

TNF- α induces TGF- β_1 expression in lung fibroblasts at the transcriptional level *via* AP-1 activation

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Abstract

Tumour necrosis factor- α (TNF- α) and transforming growth factor- β_1 (TGF- β_1) are peptides with multiple biological activities that influence neoplastic, immunologic and fibroproliferative diseases. There are clear interrelationships and overlap between the actions of TNF- α and TGF- β_1 in lung fibrosis; therefore, we postulated that TNF- α may play a significant role in regulating TGF- β_1 expression in lungs. We recently reported that TNF- α activates the extracellular regulated kinase (ERK)-specific pathway in fibroblasts resulting in stabilization of TGF- β_1 mRNA and increased expression of TGF- β_1 . In the current study, we further investigated the molecular mechanisms involved in TNF- α regulation of TGF- β_1 expression. Nuclear run-on assays showed that treatment of Swiss 3T3 fibroblasts with TNF- α increased transcription of the TGF- β_1 gene in an ERK independent manner. Pre-treatment with the activator protein-1 (AP-1) inhibitor curcumin attenuated TNF- α induced transcription of the TGF- β_1 gene. TNF- α induced increased levels of c-Jun and C-Fos in the nucleus accompanied by phosphorylation of c-Jun. In electrophoretic mobility shift assays, AP-1 binding to an AP-1 binding site found within the TGF- β_1 promoter was increased in nuclear extracts from Swiss 3T3 fibroblasts treated with TNF- α . Together, these results suggest that TNF- α induces expression and DNA binding of AP-1 resulting in increased transcription of the TGF- β_1 gene. It is essential to know which transcription pathways are activated because of the wide distribution of TNF- α and TGF- β_1 , the general lack of effective treatments for fibroproliferative disease and the possibility that targeting the correct transcription factors could be palliative.

Keywords: transforming growth factor- β_1 • tumour necrosis factor- α • activator-protein-1 • lung fibrosis

Introduction

Transforming growth factor- β_1 (TGF- β_1) is a multifunctional cytokine involved in a wide variety of biological processes such as embryonic development, neoplastic growth and differentiation, cell proliferation and apoptosis, fibrous tissue deposition and regulation of the immune response [1]. Up-regulation of TGF- β_1 expression is a consistent feature of most fibrotic diseases. Indeed, patients with pulmonary fibrosis have increased levels of TGF- β_1 in their lungs and the levels correlate with severity of lung disease [2]. In rodent models, expression of active TGF- β_1 in lungs induces a severe fibrotic response, whereas inhibitors of

TGF- β_1 expression or signalling afford protection from fibrosis [3]. Consequently, TGF- β_1 has been proposed to be a 'master switch' in induction of fibrosis [4]. In the lung, TGF- β_1 can drive epithelial-to-mesenchymal transition, fibroblast-to-myofibroblast differentiation, and is the most potent inducer of extracellular matrix production characterized to date. Despite intensive investigation into the biological activities of TGF- β_1 in the lung, the mechanisms by which expression of this growth factor are regulated are not yet fully understood.

There is increasing evidence that tumour necrosis factor- α (TNF- α) is important in the initiation and perpetuation of the fibrotic process. TNF- α is a pleiotropic cytokine produced by many cell types in response to infection or injury. In addition to its well-characterized pro-inflammatory properties, TNF- α may modulate cellular differentiation, proliferation and apoptosis. The functions of TNF- α can be both beneficial and detrimental. TNF- α expression is increased in the lungs of patients with pulmonary fibrosis, and agents with anti-TNF- α properties such as pirfenidone and etanercept

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have shown promise in treatment of some patients [5]. Furthermore, TNF- α polymorphisms indicative of high levels of TNF- α expression correlate with an increased risk of developing interstitial pulmonary fibrosis (IPF) [6, 7]. Consistent with these observations, rodents exposed to fibrogenic agents such as bleomycin, silica or asbestos show increased expression of TNF- α in their lungs [8, 9]. Infusion of anti-TNF- α antibody [10] or soluble TNF receptor [11] reduced bleomycin-induced lung injury and decreased expression of TGF- β_1 in the treated mice. Likewise, overexpression of TNF- α in lungs of normal rats induced patchy, interstitial fibrosis and was preceded by a significant increase in TGF- β_1 production [12]. We showed that mice with both the 55 and 75 kD receptors for TNF- α knocked out (TNFR-KO) fail to develop significant bleomycin-induced fibrosis, silicosis or asbestosis [9, 13, 14]. Furthermore, the TNFR-KO mice had reduced levels of TGF- β_1 in their lungs after exposure to asbestos [13]. We later showed that TGF- β_1 overexpression in TNFR-KO mice was sufficient to induce pulmonary fibrosis [15].

Since there are clear interrelationships and overlap between the actions of TNF- α and TGF- β_1 in lung fibrosis, we have postulated that TNF- α may play a significant role in regulating TGF- β_1 expression in lung. In support of this hypothesis, we showed *in vitro* that TNF- α rapidly up-regulates TGF- β_1 and collagen expression in both primary lung mesenchymal and epithelial cells [16]. In studies designed to investigate a signal transduction pathway by which TNF- α induces TGF- β_1 expression, we demonstrated that TNF- α rapidly activates the extracellular regulated kinase (ERK)-specific mitogen-activated protein kinase pathway resulting in stabilization of TGF- β_1 mRNA and increased expression of TGF- β_1 protein in primary mouse lung fibroblasts (MLFs) [17]. In the findings presented here, we show that TNF- α rapidly induces activation of the activator protein-1 (AP-1) transcription factor and increases transcription of the TGF- β_1 gene. Together, these results show that TNF- α up-regulates TGF- β_1 through a complex mechanism involving both increased transcription and mRNA stability.

Materials and methods

Cell culture

Swiss 3T3 fibroblasts were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% bovine calf serum (Invitrogen, Carlsbad, CA, USA), L-glutamine, non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 μ g/ml). Primary cultures of MLFs were established as previously described [16]. In this study, we used MLF cultures at passages 2 and 3. Fresh growth media was added every 3 to 4 days and cells were kept sub-confluent until used for experiments. Confluent monolayers were rendered quiescent in media containing 1% serum for 48 hrs before use in experiments. All experiments were performed in media containing 1% serum.

Real-time RT-PCR

Total RNA was isolated using RNeasy kit (Qiagen, Gaithersburg, MD, USA) and any contaminating DNA removed using TURBO DNA-free (Ambion, Austin, TX, USA), both according to the manufacturer's recommendations. Reverse transcription of 250 ng total RNA was performed in a total volume of 20 μ l using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. One microlitre of cDNA was PCR amplified in 20 μ l reactions containing primers at 250 nM in iQ SYBR Green Supermix (Bio-Rad) as previously described [17]. Primers for TGF- β_1 pre-mRNA were as follows: 5'-GCTACCATGCCAAGCTCTGTCT-3' (sense); and 5'-CCTACCACCCAGCCTCTG-3' (antisense) resulting in amplification of a 116 bp product. Primers for mature TGF- β_1 mRNA were as follows: 5'-GGATACCAACTATTGCTTCAGCTCC-3' (sense) and 5'-AGGCTCCAATATAGGGCAGGGTC-3' (antisense) yielding a 156 bp product. The sense primer was designed to overlap both the fifth and sixth exons to control against amplification of TGF- β_1 pre-mRNA or DNA. Primers for mouse MMP9 mRNA were: 5'-AGACCTGAAACCTCCAACCTCAC-3' (sense) and 5'-TGTTATGATGGTCCCCTGAGGC-3' (antisense), resulting in a 262 bp product [18]. Primers for mouse acidic ribosomal phosphoprotein (36B4) mRNA were as follows: 5'-CGACCTGGAAGTCCAACCTAC-3' (sense) and 5'-ATCTGCATCTGCTTG-3' (antisense) yielding a 109 bp product [19]. Negative controls, including cDNA reactions without reverse transcriptase or RNA, and PCR mixtures lacking cDNA were included in each PCR. Following amplification, specificity of the reaction was confirmed by melt curve analysis. Relative quantitation was determined using the comparative C_T method with data normalized to 36B4 and calibrated to the average C_T of untreated controls.

Nuclear run-on

To determine transcription rate, nuclear run-on assays were performed. Quiescent Swiss 3T3 cells were treated with TNF- α (10 ng/ml) or media alone for 2 hrs. Nuclei were then isolated from 1×10^7 cells using Nuclei Isolation Kit (Nuclei PURE Prep, Sigma-Aldrich, St. Louis, MO, USA). Nuclei were either stored in liquid nitrogen, or used immediately. Traditional nuclear run-on assays were performed as described [20]. Briefly, 100 μ l of nuclei were incubated with 30 μ l of 5 \times reaction buffer (25 mM Tris-HCl, pH 8, 12.5 mM MgCl₂, 750 mM KCl, 1.25 mM each of ATP, GTP and CTP and 100 μ Ci α -³²P-UTP) for 30 min. at 30°C, then 15 units of RQ1 DNase (Promega, Madison, WI, USA) were added and incubation continued for another 15 min. Labelled RNA was extracted with Trizol (Invitrogen) and radioactivity was determined by liquid scintillation counting. Each extract was normalized to radioactivity and hybridized to target sequences immobilized on a nylon membrane at 60°C overnight. The membrane was washed extensively to remove excess RNA: 2 \times saline-sodium citrate buffer (SSC) (2 \times 5 min., room temperature); 2 \times SSC, 1% SDS (15 min., 65°C); 2 \times SSC (5 min., room temperature); 2 \times SSC, 10 μ g/ml RNase A (10 min., 37°C); 2 \times SSC (5 min., room temperature); 0.1 \times SSC (3 \times 5 min., room temperature). After autoradiography, the intensity of hybridized radiolabelled mRNA was determined by Phosphor Imager analysis using ImageGauge software. The alkaline denatured target DNA sequences, murine TGF- β_1 [21] and murine 36B4 cDNAs cloned into pGEM7Z were immobilized on nylon membrane by slot blotting followed by UV cross-linking. To monitor non-specific hybridization, empty pGEM7Z vector was also blotted on the membrane.

In some experiments, PCR-based nuclear run-on assays were performed as previously described [22, 23]. Briefly, fresh or thawed nuclei

(1×10^7) were split into two aliquots. The first aliquot was incubated with $2 \times$ reaction buffer (20% (v/v) glycerol, 100 mM KCl, 10 mM MgCl₂, 4.5 mM dithiothreitol (DTT), 1.2 mM adenosine 5'-triphosphate (ATP), 0.6 mM each of cytosine 5'-triphosphate (CTP), guanine 5'-triphosphate (GTP) and uridine 5'-triphosphate (UTP), 0.5 mM spermidine, 0.15 mM spermine and 80 U/ml RNase inhibitor) for 15 min. at 22°C. The second aliquot was treated the same as the first, except that no ribonucleotides (rNTPs) were added. Nuclei were then subjected to RQ1 DNase I (20 units, Promega) digestion in the presence of 1 mM CaCl₂ for 15 min. at 37°C. Nuclear proteins were digested by adding $10 \times$ digestion buffer (100 mM Tris, pH 8.0, 10 mM ethylenediaminetetraacetic acid, 5% SDS and 100 µg/ml proteinase K) and incubation for 30 min. at 37°C. Nuclear RNA was then purified using RNeasy kit (Qiagen). Isolated RNA was treated with TURBO DNA-free (Ambion) to ensure no contaminating DNA. Real-time RT-PCR was carried out using primers that amplify TGF-β₁ pre-mRNA as described above. To quantitate the nascent TGF-β₁ pre-mRNA transcripts, values obtained from nuclear run on reactions with no rNTPs added were subtracted from reactions with rNTPs added.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed with the Gel Shift Assay System and AP-1 consensus oligonucleotides (5'-CGCTTGATGAGTCAGCCGAA-3' (Promega). The double-stranded oligonucleotides were labelled with [γ -³²P]ATP (Perkin Elmer, Waltham, MA, USA). Binding reactions were carried out with nuclear extracts prepared from TNF-α treated or untreated Swiss 3T3 cells at the indicated times using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. One microlitre of ³²P-labelled probe was incubated with 5 µg of Swiss 3T3 nuclear extract for 10 min. at 37°C. DNA-protein binding specificity was tested by competition assays in which the binding reactions were pre-incubated with 100-fold excess of unlabelled specific or non-specific competitor oligonucleotides prior to the addition of the labelled probe. Positive controls consisted of HeLa extracts provided with the Gel Shift Assay System (Promega). For the supershift experiment, 2 µg of each antibody were added to the reaction mixtures that already contained the probe and 10 µg nuclear lysates. The entire mixture was incubated for an additional 30 min. at room temperature. The antibodies used were as follows: anti-c-Jun (sc-44X, Santa Cruz, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-c-Jun (sc-822X, Santa Cruz), anti-c-Fos (sc-253X, Santa Cruz) and normal mouse IgG (PP100, Chemicon, Temecula, CA, USA). After the binding reaction was complete, the DNA-protein complexes were resolved by electrophoresis in a 6% non-denaturing acrylamide gel (Invitrogen). After electrophoresis, the gels were transferred onto 3M Whatman paper, dried and subjected to autoradiography.

Western immunoblotting

Nuclear extracts prepared as described above were separated by SDS-PAGE (12% pre-cast gels from NuPAGE, Invitrogen) followed by semi-wet electrophoretic transfer to 0.45 µm polyvinylidene difluoride (PVDF) membrane as previously described [17]. The membranes were blocked for 1 hr in tris-buffered saline with tween 20 (TBST) (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% bovine serum albumin and then hybridized overnight with one of the following primary antibodies: rabbit anti-c-Fos, rabbit anti-c-Jun, rabbit anti-phospho-c-Jun (Ser63) (all from Cell Signaling, Danvers, MA, USA) or mouse anti-β-actin (Abcam, Cambridge, MA, USA). After three washes in TBST, membranes were probed with the

appropriate secondary antibody: sheep antimouse (Amersham, Piscataway, NJ, USA) or donkey anti-rabbit IgG (Amersham), each conjugated to horseradish peroxidase (HRP). The HRP-catalysed chemiluminescence reaction was developed with Enhanced Chemiluminescence System (ECL, Amersham). The ECL-treated membranes were exposed to X-ray film to visualize the immunoreactive protein bands. The membranes were stripped and reprobed with β-actin to correct for differences in protein loading.

Statistics

All values are expressed as means \pm S.E.M., where $n = 3$ or 4. An unpaired Student's t-test was used to assess the difference between two groups. ANOVA was performed when more than two groups were compared to a single control and Tukey post-tests were used to assess differences between individual groups within the set. Differences were considered statistically significant when $P < 0.05$.

Results

De novo transcription is required for TNF-α induction of TGF-β₁

In a recent study [17] we showed that treatment of fibroblasts isolated from the lungs of adult C57 mice, or Swiss 3T3 mouse embryo fibroblasts, with recombinant, murine TNF-α resulted in a dose- and time-dependent increase in levels of TGF-β₁ mRNA through a mechanism involving mRNA stabilization. The increase in TGF-β₁ message could be detected as early as 2 hrs after addition of TNF-α suggesting that transcription of the TGF-β₁ gene may also be up-regulated. To determine if TNF-α-induction of TGF-β₁ mRNA expression requires *de novo* transcription, Swiss 3T3 fibroblasts were treated with 50 µM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), an inhibitor of RNA polymerase II transcription, for 1 hr prior to addition of TNF-α (10 ng/ml). In our previous study [17], treatment of cells with 10 ng/ml of TNF-α produced the strongest induction of TGF-β₁ expression. After 12 hrs, TGF-β₁ mRNA levels were measured by real-time RT-PCR. As a control, we also measured the effect of DRB on TNF-α induction of MMP-9 mRNA, which is known to be transcriptionally up-regulated by TNF-α in fibroblasts [24]. Figure 1 shows that DRB reduced the TNF-α induction of TGF-β₁ by 37.5% whereas TNF-α induction of MMP9 mRNA was completely blocked. These results suggest that TNF-α induces TGF-β₁ mRNA expression at least partially *via* a transcriptional mechanism.

TNF-α increases transcription of the TGF-β₁ gene

To determine if TNF-α increased transcription of TGF-β₁, we first measured the levels of pre-mRNA since these newly synthesized and unspliced RNAs have been shown to reflect transcriptional activity [25]. To measure TGF-β₁ pre-mRNA, we designed PCR

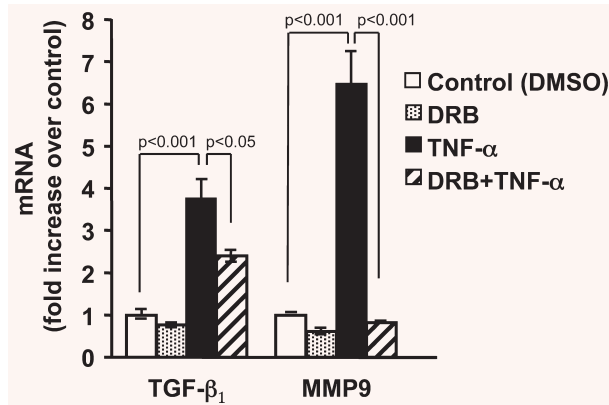


Fig. 1 Inhibition of transcription partially blocks TNF- α induction of TGF- β_1 mRNA. Quiescent Swiss 3T3 fibroblasts were pre-treated with 50 μ M 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), or vehicle (DMSO) as control, for 1 hr followed by addition of 10 ng/ml TNF- α for another 12 hrs. At the indicated times, total RNA was isolated and TGF- β_1 mRNA was measured by quantitative real-time RT-PCR. As a control, MMP9 (a gene known to be transcriptionally up-regulated by TNF- α) mRNA was also measured. Relative quantitation was determined using the comparative C_T method with data normalized to 36B4 riboprotein mRNA and calibrated to the average ΔC_T of untreated controls (fold induction = $2^{-\Delta\Delta C_T}$). Data are represented as the mean \pm S.E.M., of three independent experiments.

primers such that the sense primer is located in exon 6 and the antisense primer is located within intron 6. Processed or mature mRNA levels were measured by using a sense primer that spanned the junction of exons 5 and 6 and an antisense primer in exon 6. As shown in Fig. 2A, TGF- β_1 pre-mRNA increased dramatically between 1 and 2 hrs after treatment with TNF- α and preceded the increase of TGF- β_1 mature mRNA. Pre-treatment of the cells with DRB to inhibit transcription completely blocked the increase in TGF- β_1 pre-mRNA (Fig. 2B) suggesting that TNF- α treatment increased transcriptional activity of the TGF- β_1 promoter. Experiments using actinomycin D yielded similar results at the 1 and 2 hrs time-points; however, pronounced toxicity was observed in cultures receiving TNF- α and actinomycin D at the 4-hr time-point.

We previously reported that TNF- α failed to activate a TGF- β_1 promoter-luciferase construct (pHTG -1362/+819) transfected into Swiss 3T3 cells [17]. We considered that the promoter-reporter assay may not accurately reflect regulation of the endogenous TGF- β_1 gene where chromatin effects may represent another level of control. Therefore we performed nuclear run-on assays to determine if TNF- α increases the rate of TGF- β_1 transcription. Addition of TNF- α resulted in a greater than twofold increase in the rate of TGF- β_1 transcription when compared to addition of media alone (Fig. 3). Together, these results strongly suggest that TNF- α induces transcription of the TGF- β_1 gene.

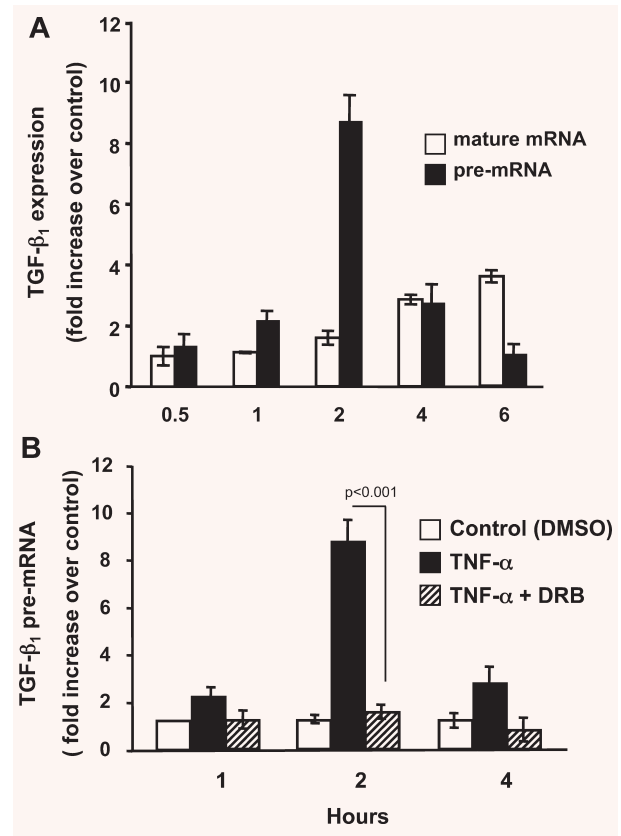


Fig. 2 TNF- α induction of TGF- β_1 mRNA is preceded by an increase in TGF- β_1 pre-mRNA and requires *de novo* transcription. (A) Quiescent Swiss 3T3 fibroblasts were treated with 10 ng/ml TNF- α for the indicated times. Expression of TGF- β_1 pre-mRNA and mature mRNA were determined by quantitative real-time RT-PCR using primers designed to amplify unprocessed mRNA (pre-mRNA) or spliced mRNA (mature mRNA) after extensive DNase treatment. (B) Quiescent Swiss 3T3 fibroblasts were pre-treated with 50 μ M DRB or vehicle (DMSO) as control for 1 hr followed by addition of 10 ng/ml TNF- α . At the indicated times TGF- β_1 pre-mRNA was measured as described above. (Mean \pm S.E.M., relative units, $n = 3$.)

ERK signalling is not required for TNF- α induced transcription of the TGF- β_1 gene

We recently showed that TNF- α rapidly activates the ERK signalling pathway resulting in stabilization of TGF- β_1 mRNA [17]. Here, we asked if TNF- α activation of ERK signalling also leads to increased transcription of the TGF- β_1 gene. Quiescent Swiss 3T3 cells were pre-treated with U0126, a highly selective inhibitor of ERK activation, followed by addition of TNF- α for 2 hrs. Nuclear run-on assays were then performed as described above. As seen in Fig. 4, inhibition of ERK activation had no effect on TNF- α induced transcription of the TGF- β_1 gene. In similar experiments, TNF- α induced increases in TGF- β_1 pre-mRNA levels were also

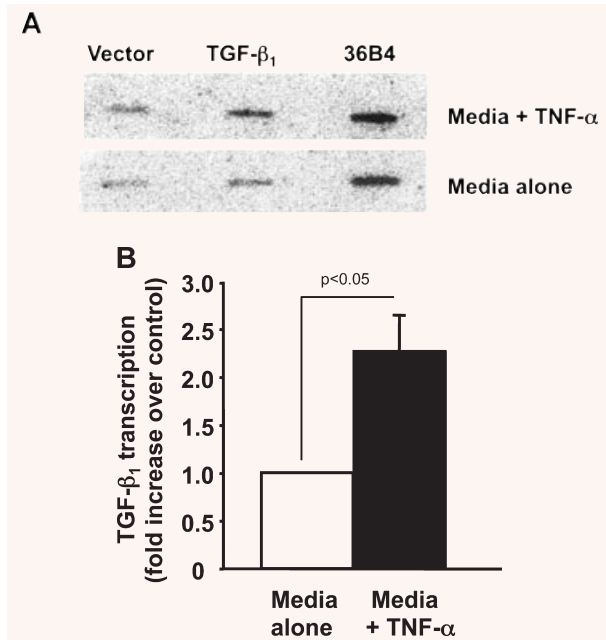


Fig. 3 TNF- α stimulates transcription of the TGF- β_1 gene in fibroblasts. Quiescent Swiss 3T3 cells were treated with TNF- α (10 ng/ml) or media alone for 2 hrs. Nuclei were then isolated and nuclear run-on transcription reactions performed in the presence of α -[³²P] labelled UTP. Radiolabelled RNA was hybridized to plasmid DNAs carrying cDNAs for murine TGF- β_1 , murine 36B4, or vector alone under stringent conditions. (A) Autoradiogram. (B) Densitometric measurements of TGF- β_1 transcription rate were performed with ImageGauge software and are expressed relative to 36B4 transcription rate after subtracting background hybridization to the vector control. In some experiments, PCR-based nuclear run-on reactions were performed. Data are representative of four experiments.

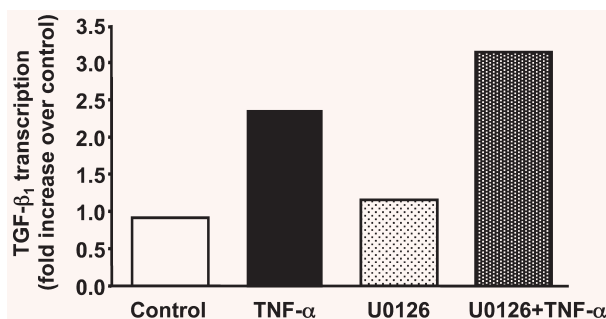


Fig. 4 Inhibition of the MEK/ERK signalling pathway does not block TNF- α stimulated transcription of the TGF- β_1 gene. Quiescent Swiss 3T3 cells were treated with 10 μ M U0126 or DMSO as vehicle control for 30 min. before stimulation with TNF- α (10 ng/ml) for an additional 2 hrs. Nuclei were then isolated and PCR-based nuclear run-on assays were performed. This experiment was repeated with similar results.

unaffected by pre-treatment with U0126 (data not shown). These results suggest that ERK signalling is not required for TNF- α induction of TGF- β_1 transcription.

AP-1 activity is required for TNF- α induction of TGF- β_1

Transcription of the human TGF- β_1 gene has been shown to be regulated primarily by AP-1 [26–28] and selective promoter factor (Sp1) [29]. To determine if either of these transcription factors is involved in TNF- α induced TGF- β_1 transcription, we pre-treated Swiss 3T3 cells with curcumin, an inhibitor of AP-1 or mithramycin, an inhibitor of Sp1. Curcumin, completely blocked TNF- α induction of TGF- β_1 pre-mRNA (Fig. 5A) and reduced mature mRNAs by 60% (Fig. 5B). In contrast, mithramycin had no effect on TGF- β_1 pre-mRNA (Fig. 5A) or mature mRNA (Fig. 5B) levels. Curcumin has been reported to block NF- κ B activation [30], therefore we also pre-treated cells with SC-514 (Calbiochem, San Diego, CA, USA), a highly selective inhibitor of IKK-2 that has been shown to specifically block NF- κ B-dependent gene expression. As shown in Fig. 6, SC-514 failed to block TNF- α induction by TGF- β_1 , even when added at 100 μ M. These results were confirmed by transfection of Swiss 3T3 cells with a cDNA encoding a dominant negative I κ B (I κ B Δ N), a cellular inhibitor of NF- κ B activation, prior to treatment with TNF- α (data not shown). Together, these results implicate AP-1 in TNF- α up-regulation of TGF- β_1 transcription.

TNF- α activates AP-1 binding to DNA

To determine whether TNF- α activated AP-1 in our cells, we performed EMSAs. The mouse TGF- β_1 promoter contains a canonical AP-1 binding site (TGAGTCA) at nucleotide 445–451 (M57902). Therefore, nuclear protein extracts prepared from Swiss 3T3 cells treated with TNF- α , or media alone as control, were incubated with radiolabelled double-stranded oligonucleotides representing the consensus binding site for AP-1 (TGAGTCA) as a probe. As shown in Fig. 7A and B, TNF- α increased DNA binding of AP-1 in a time-dependent manner reaching approximately twofold over control after 2 hrs. To determine if the observed binding was specific to the AP-1 site, we used unlabelled probe and a non-specific oligonucleotide as competitors. The addition of 100-fold molar excess of unlabelled probe completely blocked binding to the labelled probe whereas addition of an equal concentration of a non-specific oligonucleotide only slightly reduced binding. To confirm that AP-1 was a component of the complexes observed, we performed supershift assays using antibodies to c-Jun and c-Fos, components of the AP-1 transcription factor. As seen in Fig. 7C, addition of a monoclonal antibody that specifically recognizes phosphorylated c-Jun elicited a strong supershift in the nuclear extract from Swiss 3T3 fibroblasts 2 hrs after treatment with TNF- α . Polyclonal antibodies to total c-Jun and c-Fos reduced DNA binding and caused a supershift of less intensity. This is

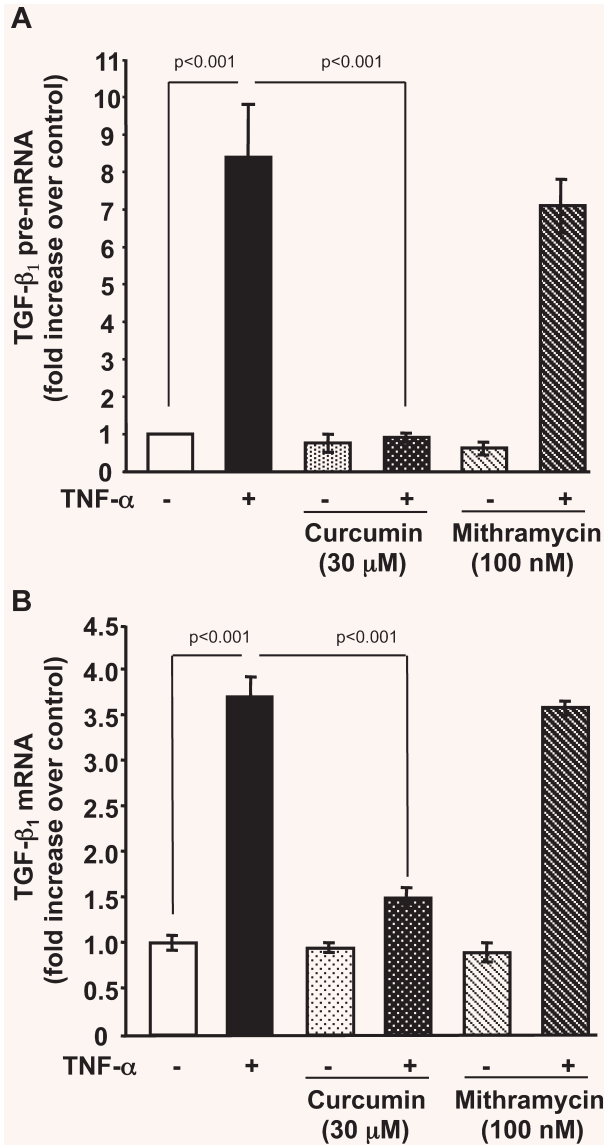


Fig. 5 Curcumin, an AP-1 inhibitor, abrogates TNF- α induction of TGF- β_1 pre-mRNA and mature mRNA. Quiescent Swiss 3T3 fibroblasts were treated with TNF- α (10 ng/ml) or media alone in the presence (+) or absence (-) of curcumin (30 μ M) or mithramycin (100 nM). **(A)** After 2 hrs, TGF- β_1 pre-mRNA was measured by quantitative real-time RT-PCR as described above. **(B)** After 6 hrs, mature TGF- β_1 mRNA was measured by quantitative real-time RT-PCR as described above. (Mean \pm S.E.M., relative units, $n = 4$.)

consistent with some antibody binding to the DNA binding regions of c-Jun and c-Fos. These data show that TNF- α induces specific binding of AP-1 to DNA.

To determine if TNF- α increases the levels of c-Jun and c-Fos in the nucleus, we performed Western blot analyses of nuclear

extracts from Swiss 3T3 cells treated with TNF- α or media alone. Incubation of quiescent Swiss 3T3 fibroblasts with TNF- α resulted in a rapid increase in nuclear c-Jun levels that was further increased at 2 hrs (Fig. 8). Moreover, TNF- α induced rapid phosphorylation of c-Jun within 1 hr of treatment (Fig. 8). Similarly, the nuclear levels of c-Fos were significantly increased in TNF- α treated cells after 1 hr and returned to near control levels by 2 hrs (Fig. 8). Together these results clearly demonstrate that TNF- α increases nuclear levels of c-Jun and c-Fos and that these transcription factors specifically bind to an AP-1 site found within the TGF- β_1 promoter.

TNF- α up-regulates transcription of TGF- β_1 mRNA in an AP-1 dependent manner in primary mouse lung fibroblasts

We previously demonstrated that the Swiss 3T3 fibroblast cell line is very similar to primary MLFs in their response to TNF- α [17]. To confirm that TNF- α induces transcription of TGF- β_1 in an AP-1 dependent manner in MLFs, we next asked whether curcumin could block TNF- α induced expression of TGF- β_1 pre-mRNA in primary lung fibroblasts isolated from adult C57Bl6 mice. As shown in Fig. 9, TNF- α induced a rapid increase in TGF- β_1 pre-mRNA that was completely blocked by the AP-1 inhibitor, curcumin. These results confirm the biological relevance of our findings in primary lung cells.

Discussion

In the present study, we show that TNF- α doubles the rate of transcription of the TGF- β_1 gene in an AP-1 dependent manner. We previously demonstrated that TNF- α also stabilizes TGF- β_1 mRNA *via* an ERK dependent pathway. Together these results demonstrate that the mechanisms by which TNF- α up-regulate TGF- β_1 are complex; they involve a combination of transcriptional and post-transcriptional events that lead to the observed three- to fourfold increase in steady-state TGF- β_1 mRNA 6–12 hrs after exposure to TNF- α . It is essential to have this information because TNF- α and TGF- β_1 are potent peptide growth factors that have been implicated in playing important roles in the development of a variety of diseases including neoplastic and fibrogenic processes. Moreover, there is increasing evidence that TNF- α influences TGF- β_1 expression. TNF- α has been shown to induce the expression of TGF- β_1 in many cell types including microglial cells [31], mature adipocytes [32], human proximal tubular cells [33] and rat pulmonary artery endothelial cells [34] *in vitro*. Adenovirus-mediated expression of TNF- α in lungs of normal rats was shown to induce TGF- β_1 production and interstitial fibrogenesis [12], suggesting a functional importance of TNF- α -induced TGF- β_1 expression *in vivo*. However, there is clear evidence that TNF- α has antifibrotic properties as well. In addition

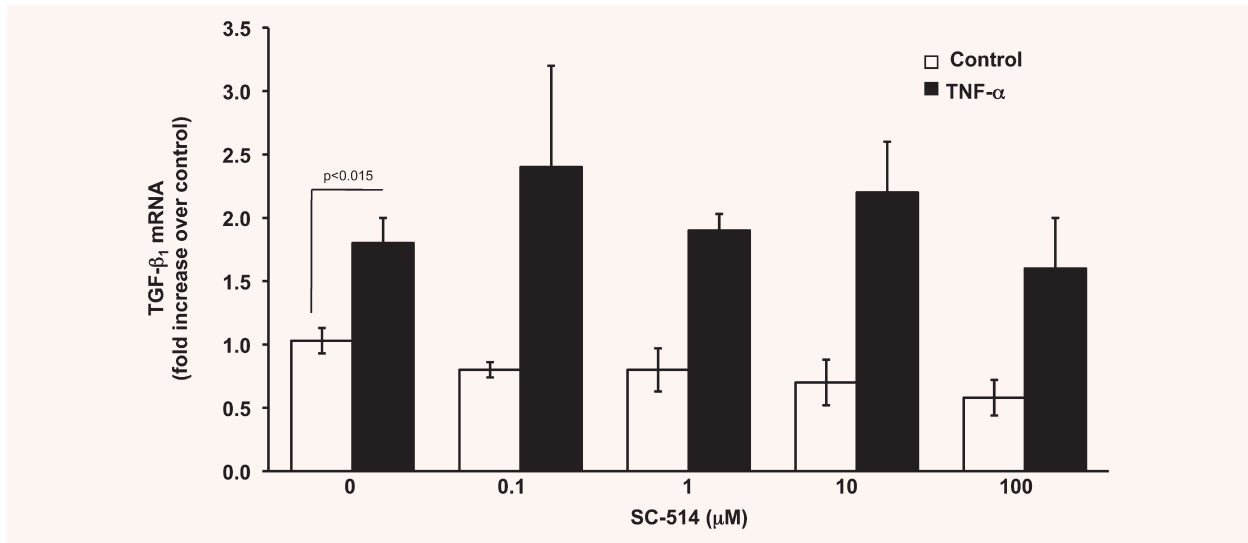


Fig. 6 NF- κ B activation is not required for TNF- α induction of TGF- β ₁. Quiescent Swiss 3T3 fibroblasts were pre-treated with increasing doses (0.1–100 μ M) of the IKK-2 inhibitor SC-514 or vehicle (DMSO) for 1 hr and then treated with TNF- α (10 ng/ml) or media alone. After 6 hrs, mature TGF- β ₁ mRNA was measured by quantitative real-time RT-PCR as described above. (Mean \pm S.E.M., relative units, $n = 3$.)

to its well known pro-inflammatory effects, TNF- α also inhibits synthesis of structural components such as type I collagen and induces production of stromal collagenases [35]. Moreover, overexpression of TNF- α diminished pulmonary fibrosis induced by bleomycin and TGF- β ₁ *in vivo* [36]. A possible explanation for the contradictory effects of TNF- α was recently provided by Oikonomou *et al.* [37]. They showed that in bleomycin-induced fibrosis, expression of the membrane-bound form of TNF- α is sufficient to elicit an inflammatory response, but does not lead to fibrosis, whereas expression of the soluble form of TNF- α is crucial for lymphocyte recruitment and a prerequisite for TGF- β ₁ expression and the development of fibrosis [37]. We propose that soluble TNF- α directly up-regulates expression of TGF- β ₁ in the lung thereby initiating or perpetuating a cytokine cascade that leads to fibrosis. The present study provides new information that clarifies the intracellular signalling mechanisms involved in TNF- α up-regulation of TGF- β ₁. This pathway is not unique to mouse fibroblasts as we have shown that fibroblasts and alveolar epithelial cells from the lungs of both mice and human beings exhibit up-regulated TGF- β ₁ expression in response to TNF- α [16, 38].

Interstitial pulmonary fibrosis afflicts millions of individuals worldwide, yet there are no effective treatments and few therapeutic approaches (5). Our findings are consistent with the thinking that blunting TNF- α expression or signalling pharmacologically may be useful in preventing or treating pulmonary fibrosis. As mentioned above, anti-TNF- α treatment with agents such as pirfenidone and etanercept have shown promise in treatment of some patients [5]. Interestingly, the AP-1 inhibitor, curcumin has been shown to inhibit the development of pulmonary fibrosis by either bleomycin [39] or amiodarone [40]. Further studies aimed

at understanding the mechanisms involved in TNF- α regulation of TGF- β ₁ may lead to new strategies for the treatment of IPF.

Numerous studies have shown that TGF- β ₁ expression may be controlled at the levels of transcription, mRNA stability, mRNA translation or secretion of preformed protein [33, 41–43]. Our results showed that TNF- α induced a rapid increase in TGF- β ₁ mRNA levels detected as early as 2 hrs after treatment (Fig. 2A). Although our previous study showed that TNF- α increased the half-life of TGF- β ₁ mRNA to more than 24 hrs, the rapid increase in TNF- α mRNA levels suggested that TNF- α may also increase transcription of the TGF- β ₁ gene. In this study, we show that *de novo* transcription is required for maximal TNF- α induction of TGF- β ₁. In an effort to separate the effects of TNF- α on TGF- β ₁ mRNA stability and transcriptional activation, we measured levels of newly transcribed unprocessed pre-mRNA, also known as heterogeneous nuclear RNA (hnRNA), by real-time RT-PCR [25] and found that TNF- α induced a significant increase in TGF- β ₁ pre-mRNA that preceded the increase in mature mRNA (Fig. 2A). Because changes in the amount of unprocessed pre-mRNA may reflect not only altered transcription but also altered processing, we confirmed these results using traditional nuclear run-on analyses (Fig. 3). Together these results show that TNF- α treatment does indeed increase the rate of transcription of the TGF- β ₁ gene as well as stabilize the mRNA.

That TNF- α regulation of TGF- β ₁ involves both transcriptional and post-transcriptional mechanisms is not surprising since several factors, including TGF- β ₂ [44] and nerve growth factor [45], regulate TGF- β ₁ in a similar manner. It is becoming apparent that carefully orchestrated transcriptional and post-transcriptional processes allow precise and rapid changes in the production of TGF- β ₁. The relative contribution of gene transcription and mRNA turnover to

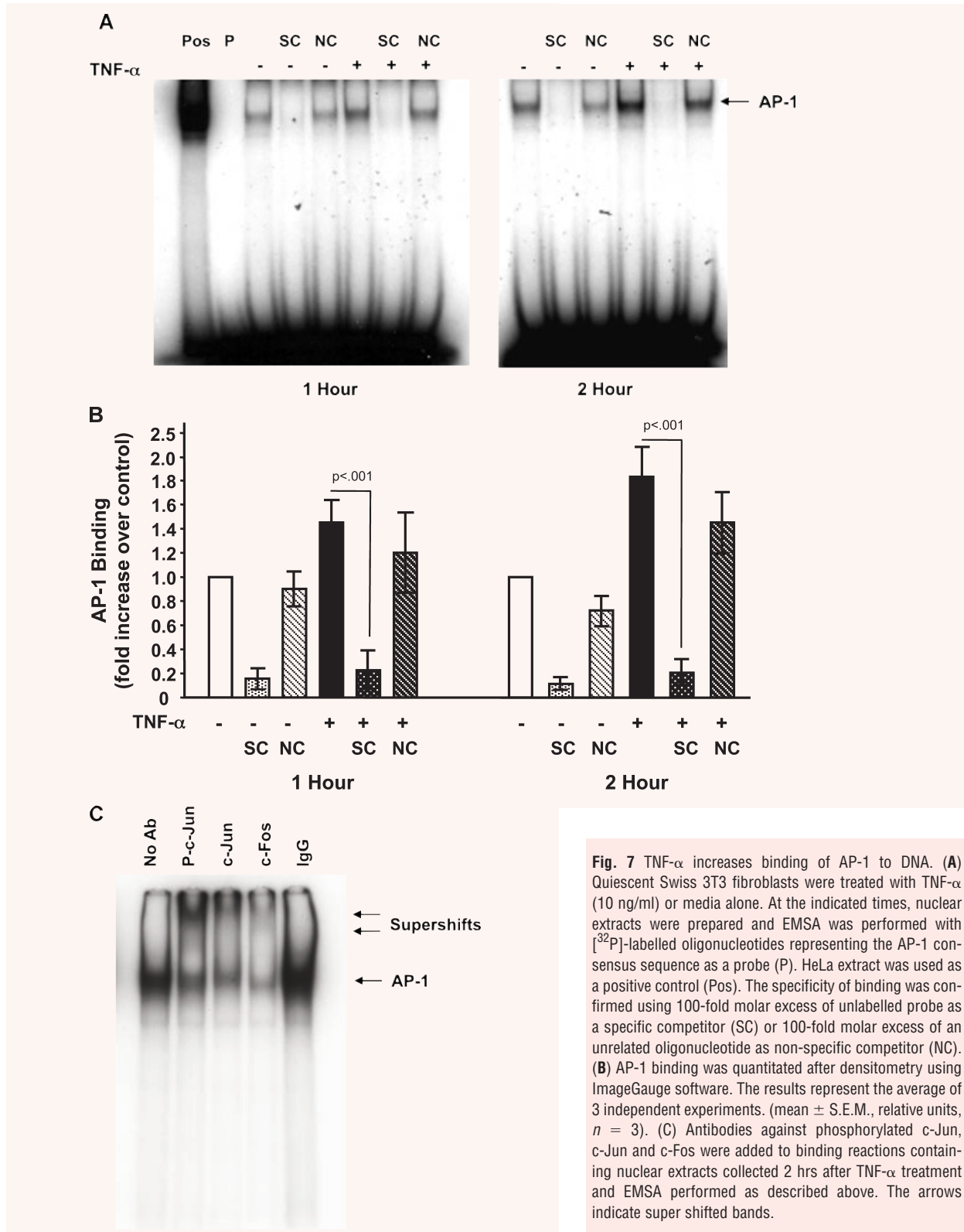


Fig. 7 TNF- α increases binding of AP-1 to DNA. **(A)** Quiescent Swiss 3T3 fibroblasts were treated with TNF- α (10 ng/ml) or media alone. At the indicated times, nuclear extracts were prepared and EMSA was performed with [32 P]-labelled oligonucleotides representing the AP-1 consensus sequence as a probe (P). HeLa extract was used as a positive control (Pos). The specificity of binding was confirmed using 100-fold molar excess of unlabelled probe as a specific competitor (SC) or 100-fold molar excess of an unrelated oligonucleotide as non-specific competitor (NC). **(B)** AP-1 binding was quantitated after densitometry using ImageGauge software. The results represent the average of 3 independent experiments. (mean \pm S.E.M., relative units, $n = 3$). **(C)** Antibodies against phosphorylated c-Jun, c-Jun and c-Fos were added to binding reactions containing nuclear extracts collected 2 hrs after TNF- α treatment and EMSA performed as described above. The arrows indicate super shifted bands.

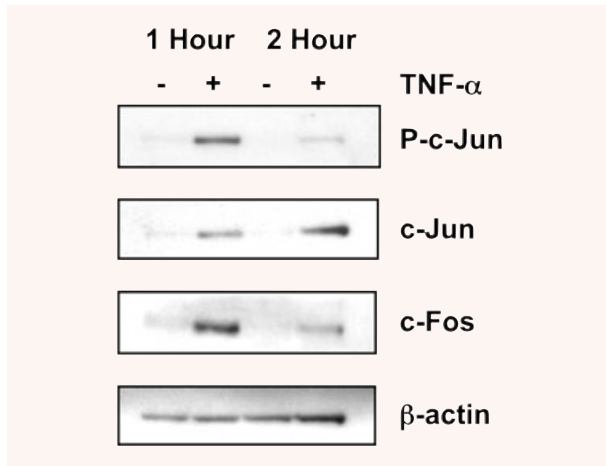


Fig. 8 TNF- α increases nuclear levels of c-Jun and c-Fos. Quiescent Swiss 3T3 fibroblasts were treated with TNF- α (10 ng/ml) or media alone for the indicated times. Nuclear extracts were prepared and analysed by Western blot for c-Jun phosphorylated at Ser63 (P-c-Jun). The blot was stripped and reprobbed for total c-Jun and then c-Fos. Human β -actin was used as an internal loading control after stripping the blot a third time. The results presented are representative of three independent experiments.

TGF- β_1 expression are likely to differ according to the stimulus and the cell type involved. In our studies, TNF- α induced TGF- β_1 expression in lung fibroblasts was inhibited ~37% when RNA polymerase II transcription was inhibited (Fig. 4), and more than 60% when ERK signalling was inhibited [17]. Thus, in lung fibroblasts, post-transcriptional events initiated by TNF- α appear to be a major contributor for the increased expression of TGF- β_1 ; however, increased transcription contributes to the over-all increase in TGF- β_1 .

The mouse and human TGF- β_1 promoters are well conserved in sequence and general structure. Both the human [46] and mouse [47] TGF- β_1 genes have two promoter regions, one located 5' to the most upstream transcriptional start site, and another located between the two major transcriptional initiation sites. Both human and mouse TGF- β_1 promoter regions contain consensus sequences for AP-1, NF- κ B and Sp1. Curcumin, an AP-1 inhibitor blocked TNF- α induced TGF- β_1 transcription (Fig. 5A) resulting in a 60% reduction in the steady state levels of TGF- β_1 mRNA, whereas inhibitors of Sp1 (Fig. 5B) or NF- κ B (Fig. 6) had no effect. These results strongly suggest that AP-1 mediates TNF- α induced TGF- β_1 transcription. Consistent with these results, TNF- α induced AP-1 binding (Fig. 7A) that was preceded by phosphorylation of c-Jun within the transactivation domain (Ser63) (Fig. 8). Activation of c-Jun is known to initiate dimerization of c-Jun with other AP-1 binding partners, such as c-Fos [48], which was also up-regulated by TNF- α in our experiments. Supershift assays confirmed that TNF- α induced binding of c-Jun and c-Fos to a sequence found in the TGF- β_1 promoter (Fig. 6C). Additional studies using chromatin immunoprecipitation will be required to demonstrate increased binding of AP-1 to the endogenous TGF- β_1 promoter in response to TNF- α and whether other transcription factors are involved.

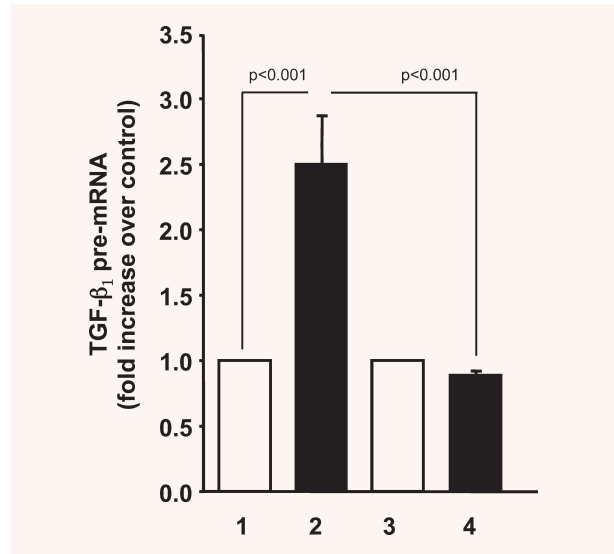


Fig. 9 TNF- α induces transcription of TGF- β_1 in primary mouse lung fibroblasts by an AP-1 dependent mechanism. Fibroblasts isolated from the lungs of adult C57 mice were rendered quiescent and treated with 10 ng/ml TNF- α (lanes 2 and 4) or media alone (lanes 1 and 3) for 2 hrs. Cells in lanes 3 and 4 were incubated with 30 μ M curcumin for 1 hr prior to treatment. TGF- β_1 pre-mRNA was measured by quantitative real-time RT-PCR as described above. (mean \pm S.E.M., relative units, $n = 4$).

Our previous results show that TNF- α activation of ERK signalling leads to stabilization of the TGF- β_1 mRNA in lung fibroblasts [17]. ERK has been shown to phosphorylate residues of the c-Jun transactivation domain, thereby facilitating AP-1 transcription factor dimerization, DNA binding, and transcriptional regulation [49]. Therefore, we postulated that TNF- α activation of the ERK pathway may mediate both AP-1 dependent induction of TGF- β_1 transcription and mRNA stabilization. However, inhibition of ERK signalling had no effect on TNF- α induction of TGF- β_1 transcription (Fig. 4), suggesting that independent signalling pathways are likely involved in up-regulation of TGF- β_1 expression *via* these two mechanisms. Although our results do not rule out the possibility that TNF- α activation of ERK signalling and AP-1 induces transcription of an intermediate that in turn up-regulates TGF- β_1 expression. Additional studies will be required to answer these fundamental questions.

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