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Early Growth Response 3 (Egr-3) Is Induced by Transforming Growth Factor-β and Regulates Fibrogenic Responses

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Members of the early growth response (Egr) gene family of transcription factors have nonredundant biological functions. Although Egr-3 is implicated primarily in neuromuscular development and immunity, its regulation and role in tissue repair and fibrosis has not been studied. We now show that in normal skin fibroblasts, Egr-3 was potently induced by transforming growth factor-β via canonical Smad3. Moreover, transient Egr-3 overexpression was sufficient to stimulate fibrotic gene expression, whereas deletion of Egr-3 resulted in substantially attenuated transforming growth factor-β responses. Genome-wide expression profiling in fibroblasts showed that genes associated with tissue remodeling and wound healing were prominently up-regulated by Egr-3. Notably, <5% of fibroblast genes regulated by Egr-1 or Egr-2 were found to be coregulated by Egr-3, revealing substantial functional divergence among these Egr family members. In a mouse model of scleroderma, development of dermal fibrosis was accompanied by accumulation of Egr-3–positive myofibroblasts in the lesional tissue. Moreover, skin biopsy samples from patients with scleroderma showed elevated Egr-3 levels in the dermis, and Egr-3 mRNA levels correlated with the extent of skin involvement. These results provide the first evidence that Egr-3, a functionally distinct member of the Egr family with potent effects on inflammation and immunity, is up-regulated in scleroderma and is necessary and sufficient for profibrotic responses, suggesting important and distinct roles in the pathogenesis of fibrosis. *(Am J Pathol 2013, 183: 1197–1208; http://dx.doi.org/10.1016/j.ajpath.2013.06.016)*

Scleroderma or systemic sclerosis is an acquired connective tissue disease of unknown etiology associated with fibrosis in the skin and internal organs.[1–3] Fibrosis is due to persistent activation of fibroblasts and α-smooth muscle actin (α-SMA)–positive myofibroblasts, resulting in excessive production and accumulation of collagen and extracellular matrix (ECM) components in target tissues. There is no effective therapy to prevent or control the progression of fibrosis in scleroderma. Transforming growth factor-β (TGF-β) is a potent inducer of ECM production, myofibroblast differentiation, and epithelial-mesenchymal transition and is implicated in physiologic and pathologic tissue repair.[4,5] Although the canonical Smad pathway is fundamental in mediating TGF-β response in fibroblasts, the complex intracellular signaling networks underlying pathologic fibrosis remain incompletely understood.

Early growth response (Egr) transcription factors regulate a wide range of biological processes. The Egr family comprises Egr-1 (NGFI-A, Krox-24), Egr-2 (Krox-20), Egr-3, and Egr-4 (NGFI-C), along with their endogenous inhibitors nerve growth factor–induced protein A (NGFI-A) binding proteins NAB1 and NAB2.[6,7] The expression of Egr proteins is induced in a variety of cell types in response to growth factors, cytokines, hypoxia, and mechanical forces associated with injury and stress. Egr-1, Egr-2, and Egr-3 share a conserved zinc-finger DNA binding domain that recognizes a 9-bp GC-rich Egr response element found in multiple target gene promoters.[8] Induction of Egr-1 is characteristically rapid

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and transient,[9] whereas induction of Egr-2 and Egr-3 is delayed and sustained.[10,11] Despite their structural similarities and shared mechanisms of regulation, these three members of the Egr family are functionally nonredundant in some systems[10,12] and redundant in others.[13,14]

To date, Egr-3 has been studied primarily in the context of central nervous system development and in muscle stretch receptor function, angiogenesis, cancer, and immunity. Egr-3 has an essential role in learning and memory processing.[15] Egr-3−deficient mice are ataxic and lack muscle stretch receptors.[16,17] Egr-3 also has a major role in immunity,[18] and its interaction with the forkhead transcription factor FoxO3a is required for T-cell anergy.[19] The previous finding that ectopic Egr-3 expression in myoblasts caused potent stimulation of the expression of TGF-β1 and collagen genes potentially implicates Egr-3 in connective tissue homeostasis and tissue repair.[20] The present studies were undertaken to explore the expression and regulation of Egr-3 in the context of fibrogenesis and its function in profibrotic TGF-β signaling. The results show that in normal fibroblasts, TGF-ß was a potent inducer of Egr-3 expression via the canonical Smad pathway, and Egr-3 elicited marked profibrotic responses in these cells. Levels of Egr-3 were significantly up-regulated in scleroderma skin biopsy samples and in lesional tissue from mice with bleomycin-induced scleroderma. Taken together, these findings identify Egr-3 as a novel TGF-ß−inducible transcription factor with potent profibrotic effects and altered expression in scleroderma, suggesting a previously unsuspected role in pathogenesis.

Materials and Methods

Cell Culture and Reagents

Primary cultures of dermal fibroblasts were established by explantation from skin biopsy samples from healthy adults or from neonatal foreskin specimens.[21] The protocols for skin biopsies were approved by the Institutional Review Board at Northwestern University (Chicago, IL). Skin fibroblasts from 4-week-old Egr-3−null mice[16] and wild-type littermates, mouse embryonic fibroblasts from Smad3-null mice,[22] and human fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland), 50 μg/mL of penicillin, and 50 μg/mL of streptomycin in a humidified atmosphere of 5% CO_2 at 37°C and were studied between passages 2 and 8.[21] At confluence, fresh serum-free media supplemented with 0.1% bovine serum albumin were added to the cultures for 24 hours before the addition of TGF-β2 (PeproTech, Rocky Hill, NJ). The ß2 isoform of TGF-β was used in these studies because we had previously shown its robust effects on induction of fibrotic gene expression in a variety of cell types.[10]

RNA Isolation and qPCR

At the end of each experiment, cultures were harvested and RNA was isolated using RNeasy Plus mini kits (Qiagen Inc., Valencia, CA) and examined by real-time quantitative PCR (qPCR).[23] One microgram of RNA was used for cDNA synthesis in 20 μL of reaction volume using cDNA Synthesis SuperMix (Quanta BioSciences, Gaithersburg, MD). Eight microliters of cDNA, 2 μL of primers (2 μmol/L each), and 10 μL of 2× Power SYBR master mix (Applied Biosystems, Foster City, CA). qPCR was performed in triplicate using an ABI 7300 thermocycler (Applied Biosystems). Data were normalized to 18S RNA, and fold change was represented as

$$2^{-\Delta\Delta Ct}\left(2^{-[(\text{Ct target}-\text{Ct 18S})\text{treatment} - (\text{Ct target}-\text{Ct 18S})\text{ non}-\text{treatment}]}\right).$$

Western Blot Analysis

At the end of each experiment, fibroblasts were harvested, and whole cell lysates were examined by Western blot analysis.[10] Antibodies to Egr-3 (Santa Cruz Biotechnology, San Francisco, CA), type I collagen (SouthernBiotech, Birmingham, AL), α-SMA (Sigma-Aldrich, St. Louis, MO), and glyceraldehyde-3-phosphate dehydrogenase (Zymed Laboratories Inc., San Francisco, CA) were used for immunoblotting. Bands were detected using electrochemiluminescence reagents (Pierce Biotechnology, Rockford, IL).

ELISAs

Supernatants from confluent cultures of fibroblasts infected with adenovirus (Ad)−Egr-3 or adenovirus containing green fluorescent protein (Ad-GFP) as control for 48 hours were collected and stored at −80°C. Samples were thawed at room temperature, and levels of secreted TGF-ß1 were determined in triplicate using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Confocal Immunocytochemical Analysis

Ten thousand fibroblasts per well were seeded onto 8-well Lab-Tek II chamber glass slides (Nalge Nunc International, Naperville, IL) and were incubated in serum-free Eagle's minimal essential medium with 0.1% bovine serum albumin for 16 hours. Fresh media with 10 ng/mL of TGF-β2 were then added, and the incubations were continued for a further 4 hours. At the end of the experiments, cells were fixed, permeabilized, and incubated with primary antibodies to Egr-3 at 1:200 dilution (Santa Cruz Biotechnology) or to type I collagen at 1:500 dilution (SouthernBiotech).[24] Cells were then washed with PBS and incubated with secondary antibodies at 1:500 dilution (Alexa Fluor 488 and 594; Invitrogen, Carlsbad, CA). Slides were examined using a Nikon C2 confocal laser scanning microscope (Nikon Corp, Tokyo, Japan).[25] Images were obtained using Nikon C2 software version 1 (Nikon Corp). Immunofluorescence signal intensities were quantified using ImageJ version 1.46r (NIH, Bethesda, MD).

Table 1 Clinical Features of Patients with Scleroderma

Study code	Age (years)/sex	Duration (years)*	Skin score†
N998	27/F	NA	NA
N999	38/M	NA	NA
N1004	37/F	NA	NA
SScMH_06	54/F	3	16
SScMH_15	63/F	5	36
SScMH_17	53/M	1	35
SScMH_37	46/F	0	13
SScReg_1213	30/F	5	4
SScReg_1204	45/F	1	23

*Duration from first non—Raynaud disease manifestation.
†Modified Rodnan skin score (range, 0—51).
F, female; M, male; NA, not applicable.

Plasmids and Transient Transfection Assays

The Col1A1-luc harbors the truncated human COL1A1 promoter with 5′ ends at −759 bp fused to luciferase.[26] α-SMA—luc harbors a 1.1-kb promoter fragment from the mouse α-SMA gene upstream of luc.[27] Fibroblasts at 70% confluence were transfected with the indicated reporter constructs using the SuperFect transfection kit (Qiagen Inc.). After 24 hours, fibroblasts were infected with Ad—Egr-3 or control adenovirus.[20] Cultures were incubated in serum-free media containing 0.1% bovine serum albumin for 24 hours. Cultures were harvested, and whole cell lysates were assayed for luciferase activities using the dual-luciferase reporter assay system (Promega Corp., Madison, WI). In each experiment, Renilla luciferase pRL-TK (Promega Corp.) was cotransfected as control for transfection efficiency.[28] Transient transfection experiments were performed in triplicate and were repeated at least twice with consistent results.

Microarray Procedures and Data Analysis

To examine genome-wide gene expression changes induced by Egr-3, serum-starved confluent fibroblasts were infected with Ad—Egr-3 or adenovirus containing green fluorescent protein (Ad-GFP) at 50 multiplicity of infection,[20] a concentration found in preliminary experiments to be optimal for achieving high levels of cellular Egr-3 expression (data not shown). After 48 hours of incubation, when close to 100% of infected fibroblasts showed strong GFP expression, cultures were harvested and total RNA was isolated using RNeasy Plus mini kits (Qiagen Inc.). The integrity of RNA was determined using Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Fluorescently labeled cDNA was prepared (Ambion, Austin, TX) and was hybridized to Illumina Human HT-12 version 3 microarray chips containing 44,000 probes (Illumina Inc., San Diego, CA). Raw signal intensities for each probe were obtained using Illumina BeadStudio data analysis software and were imported to the Bioconductor lumi package for transformation and normalization.[29—31] The data were preprocessed using a variance stabilization transformation method[31] followed by quantile normalization. Data from probes that produced signals near or below background levels (estimated based on Illumina-negative control probes) with all samples were discarded. Genes that showed >1.5-fold up- or down-regulation at 48 hours in Egr-3—expressing fibroblasts compared with control fibroblasts were subjected to further analysis.

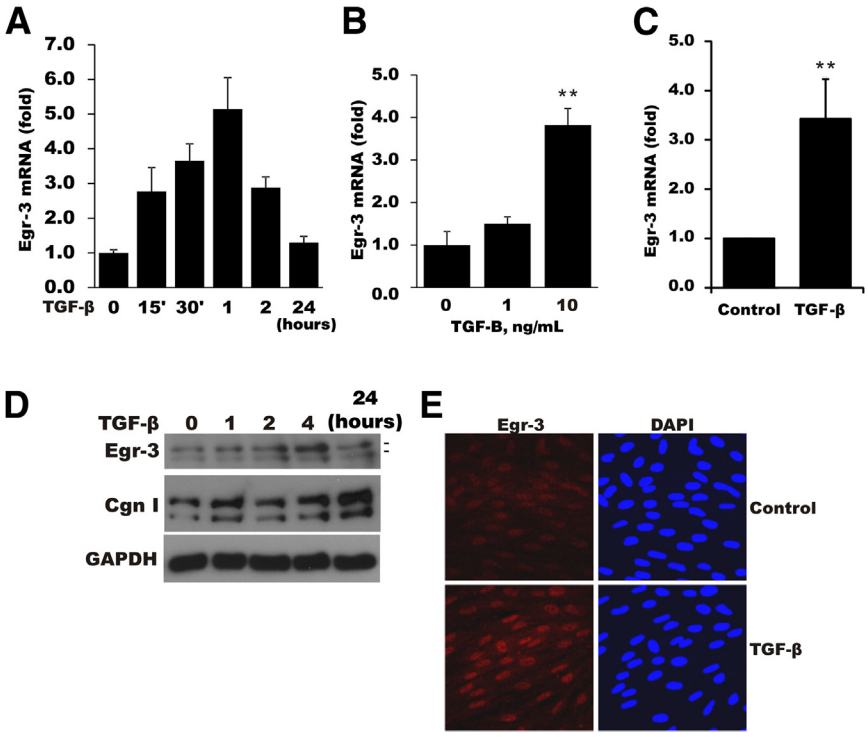

Figure 1 TGF-β stimulated Egr-3 expression. Confluent foreskin (**A, B, D,** and **E**) and healthy adult skin (**C**) fibroblasts were incubated in serum-free media with 10 ng/mL of TGF-β2 (unless otherwise indicated) for the indicated periods (**A** and **D**), 60 minutes (**B** and **C**), and 4 hours (**E**). **A—C:** Total RNA was subjected to qPCR. The results represent the means ± SEM of triplicate determinations from three independent experiments. $^{**}P < 0.01$. **D:** Whole cell lysates were subjected to Western blot analysis. Representative blot with the two Egr-3 isoforms indicated. **E:** Fibroblasts were immunostained with antibodies to Egr-3 (red) or were stained with DAPI (blue). Representative immunofluorescence photomicrographs. Original magnification, ×60.

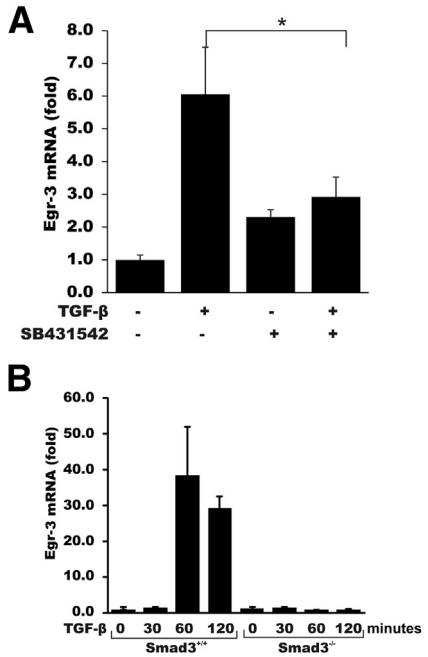

Figure 2 Smad-dependent stimulation of Egr-3 expression. **A:** Confluent skin fibroblasts were preincubated for 30 minutes with 10 μmol/L SB431542, followed by 10 ng/mL of TGF-β2 for a further 60 minutes before RNA isolation and qPCR. The results represent the means ± SEM of triplicate determinations. $*P < 0.05$. **B:** Fibroblasts from Smad3-null or wild-type mouse embryos in parallel were incubated with 10 ng/mL of TGF-β2 before RNA harvesting. The results represent the means ± SEM of triplicate determinations from three independent experiments.

To compare Egr-3–regulated genes with those regulated by Egr-1, Egr-2, or TGF-β, human skin fibroblasts were infected with Ad–Egr-1, Ad–Egr-2, or Ad–Egr-3 or were incubated with 10 ng/mL of TGF-β for 48 hours, and RNA was isolated for microarray analysis.[10,24] Bioinformatics analysis was performed to generate a list of genes showing a >1.5-fold change in expression in treated versus control cultures. Gene lists for each treatment were entered into Microsoft Excel 2010 spreadsheets (Microsoft Corp., Redmond, WA) and were analyzed using the vlookup function to generate lists of genes regulated by Egr-1, Egr-2, and Egr-3 or both Egr-3 and TGF-β.

Microarray Data Analysis of Scleroderma Skin Biopsy Samples

Expression of Egr-1, Egr-2, or Egr-3 mRNA was interrogated in publicly available scleroderma skin biopsy–derived genome-wide expression microarray data sets (Gene Expression Omnibus; *http://www.ncbi.nlm.nih.gov/geo*; accession number GSE9285).[32]

Regulation of Egr-3 Expression *in Vivo*

To examine the regulation of Egr-3 expression *in vivo*, scleroderma was induced in 8-week-old female BALB/c mice by bleomycin injections (The Jackson Laboratory, Bar Harbor, ME).[33] The animal protocols were institutionally approved by the Northwestern University Animal Care and Use Committee. Twenty micrograms of filter-sterilized bleomycin per mouse (dissolved in PBS) (Mayne Pharma, Paramus, NJ) or PBS was administered by s.c. injections for 14 days, mice were sacrificed on day 21, and lesional skin was harvested.[34] Each experimental group consisted of more than three mice. Four-micron sections were processed for immunohistochemical analysis using primary antibodies against Egr-3 (Santa Cruz Biotechnology) or α-SMA (Sigma-Aldrich) at 1:200 dilution. Secondary antibodies labeled with Alexa Fluor 488 (Invitrogen) at 1:200 or 1:500 dilution were used to detect bound antibodies. Substitution of the primary antibodies with isotype-matched irrelevant IgG at the same concentration served as negative controls. Sections were viewed under a Nikon C2 microscope,[25] and images were acquired using Nikon C2 software. Immunopositive cells were counted by a blinded observer (B.Y.) in five randomly chosen high-power fields.

To assess Egr-3 expression in scleroderma, skin biopsy samples from the affected forearm of patients with early-stage diffuse cutaneous scleroderma ($n = 6$) or healthy adults ($n = 3$) (Table 1) were obtained under protocols approved by the Institutional Review Boards for Human Studies at Northwestern University. Clinical features of the participants are shown in Table 1. Five-micron-thick paraffin-embedded or frozen sections were immunostained with primary antibody to Egr-3 (Santa Cruz Biotechnology) followed by horseradish peroxidase–labeled secondary antibody (Invitrogen). Preincubation of the slides with preimmune serum served as negative control. Images were acquired using a Zeiss Axioskop microscope with a CRi *Nuance* spectral

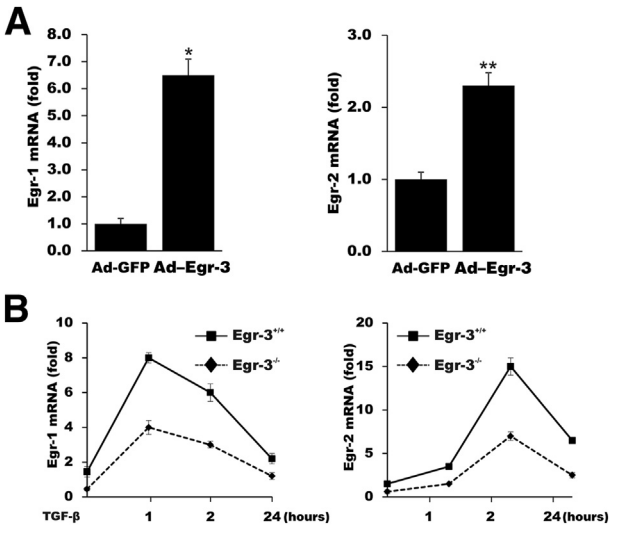

Figure 3 Egr-3 regulated Egr-1 and Egr-2 mRNA expression. **A:** Confluent fibroblasts were infected with Ad–Egr-3 or Ad-GFP. After 48 hours of incubation, fibroblasts were harvested and RNA was subjected to qPCR analysis. The results are shown as fold change compared with control Ad-GFP. $*P < 0.05$, $**P < 0.01$. **B:** Confluent wild-type ($Egr\text{-}3^{+/+}$) and $Egr\text{-}3^{-/-}$ mouse skin fibroblasts in parallel were incubated with 10 ng/mL of TGF-β2 for the indicated periods. Total RNA was subjected to qPCR. The results represent the means ± SEM of triplicate determinations from three independent experiments.

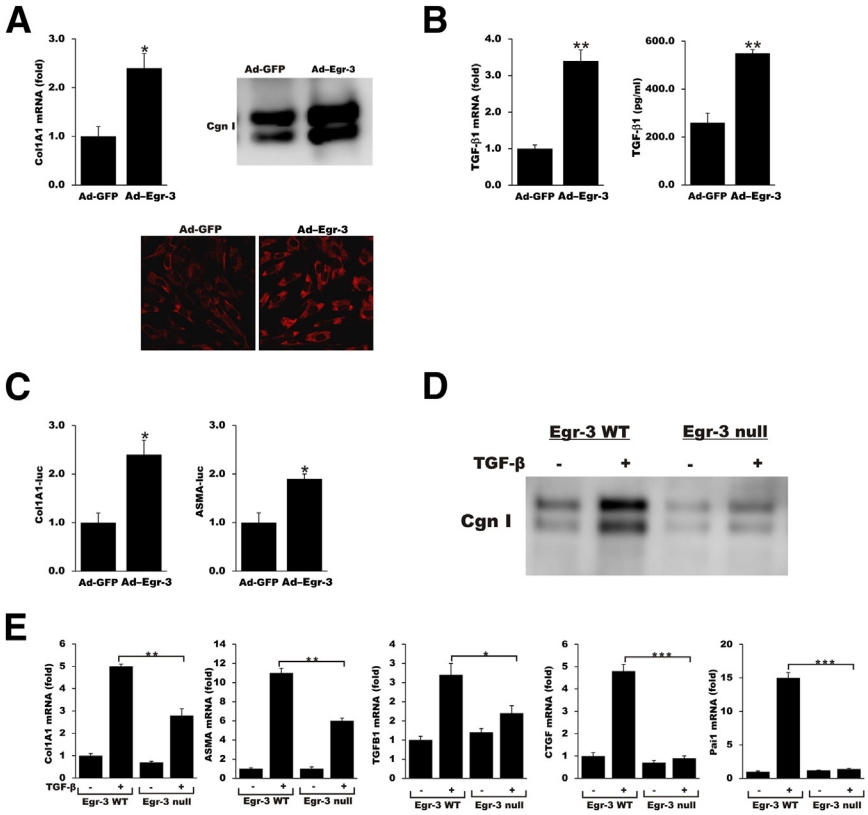

Figure 4 Egr-3 was sufficient and necessary for the stimulation of fibrotic gene expression. **A** and **B**: Egr-3 stimulates fibrotic gene expression. Confluent dermal fibroblasts were infected with Ad-Egr-3 or Ad-GFP for 48 h. **A**: Real-time qPCR (left upper panel). The results of real-time qPCR are shown as -fold change compared to control, and represent the means ± SEM of triplicate determinations from three independent experiments. Western immunoblot (right upper panel). Confocal microscopy after immunostaining with antibodies to Type I collagen and DAPI. Original magnification × 60 (lower panels). **B**: Egr-3 stimulated TGF-ß production, shown using qPCR and ELISA. **C**: Fibroblasts transfected with Col1A1-luc or α-SMA—luc were infected with Ad—Egr-3 or Ad-GFP for 48 hours, and whole cell lysate were assayed for luciferase activity. **D** and **E**: Reduced TGF-ß responses in fibroblasts lacking Egr-3. *Egr-3*—null and wild-type (WT) fibroblasts in parallel were incubated with 10 ng/mL of TGF-ß2 for 24 hours. Supernatants were analyzed by Western immunoblot (**D**) or qPCR (**E**). The results are shown as fold change compared with control and represent the means ± SEM of triplicate determinations from three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

camera (Carl Zeiss MicroImaging GmbH, Jena, Germany). The pattern and expression of Egr-3 staining was scored by a blinded observer (B.Y.) in five randomly chosen high-power fields. Preimmune serum was used in place of primary antibody as negative controls.

Statistical Analysis

Each experiment was repeated at least three times. Statistical analysis was performed using the Student's *t*-test. Results are shown as means ± SEM; $P < 0.05$ was considered statistically significant.

Results

TGF-β Stimulates *Egr-3* Gene Expression

To shed light on the regulation and function of Egr-3 in the context of fibrogenesis, neonatal foreskin fibroblasts at confluence were incubated with TGF-β for up to 48 hours. The results of qPCR analysis showed that TGF-β stimulated Egr-3 mRNA expression in a time- and dose-dependent manner (Figure 1A—C). A maximal fivefold increase was seen at 60 minutes, followed by decline to near basal levels by 24 hours. Adult skin fibroblasts incubated with TGF-β showed an identical pattern of Egr-3 stimulation (data not shown). Western blot analysis indicated a time-dependent increase in Egr-3 protein levels in TGF-β—treated fibroblasts, with a maximal greater than threefold increase at 4 hours, followed by a gradual decline (Figure 1D). The two Egr-3 isoforms representing different transcription start sites of the *Egr-3* gene[35] showed comparable responses to TGF-β. Confocal microscopy confirmed an approximately threefold increase in Egr-3 localized mostly in the nucleus (Figure 1E).

Intracellular Pathways Mediating Egr-3 Stimulation

To elucidate the intracellular signaling mechanisms underlying the stimulation of Egr-3 expression by TGF-β, the effects of pharmacologic and genetic disruption of Smad signaling were examined. Pretreatment of fibroblasts with 10 nmol/L SB431542, a selective inhibitor of ALK5-mediated Smad 2/3 phosphorylation, significantly attenuated the stimulation of Egr-3 mRNA (Figure 2A). To further investigate the role of Smad2/3 in the stimulation of Egr-3, we examined the effect of TGF-ß in mouse embryonic fibroblasts lacking Smad3. Incubation of Smad3-null mouse embryonic fibroblasts with TGF-β resulted in a dramatically reduced magnitude of Egr-3 stimulation compared with wild-type fibroblasts (Figure 2B). These results indicate a dominant role for the canonical Smad2/3 pathway in the regulation of Egr-3 by TGF-β.

Egr-3 Induces *Egr-1* and *Egr-2* Gene Expression

Members of the *Egr* gene family typically exist in functionally linked intracellular networks and coregulate each other's expression in many cell types.[10,36,37] To examine whether Egr-3 has a role in regulating the expression of other Egr genes, the regulation of *Egr-1* and *Egr-3* was examined

Table 2 Top 40 Genes Up-Regulated by Egr-3

Symbol	Description	Fold change
KISS1R	KISS1 receptor	11.2
SBSN	Suprabasin	7.6
NPW	Neuropeptide W	6.9
LFNG	LFNG O-fucosylpeptide 3-β-N-acetylglucosaminyltransferase	5.0
HES4	Hairy and enhancer of split 4 (Drosophila)	4.9
HCFC1R1	Host cell factor C1 regulator 1 (XPO1 dependent)	4.7
PENK	Proenkephalin	4.3
FGFR3	Fibroblast growth factor receptor 3	4.2
SGK223	Homolog of rat pragma of Rnd2	3.9
CST6	Cystatin E/M	3.7
CUEDC1	CUE domain containing 1	3.7
PRRX2	Paired related homeobox 2	3.6
GPR162	G protein–coupled receptor 162	3.6
STMN3	Stathmin-like 3	3.5
LOC644063	Heterogeneous nuclear ribonucleoprotein K pseudogene	3.5
TSPAN17	Tetraspanin 17	3.4
HBA2	Hemoglobin, α2	3.4
KRT86	Keratin 86	3.3
CCNY	Cyclin Y	3.2
IRF1	Interferon regulatory factor 1	3.1
RTN4R	Reticulon 4 receptor	3.1
SLCO2A1	Solute carrier organic anion transporter family, member 2A1	3.0
NPTX2	Neuronal pentraxin II	3.0
RSPRY1	Ring finger and SPRY domain containing 1	3.0
SLC7A5	Solute carrier family 7 member 5	3.0
ALPL	Alkaline phosphatase, liver/bone/kidney	3.0
SBDSP1	Shwachman-Bodian-Diamond syndrome pseudogene 1	3.0
NPAS1	Neuronal PAS domain protein 1	3.0
DNLZ	DNL-type zinc finger	3.0
C20orf27	Chromosome 20 open reading frame 27	2.9
CCL25	Chemokine (C-C motif) ligand 25	2.9
GRM2	Glutamate receptor, metabotropic 2	2.8
ARC	Activity-regulated cytoskeleton-associated protein	2.8
EIF5	Eukaryotic translation initiation factor 5	2.8
IFI6	Interferon, alpha-inducible protein 6	2.8
TRIB3	Tribbles homolog 3 (Drosophila)	2.7
SUSD2	Sushi domain containing 2	2.7
OSGIN1	Oxidative stress induced growth inhibitor 1	2.7
RAD21	RAD21 homolog (*S. pombe*)	2.7
NFIC	Nuclear factor I/C (CCAAT-binding transcription factor)	2.7

in fibroblasts overexpressing or lacking *Egr-3*. In fibroblasts infected with *Egr-3* adenovirus, qPCR results showed a more than sixfold increase in Egr-1 and an approximately twofold increase in Egr-2 mRNA levels (Figure 3A). Significantly, *Egr-3*–null skin fibroblasts incubated with TGF-β showed attenuated stimulation of *Egr-1* or *Egr-2* gene expression (Figure 3B). In contrast, neither Ad–Egr-1 nor Ad–Egr-2 regulated Egr-3 mRNA expression (data not shown).

Egr-3 Induces Fibrotic Gene Expression and Mediates TGF-β Responses

To examine the potential effect of Egr-3 on the regulation of fibrotic responses, human foreskin fibroblasts were infected with Ad–Egr-3 or Ad-GFP virus, and after 48 hours of incubation, whole cell lysates and total RNA were analyzed by Western immunoblotting and real-time qPCR. The results showed that ectopic Egr-3 induced significant stimulation of Col1A1 mRNA and protein expression (Figure 4A). Egr-3 was also shown to induce significant stimulation of TGF-β mRNA and protein expression (Figure 4B). Furthermore, transient transfection assays demonstrated that Egr-3 was able to transactivate Col1A1 and α-SMA promoters in luciferase assays (Figure 4C), indicating that the stimulatory response was mediated at the transcriptional level. To examine the potential role of Egr-3 as a mediator of the profibrotic responses elicited by TGF-β, skin fibroblasts from *Egr-3*–null mice were studied. Markedly attenuated collagen stimulation by TGF-ß2 in *Egr-3*–null fibroblasts was confirmed by Western blot analysis (Figure 4D). Moreover, qPCR showed that in fibroblasts lacking Egr-3, TGF-ß2 induction of multiple fibrotic genes (*Col1A1*, α-SMA, *TGFβ1, CTGF,* and *PAI-1*) was significantly abrogated (Figure 4E). Together, these results indicate that Egr-3 is sufficient and necessary for TGF-β–induced fibrotic responses.

To investigate the genome-wide effects of Egr-3 on fibroblast function, microarray expression profiling was undertaken. For this purpose, confluent foreskin fibroblasts were infected with Ad–Egr-3 or Ad-GFP, and after 48 hours of incubation, RNA was harvested and hybridized to Illumina microarrays. Analysis of the data showed that Egr-3 overexpression in fibroblasts resulted in a greater than twofold change ($P < 0.01$) in the expression of 638 transcripts of >48,000 probes. The top 40 Egr-3–regulated genes are shown in Table 2 and Table 3.

The set of genes showing >1.5-fold change (up or down) was defined as the "fibroblast Egr-3 signature." These genes were subjected to Gene Ontology (GO) and pathway analysis using DAVID software version 6.7 (*http://david.abcc.ncifcrf.gov*, last accessed January 18, 2013)[38] to categorize them based on their known function. The analysis identified 46 GO functional categories that were significantly enriched with Egr-3–regulated genes ($P < 0.001$). Three of the top GO categories have ascribed function related to ECM homeostasis (Figure 5A). Included in this category are genes with functions related to ECM homeostasis, tissue repair, and fibrosis. Real-time qPCR was performed to confirm changes in the expression of multiple genes listed in Table 2 (*Kiss1R* and *IRF1*) and Table 3 (*MMP-1*) and selected other genes (Figure 5B and Table 4).

Comparative Analysis of Genes Regulated by Egr-3 Versus Egr-1, Egr-2, and TGF-β

Because Egr-1, Egr-2, and Egr-3 exhibit extensive cross talk and also regulate each other's expression to some

Table 3 Top 40 Genes Down-Regulated by Egr-3

Symbol	Description	Fold change
*CFB**	Complement factor B	0.1
*CXCL6**	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	0.1
*IL8**	Interleukin 8	0.1
*CXCL6**	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	0.1
*IL8**	Interleukin 8	0.1
*SOD2**	Superoxide dismutase 2, mitochondrial	0.1
*SLC39A8**	Solute carrier family 39 (zinc transporter), member 8	0.2
SOD2	Superoxide dismutase 2, mitochondrial	0.2
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	0.2
C3	Complement component 3	0.2
CCL2	Chemokine (C-C motif) ligand 2	0.2
*SLC39A8**	Solute carrier family 39 (zinc transporter), member 8	0.2
PTGES	Prostaglandin E synthase	0.2
PTX3	Pentraxin 3, long	0.2
LOC644936	Actin, β pseudogene	0.2
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	0.2
IL6	Interleukin 6 (interferon, β 2)	0.3
TAPBP	TAP binding protein (tapasin)	0.3
LAMB1	Laminin, β 1	0.3
CXCL12	Chemokine (C-X-C motif) ligand 12	0.3
HSD11B1	Hydroxysteroid (11-β) dehydrogenase 1	0.3
MT2A	Metallothionein 2A	0.3
*COL6A2**	Collagen, type VI, α2	0.3
PABPC1	Poly(A) binding protein, cytoplasmic 1	0.3
MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	0.3
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	0.3
CTSD	Cathepsin D	0.3
HLA-B	Major histocompatibility complex, class I, B	0.3
CH25H	Cholesterol 25-hydroxylase	0.3
*COL6A2**	Collagen, type VI, α2	0.3
HLA-F	Major histocompatibility complex, class I, F	0.3
ABI3BP	ABI family, member 3 (NESH) binding protein	0.3
DLGAP4	Discs, large (Drosophila) homolog-associated protein 4	0.3
PKM2	Pyruvate kinase, muscle	0.3
SLC39A14	Solute carrier family 39 (zinc transporter), member 14	0.3
PKM2	Pyruvate kinase, muscle	0.3
COL8A1	Collagen, type VIII, α1	0.3
AKR1B1	Aldo-keto reductase family 1, member B1 (aldose reductase)	0.3
YWHAE	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	0.3
TNFAIP6	Tumor necrosis factor, α-induced protein 6	0.3

*Duplicates are the result of several probes being used for each gene. For details see raw data at GEO (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE9285).

degree,[10,36,37] we sought to compare the fibroblast Egr-3 signature with the signature of genes regulated by Egr-1 and Egr-2 in these cells using microarray analysis. Twenty-two genes were found to be regulated by all three Egr proteins (Figure 6). Among these, 5% of genes regulated by Egr-1 and 4% of genes regulated by Egr-2 were also regulated by Egr-3. One of the genes prominently regulated by all three Egr proteins is procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (*Plod1*), an enzyme that plays a key role in the generation of hydroxylysine aldehyde–derived collagen cross-links and is implicated in fibrosis.[39] Twenty-four genes were coregulated by Egr-3 and TGF-β, including *Egr-2* and α-SMA (Figure 6B).

To examine the potential roles of Egr-1, Egr-2, and Egr-3 in scleroderma, a publicly available expression microarray data set of extensively characterized scleroderma skin biopsy samples was interrogated.[32] Analysis of the data set consisting of 26 scleroderma biopsy samples indicated that Egr-3 mRNA expression was correlated with Egr-2 mRNA level in individual biopsy samples ($R^2 = 0.5205$, $P < 0.01$), whereas no correlation with Egr-1 mRNA ($R^2 = 0.0302$, $P > 0.05$) was found (Figure 6C).

Egr-3 Expression Is Elevated in a Mouse Model of Scleroderma

In light of the profibrotic activities associated with Egr-3 and its role in mediating TGF-ß responses in fibroblasts, we next sought to characterize Egr-3 expression in an experimentally induced model of fibrosis in the mouse.

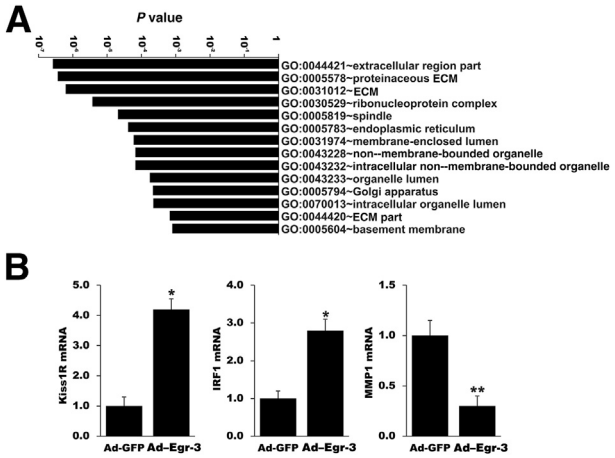

Figure 5 Egr-3 induced genome-wide changes in fibroblast gene expression. **A:** Confluent dermal fibroblasts were infected with Ad—Egr-3 or Ad-GFP for 48 hours, and RNA was hybridized to Illumina chips containing 48,000 probes. Genes showing >1.5-fold change in expression were subjected to GO analysis. Shown are the top 14 GO categories (*P* values were calculated by DAVID software version 6.7). **B:** Expression of selected genes was examined by qPCR analysis using the same RNA as used for the microarray hybridizations. The results represent the means ± SEM. $*P < 0.05$, $**P < 0.01$.

Subcutaneous injection of bleomycin induces progressive scleroderma-like skin and lung fibrosis in BALB/c mice.[40] Bleomycin-injected mice showed increased dermal thickness at 21 days that was associated with a substantial increase in Egr-3 immunostaining (Figure 7A). Examination of sequential slides showed that 26% of Egr-3—positive cells in the dermis also showed positive α-SMA immunostaining (data not shown). Double immunofluorescence staining was used to further characterize the colocalization of Egr-3 and myofibroblasts. The results showed a dramatic increase in Egr-3 nuclear accumulation and α-SMA immunostaining in the lesional skin in bleomycin-injected mice (Figure 7B). Moreover, 38% of Egr-3—positive cells were also positive for α-SMA. Egr-3 expression was also observed in α-SMA—negative cells.

Egr-3 Expression Is Up-Regulated in Scleroderma Skin Biopsy Samples

To examine the expression of Egr-3 in scleroderma, immunohistochemical analysis of skin biopsy samples from patients with scleroderma ($n = 6$) and healthy adults ($n = 3$) in parallel was performed. The clinical features of the patients and controls are shown in Table 1. In the epidermis, comparable Egr-3 staining was seen in scleroderma and control skin biopsy samples, with the most marked expression in the basal layer (Figure 8, A and B). In contrast, in the dermis, strong Egr-3 expression was noted only in scleroderma, but not control, biopsy samples (Figure 8, C and D). The specificity of the anti—Egr-3 antibody was confirmed by Western immunoblots (Figure 1C). Incubation of skin biopsy samples with preimmune serum further demonstrated the specificity of immunostaining.

To evaluate Egr-3 mRNA expression in scleroderma, a previously reported extensively characterized genome-wide expression microarray data set from skin biopsy samples[32] was interrogated. The analysis showed a positive correlation of Egr-3 mRNA levels with the modified Rodnan skin score, a validated index of skin involvement ($R^2 = 0.4213$, $P < 0.01$) (Figure 8E).

Discussion

In this study, we examined the regulation, expression, and function of the transcription factor Egr-3 in the fibrogenesis context. The results demonstrate that the expression of Egr-3 in fibroblasts is directly stimulated by TGF-ß. In contrast to Egr-2, Egr-3 shows rapid and transient induction similar to Egr-1. In normal fibroblasts, Egr-3 elicits a broad fibrogenic response, with potent up-regulation of several genes for collagens and other ECM genes. The fibroblast Egr-3 response gene signature shows only a modest overlap with the pattern of gene regulation elicited by either Egr-1 or Egr-2, highlighting its distinct functional role. In light of its potent profibrotic effects and its elevated expression in lesional scleroderma tissue, and in a mouse model of scleroderma, the present results implicate Egr-3 as a mediator of TGF-ß—regulated fibrosis important in the pathogenesis of scleroderma.

Multiorgan fibrosis causes significant morbidity and mortality in scleroderma.[1—3] Fibrosis is a complex process involving aberrant fibroblast activation, increased synthesis and deposition of collagens and ECM molecules, and differentiation and persistence of contractile myofibroblasts

Table 4 qPCR Confirmation of Differentially Expressed Genes in Egr-3—Overexpressing Fibroblasts

Gene symbol	Fold change	qPCR fold change (means ± SEM)	GO category
IRF1	3.1	2.8 ± 0.3	GO:0060337: type I interferon-mediated signaling pathway
Kiss1R	11.2	4.2 ± 0.4	GO:0051496: positive regulation of stress fiber assembly
α-SMA	2.0	1.8 ± 0.3	GO:0015629: actin cytoskeleton
CTGF	1.8	2.4 ± 0.3	GO:0031012: ECM
Plod1	1.8	2.2 ± 0.4	GO:0030198: extracellular matrix organization
Egr-2	1.8	2.3 ± 0.2	GO:0003700: sequence-specific DNA binding transcription factor activity
MMP1	0.3	0.3 ± 0.1	GO:0031012 ECM
MMP3	0.3	0.2 ± 0.08	GO:0031012 ECM

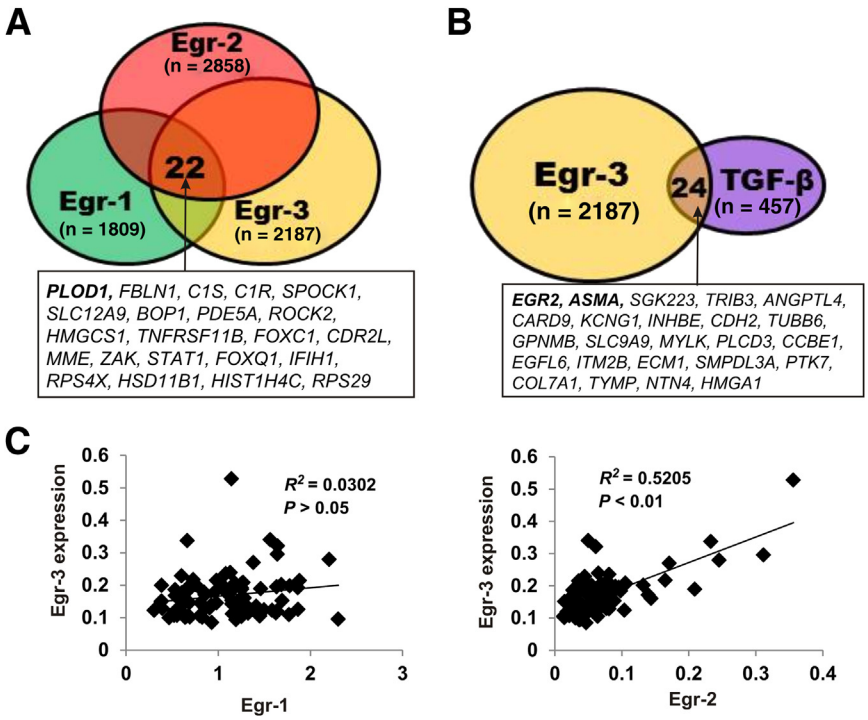

Figure 6 Correlation of Egr expression and Egr signature *in vitro* and in scleroderma biopsy samples. **A** and **B:** Human skin fibroblasts were infected with Ad—Egr-1, Ad—Egr-2, or Ad—Egr-3 or were incubated with 10 ng/mL of TGF-β for 48 hours. RNA was isolated for microarray analysis. The Venn diagrams show the overlap of genes whose expression was up- (**A**) or down-regulated (**B**) >1.5-fold by Egr-3. The boxes list the genes that overlap. **C:** mRNA levels were interrogated in publicly available scleroderma skin biopsy—derived genome-wide expression data sets.[32] Fold change in mRNA levels was normalized with average expression levels of the samples in the entire cohort.

with stiffening of the connective tissue.[41] Substantial evidence implicates TGF-ß as a key trigger that initiates and sustains fibroblast activation and myofibroblast differentiation in pathologic fibrogenesis.[4] In the past decade, a variety of intracellular signaling networks have been identified that mediate profibrotic TGF-ß responses.[42] In addition to the canonical Smad pathway, ERK, FAK, Akt, c-Abl, Egr-1, and Egr-2 have recently also been shown to function as important nodes in TGF-ß—dependent signaling networks that are dysregulated and likely to play important roles in fibrosis.[43] The present studies are the first to demonstrate that Egr-3 is a novel mediator of TGF-ß—regulated fibrogenesis and a potential target for antifibrotic therapy. We show herein that Egr-3 expression is up-regulated in the skin of mice with bleomycin-induced scleroderma and that increased expression is closely associated with myofibroblast accumulation in the lesional dermis. Moreover, elevated Egr-3 expression is observed in skin biopsy samples from patients with scleroderma, and Egr-3 mRNA levels show a positive correlation with the Rodnan skin score.[44] The results show that TGF-ß2 induced the expression of Egr-3, which, in turn, mediated broad changes in fibroblast gene expression. Of interest, these microarray studies demonstrated that the core "fibroblast Egr-3 gene signature" comprising several ECM-related genes overlaps only modestly with the genes regulated by the closely related transcription factors Egr-1 and Egr-2. Together, these observations identify Egr-3 as a functionally distinct factor with a potentially important role in the pathogenesis of scleroderma.

Early growth response genes are implicated in multiple physiologic and pathologic processes.[12] In contrast to Egr-1 and Egr-2, which have been studied in fibroblasts, the function of Egr-3 is poorly understood. Previous studies demonstrating that Egr-3 directly regulates TGF-ß expression in neural cells provided the rationale for investigating the regulation and function of Egr-3 in fibrogenesis.[20] Egr-1, Egr-2, and Egr-3 bind to similar GC-rich DNA sequences via highly homologous zinc finger DNA-binding domains and exhibit functional redundancy in some cells. Previous studies have implicated Egr-3 in inflammation, autoimmunity, angiogenesis, and cancer.[12] Although Egr-3—null mice are viable, 40% die before postnatal day 21.[17] These mice show severe abnormalities in sympathetic nervous system[45,46] and T-cell[46] development. Moreover, Egr-3 has an important role in angiogenesis, and in endothelial cells it is one of the most highly up-regulated genes in response to vascular endothelial growth factor.[47] In addition, Egr-3 has potent effects on inflammation and immunity and negatively regulates T-cell function.[36,48] In T cells, Egr-3 expression is induced by engagement of the T-cell receptor in an NFAT-dependent manner.[19,49] Egr-3—null mice are resistant to anergy induction *in vivo* and show increased susceptibility to autoimmune pneumonitis.[36,48] Indeed, Egr-3 is indispensible for preventing the development of lupus-like autoimmunity in mice.[50] At the same time, mice with lupus-like manifestations have diminished expression of Egr-3, and a peptide that ameliorated the autoimmune phenotype was shown to enhance Egr-3 expression.[51] The inhibitory effect of Egr-3 on $CD4^+$ and $CD8^+$ T-cell effector function involve a dual action of inhibiting the induction of activating genes while simultaneously promoting the expression of inhibitory genes through Sprouty 1.[52] Indeed, in T cells overexpressing Egr-3, Egr-3 binds directly to the Sprouty 1 promoter. Moreover, by

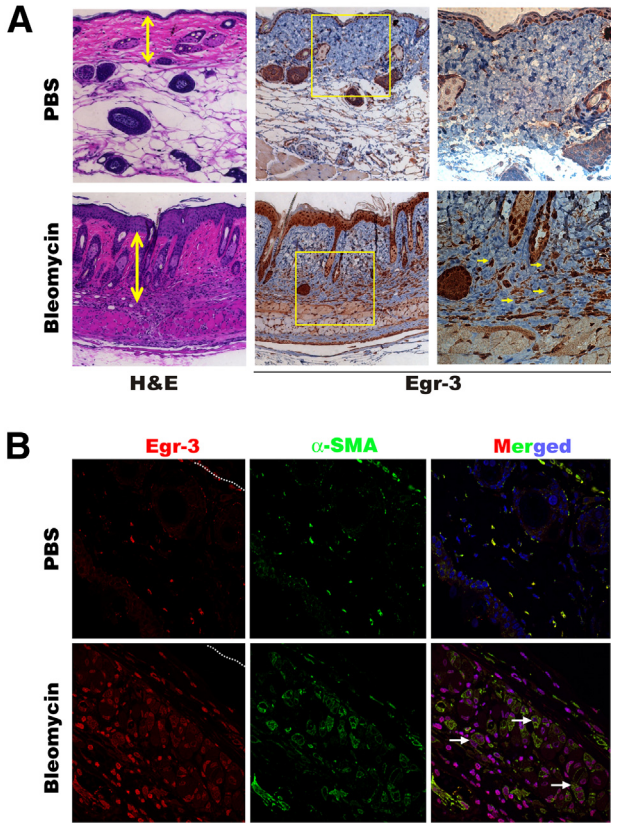

Figure 7 Elevated Egr-3 expression in fibrotic dermis in the mouse. Mice received daily s.c. injections of bleomycin or PBS, and lesional skin was harvested on day 21. **A**: H&E (**left column**) or immunohistochemical analysis using Egr-3 antibody. **Arrows** indicate the extent of the dermis. **Boxed** areas in the **middle column** correspond to higher-magnification images in the **right column**. **B**: Double-labeled immunofluorescence. Sections were immunostained with antibodies to Egr-3 (red), α-SMA (green), or DAPI (blue), and images were acquired using Nikon confocal microscopy. **Arrows** indicate α-SMA– and Egr-3–positive dermal cells. Dotted lines delineate the epidermis. Original magnification: ×100 (**main images, A**); ×400 (**boxed areas, A**); ×600 (**B**).

virtue of its ability to modulate angiogenesis, inflammation, immunity, and apoptosis, Egr-3 is strongly linked with tumorigenesis, and its expression is highly up-regulated in, and is predictive of the outcome of, prostate cancer.[53]

The expression of Egr family proteins is regulated by a variety of hormones, cytokines, and growth factors. We show herein, for the first time, that TGF-β is a potent stimulus for Egr-3 expression. Other mediators of fibrosis, including the canonical Wnt ligand Wnt3a, also stimulates Egr-3 expression (manuscript in preparation). However, TGF-ß regulation of Egr-3, unlike that of Egr-1 and Egr-2, produces a relatively sustained response that is independent of canonical Smad signaling. Moreover, our gain-of-function and loss-of-function studies demonstrated that Egr-3 is capable of modulating the expression of Egr-1 and Egr-2. To identify target genes that are regulated by Egr-3, we performed microarray analysis in normal fibroblasts overexpressing Egr-3. These studies led to the identification of a "fibroblast Egr-3–responsive gene signature" that was significantly enriched with genes implicated in ECM homeostasis. One of the genes most highly up-regulated by Egr-3 was KISS1R, also called G protein–coupled receptor 54 (GPCR54), a receptor for the Kiss ligand.[54] Kiss1R is known to be expressed in the brain (cerebellum, cerebral cortex, and brainstem)[55] and has been implicated in playing roles in metastasis[56] and fertility.[57] Interaction of Kiss1R with EGFR results in stimulation of matrix metalloprotease 9 activity and enhanced breast cancer cell invasiveness.[58] The present results demonstrate that in fibroblasts, ectopic Egr-3 induces Kiss1R, suggesting a potential role for the Kiss ligand-receptor axis in fibrogenesis. Consistent with other reports, we found that the overlap between genes regulated by Egr-3 and those regulated by Egr-1 and Egr-2 is modest,

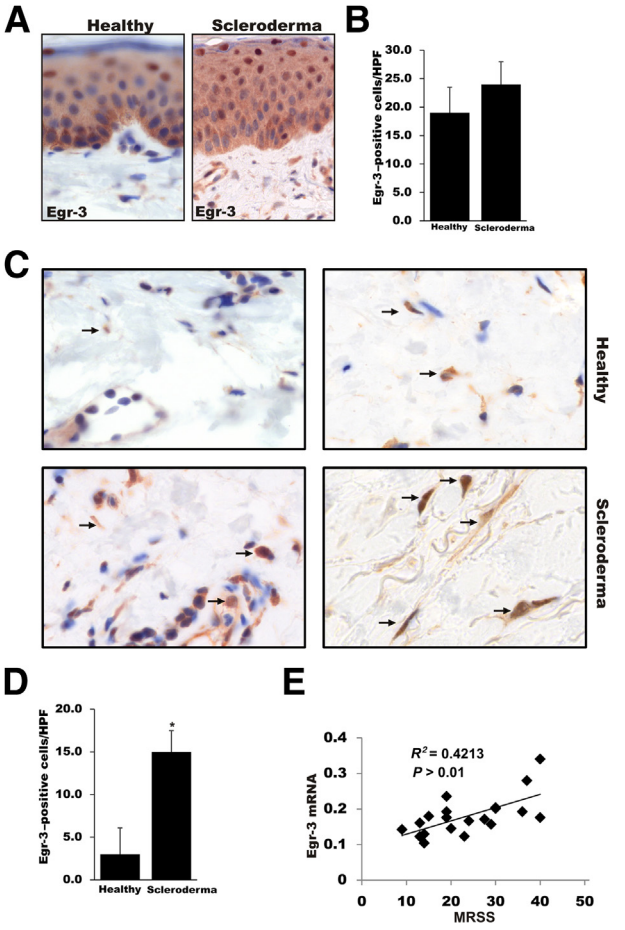

Figure 8 Elevated Egr-3 expression in scleroderma skin. Skin biopsies from healthy individuals ($n = 3$) and patients with scleroderma ($n = 6$) were examined by immunohistochemical analysis using primary antibodies to Egr-3. **A**: Representative images of the epidermis. **B**: Quantification of Egr-3–postive cells in the epidermis. Bars represent means ± SD. **C**: Representative images of dermis. **Arrows** indicate Egr-3–positive cells. **D**: Quantification of Egr-3–positive cells in the dermis. $^*P < 0.05$. **E**: Levels of Egr-3 mRNA in scleroderma skin biopsies were interrogated in a publicly available genome-wide expression microarray data set and were normalized with average expression levels in the entire cohort and correlated with the modified Rodnan skin score (MRSS) for each biopsy sample.[32] Results are the means ± SEM from five randomly chosen high-power fields (HPFs). Original magnification: ×100 (**A**); ×400 (**C**).

indicating a substantial degree of functional heterogeneity among these structurally similar transcription factors.

The present studies are the first to implicate Egr-3 in fibrosis and scleroderma. The results indicate increased expression of Egr-3 in experimental mouse scleroderma, with double-labeling studies demonstrating Egr-3 expression in the fibrotic skin primarily in interstitial myofibroblasts, contractile stromal cells with a crucial role in pathologic tissue remodeling.[59] The present studies show that the number of Egr-3–positive myofibroblasts was markedly increased in the fibrotic skin of bleomycin-treated mice. The findings are consistent with the observation that Egr-3 stimulates the transcription of α-SMA, a key determinant of the myofibroblast phenotype.[59] We also found that Egr-3 expression was markedly up-regulated in skin biopsy samples from patients with scleroderma. Because of the relatively small size of the study cohort, it was not possible in this study to establish a robust correlation between Egr-3 expression and disease activity, progression, or outcome (data not shown); future studies to examine these important questions could be highly informative.

In summary, the present results demonstrate that Egr-3 is a TGF-ß–inducible transcription factor that in fibroblasts induces fibrotic gene expression in response to TGF-β. We demonstrate aberrant Egr-3 expression in a mouse model of scleroderma and in skin biopsies from scleroderma patients. Together, these findings suggest that Egr-3 is a novel component of the intracellular signaling network that, along with Smads, Egr-2, and Eg-1, plays essential roles in orchestrating long-term fibrogenic responses elicited by TGF-ß, and other profibrotic stimuli. Egr-3 appears to have an important and previously unsuspected role in the pathogenesis of scleroderma.

Acknowledgment

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