Published in final edited form as:

Cell Mol Life Sci. 2009 December ; 66(24): 3967–3978. doi:10.1007/s00018-009-0157-5.

Doxorubicin-mediated Apoptosis in Glioma Cells Requires NFAT3

Sreelatha Gopinath¹, Sravan K. Vanamala¹, Meena Gujrati², Jeffrey D. Klopfenstein³, Dzung H. Dinh³, and Jasti S. Rao^{1,3,*}

¹Department of Cancer Biology & Pharmacology, University of Illinois–College of Medicine at Peoria, Peoria, IL, 61656, USA

²Department of Pathology, University of Illinois–College of Medicine at Peoria, Peoria, IL, 61656, USA

³Department of Neurosurgery, University of Illinois–College of Medicine at Peoria, Peoria, IL, 61656, USA

Abstract

Nuclear Factor of Activated T cells (NFAT), a family of transcription factors, has been implicated in many cellular processes, including some cancers. For the first time, the present study characterizes the role of NFAT3 in doxorubicin (DOX) mediated apoptosis, migration, and invasion in SNB19 and U87 glioma cells. This study demonstrates specific knockdown of NFAT3 results in a dramatic inhibition of the apoptotic effect, induced by DOX, and favors cell survival. Inhibition of NFAT3 activation by shNFAT3 (shNF3) significantly downregulated TNF- α induction, its receptor TNFR1, caspase 10, caspase 3 and PARP, abrogating DOX-mediated apoptosis in glioma cells. DOX treatment resulted in NFAT3 translocation to the nucleus. Similarly, shNF3 treatment in SNB19 and U87 cells reversed DOX-induced inhibition of cell migration and invasion as determined by wound healing and matrigel invasion assays. Taken together, these results indicate that NFAT3 is a prerequisite for the induction of DOX-mediated apoptosis in glioma cells.

Keywords

glioma; SNB19; U87; NFAT3; doxorubicin and apoptosis

Introduction

Despite aggressive surgery, radiotherapy, and chemotherapy, malignant brain tumors remain a therapeutic challenge [1]. An inherent resistance to apoptosis may account for the low success rate of glioma treatments [2,3]. However, because the effector arm of the apoptotic pathway often remains intact, these cells can be induced to undergo apoptosis by direct stimulation of the cell death receptors in the TNFR superfamily [4].

Nuclear Factor of Activated T cells (NFAT) is a family of transcription factors initially discovered in T cells, which play a crucial role in immunity. More recently, however, the NFAT family has been implicated in many cellular processes including malignancies [5]. Further studies indicate that the NFAT signaling is involved in the regulation of cell growth and development in a wide variety of different tissues and cell types [6,7]. NFAT3 is one of the five known isoforms of this family of transcription factors, which are known to be involved in

*Correspondence: Jasti S. Rao, Ph.D., Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine, Box 1649, Peoria, IL, 61656, USA; phone (309) 671-3445; fax (309) 671-3442; jsrao@uic.edu.

activity-dependent gene expression, linking surface receptor stimulation to nuclear events, and ultimately altering the fate of the cell. Unlike NFAT1, -2 or -4, NFAT3 is expressed in non-immune tissues, including heart, brain, and breast [8,9] where it is thought to influence such varied processes as cardiac hypertrophy, learning and memory, adipocyte differentiation, and breast cancer development [10–15].

NFAT1, -2, -3, and -4 are cytosolic proteins, constitutively expressed in resting cells, and their activation is regulated by calcium and the Ca²⁺/calmodulin-dependent serine-phosphatase, calcineurin [16]. Upon stimulation, NFATs are dephosphorylated by calcineurin, and they are then translocated to the nucleus where they bind to consensus DNA sites and control the expression of target genes including: IL-2, IL-4, IL-5, IL-13, IFN- γ , TNF- α and COX-2 [17–21]. Activation of NFAT is sensitive to calcineurin inhibitors and immunosuppressive agents, such as cyclosporin A and FK506 [16,22].

DOX is an anti-tumor drug that is being widely used in the treatment of a broad spectrum of cancers [23,24]. The pro-apoptotic effects of DOX are well established, as it can induce cell death via DNA damage through topoisomerase II inhibition, by inducing expression of the tumor suppressor protein, p53, and by free radical generation through redox reaction [25–27]. DOX has shown a marked cytotoxic effect against malignant glioma cells *in vitro* [28]. DOX induced up regulation of TNF- α , uPA, IL-8, and MCP-1 in a number of lung cancer cells was reported by Niiya, et al. [29] (2003). Highly enhanced anti-tumor effect was also reported with systemic administration of TNF- α in combination with DOX for lung tumors [30,31]. Kalivendi, et al. [31] documented that DOX activated the transcription factor NFAT, leading to the upregulation of Fas/Fas L-dependent apoptosis through a calcium/calcineurin-signaling pathway in cardiac cells.

So far, there has been no comprehensive study published on the role of DOX in NFAT activation in cancer cells. Given the importance of cell cycle, apoptosis, and angiogenesis phenomena in the development of cell malignancies, it is of considerable interest to understand the mechanisms by which NFAT affects cell growth, differentiation and function. In the present work, we studied the role of NFAT3 in DOX-mediated apoptosis in SNB19 and U87 glioma cell lines. The hypothesis that DOX causes apoptosis in these cells was tested by FACS analysis and DNA fragmentation. For the first time, we have shown the role of the NFAT3 isoform in SNB19 and U87 glioma cell lines in DOX-mediated cellular apoptosis.

Materials and Methods

Construction of a vector expressing shRNA for NFAT3

pSilencerCMV3.1 (Ambion) was used to construct a vector expressing shRNA for NFAT3 downstream of the cytomegalovirus promoter (Fig. 1A). Exons 4, 5, and 6 were targeted simultaneously by including 21 bases corresponding to each exon. The following was used for the NFAT sequence:

aatggatccGCCACTGACCCTACAGATGTTCAAGAGACATCTGTAGGGTCAGTGGCTT
CAAGAGATTCAAGAGAGGGTGAGACGGACATCGGGTTCAAGAGACCCGATGTC
CGTCTCACCTTCAAGAGATTCAAGAGACTGGTACTGACTGGCTCCATTCAAGA
GATGGAGCCAGTCAGTACCAGaagcttcg. It is 93 bases in length with *Bam*HI and *Hind*III sites incorporated at the ends, and with a nine base loop region between each 21 base molecule. The oligo was self-annealed in 6X SSC using standard protocols and ligated on to the *Bam*HI and *Hind*III site of a pSilencerCMV3.1 vector. This yielded a shRNA expression plasmid for NFAT3, which was designated shNF3. BGH poly-A terminator served as a stop signal for RNA synthesis.

Cell culture and transfection of shRNA vectors

For this study, we used the established human glioblastoma cell lines SNB19 and U87. Cells were grown in Dulbecco's modified Eagle's medium, supplemented with 1% glutamine, 100 µg/mL streptomycin, 100 units/mL penicillin, and 10% fetal bovine serum (pH 7.2–7.4) in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were sub-cultured every three to five days. Cells were grown in 100 mm dishes or six well plate for all treatment conditions and on eight-well chamber slides for immunocytochemistry and tunel assays.

Scrambled vector (pSV) and shNF3 vector were transfected into SNB19 and U87 cells independently by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Briefly, the cells were cultured in a 100 mm dish to 85–90% confluence. Then, 21 µL of lipofectamine (diluted in 100 µL of serum-free medium) was added dropwise to 7 µg of plasmid DNA (in 100 µL of serum-free medium). This mixture was incubated for 30 min and was then used to transfect each plate in the absence of serum. After six hours, the medium was replaced with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and the cells were incubated for another 12 to 24 hours. DOX (1µM/L) was added to these cells in the absence of serum and incubated for an additional 24 hours. To study the role of TNF-α in DOX induced NFAT3 mediated apoptosis, cells were also treated with TNF-α protein (200 U/ml) for 24 h.

RT-PCR analysis

A total of 2×10^5 of SNB19 and U87 cells were independently cultured in each well of six-well plates to 80% confluence. After transfection with either shNF3 or pSV and subsequent treatment with DOX, the cells were extracted with Trizol reagent (Invitrogen, USA), and the total RNA was isolated as per the manufacturer's instructions. One µg of total RNA was reverse-transcribed (Superscript II, Invitrogen), and the cDNA was subjected to PCR amplification targeting NFAT3 (forward primer 5'-AGGCCTACAGCCCCAGTG-3' and reverse primer 5'-CGCCCATTTGGAGACATAAAA-3') and GAPDH (forward primer 5'-AGCCACATCGCTCAGACACC-3' and reverse primer 5'-GTACTCAGCGGCCAGCATCG-3'). The PCR cycling conditions were: 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec and a final extension of 72°C for 10 min.

Western blot analysis

SNB19 and U87 cells were washed with ice-cold DPBS 48 hours after treatment and resuspended in 150 µL of radioimmune precipitation assay buffer (20 mmol/L Tris-HCl (pH 7.4), 2.5 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 100 mmol/L NaCl, and 100 mmol/L sodium fluoride) containing 1 mmol/L of sodium vanadate, 10 µg/mL of aprotinin, and 10 µg/mL of leupeptin. Cells were then homogenized using five ultrasonic pulses of one second each. The lysate was centrifuged at $12,000 \times g$ for 10 min at 4°C to remove cellular debris.

Equal amounts of protein were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were probed overnight with antibodies against NFAT3, phospho-NFAT3, AKT, NFAT4, phospho-AKT, JNK, phospho-JNK, Bcl2, TNF-α, Cyclin E, Cyclin B, Caspase-10, Caspase-3, PARP, and GAPDH (Santa Cruz, CA, USA). The membranes were subsequently washed three times with PBS to remove excess primary antibodies, incubated with appropriate HRP-conjugated secondary antibodies, and then developed according to enhanced chemiluminescence protocol (Amersham, Arlington Heights, IL).

NFAT3 translocation assay by immunofluorescence

NFAT expression and localization were observed by immunocytochemistry assay according to published standard protocols. Human glioma cells SNB19 and U87 grown in eight-well chamber slides were treated as described previously and were fixed (4% paraformaldehyde, 10 min at room temperature), permeabilized (cold methanol for 2 min), and rehydrated (PBS for 10 min). PBST containing 2% BSA was used for blocking the cells for one hour followed by a two-hour incubation with anti-NFAT3 antibodies (Santa Cruz, CA, USA) at a dilution of 1:300 in PBST containing 2% bovine serum albumin and 1.5% horse serum, followed by a final incubation with Texas Red conjugated secondary antibody (1:1000 in PBS/2% bovine serum albumin, 0.5% tween 20) for one hour. NFAT expression was visualized by fluorescence microscopy (Olympus IX71) and photographed.

DNA fragmentation assay

SNB19 and U87 cells were cultured in six-well culture plates at a concentration of 2×10^5 cells/well. After a 24-hour incubation period, cells were transfected with shNF3 vector or pSV. Untreated cells were also cultured under similar conditions and served as the control. Genomic DNA was extracted as per protocol described by Huang, et al. [32]. In brief, the cells were resuspended in 50 μ L of PBS, fixed in 1 mL of ice cold 70% ethanol and stored at -20°C overnight. After fixation, the mixture was centrifuged at 6000 rpm at 4°C for 5 min, and the pellet was resuspended in 50 μ L of phosphate-citrate buffer (192 parts of 0.2M Na_2HPO_4 and 8 parts of 0.1M citric acid, pH 7.8) and then left at room temperature for 30 min. After another centrifugation at 10,000 rpm for 5 min, the supernatant was transferred to 0.5 mL Eppendorf tubes and incubated at 37°C with 3 μ L of NP40 (0.23%) and 3 μ L of RNase (1mg/mL). The solution was further incubated for 30 min after adding 3 μ L of Proteinase K (1mg/mL). The DNA content in the mixture was quantified using a spectrophotometer and 10 μ g of DNA was electrophoresed on a 2% agarose gel using TAE buffer. DNA fragmentation was visualized under UV light after staining with ethidium bromide.

Matrigel invasion assay

SNB19 and U87 cells were transfected with either shNF3 or scrambled vector and treated with DOX as described above. After treatment, cells were trypsinized and 2×10^5 cells were placed into matrigel-coated transwell inserts with an 8- μ m pore size. Cells were allowed to migrate through the matrigel for 24 hours. The cells in the upper chamber were removed with a cotton swab. The cells that adhered to the outer surface of the transwell (i.e., the cells which had invaded the matrigel) were fixed, stained using the Hema-3 staining kit, and counted under a light microscope as described previously [33].

In situ terminal-deoxynucleotidyl transferase mediated dUTP nick endlabeling (TUNEL) assay

The TUNEL assay was carried out to detect apoptotic cells after the above-described treatments. SNB19 and U87 cells were cultured on eight-well chamber slides at a density of 2×10^3 per well. After 24 hours, the cells were transfected with either shNF3 vector or scrambled vector in serum-free medium. Twenty-four hours later, the cells were treated with DOX and incubated for another 24 hours. The cells were fixed after termination in 10% phosphate-buffered formalin for 15 min. TUNEL staining for detection of apoptotic cells was carried out using the TUNEL Apoptotic Detection kit (Roche Molecular Biochemicals, Indianapolis, IN) as per the manufacturer's instructions. Briefly, the fixed cells were washed three times in PBS (5 min/wash). The cells were then incubated with 0.05% Tween 20 in PBS containing 0.2% bovine serum albumin for 15 min at room temperature. The cells were washed twice in PBS and incubated with 50 μ L of terminal deoxynucleotidyl transferase end-labeling cocktail for 60 min at room temperature. The reaction was terminated, and the slides were washed three times in PBS and blocked with blocking buffer for 20 min at room temperature.

Then, the slides were incubated with 50 μ L of avidin-Texas red in the dark for 30 min at room temperature, washed three times in PBS, and mounted with anti-fading gel mount (Biomedex, Foster City, CA). Slides were allowed to dry in the dark, observed under a fluorescent microscope (Olympus IX71), and photographed.

FACS analysis

Induction of apoptosis in response to shRNA-induced downregulation of NFAT3 and DOX individually and in combination was tested using FACS analysis. FACS analysis was performed according to the manufacturer's instructions and 10,000 cells were recorded for each transfection. Cells were treated as described earlier and were trypsinised, washed with 1X PBS, and incubated for 30 min with 1 mL of propidium iodide in the dark. After the incubation period, the cells in propidium iodide were analyzed using a FACS Calibur flow cytometer (BD BioSciences, San Jose, CA) with an excitation wavelength of 488 nm and emission wavelength of 530 nm.

Wound healing assay

To study cell migration and cell interactions, cells were seeded at a density of 2×10^6 in a six-well plate and treated as described earlier with shNF3 and DOX. After the transfection and the DOX treatment was complete, a straight scratch was made in individual wells with a 200 μ L pipette tip. This point was considered as the zero hour, and the width of the wound was photographed under the microscope. After 18 to 24 hours, the cells were checked for the wound healing and again photographed under microscope. Wound healing was measured by calculating the reduction in the width of the wound after incubation.

Isolation of nuclear and cytoplasmic cell fractions

SNB19 and U87 cells cultured in 100 mm petri dishes were treated with shNF3 and DOX as described earlier. Harvested cells were washed once with 1X PBS and the cell pellet was resuspended in 300 μ L of cytoplasmic extraction buffer (10mM HEPES, pH 7.9; 1.5mM MgCl₂, 10mM KCl, 0.5mM DTT, 0.3mM sodium vanadate, 1mM PMSF, 10 μ g/mL Aprotinin and 10 μ g/mL leupeptin). This mixture was incubated for 10 min at 4°C and then centrifuged at 6000 rpm at 4°C for 10 min. The supernatant (cytoplasmic extract) was collected and the remaining pellet was suspended in 250 μ L of nuclear extraction buffer (20mM HEPES, pH 7.9; 1.5mM MgCl₂, 420mM NaCl, 0.5mM DTT, 0.2mM EDTA, 0.3mM Sodium vanadate, 1mM PMSF, 10 μ g/mL Aprotinin and 10 μ g/mL leupeptin). The fluid was incubated for 30 min on ice, centrifuged at 13,000 rpm for 5 min at 4°C and the supernatant (nuclear extract) was collected and used for western blot analysis.

Results

Knockdown of NFAT3 expression upon RNAi treatment

We transfected SNB19 and U87 cells with either pSV and/or shNF3. RT-PCR and immunoblot analysis showed significant decrease in NFAT3 expression in shNF3 treated cells compared to controls (Fig. 1C and 1D). GAPDH activity was analyzed at both mRNA and protein levels to serve as a loading control (results confirmed equal loading). Taken together, these results demonstrate the efficient downregulation of NFAT3 with the shNF3 plasmid. To determine the specificity of the shNF3 construct we have immunoblotted the proteins after treatment for other NFAT's. Figure 1D shows the unaltered NFAT4 expression after the shNF3 treatment.

DOX-induced apoptosis in glioma cells requires NFAT3

The mechanism of DOX-induced apoptosis via distinct pathways has been well elucidated in different cancers, including gliomas. To investigate the potential role of NFAT3 in DOX-

mediated apoptosis, SNB19 and U87 cells were transfected with the NFAT3 shRNA expression vector or pSV (negative control) and then with DOX. We found that DOX treatment was able to induce apoptosis in glioma cells. Importantly however, suppression of endogenous NFAT3 enhanced cell survival with shRNA alone or shRNA and DOX treatment together as compared to DOX treatment alone (Fig. 2A).

Further studies showed that the role of NFAT3 in DOX-induced apoptosis was mediated by upregulation of its target gene, TNF- α . This conclusion was based on the findings that inhibition of NFAT3 activation by shNF3 dramatically downregulated TNF- α induction, its receptor TNFR1, caspase 10, caspase 3 and PARP, sequentially, and thus led to abrogation of DOX-mediated apoptosis in glioma cells. DOX treatment led to increased expression of the aforementioned molecules whereas shNF3 treatment alone or in combination with DOX showed decreased expression in both cell lines (Fig. 2A), which indicates that NFAT3 is required for DOX-mediated apoptosis. Moreover, caspases 3, 10 and PARP (molecules that mediate the apoptotic mechanism) were cleaved and activated by DOX as shown by western blot analysis of cell lysates (Fig. 2A). Cells treated with shNF3 showed no indication of activated caspases or cleaved PARP. shNF3 inhibited apoptosis was further confirmed by the increased expression of anti-apoptotic molecule, Bcl2. Certain molecules like AIF, p53 and cleaved BID were unchanged with any of the treatments (supplement figure 1). Addition of TNF- α protein increased the expression of caspase-10 and PARP irrespective of the treatments (supplement figure 2).

DOX is known to induce cellular apoptosis by intercalating into the DNA. The results of the TUNEL assay showed increased DNA damage and fragmentation in glioma cells treated with DOX as compared to controls and cells transfected with pSV (Fig. 2B). Knockdown of NFAT3 reduced the DNA damage caused by DOX. We were also able to observe DNA fragmentation after purifying total nuclear DNA, which was electrophoretically separated on an agarose gel. Cells treated with DOX underwent DNA degradation, which indicate apoptosis, whereas shNF3 treatment in combination with DOX showed no degradation of DNA (Fig. 2C).

shNF3 treatment reversed DOX-mediated inhibition of cell invasion through matrigel

SNB19 and U87 cells transfected with shNF3 or shNF3 and DOX treatment invaded matrigel-coated filters to a greater extent than did the cells treated with DOX alone (Fig. 3A). The percent of invasive cells after the treatment with shNF3 or shNF3/DOX combination to the lower side of the membrane at 48 hours were 95% (SNB19), 82% (U87) and 90% (SNB19), and 80% (U87) respectively. Only 18% and 15% of the DOX alone treated SNB19 and U87 cells invaded to the lower side of the membrane during the same period of time (Fig. 3B). Respective controls showed 85–100% of cell invasion.

shNF3 treatment reversed DOX-mediated inhibition of cell migration in wound healing assay

shNF3 treatment in SNB19 and U87 cells also reversed DOX-induced inhibition of cell migration as determined by wound healing assay, wherein a wound was made in a sub-confluent cell monolayer and cells were allowed to migrate into the cell-free area. The distance moved by the cells in treated and untreated wells were compared (Fig. 3C). The percent of wound repaired by cell motility of shNF3 and combination of shNF3 and DOX treatment were 45% (SNB19), 52% (U87) and 35% (SNB19), and 52% (U87) respectively. In contrast, the percent of wound repair in DOX alone treatment were 5% (SNB19) and 10% (U87) (Fig. 3D). These results indicate that after knockdown of NFAT3 and treatment with DOX, the cells were able to divide and survive, suggesting an important role being played by the NFAT3 in DOX-mediated cell migration.

NFAT3 is translocated into the nucleus by DOX

It is known that NFATs in the phosphorylated form reside in cytoplasm and in dephosphorylated form in the nucleus. Immunocytochemistry showed that the shNF3 treatment of cells led to reduction of NFAT3 in the cytosol as well as in nucleus 48 hours after treatment. DOX treatment translocated NFAT3 into the nucleus, and the combination of shNF3 and DOX showed very little or no NFAT3 in cytoplasm or nucleus. In the control cells and the scrambled vector treatment NFAT3 was present only in cytoplasm (Fig. 4). This was also proved by western blot analysis of nuclear and cytoplasmic extracts from these cells. As shown in supplement figure 3, when nuclear extracts were probed with anti NFAT3 antibody, the DOX treatment showed increased expression as compared to the control and no expression with the shNF3 treatment. Similarly the cytoplasmic extracts, when probed with phospho-NFAT3, showed decreased expression of NFAT3 as compared to control and less or no expression in shNF3 treatment. These results indicate that DOX translocates cytosolic NFAT3 into the nucleus and thus leads to apoptosis.

Downregulation of NFAT3 by shNF3 reverses DOX-induced inhibition of cell cycle progression

FACS analysis of SNB19 and U87 cells treated with DOX resulted in apoptosis. Percent of cells in sub G₀/G₁ phase was as high as 61–64% and 66–68% in the cells treated with DOX alone in SNB19 and U87 respectively. Whereas, only 2–13% of cells in sub G₀/G₁ phase was found in controls and shNF3 with or without DOX treated cells (Fig. 5A). The cell cycle progression into the S phase requires the enzyme cdk2, which is inhibited by p21. In the present study, we observed that the amount of p21 expression was lower after treatment with shNF3 or shNF3 and DOX, than in the treatment with DOX alone. Similarly, increased expression of cyclin E and pcdk2 was observed, which indicates the role of NFAT3 in cell cycle progression and DOX-mediated apoptosis. Expression of cyclin D1 was unchanged (Fig. 5B). Collectively, these results provide direct evidence for the important role of NFAT3 activation in DOX-induced apoptosis by upregulation of TNF- α expression in glioma cells.

Discussion

Programmed cell death occurs in virtually all cell types and is precisely regulated at the cellular and molecular levels. NFAT proteins have been shown to bind to promoter regions and upregulate the expression of some effectors of apoptosis, such as TNF- α and FasL [16]. Most NFATs perform their function by partnering with other transcription factors and are known for their controversial properties like tumor suppression and pro-apoptotic activity. Yan, et al. [34] demonstrated that inhibition of COX-2 expression with diminishing endogenous NFAT3 blocked the transformation of C141 cells under the treatment of TNF- α . Similarly, Li et al [35] also documented the role of NFAT3 in regulation of iNOS and in suppression of cell transformation suggesting a tumor suppressor activity of NFAT3. In contrast, tumor development via angiogenesis and apoptotic resistance with the upregulation of COX-2 has also been documented in variety of human cancer cells [36–38]. This could be due to the inability of NFATs to partner with other transcription factors. Studies by Ranger et al., [39] revealed that deletion of NFAT1 in cartilage cells leads to the uncontrolled proliferation and exhibit cancer phenotypes both *in vitro* and *in vivo*, suggesting a potential tumor suppressor role of NFAT1 in this special cell lineage. NFAT3 was also found to enhance estrogen receptor transcriptional activity in breast cancer cells and play an important role in regulation of breast cancer cell growth [15,40]. These conflicting results highlight the versatility of NFAT proteins in cell fate determination, depending on their functional modulations under certain condition [6,41]. Zhang, et al. [42] demonstrated that NFAT3, as a new kind of cofactor, displays dual transcription modulation modes dependent on tissue types. NFAT3 acted as a transcription activator of estrogen receptors in breast cancer cells and inhibited their transcription activities

in all cell types derived from kidney tissue. Recently, NFAT transcriptional activity has been reported to regulate the acetylcholinesterase promoter during ionophore-induced apoptosis in HeLa cells [43].

In the present study, we demonstrated that treatment of SNB19 and U87 glioma cell lines with shNF3 resulted in downregulation of NFAT3. DOX treatment of these cells resulted in apoptosis in a dose-dependent manner (supplement figure 4). We further investigated the influence of NFAT3 on DOX-mediated apoptosis by NFAT3 downregulation in SNB19 and U87 cells. Western blot analysis showed a decrease in expression of TNF- α , a downstream element of NFAT3, which is known for its roles in immunity and cellular remodeling as well as influencing apoptosis and cell survival [44]. Data from many different studies underlined the importance of TNF- α activation in human cancers. DOX induced TNF- α , uPA, IL-8 and MCP-1 in a number of lung cancer cells with different levels of expression has been reported by [29]. Enhanced anti-tumor effect was also reported with systemic administration of TNF- α in combination with DOX for lung tumors [30]. Cells lacking NFAT1 showed decreased expression of TNF- α [45]. In the present study, decreased expression of TNF- α lowered the expression of TNFR1, caspase 10, caspase 3, and PARP. Similar results were obtained when shNF3 was used in combination of DOX. Moreover, DOX treatment alone increased the above-mentioned molecules. Addition of TNF- α also increased the expression of caspase-10 and PARP. This shows that NFAT3 has a pro-apoptotic role in glioma cells. Thus, we anticipate that NFAT3 represents a putative tumor suppressing mechanism and downregulation of NFAT3 reversed the apoptotic mechanism induced by DOX.

There were certain molecules (e.g., p53, AIF and cleaved BID) that were unchanged with either treatment. p53-mediated apoptosis is common in tumor cells when treated with DOX and is dependent on the Apaf-1/caspase-9 pathway involving cytochrome *c* release from mitochondria [46]. The p53-mediated activation of calcineurin in lung, renal, colon, and ovarian carcinoma cells and subsequent activation of NFAT to induce apoptosis has been reported as well [47]. However, in the present study, we did not observe any change in the expression of p53 or in cleaved BID, which in turn releases cytochrome *c* from mitochondria.

Under basal conditions, NFAT3 proteins are present in the cytoplasm in its phosphorylated state and, upon increase in the intracellular calcium, it translocates to nucleus by dephosphorylation at multiple serine residues [48–50]. In the present study, we found that NFAT3 translocated to the nucleus with DOX treatment and that there was little or no NFAT3 in the nucleus of control cells as well as the cells treated with shNF3 alone or in combination of DOX. This could be due to increase in the calcium levels of the cells when treated with DOX. Similar type of results where DOX activated the transcription factor NFAT, leading to the upregulation of Fas/FasL-dependent apoptosis through a calcium/calcineurin-signaling pathway, has been documented in rat cardiac cells [31]. Yaghi and Sims [51] reported NFAT3 nuclear translocation in smooth muscle cells when treated with PE or 20-HETE.

The NFAT family of proteins plays an important role in regulation of genes that control cell cycle progression, cell development and differentiation, angiogenesis, and possibly tumorigenesis [17,52–55]. Thus, in the present study the effect of shNF3 on cell cycle progression was studied. We found that DOX treatment led the cells to apoptosis. Interestingly, shNF3 treatment with or without DOX showed only 7–13% of apoptotic cells, suggesting that decreased expression of NFAT3 in the cells will lead to survival and vice-versa. It also suggests the important role played by NFAT3 in DOX-mediated apoptosis. This was proved by the western blot analysis for cell cycle-controlling genes like phospho-cdk2 and cyclin E, which were increased in expression with DOX treatment and decreased with shNF3 alone or with a combination of shNF3 and DOX. These results suggest that NFAT3 is required for DOX to inhibit cell cycle progression and has a central role in cell cycle control. Baksh, et al. [56]

demonstrated that the calcineurin/NFAT1 pathway negatively regulates the expression of cyclin-dependent kinase 4 (CDK4) in Jurkat cells. NFATs need not be always repressors of cell cycle controlling genes. Accumulating evidence suggests that the phosphatase calcineurin plays a major role in the regulation of cell cycle progression by acting during the early stages of G1 phase [57–61]. In addition, it has also been demonstrated that overexpression of NFAT2 in pre-adipocyte 3T3-L1 cells promoted cell cycle progression even under low serum concentrations and induced altered expression of cell cycle-related genes, such as cyclin D1, cyclin D2, pRB, and c-Myc [62].

Infiltrative and destructive growth patterns of malignant gliomas are characterized by their migratory and invasive properties. Infiltrative growth prevents complete tumor resection and therefore causes significant neurological morbidity and mortality [63]. Jauliac, et al. [64] reported that two dominant negative NFATs, one deleted in the carboxyl terminus of the sequence necessary for DNA-binding¹², and the VIVIT peptide¹³, which blocks the ability of calcineurin to activate NFAT inhibited carcinoma invasion when transfected into MDA-MB-435. In the present study, we found that downregulation of NFAT3 alone or in combination with DOX increased the migration and invasion when compared to DOX alone treated cells. Impaired migration in DOX treated cells could be due to active apoptosis caused by DOX as seen in Tunel assay. Nevertheless, these data implicate conventional NFATs in carcinoma migration and invasion.

NFAT3-induced, TNF- α -mediated apoptosis by DOX treatment include multiple signal molecules as described earlier. Conflicting role of TNF- α in cell cycle control [30,44] demands further investigation of whether NFAT3 role in cell cycle control is through TNF- α or mediated by other molecule. These issues are currently being investigated in our laboratory. Since, downregulation of NFAT3 is reversing the DOX induced apoptosis and DOX treatment alone is inducing the apoptosis with the available basal NFAT3, upregulation of NFAT3 may not be required for the cells to undergo apoptosis in the presence of DOX.

In summary, NFAT transcription factors are known for their versatile functions in cell cycle progression, cell development and differentiation. Here, we demonstrate the importance of NFAT3 by siRNA-mediated downregulation of NFAT3, which led to cell survival and subsequent inhibition of DOX mediated apoptosis in SNB19 and U87. Western blot analysis showed the influence of NFAT3 downregulation on apoptotic molecules in both DOX treatment alone and in combination with shNF3. These findings imply that NFAT3 possesses anti-tumorigenic properties and might play an important role in DOX-mediated apoptosis in SNB19 and U87 glioma cell lines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Shellee Abraham for helping in manuscript preparation, and Diana Meister and Sushma Jasti for manuscript review.

This research was supported by National Cancer Institute Grants CA75557, CA116708, CA138409, (to J.S.R.). The contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH.

Reference List

1. Ohgaki H, Kleihues P. Epidemiology and etiology of gliomas. *Acta Neuropathol* 2005;109:93–108. [PubMed: 15685439]

2. Campbell JW, Pollack IF, Martinez AJ, Shultz B. High-grade astrocytomas in children: radiologically complete resection is associated with an excellent long-term prognosis. *Neurosurgery* 1996;38:258–264. [PubMed: 8869052]
3. Devaux BC, O'Fallon JR, Kelly PJ. Resection, biopsy, and survival in malignant glial neoplasms. A retrospective study of clinical parameters, therapy, and outcome. *J. Neurosurg* 1993;78:767–775. [PubMed: 8468607]
4. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998;281:1305–1308. [PubMed: 9721089]
5. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev* 2004;56:185–229. [PubMed: 15169927]
6. Hogan PG, Chen L, Nardone J, Rao A. Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev* 2003;17:2205–2232. [PubMed: 12975316]
7. Horsley V, Pavlath GK. NFAT: ubiquitous regulator of cell differentiation and adaptation. *J. Cell Biol* 2002;156:771–774. [PubMed: 11877454]
8. Hoey T, Sun YL, Williamson K, Xu X. Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. *Immunity* 1995;2:461–472. [PubMed: 7749981]
9. Lyakh L, Ghosh P, Rice NR. Expression of NFAT-family proteins in normal human T cells. *Mol. Cell Biol* 1997;17:2475–2484. [PubMed: 9111316]
10. Benedito AB, Lehtinen M, Massol R, Lopes UG, Kirchhausen T, Rao A, Bonni A. The transcription factor NFAT3 mediates neuronal survival. *J. Biol. Chem* 2005;280:2818–2825. [PubMed: 15537643]
11. Bushdid PB, Osinska H, Waclaw RR, Molkentin JD, Yutzev KE. NFATc3 and NFATc4 are required for cardiac development and mitochondrial function. *Circ. Res* 2003;92:1305–1313. [PubMed: 12750314]
12. Groth RD, Mermelstein PG. Brain-derived neurotrophic factor activation of NFAT (nuclear factor of activated T-cells)-dependent transcription: a role for the transcription factor NFATc4 in neurotrophin-mediated gene expression. *J. Neurosci* 2003;23:8125–8134. [PubMed: 12954875]
13. Kim HB, Kong M, Kim TM, Suh YH, Kim WH, Lim JH, Song JH, Jung MH. NFATc4 and ATF3 negatively regulate adiponectin gene expression in 3T3-L1 adipocytes. *Diabetes* 2006;55:1342–1352. [PubMed: 16644691]
14. Mathew S, Mascareno E, Siddiqui MA. A ternary complex of transcription factors, Nishid and NFATc4, and co-activator p300 bound to an intronic sequence, intronic regulatory element, is pivotal for the up-regulation of myosin light chain-2v gene in cardiac hypertrophy. *J. Biol. Chem* 2004;279:41018–41027. [PubMed: 15272022]
15. Zhang H, Xie X, Zhu X, Zhu J, Hao C, Lu Q, Ding L, Liu Y, Zhou L, Liu Y, Huang C, Wen C, Ye Q. Stimulatory cross-talk between NFAT3 and estrogen receptor in breast cancer cells. *J. Biol. Chem* 2005;280:43188–43197. [PubMed: 16219765]
16. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol* 1997;15:707–747. [PubMed: 9143705]
17. Baksh S, Widlund HR, Frazer-Abel AA, Du J, Fosmire S, Fisher DE, DeCaprio JA, Modiano JF, Burakoff SJ. NFATc2-mediated repression of cyclin-dependent kinase 4 expression. *Mol. Cell* 2002;10:1071–1081. [PubMed: 12453415]
18. Cockerill PN, Shannon MF, Bert AG, Ryan GR, Vadas MA. The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer. *Proc. Natl. Acad. Sci. U.S.A* 1993;90:2466–2470. [PubMed: 8460159]
19. Iniguez MA, Martinez-Martinez S, Punzon C, Redondo JM, Fresno M. An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes. *J. Biol. Chem* 2000;275:23627–23635. [PubMed: 10816557]
20. McCaffrey PG, Goldfeld AE, Rao A. The role of NFATp in cyclosporin A-sensitive tumor necrosis factor-alpha gene transcription. *J. Biol. Chem* 1994;269:30445–30450. [PubMed: 7982959]
21. Shannon MF, Coles LS, Vadas MA, Cockerill PN. Signals for activation of the GM-CSF promoter and enhancer in T cells. *Crit. Rev. Immunol* 1997;17:301–323. [PubMed: 9202885]
22. Crabtree GR, Olson EN. NFAT signaling: choreographing the social lives of cells. *Cell* 2002;109:S67–S79. [PubMed: 11983154]

23. Singal PK, Iliskovic N. Doxorubicin-induced cardiomyopathy. *N. Engl. J. Med* 1998;339:900–905. [PubMed: 9744975]
24. Tan C, Etcubanas E, Wollner N, Rosen G, Gilladoga A, Showel J, Murphy ML, Krakoff IH. Adriamycin--an antitumor antibiotic in the treatment of neoplastic diseases. *Cancer* 1973;32:9–17. [PubMed: 4352019]
25. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem. Pharmacol* 1999;57:727–741. [PubMed: 10075079]
26. Hurley LH. DNA and its associated processes as targets for cancer therapy. *Nat. Rev. Cancer* 2002;2:188–200. [PubMed: 11990855]
27. Jung K, Reszka R. Mitochondria as subcellular targets for clinically useful anthracyclines. *Adv. Drug Deliv. Rev* 2001;49:87–105. [PubMed: 11377805]
28. Wolff JE, Trilling T, Molenkamp G, Egeler RM, Jurgens H. Chemosensitivity of glioma cells in vitro: a meta analysis. *J. Cancer Res. Clin. Oncol* 1999;125:481–486. [PubMed: 10480340]
29. Niiya M, Niiya K, Kiguchi T, Shibakura M, Asaumi N, Shinagawa K, Ishimaru F, Kiura K, Ikeda K, Ueoka H, Tanimoto M. Induction of TNF-alpha, uPA, IL-8 and MCP-1 by doxorubicin in human lung carcinoma cells. *Cancer Chemother. Pharmacol* 2003;52:391–398. [PubMed: 12908082]
30. Cao W, Chi WH, Wang J, Tang JJ, Lu YJ. TNF-alpha promotes Doxorubicin-induced cell apoptosis and anti-cancer effect through downregulation of p21 in p53-deficient tumor cells. *Biochem. Biophys. Res. Commun* 2005;330:1034–1040. [PubMed: 15823547]
31. Kalivendi SV, Konorev EA, Cunningham S, Vanamala SK, Kaji EH, Joseph J, Kalyanaraman B. Doxorubicin activates nuclear factor of activated T-lymphocytes and Fas ligand transcription: role of mitochondrial reactive oxygen species and calcium. *Biochem J* 2005;389:527–539. [PubMed: 15799720]
32. Huang X, Adams MD, Zhou H, Kerlavage AR. A tool for analyzing and annotating genomic sequences. *Genomics* 1997;46:37–45. [PubMed: 9403056]
33. Lakka SS, Rajan M, Gondi CS, Yanamandra N, Chandrasekar N, Jasti SL, Adachi Y, Siddique K, Gujrati M, Olivero W, Dinh DH, Kouraklis G, Kyritsis AP, Rao JS. Adenovirus-mediated expression of antisense MMP-9 in glioma cells inhibits tumor growth and invasion. *Oncogene* 2002;21:8011–8019. [PubMed: 12439751]
34. Yan Y, Li J, Ouyang W, Ma Q, Hu Y, Zhang D, Ding J, Qu Q, Subbaramaiah K, Huang C. NFAT3 is specifically required for TNF-alpha-induced cyclooxygenase-2 (COX-2) expression and transformation of C141 cells. *J. Cell Sci* 2006;119:2985–2994. [PubMed: 16803872]
35. Li J, Song L, Zhang D, Wei L, Huang C. Knockdown of NFAT3 blocked TPA-induced COX-2 and iNOS expression, and enhanced cell transformation in C141 cells. *J. Cell Biochem* 2006;99:1010–1020. [PubMed: 16475165]
36. Kuwano T, Nakao S, Yamamoto H, Tsuneyoshi M, Yamamoto T, Kuwano M, Ono M. Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. *FASEB J* 2004;18:300–310. [PubMed: 14769824]
37. Mann M, Sheng H, Shao J, Williams CS, Pisacane PI, Sliwkowski MX, DuBois RN. Targeting cyclooxygenase 2 and HER-2/neu pathways inhibits colorectal carcinoma growth. *Gastroenterology* 2001;120:1713–1719. [PubMed: 11375952]
38. Sheng H, Shao J, DuBois RN. K-Ras-mediated increase in cyclooxygenase 2 mRNA stability involves activation of the protein kinase B1. *Cancer Res* 2001;61:2670–2675. [PubMed: 11289146]
39. Ranger AM, Gerstenfeld LC, Wang J, Kon T, Bae H, Gravalles EM, Glimcher MJ, Glimcher LH. The nuclear factor of activated T cells (NFAT) transcription factor NFATp (NFATc2) is a repressor of chondrogenesis. *J. Exp. Med* 2000;191:9–22. [PubMed: 10620601]
40. Yang TT, Xiong Q, Enslen H, Davis RJ, Chow CW. Phosphorylation of NFATc4 by p38 mitogen-activated protein kinases. *Mol. Cell Biol* 2002;22:3892–3904. [PubMed: 11997522]
41. Viola JP, Carvalho LD, Fonseca BP, Teixeira LK. NFAT transcription factors: from cell cycle to tumor development. *Braz. J. Med. Biol. Res* 2005;38:335–344. [PubMed: 15761612]
42. Zhang H, Wang X, Li J, Zhu J, Xie X, Yuan B, Yang Z, Zeng M, Jiang Z, Li J, Huang C, Ye Q. Tissue type-specific modulation of ER transcriptional activity by NFAT3. *Biochem. Biophys. Res. Commun* 2007;353:576–581. [PubMed: 17194453]

43. Zhu H, Gao W, Jiang H, Wu J, Shi YF, Zhang XJ. Calcineurin mediates acetylcholinesterase expression during calcium ionophore A23187-induced HeLa cell apoptosis. *Biochim. Biophys. Acta* 2007;1773:593–602. [PubMed: 17320203]
44. Szlosarek PW, Balkwill FR. Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. *Lancet Oncol* 2003;4:565–573. [PubMed: 12965278]
45. Chuvpilo S, Jankevics E, Tyrsin D, Akimzhanov A, Moroz D, Jha MK, Schulze-Luehrmann J, Santner-Nanan B, Feoktistova E, Konig T, Avots A, Schmitt E, Berberich-Siebelt F, Schimpl A, Serfling E. Autoregulation of NFATc1/A expression facilitates effector T cells to escape from rapid apoptosis. *Immunity* 2002;16:881–895. [PubMed: 12121669]
46. Soengas MS, Alarcon RM, Yoshida H, Giaccia AJ, Hakem R, Mak TW, Lowe SW. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* 1999;284:156–159. [PubMed: 10102818]
47. Rivera A, Maxwell SA. The p53-induced gene-6 (proline oxidase) mediates apoptosis through a calcineurin-dependent pathway. *J. Biol. Chem* 2005;280:29346–29354. [PubMed: 15914462]
48. Liao W, Wang S, Han C, Zhang Y. 14-3-3 proteins regulate glycogen synthase 3beta phosphorylation and inhibit cardiomyocyte hypertrophy. *FEBS J* 2005;272:1845–1854. [PubMed: 15819880]
49. Passier R, Zeng H, Frey N, Naya FJ, Nicol RL, McKinsey TA, Overbeek P, Richardson JA, Grant SR, Olson EN. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. *J. Clin. Invest* 2000;105:1395–1406. [PubMed: 10811847]
50. Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, Crabtree GR. Identification of a putative regulator of early T cell activation genes. *Science* 1988;241:202–205. [PubMed: 3260404]
51. Yaghi A, Sims SM. Constrictor-induced translocation of NFAT3 in human and rat pulmonary artery smooth muscle. *Am. J. Physiol. Lung Cell Mol. Physiol* 2005;289:L1061–L1074. [PubMed: 16055480]
52. Caetano MS, Vieira-de-Abreu A, Teixeira LK, Werneck MB, Barcinski MA, Viola JP. NFATC2 transcription factor regulates cell cycle progression during lymphocyte activation: evidence of its involvement in the control of cyclin gene expression. *FASEB J* 2002;16:1940–1942. [PubMed: 12368232]
53. Graef IA, Wang F, Charron F, Chen L, Neilson J, Tessier-Lavigne M, Crabtree GR. Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. *Cell* 2003;113:657–670. [PubMed: 12787506]
54. Hernandez GL, Volpert OV, Iniguez MA, Lorenzo E, Martinez-Martinez S, Grau R, Fresno M, Redondo JM. Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporin A: roles of the nuclear factor of activated T cells and cyclooxygenase 2. *J. Exp. Med* 2001;193:607–620. [PubMed: 11238591]
55. Zaichuk TA, Shroff EH, Emmanuel R, Filleul S, Nelius T, Volpert OV. Nuclear factor of activated T cells balances angiogenesis activation and inhibition. *J. Exp. Med* 2004;199:1513–1522. [PubMed: 15184502]
56. Baksh S, DeCaprio JA, Burakoff SJ. Calcineurin regulation of the mammalian G0/G1 checkpoint element, cyclin dependent kinase 4. *Oncogene* 2000;19:2820–2827. [PubMed: 10851085]
57. Anraku Y, Ohya Y, Iida H. Cell cycle control by calcium and calmodulin in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 1991;1093:169–177. [PubMed: 1863597]
58. Lipskaia L, Lompre AM. Alteration in temporal kinetics of Ca²⁺ signaling and control of growth and proliferation. *Biol. Cell* 2004;96:55–68. [PubMed: 15093128]
59. Terada N, Lucas JJ, Gelfand EW. Differential regulation of the tumor suppressor molecules, retinoblastoma susceptibility gene product (Rb) and p53, during cell cycle progression of normal human T cells. *J. Immunol* 1991;147:698–704. [PubMed: 1906503]
60. Tomono M, Toyoshima K, Ito M, Amano H. Calcineurin is essential for DNA synthesis in Swiss 3T3 fibroblasts. *Biochem. J* 1996;317(Pt 3):675–680. [PubMed: 8760349]
61. Tomono M, Toyoshima K, Ito M, Amano H, Kiss Z. Inhibitors of calcineurin block expression of cyclins A and E induced by fibroblast growth factor in Swiss 3T3 fibroblasts. *Arch. Biochem. Biophys* 1998;353:374–378. [PubMed: 9606972]
62. Neal JW, Clipstone NA. A constitutively active NFATc1 mutant induces a transformed phenotype in 3T3-L1 fibroblasts. *J. Biol. Chem* 2003;278:17246–17254. [PubMed: 12598522]

63. Giese A, Bjerkvig R, Berens ME, Westphal M. Cost of migration: invasion of malignant gliomas and implications for treatment. *J. Clin. Oncol* 2003;21:1624–1636. [PubMed: 12697889]
64. Jauliac S, Lopez-Rodriguez C, Shaw LM, Brown LF, Rao A, Toker A. The role of NFAT transcription factors in integrin-mediated carcinoma invasion. *Nat. Cell Biol* 2002;4:540–544. [PubMed: 12080349]

Figure 1. Construction of shRNA molecule for NFAT3 and downregulation of NFAT3 in SNB19 and U87 using shNF3

A) Schematic representation of NFAT3 gene with its exons. Exons 4, 5 and 6 were targeted for construction of shRNA for NFAT3. **B)** Tertiary structure of the proposed shRNA showing the folds of siRNA 1, 2 and 3 deduced from M-fold software. **C)** RT-PCR analysis of glioma cells with NFAT3 primers and GAPDH primers. **D)** Immunoblot analysis of cell lysates from SNB19 and U87 cells. Fifty μ g of whole cell lysates from control SNB19 and U87 cells and cells infected with SV and shNF3 for 48 h were probed with anti-NFAT3, phospho-specific anti-NFAT3 (pNFAT) and anti-NFAT4 antibodies.

Figure 2. Apoptosis induced by DOX treatment is halted in the absence of NFAT3 expression
A) Cell lysates were collected from SNB19 and U87 after transfection with SV, shNF3 and treatment with or without DOX. Western blot analysis of 50 μ g of total cell lysates were performed to check the expression of NFAT3, pNFAT3, FADD, TNF- α , TNFR1, Bcl2, caspase 3, caspase 10 and PARP. GAPDH was used as a loading control. **B)** SNB19 and U87 cells were transfected with SV or shNF3, incubated for 16 h, treated with DOX (1 μ M) and incubated for another 24 h. Apoptosis was detected using the TUNEL assay as per manufacturer's instructions. Cells were photographed under light or fluorescent microscope. **C)** Agarose gel electrophoresis pattern of DNA obtained from SNB19 and U87 cells after transfection with either SV or shNF3 and treatment with DOX, respectively.

Figure 3. shNF3 treatment reversed DOX-mediated inhibition of cell invasion and wound healing
A) Phase micrographs on invading SNB19 and U87 cells. After transfection with SV, shNF3 and with or without DOX, cells were trypsinized and 2×10^5 cells were placed into matrigel-coated transwell inserts with an 8- μ m pore size. After 24 h the cells that adhered to the outer surface of the transwell (i.e., the cells which had invaded the matrigel) were fixed, stained using the Hema-3 staining kit, and counted under a light microscope. **B)** Number of cells from ten different fields was counted for each sample and the percent of cells invaded after transfection with SV, shNF3 and with or without DOX were analysed and compared with untreated control cells. Graph represents quantification of cell invasion through matrigel. Shown is the mean of ten randomly selected different fields. **C)** SNB19 and U87 cells were cultured in six well plate

at a concentration of 2×10^6 and transfected with SV, shNF3 and with or without DOX. Untreated cells were also maintained simultaneously. After the transfection and the DOX treatment were complete, a straight scratch was made in individual wells with a 200 μ L pipette tip. This point was considered as the zero hour, and the width of the wound was photographed under the microscope. After 24 hours, the cells were checked for the wound healing and again photographed under microscope. **D)** Quantification of cell wound healing after cells were transfected with SV or shNF3 and/or DOX. Shown are the mean values of ten randomly selected different fields.

Figure 4. DOX treatment leads to NFAT3 activation as judged by its nuclear translocation

A) Cytosolic and nuclear presence of NFAT3 in SNB19 and U87 cells that were treated with SV or shNF3 and/or DOX. After treatment the cells were fixed and checked for the expression of NFAT3 by immunofluorescence assay using anti-NFAT3 primary antibody followed by fluorescein isothiocyanate (FITC) - conjugated anti-rabbit secondary antibody. Cells were observed under a fluorescent microscope. DOX stimulates the NFAT3 translocation into the nucleus and the downregulation of NFAT3 resulted in decreased expression of NFAT3 in both cytoplasm and nucleus.

Figure 5. shNF3 transfection reverses DOX induced inhibition of cell cycle progression

A) DNA content of SNB19 and U87 cells were measured by flow cytometric analysis to determine the fate of cell cycle after transfection with SV or shNF3 and/or DOX. DOX treatment increased the number of apoptotic cells in sub G₀/G₁ phase and the cells treated with shNF3 alone and in combination of DOX resulted in very less number of cell in the sub G₀/G₁ phase. The graph shows the percent of apoptotic cells in sub G₀/G₁ phase after treating SNB19 and U87 cells with SV or shNF3 and/or DOX. Values are mean $\pm$ S.D. from three different experiments ($p < 0.001$). **B)** After 48h, total cell lysates collected from control and transfected cells with or without DOX treatment were subjected to SDS-PAGE analysis and

transferred onto nitrocellular membrane. The membranes were probed for p21, p-cdk2, cyclinE and cyclin D1 using respective antibodies. GAPDH was used as loading control.