Fli1 and Ets1 Have Distinct Roles in Connective Tissue Growth Factor/CCN2 Gene Regulation and Induction of the Profibrotic Gene Program*

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Sashidhar S. Nakerakanti, Bagrat Kapanadze, Masaomi Yamasaki, Margaret Markiewicz, and Maria Trojanowska

From the Division of Rheumatology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29425

CCN2 (connective tissue growth factor), an important regulator of angiogenesis, chondrogenesis, and wound healing, is over-expressed in a majority of fibrotic diseases and in various tumors. This study investigated regulation of CCN2 gene expression by Ets family of transcription factors, focusing on two members, Fli1 and Ets1, with deregulated expression during fibrosis and tumorogenesis. We show that Ets1 and Fli1 have opposite effects on CCN2 gene expression. Ets1 functions as an activator of CCN2 transcription, whereas Fli1 acts as a repressor. A functional Ets binding site was mapped at −114 within the CCN2 promoter. This site not only mediates stimulation by Ets factors, including Ets1, Ets2, and GABPα/β, but is also required for the transforming growth factor (TGF-β) response. The contrasting functions of Ets1 and Fli1 in regulation of the CCN2 gene were confirmed by suppressing their endogenous levels using adenoviral vectors expressing specific small interfering RNAs. Additional experiments using chromatin immunoprecipitation assays have revealed that in fibroblasts both Ets1 and Fli1 occupy the CCN2 promoter. TGF-β stimulation resulted in displacement of Fli1 from the CCN2 promoter and a transient inhibition of Fli1 synthesis. Moreover, reduction of Fli1 expression resulted in up-regulation of COL1A1 and COL1A2 genes and down-regulation of the MMP1 gene. Thus, inhibition of Fli1 recapitulated some of the key effects of TGF-β, suggesting that Fli1 suppression is involved in activation of the profibrotic gene program in fibroblasts. On the other hand, activation of the CCN2 gene downstream of Ets1 is consistent with its role in angiogenesis and extracellular matrix remodeling. This study strongly supports a critical role of Fli1 and Ets1 in the pathological extracellular matrix regulation during fibrosis and cancer.

Ets1 and Fli1 are members of the evolutionarily conserved family of transcription factors characterized by a winged helix-turn-helix DNA binding domain (ETS domain) that recognizes a purine-rich GGA(A/T) core sequence (Ets binding site (EBS)) (1). Ets1 and its close homolog Ets2 are widely expressed in many different tissues and cell types, where they primarily function as regulators of cell migration and invasion (2–4). Overexpression of Ets1 has been frequently observed in pathological conditions characterized by increased angiogenesis and extracellular matrix (ECM) remodeling, including various tumors and rheumatoid arthritis (5). Activation of Ets1 occurs in response to Ras/mitogen-activated protein kinase signaling through phosphorylation on the Thr38 within the N-terminal PNT (pointed) domain (6). Additional studies have shown that phospho-Thr38 augments interaction of Ets1 with the transcription coactivator CREB-binding protein/p300 (7). In contrast, Ca2+-dependent signaling inhibits DNA binding activity of Ets1 through phosphorylation of serine residues within the autoinhibitory module located in the C-terminal domain (8). Ets1 is also modulated by acetylation in response to TGF-β signaling (9). Interestingly, increased Ets1 acetylation correlated with the dissociation of Ets1 from p300 (9). In addition, recent studies have also demonstrated that Ets1 is modified by sumoylation that involves Lys15 within the N-terminal segment of Ets1 (10). In contrast to Ets1 phosphorylation, the functional significance of other post-translational modifications are not known.

Fli1 is preferentially expressed in hematopoietic cell lineages. It plays an important role in megakaryocytic differentiation, and it has also been implicated in myelomonocytic, erythroid, and NK cell development (11, 12). Fli1 is highly expressed in vascular endothelial cells, but its target genes and its role in the vasculature have not been fully characterized (13). Furthermore, recent studies have shown that Fli1 is expressed in dermal fibroblasts, where it may play a role in ECM gene regulation (14, 15). However, in contrast to hematopoietic cells, in which the role of Fli1 is well established, relatively little is known about Fli1 function in other cell types.

CCN2 (connective tissue growth factor) is a member of the CCN family of the multifunctional matricellular factors, which regulate angiogenesis, chondrogenesis, and wound healing (16, 17). CCN2 is expressed at low levels in the adult tissues, and its overexpression is linked to several pathological disorders, such as organ fibrosis and tumorogenesis (16, 17). Early studies by Grotendorst (18) have shown that CCN2 is induced by TGF-β

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1To whom correspondence should be addressed: Division of Rheumatology and Immunology, Medical University of South Carolina, 96 Jonathan Lucas St., Charleston, SC 29425. Tel.: 843-792-7921; Fax: 843-792-7121; E-mail: trojanme@musc.edu.

2The abbreviations used are: EBS, Ets binding site; ECM, extracellular matrix; TGF, transforming growth factor; CREB, cAMP-response element-binding protein; ERK, extracellular signal-regulated kinase; HDMVEC, human dermal microvascular endothelial cell; ChIP, chromatin immunoprecipitation; MOI, multiplicity of infection.
and that it contributes to the regulation of the profibrotic gene program in fibroblasts. CCN2 is also up-regulated by other fibrogenic cytokines, including angiotensin II, endothelin 1, and thrombin, further supporting its role in organ fibrosis (19–21). There is increasing evidence that CCN2 plays a positive role in tumorigenesis, where it may contribute to formation of reactive stroma by promoting excessive matrix remodeling and tumor angiogenesis (22). Proangiogenic factors, such as hypoxia, vascular endothelial growth factor, and S1P, are among the known inducers of CCN2 (23–25). Recent studies have begun to characterize signaling pathways involved in regulation of the CCN2 gene in response to various stimuli. It was shown that stimulation of CCN2 gene expression by TGF-β required activation of both Smad and ERK signaling pathways (26). Additional pathways implicated in CCN2 gene regulation include small GTPase Rho and protein kinase C (27, 28). Moreover, the regulation of CCN2 occurs primarily at the level of transcription, with the exception of TGF-β/Smad3 signaling, little is known about the nature of the transcription factors mediating regulation of the CCN2 gene in response to various stimuli.

Fli1 and Ets1 have been previously associated with regulation of ECM genes, including collagen type I and tenasin-C (14, 15). Importantly, reduction of Fli1 expression correlated with elevated collagen synthesis in patients with scleroderma, a systemic fibrotic disease, suggesting that absence of Fli1 may directly contribute to the process of fibrosis (29). On the other hand, elevated expression of Ets1 is often present in stromal fibroblasts and endothelial cells in various tumors (5). Given the fact that CCN2 overexpression frequently occurs under pathologic conditions that are also characterized by the aberrant expression of Fli1 and Ets1, this study was designed to determine whether Ets1 and Fli1 are involved in regulation of the CCN2 gene in fibroblasts and endothelial cells. We found that Ets1 and Fli1 play contrasting roles in CCN2 gene regulation, with Ets1 being a positive regulator and Fli1 a negative regulator of this gene.

**EXPERIMENTAL PROCEDURES**

**Cells**—Human fibroblast cultures were established from the foreskins of newborns. Briefly, the tissue was minced and digested overnight at 37 °C with collagenase (0.25%) and DNase I (0.05%) in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum and then plated. After establishing the culture, regular maintenance and propagation was carried out in Dulbecco’s modified Eagle’s medium supplemented with 10% serum. All experiments were carried out in early passage cells.

Human dermal microvascular endothelial cells (HDMVECs) were isolated from human foreskins using the protocol of Richard et al. (30). Briefly, primary cultures of human foreskins were established after the removal of epidermis. Such cultures consist of a mixture of HDMVECs, dermal fibroblasts, and some keratinocytes. Subconfluent cultures were treated with tumor necrosis factor-α for 6 h to selectively induce the expression of E-selectin in HDMVECs. HDMVECs were then purified using magnetic beads coupled to an anti-E-selectin monoclonal antibody. First passage cultures usually consist of >99% HDM-VECs. A second immunomagnetic purification step ensures homogenous population of HDMVECs suitable for long-term culturing. Purity of the HDMVEC cultures was evaluated using anti-CD31 and anti-von Willebrand factor antibodies.

**Plasmids, Transient Transfections, and Luciferase Assay**—Connective tissue growth factor promoter cloned upstream of the luciferase reporter gene in pGL2 vector (CCN2-Luc) was a gift from Dr. Gary Grotendorst (University of Miami). Site-specific mutations of the connective tissue growth factor promoter were generated using the Exsite site-directed mutagenesis kit (Stratagene) using the primers given in Table 1. pSG5Ets1, pSG5Fli1, pSG5Ets2, pSG5Ets2 dominant negative mutant, and pSG5GABPα and pSG5GABPβ expression plasmids were described earlier (15).

Transient transfections were performed in foreskin fibroblasts seeded into 12-well plates using Fugene6 (Roche Applied Science) according to the manufacturer’s instructions. After overnight incubation, some cells were treated with 2.5 ng/ml TGF-β and then further incubated for 24 h. The cells were harvested and assayed for luciferase reporter activity using the Promega luciferase assay kit according to the manufacturer’s instructions. Co-transfection with pSV-β-galactosidase control vector was used to normalize for transfection efficiency. β-Galactosidase was measured using Galacto-Light-Plus (Tropix).

**Adenoviral siRNA Vectors**—The following siRNA target sequences were used: for Ets1, CAGACACCTTGCAAGATTGA; for Fli1, GTTCACTGCTGCACTTATAAA. Annealed double-stranded oligonucleotides were cloned into pMUL and then electroporated into BJ5183 AD-1 (Stratagene) to generate recombinant Adenoeasy vector. The recombinant Adenoeasy plasmid after linearization with Pmel and then electroporated into B5183-AD-1 (Stratagene) to generate recombinant Adenoeasy vector. The recombinant Adenoeasy plasmid after linearization with Pmel enzyme was transfected into QBI-293 cells using Transfectin (Bio-Rad) for generation of adenovirus. The shuttle vector plasmid and Adenoeasy vector plasmid were sequenced to confirm the cloning. The primary adenoviral stock was then amplified and concentrated by cesium chloride density gradient centrifugation. Typical viral titer was 1 × 10¹¹ plaque-forming units/ml. Control adenovirus vector was prepared in the same manner.

In addition, an oligonucleotide targeted to 5′-GGAG GCCGGCATCACATAC-3′ sequence of the Fli1 gene was also cloned into pRNAV H1.1, as described above, to validate the effect of Fli1 suppression on the CCN2 gene. The plasmid was electroporated into foreskin fibroblasts by Amaxa kit as per the manufacturer’s instructions. To validate the effect of Ets1 suppression on the CCN2 gene, the following protocol was used: a
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### TABLE 2
Primers for quantitative PCR

| Gene         | Primers (5′–3′)                                      |
|--------------|-----------------------------------------------------|
| CCN2         | TTCGGAACGTGCACCCGGAAGAA (forward)                   |
| Fli1         | GATGGTGAACCATCTGATTGGA (reverse)                    |
| Ets1         | GGTTGATGAACCTAGAAAG (reverse)                       |
| β-Actin      | ATTCTCTGGAAGACCTTTGGTA (forward)                    |
| Collagen I A(1) | CCAAGAAGACTTGCTACTGGA (forward)                    |
| Collagen I A(2) | GAATGTCAGCTTGGCGTGAA (forward)                   |
| MMP1*       | TCGGGGTCTGTGCTCTTA (forward)                       |

* Obtained from PRIMER BAKK (available on the World Wide Web at pga.mgh.harvar.edu/primerbank/); identification number 4105215A1.

330-bp region (positions 1830–2160) specific for the Ets1 (NM_005238.2) gene cloned into the pLitsu vector (New England Biolabs) was provided by Dr. Tien Hsu (Medical University of South Carolina). The Ets1-specific region was transcribed in vitro using T7 Ribopax Express RNA interference system (Promega) as per the manufacturer’s instructions. The RNA was then digested with dicer enzyme (New England Biolabs) to generate a pool of siRNAs. siRNAs were transfected into foreskin fibroblasts using RNAiFect (Qiagen) according to the manufacturer’s protocol.

**RNA Isolation, Quantitative Reverse Transcription—PCR**

Total RNA was isolated from the fibroblasts using Trizol reagent (MRC Inc.) according to the manufacturer’s instructions. 2 μg of RNA was reverse transcribed in a 20-μl reaction volume using random primers and the Transcriptor first strand synthesis kit (Roche Applied Science) and then diluted to 40 μl. Real time quantitative PCR was carried out using IQ Sybr green mix (Bio-Rad) on an iCycler machine (Bio-Rad) using 1 μl of the cDNA in triplicates with β-actin as the internal control. The primers used for the various genes are listed in Table 2 and have been validated to β-actin. The -fold change in the levels of genes of interest was determined by 2−ΔΔCt. PCR conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, and 58 °C for 1 min. To check for the absence of secondary products, a melt curve analysis for the PCR product was carried out.

**Immunoprecipitation and Western Blotting**

Cell lysates were prepared after appropriate treatment in radiimmune precipitation buffer. For immunoprecipitation of Ets1, 2 μg of Ets1 monoclonal antibody (NCL) was added to 300 μg of pre-cleared cell lysate, and complex formation was carried out at 4 °C overnight. The protein-antibody complexes were recovered using protein G-Sepharose beads for 2 h at 4 °C. The immunoprecipitates were washed four times in radiimmune precipitation buffer, eluted by boiling for 5 min in 2X SDS sample buffer, and analyzed by Western blot with anti-Ets1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For straight Western blotting, 15–25 μg of protein was size-fractionated on SDS-PAGE and then transferred on nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk in Tween/Tris-buffered saline. The membranes were then probed with anti-Fli1 antibody (BD Biosciences), anti-collagen antibody (Southern Biotechnologies), or anti-MMP1 antibody (Chemicon) overnight at 4 °C. For CCN2 Western blot, the membranes were blocked in 2% gelatin and probed with anti-CCN2 antibody (Santa Cruz Biotechnology) overnight at room temperature. The blots were then incubated with appropriate horseradish peroxide-coupled secondary antibodies and developed using the Chemiluminescent kit (Pierce). The blots were stripped and reprobed for β-actin (Sigma).

**Chromatin Immunoprecipitation (ChiP)**—ChiP was carried out according to the protocol of Boyd and Farnham (31) with modifications. Cells either untreated or treated with TGF-β for 4 h were cross-linked by adding formaldehyde directly to cell culture medium to a final concentration of 1% and incubating for 10 min at 37 °C. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. The cells were washed with cold PBS containing protease inhibitors and then scraped. Cells were recovered by centrifugation and were suspended in cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40) containing protease inhibitors and incubated on ice for 20 min. Nuclei were collected by centrifugation at 5,000 rpm, resuspended in sonicating buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing protease inhibitor mixture, and incubated on ice for 10 min. Chromatin was sheared by sonicating to yield an average fragment size of 500 bp and then microcentrifuged at 14,000 rpm, and supernatant containing soluble chromatin was collected. The chromatin was either stored at −70 °C or processed for immunoprecipitation. The chromatin solution was precleared with protein G-Sepharose (blocked with sonicated salmon sperm DNA and 5% bovine serum albumin) for 30 min at 4 °C. Precleared chromatin from 2 × 107 cells was incubated with 10 μg of Ets1 (sc-350X; Santa Cruz Biotechnology) or Fli1 antibody (developed at the Medical University of South Carolina antibody facility) and rotated at 4 °C overnight. Recovery of immunoprecipitated chromatin was done using protein G-Sepharose beads for 2 h at 4 °C under rotation. The chromatin-antibody-protein G-Sepharose complexes were recovered by centrifugation. The beads were washed for 5 min at room temperature sequentially with the following buffers: low salt wash buffer (20 mM Tris-HCl, pH 8.1, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 0.1% SDS), high salt wash buffer (20 mM Tris-HCl, pH 8.1, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 0.1% SDS), lithium chloride wash buffer (10 mM Tris-HCl, pH 8.1, 0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA), buffer D (buffer C with 500 mM LiCl). The beads were then washed twice in TE buffer (10 mM Tris-HCl, pH 8.1, 0.1 mM EDTA). Immunocomplexes were eluted off of the beads in two rounds of 100 μl of 1% SDS, 0.1 M NaHCO3, and the cross-links were reversed by incubation for 4 h at 65 °C after the addition of NaCl to a final concentration of 0.2 M. Proteins were digested with proteinase K (40 μg/ml) for 1 h at 50 °C. DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Of the total yield of 30 μl of DNA, 2 μl was used in each PCR. Relative -fold enrichment of the DNA was determined either by regular PCR followed by agarose gel electrophoresis or by quantitative PCR. A 213-base pair region (−194 to +19) of the CCN2 promoter encompassing the putative EBSs was amplified using the following primers (5′–3′): forward, gaaggtgcccagcttttct; reverse, ggaagattggttgtggtgag. For regular PCR, 2 μl of enriched DNA and 1 μl of input DNA was used as the template in a reaction volume of 25 μl.
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RESULTS

Ets Factors Modulate CCN2 Gene Expression—To investigate the potential role of Ets factors in CCN2 gene regulation, we utilized the CCN2 promoter linked to the luciferase reporter gene (CCN2-Luc) (33). Human foreskin fibroblasts were co-transfected with CCN2-Luc and a dominant negative mutant of Ets2 (DNM-Ets2). This mutant consists of a DNA binding domain of Ets2 but lacks the transactivation domain and is effective in blocking interactions of Ets factors with the Ets binding sites (34). DNM-Ets2 inhibited basal promoter activity by 50% and completely abolished TGF-β stimulation of the CCN2 promoter, thus linking Ets factors to regulation of the CCN2 gene (Fig. 1, A and B). To begin examination of the involvement of specific Ets factors, CCN2-Luc was co-transfected with Ets1, Ets2, GABPa/β, or Fli1. Each of these factors had differential effect on the basal and TGF-β-stimulated promoter activity. Ets1 or GABPa/β and, to a lesser extent, Ets2 showed stimulation of the CCN2 promoter, whereas Fli1 inhibited basal promoter activity by ~30% (Fig. 1A). Furthermore, Ets1, Ets2, and GABPa/β cooperated with TGF-β stimulation of the CCN2 promoter, whereas Fli1 partially inhibited TGF-β stimulation (Fig. 1B). This initial experiment has suggested that various Ets family members may play distinct roles in CCN2 gene regulation in human fibroblasts.

Mapping of the EBSs in the CCN2 Promoter—Previous studies have characterized several response elements in the proximal region of the CCN2 promoter, including TβRE, Smad3, and Sp1 binding sites (35). Inspection of the −200 to +13 promoter region revealed the presence of the four putative Ets binding sites, which were termed EBS-1 to -4 (Fig. 2A). These GGAA motifs were mutated individually, and their contribution to the basal CCN2 promoter activity was determined. Mutation of EBS-2 had a dramatic effect on the basal promoter activity (90% reduction), whereas mutations in either EBS-1 or EBS-3 had a much smaller effect on the basal activity (Fig. 2B). This result suggests that EBS-2 is a key element in basal CCN2 gene regulation. Mutation of EBS-1 had a small effect on the basal promoter activity (Fig. 2B), which is consistent with the observation that ETS1, a member of the Ets family, is activated in fibroblasts by the Smad3 signaling pathway (36). In contrast, GABPA/β, which is expressed in fibroblasts, is a critical mediator of TGF-β signaling (37). These results indicate that Ets factors modulate both basal and TGF-β-induced CCN2 gene expression, and suggest that these factors play distinct roles in CCN2 gene regulation.

Statistical Analysis—Student’s t test analysis using GraphPad InStat statistics software (version 1.12) was performed to determine statistical significance. Values less than or equal to 0.05 were considered statistically significant.

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EBS-3 decreased promoter activity by ~50%. EBS-4 mutation did not consistently affect the basal promoter activity (Fig. 2B). These data further confirm that Ets factors contribute to regulation of the CCN2 gene.

_Ets Factors Stimulate the CCN2 Promoter via EBS-3—_We next determined which of the EBSs mediated TGF-β and Ets1 stimulation of the CCN2 promoter. We also assessed whether mutations of EBSs affected cooperation of Ets1 with the TGF-β signaling pathway. As shown in Fig. 3A, mutating EBS-3 had the most pronounced effect on the promoter activation. Stimulatory effects of either TGF-β or Ets1 as well as cooperative stimulatory effect of TGF-β and Ets1 were completely abrogated by this mutation. Mutation in EBS-4, a site adjacent to the Smad binding site, also completely inhibited TGF-β stimulation but had no effect on Ets1 stimulation of the CCN2 promoter. However, cooperative effect of Ets1 and TGF-β was greatly diminished by this mutation. Mutations in EBS-1 and in EBS-2 did not consistently affect responses to Ets1 and TGF-β. We next examined whether EBS-3 and EBS-4 also contributed to the Ets2 stimulation of the CCN2 promoter. Similar to Ets1, Ets2 also required an intact EBS-3 for the promoter stimulation; however, unlike Ets1, mutation in EBS-4 did not affect Ets2 and TGF-β cooperation (Fig. 3B). Whereas GABPα/β also required EBS-3 for stimulation, in contrast to Ets1 and Ets2, GABPα/β stimulation and cooperation with TGF-β were abrogated by EBS-4 mutation (Fig. 3C). These data suggest that EBS-3 mediates stimulatory effects of Ets1, Ets2, and GABPα/β on the CCN2 promoter. In addition, TGF-β stimulation requires both EBS-3 and EBS-4.

The effect of Fli1 on the activity of the promoter carrying mutated EBSs was next examined. Mutation of EBS-3 augmented the inhibitory effect of Fli1 on the CCN2 promoter (Fig. 3D); however, Fli1 turned into an activator when EBS-2 or EBS-4 was mutated. These data suggest that Fli1 may function as a weak activator of the CCN2 promoter, which exerts its inhibitory effects by competing with other more potent activator(s) (e.g. GABPα/β).

### Opposite Effects of Ets1 and Fli1 on the Expression Levels of the Endogenous CCN2 Gene in Dermal Fibroblasts—

The above promoter analyses have suggested that Ets1 and Fli1 may play distinct roles in CCN2 gene regulation. To study the role of Ets1 and Fli1 in regulation of the endogenous CCN2 gene, we employed an adenovirus-mediated RNA interference technique to knock down Ets1 or Fli1. Initial experiments have established an optimal dose and time of treatment with AdEts1 siRNA to achieve maximal inhibition of endogenous Ets1 mRNA level (data not shown). Treatment of fibroblasts with 100 MOI of AdEts1 siRNA for 72 h resulted in reduction of Ets1 mRNA and protein expression levels in foreskin fibroblasts by ~75% (Fig. 4A). This condition led to a ~50% decrease in the

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**EBS mutations were co-transfected with either empty vector or a specific Ets expression vector, where indicated, cells were stimulated with TGF-β. Transfections were normalized using pSV-β-galactosidase control vector. Luciferase activity of each promoter construct transfected with empty vector was arbitrarily set at 1. The graph represents fold change in promoter activity in response to specific Ets factor or/and TGF-β treatments. The values depicted are means ± S.E. of four independent experiments performed in duplicate. *, significant values at p < 0.05.**
basal expression levels of CCN2 mRNA and protein (Fig. 4A).

After a 72-h incubation with AdEts1 siRNA, cells were treated with TGF-β for an additional 24 h. Ets1 and CCN2 mRNA levels were determined using quantitative reverse transcription-PCR. Left, the graph represents fold change in Ets1 mRNA levels in response to Ets1-specific siRNA treatment in comparison with Ets1 mRNA levels in cells treated with control virus, which were arbitrarily set at 1. Right, Ets1 protein levels were determined by IP-Western blot as described under “Experimental Procedures.” B, basal expression levels of CCN2 mRNA (left) and protein (right) in cells treated with control and Ets1 siRNA adenoviruses. CCN2 protein levels were determined by Western blot. C, TGF-β-induced CCN2 mRNA (left) and protein (right) levels in cells treated with control and Ets1 siRNA adenoviruses. The values depicted are means ± S.E. of three independent experiments performed in duplicate. *, significant values at p < 0.05.

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expression levels. Fli1 mRNA and protein levels were decreased by ~70% by siRNA treatment (Fig. 5A). This resulted in an increase in CCN2 mRNA and protein levels (Fig. 5B). TGF-β treatment decreased endogenous Fli1 mRNA levels by 40% in cells transduced with control adenovirus. Combination of AdFli1 siRNA and TGF-β treatment further reduced endogenous Fli1 mRNA levels to about 20% of the level present in untreated cells (Fig. 5A). Under these conditions, TGF-β stimulation of CCN2 mRNA increased about 2-fold, whereas TGF-β stimulation of CCN2 protein was not consistently affected (Fig. 5C). A more pronounced effect of Fli1 inhibition on CCN2 gene expression at the basal level is consistent with the down-regulation of Fli1 by TGF-β treatment. These observations were confirmed with an adenoviral vector carrying a different Fli1 siRNA sequence (not shown).
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Reduction of Fli1 gene expression by TGF-β suggested that the presence of Fli1 might interfere with TGF-β/Smad signaling. To test this possibility, we used previously generated adenoviral vector carrying Fli1 (AdFli1) (29). Cells were transduced with 10 MOI of AdFli1, which resulted in 8–10-fold induction of the Fli1 mRNA level above the endogenous levels. Under these conditions, basal expression levels of CCN2 were almost completely inhibited, and TGF-β stimulation was markedly reduced (Fig. 6, A and B). Together, these experiments indicate that in dermal fibroblasts endogenous Fli1 functions as a repressor of the CCN2 gene. Thus, Ets1 and Fli1 play opposite roles in regulation of the CCN2 gene in human fibroblasts.

Regulation of the CCN2 Gene by Ets1 and Fli1 Depends on the Cellular Context—Both Ets1 and Fli1 are expressed by endothelial cells in vivo. Therefore, it was important to determine whether they are involved in CCN2 gene regulation in these cells. Endogenous Ets1 or Fli1 was inhibited in HDMVECs using appropriate adenoviral vectors. Inhibition of Ets1 expression levels by 75% did not affect basal mRNA levels of the CCN2 gene, whereas similar inhibition of the endogenous Fli1 gene resulted in a 3-fold increase of CCN2 mRNA (Fig. 7, A and B). Similar to fibroblasts, TGF-β (2.5 ng/ml) potently stimulated CCN2 mRNA levels. Reduction of either Ets1 or Fli1 mRNA levels greatly diminished this response (Fig. 7, A and B). Thus, Fli1 functions as a repressor of CCN2 gene both in dermal fibroblasts and in HDMVECs; however, unlike fibroblasts, Ets1 does not seem to be involved in the basal expression of the CCN2 gene in HDMVECs. However, Ets1 is required for TGF-β stimulation of the CCN2 gene in HDMVECs. In contrast to fibroblasts, Fli1 also contributes to TGF-β stimulation in HDMVECs. These data suggest that the functions of Ets1 and Fli1 are dependent on cellular context.

TGF-β Affects Promoter Occupancy of Fli1 but Not Ets1—So far we have demonstrated that both Ets1 and Fli1 contribute to the basal expression of the CCN2 gene. To determine whether Ets1 and Fli1 directly bind to the endogenous CCN2 promoter, we performed ChIP assays. After immunoprecipitations with either Ets1 or Fli1 antibody, enrichment of endogenous CCN2 promoter was analyzed by PCR. In untreated human fibroblasts, both Ets1 and Fli1 occupied the −194 to +13 region of the CCN2 promoter (Fig. 8A). 4-h TGF-β treatment did not affect Ets1 promoter occupancy, whereas the presence of Fli1 was no longer detectable (Fig. 8A). We also employed quantitative PCR to measure the -fold enrichment of the CCN2 promoter after immunoprecipitation with specific antibodies (Fig. 8B). A 2.8-fold enrichment with Ets1 antibody and 2.1-fold enrichment with the Fli1 antibody of the CCN2 promoter was achieved as compared with the no antibody control (set at 1.0). However, after TGF-β treatment for 4 h, only Ets1 antibody enriched the CCN2 promoter (2.1-fold), whereas Fli1 antibody did not, as compared with the control. This confirmed the results obtained with regular PCR. These data suggest that Fli1 is displaced from the CCN2 promoter by TGF-β treatment, whereas Ets1 promoter occupancy is not affected by TGF-β.

Ets1 and Fli1 Have Overlapping and Distinct Roles in Regulation of TGF-β-dependent Genes—Ets1 is known to activate MMP1 gene transcription in various cell types through a composite Ets1-AP1 binding site (36). Since TGF-β inhibits MMP1 gene expression, we wished to determine whether Fli1 is also
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involved in MMP1 gene regulation in dermal fibroblasts. Ets1 and Fi1 were inhibited using respective adenoviral siRNAs. As expected, inhibition of Ets1 resulted in a marked reduction of basal MMP1 mRNA and protein levels (80% reduction) (Fig. 9A). Similar reduction of MMP1 expression was observed in response to TGF-β treatment. Interestingly, the inhibitory effect of TGF-β was partially reversed when Ets1 levels were reduced, suggesting that Ets1 might be required for TGF-β-mediated suppression of MMP1. Inhibition of Fi1 resulted in an over 60% reduction of MMP1 mRNA and protein levels, suggesting that similarly to Ets1, Fi1 is a positive regulator of the MMP1 gene in dermal fibroblasts. In addition, the inhibitory effect of TGF-β was enhanced in cells with reduced levels of Fi1. These results suggest a novel role for Fi1 in regulating MMP1 gene expression.

Previous studies using embryonic fibroblasts from Fi1−/− mice have demonstrated inverse relationship between Fi1 and collagen type I expression levels (29). To confirm these observations in human dermal fibroblasts, COL1A1 and COL1A2 mRNA levels were examined in cells treated with AdFi1 siRNA. As shown in Fig. 10, both COL1A1 and COL1A2 mRNAs were significantly increased (8- and 6-fold, respectively). Collagen protein levels were also significantly increased, thus confirming that Fi1 is a repressor of type I collagen. In comparison, TGF-β stimulation resulted in a 2-fold increase of the collagen mRNAs, and there was no further effect of Fi1 inhibition on this response. On the other hand, reduction of Ets1 levels resulted in inconsistent effects on collagen gene expression (data not shown).

The above results have suggested that Ets1 and Fi1 may also affect expression of other TGF-β-regulated genes. We therefore tested the effects of Fi1 and Ets1 siRNA treatment on the mRNA expression levels of PAI1, fibronectin, α-SMA, SMAD7, and thrombospondin (Table 3). Interestingly, inhibition of either Ets1 or Fi1 siRNAs had a similar effect on expression of PAI1 and fibronectin, with a marked increase at the basal level and synergistic increases in response to TGF-β treatment. Expression of α-SMA was also markedly increased at the basal level; however, TGF-β stimulation of α-SMA was not affected. In contrast, Smad7, which is potently stimulated by TGF-β, was only modestly up-regulated at the basal level by inhibition of Fi1 and was not affected by inhibition of Ets1. Suppression of either Ets1 or Fi1 levels did not affect the basal level expression of thrombospondin, whereas TGF-β-stimulated expression was inhibited when Fi1 was inhibited and suppression of Ets1 had no appreciable effect. Together, these data suggest that Fi1 and Ets1 have both overlapping and distinct functions in regulating matrix-related genes.

DISCUSSION

Ets factors constitute a relatively large gene family with 27 members encoded by the human genome (37). The identification of the physiologic target genes for the specific Ets factor is challenging, since with few exceptions Ets genes are ubiquitously expressed (38). Furthermore, because of the presence of the conserved DNA binding domain, multiple Ets factors recognize the same consensus sequence containing the 5’-GGA(A/T)-3’ core motif (38). Interestingly, however, some of the recent studies imply that such DNA binding redundancy of distinct Ets factors may serve as a molecular switch to turn on and off a subset of target genes under different physiologic conditions (39). Furthermore, it was postulated that such “Ets conversion” programs might represent a common theme of the regulatory mechanisms involving Ets factors (2). The results of the current
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Whereas this study has mainly focused on Ets1 and Fli1, our results suggest that other Ets family members are involved in CCN2 gene regulation. Specifically, the mutation in EBS-2 that resulted in a significant inhibition of the basal activity of the CCN2 promoter suggests the existence of another positive regulator of this gene. Furthermore, our data suggest that GABPα/β contributes to TGF-β stimulation of the CCN2 promoter through EBS-4. GABPα and -β have been previously shown to interact with Smad3/Smad4 in B lymphocytes and to mediate TGF-β stimulation of the murine mammary tumor virus long terminal repeat in these cells (41). Recent studies have suggested that TGF-β stimulation of the CCN2 gene requires both Smad3 and ERK1/2 signaling pathways (26). Since Ets factors are the effectors of the mitogen-activated protein kinase pathway, it is plausible that GABPα or another putative Ets factor interacting with EBS-4 is targeted by ERK1/2. However, it is unlikely that it is Ets1, since we have previously shown that Ets1 phosphorylation is not modulated by TGF-β in dermal fibroblasts (9). Furthermore, it is known that Ets1 phosphorylation requires a prolonged ERK1/2 activation, whereas in dermal fibroblasts TGF-β causes only a transient activation of ERK1/2. We have recently shown that in dermal fibroblasts Ets1 phosphorylation on Thr384 occurs in response to tumor necrosis factor-α in an ERK1/2-sphingosine kinase-dependent manner (42). The additional Ets family members that may contribute to CCN2 gene regulation remain to be characterized.

Ets1 has been implicated in regulation of a number of genes in various cell types; however, most of these studies were based on promoter analyses (43). Furthermore, because of the limited life span of primary cells, which renders genetic manipulations impractical, the majority of the previous studies utilized immortalized or transformed cell lines. To overcome these problems and to efficiently block endogenous expression of Ets1 and Fli1, we developed adenoviral vectors carrying specific siRNA sequences. Blockade of Ets1 expression resulted in a significant decrease of basal and TGF-β-stimulated levels of CCN2 mRNA in dermal fibroblasts, thus verifying the contribution of Ets1 to CCN2 gene activation. In HDMVECs the basal expression levels of CCN2 mRNA were not influenced by Ets1 reduction; however, TGF-β stimulation of Ets1 was almost completely abrogated in the absence of Ets1, suggesting that in these cells Ets1 functions primarily as a component of the TGF-β signaling pathway. In general, these studies confirmed that Ets1 functions as an activator of the CCN2 gene with distinct effects in different cellular contexts.

In contrast to Ets1, blockade of the endogenous Fli1 gene resulted in increased basal and TGF-β-stimulated CCN2 mRNA levels in dermal fibroblasts. An even more pronounced up-regulation of basal CCN2 levels was observed in HDM-
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VECs. These results verified the role of Fli1 as a repressor of the endogenous CCN2 gene. Interestingly, however, blockade of Fli1 resulted in a reduced TGF-β stimulation of CCN2 mRNA in HDMVECs. We have also observed that Fli1 had a stimulatory effect on the promoter carrying substitution mutations in some of the EB5s, suggesting that Fli1 may play a dual role in regulation of the CCN2 gene, depending on the presence of other Ets factors interacting with the CCN2 promoter. These results further underscore the importance of cellular context in modulating function of Ets1 and Fli1.

This study illustrates that Ets1 and Fli1 have both distinct and overlapping functions in regulation of ECM-related genes and that the aberrant expression of these factors may directly contribute to pathological ECM remodeling. In particular, down-regulation of Fli1 is sufficient to mimic some of the key effects of TGF-β in dermal fibroblasts and in other Ets factors interacting with the CCN2 promoter. These results further underscore the importance of cellular context in modulating function of Ets1 and Fli1.

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