutively active NFATc1 mutant causes the sustained expression of D-type cyclins and myc, which thereby affects the cell cycle progression and contributes to the immortalization of the 3T3-L1 preadipocyte cell line (Neal and Clipstone, 2003). Further study of these issues will greatly facilitate our understanding of the complicated process of cell transformation.

In summary, we demonstrated that TNF- $\alpha$  was able to induce NFAT activation, subsequently leading to COX-2 expression, by which cell transformation was mediated. These findings imply that NFAT3/COX-2 may act as a link between inflammation and carcinogenesis by being involved in the tumor promotion stage. They may also provide some evidence to support the notion that NSAIDs and COX-2-selective inhibitors can be used for tumor chemoprevention in patients with chronic inflammation, such as UV radiation, hepatitis and gastrointestinal inflammation.

## Materials and Methods

### Cell culture and construction of siRNA vectors

JB6 P<sup>+</sup> mouse epidermal cell line, C141, and its stable transfectant with a NFAT-Luc reporter containing three NFAT binding sites as described in previous studies (Huang et al., 2000; Rincon and Flavell, 1996), were 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, Rockville, MD) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in the air. The specific small interference RNAs that targeted mouse NFAT3/NFATc4 and mouse COX-2 were designed with a siRNA converter on the website of Ambion Inc., according to the gene sequence in GenBank and guideline of siRNA, and synthesized by Invitrogen. The target sequences were 5'-gccattgactctgcagatg-3' (bases 1409-1427 of NM023699, mouse NFAT3 mRNA) and 5'-agacagatcataagcgagga-3' (bases 686-705 of BC052900, mouse COX-2 mRNA). The siRNA sequences were controlled via BLAST search and did not show any homology to other known human genes. The siRNAs were inserted into pSuppressor vector and verified by DNA sequencing. The siRNA vectors were designated as siNFAT3 and siCOX-2, respectively.

### Stable transfection

The COX-2-luciferase reporter plasmid contains the upstream 5'-flanking region (1432/+59) of the human COX-2 gene promoter linked to the luciferase reporter, as described in previous studies (Subbaramaiah et al., 2001). The iNOS-luciferase reporter plasmid containing 7.2 kb of upstream 5'-flanking region of human iNOS promoter was kindly provided by D. A. Geller (de Vera et al., 1996). COX-2 reporter and iNOS reporter in combination with mock vector or siNFAT3 vector, and siCOX-2 vector were transfected into C141 cells by Lipofectamine 2000 reagent (Gibco BRL, Rockville, MD) according to the manufacturer's instruction. Briefly, C141 cells were cultured in a 6-well plate to 85-90% confluence. Five  $\mu$ g of plasmid DNA (4  $\mu$ g reporter plasmid DNA and 1  $\mu$ g NFAT3 siRNA vector for co-transfection), being mixed with 10  $\mu$ l of Lipofectamine 2000 reagent, were used to transfect each well in the absence of serum. After 6 hours, the medium was replaced with 5% FBS MEM. Approximately 36-48 hours after the beginning of the transfection, the medium was replaced with 5% FBS MEM containing 500  $\mu$ g/ml G418 (Gibco BRL, Rockville, MD). After selection for 28-45 days with G418, the stable transfectants with reporter genes were identified by measuring the basal level of luciferase activity, and the stable transfectants with siRNA were verified by analyzing the downregulation of targeted gene expression by western blot. To exclude any bias from the pressure of cell single-clone selection, we generated 'mass stable clones.' After transfection with plasmids in each well of a 6-well plate and selecting with G418, more than 15 colonies per well were grown. Instead of ring cloning these cells, all colonies in each well were grown as a 'mass stable culture'. Stable transfectants, C141-siNFAT3/COX-2 mass1, C141-siNFAT3/iNOS mass1 and mass2, C141-COX-2 mass1, C141-iNOS mass1, C141-mock and C141-siCOX-2 were established and cultured in G418-free 5% FBS MEM for at least two passages before each experiment.

### COX-2, iNOS and NFAT reporter assays

Confluent monolayer cells of the transfectants were trypsinized, and 8 $\times$ 10<sup>3</sup> viable cells suspended in 100  $\mu$ l of 5% FBS MEM were added to each well of a 96-well

plate. The plates were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air. After the cell density reached 80-90%, the cells were exposed to 20 U/ml of TNF- $\alpha$  for COX-2 and iNOS induction, and to 5 or 20 U/ml of TNF- $\alpha$  in combination with or without 10  $\mu$ M cyclosporine A (CSA) for NFAT induction. The luciferase activity was determined by the luciferase assay at different periods of time of incubation with TNF- $\alpha$  (Promega, Madison, WI) using a luminometer (Wallac 1420 Victor 2 multilable counter system) after the addition of 50  $\mu$ l of lysis buffer for 30 minutes at 4°C. The results were expressed as relative luciferase units (RLU) or COX-2 or iNOS transcriptional induction relative to medium control (relative COX-2 or iNOS transcriptional induction). The Student's *t*-test was used to determine the significance of the differences of COX-2 and iNOS induction. The differences were considered significant at a *P*  $\leq$  0.05.

### COX-2 expression assay

A total of 2 $\times$ 10<sup>5</sup> C141 and its transfectants were cultured in each well of a 6-well plate to 80% confluence. After exposure to TNF- $\alpha$  for 24 hours and/or 48 hours, the cells were washed once with ice-cold PBS and then extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with a rabbit-specific antibody against COX-2 (Cayman Chemical, Ann Arbor, MI). The protein band, specifically bound to the primary antibody, was detected using an anti-rabbit IgG-AP-linked and an ECF western blotting system (Amersham Biosciences, Piscataway, NJ).

### RT-PCR

2 $\times$ 10<sup>5</sup> of C141 cells were cultured in each well of a 6-well plate to 80% confluence. After exposure to TNF- $\alpha$ , the cells were extracted with Trizol reagent (Invitrogen, USA), and the total RNA was isolated following the manufacturer's instructions. 1  $\mu$ g of total RNA was reverse-transcribed (Superscript II, Invitrogen), and cDNA was subjected to PCR amplifications using primer pairs specific for mouse iNOS (forward: 5'-cct tgt tca gct acg cct tc-3'; backward: 5'-ctg agg gct ctg ttg agg tc-3'), mouse COX-2 (forward: 5'-tcc tcc tgg aac atg gac tc-3'; backward: 5'-gct cgg ctt cca gta ttg ag-3') and  $\beta$ -actin (forward: 5'-cat ceg taa aga cct cta tgc c-3'; backward: 5'-acg cag ctc agt aac agt cc-3'), which served as the internal control. The PCR cycling conditions were: 30 cycles of 95°C for 45 seconds, 58°C for 45 seconds and 72°C for 45 seconds.

### PGE2 production measurement

PGE2 levels were determined in the 48 hour culture medium by ELISA according to the instructions of the manufacturer (Cayman Chemical, Ann Arbor, MI). Values were expressed as the mean  $\pm$  s.d. of triplicate samples.

### NFAT3 translocation assay

C141 cells were treated with 20 U/ml of TNF- $\alpha$  for 12 hours, and then extracted by using CellLytic™ NuCLEAR™ Extraction Kit (Sigma, Missouri, USA) according to the manufacturer's instruction. Briefly, the cells were incubated in the hypotonic buffer for 15 minutes. The swollen cells were lysed by adding 10% IGEPAL CA-630 solution to a final concentration of 0.6%, and then centrifuged. The supernatant is the cytoplasm fraction. The pellet was resuspended in Extraction Buffer, and agitated for 30 minutes on a vortex mixer. After centrifugation, the supernatant was collected (nuclear extracts). The extracts were quantified with Dc protein assay kit (Bio-Rad, CA), separated on polyacrylamide-SDS gels, and analyzed by western blot with the antibody against NFAT3 (Santa Cruz Biotechnology, Santa Cruz, CA) as described above.

### Anchorage-independent colony growth assays

Soft agar colony formation assays were performed as described previously (Huang et al., 1999). Briefly 2.5 ml of 0.5% agar in MEM supplemented with 10% fetal bovine serum and stimulus reagents of interest was laid onto each well of a 6-well tissue culture plate. 1 ml of C141 cells was mixed with 2 ml of 0.5% agar MEM and layered on top of the 0.5% agar layer. The plates were incubated at 37°C in 5% CO<sub>2</sub> for three weeks, then the colonies were counted under microscopy, and colonies with more than 16 cells were scored.

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