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Targets of Transcriptional Regulation by Transforming Growth Factor-β: Expression Profile Analysis Using Oligonucleotide Arrays

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Transforming growth factor-βs (TGF-βs) are potent inhibitors of cell proliferation, and disruption of components of the TGF-β signaling pathway leads to tumorigenesis. Mutations of transmembrane receptors and Smads mediating intracellular signaling have been reported in various cancers. To identify transcriptional targets of TGF-β, we conducted an expression profile analysis. HaCaT cells derived from human keratinocytes and highly sensitive to TGF-β were treated with TGF-β in the absence or presence of cycloheximide (CHX). mRNAs extracted from the HaCaT cells were used for hybridization of oligonucleotide arrays representing approximately 5600 human genes. TGF-β increased the expression of PAI-1, junB, p21 cdk inhibitor, Smad7, βIG-H3, and involucrin that have been reported to be up-regulated by TGF-β, validating the usefulness of this approach. The induction of βIG-H3 by TGF-β was completely abolished by CHX, suggesting that the transcription of βIG-H3 is not directly regulated by TGF-β. Unexpectedly, we identified more genes down-regulated by TGF-β than up-regulated ones. TGF-β repressed the expression of epithelial specific Ets that may be involved in breast and lung tumorigenesis, which could contribute to tumor suppression by TGF-β. Among a panel of cell cycle regulators, TGF-β induced the expression of p21 cdk inhibitor; however, the induction of other cdk inhibitors was not significant in the present study. Taken together, the results suggest that TGF-β may suppress tumorigenesis through positive and negative regulation of transcription.

Key words: TGF-β — DNA chip — HaCaT — p21 — Ets

Transforming growth factor-βs (TGF-βs) belong to a large family of secreted polypeptides that include activins, bone morphogenetic proteins (BMPs), and other ligands. Members of the TGF-β superfamily exert a wide variety of biological activities, and govern cell fate, such as growth, apoptosis, and differentiation.[1)] TGF-βs invoke varying cellular responses depending upon the cell type and environment. TGF-βs inhibit cell growth and arrest cells at the G1/S boundary in the cell cycle.[2)] Thus, TGF-βs are negative regulators of cell growth and suppress tumorigenesis.[3)] In a different context, however, TGF-βs promote cell proliferation. This is thought to be an indirect effect via induction of secretion of other growth factors. TGF-β1 was originally identified as a factor that induces anchorage-independent growth of normal cells. Thus once tumor cells are rendered insensitive to TGF-β, TGF-β may support tumor invasion through promotion of cell adhesion, angiogenesis, and immunosuppression.[4)]

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The TGF-β superfamily members bind to two types of transmembrane receptors with serine/threonine kinase activity.[1)] Type II receptors are constitutively active kinases, and transphosphorylate type I receptors upon ligand binding. Type I receptors subsequently phosphorylate intracellular substrates. Signaling from the cell membrane to the nucleus is propagated by Smad proteins.[5,6)] Smads are classified into three types depending upon structure and function. Receptor-regulated Smads (R-Smads) are phosphorylated by activated type I receptors. R-Smads then associate with common mediator Smads (Co-Smads). The heteromeric complexes translocate from the cytoplasm to the nucleus, and regulate transcription of target genes. R-Smads and Co-Smads can bind directly to DNA, although the affinity is relatively low. Thus stable DNA binding with strict sequence specificity is achieved by interaction with other sequence-specific DNA binding partners.[7)] Smads also interact with transcriptional co-activators such as p300 and CBP that possess histone acetyltransferase activity.[8–10)] Recently, Smads have been shown to interact with TGIF and c-Ski that recruit histone

deacetylase.[11–14] p300 and CBP neutralize the positive charge of histones and loosen chromatin structure, resulting in activation of transcription. In contrast, histone deacetylases tighten chromatin structure and repress transcription. Thus Smads are involved in both positive and negative regulation of transcription by the TGF-β superfamily members. Inhibitory Smads (I-Smads) antagonize signaling by R-Smads and Co-Smads at least by inhibiting phosphorylation of R-Smads.

Eight mammalian Smads have been identified.[5, 6] Smad2 and Smad3 are activated by TGF-β and activin type I receptors. Smad1, Smad5, and Smad8 mediate BMP signaling. Smad4 is the only Co-Smad found in mammals. Smad4 was originally identified as DPC4, a tumor suppressor gene product in pancreas cancers.[15] Smad6 and Smad7 are I-Smads. Smad6 preferentially inhibits BMP signaling, whereas Smad7 antagonizes TGF-βs, activins and BMPs.

Components of the TGF-β signaling pathway are altered in cancer cells.[3] The *TGF-β type II receptor* gene contains a consecutive stretch of 10 adenines that correspond to amino acids 125–128 within the extracellular region of the receptor. In cases of hereditary non-polyposis colorectal cancer (HNPCC) with mismatch repair defect, this adenine stretch is frequently mutated to give rise to truncated receptors.[16] It was also reported that a case of HNPCC without mismatch repair defect suffers from a germline mutation in the *TGF-β type II receptor* gene.[17] Repression of TGF-β type II receptor was shown to be responsible for oncogenesis of Ewing sarcomas.[18] Although the number is less than the type II receptor, alterations of the TGF-β type I receptor have been reported.[3, 19] As mentioned above, Smad4 was identified as a tumor suppressor in pancreas cancers. Mutations of *Smad4* are also found in colon, lung, and other cancers.[20] *Smad2* was found to be mutated in colon and lung cancers.[21, 22] In an animal model, heterozygotic compound mutation of *APC* and *Smad4* gave rise to invasive colon cancers.[23] Polyps with loss of heterozygosity of the *Smad4* gene grew in mice with heterozygous loss of *Smad4*.[24] Smad3 knock-out mice frequently developed invasive colon cancers.[25] Mice with heterozygous deletion of the *TGF-β1* gene exhibited accelerated tumorigenesis by chemical carcinogens compared to wild type mice.[26] All of these observations are consistent with the idea that TGF-β is a tumor suppressor.

It is thus important to identify targets of TGF-β in growth regulation. Recent advances in the DNA chip technology have enabled comprehensive survey of such target genes. We conducted oligonucleotide microarray analysis using HaCaT cells derived from human keratinocytes. TGF-β increased the expression of p21 cdk inhibitor. On the other hand, TGF-β repressed the expression of epithelial specific Ets that may be involved in breast and lung tumorigenesis.[27, 28] Our results indicate that TGF-β may suppress tumorigenesis through positive and negative regulation of transcription.

MATERIALS AND METHODS

Cell culture HaCaT cells were provided by Nobert E. Fusenig (DKFZ, Heidelberg, Germany), and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics. Mv1Lu cells were obtained from American Type Culture Collection (Bethesda, MD), and cultured in DMEM with 10% FBS and antibiotics.

Growth inhibition assay Cells were seeded in 24-well plates at a density of 5×10^4 cells per well, and treated with various concentrations of TGF-β. [^{3}H]Thymidine incorporation was assayed as previously described.[29]

RNA extraction and northern blotting HaCaT cells were treated with 400 p*M* of TGF-β for the indicated time periods. When cells were cultured in the presence of cycloheximide (Sigma, St. Louis, MI), 20 μg/ml of the drug was added to the medium 1 h before the addition of TGF-β. Total RNA was extracted from the cells with Isogen (Wako, Osaka). Ten micrograms of RNA was electrophoresed and blotted onto a membrane. Radioactive probes were made using Ready-To-Go Kit (Amersham

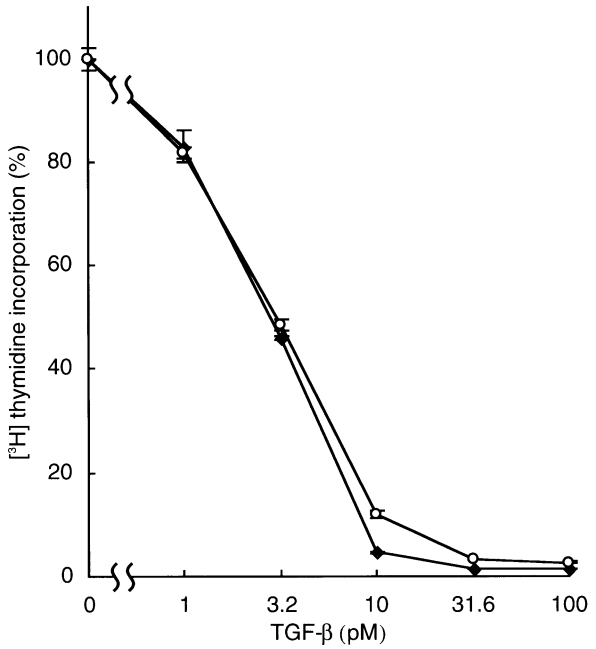

Fig. 1. Growth inhibition by TGF-β. The growth of HaCaT (○) and Mv1Lu (◆) cells was assayed in terms of [^{3}H]thymidine incorporation in the presence of various concentrations of TGF-β. The experiment was done in duplicate, and error bars represent standard deviation.

Pharmacia Biotech, Piscataway, NJ). Membranes were hybridized, washed, and subjected to Fuji BAS imaging as described.[30] mRNA was purified from total RNA using Oligotex dT-30 Super latex beads (TaKaRa Biochemicals, Tokyo). Northern blotting was performed to monitor the quality of mRNA (unpublished results).

Oligonucleotide microarray analysis Oligonucleotide microarray "GeneChip" (Affymetrix, Santa Clara, CA) analysis was performed essentially as described.[31] Aliquots of the mRNA carefully examined by northern blotting were used for the preparation of biotinylated probes. The first strand cDNA was synthesized from 2 μg of mRNA with an oligo(dT) primer containing a T7 RNA polymerase promoter sequence at its 5′ end using SuperScript Choice System (Gibco BRL, Rockville, MD). The second strand cDNA was synthesized by *Escherichia coli* DNA polymerase I and ligase. One microgram of cDNA was used for the following *in vitro* transcription. The reaction was performed in the presence of biotinylated ribonucleotides using EnZo BioArray High Yield RNA Transcript Labelling Kit (Affymetrix). Synthesized cRNA was cleaned with RNeazy (Qiagen, Valencia, CA), and fragmented by incubation at 94°C for 35 min in buffer containing 40 m*M* Tris-acetate (pH 8.1), 100 m*M* potassium acetate, and 30 m*M* magnesium acetate. Hybridization of a GeneChip array (HuGeneFL) was performed for 16 h. Washing and staining were done as described.[31] GeneChip arrays were scanned by a confocal scanner.

The data collected from scanning were processed by using GeneChip software supplied by Affymetrix,[32, 33] and "Average Difference" intensities and fold changes were calculated. Note that fold change does not necessarily match the ratio of intensities because the formula for fold induction is not the simple ratio of intensities, but takes other factors into consideration. In extracting genes that show significant change (Tables I and II, but not Table III), we set the criterion that fold change is greater than or equal to 3 at 2 or 6 h of TGF-β treatment. In addition, we excluded genes whose intensity is lower than the background level after increase or before decrease.

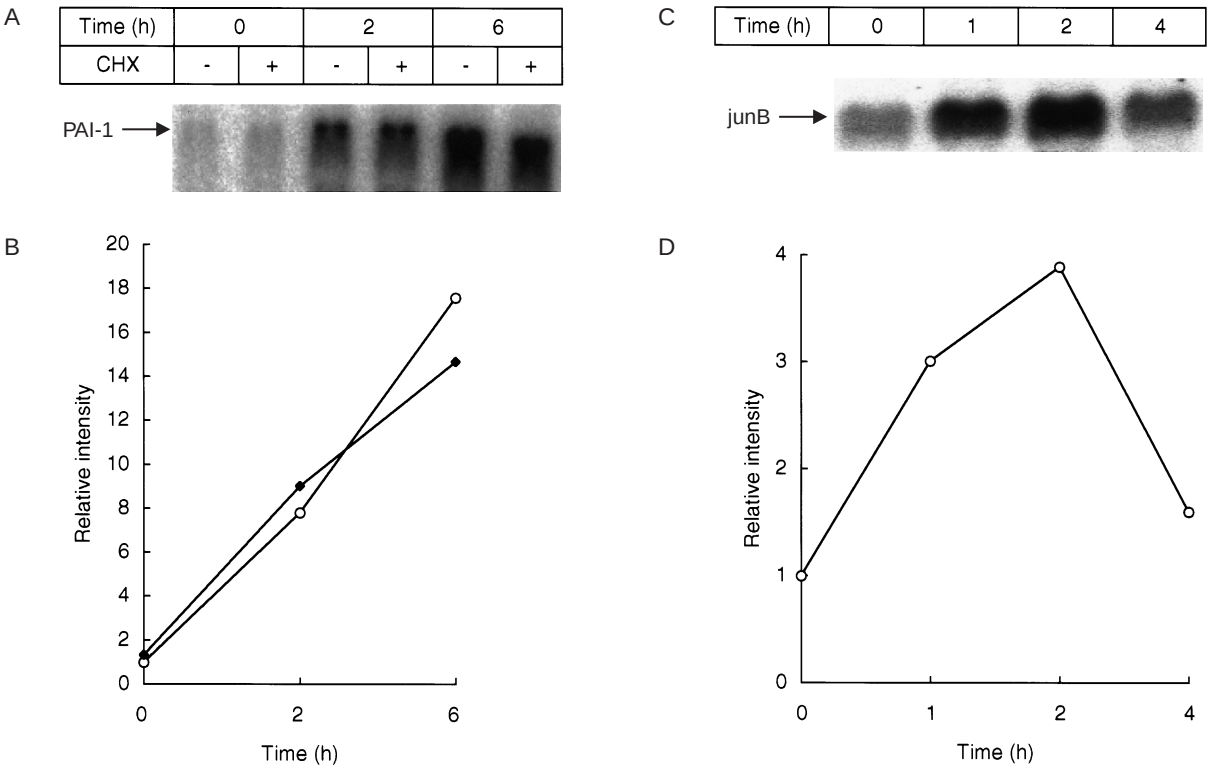

Fig. 2. Northern blotting of TGF-β-inducible genes. HaCaT cells were treated with 400 p*M* of TGF-β for the time periods indicated. Total RNA was extracted from the cells, and subjected to northern blotting. The probes used were PAI-1 (A) and junB (C). In the experiment (A), cells were cultured in the absence or presence of 20 μg/ml cycloheximide (CHX). In the experiment (C), CHX was not added. The intensities of the bands were quantified for PAI-1 (B) and junB (D). The values were normalized against the intensity at time 0 in the absence of CHX. (B) ○ CHX (−), ◆ CHX (+).

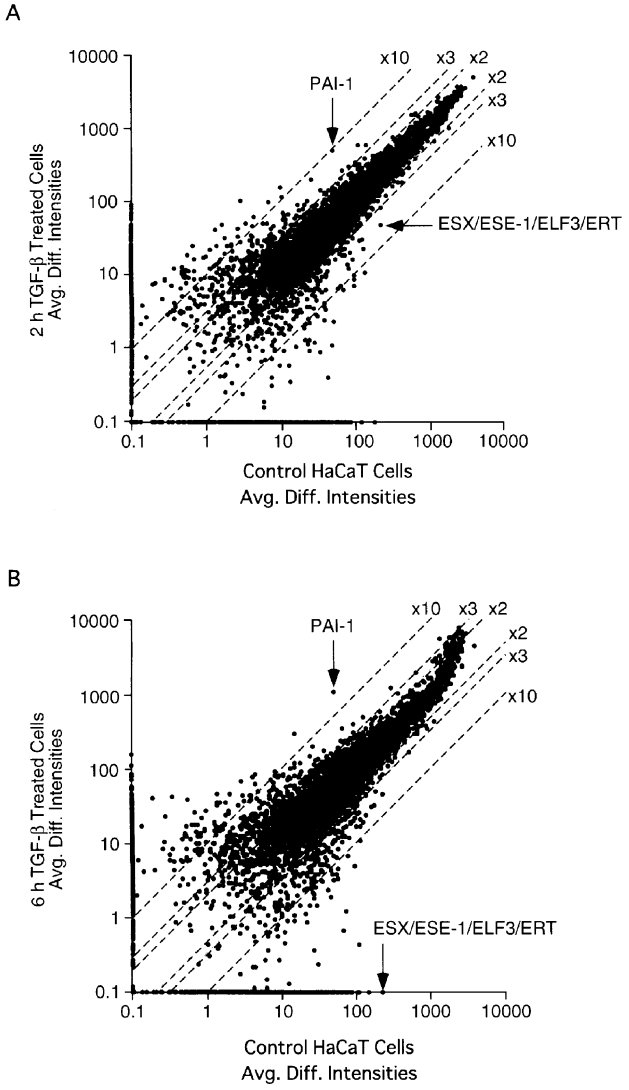

Fig. 3. Global gene expression profile of HaCaT cells upon TGF-β treatment. The average difference (Avg. Diff.) intensities of the genes examined in the absence of CHX were plotted for 2 h vs. 0 h (A) and 6 h vs. 0 h (B). The horizontal axes represent time 0 h, whereas the vertical axes represent time after TGF-β treatment. Spots corresponding to PAI-1 or ESX/ESE-1/ELF3/ERT are marked with an arrow. Changes in intensity of 2, 3, and 10 fold are delineated by lines parallel to the diagonal line. Spots with an intensity below 0.1 are collected on the axes.

RESULTS AND DISCUSSION

Responses of HaCaT cells to TGF-β To identify genes transcriptionally regulated by TGF-β, we used HaCaT cells derived from human keratinocytes.[34] We confirmed the inhibitory effect of TGF-β on the growth of HaCaT cells in comparison with Mv1Lu mink lung epithelial cells as a reference (Fig. 1).[35] The DNA synthesis of HaCaT cells was almost completely inhibited by TGF-β at the concentration of 100 p*M*. Thus, HaCaT cells are highly sensitive to TGF-β, at least in growth inhibition assay. We used cycloheximide (CHX) in an attempt to identify genes directly regulated by TGF-β. In a previous study, 10 μg/ml of CHX was used to inhibit *de novo* protein synthesis.[36] We used 20 μg/ml of CHX. This concentration of CHX caused no morphological change of HaCaT cells for 24 h (unpublished results). We next performed northern blotting to see the time course of expression of TGF-β-inducible genes (Fig. 2). The expression of plasminogen activator inhibitor-1 (PAI-1) continuously increased for at least 6 h, and CHX treatment caused a slight decrease at 6 h. On the other hand, junB transcripts reached a peak at around 2 h, and then decreased at 4 h as reported previously in NRK cells.[37] Based upon these observations, we treated HaCaT cells with 400 p*M* TGF-β for 2 and 6 h in the presence or absence of CHX, and extracted mRNA.

Genes up-regulated by TGF-β We conducted an expression profile analysis using oligonucleotide arrays, the GeneChip system developed by Affymetrix. We first monitored the quality of the extracted mRNAs using test chips containing control genes such as glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) and β-actin (unpublished results), and confirmed that the mRNAs are intact enough to perform hybridization of oligonucleotide arrays of approximately 5600 human genes. Global expression patterns of 2 and 6 h TGF-β treatment are shown in Fig. 3.

Genes up-regulated by TGF-β are listed in Table I. In selecting the genes, we employed a relatively stringent criterion. According to the manufacturer's specification, a 2-fold difference of hybridization intensity can be significant. We adopted 3-fold difference as the cut-off threshold either at 2 or 6 h, and identified 32 genes that account for 0.6 % of the 5600 genes examined. When we took a 2-fold change as the threshold, approximately 200 genes were selected (unpublished results). As previously reported, PAI-1, junB, p21 cdk inhibitor, Smad7, βIG-H3, and involucrin exhibited increase.[37–42] gadd45, which was shown to be induced by TGF-β,[43] also increased 2.7 fold at 2 h in the absence of CHX (unpublished results). The induction of PAI-1 in GeneChip analysis correlated well with the result of northern blotting both in the absence and presence of CHX (Fig. 2B). Besides these genes, it was revealed that expression of many other genes was also induced upon TGF-β stimulation. Although the relevance of these genes to the action of TGF-β is not clear at present, future studies should shed light on this subject. Intriguingly, nma is a human homolog of BAMBI.[44, 45] BAMBI was identified in *Xenopus*, and was shown to form inactive complexes with receptors for members of the TGF-β superfamily. nma thus may act as a negative feedback component in TGF-β signaling. TGF-β up-regu-

Table I. Genes Up-regulated by TGF-β (400 p*M*)[a)]

Genbank accession no.	Description	Function	Control intensity (0 h)		TGF-β (2 h)				TGF-β (6 h)			
			Intensity		Intensity		Fold change		Intensity		Fold change	
		CHX	−	+	−	+	−	+	−	+	−	+
J03764	PAI-1	extracellular matrix	52	96	466	520	9.1↑	5.6↑	1074	682	20.5↑	7.1↑
L07919	Dlx-2	transcription factor	2	5	46	57	>6.9↑[b)]	>4.4↑	−6	149	<1.1↓[c)]	>11.1↑
U62800	cystatin M	proteinase inhibitor	−13	−9	37	49	>6.2↑	>4.8↑	79	251	>5.8↑	>16.1↑
U23070	nma (Bambi homologue)	TGF-β-family pseudoreceptor	25	66	98	98	4.9↑	1.5↑	148	159	5.2↑	2.4↑
U20734	junB	proto-oncogene, transcription factor	43	209	172	660	4.5↑	3.2↑	89	650	2.4↑	3.1↑
X16707	fra-1	transcription factor	38	253	161	643	4.2↑	2.5↑	−100	1212	<10.7↓	4.8↑
L43821	HEF1	docking protein	−3	18	25	44	>3.8↑	>2.4↑	15	51	>1.8↑	2.8↑
U67784	RDC1	G protein-coupled receptor	12	8	48	8	>3.7↑	>1.2↑	23	20	>1.7↑	>2.1↑
U90546	BTF4	glycoprotein	12	6	44	14	3.7↑	>1.4↑	36	0	>2.2↑	>1.1↑
L22846	E2F-2	transcription factor	36	52	43	63	3.6↑	1.2↑	42	53	>2.4↑	1.0
D13540	SHP-2	tyrosine phosphatase	−13	10	11	−1	>3.5↑	<1.6↓	2	1	>1.7↑	<1.6↓
U73936	Jagged1	Notch ligand	11	28	31	39	>3.4↑	1.4↑	25	57	>1.8↑	2.1↑
M16364	creatine kinase-B	protein kinase	−6	−16	17	−37	>3.4↑	<2.1↓	−8	24	<1.1↓	>3.8↑
J04102	ets-2	proto-oncogene, transcription factor	30	50	62	187	3.3↑	3.2↑	131	211	4.4↑	4.3↑
Z79693	protein tyrosine phosphatase receptor type R	tyrosine phosphatase receptor	1	17	24	21	>3.3↑	>1.3↑	3	56	>1.1↑	3.4↑
M16750	pim-1	oncogene, serine/threonine kinase	38	77	81	62	3.3↑	1.4↑	61	169	1.6↑	2.5↑
J04111	c-jun	proto-oncogene, transcription factor	9	82	31	177	>3.2↑	2.2↑	36	122	>2.0↑	1.5↑
X02612	cytochrome P-450	cytochrome	22	40	60	194	3.2↑	5.9↑	30	1727	1.4↑	43↑
AF010193	Smad7	TGF-β signaling inhibitor	87	291	290	644	3.1↑	2.7↑	251	408	2.9↑	1.4↑
U09579	p21	CDK inhibitor	109	182	334	349	3.1↑	2.5↑	216	496	2.5↑	3.5↑
M93143	plasminogen-like protein	extracellular matrix	11	20	36	28	3.1↑	1.4↑	19	32	>1.4↑	1.6↑
X17025	human homolog of yeast IPP isomerase	biosynthetic enzyme	43	57	129	82	3.0↑	1.4↑	137	135	1.6↑	1.6↑
L27624	tissue factor pathway inhibitor-2	proteinase inhibitor	22	50	66	8	3.0↑	<3.1↓	43	22	1.9↑	2.2↓
L29219	CDC-like kinase 1	protein kinase	4	15	24	24	>3.0↑	>1.4↑	20	5	>1.5↑	<1.7↓
L13286	mitochondrial 1,25-dihydroxyvitamin D3 24-hydroxylase	mitochondrial protein	0	10	24	27	>2.5↑	>1.9↑	55	26	>3.1↑	>2.6↑
M24351	parathyroid hormone-like protein A (PTHLH)	parathyroid hormone	130	226	280	139	2.1↑	1.6↓	501	135	3.8↑	−1.7↓
M77349	βIG-H3 (TGF-β induced gene product)	unknown	1389	1804	2491	1657	1.8↑	1.0	5698	1755	4.1↑	1.1↑
M13903	involucrin	membrane-bound protein	314	322	518	540	1.7↑	1.7↑	1207	530	3.8↑	1.6↑
M62324	modulator recognition factor I (MRF-1)	unknown	19	41	32	24	1.7↑	1.1↑	85	31	>4.2↑	1.4↑
M63262	5-lipoxygenase activating protein (FLAP)	biosynthetic enzyme	8	20	29	8	1.7↑	1.2↑	53	36	>3.2↑	1.8↑
Z37976	latent transforming growth factor-β binding protein (LTBP-2)	extracellular matrix	28	44	36	55	1.3↑	1.6↑	116	73	3.5↑	2.1↑
X80822	ribosomal protein L18a	ribosomal protein	1372	2474	1702	3619	1.2↑	1.5↑	4219	3165	3.1↑	1.3↑

a) Genes were listed according to the magnitude of the fold increase at 2 h in the absence of CHX. Upward and downward arrows represent increase and decrease, respectively.

b) A greater than sign (>) indicates that the fold change likely represents an overestimation, since the intensity of the gene was below a certain threshold in the TGF-β-untreated control sample, and, consequently, the fold change was increased to an arbitrary, low value by the GeneChip software.

c) A less than sign (<) indicates that the fold change likely represents an underestimation, since the intensity of the gene was below a certain threshold in the TGF-β-untreated control sample, and, consequently, the fold change was decreased to an arbitrary, high value by the GeneChip software.

Table II. Genes Down-regulated by TGF-β (400 p*M*)[a)]

Genbank accession no.	Description	Function	Control intensity (0 h)		TGF-β (2 h)				TGF-β (6 h)			
			Intensity		Intensity		Fold change		Intensity		Fold change	
		CHX	−	+	−	+	−	+	−	+	−	+
X04500	prointerleukin 1β	cytokine	142	532	11	892	13.2↓	1.7↑	24	2033	6.0↓	3.8↑
U41163	creatine transporter (SLC6A10)	transporter	16	−11	−52	−40	<8.0↓[b)]	<2.5↓	−52	14	<4.3↓	>2.8↑[c)]
M29550	calcineurin A1	protein phosphatase	67	42	7	27	<7.9↓	1.6↓	78	35	1.2↑	2.2↓
X57522	RING4	transporter	50	6	55	90	<7.8↓	<2.1↓	35	89	<5.5↓	>6.7↑
U60276	arsenite-stimulated human ATPase	anion-transporting ATPase	56	15	2	−6	<6.4↓	<2.1↓	−13	14	<4.3↓	1.0
U90543	butyrophilin (BTF1)	glycoprotein	−6	−48	−58	−52	<6.3↓	<1.2↓	−31	−56	<2.2↓	<1.6↓
U73843	epithelial-specific Ets (ESE-1b)	transcription factor	240	823	46	1074	4.9↓	1.7↑	−21	2097	<9.2↓	2.9↑
D38037	FKBP-12	peptidyl-prolyl *cis-trans* isomerase	1	−31	−36	12	<4.8↓	>5.6↑	−54	−6	<3.6↓	>6.0↑
M27492	interleukin-1 receptor	cytokine receptor	36	13	−5	17	<4.6↓	<1.2↓	18	−3	<1.9↓	<2.1↓
L40386	DP-2	transcription factor	33	2	3	20	<4.1↓	>1.9↑	35	−7	1.6↓	<1.6↓
D86961	KIAA0206 gene	unknown	32	36	3	48	<4.0↓	1.3↓	33	11	<1.8↓	<3.7↓
U02031	sterol regulatory element binding protein-2	transcription factor	15	−3	9	13	<3.8↓	<1.6↓	−49	12	<4.1↓	<1.8↓
M30703	amphiregulin	growth factor	30	38	3	61	<3.7↓	1.6↑	−28	101	<3.1↓	2.7↑
L25270	XE169	unknown	−7	−12	−33	−20	<3.6↓	>2.9↑	−25	15	<1.9↓	>5.9↑
X86163	B2-bradykinin receptor 3	G-protein coupled receptor	27	10	1	20	<3.6↓	1.0	19	24	<1.4↓	>1.3↑
U94836	ERPROT 213-21	unknown	52	−23	−34	45	3.6↓	1.6↑	3	63	<3.4↓	2.2↑
U30313	diadenosine tetraphosphatase	nucleotide pyrophosphatase	26	−13	1	8	<3.5↓	>2.1↑	−17	−1	<3.0↓	>1.8↑
M31525	MHC class II lymphocyte antigen (HLA-DNA)	lymphocyte antigen	132	123	30	159	<3.4↓	1.3↑	170	131	1.3↑	1.1↑
U53003	GT335	unknown	32	9	9	10	<3.4↓	<1.4↓	6	37	<2.2↓	1.3↑
X74795	cdc46	DNA replication licensing factor	521	444	192	568	3.4↓	1.3↑	558	563	1.1↑	1.3↑
U72661	ninjurin 1	adhesion molecule	47	15	14	125	3.3↓	>6.6↑	−11	391	<3.8↓	>29.0↑
D49490	protein disulfide isomerase related protein (PDIR)	oxidoreductase	14	−5	−7	17	<3.2↓	>2.1↑	41	78	>2.3↑	>1.6↑
X04325	gap junction protein	unknown	19	1	−2	−8	<3.2↓	>1.5↓	−25	−4	<3.1↓	<1.4↓
Y11215	SKAP55	Src kinase-associated phosphoprotein	23	19	2	31	<3.2↓	>2.6↑	27	15	1.2↑	2.6↑
D84307	phosphoethanolamine cytidylyltransferase	biosynthetic enzyme	67	62	22	29	3.1↓	2.2↓	73	66	1.3↓	<1.5↓
X55448	glucose-6-phosphate dehydrogenase	biosynthetic enzyme	83	45	27	60	3.1↓	1.3↑	60	73	1.4↓	1.6↑
L11372	protocadherin 43	adhesion molecule	24	−7	6	6	<3.0↓	<1.2↓	21	−6	1.2↓	<2.2↓
U38864	C2H2-150	transcription factor	−49	−86	−65	−126	<2.7↓	<3.1↓	−116	−61	<4.2↓	>2.7↑
L26081	semaphorin-III (Hsema-I)	ligand	37	37	15	28	2.6↓	1.3↓	5	−15	<3.2↓	<4.9↓
M55621	N-acetylglucosaminyl-transferase I (GlcNAc-TI)	biosynthetic enzyme	85	124	34	88	2.5↓	1.0	23	53	3.6↓	<1.4↓
AB003698	Cdc7-related kinase	protein kinase	58	33	33	31	2.1↓	1.1↓	20	27	<3.4↓	<1.2↓
D85418	phosphatidylinositol-glycan-class C (PIG-C)	biosynthetic enzyme	80	40	39	86	2.1↓	2.1↑	7	79	<4.5↓	1.9↑
U77664	RNaseP protein p38 (RPP38)	nucleotide processing enzyme	73	60	37	86	2.0↓	1.4↑	12	124	<3.5↓	2.1↑
U90549	non-histone chromosomal protein (NHC)	chromosomal protein	68	67	26	49	2.0↓	1.4↓	15	45	<3.6↓	<1.5↓

Table II. (Continued)

Genbank accession no.	Description	Function	Control intensity (0 h)		TGF-β (2 h)				TGF-β (6 h)			
					Intensity		Fold change		Intensity		Fold change	
		CHX	−	+	−	+	−	+	−	+	−	+
X99720	TPRC	unknown	69	53	35	47	2.0↓	1.1↓	10	54	<3.8↓	1.0
L20859	leukemia virus receptor 1 (GLVR1)	transporter	66	119	35	126	1.9↓	1.1↑	20	97	<3.2↓	1.2↓
M58286	tumor necrosis factor receptor	cytokine receptor	130	133	69	170	1.9↓	1.3↑	98	45	3.4↓	3.0↓
M83667	NF-IL6-β protein	transcription factor	67	94	52	84	1.9↓	1.2↑	21	373	3.2↓	4.0↑
U80034	mitochondrial intermediate peptidase precursor (MIPEP)	mitochondrial protein	52	54	31	36	1.7↓	1.0	−14	18	<3.1↓	1.2↓
D78586	CAD	biosynthetic enzyme	139	110	86	85	1.6↓	1.3↓	45	16	3.1↓	6.9↓
U52513	RIG-G	unknown	234	189	143	139	1.6↓	1.1↑	45	185	4.1↓	1.0
M59371	protein tyrosine kinase	protein kinase	66	125	40	184	1.5↓	2.1↑	−8	264	<4.5↓	2.5↑
U35113	metastasis-associated mta1	unknown	37	29	25	49	1.5↓	2.3↓	20	32	<4.5↓	1.8↓
L19871	ATF3	transcription factor	109	229	60	451	1.4↓	2.0↑	−2	421	<4.1↓	1.8↑
M24594	interferon-inducible 56 Kd protein	unknown	128	138	92	89	1.4↓	1.3↓	19	50	<5.1↓	2.4↓
U26266	deoxyhypusine synthase	biosynthetic enzyme	88	50	64	63	1.4↓	1.3↑	19	104	<4.3↓	2.1↑
D86973	KIAA0219 gene (GCN1 human homolog)	transcription factor	115	51	91	60	1.3↓	1.2↑	−1	36	<6.6↓	1.4↓
D87120	cancellous bone osteoblast	unknown	94	65	56	27	1.3↓	2.4↓	21	7	<3.5↓	<5.0↓
X63417	irlB	transcription factor	60	48	46	68	1.3↓	1.4↑	1	70	<3.5↓	1.5↑
U15641	E2F-4	transcription factor	107	101	84	89	1.3↓	1.1↓	25	78	4.3↓	1.3↓
D38305	Tob	tumor suppressor	50	47	42	36	1.2↓	1.3↓	−1	28	<3.4↓	1.7↓
U10324	nuclear factor NF90	transcription factor	93	137	121	39	1.2↓	1.5↓	−8	46	<5.9↓	1.4↓
D43947	KIAA0100 gene	unknown	53	54	58	30	1.1↓	<2.9↓	11	16	<3.0↓	<5.0↓
L08238	Mg44	unknown	87	−137	82	−77	1.1↓	<2.3↓	−451	−213	<16.9↓	<3.6↓
U37408	CtBP	transcription factor	61	80	56	20	1.1↓	4.0↓	33	8	<4.4↓	<3.7↓
U84720	RAE1	transporter	189	175	171	145	1.1↓	1.2↓	49	277	3.8↓	1.6↑
Z24724	polyA site DNA	unknown	60	40	56	35	1.1↓	1.2↓	15	15	<3.1↓	2.7↓
Y12711	putative progesterone binding protein	steroid membrane receptor	88	92	88	42	1.0	2.2↓	25	28	3.0↓	3.2↓
D42040	KIAA9001 gene	unknown	84	340	81	189	1.0	1.4↓	12	211	<4.5↓	1.3↓
U12128	tyrosine phosphatase 1	protein phosphatase	60	57	59	61	1.0	1.1↑	19	20	<3.0↓	2.9↓
L08488	inositol polyphosphate 1 phosphatase	biosynthetic enzyme	103	111	106	94	1.0	1.5↓	50	104	3.8↓	1.1↓
X77366	HBZ17	transcription factor	99	102	103	94	1.0	1.1↓	22	109	4.2↓	1.1↑
X04470	antileukoprotease (ALP)	protease inhibitor	235	129	239	209	1.0	1.6↑	20	276	<8.2↓	1.5↑
D42053	KIAA0091 gene	unknown	73	81	83	52	1.1↑	1.1↑	39	62	<4.9↓	1.4↓
L77213	phosphomevalonate kinase	metabolic enzyme	54	106	118	8	1.1↑	<3.5↓	11	31	<3.1↓	1.8↓
U03688	dioxin-inducible cytochrome P450 (CYP1B1)	cytochrome	81	87	100	109	1.2↑	1.6↑	23	229	3.5↓	3.3↑
X74262	RbAp48	chromosomal protein	102	111	123	72	1.2↑	1.8↓	33	53	3.1↓	2.4↓
U16799	Na,K-ATPase β-1 subunit	biosynthetic enzyme	110	89	167	82	1.5↑	1.7↓	37	122	3.0↓	1.4↑
M21388	unproductively rearranged Ig mu-chain mRNA V-region	unknown	548	285	833	407	1.5↑	1.4↑	167	−13	3.3↓	<1.3↓
X16707	fra-1	transcription factor	38	253	161	643	4.2↑	2.5↑	−100	1212	<10.7↓	4.8↑

a) Genes were listed according to the magnitude of the fold decrease at 2 h in the absence of CHX. Upward and downward arrows represent increase and decrease, respectively.
b) A less than sign (<) indicates that the fold change likely represents an underestimation as described in Table I.
c) A greater than sign (>) indicates that the fold change likely represents an overestimation as described in Table I.

lated Dlx-2 whose expression is regulated by BMP-4 as well.[46] TGF-β transiently induced the expression of Fra-1, a Fos-related gene,[47] with kinetics similar to that of junB. These two proteins belong to the AP-1 family, and may mediate early responses to TGF-β.

Effect of CHX In a number of cases, CHX itself exhibited moderate induction of mRNA, as exemplified in the induction of PAI-1. CHX may inhibit synthesis of proteins involved in mRNA degradation. HaCaT cells treated with CHX exhibited a higher level of PAI-1 at 0 and 2 h than in

Table III. Transcriptional Regulation of Cell Cycle Regulators by TGF-β[a]

Genbank accession no.	Description	Control intensity (0 h)		TGF-β (2 h)				TGF-β (6 h)			
				Intensity		Fold change		Intensity		Fold change	
	CHX	−	+	−	+	−	+	−	+	−	+
X05360	CDC2	128	112	133	72	1.0	1.3↓	95	57	1.2↓	1.6↓
M37712	CDC2 like 1, (PITSLRE)	23	55	47	12	2.1↑	<2.6↓[b]	32	11	1.4↑	<2.5↓
U77949	CDC6	142	82	71	85	1.7↓	1.0	44	72	2.8↓	1.2↓
AB003698	CDC7	58	33	33	31	2.1↓	1.1↓	20	27	<3.4↓	1.2↓
U18291	CDC16	73	69	54	56	1.3↓	1.2↓	52	50	2.2↓	1.4↓
M81933	CDC25A	67	74	44	59	1.5↓	1.3↓	46	59	1.4↓	1.6↓
S78187	CDC25B	671	497	476	481	1.4↓	1.0↑	364	477	1.8↓	1.0
L26584	CDC25C	10	13	25	48	2.5↑	>2.0↑[c]	48	46	>2.8↑	>2.1↑
L10844	CDC42	−37	−52	−47	−29	<2.1↓	>2.2↑	−64	−42	<2.3↓	>1.7↑
X51688	cyclin A	147	67	147	73	1.0	<1.2↓	128	21	1.1↑	5.5↓
M25753	cyclin B1	353	323	318	224	1.1↓	<1.4↓	286	193	1.2↓	1.7↓
M74091	cyclin C	9	10	6	12	<1.3↓	<1.3↓	4	−1	<1.3↓	<1.8↓
X59798	cyclin D1	862	782	931	1150	1.2↑	1.6↑	1028	1531	1.3↑	2.0↑
D13639	cyclin D2	461	552	175	455	2.4↓	1.0	325	498	1.3↓	1.1↓
M92287	cyclin D3	158	129	108	113	1.3↓	1.1↑	109	87	1.2↓	1.2↓
X95406	cyclin E1	−64	−66	−41	−30	<2.1↓	<2.1↓	−51	−64	<2.7↓	>1.1↑
Z36714	cyclin F	101	90	39	154	2.6↓	1.1↑	167	113	1.1↓	1.2↓
X77794	cyclin G1	116	65	91	52	1.3↓	1.3↓	40	42	2.1↓	1.5↓
U11791	cyclin H	216	202	166	167	1.3↓	1.1↓	162	176	1.2↓	1.2↓
D50310	cyclin I	487	349	472	367	1.0	1.0	505	212	1.0	1.6↓
M68520	CDK2	115	69	81	47	1.0	1.5↓	55	49	1.5↓	1.4↓
U37022	CDK4	354	291	279	256	1.3↓	1.0	212	154	1.5↓	1.4↓
X66365	CDK6	−16	−59	−92	−86	<8.7↓	<2.4↓	−110	−80	<5.5↓	<2.5↓
L36844	p15/Ink4b	41	51	43	66	1.1↑	1.3↑	53	60	1.9↑	1.2↑
U26727	p16/Ink4a	107	90	93	123	1.1↓	1.3↑	218	124	2.0↑	1.4↑
U40343	p19/Ink4d	43	68	35	47	1.3↓	1.5↓	31	88	1.4↓	1.0
U09579	p21	109	182	334	349	3.1↑	2.5↑	216	496	2.5↑	3.5↑
U10906	p27/Kip1	−11	34	−10	22	<1.3↓	2.1↓	−15	25	<2.5↓	<1.9↓
X80343	p35 regulatory subunit of cdk5 kinase	−24	−71	−49	−53	<3.5↓	>1.9↑	−73	−49	<3.3↓	>2.5↑
U22398	p57/Kip2	−7	−9	−9	−19	<1.2↓	<1.2↓	0	−2	1.0	>1.3↑
M22898	p53	394	372	327	335	1.2↓	1.1↓	351	325	1.1↓	1.1↓
L41870	RB	43	33	37	32	1.2↓	1.0	45	1.1	1.1↑	<1.9↓
L14812	p107	40	46	45	29	1.1↑	1.6↓	40	29	1.0	1.6↓
X76061	p130	8	12	16	2	>1.9↑	<1.5↓	14	4	>1.3↑	<1.6↓
U47677	E2F-1	8	9	26	45	>2.8↑	1.0	66	48	>1.1↑	<1.2↓
L22846	E2F-2	36	52	43	63	3.6↑	1.2↑	42	53	>2.4↑	1.0
D38550	E2F-3	94	72	72	46	1.3↓	1.0	64	28	1.3↓	2.6↓
U15641	E2F-4	107	101	84	89	1.3↓	1.1↓	25	78	4.3↓	1.3↓
U31556	E2F-5	16	20	2	22	1.3↓	1.1↑	−14	35	<1.8↓	1.7↑
L23959	DP-1	−6	9	22	−4	>3.9↑	<4.5↓	−52	−67	<3.2↓	<10.0↓
L40386	DP-2	33	2	3	20	<4.1↓	>1.9↑	35	−7	1.6↓	<1.6↓
M15796	PCNA	455	429	313	312	1.5↓	1.4↓	489	133	1.1↑	3.2↓
L00058	c-Myc	129	78	48	92	2.7↓	1.2↓	54	57	2.4↓	1.7↓
U92436	PTEN	21	36	18	35	1.2↓	1.0	31	23	1.1↑	1.3↓
X62048	Wee1	56	51	47	32	1.2↓	1.4↓	7	37	<2.7↓	1.4↓

a) Upward and downward arrows represent increase and decrease, respectively.
b) A less than sign (<) indicates that the fold change likely represents an underestimation as described in Table I.
c) A greater than sign (>) indicates that the fold change likely represents an overestimation as described in Table I.

the absence of CHX. At 6 h, however, the level of PAI-1 was less in the presence of CHX than that in the absence of CHX. The result reflects complex regulation of the transcription of PAI-1. In an earlier phase, the induction of PAI-1 may not require *de novo* protein synthesis, whereas the expression at a later phase may depend on protein synthesis. junB showed transient induction by TGF-β, as was found by northern blotting (Fig. 2, C and D). CHX almost completely suppressed the marked decrease of junB expression from 2 to 6 h (note the changes of the intensities in Table I). A similar pattern was observed with Dlx-2 and Fra-1 that are also transiently induced by TGF-β. The expression of βIG-H3 increased for 6 h in the absence of CHX, whereas the induction by TGF-β was completely abolished by the presence of CHX. Thus βIG-H3 is unlikely to be a direct target of TGF-β, and the induction requires synthesis of other protein(s).

Genes down-regulated by TGF-β Unexpectedly, we observed many genes down-regulated by TGF-β (Table II). This could be due to the induction of proteases. The number of the repressed genes is 70, which is 1.3% of the genes examined. When we took 2-fold change as the threshold, approximately 700 genes were selected (unpublished results). TGF-β repressed the expression of prointerleukin-1β, which was antagonized by CHX. Interleukin-1β, on the other hand, induces the expression of Smad7, thereby inhibiting TGF-β signaling.[48] Interestingly, TGF-β also down-regulated interleukin-1 receptor. TGF-β repressed expression of genes induced by interferon, RIG-G and 56 kd protein.[49, 50] Thus, TGF-β seems to affect the actions of various cytokines through transcriptional regulation.

TGF-β markedly repressed expression of epithelial specific Ets (ESX/ELF3/ESE-1/ERT). ESX was shown to be overexpressed at an early stage of human breast cancer development.[27] Furthermore, ELF3 expression was shown to be increased in lung carcinoma.[28] Thus, the repression of ESX/ELF3/ESE-1/ERT may contribute to the tumor suppressive activity of TGF-β. ERT, however, was identified as a transcription factor that induces the expression of TGF-β type II receptor, and loss of ERT may be responsible for oncogenesis in a different context.[51, 52]

Smad2 and Smad3 interact with transcriptional coactivators such as p300 and CBP.[8–10] Recently, however, Smad2 and Smad3 have been shown to associate with TGIF and c-Ski that recruit histone deacetylase.[11, 13, 14, 53] Thus, TGF-β seems to both activate and repress transcription through Smad proteins, depending on cellular conditions. Taken together, the results indicate that TGF-β may suppress tumorigenesis through positive and negative regulation of transcription.

Transcription of cell cycle regulators TGF-β is a potent inhibitor of cell growth. The transcriptional regulation of various cell cycle regulators by TGF-β is summarized in Table III. It has been reported that TGF-β induces the expression of p15 and p21 cdk inhibitors in HaCaT cells.[39, 54] TGF-β, on the other hand, represses the expression of c-myc, cdk4, and cdc25A.[55–57] It has been suggested that targets of growth inhibition by TGF-β may vary depending on the cell type.[57, 58] In our analysis, p21 increased 3.1 fold at 2 h upon treatment by TGF-β. p15, however, increased only 1.1 and 1.9 fold at 2 and 6 h, respectively. Northern blot analysis showed a more significant increase of p15 (unpublished results).[54] The reason for the discrepancy between the northern blotting and the GeneChip analysis is not clear at present. The intensity of Cdk4 decreased from 354 to 279 and 212 at 2 and 6 h, respectively. Cdk6 exhibited a more marked decrease, which may contribute to cell cycle arrest by TGF-β. p16 increased 2.0 fold at 6 h. The levels of p19, p27 and p57 cdk inhibitors remained rather constant. p18 is not contained in the DNA chip. It was reported that TGF-β does not directly affect the expression of cyclin D's, whereas it inhibits increase of cyclin E and A in cycling HaCaT cells.[59] In the present analysis, the intensity of cyclin E1 decreased moderately at 2 and 6 h, whereas the levels of cyclin A and cyclin B1 remained almost constant. The reason for this is probably that most of the cells were still cycling and did not reach the G1/S arrest, which would eventually be caused by TGF-β treatment,[60] during the relatively short TGF-β treatment used in our experiment. The result also suggests that cyclins are unlikely to be direct targets of growth arrest by TGF-β. c-myc and cdc25A decreased about 2.7 and 1.5 fold at 2 h, respectively. Interestingly, TGF-β exerted varying effects on members of the E2F family. TGF-β down-regulated E2F-4 and DP-2, whereas it up-regulated E2F-2.

Targets of TGF-β The identification of previously reported TGF-β inducible genes in the present study validates the usefulness of the GeneChip analysis in the investigation of transcriptional regulation by TGF-β. We have identified many genes that have not yet been reported to be regulated by TGF-β. The results provide important clues about the mechanisms of the biological activities of this pleiotropic growth/differentiation factor. The oligonucleotide arrays contain approximately 5600 genes, but the human genome is thought to code approximately 30 000 genes. The DNA microarray analysis of the uncharacterized genes will almost certainly reveal novel targets of TGF-β, which may play critical roles in tumor suppression by the factor.

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