

Matrix Pathobiology

# Smad-Independent Transforming Growth Factor- $\beta$ Regulation of Early Growth Response-1 and Sustained Expression in Fibrosis

## Implications for Scleroderma

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**Transforming growth factor- $\beta$  (TGF- $\beta$ ) plays a key role in scleroderma pathogenesis. The transcription factor early growth response-1 (Egr-1) mediates the stimulation of collagen transcription elicited by TGF- $\beta$  and is necessary for the development of pulmonary fibrosis in mice. Here, we report that TGF- $\beta$  causes a time- and dose-dependent increase in Egr-1 protein and mRNA levels and enhanced transcription of the Egr-1 gene via serum response elements in normal fibroblasts. The ability of TGF- $\beta$  to stimulate Egr-1 was preserved in Smad3-null mice and in explanted Smad3-null fibroblasts. The response was blocked by a specific mitogen-activated protein kinase kinase 1 (MEK1) inhibitor but not by an ALK5 kinase inhibitor. Furthermore, MEK1 was phosphorylated by TGF- $\beta$ , which was sufficient to drive Egr-1 transactivation. Stimulation by TGF- $\beta$  enhanced the transcriptional activity of Elk-1 via the MEK-extracellular signal-regulated kinase 1/2 pathway. Bleomycin-induced scleroderma in the mouse was accompanied by increased Egr-1 accumulation in lesional fibroblasts. Furthermore, biopsies of lesional skin and lung from patients with scleroderma showed increased Egr-1 levels, which were highest in early diffuse disease. Moreover, both Egr-1 mRNA and protein**

**were elevated in explanted scleroderma skin fibroblasts *in vitro*. Together, these findings define a Smad-independent TGF- $\beta$  signal transduction mechanism that underlies the stimulation of Egr-1, demonstrate for the first time sustained Egr-1 up-regulation in fibrotic lesions and suggests that Egr-1 has a role in the induction and progression of fibrosis. (Am J Pathol 2008, 173:1085–1099; DOI: 10.2353/ajpath.2008.080382)**

Scleroderma or systemic sclerosis (SSc), a potentially fatal disease of unknown cause, is characterized by fibrosis with collagenous scar tissue formation in the skin and lungs.<sup>1</sup> The pathogenesis of fibrosis remains incompletely understood, and effective treatments are lacking.<sup>2</sup> As the primary collagen-producing cells activated by paracrine and autocrine transforming growth factor- $\beta$  (TGF- $\beta$ ), fibroblasts are the key cellular effectors of fibrosis.<sup>3</sup> Fibroblast responses elicited by TGF- $\beta$  involve both Smad-dependent and -independent signal transduction. In the Smad pathway, activated ALK5 phosphorylates Smad2/3, inducing interaction with Smad4, translocation into the nucleus, and binding to consensus Smad-binding DNA elements of target genes, such as COL1A2.<sup>4</sup> In addition, TGF- $\beta$  also activates non-Smad pathways in a cell- and context-dependent manner.<sup>5</sup>

The early growth response-1 (Egr-1) transcription factor was originally identified as an early-immediate gene product induced by environmental stress.<sup>6</sup> In addition to Egr-1, the early response gene family also includes Egr-2, Egr-3, and Egr-4, along with their endogenous

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inhibitors NAB1 and NAB2. The Egr-1 gene codes for an 82-kDa zinc finger protein that binds to GC-rich regulatory DNA elements found in the promoter region of many genes. Although negligible in normal cells, Egr-1 expression is rapidly and transiently induced during acute injury by growth factors, cytokines, oxidant stress, hormones, neurotransmitters, UV light, and mechanical forces.<sup>7,8</sup> As a transcriptional master regulator that integrates converging signaling pathways, Egr-1 has a central role in orchestrating adaptive cellular response to injury.<sup>9</sup>

Because of its significance in cardiovascular pathobiology, the regulation of Egr-1 in the vascular system has been investigated extensively. In vascular smooth muscle cells, TGF- $\beta$  was shown to induce Egr-1 via the extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) pathway.<sup>10</sup> In contrast, the regulation of Egr-1 expression in the context of fibrogenesis has not been characterized to date, despite an emerging recognition of the importance of Egr-1 in the pathogenesis of fibrosis. We had previously demonstrated that TGF- $\beta$  stimulation of normal fibroblasts resulted in enhanced Egr-1 DNA-binding activity specific for a GC-rich sequence within the promoter region of COL1A2.<sup>11</sup> Furthermore, Egr-1 was shown to be both necessary and sufficient to stimulate type I collagen production *in vitro*, suggesting a fundamental role for Egr-1 in mediating TGF- $\beta$ -dependent profibrotic responses. In accordance with these observations, Egr-1-null mice were shown to be protected from pulmonary fibrosis induced by TGF- $\beta$ .<sup>12</sup>

In the present study, we investigated the mechanism of Egr-1 stimulation by TGF- $\beta$ . The results indicate that TGF- $\beta$  caused rapid and transient up-regulation of Egr-1 via a Smad-independent pathway involving mitogen-activated protein kinase kinase 1 (MEK1)-ERK1/2-Elk-1. The expression of Egr-1 was markedly up-regulated in fibrotic lesions in a mouse model of scleroderma, and elevated Egr-1 was noted in skin and lung biopsies from patients with active scleroderma. Because Egr-1 is known to mediate stimulation of collagen by TGF- $\beta$  and to induce the production of fibrogenic signals and their receptors, our findings suggest that sustained up-regulation of Egr-1 in scleroderma might contribute to amplification or persistence of the TGF- $\beta$ -driven fibrotic process. Accordingly, targeting Egr-1 regulation and biological activity represents a novel therapeutic strategy to block TGF- $\beta$ -dependent pathological fibrogenesis.

## Materials and Methods

### Cell Culture and Reagents

Cultures of normal human dermal fibroblasts were established by explant from neonatal foreskin or forearm skin biopsies of healthy adults and studied at early (<8) passage. Dermal fibroblasts were obtained by biopsy of the affected dorsal forearm and/or from clinically unaffected skin from four patients with early diffuse cutaneous SSc<sup>13</sup> and studied in parallel with fibroblasts from three healthy donors. All patients fulfilled the American College of Rheumatology criteria for SSc. Cultures of murine dermal

fibroblasts were established from newborn female Smad3-null mice and their wild-type littermates.<sup>14</sup> Murine NIH3T3 fibroblasts were from the American Type Culture Collection (Manassas, VA). All fibroblasts were maintained in modified Eagle's minimal essential medium or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% vitamins, and 2 mmol/L L-glutamine and studied at early confluence. Culture media and tissue culture reagents were from Biowhittaker (Walkersville, MD). For experiments, fresh serum-free media containing 0.1% bovine serum albumin was added to cultures for 24 hours before TGF- $\beta$ . In selected experiments, fibroblasts were preincubated in media containing the kinase inhibitors SB431542 (Sigma-Aldrich, St. Louis, MO) or U0126 (Cell Signaling, Beverly, MA) for 30 minutes, followed by 12.5 ng/ml TGF- $\beta$ 1 (PeproTech, Rocky Hill, NJ) for up to 48 hours. Expression plasmids for constitutively active MEK1, Gal4-dbd-Elk-1, and its control vector and the reporter plasmid 5xGAL4-luciferase were all from Stratagene (La Jolla, CA).

### Northern Analysis and PCR

Total RNA was isolated from confluent fibroblasts using TRIzol Reagent (Life Technologies, Grand Island, NY). For Northern analysis, total RNA was examined using [ $\alpha$ -<sup>32</sup>P]dCTP-labeled cDNA probes for human Egr-1, glyceraldehyde-3-phosphate dehydrogenase, and 18S. After extensive washing of the nitrocellulose membranes, cDNA-mRNA hybrids were visualized by autoradiography. Signal intensities were quantitated by densitometry, and results were normalized with levels for glyceraldehyde-3-phosphate dehydrogenase mRNA or 18S RNA in each sample. For PCR analysis, total RNA (1  $\mu$ g) from cultured fibroblasts or lesional skin tissue from mice was reverse-transcribed to cDNA using AMV Reverse Transcriptase System (Promega, Madison, WI) and subjected to amplification. PCR products were separated by electrophoresis in 1.5% agarose gels. For real-time quantitative PCR analysis, total RNA was reverse-transcribed, and the products (50 ng) were amplified with the oligonucleotide primers shown in Table 1 using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the ABI 7500 Prism Sequence Detection System machine.

### Western Analysis

At the end of the experiments, fibroblasts were harvested, whole-cell lysates were prepared, and equal amounts of proteins (20 to 50  $\mu$ g/lane) were subjected to electrophoresis in 8% SDS polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA), and membranes were probed sequentially with primary antibodies specific for Egr-1 (C19), MEK1 (C18), phospho-MEK1(Ser<sup>298</sup>), and Actin (C2) (all from Santa Cruz Biotechnology, Santa Cruz, CA); Smad3 (Zymed Laboratories, South San Francisco, CA); or phospho-ERK1/2 (Thr<sup>202</sup> Tyr<sup>204</sup>), ERK1/2, or phospho-Smad2 (Ser<sup>465,467</sup>) (all from Cell Signaling), as indicated. Membranes were then incubated with appropriate secondary

**Table 1.** Sequences Used for RT-PCR and Real-Time Quantitative PCR

Gene	cDNA sequence
<b>RT-PCR</b>	
Egr-1	Forward: 5'-GACAGCAACCTTTTCTCC CAGG-3'
	Reverse: 5'-GTTAGGTCTCCTCACTTGGG GGAA-3'
Egr-2	Forward: 5'-CCGCCAAGGCCGTAGACA AAA-3'
	Reverse: 5'-GGGTCAATGGAGAACTT GCCC-3'
Egr-3	Forward: 5'-GAGAAGCTGCCGGTGACC ATGA-3'
	Reverse: 5'-AGTTGGAAGGGGAGTCGAA GGC-3'
Nab1	Forward: 5'-TGCTGACAAGAAGAGA TGAG-3'
	Reverse: 5'-TCCTGGTTTCCACAGA CTAC-3'
<b>Quantitative PCR</b>	
Egr-1	Forward: 5'-TGCGGCAGAAGGACAAGA AAGC-3'
	Reverse: 5'-TGAGGAAGGGAAGCTGCT GACC-3'
Egr-2	Forward: 5'-CCGCCAAGGCCGTAGACA AAAT-3'
	Reverse: 5'-GGGTCAATGGAGAACTTG CCCA-3'
Egr-3	Forward: 5'-GAGAAGCTGCCGGTGACC ATGA-3'
	Reverse: 5'-AGTTGGAAGGGGAGTCGA AGGC-3'
Nab2	Forward: 5'-TGACAGCCAGAAGGAAG AGGA-3'
	Reverse: 5'-AGGTGCTCTCTCTCGGGCT ACTT-3'
Actin	Forward: 5'-AATGTCGCGGAGGACTT TGAT-3'
	Reverse: 5'-AGGATGGCAAGGACTT CCTG-3'
Mouse Egr-1	Forward: 5'-TTTGCTCCGTTCCA CCTGC-3'
	Reverse: 5'-TGCCAACTTGATGGTCAT GCGC-3'

antibodies and subjected to enhanced chemiluminescence detection using ECL Reagent (Amersham Pharmacia, Piscataway, NJ).

### Transient Transfection Assays

Plasmids harboring 1.2 kb of the mouse Egr-1 gene promoter or 5'-truncated fragments of the promoter<sup>15</sup> fused to luciferase gene were used for transient transfection assays. At early confluence, fibroblasts were transfected using SuperFect Transfection kit (Qiagen, Valencia, CA). Twenty-four hours later, fresh serum-free media containing 0.1% bovine serum albumin and indicated kinase inhibitors (10  $\mu$ mol/L) were added, followed 30 minutes later by TGF- $\beta$ 1. At the end of the experiments cultures were harvested, and cell lysates were assayed for their luciferase activities using the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase pRL-TK (Promega) was cotransfected along with the re-

porter constructs, and luciferase activity was used to monitor transfection efficiency. Experiments were performed in triplicate, and the results were normalized for protein concentrations. To assess Elk-1 transactivation, NIH3T3 fibroblasts were cotransfected with Gal4-dbd-Elk-1 along with 5xGAL4-luciferase reporter plasmids, and after incubation with TGF- $\beta$ , luciferase activities were quantified as above.

### Immunofluorescence

The regulation of endogenous Egr-1 expression was further examined by immunocytochemistry and fluorescence confocal microscopy.<sup>16</sup> Briefly, foreskin fibroblasts (10<sup>4</sup> cells/well) incubated in serum-free media with 0.1% bovine serum albumin were exposed to 1 ng/ml TGF- $\beta$ 1 for 30 minutes. Cultures were then fixed with 100% methanol and incubated with rat anti-human Egr-1 antibodies (C19) at a 1:100 dilution, followed by fluorescein isothiocyanate-labeled secondary antibodies. Nuclei were identified using 4,6-diamidino-2-phenylindone. Subcellular distribution of immunofluorescence was then evaluated under a Zeiss LSHS10 laser scanning confocal microscope.

### Egr-1 Expression in Mouse Skin in Vivo

Six-week-old female Smad3-null mice and wild-type Back Swiss  $\times$ 129 SJ littermates (Taconic, Germantown, NY) weighing 15 to 20 g were administered 250 ng TGF- $\beta$ 1 s.c. or PBS s.c. One or 24 hours later, mice were euthanized, and the injected skin was processed for immunohistochemical analysis.<sup>17</sup> Four-micrometer sections from lesional skin were deparaffinized, rehydrated, and immersed in Tris-buffered saline-0.1%Tween 20 buffer followed by target retrieval solution (DAKO, Carpinteria, CA). After incubation of the slides with rabbit anti-mouse Egr-1 polyclonal antibodies (C-19; Santa Cruz Biotechnology) at a dilution of 1:300, donkey anti-rabbit Ig as secondary antibodies (Promega) at 1:1000 dilution were applied. Bound antibodies were detected using DAKO Envision + System according to the manufacturer's instructions. After counterstaining with hematoxylin, sections were mounted with Permount (Fisher Scientific, Pittsburgh, PA) and viewed under an BH-2 microscope (Olympus, Tokyo, Japan). Skin samples from Egr-1-null C57/BL6 mice<sup>18</sup> were stained in parallel as negative controls. An additional negative control consisted of substituting nonspecific rabbit IgG (Invitrogen, Carlsbad, CA) for the primary antibody. To quantify Egr-1 expression in lesional skin, at least 30 fibroblastic cells (identified based on their characteristic spindle-shaped morphology) from multiple microscopic fields were scored as clearly immunopositive or negative for Egr-1 by three independent examiners blinded to the treatment, and the ratio of positive cells to total cells was calculated. In other experiments, 6-week-old BALB/c mice weighing 20 g were given s.c. bleomycin or PBS daily for up to 28 days.<sup>17</sup> At the end of the experiments, mice were sacrificed, and lesional skin was processed for quantitative PCR or for immunohistochemistry as above. Each experimental group contained at least five mice, and three separate experiments were performed with similar

results. All animal studies were performed using protocols and guidelines approved by the Northwestern University Animal Care and Use Committee.

### *Egr-1 Expression in SSc Lesional Tissue*

Skin tissue samples were obtained by biopsy from the affected forearm from 23 patients with SSc (16 patients with disease duration <3 years, early stage; and seven patients with disease duration  $\geq$ 3 years, late stage) and five healthy adult controls. The protocols for tissue collection were approved by the Institutional Review Boards for Human Studies at Northwestern University, the University of Pittsburgh, and Kanazawa University. Some of the SSc tissues were processed following exactly the same procedure as described under Egr-1 Expression in Mouse Skin in Vivo, where the slides were counterstained with hematoxylin. For other tissues, methyl green was used for counterstain. Paraffin-embedded or frozen tissue sections were subjected to immunostaining using primary anti-Egr-1 antibody (C19; Santa Cruz Biotechnology) at 1:300 dilution, followed by incubation with biotinylated anti-rabbit IgG secondary antibodies (Santa Cruz sc-2089) at 1:100 dilution. Slides were then reacted with avidin-biotin peroxidase (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and peroxidase activity was detected with diaminobenzidine substrate (brown color). To quantify Egr-1 in lesional dermis, Egr-1-positive fibroblasts were counted by blinded examiners, and the ratio of positive cells to total cells was calculated. Tissue sections from prostate cancer were used as positive control, and substitution of primary antibody with nonspecific rabbit IgG (Invitrogen) served as negative control. Lung tissue was obtained from four patients with SSc-associated pulmonary fibrosis who were undergoing lung transplant surgery, and from unused normal donor lungs from three patients. Tissues were processed as above, the slides were counterstained with hematoxylin. To quantify Egr-1 expression, the numbers of cells in several microscopic fields were scored as clearly immunopositive or negative for Egr-1 by two independent examiners, and the ratio of positive cells to total cells was calculated. Tissue sections from a colon cancer were used as a positive control, and nonspecific rabbit IgG was used as a negative control.

### *Statistical Analysis*

Statistical significance was determined using the unpaired Student's *t*-test. Values of  $P \leq 0.05$  were considered significant.

## **Results**

### *TGF- $\beta$ Stimulates Egr-1 Expression in Normal Fibroblasts*

To characterize Egr-1 regulation by TGF- $\beta$ , quiescent foreskin fibroblasts were stimulated with increasing concentrations of TGF- $\beta$ 1. The results of Western and Northern analysis showed rapid (30 minutes) and dose-depen-

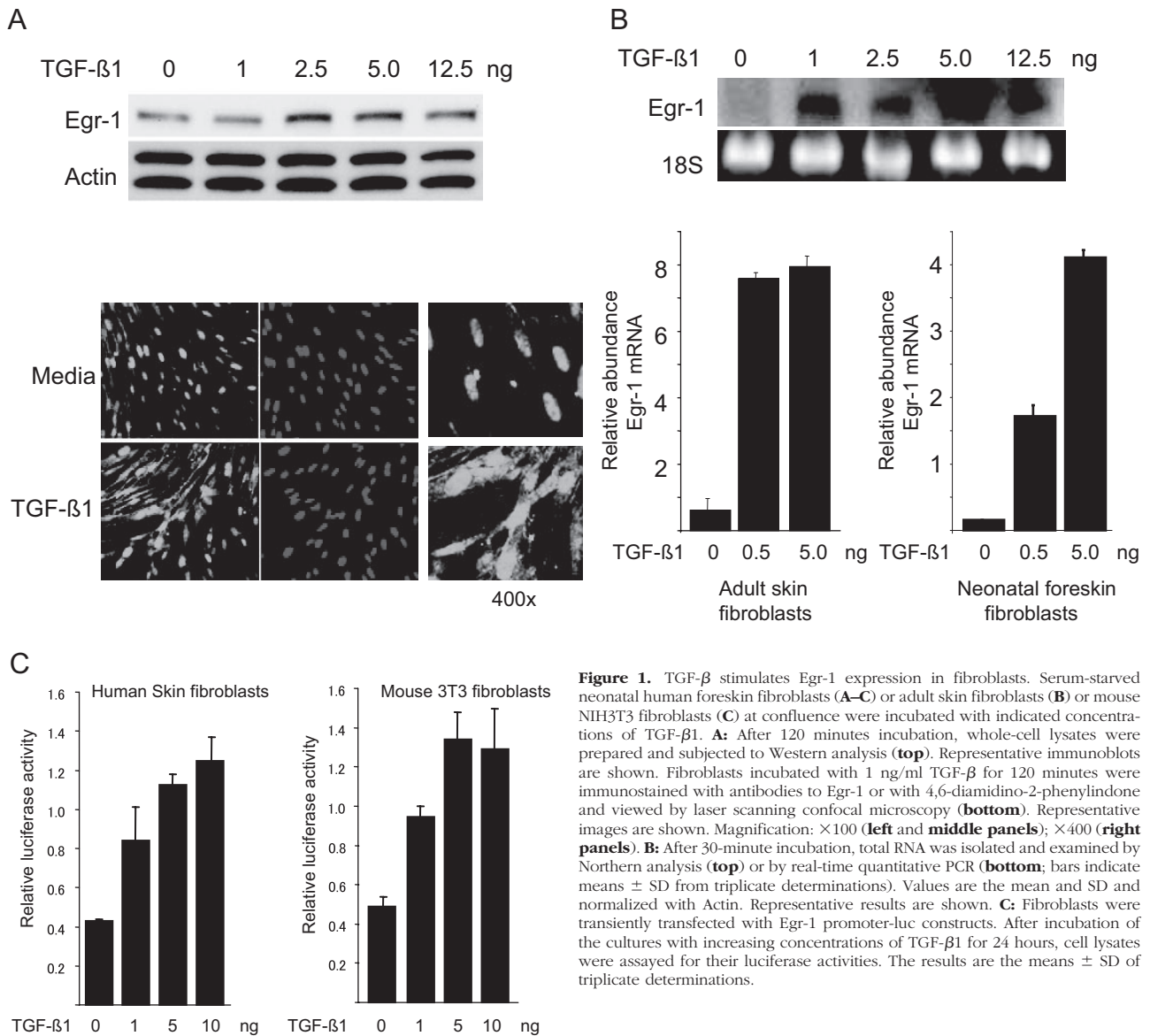
dent increase in Egr-1 protein (Figure 1A, top) and mRNA (Figure 1B, top) expression. Immunofluorescence confocal microscopy indicated little detectable Egr-1 in the absence of TGF- $\beta$  and marked accumulation in both cytoplasm and nucleus in TGF- $\beta$ -treated fibroblasts (Figure 1A, bottom). Real-time quantitative PCR analysis confirmed the dose-dependent stimulation of Egr-1 mRNA in neonatal and adult dermal fibroblasts (Figure 1B, bottom).

To characterize the level of regulation of Egr-1, quiescent fibroblasts were transiently transfected with a plasmid containing the proximal 1.2 kb of the human Egr-1 gene promoter linked to the luciferase reporter and then incubated in media with increasing concentrations of TGF- $\beta$ 1. Twenty-four hours later, the cultures were harvested, and cell lysates were assayed for their luciferase activities. The results showed that TGF- $\beta$  induced a dose-dependent increase in Egr-1 promoter activity in transfected primary foreskin fibroblasts (Figure 1C, left) and in NIH3T3 fibroblasts (Figure 1C, right). In both human and mouse fibroblasts, TGF- $\beta$  at a concentration of 10 ng/ml induced a  $\sim$ threefold stimulation of Egr-1 promoter activity.

In addition to Egr-1, the family of early growth response transcription factors also includes Egr-2, Egr-3, and Egr-4. Because little is known about the function or regulation of the Egr moieties in the context of fibrosis, foreskin fibroblasts were incubated with TGF- $\beta$  for indicated periods, and mRNA expression was evaluated by RT-PCR analysis. The results showed that TGF- $\beta$ 1 caused rapid stimulation of Egr-3 and Egr-1 mRNA in these cells (Figure 2). Marked increase was seen at 60 minutes, followed by decline after 120 minutes. The expression of Egr-2 mRNA was also stimulated, but in contrast to Egr-1, the response was relatively sustained, with elevated mRNA expression persisting at 4 hours. We also examined TGF- $\beta$  regulation of Nab1 and Nab2, which are inhibitory members of the early growth response family that bind to Egr-1 and regulate its activity.<sup>19</sup> The results of PCR analysis showed that Nab1 was constitutively expressed in fibroblasts, as it is in most cell types, and was further increased by TGF- $\beta$ . In contrast, Nab2 mRNA was undetectable in the absence of stimulation but was strongly induced by TGF- $\beta$ ; however, compared with the rapid stimulation of Egr-1 and Egr-3, the Nab2 response was delayed, peaking at 4 hours (Figure 2). Thus, endogenous inhibitors of Egr-1 are induced by the same environmental signals that stimulate Egr-1 and, at least in the case of Nab2, show delayed response compatible with a negative feedback function. The stimulation of mRNA of each of the Egr moieties by TGF- $\beta$  was confirmed by real-time quantitative PCR (data not shown).

### *Delineation of the Egr-1 Promoter TGF- $\beta$ Response Region*

As illustrated schematically (Figure 3, left), the proximal promoter of the Egr-1 gene harbors multiple potential regulatory elements. These include binding sites for AP-1, GC-rich elements, and five serum-responsive ele-



**Figure 1.** TGF- $\beta$  stimulates Egr-1 expression in fibroblasts. Serum-starved neonatal human foreskin fibroblasts (**A–C**) or adult skin fibroblasts (**B**) or mouse NIH3T3 fibroblasts (**C**) at confluence were incubated with indicated concentrations of TGF- $\beta$ 1. **A:** After 120 minutes incubation, whole-cell lysates were prepared and subjected to Western analysis (**top**). Representative immunoblots are shown. Fibroblasts incubated with 1 ng/ml TGF- $\beta$  for 120 minutes were immunostained with antibodies to Egr-1 or with 4,6-diamidino-2-phenylindone and viewed by laser scanning confocal microscopy (**bottom**). Representative images are shown. Magnification:  $\times 100$  (**left and middle panels**);  $\times 400$  (**right panels**). **B:** After 30-minute incubation, total RNA was isolated and examined by Northern analysis (**top**) or by real-time quantitative PCR (**bottom**); bars indicate means  $\pm$  SD from triplicate determinations. Values are the mean and SD and normalized with Actin. Representative results are shown. **C:** Fibroblasts were transiently transfected with Egr-1 promoter-luc constructs. After incubation of the cultures with increasing concentrations of TGF- $\beta$ 1 for 24 hours, cell lysates were assayed for their luciferase activities. The results are the means  $\pm$  SD of triplicate determinations.

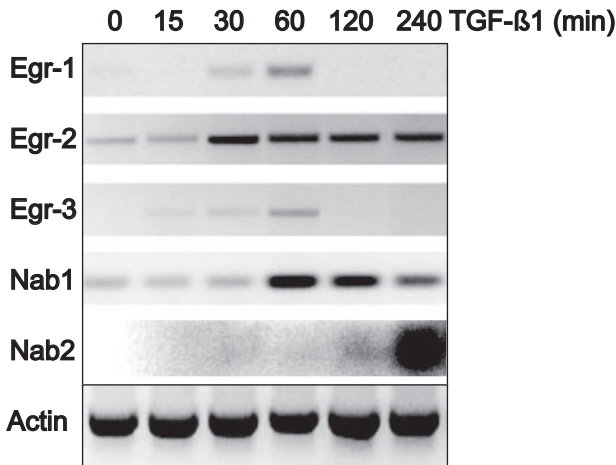
ments (SREs). Transient transfection assays were performed to localize the functional TGF- $\beta$  response regions of the Egr-1 gene. For this purpose, mouse NIH3T3 fibroblasts were transfected with the indicated deletion or truncation mutants of the mouse Egr-1 promoter linked to the luciferase reporter gene. Incubation of transfected fibroblasts with TGF- $\beta$  resulted in  $\sim$ twofold stimulation of luciferase activity driven by the full-length Egr-1 promoter (Figure 3). The activity of luciferase fused to a promoter mutant lacking all five SRE sites (B2) showed a substantial decrease in basal activity and complete lack of stimulation by TGF- $\beta$ .

Deletion of neither the region containing the distal AP-1 and Sp1 binding sites (C21) nor the two proximal SRE sites (D7) significantly affected either basal promoter activity or the TGF- $\beta$  response. In contrast, truncation of the three distal SRE sites (AA) resulted in complete abrogation of TGF- $\beta$  response, indicating that the distal SRE sites mediate TGF- $\beta$ -induced Egr-1 promoter transactivation.

### Role of Smad3 in Egr-1 Stimulation

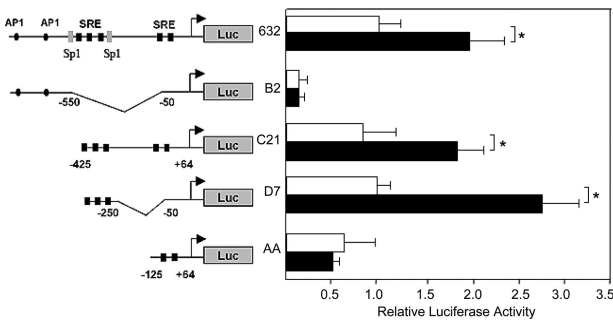
Downstream targets for activated ALK5, Smad2, and Smad3 are the primary signal transducers for most TGF- $\beta$  responses in fibroblasts.<sup>20</sup> To evaluate their role as potential mediators of Egr-1 stimulation, SB431542, a potent and specific inhibitor of ALK5-dependent TGF- $\beta$  signaling, was used.<sup>21</sup> Confluent serum-starved human fibroblasts were preincubated with 10  $\mu$ mol/L SB431542 for 30 minutes, followed by TGF- $\beta$ 1 for indicated periods. The results of Northern analysis showed that SB431542 pretreatment failed to prevent rapid and transient stimulation of Egr-1 mRNA induced by TGF- $\beta$  (Figure 4A). Under these experimental conditions, SB431542 significantly prevented the phosphorylation of Smad2 induced by TGF- $\beta$  (Figure 4B, right).

Complementary experiments used primary dermal fibroblasts from Smad3-null mice. We have shown previously that Smad3-null fibroblasts displayed impaired



**Figure 2.** TGF- $\beta$  regulation of the expression of Egr moieties. Confluent foreskin fibroblasts were incubated with TGF- $\beta$ 1 (12.5 ng) for indicated periods. Total RNA was isolated and subjected to RT-PCR analysis. The results are representative of at least two independent experiments.

TGF- $\beta$  responses *in vitro*, with substantially reduced stimulation of collagen.<sup>14</sup> Confluent fibroblasts from Smad3-null mice and their wild-type littermates were incubated in parallel with TGF- $\beta$ 1 (12.5 ng) for up to 120 minutes. The results of Western analysis showed that Egr-1 was rapidly induced by TGF- $\beta$ 1 in wild-type murine fibroblasts in a manner comparable with that observed in normal human fibroblasts (Figure 4B). Smad3-null fibroblasts showed virtually identical early induction of Egr-1. Interestingly, basal levels of Egr-1 were found to be modestly elevated. In agreement with results from Western analysis, examination of Egr-1 mRNA levels showed that TGF- $\beta$ 1 caused comparable stimulation in both wild-type fibroblasts and Smad3-null fibroblasts (data not shown; Figure 5B). Taken together, the results from experiments using ALK5 inhibition and Smad3-deficient mouse fibroblasts indicated that stimulation of Egr-1 by TGF- $\beta$  occurred largely independent of the ALK5/Smad3 signaling axis.



**Figure 3.** Delineation of TGF- $\beta$  response region of the Egr-1 gene promoter. NIH3T3 fibroblasts transiently transfected with indicated 5' deletion or truncation mutants of the mouse Egr-1 gene promoter linked to the luciferase reporter were incubated with TGF- $\beta$ 1 for 24 hours. Cell lysates were assayed for their luciferase activities. The results, normalized with Renilla luciferase activities, are the means  $\pm$  SD of triplicate determinations from at least two independent experiments.  $\square$ , untreated fibroblasts;  $\blacksquare$ , TGF- $\beta$ -treated fibroblasts. \* $P$  < 0.005.

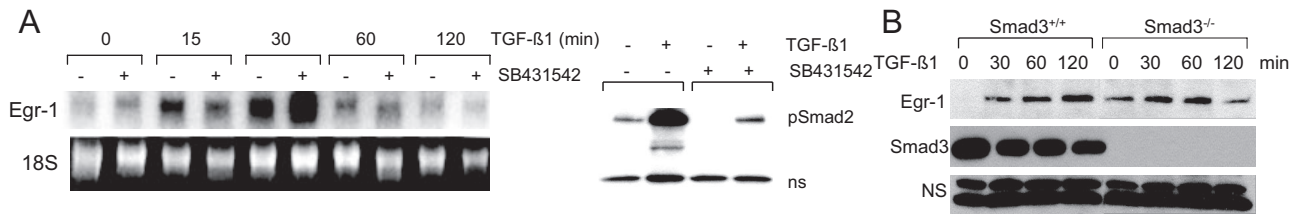
### MAPK Pathway Mediates Egr-1 Induction

Because stimulation of Egr-1 appeared to be a Smad3-independent TGF- $\beta$  response, we proceeded to examine the functional role of non-Smad TGF- $\beta$  signaling pathways. For this purpose, confluent foreskin fibroblasts were pretreated with the selective MEK1/2 kinase inhibitor U0126 for 30 minutes, followed by incubation with TGF- $\beta$ 1 for up to 120 minutes. Results from Northern analysis showed that the rapid stimulation of Egr-1 mRNA was completely abolished by pretreatment of the cultures with U0126 (Figure 5A, left). Under the experimental conditions used here, U0126 had no demonstrable cellular toxicity. Consistent results were obtained in separate experiments using real-time quantitative PCR analysis (Figure 5A, right). Furthermore, in contrast to the ALK5 inhibitor SB431542 (Figure 5B) or inhibitors of p38 (data not shown), pretreatment of the cultures with U0126 abrogated the stimulation of Egr-1 in both wild-type and Smad3-null dermal fibroblasts (Figure 5B). These results suggested that MEK1/2, which is upstream of, and directly activates, ERK1/2, and which is induced by a variety of growth factors and cytokines, might serve as a mediator for TGF- $\beta$  stimulation of Egr-1.

To examine the effect of TGF- $\beta$  on MEK1/2, serum-starved fibroblasts were incubated with TGF- $\beta$  for indicated periods and subjected to Western analysis. The results showed that TGF- $\beta$  stimulation induced rapid phosphorylation of cellular MEK1/2 (Figure 6A). Phosphorylation of ERK1/2 and Smad2 also occurred in parallel with unaltered ERK1/2. To examine the functional contribution of MEK1 in Egr-1 stimulation, NIH3T3 fibroblasts were cotransfected with expression vector for a constitutively active MEK1 mutant, along with Egr-1 promoter-luc reporter, and incubated in the presence or absence of TGF- $\beta$  for a further 24 hours. The results of transient transfection assays showed that constitutively active MEK1 was by itself capable of causing dose-dependent stimulation of Egr-1 promoter activity (Figure 6B, left) and endogenous Egr-1 protein accumulation (Figure 6C). No stimulation occurred in NIH3T3 fibroblasts transfected with empty vector. Pretreatment of the cultures with the MEK1 kinase inhibitor U0126 significantly attenuated the stimulation of Egr-1 promoter activity induced by MEK1 (Figure 6B, right). Taken together, these results demonstrate that MEK1 is activated by TGF- $\beta$ 1 in fibroblasts and plays a major functional role in mediating the stimulation of Egr-1 promoter activity and Egr-1 protein accumulation in these cells.

### TGF- $\beta$ Stimulates Elk-1 Transcriptional Activity

We next turned our attention to downstream effectors of the MAPK-mediated TGF- $\beta$  response. Deletion analysis identified the distal SRE sites of the Egr-1 promoter as the elements responsible for stimulation by TGF- $\beta$ . Serum response elements are known to bind to multicomponent complexes that contain the Ets-like transcription factor Elk-1.<sup>22</sup> Elk-1 is a direct ERK1/2 substrate in the MAPK signaling cascade, and its phosphorylation has been



**Figure 4.** Smad3-independent TGF- $\beta$  stimulation of Egr-1. **A:** Confluent serum-starved foreskin fibroblasts were pretreated with 10  $\mu$ mol/L SB431542 for 30 minutes, followed by TGF- $\beta$ 1 for indicated periods. At the end of the incubation, total RNA was isolated and examined by Northern analysis. The results are representative of two independent experiments. **B:** Time-dependent Egr-1 stimulation in mouse skin fibroblasts. Confluent serum-starved fibroblasts explanted from Smad3-null mice and wild-type littermates in parallel were incubated with TGF- $\beta$ 1 for indicated periods. Whole-cell lysates were examined by Western analysis. NS, nonspecific band. Representative immunoblots are shown.

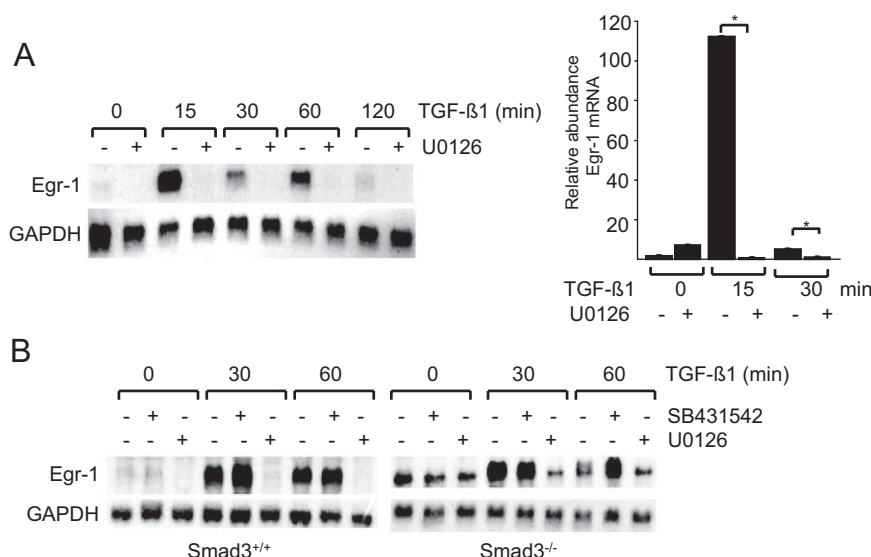
shown to enhance its binding to SRE sequences. In fibroblasts, TGF- $\beta$  stimulation induced activation of the MEK-ERK1/2 pathway (Figure 6A). Furthermore, in HaCaT cells, TGF- $\beta$  has been shown to increase Elk-1 transcriptional activity.<sup>23</sup> Because the distal SRE sites appear to serve as TGF- $\beta$  response elements in the Egr-1 promoter and because Elk-1 is known to mediate the induction of Egr-1 by inflammation-associated stimuli, we examined the functional role of Elk-1 in mediating the TGF- $\beta$  response using heterologous Gal4-Elk-1 transactivation assays. For this purpose, NIH3T3 fibroblasts were co-transfected with a reporter construct containing five tandem repeats of the yeast Gal4 DNA binding element linked to the luciferase reporter along with pGAL4-DBD-Elk-1, which expresses a chimeric protein that contains the GAL4 DNA binding domain fused to the transactivation domain of Elk-1. Transfected cells were incubated with TGF- $\beta$  overnight, and cell lysates were assayed for their luciferase activities. The results showed that TGF- $\beta$  induced a ~twofold increase in Elk-1-dependent transcription (Figure 7). No induction was observed in fibroblasts transfected with a plasmid that expresses only the pGAL4-binding domain (data not shown). Pretreatment of the cultures with U0126 completely abrogated the TGF- $\beta$  response. We conclude therefore that TGF- $\beta$  stimulation leads to Elk-1 activation via the MEK-ERK1/2 pathway.

### TGF- $\beta$ Induces Egr-1 Expression *in Vivo*

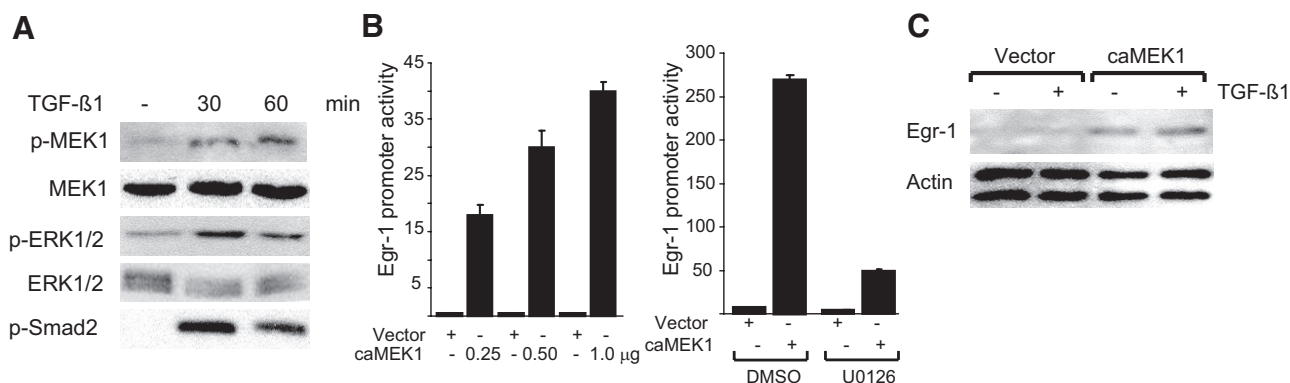
To examine the *in vivo* regulation of Egr-1 in skin fibroblasts, young Smad3-null mice and wild-type littermates received a single s.c. injection of 250 ng of TGF- $\beta$ 1 or PBS in parallel, and 1 or 24 hours later, Egr-1 expression in the lesional dermis was examined. The results of immunohistochemistry indicated that by 24 hours, TGF- $\beta$ 1 induced a marked increase in Egr-1 in fibroblastic cells (Figure 8A). Quantitation of the results from multiple determinations indicated a reproducible two- to threefold increase in skin Egr-1 expression in both Smad3-null mice and wild-type littermates (Figure 8B). Parallel examination of lesional skin from Egr-1-null mice showed no detectable Egr-1 expression, indicating the specificity of the immunostaining (Figure 8C). Therefore, TGF- $\beta$  induces Egr-1 expression in a Smad3-independent manner *in vivo* and as *in vitro*.

### Bleomycin-Induced Mouse Scleroderma Is Associated with Egr-1 Up-Regulation

By inducing fibroblast activation and extracellular matrix accumulation, TGF- $\beta$  plays a key role in fibrosis. In light of the present results showing that Egr-1 is stimulated by



**Figure 5.** MEK1-dependent stimulation of Egr-1 mRNA. Confluent serum-starved human (**A**) or mouse (**B**) skin fibroblasts were preincubated with protein kinase inhibitors (10  $\mu$ mol/L) for 30 minutes, followed by TGF- $\beta$ 1 for indicated periods. **A:** Cultures were harvested, and total RNA was subjected to Northern analysis (**left**) or real-time quantitative PCR analysis (**right**). For real-time PCR, values are the mean and SD and normalized with Actin. \* $P < 0.05$ . **B:** RNA from Smad3-null dermal fibroblasts or wild-type control fibroblasts was subjected to Northern analysis. Representative autoradiograms are shown.

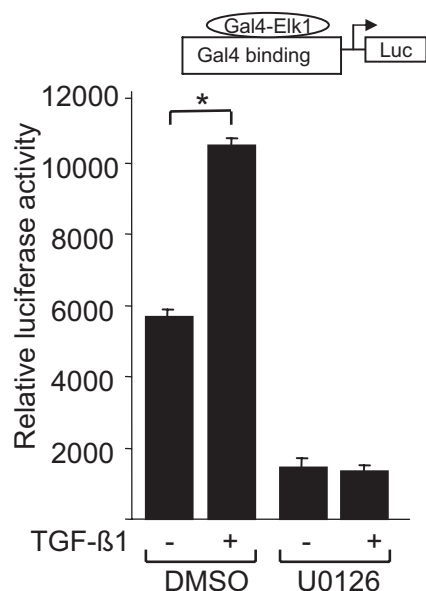


**Figure 6.** MEK1 is activated by TGF- $\beta$  and induces Egr-1. **A:** Foreskin fibroblasts were incubated with TGF- $\beta$  for indicated periods and whole-cell lysates were subjected to Western analysis. Representative autoradiograms are shown. **B:** NIH3T3 fibroblasts were cotransfected with indicated concentrations of constitutively active MEK1 (caMEK1) or empty vector along with Egr-1-luc (left). After incubation for 24 hours, cultures were harvested, and cell lysates were assayed for their luciferase activities. Transfected NIH3T3 fibroblasts were incubated in media containing U0126 for 24 hours (right). Results, normalized with Renilla luciferase, are expressed as means  $\pm$  SD of triplicate determinations from two independent experiments. **C:** Whole-cell lysates prepared from transfected NIH3T3 fibroblasts incubated with TGF- $\beta$  for 24 h were examined by Western analysis. Representative immunoblots are shown.

TGF- $\beta$  and is involved in mediating TGF- $\beta$  responses downstream of the activated TGF- $\beta$  receptors, we proceeded to evaluate Egr-1 expression in a murine model of skin fibrosis. In agreement with our previous findings,<sup>14</sup> s.c. administration of bleomycin in BALB/c mice resulted in scleroderma-like dermal fibrosis and local TGF- $\beta$  signaling (data not shown). Immunohistochemical examination showed an almost threefold increase in Egr-1 expression in lesional dermis at day 21 compared with PBS-treated control mice (Figure 9A). Immunostaining was localized mostly within the nucleus of fibroblastic cells. The levels of Egr-1 mRNA in lesional skin were increased in parallel with Egr-1 protein (Figure 9B).

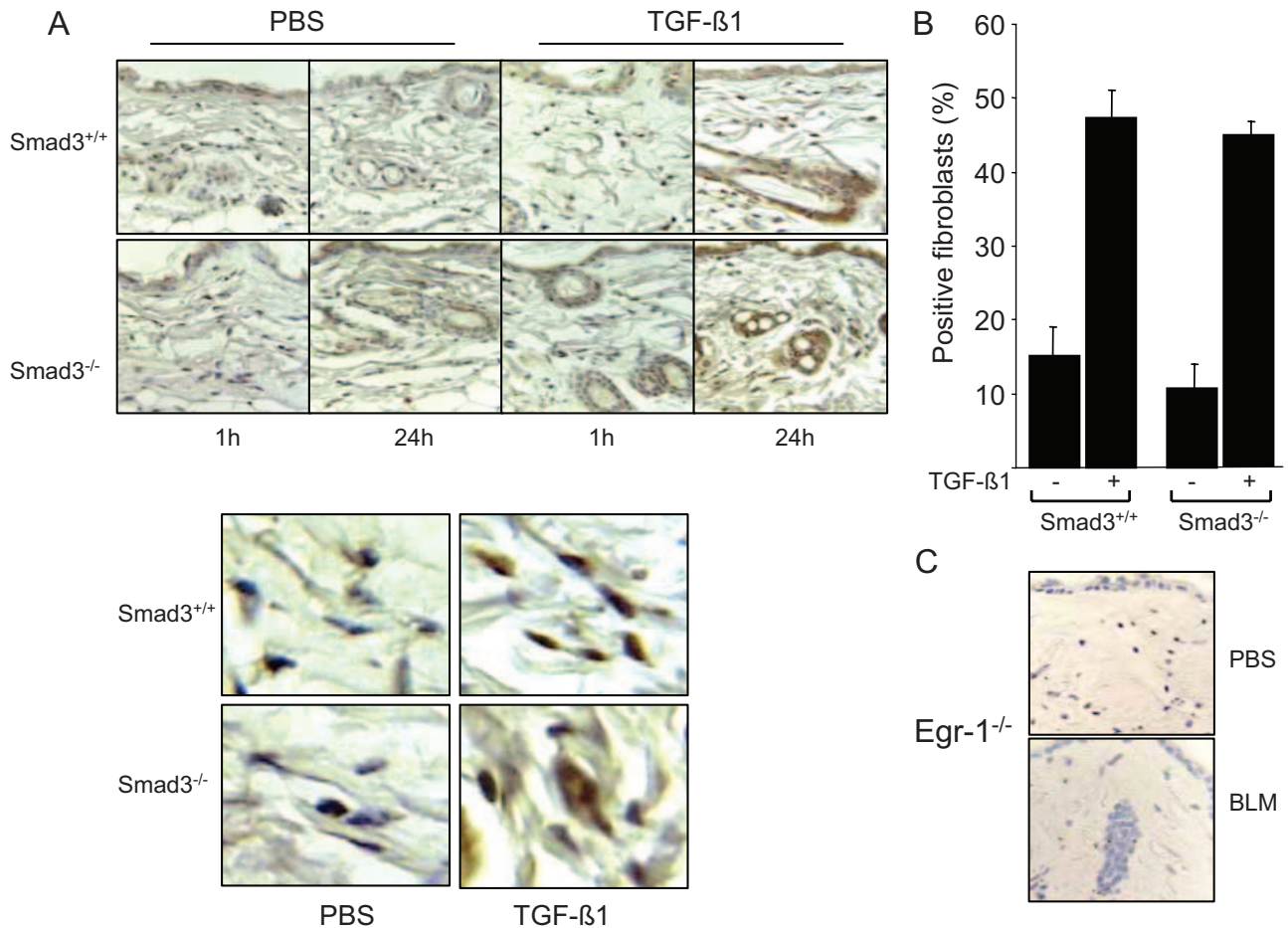
### Elevated Egr-1 Expression in Lesional SSc Skin and Lungs

Although Egr-1 is normally low or undetectable in most tissues, rapid and transient Egr-1 accumulation takes place in response to injury, ischemia, and stress. We have shown previously that Egr-1 can directly transactivate COL1A2 transcription and mediates stimulation of collagen production and other fibrotic responses elicited by TGF- $\beta$ .<sup>11</sup> Because TGF- $\beta$  is known to play a pivotal role in the pathogenesis of SSc and its activity is up-regulated in lesional tissues, aberrant Egr-1 regulation in response to chronic TGF- $\beta$  signaling in lesional fibroblasts might contribute to the development or persistence of fibrosis. To further explore this notion, the expression of Egr-1 in SSc lesional tissue was examined by immunohistochemistry. For this purpose, tissue samples from 23 patients with SSc and 5 matched healthy controls were studied in parallel. The clinical characteristics of the patients are presented in Table 2. The results showed that in contrast to skin tissue from healthy individuals that had little or no detectable Egr-1, a significant proportion of fibroblastic cells in SSc lesional dermis showed positive Egr-1 immunostaining (Figure 10A). Almost all SSc skin biopsies demonstrated intracellular Egr-1 that was detectable in the papillary and the reticular dermis and at the dermis-adipose layer interface; the epidermis in both SSc samples and healthy controls showed Egr-1 expression, which was most pronounced in the basal layer. Immunostaining was specific, because substitution of the primary antibody resulted in the absence of brown staining. Quantitative analysis showed that SSc patients with relatively early-stage disease (<3 years duration) had a ~20-fold increase in the proportion of Egr-1-positive fibroblasts in the lesional dermis and that patients with late-stage disease (>3 years) had a ~10-fold elevation compared with healthy controls (Figure 10A, right). Moreover, a trend toward a negative correlation between the proportion of Egr-1-positive fibroblasts and disease duration was noted ( $r = -0.538, P = 0.0072$ ).



**Figure 7.** TGF- $\beta$  stimulates Elk-1-dependent transcription. Subconfluent NIH3T3 fibroblasts were cotransfected with the expression vector pGal4-dbd-Elk-1 along with the reporter construct pGal4-DNA-luc. Transfected fibroblasts were preincubated in the presence or absence of 10  $\mu$ mol/L U0126 for 30 minutes before incubation with TGF- $\beta$  for 24 hours. Cell lysates were assayed for their luciferase activities. The results, shown as the means  $\pm$  SD of triplicate determinations, are representative of three independent experiments. \* $P < 0.01$ .





**Figure 8.** TGF- $\beta$  induces Egr-1 expression *in vivo*. Six-week-old female Smad3-null mice and wild-type littermate mice received a single s.c. injection of 250 ng TGF- $\beta$ 1 or PBS. Lesional skin was harvested 1 or 24 hours later and examined by immunohistochemistry using antibodies against Egr-1. **A:** Representative photomicrographs; **bottom panels**, higher magnification. **B:** The proportion of Egr-1-positive fibroblasts in the lesional dermis (at 24 hours) was determined. The results represent the means  $\pm$  SE. **C:** Negative control. Skin tissue from Egr-1-null mice was processed in parallel.

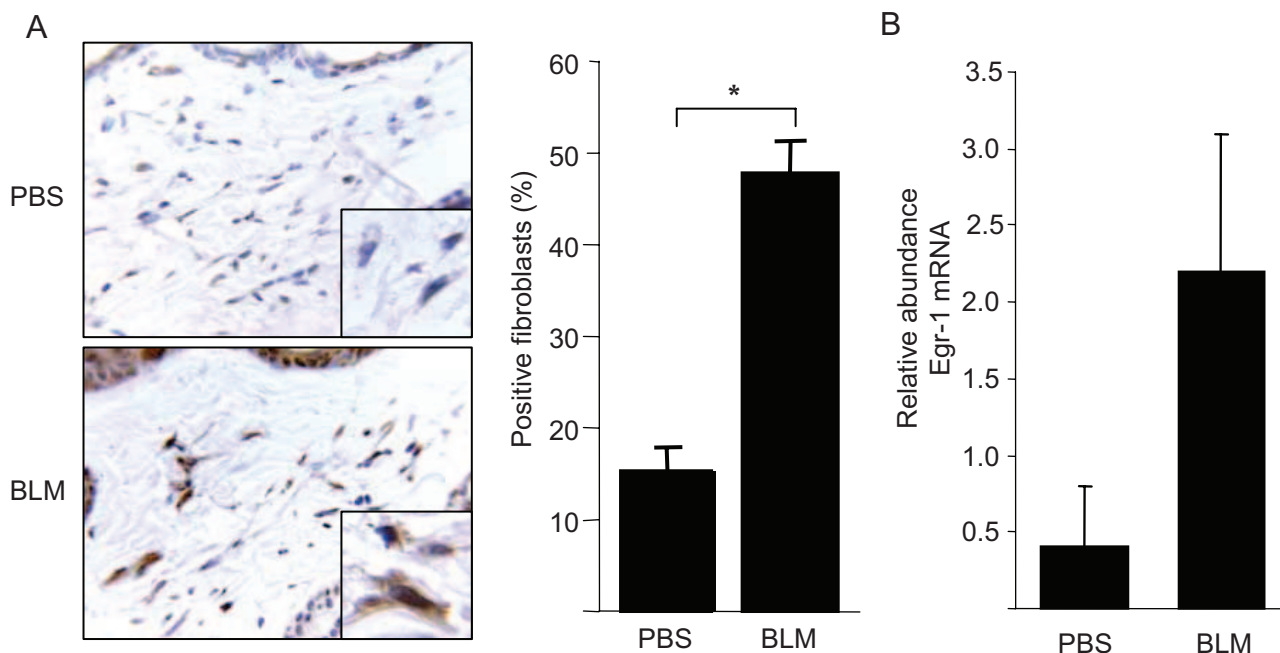
Next, Egr-1 expression was examined in SSc dermal fibroblasts explanted from the lesional skin. For this purpose, fibroblasts from four patients with early and untreated SSc, and three matched healthy controls were studied at low passage (<5). Confluent cultures were incubated in serum-free Dulbecco's modified Eagle's medium for 48 hours, and total RNA or whole-cell lysates were examined by real-time quantitative PCR analysis or Western analysis, respectively. Quantitative PCR analysis showed elevated Egr-1 mRNA levels in all four SSc fibroblast lines, with a ~fivefold (mean) increase compared with healthy control fibroblasts studied in parallel (Figure 10B, left). Western analysis showed that Egr-1 protein was elevated in unstimulated SSc fibroblasts explanted from both clinically involved and uninvolved skin, compared with healthy control fibroblasts (Figure 10B, right). Examination of lesional fibroblasts from an additional five patients indicated a two- to fourfold elevation in Egr-1 protein (data not shown).

Fibrosis in SSc commonly affects the lungs, and the process is associated with fibroblast activation and local TGF- $\beta$  signaling.<sup>24</sup> To examine Egr-1 expression in the lung in SSc, samples from four patients with SSc-associated pulmonary fibrosis and normal donor lungs were

examined by immunohistochemistry. The results showed low Egr-1 expression in normal donor lung tissue, detected primarily in alveolar macrophages and in occasional type I and type II pneumocytes (Figure 10C, left). In contrast, lung tissues from all four SSc patients showed strong Egr-1 expression at fibrotic loci. In these regions, Egr-1 immunostaining was detected in epithelial cells and mesenchymal cells. Quantitation confirmed a marked increase in the numbers of Egr-1-positive cells in SSc lungs (Figure 10C, right).

### Discussion

Stimulation of collagen transcription by TGF- $\beta$  involves both canonical Smad signaling and intracellular pathways that operate through cross-talk with the Smad axis or in a Smad-independent manner via ERK, c-Jun N-terminal kinase, p38, cyclin-dependent kinase 2/4, Rho, focal adhesion kinase, c-Abl, and phosphatidylinositol 3-kinase.<sup>25-28</sup> We had previously identified Egr-1 as a novel TGF- $\beta$  mediator with a significant role in collagen regulation.<sup>10</sup> Whereas induction of Egr-1 by acute injury has been investigated extensively, its regulation by



**Figure 9.** Bleomycin-induced skin fibrosis associated with Egr-1 expression in vivo. Six-week-old female BALB/C mice received daily s.c. injections of bleomycin (BLM) or PBS. **A:** At day 21, lesional skin was harvested. Immunohistochemistry using antibodies against Egr-1. Hematoxylin counterstain. Representative images are shown. Magnification:  $\times 400$ ;  $\times 1000$  (inset, left). The proportion of immunopositive fibroblasts in the lesional dermis was determined (right). Results represent means  $\pm$  SD. **B:** Total RNA from lesional skin was analyzed by real-time quantitative PCR. Results are the means  $\pm$  SD of duplicate determinations from three mice/group. \* $P < 0.005$ .

TGF- $\beta$  in the context of fibrogenesis and its role in fibrosis remain poorly understood. In this study, we therefore explored the molecular basis of Egr-1 stimulation by TGF- $\beta$  in fibroblasts. We find that the response involves Smad-independent transcriptional regulation via the MEK1-ERK1/2-Elk-1 signaling cascade. The expression of Egr-1 was up-regulated in skin and lung tissues and in explanted dermal fibroblasts from patients with active scleroderma and in fibrotic tissue from mice with bleomycin-induced scleroderma. These findings suggest that unchecked TGF- $\beta$  activity associated with pathological fibrogenesis causes MAPK-dependent up-regulation and persistent local expression of Egr-1 that in turn might contribute to the progression of fibrosis.

Egr-1, a prototypic member of the early-immediate growth response gene family, couples acute perturbations in the extracellular milieu to short-lived changes in the target gene expression.<sup>29</sup> In immortalized vascular endothelial cells, ectopic Egr-1 expression resulted in up-regulation of >300 genes, including transcriptional regulators, signaling proteins, cell cycle regulators, cytokines, and extracellular matrix components,<sup>30</sup> whereas in normal skin fibroblasts, Egr-1 altered the expression of >100 genes involved in inflammation, apoptosis, cell cycle, cell growth, cell migration, and extracellular matrix (S. Bhattacharyya, S. Lin, and J. Varga, unpublished data). Normal tissue has low Egr-1, but expression is rapidly and transiently induced in acute injury. In turn, Egr-1 up-regulates tumor necrosis factor- $\alpha$ , TGF- $\beta$ , platelet-derived growth factor, CD44, and intercellular adhesion molecule-1, indicating a role in the cellular stress response program, and the tumor suppressors PTEN and p53, consistent with a role in cell proliferation, apoptosis,

and tumor suppression.<sup>31</sup> Additional biological roles of Egr-1 include regulation of synaptic plasticity and reproduction.

Emerging evidence implicates Egr-1 in physiological wound healing on the one hand and pathological tissue repair on the other. The list of Egr-1 targets relevant to chronic tissue injury and pathological fibrosis includes plasminogen activator inhibitor-1,<sup>32,33</sup> platelet-derived growth factor,<sup>34,35</sup> Snail,<sup>36</sup> fibronectin,<sup>37,38</sup> TGF- $\beta$ ,<sup>39</sup> type 2 TGF- $\beta$  receptor,<sup>40</sup> basic fibroblast growth factor,<sup>41</sup> matrix metalloproteinase-2,<sup>42,43</sup> tissue inhibitor of metalloproteinases-1,<sup>44</sup> and type I collagens.<sup>10</sup> The importance of Egr-1 in fibrosis is highlighted by its elevated expression and colocalization with connective tissue growth factor in lung tissue from smokers<sup>42</sup> and by protection of Egr-1-null mice from lung fibrosis induced by hypoxia,<sup>45</sup> by TGF- $\beta$ <sup>12</sup> or IL-13.<sup>46</sup> Our present results demonstrate that Egr-1 is induced by TGF- $\beta$  in skin and lung fibroblasts and NIH3T3 cells. The response is characterized by time- and dose-dependent Egr-1 protein accumulation, mRNA expression, and promoter activation. Stimulation by TGF- $\beta$  also caused rapid induction of other members of the early growth response gene family. In contrast to Egr-1, its endogenous inhibitor Nab2 followed more delayed kinetics, showing maximal induction at 4 hours. The discrepant time course for the induction of Egr-1 and Nab2, also reported in vascular smooth muscle cells, is consistent with the putative role of Nab2 as a negative feedback on Egr-1 activity.<sup>47</sup>

In contrast to other TGF- $\beta$ -inducible profibrotic genes, Egr-1 regulation by TGF- $\beta$  appears to be largely Smad-independent and parallels Egr-1 induction by inflammatory signals, such as platelet-derived growth factor, lipo-

**Table 2.** Clinical Characteristics of SSc Patients Studied

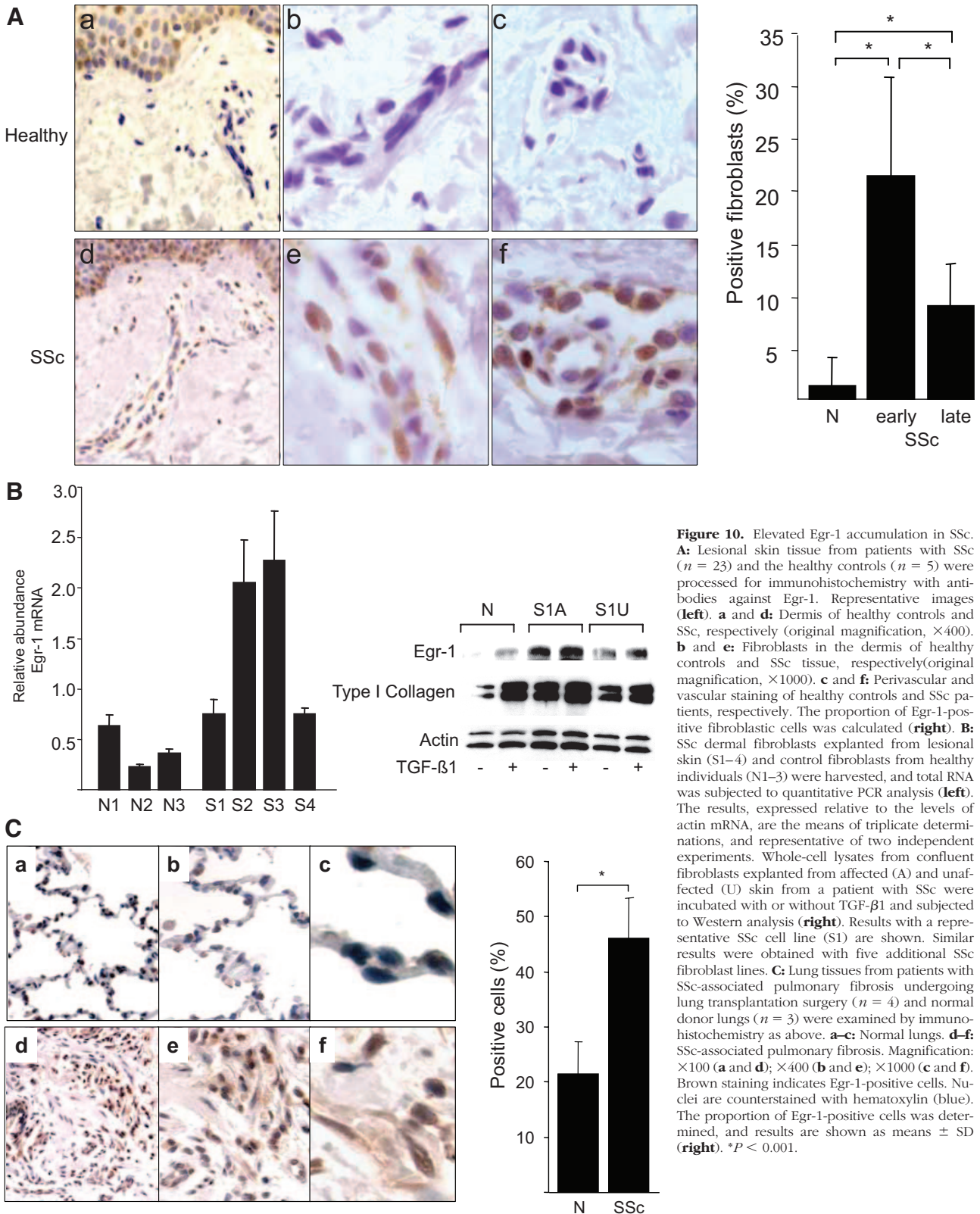
Sample	Age	Sex	Disease duration (years)	SSc subset
Skin tissue immunostaining				
SSc1	59	F	0.40	dcSSc
SSc2	44	F	1.80	dcSSc
SSc3	21	F	6.00	dcSSc
SSc4	42	F	0.58	dcSSc
SSc5	44	F	0.40	lcSSc
SSc6	49	F	2.00	lcSSc
SSc7	54	M	3.00	dcSSc
SSc8	64	M	0.17	dcSSc
SSc9	30	F	2.5	dcSSc
SSc10	24	F	9.00	dcSSc
SSc11	73	F	0.20	dcSSc
SSc12	43	F	10.00	dcSSc
SSc13	31	F	15.00	lcSSc
SSc14	57	F	10.00	dcSSc
SSc15	54	F	12.00	dcSSc
SSc16	25	F	1.00	dcSSc
SSc17	16	F	7.00	dcSSc
SSc18	55	F	0.30	lcSSc
SSc19	67	M	0.25	dcSSc
SSc20	57	F	3.00	dcSSc
SSc21	30	F	2.50	dcSSc
SSc22	43	M	0.08	dcSSc
SSc23	63	F	0.90	lcSSc
Lung tissue immunostaining				
SSc25	57	M	N/A	dcSSc
SSc29	60	F	N/A	dcSSc
SSc30	58	F	N/A	lcSSc
SSc31	35	M	N/A	SSc sine scleroderma
Explanted skin fibroblasts				
S10	36	F	0.91	lcSSc
S11	46	F	1.00	dcSSc
S12	54	M	1.00	dcSSc
S34	38	F	1.00	dcSSc
SU9	N/A	N/A	<5.00	dcSSc

lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc.

polysaccharide, oxidative stress, ischemia reperfusion, cigarette smoke, and mechanical stretch.<sup>33,34,37,42</sup> The proximal 1 kb of the Egr-1 gene promoter, sufficient to direct tissue-specific Egr-1 expression *in vivo*,<sup>48</sup> harbors five CarG elements, also known as serum response elements (SREs), in addition to binding sites for Sp1, NF- $\kappa$ B, and Smad2/3.<sup>6</sup> The present results identify the distal SRE sites as a principal TGF- $\beta$  response elements for Egr-1. The SRE sites are recognized by serum response factor and ternary complex factors, such as Elk-1 and related Ets family oncogenes.<sup>22</sup> Phosphorylation of cellular Elk-1 by ERK results in enhanced DNA binding activity and SRE-mediated transcription. To induce transcription, the ternary complex factors must bind both to serum response factor and to SRE sequences, and induction of Egr-1 typically depends on cooperative interaction between serum response factor and one or more ternary complex factors. Interestingly, the Egr-1 promoter also contains an Egr-1 binding site, enabling Egr-1 to down-regulate its transcription. Thus, Egr-1 expression is tightly regulated via the positively acting transcription factors serum response factor and Elk-1 and negatively acting factors, including Egr-1, thereby establishing a negative feedback loop restricting the duration of Egr-1 expression.

To better understand Egr-1 regulation in the context of fibrosis, we examined Egr-1 expression in bleomycin-induced murine scleroderma.<sup>49</sup> We noted that whereas Egr-1 protein and mRNA were essentially undetectable in BS-treated control mice, levels were elevated in lesional skin from bleomycin-treated mice, with expression localized principally to fibroblastic cells. Although the pathogenesis of skin fibrosis in this mouse model of scleroderma is not fully understood, transient local inflammation and TGF- $\beta$  production and activity are considered to be important, and fibrosis is attenuated in Smad3-null mice.<sup>14</sup> Therefore, elevated Egr-1 expression in lesional skin may be a reflection of local TGF- $\beta$  activity or persistent fibroblast stimulation. In addition to TGF- $\beta$ , cytokines such as IL-13 are also known to contribute to bleomycin-induced fibrogenesis, and IL-13 is a potent inducer for Egr-1 gene expression.<sup>50</sup> Chronic tissue hypoxia and ischemia reperfusion are prominent features of scleroderma that are also capable of stimulating Egr-1, and thus may account for the sustained expression of Egr-1 in fibrotic lesions.<sup>51,52</sup> Additionally, bleomycin itself may directly regulate Egr-1 expression.<sup>52</sup>

Little or no Egr-1 expression was detected in normal adult skin or nonfibrotic lung tissue. Normally, Egr-1 expression and activity are tightly regulated by endogenous



inhibitors, such as NAB2, which is induced by the same signals that also regulate Egr-1, and by Egr-1 itself which can repress its own transcription. In scleroderma lesional tissues, Egr-1 was consistently detected, with highest

levels in patients with early and progressive disease, supporting a functional role for Egr-1 in the development and progression of fibrosis. Transcriptional profiling of lesional skin tissue from patients with early-stage sclero-

derma using cDNA microarrays showed elevated expression of Egr-1 mRNA (F. Tan, unpublished information).<sup>53</sup> Whereas lungs from patients without fibrosis showed only low levels of Egr-1, detected in alveolar macrophages and occasional lining cells, fibrotic lesions from scleroderma lungs consistently showed strong Egr-1 staining in stromal cells.

Scleroderma skin fibroblasts displayed elevated Egr-1 mRNA and protein *in vitro*, although heterogeneity from one cell line to another was observed. A previous study similarly demonstrated overexpression of Egr-1 associated with increased levels of TGF- $\beta$  and connective tissue growth factor in lung fibroblasts from patients with chronic obstructive pulmonary disease.<sup>43</sup> It has been proposed that the scleroderma fibroblast phenotype reflects constitutive autocrine stimulation by TGF- $\beta$ .<sup>54,55</sup> Thus, an autocrine TGF- $\beta$  stimulatory loop might underlie the persistent expression of Egr-1 in explanted scleroderma fibroblasts. Moreover, Egr-1, by virtue of its capacity to directly stimulate TGF- $\beta$  production and expression of type 2 TGF- $\beta$  receptor, might itself further contribute to autocrine TGF- $\beta$  signaling. However, the strong TGF- $\beta$  activation signature detected in transcriptional analysis of SSc skin biopsies was largely extinguished in cultured fibroblasts explanted from the same lesions.<sup>53,56</sup> Currently, we are exploring the mechanisms for constitutive Egr-1 up-regulation in scleroderma fibroblasts.

Although most tissues demonstrate low basal Egr-1 expression, Egr-1 is readily inducible by acute injury and stress as an early and transient response. The same stimuli that induce Egr-1 expression normally also induce its inhibitors, such as Nab2 and Egr-1, and this negative feedback on Egr-1 activity ensures its rapid extinction. Although short-lived Egr-1 expression and activity orchestrate adaptive tissue response to acute injury, persistent Egr-1 expression results in unchecked target gene activation that contributes to tissue damage. For instance, up-regulated Egr-1 expression in emphysema might be involved in aberrant tissue remodeling,<sup>57</sup> persistent Egr-1 expression in vascular smooth muscle cells contributes to atherosclerotic injury,<sup>58</sup> and in bowel mucosal cells, it plays a role in chronic inflammation in inflammatory bowel disease.<sup>59</sup> Furthermore, in contrast to acute Egr-1 induction that stimulates angiogenesis, sustained Egr-1 expression blocks it.<sup>41,60</sup> We have recently found that Egr-1-null mice are partially protected from fibrosis in the bleomycin-induced model of scleroderma (unpublished data). Therefore, persistent Egr-1 expression or activity in lesional tissue might contribute to progression of TGF- $\beta$ -induced fibrosis and could also suppress local vascular repair contributing to vascular rarefaction characteristic of scleroderma.<sup>61</sup>

In summary, the present results show that TGF- $\beta$  is a potent inducer of Egr-1 transcription and Egr-1 accumulation. Stimulation involves a Smad-independent MAPK signaling pathway comparable with Egr-1 regulation associated with acute cellular stress. The upstream components of this pathway that link the activated TGF- $\beta$  receptors to MEK1 are currently unknown. It is relevant to note in this regard that induction of Egr-1 by oxidative

stress was shown to involve the nonreceptor tyrosine kinase c-Abl.<sup>62</sup> Because c-Abl can also be activated by TGF- $\beta$  (W. Ishida, S. Bhattacharyya, and J. Varga, unpublished data), this tyrosine kinase therefore might be responsible for coupling TGF- $\beta$  to the MEK-ERK-Elk-1 pathway for inducing Egr-1. We are currently exploring this mechanism. The expression of Egr-1, normally tightly regulated, was elevated in bleomycin-induced murine scleroderma, in explanted SSc dermal fibroblasts, and in fibrotic skin or lung tissue from patients with SSc. The inducibility of Egr-1 by TGF- $\beta$  and by IL-13; its capacity to stimulate production of extracellular matrix genes (collagen and fibronectin), enzymes (matrix metalloproteinases and plasminogen activator inhibitor-1), and fibrogenic cytokines (TGF- $\beta$  and platelet-derived growth factor); and its persistent expression in fibrotic lesions all point to its potential importance in fibrosis. Blocking Egr-1 expression or biological activity appears to be a potential strategy to control pathological fibrogenesis. Significantly, several drugs in current clinical use have potent inhibitory effects on Egr-1 expression or activity. These drugs include mycophenolate mofetil,<sup>63</sup> cyclosporine,<sup>64</sup> simvastatin,<sup>65</sup> and insulin-sensitizing peroxisome proliferator-activated receptor- $\gamma$  ligands, such as rosiglitazone (M. Wu and J. Varga, unpublished data).<sup>65</sup> Because Egr-1-null mice have no obvious phenotype, it might be feasible to use such drugs to therapeutically target abnormal Egr-1 expression or function in fibrosis with little ill effect.

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