The transcription factor ST18 regulates proapoptotic and proinflammatory gene expression in fibroblasts

Julia Yang,* Michelle F. Siqueira,* Yugal Behl,* Mani Alikhani,† and Dana T. Graves*,[1]

*Department of Periodontology and Oral Biology, Boston University School of Dental Medicine, Boston, Massachusetts, USA; and †Department of Orthodontics, New York University College of Dentistry, New York, New York, USA

ABSTRACT **Suppression of tumorigenicity 18 (ST18) and the homologues neural zinc-finger protein-3 (NZF3) and myelin transcription factor 3 (Myt3) are transcription factors with unknown function. Previous studies have established that they repress transcription of a synthetic reporter construct consisting of the consensus sequence AAAGTTT linked to the thymidine kinase promoter. In addition, ST18 exhibits significantly reduced expression in breast cancer and breast cancer cell lines. We report here for the first time evidence that ST18 mediates tumor necrosis factor (TNF) -α induced mRNA levels of proapoptotic and proinflammatory genes in fibroblasts by mRNA profiling and silencing with ST18 small interfering RNA (siRNA). Gene set enrichment analysis and mRNA profiling support this conclusion by identifying several apoptotic and inflammatory pathways that are downregulated by ST18 siRNA. In addition, ST18 siRNA reduces TNF-induced fibroblast apoptosis and caspase-3/7 activity. Fibroblasts that overexpress ST18 by transient transfection exhibit significantly increased apoptosis and increased expression of TNF-α, interleukin (IL) -1α, and IL-6. In addition, cotransfection of ST18 and a TNF-α or IL-1α reporter construct demonstrates that ST18 overexpression in fibroblasts significantly enhanced promoter activity of these genes. Taken together, these studies demonstrate that the transcription factor ST18/NZF3 regulates the mRNA levels of proapoptotic and proinflammatory genes in revealing a previously unrecognized function.—Yang, J., Siqueira, M. F., Behl, Y., Alikhani, M., and Graves, D. T. The transcription factor ST18 regulates proapoptotic and proinflammatory gene expression in fibroblasts. *FASEB J.* 22, 3956–3967 (2008)**

Key Words: cytokines · cell death · inflammation · mRNA profiling · microarray

The pathophysiology of many disease processes can be understood in terms of changes in gene expression. Transcription factors that control inflammation are particularly important in this regard. They include factors such as activating protein 1 (AP-1), cAMP responsive element binding (CREB), and NF-κB, which control genes that regulate inflammatory responses and protect the host from microbial infection, initiate processes such as wound healing, and participate in a number of pathological processes (1–3). Inappropriate activity of transcription factors has been shown to promote a number of pathologies, including atherosclerosis, cancer, arthritis, and diabetes (4–7). In some cases, inhibiting a transcription factor such as NF-κB leads to reduced formation of atherosclerotic lesions (6).

Like inflammation, apoptosis is also influenced by transcription factors that regulate the expression of pro- or antiapoptotic genes. For example, CREB influences apoptosis by enhancing expression of antiapoptotic genes that promote cell survival (8). NF-κB is also antiapoptotic; it promotes expression of antiapoptotic bcl-2 family members such as cellular FLICE-like inhibitory protein (c-FLIP) and inhibitors of apoptosis (c-IAPs) (9, 10). The expression of antiapoptotic factors can enhance tumor cell survival and diminish the effectiveness of antitumor chemotherapy (11, 12). Rb and p53 are two well-defined proapoptotic transcription factors (13, 14). Defects in p53 or Rb family members may contribute to the pathogenesis and progression of different cancers and resistance of malignant cells to chemotherapy (15). The forkhead family of transcription factors function similarly to the tumor suppressor p53 and Rb in that both promote apoptosis and affect cell cycle progression (16). One member, forkhead box O1 (FOXO1), has been shown to play an important role in tumor necrosis factor (TNF) -α-induced apoptosis by global up-regulation of proapoptotic genes that encode many different functional proteins (17).

To investigate other transcription factors that may mediate TNF-induced proinflammatory and proapoptotic genes, a pilot study was performed using a transcription factor array from Panomics (Fremont, CA, USA). Suppression of tumorigenicity 18 (ST18) was identified as a putative regulator. Human ST18 is 86%

[1] Correspondence: Boston University School of Dental Medicine, 700 Albany St. W- 202 D, Boston, MA 02118, USA. E-mail: dgraves@bu.edu

doi: 10.1096/fj.08-111013

0892-6638/08/0022-3956 © FASEB

homologous with neural zinc-finger protein-3 (NZF3) (18). ST18/NZF3 is a member of the myelin transcription factor 1 (MyT1) family of transcription factors that contain zinc-finger DNA-binding domains (19). It has six C_2HC-type zinc fingers arranged in two main clusters, each of which binds DNA and recognizes a core consensus sequence. In addition to the zinc-finger domain, the carboxyl terminus of ST18 also displays a high degree of homology to MyT1 and NZF1 (18). ST18 is constitutively expressed in the brain, with little constitutive expression in the heart, liver, kidney, skeletal muscle, pancreas, testis, ovary, or prostate tissue (20). It is found at low levels in normal breast tissue, but in a majority of primary breast tumors examined and in breast cancer cell lines, ST18 mRNA is significantly downregulated (20). Moreover, the same report demonstrates that ectopic ST18 expression inhibits xenograft tumor formation and colony formation in soft agar. Interestingly, ST18 is expressed at 50 higher than normal levels in acute myeloid leukemia and has been proposed as one of 7 markers that have diagnostic potential (21). However, it remains to be demonstrated that ST18 plays a role in myeloid leukemia development or whether the high level of expression is a secondary event. Despite the potential importance of ST18 in regulating biologically important behavior, little is known about its function or its gene targets. We report here that ST18 plays an important role in regulating the mRNA levels of proapoptotic and proinflammatory genes and that its overexpression significantly enhances apoptosis.

MATERIALS AND METHODS

Cell culture

Primary human adult dermal fibroblasts were purchased (Cambrex, East Rutherford, NJ, USA) and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum at 37°C. For most assays, fibroblasts were plated in 6-well plates and assayed in serum-free medium with or without recombinant human TNF-α (20ng/ml).

Electrophoretic mobility shift assay (EMSA)

Fibroblasts were stimulated with TNF-α (20 ng/ml) for the indicated time. Nuclear protein extracts were obtained using a nuclear extraction kit supplemented with a protease and phosphatase inhibitor cocktail (all from Pierce Biotechnology, Rockland, IL, USA). Nuclear protein concentrations were measured using BCA Protein Assay Kit (Pierce Biotechnology). EMSA was performed using a labeled ST18 DNA binding probe GATCCGGAAAGTTTGCAAAGTTTGA purchased from Panomics. A positive control cell lysate (Panomics) was used in some experiments, and excess unlabeled ST18 or noncompetitive DNA probe was tested for inhibition of DNA binding. Employing the LALIGN and PLALIGN programs, 3 potential ST18 DNA binding sites in the TNF promoter were identified by setting the parameter of variation to be not more than 3 bases. Three individual unlabeled oligonucleotides were constructed: T1, ATGGGTTTCTCCACCAAGG**AAGTTT**TCCGC (−225 to −196 bp), T2, GCATCCTGTCTGG**AAGTTA**GAAGGAAAC (−516 to −489 bp), and T3, AGGGACCCC**AGAGTTC**CTTGGAAGCCAA (−841 to −813 bp). In these experiments, fibroblasts were first transfected with ST18 expression vector, described below, for 20 h, and nuclear extracts were examined by EMSA with or without 50-fold excess unlabeled T1, T2, or T3 DNA probes. Dose response to T1 was then assayed using 10-, 50-, and 100-fold excess.

Cloning of ST18 and ST18 overexpression

The ST18 clone (5.9 kb) containing the open reading frame in a pCMV6-XL4 vector was purchased from Origenes Technologies Company (Rockville, MD, USA). Sequencing was performed to confirm isolation of ST18. The open reading frame of the ST18 (3.1 kb) was replicated by polymerase chain reaction (PCR) and the PCR product was purified and inserted into a cDNA 3.1 V5/His TOPO expression vector (Invitrogen, Carlsbad, CA, USA). Cloning of ST18 was again confirmed by DNA sequencing. To overexpress ST18, fibroblasts cells were plated at 80% confluence in 6-well plates. ST18 expression vector was mixed with lipofectamine2000 (Invitrogen) and incubated with cells (0.5 μg/ml) in serum-free medium for 16 h. Total RNA was then isolated, and mRNA levels of genes of interest were quantified by real-time PCR. To investigate the direct role of ST18 in fibroblast apoptosis, transient transfection was performed, and apoptosis was measured 24 h later using a cell death detection ELISA kit (Roche, Indianapolis, IN, USA) following the manufacture's protocol. In some experiments, ST18 was cotransfected with luciferase promoter constructs to investigate the effect of ST18 on promoter activity. The reporter construct for TNF-α contained sequences −991 to +1 of the human TNF-α gene and has been described elsewhere (22). Luciferase reporter constructs for human interleukin (IL) -1α (−816 to +177) were purchased from Panomics. A plasmid expressing renilla luciferase was used to control for transfection efficiency (Promega, Madison, WI, USA) and cell lysates were assayed for both firefly and renilla luciferase activity using a dual-reporter assay system (Promega).

Silencing of ST18

ST18 was silenced in order to investigate its functional role in TNF-α-stimulated gene expression and apoptosis. For the latter, human adult dermal fibroblasts were incubated in 25 cm^2 flasks with 19.3 μg of small interfering RNA (siRNA) mixed with RNAiFect Reagent (Qiagen, Valencia, CA, USA). Two ST18 siRNAs were used: siRNA-1 (targeted sequence TCCAATAGGATTTAAATAGAA) and siRNA-2 (targeted sequence CTGGTCAAATCCAAGAAA), both of which had similar effects on cells. However, siRNA-2 was slightly more effective and was used in most of the experiments presented. Scrambled siRNA (AATTCTAAGAACGTGTCACGT) was used as a negative control (csiRNA). Cells were incubated with the transfection complexes overnight in serum free media. After that, the cells were incubated in serum free media for an additional 24 h and then stimulated with TNF-α (20 ng/ml) for 6 h.

Real-time PCR

Total RNA was isolated using an RNAeasy Mini Kit (Qiagen). cDNA was prepared by reverse transcription (Applied Biosystems, Foster City, CA, USA) and real-time PCR was performed using TaqMan primers and probe sets (Applied Biosystems). Results were normalized to the values obtained for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each experiment was performed 3 times, and the results from the 3 experiments were combined.

Apoptosis and caspase-3/7 activity

Cells were stimulated with TNF for ~20 h following transfection with siRNA as described above. Apoptosis was measured by ELISA for cytoplasmic histone-associated DNA (Roche). To assess caspase-3/7 activity, cells were transfected with siRNA and stimulated with TNF as described for the apoptosis assay. Cytoplasmic proteins were extracted using a protein isolation kit from Pierce Biotechnology, and protein concentration was determined using the BCA assay (Bio-Rad Laboratories, Hercules, CA, USA). Cytoplasmic proteins were tested for caspase-3/7 activity measured with a Caspase-Glo™ 3/7 kit from Promega. For each experiment triplicate samples were assessed. Each assay was carried out 3 times and the value represents the mean values ± SE of the 3 experiments.

RNA profiling

Microarray analysis was performed using GeneChip human genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Fibroblasts were transfected with ST18 siRNA or csiRNA as described above and incubated with 20 ng/ml TNF-α for 6 h. Total RNA was extracted, and the integrity, purity, and quantity of the samples were determined by agarose gel electrophoresis and optical density readings (A_{260}/A_{280}), which were within the range of 1.9–2.1. RNA was subsequently submitted for microarray analysis at the Harvard Medical School-Partners Healthcare System Center for Genetics and Genomics (Cambridge, MA, USA). Three separate arrays were carried out for each group. The values for each gene were normalized using probe logarithmic intensity error estimate (PLIER; Affymetrix). To be considered as modulated by ST18 siRNA, the intensity value threshold was set at a 2.0-fold increase or decrease compared to csiRNA, with a significance level set at $P \leq 0.05$, determined by Student's t test. The activation of inflammatory and apoptotic pathways was assessed by gene set enrichment analysis as described (23). Statistical significance between the two groups, csiRNA *vs.* ST18 siRNA for a given gene set, was established by setting the false discovery rate at 0.25 and $P < 0.05$ using the open source software package described in ref. 23.

Statistical analysis

Each experiment was carried out with replicate samples per group, and each experiment was performed 3 times. Each value represents the mean ± SE of 3 independent experiments (n=3). Significance was tested by nonparametric analysis at the $P < 0.05$ level by the Mann-Whitney test.

RESULTS

ST18 was identified in a transcription factor activation array (Panomics) as strongly activated in fibroblasts stimulated with TNF-α (data not shown). To confirm ST18 activation by TNF-α, EMSA was performed. TNF-α stimulated an increase in ST18 DNA binding activity at 1 h compared to unstimulated cells (**Fig. 1*A***, left panel). Specificity of binding was demonstrated by the competitive inhibition with excess unlabeled probe (Fig. 1*A*, right panel). EMSA was also carried out to investigate potential binding sites in the TNF promoter for ST18. Three potential sequences in the TNF promoter were identified

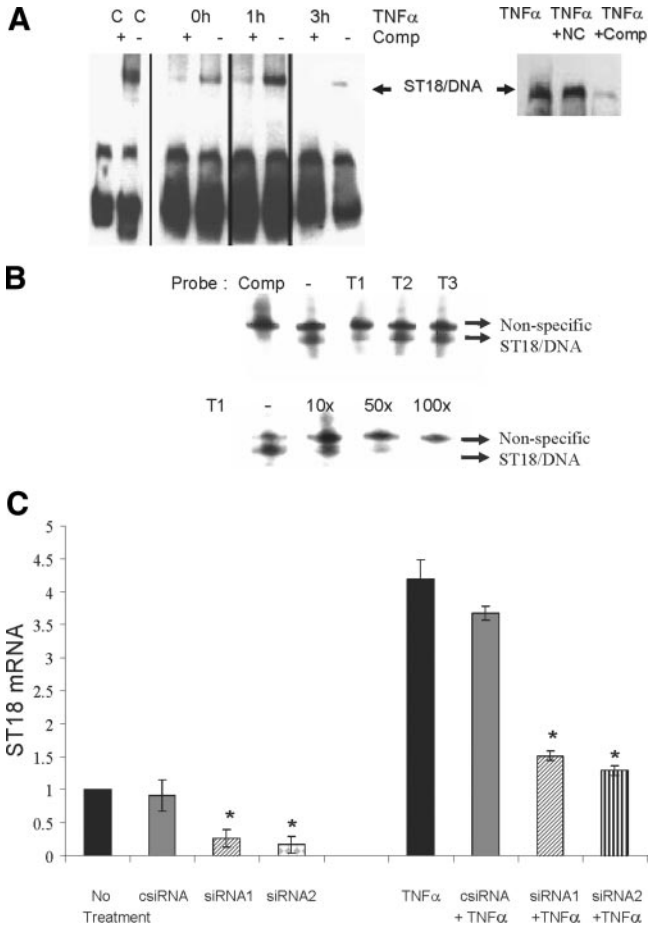

Figure 1. TNF-α stimulates ST18 DNA binding and mRNA levels. *A*) Human adult dermal fibroblasts were plated in 6-well plates. Cells were stimulated *in vitro* with 20 ng/ml of recombinant human TNF-α for 0, 1, and 3 h. Nuclear proteins were extracted, and EMSA was performed to evaluate DNA binding. A positive control cell lysate (C) is shown in the two left lanes (left panel). Specificity was demonstrated by competition with excess unlabeled probe (comp) and lack of competition with scrambled oligonucleotide (NC; right panel). Results are representative of 3 experiments *B*) Human adult dermal fibroblasts were transfected with ST18 expression vector, and nuclear extracts were examined by EMSA as described in *A*. Top panel: nuclear extract and labeled oligonucleotide were incubated with 50-fold excess unlabeled oligonucleotide T1, T2, or T3, as indicated. T1, T2, and T3 represent different potential ST18 binding sites in the TNF promoter, as described in Materials and Methods. Bottom panel: excess unlabeled T1 oligonucleotide was added as indicated. *C*) Real-time PCR was used to quantify the silencing effect of ST18 siRNA. Cells were transfected with two different ST18 siRNAs (siRNA1 or siRNA2) or control scrambled siRNA (csiRNA), and 48 h later were stimulated with or without recombinant human TNF-α for 6 h. The results were normalized by GAPDH. Each value represents the mRNA level of the experimental group as the fold difference compared to the no treatment group. Data are expressed as means ± SE of 3 experiments. *$P < 0.05$ *vs.* csiRNA.

and termed T1, T2, and T3, as described in Materials and Methods. In these experiments, fibroblasts were first transfected with the ST18 expression vector, and nuclear extracts were incubated with the labeled DNA probe,

consisting of the consensus sequence and unlabeled competitive DNA T1, T2, and T3, and examined by EMSA (Fig. 1*B*, top panel). Only the T1 probe had the ability to compete with the labeled probe, whereas the T2 and T3 showed no effect. T1 also competed with labeled probe in a dose-dependent manner (Fig. 1*B*, bottom panel).

Real-time PCR was then performed to determine whether TNF-α induced ST18 mRNA and whether ST18 could be silenced with siRNA (Fig. 1*C*). TNF-α stimulated a 4.2-fold increase in ST18 mRNA levels. Cells were transfected with two different ST18 siRNA oligonucleotides (siRNA1 and siRNA2) or csiRNA (Fig. 1*C*). In unstimulated cells, siRNA1 and siRNA2 decreased ST18 mRNA 70 and 80%, respectively, both of which were significant ($P<0.05$). In TNF-α-stimulated cells, siRNA1 and siRNA2 reduced ST18 mRNA levels 60 and 65%, respectively, compared to csiRNA ($P<0.05$). csiRNA had no effect when compared to TNF stimulation alone ($P>0.05$). In several experiments it was shown that csiRNA + TNF had no effect on gene expression compared to TNF alone; in subsequent experiments the effect of ST18 siRNA was compared to csiRNA.

The role of ST18 in mediating TNF-induced mRNA levels was investigated using ST18 siRNA and by overexpression of ST18. TNF-α-stimulated TNF-α mRNA levels were reduced by 54% by ST18 siRNA compared to csiRNA, demonstrating that ST18 plays a role in stimulated TNF-α expression ($P<0.05$) (**Fig. 2*A***). To examine the effect of ST18 overexpression, ST18 was cloned into a pcDNA 3.1 TOPO expression vector and transiently transfected into fibroblasts. Compared with transfection of vector alone, ST18 up-regulated TNF-α mRNA 4.6-fold ($P<0.05$) (Fig. 2*B*).

To demonstrate that ST18 stimulated transcription factor activity, ST18 was cotransfected with a TNF-α promoter/luciferase reporter construct (Fig. 2*C*). In the absence of cotransfection, luciferase values were low. Transfection of ST18 and the reporter construct increased luciferase activity 4.5-fold compared with empty vector and reporter. A positive control, phorbol 12-myristate 13-acetate (PMA) 10 ng/ml, stimulated a similar level of TNF promoter activity compared with transfection of ST18.

The relation between ST18 and gene expression was further investigated by RNAi studies carried out in conjunction with mRNA profiling using Affymetrix microarrays (**Table 1**). ST18 siRNA was considered to up- or down-regulate TNF-induced mRNA levels if the values were increased or decreased by at least 1.7-fold, with a value of $P \leq 0.05$ compared to cells incubated with TNF + csiRNA. Of the 579 apoptosis-related genes identified by PathwayArchitect software (Stratagene, La Jolla, CA, USA) using the Gene Ontology database (http://www.geneontology.org) (Table 1), 19.34% were significantly down-regulated by silencing of ST18, 7.42% were significantly up-regulated, and 73.24% did not meet the criteria for being modulated by ST18 siRNA. Seventy-nine proapoptotic and 33 antiapoptotic genes were down-regulated by ST18 siRNA, including many with diverse functions such as ligands, receptors, adapter molecules, transcription factors, *etc.* (Table 1). In comparison, 34 proapoptotic and 9 antiapoptotic genes were up-regulated by ST18 siRNA (Table 1). Apoptotic genes not regulated by ST18 siRNA are found in Supplemental Table 1. Because the change in individual genes does not necessarily mean that a given pathway is affected, gene set enrichment analysis was performed to establish whether ST18 modulated sets of genes that regulated apoptosis. The threshold was set at a false discovery rate of 0.25 and a value of $P \leq 0.01$, as recommended by Subramanian *et al.* (23). Several proapoptotic pathways had mRNA values reduced by ST18 siRNA, demonstrating that ST18 plays a role in modulating apoptotic gene sets (**Table 2**). These in-

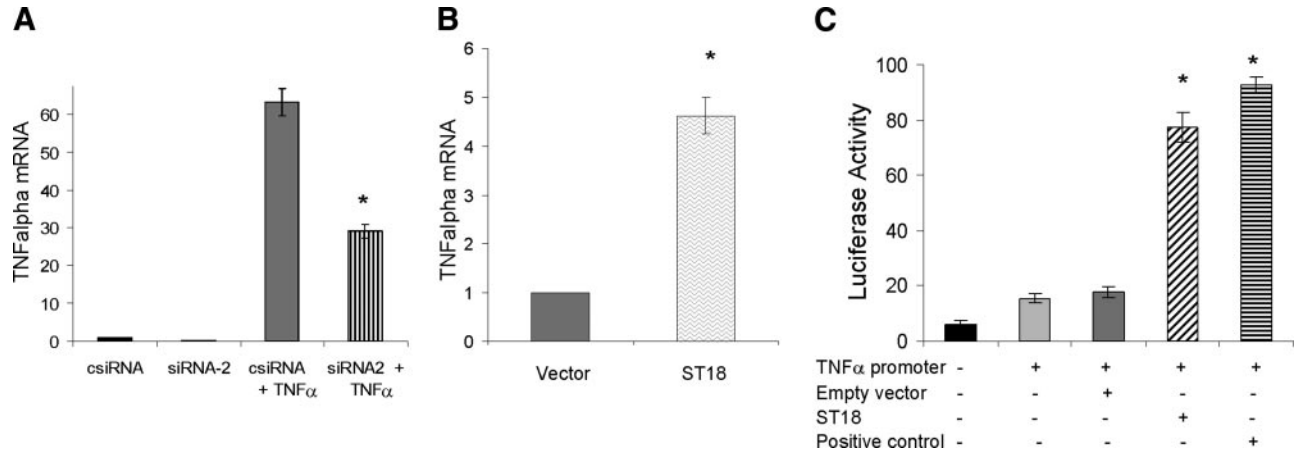

Figure 2. ST18 enhances TNF-α mRNA levels and promoter activity. *A*) Fibroblasts were transfected with ST18 siRNA2 or csiRNA, followed by stimulation with TNF-α (20 ng/ml) for 6 h as indicated. TNF-α mRNA levels were measured by real-time PCR. *B*) Fibroblasts were transfected with empty vector or ST18 expression vector for 24 h. TNF-α mRNA levels were measured by real time PCR. *C*) Fibroblasts were unstimulated or transfected with ST18 expression vector, empty vector, TNF-α promoter/luciferase reporter construct, or positive control, cells stimulated with PMA (10 ng/ml). Luciferase activity was measured. Data are expressed as means ± SE of 3 experiments. *Significantly increased by cotransfection; $P < 0.05$.

TABLE 1. *Apoptotic genes regulated by ST18 siRNA*

Name	Description	ST18 siRNA/csiRNA	*P* value
Down-regulated proapoptotic genes			
IL1A	Interleukin 1, alpha	0.00	<0.01
TNF	Tumor necrosis factor (TNF superfamily, member 2)	0.01	<0.01
IL1A	Interleukin 1, alpha	0.00	<0.01
TNF	Tumor necrosis factor (TNF superfamily, member 2)	0.01	<0.01
IL1B	Interleukin 1, beta	0.02	<0.01
BTG2	BTG family, member 2	0.03	<0.01
TNFSF9	Tumor necrosis factor (ligand) superfamily, member 9	0.03	<0.01
TRAF1	TNF receptor-associated factor 1	0.03	<0.01
GADD45B	Growth arrest and DNA-damage-inducible, beta	0.03	<0.01
INHBA	Inhibin, beta A (activin A, activin AB alpha polypeptide)	0.04	<0.01
TNFSF7	Tumor necrosis factor (ligand) superfamily, member 7	0.04	<0.01
ESPL1	Extra spindle poles like 1 (*Saccharomyces cerevisiae*)	0.04	<0.01
NR4A1	Nuclear receptor subfamily 4, group A, member 1	0.05	<0.01
IL6	Interleukin 6 (interferon, beta 2)	0.05	<0.01
ERN1	Endoplasmic reticulum to nucleus signalling 1	0.06	<0.01
IRAK2	Interleukin-1 receptor-associated kinase 2	0.06	<0.01
ADORA2A	Adenosine A2a receptor	0.06	<0.01
AXUD1	AXIN1 up-regulated 1	0.08	<0.01
RIPK2	Receptor-interacting serine-threonine kinase 2	0.09	<0.01
STK17B	Serine/threonine kinase 17b (apoptosis-inducing)	0.10	<0.01
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	0.10	<0.01
PHLDA1	Pleckstrin homology-like domain, family A, member 1	0.11	<0.01
BBC3	BCL2 binding component 3	0.11	<0.01
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	0.11	<0.01
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	0.11	<0.01
GADD45A	Growth arrest and DNA-damage-inducible, alpha	0.12	<0.01
IFNB1	Interferon, beta 1, fibroblast	0.15	<0.01
TP53BP2	Tumor protein p53 binding protein, 2	0.15	<0.01
CLEC2D	C-type lectin superfamily 2, member D	0.16	<0.01
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	0.16	<0.01
SPHK1	Sphingosine kinase 1	0.16	<0.01
ZNF443	Zinc finger protein 443	0.17	<0.01
PPARD	Peroxisome proliferative activated receptor, delta	0.20	0.01
CARD15	Caspase recruitment domain family, member 15	0.21	<0.01
PLAGL2	Pleiomorphic adenoma gene-like 2	0.21	<0.01
CD40	CD40 antigen (TNF receptor superfamily member 5)	0.22	<0.01
IL18	Interleukin 18 (interferon-gamma-inducing factor)	0.23	0.01
IRAK3	Interleukin-1 receptor-associated kinase 3	0.25	<0.01
IRAK3	Interleukin-1 receptor-associated kinase 3	0.25	<0.01
SERPINB2	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	0.25	<0.01
TRAF4	TNF receptor-associated factor 4	0.25	<0.01
RFFL	Rififylin	0.26	<0.01
SIAH1	Seven in absentia homolog 1 (*Drosophila*)	0.29	<0.01
BRAF	V-raf murine sarcoma viral oncogene homolog B1	0.29	0.01
TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	0.31	<0.01
MAP3K5	Mitogen-activated protein kinase kinase kinase 5	0.32	<0.01
TNFSF18	Tumor necrosis factor (ligand) superfamily, member 18	0.33	<0.01
RARA	Retinoic acid receptor, alpha	0.33	<0.01
FAS	Fas (TNF receptor superfamily, member 6)	0.33	<0.01
AHR	Aryl hydrocarbon receptor	0.34	<0.01
RELA	V-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	0.34	<0.01
DAXX	Death-associated protein 6	0.35	<0.01
ITGB2	Integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)	0.37	0.05
WWOX	WW domain containing oxidoreductase	0.38	0.01
CIDEC	Cell death-inducing DFFA-like effector c	0.39	0.01
TRAF3	TNF receptor-associated factor 3	0.39	0.04
FOXO3A	Forkhead box O3A	0.40	0.04
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	0.41	<0.01
FOXO1A	Forkhead box O1A (rhabdomyosarcoma)	0.42	0.02

continued on next page

TABLE 1. *(continued)*

Name	Description	ST18 siRNA/csiRNA	*P* value
HSPA1B	Heat shock 70 kDa protein 1B	0.43	<0.01
P2RX1	Purinergic receptor P2X, ligand-gated ion channel, 1	0.43	<0.01
RHOB	Ras homolog gene family, member B	0.44	<0.01
MITF	Microphthalmia-associated transcription factor	0.44	0.02
BCL10	B-cell CLL/lymphoma 10	0.44	<0.01
TRAF6	TNF receptor-associated factor 6	0.45	0.01
PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	0.47	0.05
HELLS	Helicase, lymphoid-specific	0.48	0.04
MTP18	Mitochondrial protein 18 kDa	0.49	0.02
MKL1	Megakaryoblastic leukemia (translocation) 1	0.49	<0.01
BCL2L12	BCL2-like 12 (proline rich)	0.49	0.05
CYCS	Cytochrome c, somatic	0.50	<0.01
DAPK3	Death-associated protein kinase 3	0.51	0.01
BCLAF1	BCL2-associated transcription factor 1	0.53	<0.01
MSH6	mutS homolog 6 (*Escherichia coli*)	0.54	0.01
IFIH1	Interferon induced with helicase C domain 1	0.54	0.01
CREB1	CAMP responsive element binding protein 1	0.55	0.03
RYBP	RING1 and YY1 binding protein	0.55	<0.01
PHLPP	PH domain and leucine rich repeat protein phosphatase	0.55	0.03
CTNNAL1	Catenin (cadherin-associated protein), alpha-like 1	0.56	<0.01
NALP1	NACHT, leucine rich repeat and PYD (pyrin domain) containing 1	0.56	0.03
PEA15	Phosphoprotein enriched in astrocytes 15	0.58	0.03
Down-regulated antiapoptotic genes			
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	0.04	<0.01
IER3	Immediate early response 3	0.04	<0.01
BCL2A1	BCL2-related protein A1	0.05	<0.01
BIRC3	Baculoviral IAP repeat-containing 3	0.06	<0.01
SOCS2	Suppressor of cytokine signaling 2	0.06	<0.01
GADD45G	Growth arrest and DNA-damage-inducible, gamma	0.10	0.01
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	0.12	<0.01
SOCS3	Suppressor of cytokine signaling 3	0.13	<0.01
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.15	<0.01
CLCF1	Cardiotrophin-like cytokine factor 1	0.15	<0.01
BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)	0.17	<0.01
STAT5A	Signal transducer and activator of transcription 5A	0.20	<0.01
SNAI2	Snail homolog 2 (*Drosophila*)	0.23	<0.01
SERPINB2	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	0.25	<0.01
PRKCD	Protein kinase C, delta	0.26	<0.01
CCL2	Chemokine (C-C motif) ligand 2	0.27	<0.01
BCL2L1	BCL2-like 1	0.30	<0.01
TNFRSF4	Tumor necrosis factor receptor superfamily, member 4	0.31	0.05
CFLAR	CASP8 and FADD-like apoptosis regulator	0.33	<0.01
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	0.36	<0.01
BDNF	Brain-derived neurotrophic factor	0.38	0.01
SQSTM1	Sequestosome 1	0.38	<0.01
AMIGO2	Amphoterin induced gene 2	0.40	<0.01
CBX4	Chromobox homolog 4 (Pc class homolog, *Drosophila*)	0.41	0.02
BIRC4	Baculoviral IAP repeat-containing 4	0.41	0.01
NTF3	Neurotrophin 3	0.42	0.01
BNIP1	BCL2/adenovirus E1B 19 kDa interacting protein 1	0.44	<0.01
BIRC2	Baculoviral IAP repeat-containing 2	0.47	<0.01
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3	0.53	<0.01
ANGPTL4	Angiopoietin-like 4	0.54	0.04
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1	0.57	0.02
VHL	Von Hippel-Lindau tumor suppressor	0.58	0.04
DIABLO	Diablo homolog (*Drosophila*)	0.58	<0.01
Up-regulated proapoptotic genes			
WIG1	p53 target zinc finger protein	6.68	<0.01
TP53INP1	Tumor protein p53 inducible nuclear protein 1	5.77	<0.01
PRKCA	Protein kinase C, alpha	5.11	<0.01
RIPK5	Receptor interacting protein kinase 5	4.62	0.01

continued on next page

TABLE 1. *(continued)*

Name	Description	ST18 siRNA/csiRNA	*P* value
MAGEH1	Melanoma antigen family H, 1	4.02	<0.01
ATM	Ataxia telangiectasia mutated (includes complementation groups A, C, and D)	3.84	0.01
BNIP3L	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	3.40	<0.01
TNFRSF19	Tumor necrosis factor receptor superfamily, member 19	3.27	<0.01
TRIAD3	TRIAD3 protein	3.07	<0.01
GSPT1	G1 to S phase transition 1	3.00	0.01
MDM4	Mdm4, transformed 3T3 cell double minute 4, p53 binding protein (mouse)	2.76	0.03
GNAQ	Guanine nucleotide binding protein (G protein), q polypeptide	2.61	0.02
TIA1	TIA1 cytotoxic granule-associated RNA binding protein	2.55	0.01
PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)	2.47	<0.01
SH3GLB1	SH3-domain GRB2-like endophilin B1	2.46	<0.01
F2R	Coagulation factor II (thrombin) receptor	2.46	<0.01
APAF1	Apoptotic protease activating factor	2.34	<0.01
CAMK1D	Calcium/calmodulin-dependent protein kinase ID	2.27	0.01
MOAP1	Modulator of apoptosis 1	2.23	0.01
CUL5	Cullin 5	2.16	0.01
ANK2	Ankyrin 2, neuronal	2.14	<0.01
NOTCH2	Notch homolog 2 (*Drosophila*)	2.02	<0.01
PPM1F	Protein phosphatase 1F (PP2C domain containing)	2.01	<0.01
EBAG9	Estrogen receptor binding site associated, antigen, 9	1.99	<0.01
JMY	Junction-mediating and regulatory protein	1.95	0.03
APPBP1	Amyloid beta precursor protein binding protein 1, 59 kDa	1.93	<0.01
NCKAP1	NCK-associated protein 1	1.92	<0.01
CUL3	Cullin 3	1.84	<0.01
STK4	Serine/threonine kinase 4	1.81	0.02
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	1.78	<0.01
OPA1	Optic atrophy 1 (autosomal dominant)	1.76	0.02
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	1.75	<0.01
APG12L	APG12 autophagy 12-like (*S. cerevisiae*)	1.72	<0.01
EI24	Etoposide induced 2.4 mRNA	1.70	<0.01
Up-regulated antiapoptotic genes			
IGF1R	Insulin-like growth factor 1 receptor	6.76	<0.01
BNIP2	BCL2/adenovirus E1B 19 kDa interacting protein 2	4.41	<0.01
HTATIP2	HIV-1 Tat interactive protein 2, 30 kDa	1.93	0.01
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	1.92	<0.01
PTMA	Prothymosin, alpha (gene sequence 28)	1.90	<0.01
VCP	Valosin-containing protein	1.77	0.02
SFRP1	Secreted frizzled-related protein 1	1.75	<0.01
BAG5	BCL2-associated athanogene 5	1.72	0.01
GSK3B	Glycogen synthase kinase 3 beta	1.71	<0.01

mRNA profiling was carried out in adult human fibroblasts transfected with ST18 siRNA or csiRNA and then stimulated with TNF-α for 6 h. Three separate arrays were carried out for csiRNA and ST18 siRNA, and the values were normalized using a probe logarithmic intensity error estimate (PLIER). To be considered as modulated by ST18siRNA, the intensity values were at least 1.7-fold different compared to csiRNA, with $P \leq 0.05$.

cluded a pathway designated "apoptosis," which had a very low false-discovery rate and consisted of 67 genes. Pathways consistent with death receptor stimulation included caspase, TNF, and Fas pathways. ST18 siRNA also down-regulated several pathways involved in mitochondrial signaling, even though this pathway does not play a prominent role in TNF-induced apoptosis in fibroblasts (24), such as mitochondrial pathway, the BCL2 family, BRAC2BRAC1, and ceramide pathways. Antiapoptotic pathways that were negatively modulated by ST18 siRNA included Akt and NF-κB. No apoptotic pathways were up-regulated by ST18 siRNA. The GSEA analysis is consistent with the results from individual genes that demonstrate that the effect of ST18 on mRNA levels is overwhelmingly proapoptotic.

Because ST18 promoted proapoptotic gene expression in fibroblasts, its role in apoptosis was assessed by examining the effect of ST18 siRNA on TNF-induced apoptosis and by overexpression of ST18. Without TNF-α stimulation, there was little apoptosis detected, and ST18 siRNA had no effect on basal apoptosis (**Fig. 3*A***). TNF-α stimulated a large increase in fibroblast apoptosis. TNF-α-stimulated apoptosis was significantly reduced by ST18 siRNA, 66% by siRNA2 and 51% by siRNA1 compared to csiRNA ($P<0.05$). The effect of ST18 siRNA and control siRNA is consistent with the degree of silencing observed in Fig. 1*C*. The role of ST18 in TNF-induced apoptosis was further evaluated by measuring caspase-3/7 activity. TNF-α induced a 3.5-fold increase in caspase 3/7 activity, which was reduced by 65% with ST18 siRNA2 compared

TABLE 2. *Apoptotic pathways modulated by ST18 siRNA*

Pathway	*P* value	FDR	Number of genes
Apoptosis	<0.001	0.05	67
Caspase pathway	<0.001	0.07	22
Tumor necrosis factor pathway	<0.001	0.05	28
FAS signaling pathway	<0.001	0.05	57
Passerini oxidation	<0.001	0.14	19
P53 signaling	<0.001	0.05	91
Mitochondrial pathway	<0.001	0.07	19
BCL2 family and reg network	<0.001	0.07	20
BRCA2 BRCA1	<0.001	0.07	40
Ceramide pathway	<0.001	0.09	22
AKT pathway	<0.001	0.05	17
NF-κB pathway	<0.001	0.05	23

Gene set enrichment analysis was performed with the threshold false discovery rate set at 0.25 and $P < 0.05$. The genes defined within a given pathway can be found at http://www.broad.mit.edu/gsea.

to csiRNA ($P<0.05$). The effect of ST18 overexpression was tested by transiently transfecting fibroblasts with ST18 expression vector and comparing the results to vector alone and to a positive control, cells treated with TNF-α. Overexpression of ST18 caused fibroblasts to undergo a 3-fold increase in apoptosis compared to empty-vector transfected cells (Fig. 3*C*). This increase was similar to the positive control TNF-α tested under the same conditions (Fig. 3*C*). These results indicate that ST18 functions to promote apoptosis.

The role of ST18 in regulating mRNA levels of inflammatory genes was examined by RNAi using the same approach described for apoptotic genes. Of the 242 inflammation-related genes identified in the Gene Ontology database (**Table 3**), 19.83% were significantly down-regulated by silencing of ST18, 1.65% were significantly up-regulated, and 78.52% were unchanged. The 48 inflammatory genes down-regulated by ST18 siRNA included cytokines and chemokines, their receptors, transcription factors, *etc.* (Table 3). In contrast, only 4 inflammatory genes were up-regulated by ST18 siRNA (Table 3). Inflammatory genes not affected by ST18 are shown in Supplemental Table 2. The effect of inflammatory gene expression was explored further by gene set enrichment analysis (**Table 4**). A pathway designated "inflammation" consisting of 29 genes and one designated "cytokine" were significantly down-regulated by ST18 siRNA. Several more specific proinflammatory pathways were down-regulated by ST18 siRNA, such as toll, NF-κB, TNF, IL-1 receptor signaling, IL-6, IL-17, interferon, iNOS, NO2/IL-12, and prostaglandin synthesis pathways. No inflammatory pathways were up-regulated by ST18 siRNA. Thus, ST18 modulates several inflammatory pathways stimulated by TNF-α.

Because mRNA profiling indicated that ST18 mediated TNF-induced mRNA levels of proinflammatory genes, the ability of ST18 to modulate IL-1α mRNA levels and promoter activity was examined. ST18 siRNA reduced TNF-α induced IL-1α mRNA levels by 50% compared to csiRNA ($P<0.05$) (**Fig. 4*A***). When ST18 was overexpressed, mRNA levels of IL-1α were increased 6.4-fold compared to cells transfected with vector alone ($P<0.05$) (Fig. 4*B*). To establish whether ST18 enhanced IL-1α promoter activity, cells were cotransfected with ST18 and an IL-1α promoter/luciferase reporter construct (Fig. 4*C*). Cotransfection with ST18 enhanced luciferase activity 3.3-fold compared to cotransfection with vector alone. This was similar to the positive control, IL-1α promoter activity in cells stimulated with PMA.

Experiments were also performed to investigate the role of ST18 in modulating another inflammatory mediator, IL-6. Silencing ST18 with siRNA reduced TNF-α-induced IL-6 mRNA levels by 70% ($P<0.05$) (**Fig. 5*A***). Overexpressed ST18 up-regulated the mRNA levels of IL-6 5.9-fold compared to transfection with vector alone ($P<0.05$) (Fig. 5*B*). To examine whether silencing of ST18 also affected IL-6 expression at the protein level, the supernatants were examined for IL-6

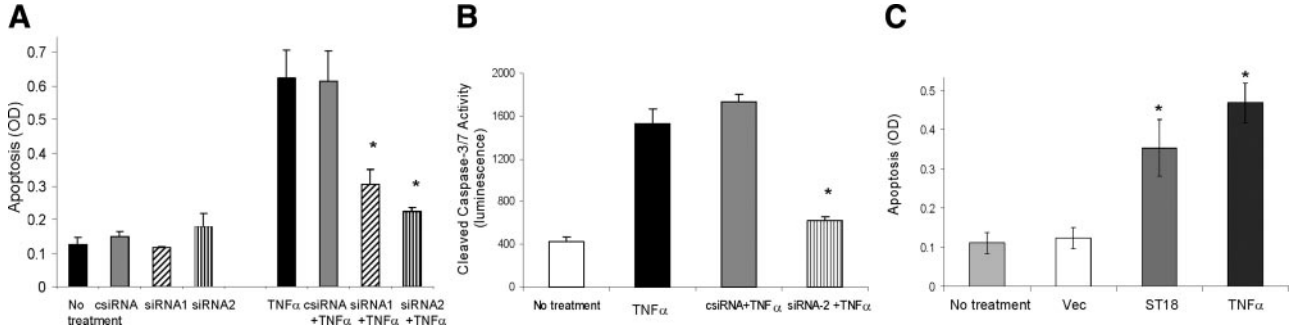

Figure 3. ST18 promotes apoptosis. *A*) Fibroblasts were transfected with ST18 siRNA (siRNA1 or siRNA2) or csiRNA, followed by stimulation with TNF-α (20 ng/ml) for 24 h as described in Materials and Methods. Cells were lysed and apoptosis was determined by ELISA, measuring the amount of histone-associated cytoplasmic DNA fragments. *B*) Fibroblasts were transfected with ST18 siRNA2 or csiRNA and stimulated with TNF-α as described in *A*. Cytoplasmic protein was extracted, and cleaved caspase-3/7 activity was measured with a luminescent substrate. *C*) Fibroblasts were transfected with culture media alone (no treatment), empty vector, ST18 expression vector, or TNF-α (20 ng/ml), and apoptosis was measured 24 h later as in *A*. Data are expressed as means ± SE of 3 experiments. *Significantly reduced by ST18 siRNA (*A*, *B*); significantly increased *vs.* no treatment (*C*); $P < 0.05$.

TABLE 3. *Inflammatory genes regulated by ST18 siRNA*

Name	Description	ST18 siRNA/csiRNA	*P* value
Down-regulated genes			
CCL20	Chemokine (C-C motif) ligand 20	0.00	<0.01
IL1A	Interleukin 1, alpha	0.00	<0.01
IL8	Interleukin 8	0.00	<0.01
SELE	Selectin E (endothelial adhesion molecule 1)	0.01	<0.01
CXCL3	Chemokine (C-X-C motif) ligand 3	0.01	<0.01
TNF	Tumor necrosis factor (TNF superfamily, member 2)	0.01	<0.01
CXCL2	Chemokine (C-X-C motif) ligand 2	0.01	<0.01
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	0.02	<0.01
IL1B	Interleukin 1, beta	0.02	<0.01
CCL4	Chemokine (C-C motif) ligand 4	0.05	<0.01
IRAK2	Interleukin-1 receptor-associated kinase 2	0.06	<0.01
ADORA2A	Adenosine A2a receptor	0.06	<0.01
IL1RN	Interleukin 1 receptor antagonist	0.08	<0.01
RIPK2	Receptor-interacting serine-threonine kinase 2	0.09	<0.01
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	0.10	<0.01
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	0.10	<0.01
CHST2	Carbohydrate (*N*-acetylglucosamine-6-O) sulfotransferase 2	0.10	<0.01
BDKRB1	Bradykinin receptor B1	0.11	<0.01
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	0.12	<0.01
HDAC9	Histone deacetylase 9	0.13	<0.01
ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)	0.13	<0.01
BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)	0.17	<0.01
CX3CL1	Chemokine (C-X3-C motif) ligand 1	0.18	<0.01
STAT5A	Signal transducer and activator of transcription 5A	0.20	<0.01
MAP2K3	Mitogen-activated protein kinase kinase 3	0.21	<0.01
CD40	CD40 antigen (TNF receptor superfamily member 5)	0.22	<0.01
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	0.23	<0.01
CCL5	Chemokine (C-C motif) ligand 5	0.24	<0.01
CCL2	Chemokine (C-C motif) ligand 2	0.27	<0.01
C3	Complement component 3	0.28	<0.01
IL4R	Interleukin 4 receptor	0.29	<0.01
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	0.30	<0.01
TNFRSF4	Tumor necrosis factor receptor superfamily, member 4	0.31	0.05
CXCL5	Chemokine (C-X-C motif) ligand 5	0.34	0.05
PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	0.35	0.01
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	0.35	0.01
ITGB2	Integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)	0.37	0.05
CCL11	Chemokine (C-C motif) ligand 11	0.37	<0.01
HRH1	Histamine receptor H1	0.40	0.02
BDKRB2	Bradykinin receptor B2	0.46	<0.01
ITCH	Itchy homolog E3 ubiquitin protein ligase (mouse)	0.51	<0.01
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	0.52	<0.01
NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	0.52	<0.01
HDAC4	Histone deacetylase 4	0.55	0.03
ZYX	Zyxin	0.57	<0.01
ABCF1	ATP-binding cassette, sub-family F (GCN20), member 1	0.57	<0.01
LTB4R	Leukotriene B4 receptor	0.59	0.04
CXCL11	Chemokine (C-X-C motif) ligand 11	0.59	<0.01
Up-regulated genes			
PRKCA	Protein kinase C, alpha	5.11	<0.01
DOCK2	Dedicator of cytokinesis 2	4.20	<0.01
CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	4.02	<0.01
ATRN	Attractin	2.77	0.01

mRNA profiling was carried out in adult human fibroblasts transfected with ST18 siRNA or csiRNA and then stimulated with TNF-α for 6 h. Three separate arrays were carried out for each group, and the values for each gene were normalized using a probe logarithmic intensity error estimate (PLIER). To be considered as modulated by ST18siRNA, the intensity values were at least 1.7-fold different compared to csiRNA, with $P \leq 0.05$.

TABLE 4. *Gene set enrichment analysis of apoptotic and inflammatory pathways*

Pathway	*P* value	FDR q value	Genes
Inflammatory pathway	<0.001	0.06	29
Cytokine pathway	<0.001	0.07	20
Toll pathway	<0.001	0.05	29
NFKB pathway	<0.001	0.05	23
TNF pathway	<0.001	0.05	28
IL1 receptor pathway	<0.001	0.05	30
IL6 pathway	<0.001	0.05	21
IL17 pathway	<0.001	0.07	14
IFN	<0.001	0.05	18
INOS	<0.001	0.05	52
NO2/IL12 pathway	<0.001	0.16	15
Prostaglandin synthesis	<0.001	0.07	28

GSEA analysis was performed as described in Table 2.

protein level by ELISA (Fig. 5*C*). On TNF-α stimulation, IL-6 protein levels were elevated 2.2-fold in comparison with the unstimulated cells. However, knockdown of ST18 with siRNA resulted in a 50% reduction of IL-6 protein compared to csiRNA. This result correlated well with the degree of down-regulation of IL-6 mRNA level by ST18 siRNA.

DISCUSSION

To better understand how TNF affects fibroblasts to modulate inflammatory or apoptotic gene expression, we identified ST18 as a transcription factor that was induced by TNF stimulation. ST18 is expressed at relatively high levels in brain and at lower levels in other tissues under normal conditions. However, its function has not been elucidated. We report here for the first time that ST18 plays an important role in stimulating proapoptotic and proinflammatory gene expression. The proinflammatory effect of ST18 was demonstrated by 3 approaches. By silencing ST18 with siRNA, it was shown that ST18 had a significant effect on the mRNA levels of almost 20% of proinflammatory genes examined. Approximately 16% of the genes identified by PathwayArchitect as being cytokines were down-regulated by ST18 siRNA, whereas 2.3% of this group were up-regulated. TNF-α induces a wide variety of proinflammatory cytokines, including IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ, and TGF-β that involves NF-κB activation (25). Microarray results showed that many target genes of NF-κB are also affected by ST18 siRNA. Thus, the overall effect of ST18 is to enhance the expression of genes that promote inflammation.

Gene set enrichment analysis was carried out to further establish inflammatory and apoptotic gene sets that are regulated by ST18. Several proinflammatory pathways were shown to be down-regulated by ST18 siRNA, including well-known pathways such as toll, NF-κB, TNF, IL-6, iNOS, and prostaglandin synthesis. ST18 overexpression significantly increased TNF-α, IL-1α, and IL-6 mRNA levels, and their mRNA levels were reduced when ST18 was knocked down by siRNA. Furthermore, by cotransfection with reporter constructs, ST18 overexpression was shown to stimulate TNF-α and IL-1α reporter activity. ST18 also repressed promoter activity of HOXA5 (data not shown), consistent with a report that cotransfection of ST18 suppresses transcription activity of a synthetic reporter construct that contains the core consensus AAAGTTT upstream of the thymidine kinase promoter (18).

Results obtained with microarrays were consistent with data obtained using real-time PCR in studies with siRNA or ST18 overexpression. For example, TNF-α,

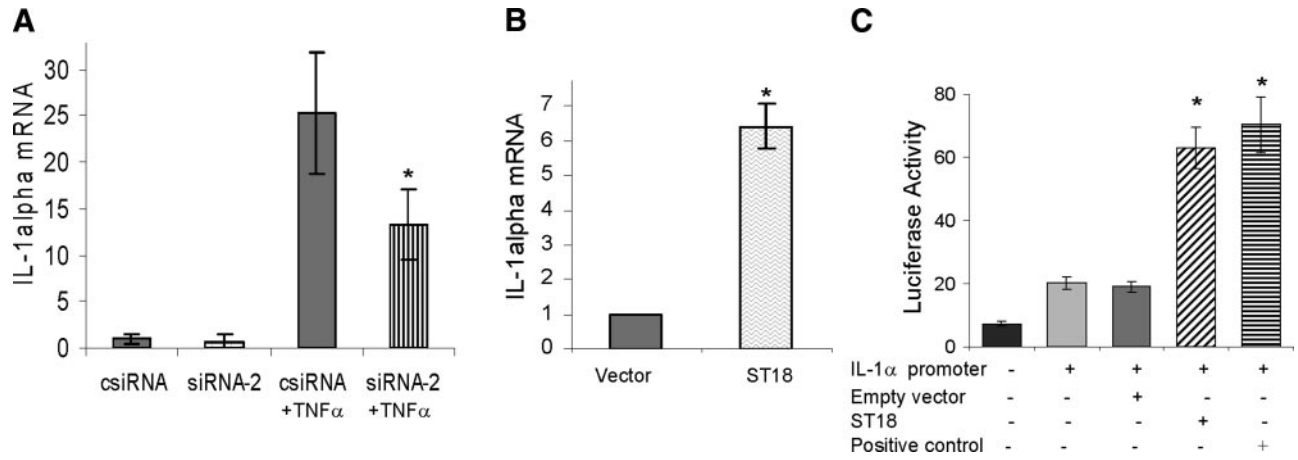

Figure 4. ST18 enhances IL-1α mRNA levels and promoter activity. *A*) Fibroblasts were transfected with ST18 siRNA2 or csiRNA with or without subsequent incubation with TNF-α (20 ng/ml) for 6 h. IL-1α mRNA levels were measured by real-time PCR. *B*) Fibroblasts were transfected with empty vector or ST18 expression vector for 24 h. IL-1α mRNA levels were measured by real-time PCR. *C*) Fibroblasts were unstimulated or transfected with ST18 expression vector, empty vector, IL-1α promoter/luciferase reporter construct, or positive control, cells stimulated with PMA (10 ng/ml). Luciferase activity was measured. Data are expressed as means ± SE of 3 experiments. *Significantly reduced by ST18 siRNA (*A*); significantly increased by ST18 expression vector or PMA (*B*, *C*); $P < 0.05$.

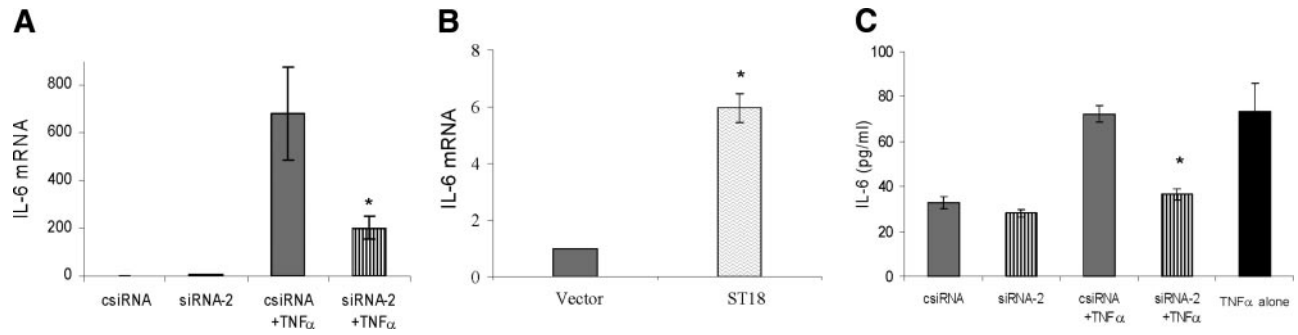

Figure 5. ST18 enhances IL-6 expression. *A*) Fibroblasts were transfected with ST18 siRNA2 or csiRNA and then incubated with or without TNF-α (20 ng/ml) for 6 h. IL-6 mRNA levels were measured by real-time PCR. *B*) Fibroblasts were transfected with empty vector or ST18 expression vector for 24 h. IL-6 mRNA levels were measured by real-time PCR. *C*) Fibroblasts were transfected with siRNA as described in *A* and then stimulated with TNF-α (20 ng/ml) for 6 h. IL-6 protein levels were measured by ELISA. Data are expressed as means ± SE of 3 experiments. *Significantly reduced by ST18 siRNA *vs.* csiRNA (*A*, *C*); significantly higher than empty vector (*B*); $P < 0.05$.

IL-1α, and IL-6 were down-regulated by ST18 siRNA whether assessed by microarray or real-time PCR. Of the apoptotic genes regulated by ST18, 70% were proapoptotic, suggesting that ST18 causes an overall shift in gene expression that is proapoptotic. This agreed well with enhanced apoptosis when cells were transfected with ST18 and reduced apoptosis when ST18 was silenced with ST18 siRNA in TNF-α-stimulated cells. The proapoptotic genes down-regulated by ST18 siRNA included ligands, receptors, adapter molecules, several apoptotic genes known to be modulated by p53 (*e.g.*, c-Fos, c-Jun, Bax, LAT32, and MDM2; ref. 26, 27), and many proinflammatory genes that are indirectly apoptotic.

TNF-α activates several intracellular pathways to induce cellular responses that include the production of inflammatory mediators and cell death (25, 28, 29). Fibroblasts participate in the early response to trauma or microbial invasion by the up-regulation of proinflammatory genes, particularly those involved in innate immunity (30). However, the inflammatory response can also result in the death of fibroblasts. For example, fibroblast cell death in response to bacterial infection under some circumstances is due to the induced expression of TNF (31). It has been shown that enhanced fibroblast apoptosis during diabetic healing is due in part to greater levels of TNF and that when fibroblast apoptosis is reduced, healing is improved, which may represent a mechanism for impaired wound healing in diabetic patients (32, 33). Thus, the effect of TNF on fibroblasts is both important in orchestrating a host response and, in pathological situations, potentially deleterious through excessive apoptosis.

ST18 was first isolated from rat brain tissue. It was identified and characterized using the degenerate primers corresponding to two stretches of conserved sequences in MyT1 and NZF1 from the NZF/MyT family. The cDNA sequence predicts a protein of 1032 amino acids with 6 zinc fingers of the C_2HC type arranged in 2 clusters, similar to MyT1 and NZF1. Similar to other members of this family, ST18 is shown to be expressed primarily in the nervous system. It has also been reported that ST18 mRNA is significantly down-regulated in breast cancer cell lines and in the majority of primary breast tumors (20). The finding that ST18 is required for TNF-induced apoptosis suggests a mechanism whereby cells that have reduced ST18 expression may be more resistant to apoptosis. Thus, ST18 may function in a fashion similar to Rb and p53, where reduced expression renders certain cell types resistant to killing by immune surveillance or from chemotherapeutic agents. FJ

We thank Alicia Ruff for help in preparing this manuscript. This work was supported by U.S. National Institute of Health grants DE07559 and DE17732.

REFERENCES

1. Wong, D. J., and Chang, H. Y. (2005) Learning more from microarrays: insights from modules and networks. *J. Invest. Dermatol.* **125,** 175–182
2. Chen, F., Castranova, V., Shi, X., and Demers, L. M. (1999) New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. *Clin. Chem.* **45,** 7–17
3. Matt, T. (2002) Transcriptional control of the inflammatory response: a role for the CREB-binding protein (CBP). *Acta Med. Austriaca* **29,** 77–79
4. Eferl, R., and Wagner, E. F. (2003) AP-1: a double-edged sword in tumorigenesis. *Nat. Rev. Cancer* **3,** 859–868
5. Monks, N. R., Biswas, D. K., and Pardee, A. B. (2004) Blocking anti-apoptosis as a strategy for cancer chemotherapy: NF-kappaB as a target. *J. Cell. Biochem.* **92,** 646–650
6. De Winther, M. P., Kanters, E., Kraal, G., and Hofker, M. H. (2005) Nuclear factor kappaB signaling in atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **25,** 904–914
7. Liu, H., and Pope, R. M. (2003) The role of apoptosis in rheumatoid arthritis. *Curr. Opin. Pharmacol.* **3,** 317–322
8. Kinjo, K., Sandoval, S., Sakamoto, K. M., and Shankar, D. B. (2005) The role of CREB as a proto-oncogene in hematopoiesis. *Cell Cycle* **4,** 1134–1135
9. Beg, A., and Baltimore, D. (1996) An essential role for NF-kB in preventing TNF-α induced cell death. *Science* **274,** 782–784
10. Wang, C. Y., Guttridge, D. C., Mayo, M. W., and Baldwin, A. S., Jr. (1999) NF-kappaB induces expression of the Bcl-2 homo-

logue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. *Mol. Cell. Biol.* **19,** 5923–5929
11. Bharti, A. C., and Aggarwal, B. B. (2002) Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochem. Pharmacol.* **64,** 883–888
12. Lavon, I., Pikarsky, E., Gutkovich, E., Goldberg, I., Bar, J., Oren, M., and Ben-Neriah, Y. (2003) Nuclear factor kappa B protects the liver against genotoxic stress and functions independently of p53. *Cancer Res.* **63,** 25–30
13. Fuster, J. J., Sanz-Gonzalez, S. M., Moll, U. M., and Andres, V. (2007) Classic and novel roles of p53: prospects for anticancer therapy. *Trends Mol. Med.* **13,** 192–199
14. Du, W., and Pogoriler, J. (2006) Retinoblastoma family genes. *Oncogene* **25,** 5190–5200
15. Pommier, Y., Sordet, O., Antony, S., Hayward, R. L., and Kohn, K. W. (2004) Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. *Oncogene* **23,** 2934–2949
16. Birkenkamp, K., and Coffer, P. (2003) FOXO transcription factors as regulators of immune homeostasis: molecules to die for? *J. Immunol.* **171,** 1623–1629
17. Alikhani, Z., Alikhani, M., Boyd, C. M., Nagao, K., Trackman, P. C., and Graves, D. T. (2005) Advanced glycation end products enhance expression of pro-apoptotic genes and stimulate fibroblast apoptosis through cytoplasmic and mitochondrial pathways. *J. Biol. Chem.* **280,** 12087–12095
18. Yee, K. S., and Yu, V. C. (1998) Isolation and characterization of a novel member of the neural zinc finger factor/myelin transcription factor family with transcriptional repression activity. *J. Biol. Chem.* **273,** 5366–5374
19. Jiang, Y., Yu, V. C., Buchholz, F., O'Connell, S., Rhodes, S. J., Candeloro, C., Xia, Y.-R., Lusis, A. J., and Rosenfeld, M. G. (1996) A novel family of Cys-Cys, His-Cys zinc finger transcription factors expressed in developing nervous system and pituitary gland. *J. Biol. Chem.* **271,** 10723–10730
20. Jandrig, B., Seitz, S., Hinzmann, B., Arnold, W., Micheel, B., Koelble, K., Siebert, R., Schwartz, A., Ruecker, K., Schlag, P. M., Scherneck, S., and Rosenthal, A. (2004) ST18 is a breast cancer tumor suppressor gene at human chromosome 8q11.2. *Oncogene* **23,** 9295–9302
21. Steinbach, D., Schramm, A., Eggert, A., Onda, M., Dawczynski, K., Rump, A., Pastan, I., Wittig, S., Pfaffendorf, N., Voigt, A., Zintl, F., and Gruhn, B. (2006) Identification of a set of seven genes for the monitoring of minimal residual disease in pediatric acute myeloid leukemia. *Clin. Cancer Res.* **12,** 2434–2441
22. Tang, X., Fenton, M. J., and Amar, S. (2003) Identification and functional characterization of a novel binding site on TNF-alpha promoter. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4096–4101
23. Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., and Mesirov, J. P. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 15545–15550
24. Alikhani, M., Alikhani, Z., Raptis, M., and Graves, D. T. (2004) TNF-alpha in vivo stimulates apoptosis in fibroblasts through caspase-8 activation and modulates the expression of pro-apoptotic genes. *J. Cell. Physiol.* **201,** 341–348
25. Gaur, U., and Aggarwal, B. (2003) Regulation of proliferation, survival and apoptosis by member of the TNF superfamily. *Biochem. Pharmacol.* **66,** 1403–1408
26. Choisy-Rossi, C., Reisdorf, P., and Yonish-Rouach, E. (1998) Mechanisms of p53-induced apoptosis: in search of genes which are regulated during p53-mediated cell death. *Toxicol. Lett.* **102-103,** 491–496
27. Sun, Y. (2006) p53 and its downstream proteins as molecular targets of cancer *Mol. Carcinog.* **45,** 409–415
28. Varfolomeev, E. E., and Ashkenazi, A. (2004) Tumor necrosis factor: an apoptosis JuNKie? *Cell* **116,** 491–497
29. Taylor, P. C. (2001) Anti-TNF therapy for rheumatoid arthritis and other inflammatory diseases. *Mol. Biotechnol.* **19,** 153–168
30. Eming, S. A., Krieg, T., and Davidson, J. M. (2007) Inflammation in wound repair: molecular and cellular mechanisms. *J. Invest. Dermatol.* **127,** 514–525
31. Graves, D., Oskoui, M., Volejnikova, S., Naguib, G., Cai, S., Desta, T., Kakouras, A., and Jiang, Y. (2001) Tumor necrosis factor modulates fibroblast apoptosis, PMN recruitment, and osteoclast formation in response to *P. gingivalis* infection. *J. Dent. Res.* **80,** 1875–1879
32. Darby, I., Bisucci, T., Hewitson, T., and MacLellan, D. (1997) Apoptosis is increased in a model of diabetes-impaired wound healing in genetically diabetic mice. *Int. J. Biochem. Cell Biol.* **29,** 191–200
33. Kane, C. D., and Greenhalgh, D. G. (2000) Expression and localization of p53 and bcl-2 in healing wounds in diabetic and nondiabetic mice. *Wound Repair Regen.* **8,** 45–58

Received for publication March 27, 2008.
Accepted for publication July 2, 2008.

The transcription factor ST18 regulates proapoptotic and proinflammatory gene expression in fibroblasts

Julia Yang, Michelle F. Siqueira, Yugal Behl, et al.

FASEB J 2008 22: 3956-3967 originally published online August 1, 2008
Access the most recent version at doi:10.1096/fj.08-111013