# Global expression analysis of the fibroblast transcriptional response to $TGF\beta$

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#### **ABSTRACT**

Objectives. Transforming Growth Factor- $\beta$  (TGF $\beta$ ) is the predominant cytokine in all forms of fibrotic reactions. As well as being secreted by immune modulators of fibrosis such as macrophages, it is involved in an autocrine feedback loop of fibroblast stimulation whose regulation is still poorly understood. We wished to gain some insight into the mechanisms of the fibroblast response to TGF $\beta$ .

**Methods.** We undertook an exhaustive transcript profiling experiment using a widely validated restriction enzyme based method for identifying differentially expressed genes (GeneCalling<sup>TM</sup>). Transcriptional responses throughout a 24-hour time course were examined at multiple time points and classified.

**Results.** By 24 hours of TGF treatment over 1000 bands, representing a large number of transcripts, were down- or upregulated greater than 2-fold. All of the known genes responsive to TGFβ, such as collagen and connective tissue growth factor, were upregulated.

Conclusions. This encyclopedic method revealed many unknown transcriptional responses to  $TGF\beta$  including the upregulation of a variety of less expected cytoskeletal and matrix components, as well as interactions between the  $TGF\beta$  and tumor necrosis factor (TNF) pathways and alterations in cell deathrelated pathways. These may in part explain the idiosyncratic responses of mesenchymal cells to  $TGF\beta$ .

#### Introduction

TGF plays a key role in a variety of fibrotic reactions including normal wound healing as well as pathologic fibrosis. It is released by a variety of immune cells, including macrophages and T cells, and is also released by platelets at sites of vascular injury. TGF is also involved

in a fibroblast autocrine feedback loop whose regulation is poorly understood. TGF is striking in the apparently opposite effects induced in fibroblast cell types, where it is pro-proliferative and induces apoptosis resistance, and in epithelial cells and leukocytes, where it is growth inhibitory and in some instances promotes apoptosis. TGF also causes significant changes in extracellular matrix synthesis by fibroblasts, including upregulation of various collagens, and downregulation of collagenolytic activities.

In order to complete the catalogue of TGF induced responses in fibroblasts, with the hope of gaining some insight into the mechanisms of the fibroblast response, we undertook an exhaustive transcript profiling experiment using Gene Calling<sup>TM</sup> technology (1), in parallel with an identical study on lesional fibroblasts from patients with scleroderma (manuscript in preparation). This study revealed striking changes in fibroblast transcripts, with a dramatic alteration in matrix synthesis, accompanying changes in metabolic pathways, cytokine and receptor transcription, and changes in cell cycle regulators which might account for the proliferative response of fibroblasts to TGF.

#### Methods

Cell culture

Fibroblast cell strains were established from punch biopsies of dermal skin as previously described (2). Cells were used at the 3rd to 6th passage. 15 cm dish cultures from 5 different cell strains were serum starved for 24 hr prior to the addition of TGF . Serum deprivation was used to accentuate the TGF response, with the inevitable consequence that the effects of serum starvation themselves were overlaid on the

TGF response. At 0 (control) 1, 4, 8, and 24 hours, cells were harvested and fibroblasts from the five strains pooled. In order to determine the effects of serum starvation explicitly, cells untreated with TGF were retained for the course of the experiment (24 hours) and were also assayed. RNA was recovered with trizol, and subjected to differential gene expression analysis.

Differential gene expression analysis GeneCalling reactions were performed essentially as described (3). Briefly, following double-stranded cDNA synthesis of polyA+ RNA, cDNA was separated into 96 different pools and each was digested with a different pair of restriction enzymes. The resulting fragments were ligated to adaptors, and amplified by PCR with complementary primers, one labeled with biotin and the other with flouorescamine (FAM). After affinity purification on streptavidin and separation by polyacrylamide gel electrophoresis, the FAM-labeled fragments were detected by laser excita-

A composite restriction fragment profile was generated for each sample, based on average peak height and variance of nine separate restriction enzyme digestions (each sample triplicate was subjected to 3 separate restriction enzyme digestions). The restriction fragment profiles of two samples were compared and differentially expressed fragments were identified. Linkage of a differentially expressed cDNA fragment to a gene was made through knowledge of the restriction enzymes used to generate the fragment ends, and hence 6bp of sequence, and the length of the fragment itself. A species-specific query of databases reveals genes that fit these criteria, with gene identification confirmed by competitive PCR using gene-specific oligonucleotides.

Semi-quantative RT-PCR analysis cDNA samples were prepared according to manufacturer's instructions (Promega). Each sample was tested for genomic DNA contamination by performing a minus reverse transcriptase control with GAPDH primers. PCR amplification from the cDNA was per-

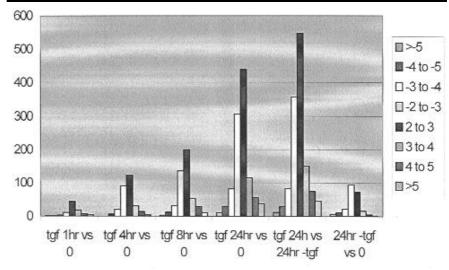
formed on 0.25 µg RNAequivalent, using the Promega Taq system with 1.5 mM MgCl<sub>2</sub> and 0.2 mM dNTPs in a total volume of 25 µl. Cycles were as follows: 95°C, 60 second denaturation, cycles of 95°C, 20 seconds, 58°C, 45 seconds, 72°C, 90 seconds. Primer pairs and optimal cycle numbers for detection within the linear range were as follows: COL11A1; Fw-tttcccctctccctc cccaat Rv-ggttgttacggtgaaatcccagagc 208bp, 29 cycles. COMP-1; Fw-acacg gacgaggacaagtgg Rv-gcatctcccacaaa gtcgtg 274bp, 28 cycles. LAMA3 Fwatgggatggctgtggatctttgg Rv-ccgtccg gtatacaagcctttatga 189 bp, 31 cycles. CCR11, Fw-aggttcagggagcagaggtata gcc Rv-tggggtgttactacgcagatggaaa 285 bp, 29 cycles. GAPDH; Fw-accaggtg gtctcctctgacttcaa Rv-tactccttggaggc catgtggg 172 bp, 27 cycles. PERI-PLAKIN; Fw-catcgtggacacagaggcc Rv-gaagtatggccctgacttcaa 900bp, 37 cycles. 10 µl of each sample was electrophoresed on a 2% agarose TAE gel. To achieve even and selective staining of the DNA, the gel was washed in 0.1 µg/ml ethidium bromide in 1xTAE, destained in 1xTAE, 30 minutes and then visualised under UV and photographed using a Stratagene eagle eye. Band intensities were quantified using Tiffany software. This quantitation method proved to be directly comparable to radioactive methods.

#### Results

Overall changes in gene expression by normal fibroblasts exposed to TGF\(\beta\) There was a steady increase in the number of differentially expressed bands detected over the time course of exposure to TGF (Fig. 1), reflecting a cascade of signal transduction events downstream of the initial events. Overall more bands were upregulated than downregulated, with 820 bands upregulated and 481 downregulated by greater than two-fold in cells at 24 hours post TGF, compared to the untreated cells at the same time. The predominance of upregulation was more striking when looking at bands showing a high level of TGFB regulation: 46 bands were upregulated more than five-fold while 12 were downregulated to the same extent. There were changes in a small number of genes over 24 hours in cells unstimulated by TGF (last column).

# Early changes in gene expression

Within one hour, there were marked increases in the levels of the transcripts for three transcription regulators: JunB, a component of the AP1 complex; and Id1 and Id3, two dominant negative inhibitors of transcriptional complexes upregulated in B cells, and induced by TGF (4, 5) Interestingly, protein tyrosine phosphatase D1, a known activator of Stat3, was also upregulated, and Stat



**Fig. 1.** Overview of bands up and downregulated in response to TGF . The total number of called bands and their fold change was quantitated at each time point in the experiment. Many bands may represent one gene. Overall, more bands are upregulated than downregulated.

3 is known to cause the upregulation of Id1 levels (6). At the same time, upregulation of two antiproliferative signals – insulin-like growth factor binding protein 6, and the cell cycle inhibitor CDKN2C – occurred. Accompanying this antiproliferative chorus was upregulation of the transcript for the long form of Bcl-X, an inhibitor of apoptosis. This upregulation marks a difference between the fibroblast response to TGF and the epithelial response, as Hep3B cells downregulate Bcl-XL in response to TGF (7), on the way to their death.

In the cytoskeleton, the non-muscle tropomyosin, tropomyosin 3, showed a rapid and sustained downregulation first evident at 1 hour, while there was a reciprocal upregulation of the skeletal muscle alpha-tropomyosin 1 by 4 hours. This may represent the first sign of acquisition of the myofibroblast phenotype in response to TGF . Lastly, upregulation of two well known targets of TGF stimulation, plasminogen activator inhibitor-1 and collagen I, was already evident at 1 hour.

#### Extracellular matrix

Changes in extracellular matrix component transcription were extensive. Early events included the upregulation of collagen I, with other collagens following suit, including fibrillar collagens V and XI, basement membrane collagens IVand VIII, and the facit collagen XVI. The transmembrane collagen XIII was significantly downregulated, and the endostatin precursor collagen XVIII was slightly so. Parallel to the increase in collagens was upregulation of the collagen modifying enzymes prolyl hydroxylase and lysyl oxidase. Concomitant with upregulated collagen I was an increase in expression of fibrillin, as well as the matrix regulatory proteins thrombospondin I (but a downregulation of its relative punctin), tenascin C, and SPARC, and the proteoglycans versican and perlecan. In contrast, decorin, which has been observed to be associated with antifibrotic events (8), was not detected. One remarkable finding, consistent with previous observations (9) was a striking and sustained upregulation of Cartilage Oligomeric Matrix Protein (COMP), a pentameric extracellular protein whose intracellular accumulation is associated with chondrocyte malfunction (10), but whose normal function in cartilage has not been clearly elucidated. Recent data suggests that COMP may be an accessory component involved in accretion of collagen monomers to promote fibrogenesis (K. Rosenberg, personal communication), and thus an upregulation induced by TGF, in parallel with increased collagen synthesis, would be a logical outcome.

#### Proteases

In general, protease changes were consistent with a profibrotic condition. The collagenase MMP1 was downregulated, consistent with previous observations (11), as well as the gelatinase MMP9 and the inhibitor/coactivator TIMP2. The most striking upregulation in this compartment was PAI-1, which has been recently implicated in fibrosis through a variety of mechanisms, and whose absence, in the PAI1 knockout mouse, confers fibrosis resistance (12).

## Intercellular signalling

As would be expected, TGF stimulation resulted in a sustained increase in connective tissue growth factor (CTGF) expression, along with autocrine upregulation of TGF itself. Another CTGF family member, WISP2, was downregulated. It is noteworthy that WISP2 appears to be highest in adult dermal fibroblasts and lowest in fetal isolates (13) and thus has a reciprocal expression pattern to CTGF.

Other growth factor changes included a reduction in Keratinocyte Growth Factor and an upregulation of vascular endothelial growth factor (VEGF), suggesting that the TGF response includes support for angiogenesis in the mesenchyme. After a transient increase, VEGF receptor expression was markedly reduced; the role for VEGF receptor in the fibroblasts themselves is not clear but may relate to its function as neuropilin, a semaphorin receptor (see below).

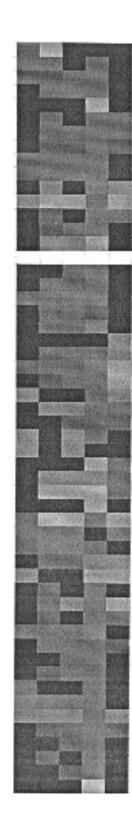
There was also an early (4 hour) down-regulation of the tumor necrosis factor-

(TNF ) target, TNF inducible protein A20, as well as a small downregulation of a TNF receptor, TNFR1 TNF is known to inhibit the TGF pathway by the partial blockade of Smad-mediated transcription (14). Strands of evidence suggest that TGF can reduce TNF -induced fibroblast death (15), and we have previously shown TGF exposure induces resistance to apoptosis (2), but the direct downregulation of components of the TNF pathway was not previously implicated in the process. Given a reduction of these components at the mRNA level, it is possible that the cells are primed to ignore the TNF pathway.

#### Cell surface molecules

There was a striking downregulation in the chemokine receptor CCR11 (presumably expressed only in a subset of fibroblast samples, see below), the receptor for TECK. Semaphorin E, best known for a role in neuronal guidance, was upregulated, whereas neuropilin, the VEGF/semaphorin 3 receptor found on neurons, endothelium, and myoepithelial cells, was downregulated. Other surface molecules previously associated with neurons were downregulated, including the intracellular adhesive protocadherin gamma C3 and the myelin-associated tight junction protein Claudin 11. Despite cytoskeletal changes associated with the myofibroblast transition, prostaglandin F2 receptor, involved in uterine contraction, was downregulated. Interestingly, plateletderived growth factor receptordownregulated while the form was upregulated. This has also been observed at the protein level (16), and raises unanswered questions about the different roles of these two receptors.

Several integrin changes were noted, including the upregulation of 6, a laminin receptor component, with reciprocal downregulation of 3, an alternative integrin 1 partner and laminin receptor component. Integrin 11, a recently identified 1 partner and collagen receptor (17), along with the major collagen binding integrin subunit 2 (18), were also upregulated. Many other integrin alpha subunits are known to be upregulated in surface expression in



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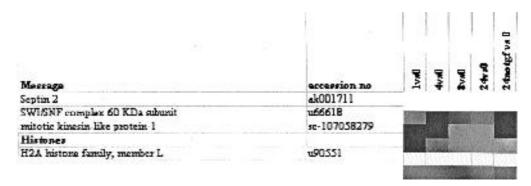


Fig. 2. Expression changes in annotated genes in response to TGF . Genes are classified according to percieved cellular function. Genes in italics are also listed in (14). Expression changes at 1, 4, 8, and 24 hours +TGF versus time = 0 are indicated at the right, followed by 24hr-TGF versus time = 0. The latter is presented as a control for the effects of serum starvation and cell culture. Values are shown as a heat map, from bright red indicating more than 4.5-fold upregulation to bright green indicating more than 4.5-fold downregulation.

response to TGF (19), including subunits 1 through 5. The absence of calls from these molecules in the data set may reflect relatively low levels, in general, of integrin message. Indeed, they are notoriously difficult to detect by Northern analysis.

#### Intracellular signalling

Many of the key steps in intracellular signalling are phosphorylations, and therefore the direct interpretation of intracellular events by transcript profiling is impossible. Nonetheless, it is possible that the determination of which components of the signalling machinery are transcribed may be a guide to the pathways favored. There was a striking downregulation, by 24 hours, of MAPkinase kinase 3b, which is responsible for activating the p38 pathway. As p38 activation is a consequence of integrin alpha2 activation and collaborates with TGF in inducing collagen synthesis (20), it is likely that this downregulation is, like the even more dramatic downregulation of SMAD2, a consequence of negative feedback.

Our finding of SMAD2 downregulation contrasts recent studies in hepatic stellate cells, where the message was unchanged by TGF at 24 hours (21). It has been suggested that SMAD3, whose message was not changed in our study, is primarily responsible for fibrosis (22), whereas in some systems SMAD2 is more responsible for epithelial cell death (23) [and its loss is associated

with tumor survival (24)]. It is possible therefore that the downregulation of SMAD2 but maintenance of SMAD3 reflects the death resistance of fibroblasts in response to TGF. No transcriptional upregulation of the inhibitory SMADs 7 and 8 was seen in the fibroblasts, in contrast to some epithelial cell types where their upregulation presumably contributes to a regulatory negative feedback loop (25). Another notable finding was the upregulation of Sprouty 4, an inhibitor of ras signalling that is upregulated in activated smooth muscle (26).

# Metabolic pathway changes

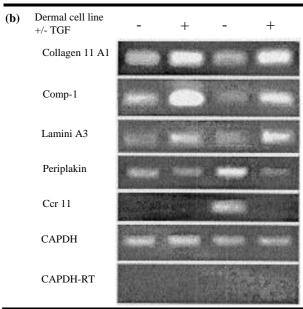
A variety of metabolic enzymes were upregulated, undoubtedly reflecting the increased synthetic activity of the cells. One of the most interesting changes was in the ratio of transcript for the glutaminyl and methionyl tRNA synthetases, where the former was two-fold downregulated and the latter was twofold upregulated. The average ratio of methionine to glutamine in proteins is approximately 0.5 (27), while actins, strongly upregulated in the contractile TGF treated fibroblast, have a met/glu ratio of ~1.5. Other metabolic changes included the upregulation of N-acetylglucosamide-(beta 1-4)-galactosyltransferase, presumably reflecting increased proteoglycan synthesis, and members of the pyruvate dehydrogenase complex. In concert with this were increases in expression of the water channel aquaporin 1, known to be induced

during hypertonic stress (28), and the VLDLreceptor, which is capable of internalizing UPA-PAI-1 complexes (29).

#### Cytoskeletal changes

The fibrotic pathway proceeds along an axis which includes, in some (usually pathological) circumstances, a transition in the fibroblast phenotype into a more contractile, smooth muscle-like cell, the myofibroblast. Consistent with this was a striking upregulation in tropomysin 1 with a reciprocal downregulation of tropomyosin 3, as well as the upregulation of smooth muscle actin and filamin 2. Paxillin was notably downregulated, suggesting a reduction in cell motility (30) reciprocal to the increased contractility of the myofibroblast state. There was a striking downregulation of the protein periplakin. This protein appears to be predominantly an epidermal component, and may connect keratin filaments to the hemidesmosomal component collagen XVII, although it is known to be expressed at low levels in fibroblasts. Desmoplakin, usually regarded as a desmosomal component, was by contrast upregulated, as was the simple epithelial keratin, keratin 7. To our knowledge, expression of keratins has not been previously noted in fibroblasts. However, the canonical distributions of intermediate filaments have been challenged by "unexpected" observations; in particular, vimentin is expressed by epithelial cells in some inflammatory conditions (31).

(a)		GeneCalling result	RT-PCR result			
Gene Id	Gene definition	Five pooled samples	Sample 1	Sample 2	Mean	
gbh_l32137	Human cartilage oligomeric matrix protein (COMP) mRNA	2.68	2.83	2.57	2.7	
gbh_af101079	Homo sapiens collagen type XI alpha-1 (COL11A1) gene	1.83	1.63	1.57	1.6	
gbh_x77598	Homo sapiens LAMA3 mRNA	-2.08	2.33	2.33	2.33	
gbh_af193507	Homo sapiens chemokine receptor (CCR11) gene	-4.07	nd	-5	-5	
gbh_af013717	Homo sapiens periplakin (PPL) mRNA, partial cds	-9.12	-1.43	-3.125	-2	



**Fig. 3.** Validation by RT-PCR. (**A**) Comparison of GeneCalling and RT-PCR data. Fold changes are expressed as log(2)[level treated/level untreated]. (**B**) Photograph of representative RT-PCR experiment, ethidium stained gel.

#### Transcriptional regulation

As described above the immediate-early transcription factor junB was rapidly upregulated and detected within 1 hour of TGF stimulation, as were ID1 and ID3, two anti-differentiation factors best characterized in the hematopoietic system. ID1 has been recently shown to be upregulated in TGF -treated embryonic fibroblasts, as well as in fibrotic myofibroblasts in experimental lung injury (32), and ID3 is known to be upregulated by TGF and is involved in the apoptotic pathway in lymphocytes (4). Alterations of Ets-1 mRNA [a known transcriptional activator of collagen synthesis (33)], were not detect-

## Death, shock, and proliferation

A constellation of transcriptional events appeared to conspire to protect the fibroblasts from cell death. These included upregulation of the cytoprotective hsp70 family member ORP150; upregulation of the survival factor Bcl-XL and downregulation of BAD (which is pro-apoptotic by displacing bax from bcl-x), and upregulation of

the survival factor and NFkappaB antagonist Bcl3 (34). One exception was the transient upregulation of DATF1, which has been shown to be death-inducing in mouse tissues.

On the other hand, a variety of transcripts were altered in a fashion consistent with cell cycle arrest, including the upregulation of cyclin-dependent kinase inhibitor 2B, a known TGF target, and downregulations of cyclins G and A. Thus, the proproliferative effect of TGF on fibroblasts cannot be accounted for by the data seen here.

# Confirmation of results using semiquantitative RT-PCR

In general, the effects of TGF on fibroblasts that were highly consistent with those previously noted in the literature, with the upregulation of collagen I, connective tissue growth factor, and accessory matrix proteins. This gave us confidence in the less anticipated results. To augment our confidence further we performed RT-PCR quantitation for five of the unanticipated results on two independent fibroblast isolates from different individuals, treated with

or without TGF for 24 hours (Fig. 3). Of the five, four were replicated with RT-PCR. The fifth, laminin A3, appeared to be upregulated by RT-PCR, but downregulated by gene calling. We ensured that the primers chosen were unique for laminin A3. The RT-PCR result seen with CCR11, where only one untreated sample appears to contain the transcript, suggests that Gene-Calling (TM) is sufficiently sensitive to pick up changes which are only present in a subset of the pooled samples.

There have been a variety of other recent gene profiling studies of fibroblasts. Most relevant to this work are the studies of Verecchia et al. (14), and Chambers et al. (32). Our data overlap incompletely with the findings of Verecchia et al., who used a nylon-based array. Among the genes in agreement are collagens I, III and XVI, versican, perlecan, sparc, thrombospondin, fibronectin and PAI-1, but several genes upregulated in their study, including MMP1 and proto-cadherin 3 were downregulated in ours. Differences in general methodology and the fibroblast source may account for this. Our findings of upregulation in ID1 and ID3 concur with the most striking observation of Chambers *et al.* (32).

#### **Conclusions**

Our studies demonstrate a change in the transcriptional repertoire of fibroblasts following TGF treatment. The changes seen are coherent and consistent with the previously reported effects of TGF on fibroblast metabolism. In general, the changes seen reflected the promotion of matrix protein synthesis, changes in cytoskeletal protein mRNAs compatible with myofibroblast transformation, and the upregulation of anti-apoptotic proteins. The latter findings are consistent with our previous studies which show that TGF induces resistance to apoptosis in dermal fibroblasts (2).

It is interesting that fibroblasts treated with TGF demonstrate a similar mRNAprofile to that of fibroblasts isolated from tissues of patients with fibrotic disease. Thus, fibroblasts from patients with scleroderma demonstrate increased mRNA for collagen and fibronectin, increased myofibroblast transformation and resistance to apoptosis. A more extensive comparison of the profile of TGF -treated fibroblasts with fibroblasts from pathologic fibrosis will elucidate whether TGF alone is responsible for phenotypic transformation in these disorders.

TGF induces fibroblast proliferation; nonetheless, at least at the 24-hour time point, most transcriptional changes appear to favor cytostasis over proliferation. Therefore the well established proliferative response of fibroblasts to TGF must be accounted for by later and possibly indirect events. Like many other transcription profiling studies, this one has revealed the presence and regulation of a variety of transcripts hitherto associated with other tissue types, particularly "neural" and "epithelial" genes such as semaphorin and claudin. It seems likely that many signals known to regulate neurons are also used by fibroblasts.

In summary, the effects of TGF on fibroblasts appear to be prosynthetic, while being protective against many apoptotic signals and pathways usually associated with inflammation and the acute phase response. A comparison of these results with the effects of TGF on epithelial cells, endothelial cells and smooth muscle cells will likely be very instructive and provide insight into mechanisms of both wound healing and pathologic fibrosis.

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