SPANDIDOS PUBLICATIONS

Expression pattern of matrix metalloproteinase and TIMP genes in fibroblasts derived from Ets-1 knock-out mice compared to wild-type mouse fibroblasts

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Abstract. Matrix-degrading proteases play a key role in normal development, wound healing, many diseases such as rheumatoid arthritis and, in particular, tumour invasion. In invasive tumours, these enzymes are expressed by fibroblasts of the tumour stroma. Their expression and activity are tightly regulated at several levels, an important one being transcription. Previous *in vitro* and *in vivo* findings pointed to a major role of the Ets-1 transcription factor for this level of regulation. In the present study, we tried to prove this role in fibroblasts. We stimulated wild-type mouse fibroblasts with physiological doses of basic fibroblast growth factor (bFGF, known to induce different proteases and expressed by tumour cells) and compared the results to those obtained in Ets-1 -/- fibroblasts derived from Ets-1 knock-out mice. We found that basal Ets-1 levels are necessary not only for a fast induction of MMPs 2, 3 and 13 by bFGF but also for maintenance of the bFGF-induced expression of tissue inhibitors of metalloproteinases (TIMPs) 1, 2 and 3, which are known not only to inhibit but also participate as activators of certain pro-MMPs.

Introduction

Apart from neoplastic cells, the tumour stroma is of pivotal importance for tumour development and progression (1-4). It is induced by tumour and stromal cell-derived factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and tumour necrosis factor α (TNFα), and it carries out many different roles. Among the most significant are tumour vascularization, the prerequisite for continuous tumour growth, and the promotion of tumour invasion, through the secretion of matrix-degrading metalloproteinases (MMPs) by stromal fibroblasts or myofibroblasts (1,5-7). Expression and activity of these enzymes are controlled by a fine balance between induction by different growth factors and cytokines, proteolytic activation through the plasminogen/plasmin system and inhibition by several tissue inhibitors of metalloproteinases (TIMPs) (8-17). An important level of regulation of MMPs is transcription and several transcription factors, such as AP-1, SP-1 and members of the ETS-family, have been shown to transactivate or transrepress different protease genes alone or in combination (18-25). Several lines of evidence point to major roles of the Ets-1 transcription factor for this level of regulation (26-28). Ets-1 is the prototype of the ETS transcription factor family (29); all members share a specific DNA-binding domain, the ETS domain, which consists of approximately 80 amino acids with four tryptophane repeats (30). It has been shown that two-thirds of the 27 human *ets* genes are expressed ubiquitously and that the DNA binding of ETS proteins has overlapping specificities (31). In biological terms, ETS-family members are expressed by many different cell types and participate in the regulation of a number of basic cellular functions, such as proliferation, apoptosis, differentiation and migration (32-39).

Combined co-transfection assays and deletion studies of promoters have shown that Ets-1 binding sites, in particular, are required for the transactivation of several genes encoding matrix degrading proteases. They include collagenase IV (MMP 2 and 9), stromelysin I (MMP 3) and the urokinase-type plasminogen activator (uPA) which initiates the cascade of proteolytic activation of these enzymes (11,40-42). The presence of an Ets-1 binding site has recently also been found in the promoter region of the *mmp13* gene encoding collagenase III (43). *In vivo*, patterns of co-expression of matrix metalloproteinases together with Ets-1 have been observed during pathological processes, especially in tumours (18,44-46) where these proteases are expressed together with Ets-1 in stromal fibroblasts during tumour invasion (27,47-49). Non-invasive lesions of the same tissue are, in contrast, negative for both Ets-1 and MMPs (18,27,45,46). *In vitro,* Ets-1 and several MMPs are induced by the same cytokines and growth factors in cultured fibroblasts (2,27,42). These observations suggest that Ets-1 is causally involved in MMP expression in fibroblasts.

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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
Ets-1	5'-CGATCTCAAGCCGACTCTCA-'3	5'-GAAGCTGGGCTCTGAGAACTC-'3
MMP 2	5'-GTACTGGGTCTATTCTGCTAG-'3	5'-CACTTCATTGTATCTCCAG-'3
MMP 3	5'-ACGAGGGCACGAGGAGCTAGC-'3	5'-GGGTCAAATTCCAACTGAGAAG-'3
MMP 9	5'-GTTCCCACTTACTATGGAAAC-'3	5'-CGTGCTCCGTGTAGAGTCTC-'3
MMP 13	5'-GAATGGTTATGACATTCTGGA-'3	5'-GATACTGTATTCAAACTGTATGG-'3
uPA	5'-AGTGCATGGTGCATGACTGC-'3	5'-CCAAAGCCAGTGATCTCACA-'3
TIMP-1	5'-CATGGAAAGCCTCTGTGGATATG-'3	5'-AAGCTGCAGGCACTGATGTG-'3
TIMP-2	5'-CCAGAAGAAGAGCCTGAACCA-'3	5'-GTCCATCCAGAGGCACTCATC-'3
TIMP-3	5'-GGCCTCAATTACCGCTACCA-'3	5'-CTGATAGCCAGGGTACCCAAAA-'3
TIMP-4	5'-TGCAGAGGGAGAGCCTGAA-'3	5'-GGTACCCATAGAGCTTCCG-'3

The aim of the present work was to prove this causal relationship. For this purpose, we investigated the expression of different proteases in Ets-1 -/- fibroblasts from an Ets-1 knock-out mouse in which the *ets-1* gene has been specifically inactivated (50). We compared the results to those obtained in Ets-1 +/+ fibroblasts. In order to induce expression of proteases in a physiological manner, we stimulated the cells with physiological concentrations of bFGF known to induce different proteases in fibroblasts and to be expressed by tumour cells (2,18,27,44). Previous studies investigating the roles of ETS-family members for transactivation or transrepression of protease genes have, in contrast, used co-transfection assays with maximal expression of the transcription factors in question (41,51,52). Since it has been published that genes of negative regulators of metalloproteinases (tissue inhibitors of metalloproteinases, TIMPs) are also Ets-1 target genes (17,53-55), we also examined the expression of TIMPs 1 to 4.

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
ets-1	94˚C for 30 sec	65˚C for 30 sec	72˚C for 60 sec
mmp 2	94˚C for 30 sec	57˚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
mmp 9	94˚C for 30 sec	64˚C for 30 sec	72˚C for 30 sec
mmp 13	94˚C for 30 sec	59˚C for 30 sec	72˚C for 30 sec
uPA	94˚C for 30 sec	56˚C for 30 sec	72˚C for 90 sec
timp 1	94˚C for 30 sec	60˚C for 30 sec	72˚C for 30 sec
timp 2	94˚C for 30 sec	60˚C for 30 sec	72˚C for 30 sec
timp 3	94˚C for 30 sec	60˚C for 30 sec	72˚C for 30 sec
timp 4	94˚C for 30 sec	60˚C for 30 sec	72˚C for 30 sec

Materials and methods

Cell culture. Mouse embryonal fibroblasts (MEF) were obtained from RAG-2-deficient mice and from Ets-1$^{-/-}$-RAG-2$^{-/-}$ chimeric mice, respectively (50). We used fibroblasts from two Ets-1$^{-/-}$-RAG-2$^{-/-}$ mice (k.o.8 and k.o.9) and from one RAG-2$^{-/-}$ mouse (wild type) as a control. Isolated MEF 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).

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 20 min or 16 h, respectively. Unstimulated cells were used as controls.

Total cellular RNA was extracted from cell monolayers by the RNeasy kit (Qiagen, Hilden, Germany). 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, Karlsruhe, Germany) were added. After incubation at 42˚C for 2 min, 1 μl of Superscript II reverse transciptase (50 units/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 final step, RNA was removed by addition of 1 μl RNAseH and incubation at 37˚C for 20 min.

PCR. For analysis of gene expression, polymerase chain reaction (PCR) amplification was 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

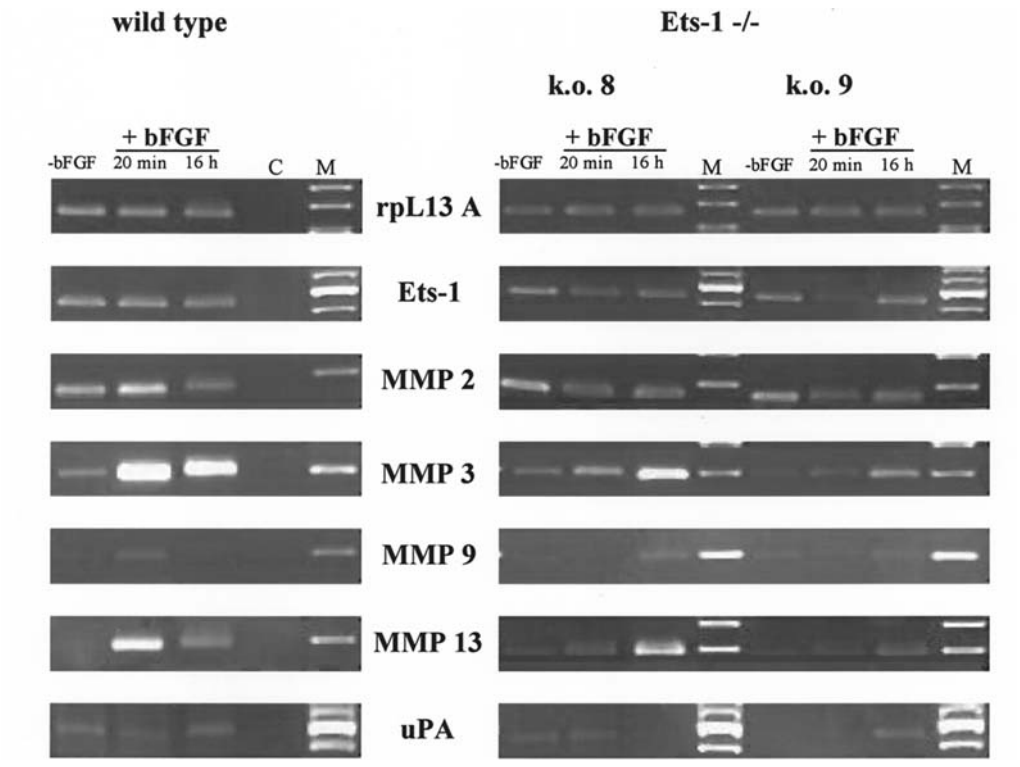

Figure 1. RT-PCR analysis of Ets-1 and protease expression in wild-type fibroblasts compared to Ets-1 -/- fibroblasts. Expression of transcripts was analysed in 2% agarose gels in wild-type and Ets-1 -/- fibroblasts derived from two different animals (k.o. 8 and k.o. 9) without and after induction with bFGF (10 ng/ml) for 20 min and 16 h. The negative control (C) for any PCR is shown at the end of every line. It is evident that *ets-1* transcripts are not induced by bFGF and that basal *ets-1* levels are necessary for early induction of MMP 2, 3 and 13. Fragment sizes were evaluated with a PCR-marker (M); gene specific products are 251-bp for rpL13A, 536-bp for Ets-1, 166-bp for MMP 2, 280-bp for MMP 3, 188-bp for MMP 9, 295-bp for MMP 13 and 525-bp for uPA.

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) TaqDNA polymerase, 0.5 μl cDNA template and sterile RNAse-free water added to a total volume of 25 μl. All PCR reagents were from Invitrogen.

We first amplified a housekeeping gene, encoding the constitutively expressed ribosomal protein, L13A (rpL13A) (56), 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. Every 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. We studied mRNA expression after bFGF stimulation at two time points: 20 min after stimulation, reflecting an early cellular response, and 16 h after stimulation, representing the situation after a long and permanent signal.

Results

We studied mRNA expression after 20 min and 16 h of bFGF stimulation, representing an early and late response of fibroblasts, and assessed Ets-1 specific effects on gene transcription by comparing the mRNA expression of the different genes between unstimulated and bFGF-stimulated fibroblasts derived from either wild-type or Ets-1 -/- mice. The results of the PCR amplifications are shown in Figs. 1 and 2. The mRNA of the housekeeping gene, rpL13A, was amplified in all probes in the same range. We obtained the following findings:

A) Ets-1 expression after bFGF stimulation. According to our previous findings, Ets-1 transcription can be induced in cultured human foreskin fibroblasts through stimulation with 10 ng/ml of bFGF. In contrast, the wild-type mouse fibroblasts used in the present experiments exhibited a basal Ets-1 transcription which was not influenced by bFGF stimulation after either 20 min or 16 h (Fig. 1). In Ets-1 -/- fibroblasts, basal Ets-1 mRNA levels decreased after 20 min of bFGF stimulation. However, an inactive Ets-1 protein is translated from this message because an inactivating cassette has been introduced into the *ets-1* gene (50). Thus, differences in gene expression found between wild-type and Ets-1 -/- cells in the present study must be attributed to basal Ets-1 levels and their blockade.

B) Effects of basal Ets-1 levels on bFGF induced protease expression. In both wild-type and Ets-1 -/- fibroblasts, a basal transcription of MMP 2 and MMP 3 as well as a very weak basal level of uPA mRNA were found without bFGF stimulation. bFGF induced MMP 2 transcripts in wild-type cells after 20 min. Transcripts returned to basal levels after 16 h. In contrast, bFGF stimulation of Ets-1 -/- cells resulted in a slight decrease of MMP 2 expression after 20 min and 16 h.

Strong induction of MMP 3 mRNA was observed after 20 min and 16 h of bFGF stimulation of wild-type fibroblasts. Comparable MMP 3 expression after 16 h of bFGF stimulation

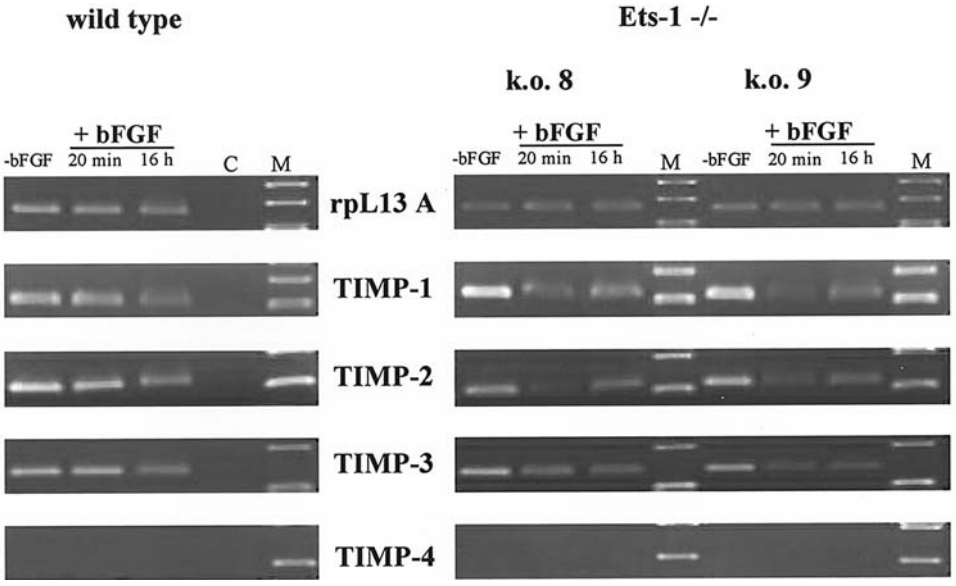

Figure 2. RT-PCR analysis of tissue inhibitors of metallomatrix proteinases (TIMPs 1-4) in wild-type fibroblasts compared to Ets-1 -/- fibroblasts. Expression of TIMPs 1-4 was assessed in wild-type and Ets-1 -/- fibroblasts derived from two different animals (k.o. 8 and k.o. 9) without and after induction with bFGF (10 ng/ml) for 20 min and 16 h as shown for Fig. 1. Basal *ets-1* levels are required for maintenance of TIMP-1 to -3 expression. The negative control (C) for any PCR is shown at the end of every line. Gene specific products are 251-bp for rpL13A, 108-bp for TIMP-1, 122-bp for TIMP-2, 135-bp for TIMP-3 and 130-bp for TIMP-4, evaluated by PCR-marker (M).

was present in fibroblasts from Ets-1 -/- animal 8. An analogous but slight induction after 16 h was demonstrated in animal 9. In both Ets-1 -/- mice, bFGF induced expression of MMP 3 was much weaker than in wild-type fibroblasts. MMP 9 was only slightly induced after 20 min of bFGF stimulation in wild-type cells and after 16 h in Ets-1 -/- fibroblasts. For MMP 13, a strong inducing effect of bFGF was again found after 20 min of stimulation of wild-type cells. Expression had considerably diminished after 16 h. In contrast, bFGF stimulated MMP 13 expression in the Ets-1 -/- cells of animal 8 only after 16 h and only a weak induction after 16 h was observed in animal 9. No clear effects of bFGF on uPA expression were evident in wild-type and Ets-1 -/- cells.

C) Effects of basal Ets-1 levels on TIMP-1 to -4 expression. Both wild-type and Ets-1 -/- cells exhibited basal expression of TIMP-1, -2 and -3 transcripts (Fig. 2). Expression of all three TIMPs was slightly decreased after 16 h of bFGF stimulation in wild-type cells. In contrast, bFGF stimulation of Ets-1 -/- cells diminished TIMP-1, -2 and -3 expression after both 20 min and 16 h. No expression of TIMP 4 was found in either wild-type or Ets-1 -/- cells.

Discussion

Matrix metalloproteinases (MMPs) are a family of zinc metallo-endopeptidases secreted by normal and tumour cells. According to their substrate specificities they can be subdivided into several classes, such as collagenases (MMPs 1, 8, 13 and 18), gelatinases (MMPs 2 and 9) and stromelysins (MMPs 3, 10 and 11) (58). These enzymes are responsible for much of the turnover of extracellular matrix components (57). MMPs are involved in a wide range of proteolytic events under normal and pathological circumstances, such as tumour invasion (57). In invasive tumours, proteases are intensely expressed by the fibroblast of the tumour stroma considered to contribute to invasion (2,27). Expression and activity of MMPs is strictly controlled by transcription, proteolytic activation through uPA and the plasminogen/plasmin system and by inhibition through different tissue inhibitors of metalloproteinases (TIMPs) (reviewed in refs. 2,58,59).

Several lines of evidence point to a major role of Ets-1 for the transcriptional regulation of both proteases and TIMPs. Ets-1 binding sites are present in the promoters of several MMP- and TIMP-encoding genes and co-transfection assays have shown that Ets-1 is able to transactivate MMP 2, 3, 9, uPA and TIMP-1 genes (11,17,40-42,53,60,61). Like MMPs, TIMPs can be produced by stromal fibroblasts of invasive tumours (62-65).

In previous studies, we found that MMPs and uPA are topographically co-expressed with Ets-1 within stromal fibroblasts of different invasive human carcinomas and that bFGF is a growth factor which induces both Ets-1 and these proteases in cultured human fibroblasts (27). In the present study, we tried to show a causal role of Ets-1 for protease expression in mouse fibroblasts after physiological stimulation by bFGF. Surprisingly, we found no Ets-1 induction by bFGF in mouse fibroblasts but basal Ets-1 expression in both wild-type and Ets-1 -/- cells. A functional Ets-1 protein is not translated in Ets-1 -/- fibroblasts because an inactivating cassette has been inserted into the *ets-1* gene of these cells. bFGF induced *mmp* 2, 3, 9 and 13 transcripts with an early effect in wild-type cells. This effect was abolished in Ets-1 -/- fibroblasts. These results demonstrate that basal expression levels of ets-1 mRNA are necessary for early induction of MMP 2, 3, 9 and 13 transcripts in mouse fibroblasts. No clear effect of bFGF stimulation was discernable on expression of uPA in either wild-type or Ets-1 -/- cells.

stimulation only slightly decreased the expression of TIMP 1-3 after 16 h in wild-type cells. In contrast, in Ets-1 -/- cells, bFGF diminished TIMP 1-3 expression at both 20 min and 16 h. This shows that basal Ets-1 levels prevent early bFGF induced decrease in TIMP 1-3 expression in mouse fibroblasts which are negative for TIMP 4. The latter finding was unexpected because it has been reported that, after transient transfection, the TIMP-4 promotor contains functional Ets-1 binding sites in C3H10T1/2 mouse fibroblasts (17). On the other hand, it is also known that TIMP 4 shows a restricted pattern of expression, with the highest mRNA levels found in the brain, heart and testes (17). Thus, the missing TIMP-4 expression in the present study could be due to differences between embryonic mouse fibroblasts and the C3H10T1/2 fibroblast cell line.

In summary, our results show that basal Ets-1 levels are required for early bFGF-induced expression of MMPs and for more or less constant maintenance of TIMP expression, suggesting a net increase in MMP activity after bFGF-stimulation. bFGF-induced rapid decrease of TIMP expression in Ets-1 -/- cells suggests an effect through a transrepressor antagonized by basal Ets-1 activity. This is in line with the current view that an array of transcriptional activators, repressors and co-regulators determine the net activity of transcription in living cells (reviewed in refs. 66,67). It is well established that genes encoding proteases and TIMPs are regulated by many transcription factors, including AP-1, Sp-1 and also different Ets family members. These factors can substitute each other or have synergistic or antagonistic effects (13,31,57,58). Several of them have probably been induced by bFGF in the present experiment. However, a novel conclusion that can be drawn from the present results is that constitutive and basal levels of transcription factor Ets-1 are necessary for effects on gene transcription induced in a physiological manner, even when the transcription factor itself is not induced. By using Ets-1 -/- fibroblasts, we could specifically address the roles of one sole Ets family member and study Ets-1 specific regulation. In contrast to the present experiments, most co-transfection assays do not reproduce physiological conditions because the transcription factors to be tested are expressed at maximal levels. Also, possible side effects caused by transfection or by the chemicals used can be circumvented.

At present, little is known about the transcriptional control of TIMP promoters but Ets binding sites are present in all of them. A transactivating activity of these sites has been proven for TIMP 1 (17,53,54).

Besides their inhibitory effects on MMPs, it is known that TIMPs are also able to form noninhibitory complexes with some proMMPs. TIMP 2 binds tightly to the zymogen of MMP 2 (proMMP 2), forming a complex that is important in cell-surface activation of proMMP 2 (68,69). The effects of bFGF on MMP 2 and TIMP-2 expression seen in the present study could lead to MMP 2 activation. TIMP 1 forms a specific complex with proMMP 9 which could be involved in proMMP 9 activation (70). Thus, the basal level of TIMPs found in our study could be necessary for creating functional MMPs. Furthermore, according to the present results, basal Ets-1 expression is necessary for both early bFGF induction of MMP 2, 3 and 13 as well as for early maintenance of TIMP-1, -2 and -3 expression after bFGF stimulation.

In conclusion, our results suggest an important role of basal Ets-1 levels in rapid transcriptional activation of several matrix-degrading proteases in fibroblasts. The findings are in line with our previous results suggesting that Ets-1 translates tumour-derived signals such as bFGF onto fibroblasts into an increased expression of matrix degrading enzymes necessary for tumour invasion (27). To our knowledge, this is the first time that transcriptional regulation of the MMP 13 gene by Ets-1 has been demonstrated in fibroblasts. At present, Ets-1-induced MMP 13 activity was only demonstrated in osteoblasts (43). Therefore, MMP 13 must be included in the growing list of MMPs induced by Ets-1 in fibroblasts. Further studies might enlighten the role of MMP 13 in angiogenesis and tumour vascularization.

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