Identification of ETS-1 target genes in human fibroblasts

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Abstract. The transcription factor Ets-1 plays several distinct critical roles in tumour development and progression by acting both in neoplastic cells and in the tumour stroma. Increased expression of Ets-1 in tumours is often associated with a worse prognosis. Stromal fibroblasts attribute an important part to the behaviour of malignant tumours. In this study we investigated the role of Ets-1 in the tumour stroma. It is well known that ets-1 expression in fibroblasts - one of the main components of the tumour stroma - can be induced by basic fibroblast growth factor (bFGF). We applied suppression subtractive hybridization (SSH) to identify genes that are differentially expressed between bFGF stimulated wild-type fibroblasts and fibroblasts with reduced Ets-1 expression. We selected clones up- or down-regulated in bFGF stimulated wild-type fibroblasts using SSH and functionally characterized them by reference to public databases using NCBI BLAST tools. Expression levels of genes corresponding to subtracted clones were analyzed using RT-PCR. Known genes were associated with diverse functions; novel Ets-1 regulated genes identified by SSH not only encoded components involved in matrix degradation (as cathepsin and PAI-2) but also constituents of the extracellular matrix (ECM) including α-2-Type I collagen, TGF-β induced protein, lumican and decorin. Our findings identify several potential novel target genes of Ets-1, and they provide potentially important insights into the role of Ets-1 in stromal fibroblasts for both remodelling and different functionalities of the ECM.

Introduction

Fibroblasts are important for synthesis of extracellular matrix (ECM) (1). ECM provides structure and elasticity for tissues, comparmentalized different cell types and serves as reservoir for growth factors by sequestering and protecting them in the microenvironment (2). Components of the ECM further interact with specific adhesion receptors on cell surfaces and are involved in regulation of multiple cell functions, including adhesion, migration, proliferation and differentiation (3). ECM also plays a key role in many properties of cancers (4). During the onset of angiogenesis ECM is degraded by proteinases especially by matrix metalloproteinases (MMPs) (5,6). ECM is part of the tumour stroma and serves, as angiogenesis proceedes, essential functions in supporting key signalling events involved in regulating migration, invasion, proliferation and survival (4,7-10).

The transcription factor Ets-1 is one of the critical mediators of ECM remodelling, but up to now Ets-1 is mainly associated with the regulation of MMPs and therefore connected to ECM degradation. The regulation of matrix degrading proteases by Ets-1 is well investigated especially in tumour invasion and metastasis (11-15). Since evolution has developed a restricted number of transcription factors for many gene regulatory networks it is probable that Ets-1 target genes are not restricted to those proteins involved in matrix-degradation in fibroblasts. The aim of the present work was therefore to identify further Ets-1 target genes in human fibroblasts and to enlighten the role and function of this transcription factor in the tumour stroma. For this purpose wild-type fibroblasts and fibroblasts with a reduced Ets-1 expression were stimulated with bFGF, a cytokine known to induce Ets-1 in fibroblasts and to be expressed by many tumour types (11,14,16). Subtractive suppression hybridization (SSH) was performed with the stimulated cells to identify the differences in gene expression.

Materials and methods

Cell culture and stable transfections. Human foreskin fibroblasts (HFF) were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. All reagents for cell culture were purchased from Invitrogen NV (Leek, The Netherlands).

The plasmid cDNA3-Ets-1-invers was transfected into the HFF cells by the calciumphosphate method, and 24 h after transfection, selection was started using 200 μg/ml G418 (Life Technologies).

Western blot analysis. Cells were lysed by 5 freeze (on dry ice)-thaw (room temperature) cycles. Lysates containing 15-20 μg

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Table I. Primers used for RT-PCR.

Gene	Forward primer	Reverse primer
RPL-13 A	5'-TACGCTGTGAAGGCATCAAC-3'	5'-CACCATCCGCTTTTTCTTGT-3'
MMP 1	5'-CTACACGGATACCCCAAGGA-3'	5'-AACTTTGTGGCCAATTCCAG-3'
MMP 3	5'-ACGAGGGCACGAGGAGCTAGC-3'	5'-GGGTCAAATTCCAACTGAGAAG-3'
PAI-2	5'-ATGGAGGATCTTTGTGTGG-3'	5'-ATGGGGGTAACTGCATTGGC-3'
Cathepsin	5'-CAGGATGTGGGGGCTCAAGG-3'	5'-GGGTGTCATTACTGCGGGAA-3'
α-2-Type I collagen	5'-GTGGGAACTTTGCTGCTCAG-3'	5'-AACAACTCCTCTCTCACCTG-3'
TGF-β induced protein	5'-GCTGTCCAGCAGCCCTACCA-3'	5'-GCTTTCAGCAGCCGGGCACA-3'
Lumican	5'-GGCCAGTACTATGATTATGA-3'	5'-GCAGCTTCTTCAGTTGTTTCAA-3'
Decorin	5'-ATGAAGGCCACTATCATCCTC-3'	5'-GAGAATTACTTATAGTTTCC-3'
Gremlin	5'-ACTCAGAGCAGACTCAGTCG-3'	5'-CTGTTGCAGCCTTCCTCGTG-3'
Retinoblastoma associated factor 600	5'-ACCCGAGCAACTTCCCGAGA-3'	5'-CGGAATAGTCCTTCACTGCC-3'
HSP-90	5'-ACTCTCACTATTGTGGATAC-3'	5'-CATCATCGTTATGTTTGGTGA-3'
TNF-receptor superfamily 11B	5'-ATTAAGTGGACCACCCAGGA-3'	5'-GCTCCTATGTTTCAAGCAGA-3'
AXL receptor tyrosine kinase	5'-CTCACGGGCACCCTTCGGTG-3'	5'-GTGACCTGGAGCCGTGGCCAG-3'
MET receptor tyrosine kinase	5'-CTGTGCTTGCACCTGGCATC-3'	5'-CTGTTGACGCTGCCACAGCTA-3'
β-2-adrenergic receptor	5'-GGAAGCCATGCGCCGGACCA-3'	5'-GGTGGGTGGCCCTGTACCAG-3'
Stromal cell derived factor receptor 1	5'-TGGCCCTCTCGCTGTTGCTG-3'	5'-GATGAAGAAGCGGCCAGAGG-3'
Insulin-like growth-factor binding protein 5	5'-CCTCTGTGGCCTCCATGGTA-3'	5'-GTACTGTCCCACCTTGAGGG-3'
Fli-1	5'-AGTGAGGGTCAACGTCAAGC-3'	5'-ACACAGTTCCTTGCCATCCA-3'
MMTV-5A	5'-AGGTTGTAATTGAAGCCAATTC-3'	5'-ATTCTTTGATGCCTGTCTTCGC-3'
Rho-8/RAS GTPase	5'-AAGGAGAGAAGAGCCAGCCAG-3'	5'-CGGACATTGTCATAGTAAGG-3'
ATP5G3	5'-TGATCCGAGCTGGATCCAGA-3'	5'-GAATGCCAACATGTCAAGCA-3'
5-tubulin	5'-TGCACATCCAGGCTGGTCAG-3'	5'-AGCTCGGCGCCCTCTGTGTA-3'
Serine/threonine-kinase 6	5'-CTGTTAAGGCTACAGCTCCA-3'	5'-AGACAGGATGAGGTACACTG-3'
Anilin, actin binding protein	5'-CAGACAGTGATCTATCAAGC-3'	5'-CTGATCCTTTGGATGGCATA-3'
Thioredoxin	5'-GTGAAGCAGATCGAGAGCAA-3'	5'-GAAAACATGATTAGACTAATTC-3'

of total protein were analysed by 10% SDS-polyacrylamide gel (SDS-PAGE) electrophoresis and the proteins transferred onto nitrocellulose (Bio-Rad, München, Germany). After blocking with 5% non-fat dry milk in phosphate-buffered saline (PBS, Life Technologies, Darmstadt, Germany) for 2 h at room temperature, the filters were incubated in TBS-T (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) and with anti-Ets-1 rabbit polyclonal serum (1:1000 dilution; Transduction Laboratories, Lexington, USA), then with a horseradish peroxidase-conjugated secondary antibody (1:5000; Amersham Biosciences, Freiburg, Germany). The antibodies were detected using the ECL reagents (Amersham Biosciences). The housekeeping protein β-actin was detected using a mouse monoclonal anti-β-actin antibody (1:5000; Sigma, Taufkirchen, Germany).

RNA isolation and c-DNA synthesis. Prior to RNA isolation cultures were stimulated with 10 ng basic fibroblast growth factor (bFGF; Roche Diagnostics GmbH, Mannheim, Germany) per ml culture medium for 16 h. Total cellular RNA was extracted from cell monolayers by the RNeasy kit (Qiagen, Hilden, Germany). The RNA quality was determined by Agilent Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) measurement. Only preparations with a RIN factor ≥9 were used for further analysis.

Generation of cDNAs by reverse transcriptase was performed in 10 μl reaction volume containing 2 μg of total cellular RNA, 1 μl of dNTPs (10 mM), 1 μl of oligo(dT)$_{12-18}$ primer (10 mM), and RNAse-free water. After incubation at 65˚C for 5 min the reaction mixture was placed on ice for 1 min. Then 2 μl of 10X RT buffer (Invitrogen, Karlsruhe, Germany), 4 μl $MgCl_2$ (25 mM), 2 μl of 0.1 M DTT, and 1 μl RNAseOUT™ recombinant RNAse inhibitor (Invitrogen) were added. After incubation at 42˚C for 2 min 1 μl of Superscript II reverse transciptase (50 U/μl; Invitrogen) was added for reverse transcription at 42˚C for 1 h. Inactivation of the enzyme was performed by heating at 70˚C for 15 min. In a last step RNA was removed by addition of 1 μl RNAseH and incubation at 37˚C for 20 min.

Suppression subtractive hybridization (SSH). After stimulation with 10 ng bFGF (Roche Diagnostics GmbH) per ml culture medium for 16 h, SSH was performed between HFF and HFF-Ets-1-invers cells in both directions using the PCR-Select

Table II. PCR conditions used for amplification with 25 cycles of denaturation, annealing and extension.

	Denaturation	Annealing	Extension
rpL13A	94˚C for 30 sec	66˚C for 30 sec	72˚C for 30 sec
mmp 1	94˚C for 30 sec	56˚C for 30 sec	72˚C for 30 sec
mmp 3	94˚C for 30 sec	65˚C for 30 sec	72˚C for 30 sec
PAI-2	94˚C for 30 sec	66˚C for 30 sec	72˚C for 30 sec
Cathepsin	94˚C for 30 sec	68˚C for 30 sec	72˚C for 30 sec
α-2-Type I collagen	94˚C for 60 sec	67˚C for 30 sec	72˚C for 60 sec
TGF-β induced protein	94˚C for 60 sec	72˚C for 30 sec	72˚C for 60 sec
Lumican	94˚C for 30 sec	55˚C for 30 sec	72˚C for 30 sec
Decorin	94˚C for 30 sec	53˚C for 30 sec	72˚C for 30 sec
Gremlin	94˚C for 60 sec	62˚C for 30 sec	72˚C for 60 sec
Retinoblastoma associated factor 600	94˚C for 60 sec	63˚C for 30 sec	72˚C for 60 sec
HSP-90	94˚C for 60 sec	53˚C for 30 sec	72˚C for 60 sec
TNF-receptor superfamily 11B	94˚C for 60 sec	62˚C for 30 sec	72˚C for 60 sec
AXL receptor tyrosine kinase	94˚C for 60 sec	72˚C for 30 sec	72˚C for 60 sec
MET receptor tyrosine kinase	94˚C for 60 sec	70˚C for 30 sec	72˚C for 60 sec
β-2-adrenergic receptor	94˚C for 60 sec	72˚C for 30 sec	72˚C for 60 sec
Stromal cell derived factor receptor 1	94˚C for 60 sec	65˚C for 30 sec	72˚C for 60 sec
Insulin-like growth-factor binding protein 5	94˚C for 60 sec	67˚C for 30 sec	72˚C for 60 sec
Fli-1	94˚C for 60 sec	67˚C for 30 sec	72˚C for 60 sec
MMTV-5A	94˚C for 60 sec	63˚C for 30 sec	72˚C for 60 sec
Rho-8/RAS GTPase	94˚C for 60 sec	68˚C for 30 sec	72˚C for 60 sec
ATP5G3	94˚C for 60 sec	66˚C for 30 sec	72˚C for 60 sec
5-tubulin	94˚C for 60 sec	72˚C for 30 sec	72˚C for 60 sec
Serine/τhreonine-kinase 6	94˚C for 60 sec	62˚C for 30 sec	72˚C for 60 sec
Anilin, actin binding protein	94˚C for 60 sec	58˚C for 30 sec	72˚C for 60 sec
Thioredoxin	94˚C for 60 sec	53˚C for 30 sec	72˚C for 60 sec

cDNA Substraction Kit (Clontech, Heidelberg, Germany) according to the manufacturer's instructions. In brief, 2 μg of mRNA from the tester and the driver was subjected to cDNA synthesis. Tester and driver cDNA were digested with RsaI. The tester cDNA was split into two groups, and each was ligated with different cDNA adapter. In a first hybridization reaction, an excess of driver was added to each sample of tester. The samples were heat-denaturated and allowed to anneal. Because of the second-order kinetics of hybridization, the concentration of high-and low-abundance sequences is equalized among the single-stranded tester molecules. At the same time, single-stranded tester molecules were significantly enriched for differentially expressed sequences. During a second hybridization, the two primary hybridization samples were mixed together without denaturation. Only the remaining equalized and substracted single-stranded tester cDNAs can reassociate forming double-stranded tester molecules with different ends. After filling in the ends with DNA polymerase, the entire population of molecules was subjected to nested PCR with two adapter-specific primer pairs. After that, secondary PCR products were used as templates for PCR amplification of the housekeeping gene GAPDH at 18, 23, 28 and 33 cycles to assure substraction efficiency.

Products of these amplified overhangs containing a substracted cDNA library (6 μl) were ligated into a pcDNA vector (Invitrogen, Karlsruhe, Germany). Subsequently, the plasmid was transformed into *E. coli* strain DH5α. Bacteria were plated onto agar plates containing ampicillin (100 μl/ml) and incubated overnight at 37˚C. Single colonies were used for plasmid preparation (QIAprep® Spin Minprep Kit, Qiagen, Hilden, Germany). The cDNA inserts were identified by sequencing (Entelechon, Regensburg, Germany) and subsequent nucleic acid homology searches of the obtained sequences were performed using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/).

PCR. For analysis of gene expression polymerase chain reaction (PCR) amplifications were performed using GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). All primers were synthesised by TIB MolBiol (Berlin, Germany). The primer sequences used are given in Table I. PCR amplification of cDNA was performed in a reaction containing 2.5 μl of 10X polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM of each desoxynucleoside triphosphate, 25 pmol 5' primer and 3' primer, 0.1 μl (0.5 U) *Taq* DNA polymerase, 0.5 μl cDNA template and sterile RNAse-free water added to a total volume

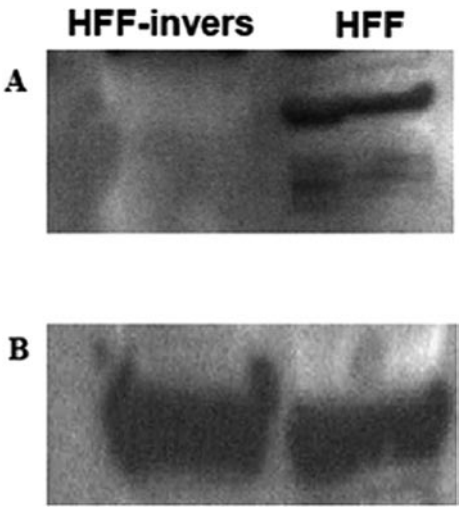

Figure 1. Analysis of Ets-1 expression. Cell extracts (20 μg) were separated by SDS-PAGE and the proteins analysed by Western blotting using an anti-Ets-1 antibody (A). HFF cells show detectable amounts of Ets-1 and HFF-Ets-1-invers have decreased level of expression of the protein. Actin was used as internal standard (B).

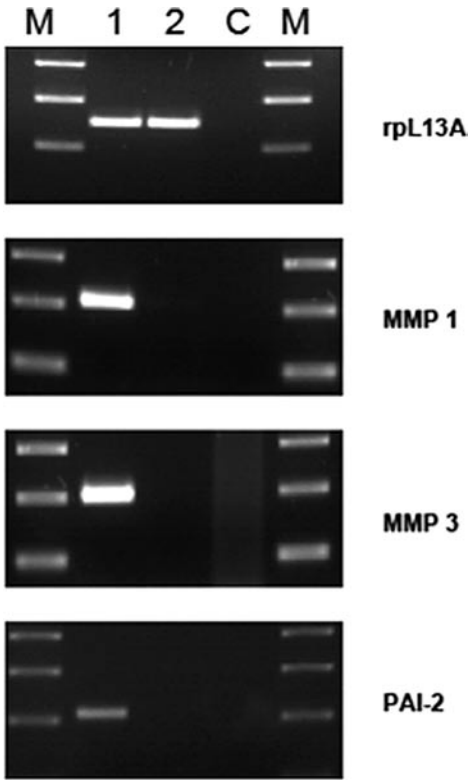

Figure 2. RT-PCR-analysis of known Ets-1 target genes in wild-type-fibroblasts compared to fibroblasts with reduced ETS-1 expression. Expression of transcripts was analysed in 2% agarose gels in HFF wild-type (lane 1) and HFF cells with a reduced Ets-1 expression (HFF-Ets-1-invers; lane 2) after induction with bFGF (10 ng/ml) for 16 h. The negative control (C) for any PCR is shown at the end of each line. Fragment sizes were evaluated with a PCR-marker (M); gene specific products are 251-bp for rpL13A, 280-bp for MMP 1, 280-bp for MMP 3 and 212-bp for PAI-2.

of 25 μl. All PCR reagents were from Invitrogen (Karlsruhe, Germany).

We first amplified a housekeeping gene, encoding the constitutively expressed ribosomal protein L13A (rpL13A) in order to monitor RNA quality and cDNA synthesis and to ensure that equivalent amounts of cDNA were used in all PCR amplifications. All PCR reactions were preceded by a denaturation step at 94˚C for 1 min followed by 25 cycles of denaturation, annealing and extension; PCR conditions for the different cDNAs are shown in Table II.

Each PCR reaction was finished by a final elongation step at 72˚C for 10 min. All PCR products were analysed by separation on a 2% agarose gel stained with ethidium bromide.

Results

A stable HFF cell line was established following transfection with an Ets-1-anti-sense expression vector. Ets-1-under-expressing (HFF-Ets-1-invers) cell line was selected from the pooled population of transfected cells, in order to avoid clonal variations. Ets-1 expression levels corresponded to the expected variations as shown by Western blotting with anti human Ets-1 antibody; while wild-type HFF cells expressed detectable amounts of Ets-1, the expression of the protein was not detectable in HFF-Ets-1-invers cells (Fig. 1A).

Subtractive suppression hybridization (SSH) has been demonstrated to be an effective method to identify new target genes in different systems (17,18). Here we used the SSH approach for the identification of Ets-1 target genes in human fibroblasts. SSH was performed with bFGF stimulated wild-type HFF cells and HFF-Ets-1-invers cells in both directions. This substraction, described in Materials and methods, generated both a forward- and a reverse-substracted cDNA pool. Thus it was possible to identify genes that are activated as well as genes that are repressed by Ets-1.

Two hundred clones obtained from the cDNA library were analysed by sequencing and subsequent homology searches. Differences in gene expression were verified by semiquantitative RT-PCR. As expected for a transcription factor a broad variety of genes involved in different cellular functions are regulated by Ets-1.

The capacity of the SSH method is demonstrated by identification of known Ets-1 target genes MMP-1, MMP-3 and PAI-2 in our experiments which were strongly expressed in wild-type HFF cells (Fig. 2). As further Ets-1 regulated genes in fibroblasts, genes coding for cathepsin, α-2-Type I collagen, TGF-β induced protein, lumican, decorin and gremlin were identified (Fig. 3). All these genes are involved in the build-up or the remodelling of the ECM. Transcripts of cathepsin, α-2-Type I collagen, lumican, decorin and gremlin are expressed in wild-type HFF but not in HFF-invers cells. Therefore Ets-1 seems to be an essential activator for expression of these genes.

In contrast the gene coding for TGF-β induced protein was expressed at a higher level in HFF-invers than in wild-type HFF cells. This gene is therefore probably repressed by Ets-1. Further genes that are regulated by Ets-1 in HFF cells are summarized in Fig. 4. For HSP-90, TNF-receptor-superfamily 11B (TNFRSF11B), insulin-like growth-factor binding protein 5 (Igfbp5), Fli-1 and MMTV-5A the transcription factor Ets-1 seems to be a potential activator. In contrast, Ets-1 seems to act as a repressor in HFF cells for retinoblastoma associated factor 600 (RBAF600), AXL-receptor-tyrosine kinase (AXL),

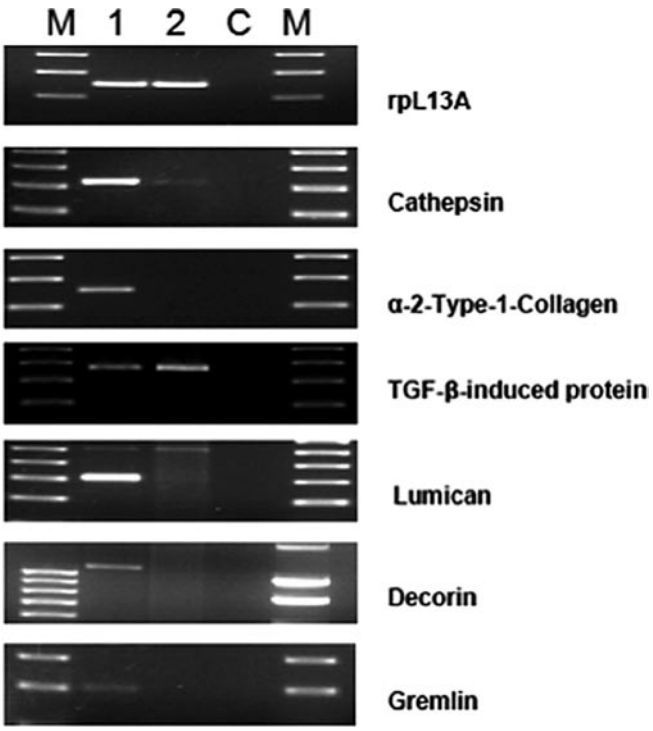

Figure 3. RT-PCR-analysis of Ets-1 target genes involved in building-up or remodelling of ECM. Expression of cathepsin, α-2-type I collagen, TGF-β induced protein, lumican, decorin and gremlin was assessed in HFF wild-type (lane 1) and HFF cells with a reduced Ets-1 expression (HFF-Ets-1-invers; lane 2) after induction with bFGF (10 ng/ml) for 16 h. The negative control (C) for any PCR is shown at the end of every line. Gene specific products are 251-bp for rpL13A, 320-bp for cathepsin, 259-bp for α-2-Type I collagen, 373-bp for TGF-β induced protein, 313-bp for lumican, 1085-bp for decorin and 202-bp for gremlin, evaluated by PCR-marker (M).

MET-tyrosine kinase (MET), β-2-adrenergic receptor (β-2AR), stromal cell derived factor receptor 1 (CXCR4), ATP5G3, 5-tubulin, serine-threonin-kinase 6 (STK6) and anilin/actin binding protein (Anilin).

Discussion

Since a limited number of transcription factors controls many gene regulatory networks it is probably that also Ets-1 target genes in fibroblasts are not restricted to those playing a role in matrix-degradation. In order to identify novel Ets-1 target genes we have analysed gene expression after bFGF stimulation of wild-type and Ets-1 repressed fibroblasts using SSH. In line with earlier studies we identified several known Ets-1 target genes involved in matrix remodelling (such as genes encoding MMP-1, MMP-3, PAI-2 and α-2-Type I collagen) in these experiments (11-14,19). In addition we could demonstrate several novel genes modulated by Ets-1 in human fibroblasts. As expected for a conserved transcription factor they comprise a broad variety of genes involved in different cellular functions. These genes encode cathepsin, TGF-β induced protein, lumican, decorin, gremlin, HSP-90, TNF-receptor-superfamily 11B, insulin-like growth-factor binding protein 5, Fli-1, MMTV-5A, retinoblastoma associated factor 600, AXL-receptor-tyrosine kinase, MET-tyrosine kinase, β-2-adrenergic receptor, stromal cell derived factor receptor 1, ATP5G3, 5-tubulin, serine-threonin-kinase 6 and anilin/actin binding protein.

The largest group among Ets-1 target genes in HFF identified in this investigation belongs to genes involved in the remodelling of the ECM. Genes encoding MMP-1, MMP-3, PAI-2 (SERPIN B2) and cathepsin all play a role in matrix

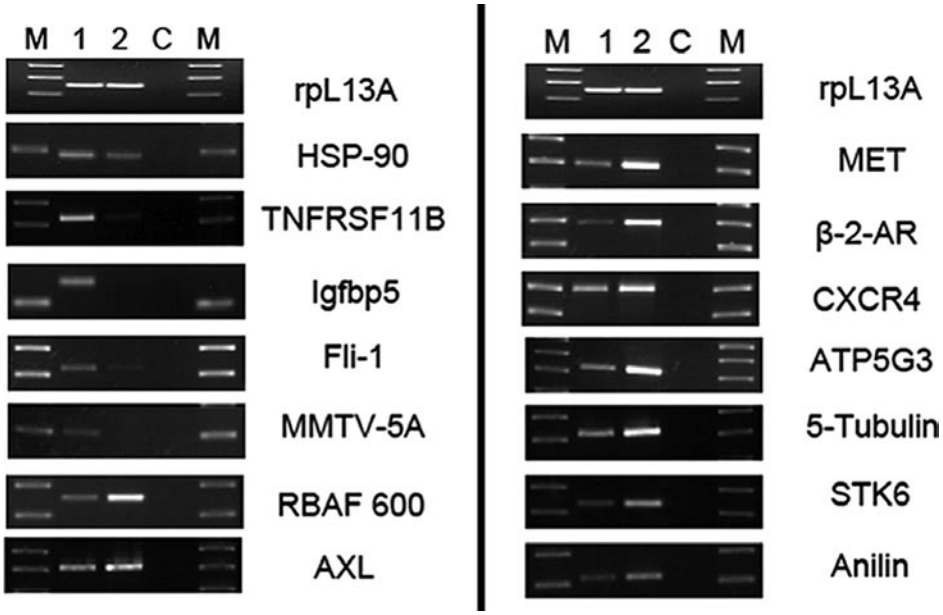

Figure 4. RT-PCR-analysis of further Ets-1 target genes identified by SSH. Expression of HSP-90, TNF-receptor-superfamily 11B (TNFRSF11B), insulin-like growth-factor binding protein 5 (Igfbp5), Fli-1, MMTV-5A, retinoblastoma associated factor 600 (RBAF600), AXL-receptor-tyrosine kinase (AXL), MET-tyrosine kinase (MET), β-2-adrenergic receptor (β-2AR), stromal cell derived factor receptor 1 (CXCR4), ATP5G3, 5-tubulin, serine-threonin-kinase 6 (STK6) and anilin/actin binding protein (Anilin) was assessed in HFF wild-type (lane 1) and HFF cells with a reduced Ets-1 expression (HFF-Ets-1-invers; lane 2) after induction with bFGF (10 ng/ml) for 16 h. The negative control (C) for any PCR is shown at the end of every line. Gene specific products are 251-bp for rpL13A, 211-bp for HSP-90, 324-bp for TNF-receptor superfamily 11B, 140-bp for insulin-like growth-factor binding protein 5, 322-bp for Fli-1, 198-bp for MMTV-5A, 256-bp for retinoblastoma associated factor 600, 411-bp for AXL receptor tyrosine kinase, 400-bp for MET receptor tyrosine kinase, 490-bp for β-2-adrenergic receptor, 497-bp for stromal cell derived factor receptor 1, 420-bp for ATP5G3, 322-bp for 5-tubulin, 231-bp for serine/threonine-kinase 6 and 220-bp for anilin, evaluated by PCR-marker (M).

Table III. The promoter regions of the different genes from public available sequences (National Center for Biotechnology Information or Ensembl Genome Browser) were used for searching potential Ets-1 binding sites with the PATCH 1.0 public program.

	Number of potential Ets-1 binding sites in the promoter region	Described Ets-1 binding sites in the literature
MMP 1	19	(13,48)
MMP 3	60	(13,48)
PAI-2	51	(49)
Cathepsin	3	
α-2-Type I collagen	4	
TGF-β induced protein	5	
Lumican	1	
Decorin	3	
Gremlin	39	
Retinoblastoma associated factor 600	1	
HSP-90	13	
TNF-receptor superfamily 11B	16	
AXL receptor tyrosine kinase	20	
MET receptor tyrosine kinase	6	(24)
β-2-adrenergic receptor	2	
Stromal cell derived factor receptor 1	20	
Insulin-like growth-factor binding protein 5	20	
Fli-1	2	(25)
MMTV-5A	8	
Rho-8/RAS GTPase	6	
ATP5G3	16	
5-tubulin	1	
Serine/threonine-kinase 6	3	
Anilin, actin binding protein	5	
Thioredoxin	56	

degradation (20-23). MET-tyrosine kinase and Fli-1 have already been described as Ets-1 target genes in human gastric cancer and in endothelial cells, respectively (24,25). However, genes encoding the ECM components α-2-Type I collagen, TGF-β induced protein (BIGH3), lumican and decorin are also target genes of Ets-1.

The ECM is product of the resident cells (including fibroblasts) of each tissue and organ and serves both structural and functional roles (26). ECM exists in a state of dynamic equilibrium with its adjacent cell populations and is responsive to changing microenvironmental conditions and functional demands of the cells and their parental tissue or organ (27). The molecular composition of ECM is known to play a critical role for fundamental processes such as cellular migration and differentiation (3).

The transcription factor Ets-1 is considered one of the critical mediators of ECM remodelling by ECM degradation especially via regulation of MMPs (28-30). This issue has particularly been addressed in tumour angiogenesis, invasion and metastasis (11-15). ECM is degraded by proteinases, especially by MMPs during the onset of angiogenesis (5,6). ECM participates in the regulation of endothelial cell migration, invasion, proliferation and survival (4,7,31). For HSP-90 the presence and function in the extracellular space has been described (32). There HSP-90 assists in activation of MMPs resulting in increased tumour invasiveness (32). It has also been shown by promoter deletion and mutational analysis that an Ets-1-like site in the promoter of the *pai-2* gene is essential for bFGF induced pai-2 expression in endothelial cells (33).

Data of this study suggest a role of Ets-1 not only for degradation but also for build-up of ECM. According to our results Ets-1 plays a role not only in expression of MMP-1, MMP-3, PAI-2 and cathepsin but also in expression of lumican, decorin and α-2-Type I collagen. It has recently been reported that Ets-1 stimulates α-2-Type I collagen promoter activity in dermal fibroblasts fitting in well with our observations (19). Lumican and decorin, induced by Ets-1 according to our experiments, are both members of the leucine-rich proteoglycan family (34,35). They both bind to collagen I and II and are able to fix and sequester growth factors (34-37). Besides modulation of growth factors and cytokine activities, proteoglycans are considered to regulate the water balance of

the ECM, influence tissue biomechanics and facilitate cellular adhesion, proliferation as well as migration (38). It has been previously described that Ets-1 is also critical to bone and cartilage development (39) and we identified gremlin as another possible Ets-1 target gene in fibroblasts. This protein is known to be involved in bone development as a regulator of bone morphogenetic proteins. In addition gremlin has further roles in growth, differentiation and development (40). Gremlin has also been found to be over-expressed in human cancers (41). Recently it was shown that gremlin is produced by the stroma of human tumours and that it acts as proangiogenic factor (42). In the present study we did not demonstrate experimentally effects of Ets-1 upon the promoters of potential Ets-1 target genes we identified. However, bioinformatics promoter analysis reveals in all genes the presence of potential Ets-1 binding sites in the promoter regions (Table III) rendering possible direct effects of Ets-1 on expression of these genes. This has already been shown for MMP-1 and MMP-3 genes in previous studies (11,43). Nevertheless indirect or combined effects of Ets-1 are likewise possible in view of our finding that Ets-1 induces in human fibroblasts other transcription factors such as the ETS family member Fli-1 or MMTV-5A.

In conclusion our results suggest that the expression level of Ets-1 within fibroblasts seems to be of pivotal importance for ECM composition. Ets-1 expression in fibroblasts may play many roles upon remodelling and different functional properties of ECM which is particularly interesting with respect to the importance attributed to stromal fibroblasts for behaviour of malignant tumours (8,44-46). Ets-1 may function as activator or as repressor via association with specific cofactors and in combination with other transcription factors depending on promoter context (47). But until today a precise understanding of the role of Ets-1 in the complex network governing the expression of ECM proteins and the enzymes that degrade them is missing. Further work will be required to define the precise roles of Ets-1 for regulation of the genes identified in this study.

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