FOXO1 Functions as a Master Switch That Regulates Gene Expression Necessary for Tumor Necrosis Factor-induced Fibroblast Apoptosis*

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Tumor necrosis factor-α (TNF-α) is a potent pro-inflammatory and pro-apoptotic mediator that plays an important role in several normal and disease processes. TNF-induced cell death is one of the principal mechanisms by which cells are removed. Although TNF-mediated apoptosis has been the subject of intense investigation, the transcriptional mechanisms through which it promotes apoptosis are not well understood and, paradoxically, the archetypal TNF-induced nuclear factor NFκB is anti-apoptotic. To identify a potential master transcriptional regulator of apoptosis, we examined an array of TNF-α-activated transcription factors. Forkhead box class-O 1 (FOXO1) was strongly activated, which was confirmed in vitro and in vivo by electrophoretic mobility shift assay. The central importance of FOXO1 was established in experiments with small inhibitory RNA (siRNA) that specifically silenced FOXO1. When FOXO1 was silenced, fibroblast apoptosis was reduced 76%. Other siRNAs that partially inhibited FOXO1 expression were proportionately effective in reducing apoptosis. Transcriptional profiling was then carried out in conjunction with siRNA to establish mechanisms by which FOXO1 modulated apoptosis. In the absence of FOXO1, TNF-α failed to up-regulate a large number of pro-apoptotic gene families including ligands, receptors, adapter molecules, mitochondrial proteins, and caspases. siRNA silencing also blocked down-regulation of anti-apoptotic genes. These results indicate that TNF induces activation of the FOXO1 transcription factor, which acts as a master switch to control apoptosis.

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The abbreviations used are: TNF, tumor necrosis factor; EMSA, electrophoretic mobility shift assay; FOXO, forkhead box class-O; NFκB, nuclear factor κB; RT, reverse transcription; TNFR, TNF receptor; TRADD, the TNFR1-associated death domain protein.

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EXPERIMENTAL PROCEDURES

Cell Culture—Primary human adult dermal fibroblasts were purchased from Cambrex (Walkersville, MD). Cells were propagated and maintained in Dulbecco’s modified Eagle’s medium (Cambrex Com-
Silencing effect of different siRNA design on FOXO1 expression

 Six different siRNAs for FOXO1 (siRNA-890, siRNA-1059, siRNA-1674, siRNA-800, siRNA-755, and siRNA-663) were designed based on standard criteria and named based on the position of the first base pair in FOXO1 mRNA. Adult human dermal fibroblasts were transfected with different siRNAs. After 48 h, FOXO1 gene expression was measured by real time PCR. The percent decrease in FOXO1 expression in comparison to control (cells that did not receive siRNA) is displayed. Each value represents the mean of five replicates ± S.E.

| siRNA     | Sequence                  | Percent silencing |
|-----------|---------------------------|-------------------|
| siRNA-663 | 5'-AAGGTTTCATCCTGTGAGCAGAT-3' | 41 ± 3 |
| siRNA-755 | 5'-AAGGACCTGTCACATGGAGCACA-3' | 38 ± 4 |
| siRNA-800 | 5'-AAGGCCGAGCTGGTAAAGGAAA-3' | 23 ± 6 |
| siRNA-890 | 5'-AAGGCCCTGGCTACTGCAAGCACA-3' | 83 ± 3 |
| siRNA-1059| 5'-AAGATGGCCTCTACTTCCCACC-3' | 3 ± 1  |
| siRNA-1674| 5'-AAGACACCCTGTACTACAGCC-3'  | 4 ± 2  |

FIG.1. TNF stimulates activation of FOXO1 in vitro. Adult human dermal fibroblasts were stimulated with TNF-α (20 ng/ml) for 0–6 h. After nuclear extraction, activation of FOXO1 was measured in response to TNF stimulation. Unlabeled FOXO1 probe in excess was used as a competitive inhibitor. A probe with a nonspecific sequence was used as a negative control. Bottom arrows indicate probe alone. The experiment was performed three times with similar results.

FIG.2. TNF stimulates Activation of FOXO1 in vitro. FOXO1 activation was measured by EMSA in nuclear extracts of the scalp of mice 1 h following TNF injection. Unlabeled FOXO1 probe in excess was used as a competitive inhibitor. A probe with a nonspecific sequence was used as a negative control. Top arrows indicate activation complex with FOXO1 or NFκB bound to the labeled probe. Bottom arrows indicate probe alone. The experiment was performed three times with similar results.

FIG.3. TNF increases expression of FOXO1. Adult human dermal fibroblasts were transfected with siRNA (siR)-890, -1054 or -1674 for 48 h, and the effect of TNF-α on FOXO1 expression and activation was measured by real time PCR and EMSA. A, 48 h after siRNA treatment cells were incubated with TNF-α (20 ng/ml) for 6 h. Real time PCR was carried out to assess FOXO1 mRNA levels. The percent change in FOXO1 expression relative to unstimulated cells is shown. Each value represents the mean of five replicates ± S.E. B, cells were treated with siRNA for 48 h followed by incubation with TNF-α or TNF for 1 h. Activation of FOXO1 was evaluated by EMSA. An unlabeled FOXO1 probe in excess was used as a competitive inhibitor, and a nonspecific probe was used as a negative control. A description of each siRNA can be found in Table I. There were six mice (n = 6). Recombinant murine TNF-α was purchased from R&D Systems ( Minneapolis, MN). Experiments with TNF-α were performed in a culture medium supplemented with 0.5% serum. Assays were performed when the cultures reached 75–85% confluence. Apoptosis of fibroblasts was determined by measuring cytoplasmic histone-associated-DNA (Roche Applied Science) following the manufacturer's instructions. Statistical difference between samples was determined by analysis of variance followed by Tukey’s multiple comparison test.

In Vivo Specimens—Wild type CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). For each data point there were six mice (n = 6). Recombinant murine TNF-α was purchased from R&D Systems (Minneapolis, MN). TNF-α (200 ng) was inoculated adjacent to the periosteum at a point on the midline of the skull located between the ears as we have described previously (17). Mice were euthanized for 1 h following injection, and the scalp tissue was obtained and snap frozen in liquid nitrogen. Nuclear proteins were extracted from pulverized frozen tissue using a nuclear protein extraction kit (Pierce) following the manufacturer’s instructions. Concentrations of nuclear proteins were measured by using a BCA protein assay (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)—Fibroblast cultures
FOXO1 Is a Master Switch for TNF-induced Apoptosis

TABLE II
Effect of different siRNA on apoptosis

| siRNA          | Apoptosis without TNF (OD) | Apoptosis with TNF (OD) | Percent inhibition of TNF-induced Apoptosis |
|---------------|---------------------------|------------------------|--------------------------------------------|
| Control       | 0.197 ± 0.06              | 1.701 ± 0.19           |                                             |
| siRNA 663     | 0.221 ± 0.05              | 1.046 ± 0.21           | 38%                                        |
| siRNA 755     | 0.239 ± 0.06              | 1.127 ± 0.17           | 33%                                        |
| siRNA 800     | 0.207 ± 0.08              | 1.358 ± 0.22           | 20%                                        |
| siRNA 890     | 0.233 ± 0.08              | 0.391 ± 0.12           | 76%                                        |
| siRNA 1059    | 0.226 ± 0.07              | 1.677 ± 0.19           | 2%                                         |
| siRNA 1674    | 0.203 ± 0.09              | 1.585 ± 0.17           | 6%                                         |

Fig. 4. Inhibition of NFκB enhances TNF-induced apoptosis. Adult human dermal fibroblasts were transfected with different siRNA for 48 h. Cells were then stimulated by TNF-α for 24 hours. Apoptosis was determined by enzyme-linked immunosorbent assay. Each value represents the mean of five replicates ± S.E. and is representative of four experiments.

Fig. 5. Inhibition of FOXO1 by siRNA reduces TNF-stimulated caspase activity. Adult human dermal fibroblasts were transfected with siRNA (sRN-890 or siRNA-1059 and stimulated with TNF-α (20 ng/ml) for 24 h. Cell lysates were assessed for caspase-3, -8, and -9 activity by fluorimetric assay. Each value represents the mean of six replicates ± S.E. The experiment was performed three times with similar results.
Expression of apoptotic genes in response to TNF-α stimulation in siR890-transfected cells compared with siR1059-transfected fibroblasts

Adult human dermal fibroblasts were transfected with siRNA-890 that silences FOXO1 or with control siRNA-1059. After 48 h cells were incubated with TNF-α (20 ng/ml) for 6 h. Total RNA was isolated and subjected to microarray analysis. Genes whose expression was changed >2-fold by siRNA-890 are shown in the left columns. Genes whose expression was previously shown to be modulated by TNF (20) but not altered >2-fold by siRNA-890 are shown in columns on the right. Each value represents the mean of two microarrays. Anti-apoptotic genes are shown in boldfaced type. NC denotes no change in expression, i.e., change in mRNA levels were less than the 2-fold threshold. Expansions of certain gene designations are as follows: Bcl-2, B-cell lymphoma-2; CD27L, CD27 ligand; CD30L, CD30 ligand; CD40L, CD40 ligand; FADD, Fas-associated protein with death domain; Foxo1, Fas ligand; 4-IBB, 4-IBB ligand; FL-18, interleukin 18; OX40L, OX40 ligand; RIP, receptor-interacting protein; TRAF, TNFR-associated factor; TRAIL, TNF-related death-inducing ligand.

| Category   | Gene name | mRNA ratio (siRNA890/siRNA1059) | Gene name | mRNA ratio (siRNA890/siRNA1059) |
|------------|-----------|---------------------------------|-----------|---------------------------------|
| Ligands    | CD27L     | 0.21                            | LT-B      | NC                              |
|            | 4-IBB     | 0.26                            | TNFα      | NC                              |
|            | TRAIL     | 0.31                            | OX40L     | NC                              |
|            | FASL†     | 0.33                            | CD30L     | NC                              |
|            | IL-18     | 0.13                            | CD40L     | NC                              |
|            |           |                                 | Lta       | NC                              |
| Receptors  | 4-IBB     | 0.33                            | CD27      | NC                              |
|            | Fas       | 0.27                            | OX40      | NC                              |
|            | CD40      | 0.31                            | TNFR1     | NC                              |
|            | LTBR      | 0.37                            | TNFR2     | NC                              |
|            |           |                                 | DR3       | NC                              |
|            |           |                                 | TNFRSF14  | NC                              |
| Mitochondrial | BID     | 0.29                            | MCL-1     | NC                              |
|            | BAX       | 0.31                            | BLK       | NC                              |
|            | BFL1      | 0.33                            | BAK       | NC                              |
|            | BCL2L13   | 0.4                             | BAD       | NC                              |
|            | Bcl-2     | 3.33                            | BOK       | NC                              |
| Caspases   | Caspase-8† | 0.38                           | Caspase-9 | NC                              |
|            | Caspase-6 | 0.38                            | Caspase-3 | NC                              |
|            | Caspase-14| 0.4                             | Caspase-1 | NC                              |
|            | Caspase-2 | 0.47                            | Caspase-7 | NC                              |
|            |           |                                 | Caspase-4 | NC                              |
| Adaptors   | TRADD†    | 0.26                            | RIP       | NC                              |
|            | TRAF1     | 0.35                            | FADD      | NC                              |
|            | TRAF6     | 0.37                            | FLASK     | NC                              |
|            | TRAF2     | 0.47                            | APAF-1    | NC                              |
|            |           |                                 | CRIADD    | NC                              |
|            |           |                                 | TRAF4     | NC                              |
| Others     | XIAP      | 5                               | MDM2      | NC                              |
|            | JUN       | 0.25                            | RPA       | NC                              |
|            | P53       | 0.43                            | CIDE-A    | NC                              |
|            | NFαB (p65)| 0.24                            | NOP30     | NC                              |
|            | BIRC5     | 3.33                            | ATM       | NC                              |
|            | GADD45†   | 0.14                            |           |                                  |
|            | CASPERS†  | 2.51                            |           |                                  |
|            | HUS1      | 0.35                            |           |                                  |

* A gene that other investigators have shown to be regulated by FOXO1 transcription factors (15, 41–43).

RNA Profiling—Fibroblasts were transfected with siRNA-890 or siRNA-1059 for 48 h followed by stimulation with TNF-α (20 ng/ml) or vehicle alone for 6 h, and total RNA was isolated as described above. RNA was converted to cDNA using a reverse transcription kit (Applied Biosystems, Foster City, CA). Genes of interest were amplified using a PCR kit (SuperArray Bioscience Corporation, Bethesda, MD) that has been optimized so that the internal standard, glyceraldehyde-3-phosphate dehydrogenase, is amplified for the same number of cycles as the gene of interest. For each experiment the reaction was performed in duplicate and stopped after 23 or 28 cycles. When the PCR was complete, samples were electrophoresed on 2% agarose gels. The optical density of each band was normalized by the value of internal glyceraldehyde-3-phosphate dehydrogenase control in the same lane, and the results were analyzed at both 23 and 28 cycles. Each experiment was carried out three times with similar results. Statistical difference between cells that were transfected with siRNA 890 or siRNA 1059 was determined by analysis of variance followed by Tukey’s multiple comparison test.

RT-PCR—Fibroblasts were transfected with siRNA-890 or siRNA-1059 for 48 h followed by stimulation with TNF-α (20 ng/ml) or vehicle alone for 6 h, and total RNA was isolated as described above. RNA was converted to cDNA using a reverse transcription kit (Applied Biosystems, Foster City, CA). Genes of interest were amplified using a PCR kit (SuperArray Bioscience Corporation, Bethesda, MD) that has been optimized so that the internal standard, glyceraldehyde-3-phosphate dehydrogenase, is amplified for the same number of cycles as the gene of interest. For each experiment the reaction was performed in duplicate and stopped after 23 or 28 cycles. When the PCR was complete, samples were electrophoresed on 2% agarose gels. The optical density of each band was normalized by the value of internal glyceraldehyde-3-phosphate dehydrogenase control in the same lane, and the results were analyzed at both 23 and 28 cycles. Each experiment was carried out three times with similar results. Statistical difference between cells that were transfected with siRNA 890 or siRNA 1059 was determined by analysis of variance followed by Tukey’s multiple comparison test.
FOXO1 Is a Master Switch for TNF-induced Apoptosis

Real-time PCR was used to monitor FOXO1 mRNA levels. After reverse transcription of RNA from siRNA-transfected cells, amplification was performed using reagents from Applied Biosystems. Primers (forward, 5'-AGAGTTACAGCCCATAGCAAG-3'; reverse, 5'-TTGCTTGGCAGATGATGAC-3') and TaqMan MGB probe (FAMAAATTTTACAGCCCATAGCAAG-3') were chosen using Primer Express software to select at least one intron-spanning primer for each.

RESULTS

TNF-α Activates FOXO1 in Vitro—A transcription factor array was carried out twice in which fibroblasts were stimulated by TNF (20 ng/ml) for 1 h using a kit developed by Panomics. FOXO1 was the most strongly induced and was identified as a candidate for additional studies (data not shown). Activation of FOXO1 was confirmed by measuring FOXO1 DNA binding activity through EMSA (Fig. 1). Nuclear extracts were prepared from human adult primary fibroblasts 0, 1, 3, and 6 h after TNF stimulation. Previously, we established that an optimal TNF-α concentration for stimulating apoptosis of fibroblasts is 20 ng/ml (20). As shown in Fig. 1A, TNF-α stimulated a marked increase in FOXO1 DNA binding activity in comparison with that of nuclear extracts from untreated cells. Activation of FOXO1 was higher at 1 h in comparison with its activation at 3 and 6 h. Specificity of the binding activity was demonstrated by competitive inhibition with excess unlabeled oligonucleotide. In contrast, an oligonucleotide with a nonspecific sequence did not interfere with the binding of nuclear extract to a labeled DNA probe.

To investigate whether NFκB was activated under the same conditions, EMSA was performed using a probe specific for NFκB (Fig. 1B). Nuclear extracts were prepared from human adult primary fibroblasts stimulated for 0, 1, 3, and 6 h with TNF-α. TNF-α-stimulated activation of NFκB DNA binding activity, whereas nuclear extracts from untreated cells showed little binding activity. Similar to the FOXO1 results, activation of NFκB was higher at 1 h compared with its activation at 3 and 6 h. The specificity of the reaction was verified by competitive probe (excess unlabeled probe) and lack of competition with excess non-competitive probe. These results indicate that TNF-α is able to simultaneously activate both FOXO1 and NFκB in fibroblasts in vitro.

TNF-α Activates FOXO1 in Vivo—To verify that TNF stimulation activates FOXO1 in vivo, TNF-α was inoculated subcutaneously into the connective tissue of the scalp in the mouse. Previously, we showed that injection of 200 ng of TNF-α at this location induces a moderate level of fibroblast apoptosis, whereas an equivalent injection of vehicle alone (50 μl of sterile phosphate-buffered saline) does not (17). One hour after injection, the tissue was obtained and analyzed by EMSA. TNF-α in comparison with vehicle alone significantly increased FOXO1 binding to DNA (Fig. 2). These results indicate that TNF-α is able to simultaneously activate both FOXO1 and NFκB in fibroblasts in vivo.

Identification of siRNA That Inhibits FOXO1 Expression—Human adult dermal fibroblasts were transfected with siRNA for 24 h. After RNA isolation, expression of FOXO1 was examined by real-time PCR. Several different siRNA constructs were tested (Table I). siRNA-890 decreased FOXO1 mRNA by 83%, whereas siRNA-1059 and -1674 had virtually no effect and were used as negative controls in further experiments. siRNA-663, -755, and -800 reduced FOXO1 mRNA levels by 23–41%, considerably less than siRNA-890. As an additional control, siRNA-890 was shown not to affect the mRNA level of a related FOXO family member, FOXO3 (data not shown). The above experiments were carried out in the absence of TNF stimulation. The effect of siRNA-890, -1059, or -1674 on FOXO1 mRNA levels was also measured after cells had been incubated with TNF-α. siRNA-890 but not siRNA-1059 and -1674 blocked TNF induction of FOXO1 mRNA levels (p < 0.05) (Fig. 3A). To investigate the effect of siRNA on the activation of FOXO1, cells were transfected with siRNA for 48 h followed by stimulation with TNF-α for 1 h and analyzed by EMSA (Fig. 3B). siRNA-890 blocked EMSA detection of FOXO1 activation in response to TNF-α, whereas siRNA-1059 and -1674 did not. These results demonstrate that TNF increases the expression of FOXO1 mRNA and establishes that siRNA-890 is able to inhibit FOXO1 expression at the mRNA level and block detection by EMSA at the protein level.

FOXO1 Activation Is Necessary for TNF-induced Apoptosis—The role of FOXO1 in promoting TNF-induced apoptosis was examined in fibroblasts transfected with different siRNA without or with TNF stimulation (Table II). Without TNF there was little apoptosis detected, and siRNA alone had virtually no effect. In cells stimulated with TNF-α there was a 7.5-fold increase in fibroblast apoptosis (p < 0.05). In cells transfected with siRNA-890 there was a 76% reduction in TNF-stimulated apoptosis. Cells transfected with siRNA-663, -755, and -800 inhibited TNF-induced apoptosis 38, 32, and 21% respectively consistent with their reduced capacity to silence FOXO1 compared with siRNA-890. siRNA-1059 and -1674 did not affect TNF-stimulated apoptosis.

Inhibition of NFκB Does Not Reduce Apoptosis in Vitro—As noted above, TNF activated both FOXO1 and NFκB in the same time frame. The impact of inhibiting NFκB with SN50, a specific NFκB inhibitor, was tested (21, 22) by EMSA, and the extent of apoptotic activity was determined by ELISA. SN50, but not the matched control SN50M, prevented NFκB activation in response to TNF-α (Fig. 4A). However, the inhibition of NFκB did not suppress fibroblast apoptosis. In fact, the level of apoptosis was 52% higher in the presence of the SN50 inhibitor (Fig. 4B) (p < 0.05). In control experiments it was determined that the NFκB inhibitor SN50 did not affect TNF-induced FOXO1 activation and that FOXO1 siRNA-890 did not enhance TNF-stimulated NFκB activity (data not shown).

Silencing of FOXO1 by siRNA Inhibits TNF-induced Caspase Activity—We have recently demonstrated that TNF induces fibroblast apoptosis through activation of caspase-3 by both caspase-8- and caspase-9-dependent pathways (17, 20). We further investigated the impact of FOXO1 in mediating TNF-induced apoptosis by examining its effect on caspase activity in fibroblasts (Fig. 5). As expected, TNF strongly induced an increase in caspase-3 (790%), caspase-8 (609%), and, to a lesser extent, caspase-9 activity (184%). siRNA-890 reduced TNF-enhanced caspase-3 activity by 79%, caspase-8 activity by 81%, and caspase-9 activity 86% (p < 0.05). In contrast the control siRNA-1059 had little effect on TNF-α-induced caspase activity.

FOXO1 Controls Expression of Many Apoptotic Genes in Response to TNF Stimulation in Vitro—Experiments with microarrays were carried out to study the impact of FOXO1 on the expression of apoptosis-related genes. Fibroblasts were transfected with siRNA-890 or siRNA-1059 followed by stimulation with TNF-α treatment. To determine which genes were dependent upon FOXO1, the effect of TNF stimulation on cells transfected with siRNA-890 was compared with cells transfected with siRNA-1059. Of 59 pro-apoptotic genes that we reported previously to be induced by TNF-α (20), siRNA-890 blocked the induction of 26 genes (Table III) using a 2-fold difference as a threshold. These represent genes from several different functional families, including apoptotic ligands (5), receptors (5), adaptor molecules (4), mitochondrial proteins (5), caspases (4), transcription factors, and other genes. In addition, we reported previously that TNF inhibited the induction of nine anti-apoptotic genes. siRNA-890 reduced TNF-associated inhibition of five of these genes, which are denoted in boldface.
FOXO1 Is a Master Switch for TNF-induced Apoptosis

DISCUSSION

TNF signaling pathways can result in cell survival or cell death. It has been demonstrated previously that decreased apoptosis of cells may result from enhanced expression of survival factors induced by activation of the transcription factor NFκB (9, 23). Considerably less is known about the role of pro-apoptotic transcription factors in mediating apoptosis induced by mediators such as TNF-α.

TNF stimulates apoptosis by engagement with TNFR1, which stimulates the recruitment and assembly of adapter proteins such as TRADD and the Fas-associated protein with death domain that eventually activate initiator caspases-8 or -9 and, finally, caspase-3, an executioner caspase (24–26). The accuracy of this model has been proven by caspase inhibitor studies, which clearly demonstrate that deletion of TNFR1 or inhibition of caspase-8 or caspase-3 prevents the apoptotic effect of TNF (27–31). Although this model identifies pathways through which TNF induces apoptosis, it does not explain why cells that have activation of TNFR1 do not necessarily undergo cell death, particularly because most cells have already expressed the machinery for cell death (32, 33). Thus, apoptosis clearly reflects the balance between pro- and anti-apoptotic factors, which suggests a potential role for transcription factors in modulating the progression of apoptotic events.

Clues to the importance of transcriptional regulation in apoptosis can also be found in temporal patterns. Although apoptosis is a very rapid cellular event taking place within 2 h, the majority of cells stimulated by TNF in vitro do not go through apoptosis until several hours after TNF stimulation (17, 34–36). Thus, there is an unexplained time lag that cannot be accounted for simply by the post-translational modifications that occur between the period of TNF signaling and caspase-mediated cell death. A possibility is that TNF must first activate a transcription factor leading to the wholesale regulation of genes that promote apoptosis. Thus, TNF-stimulated apoptosis may require two steps, one being transcriptional and the other post-translational. The requirement for a transcriptional event would explain the time lag between TNF stimulation and apoptosis.

We undertook experiments to determine whether there was a central pro-apoptotic regulator analogous to the anti-apoptotic factor, NFκB. Based upon the results of a transcription factor array which was confirmed by EMSA, we identified FOXO1 as a potential regulator of TNF-stimulated, pro-apoptotic gene expression. Studies in mammalian cells have shown that the overproduction of members of the FOXO family of transcription factors induces either apoptosis or cell cycle arrest (13, 37–39). To functionally establish the role of FOXO1 in mediating TNF-induced apoptosis, siRNA experiments were carried out. When FOXO1 was silenced by siRNA-890, the capacity of TNF-α to stimulate apoptosis was decreased by 76%. When FOXO1 was partially silenced by less effective siRNA, TNF-stimulated apoptosis was also partially inhibited. Thus, the degree to which a given siRNA decreased FOXO1 mRNA levels was proportional to its capacity to inhibit TNF-induced apoptosis. Moreover, decreased apoptosis by siRNA-890 was accompanied by a decrease in caspase-3, -8, and -9 activities. These results demonstrate that silencing FOXO1 can interrupt the post-translational pathways through which TNF stimulates apoptosis.

Previously, we demonstrated that TNF acts in a global way to enhance the expression of genes that promote apoptosis and diminish the expression of several that inhibit apoptosis (20). Silencing FOXO1 dramatically reduced the capacity of TNF to alter the expression of genes that promote or inhibit apoptosis. Whereas inhibition of FOXO1 prevented apoptosis, inhibition of NFκB had the opposite effect and increased apoptosis. This

![Graph](image-url)

**Fig. 6.** TNF stimulation modifies the expression of apoptotic genes in vitro as measured by RT-PCR. Expression of selected apoptotic genes was measured by RT-PCR on the total RNA extracted from transfected fibroblast cultures stimulated with TNF (20 ng/ml) or phosphate-buffered saline for 6 h. RT-PCR was carried out using primer pairs in which an internal standard, glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**), is run for the same number of cycles as the gene of interest. The expression of each gene is shown after 28 cycles of PCR. The experiment was performed three times with similar results. IL-18, interleukin-18; CD27L, CD27 ligand; 4–1BBL, 4-IBB ligand; FASL, Fas ligand.

**TABLE IV**

| Gene name  | 23 Cycle | 28 Cycle |
|-----------|----------|----------|
| IL-18     | 0.13     | 0.12     |
| CD27      | 0.20     | 0.19     |
| FASL      | 0.24     | 0.28     |
| 4-1BBL    | 0.23     | 0.23     |
| Bax       | 0.26     | 0.28     |

**Table IV**

Primary human dermal fibroblasts were transfected with siRNA-890 or siRNA-1059. 48 h after transfection cells were incubated with 20 ng/ml TNF-α. Total RNA was isolated 6 h after stimulation and subjected to RT-PCR with an internal standard, glyceraldehyde-3-phosphate dehydrogenase. Each value represents the average fold change in the experimental group (siRNA 890) to the control group (siRNA 1059) in response to TNF stimulation that is obtained in three separate experiments amplified for 23 or 28 cycle. Expansions of certain gene designations are as follows: FASL, Fas ligand; 4–1BBL, 4-IBB ligand; IL-18, interleukin 18.
is in agreement with previous findings that NFκB plays an important role in survival (2, 3). Furthermore, FOXO1 siRNA-890 did not reduce apoptosis by enhancing NFκB activity, and the NFκB inhibitor SN50 did not modulate FOXO1 activation.

Based on the above findings, TNF stimulation of fibroblasts leads to activation of both NFκB and FOXO1 transcription factors. Under the experimental conditions tested, FOXO1 predominated because apoptosis proceeded in the presence of NFκB as long as FOXO1 expression was not blocked. FOXO1, like other FOXO family members, modulates apoptosis through regulating the expression of apoptotic genes (14–16, 40, 41). This is in agreement with findings that the capacity of TNF to induce 26 of 59 pro-apoptotic genes or inhibit 5 of 9 anti-apoptotic genes was dependent upon FOXO1. Thus, FOXO1 serves as a “master regulator” of genes modulated by TNF that promote apoptosis. Expression of this master regulator is a virtual requirement for TNF-induced apoptosis.

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