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Association Between Enhanced Type I Collagen Expression and Epigenetic Repression of the *FLI1* Gene in Scleroderma Fibroblasts

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Objective. **Scleroderma (systemic sclerosis; SSc) is an autoimmune disease characterized by vasculopathy and widespread organ fibrosis. Altered fibroblast function, both in vivo and in vitro, is well documented and illustrated by augmented synthesis and deposition of extracellular matrix proteins. We undertook this study to investigate the possibility that epigenetic mechanisms mediate the emergence and persistence of the altered SSc fibroblast phenotype.**

Methods. **The effects of DNA methyltransferase and histone deacetylase inhibitors on collagen expression and the level of epigenetic mediators in fibroblasts were examined. The effects of transient transfection of SSc fibroblasts with *FLI1* gene and normal cells with *FLI1* antisense construct on collagen expression were determined. The methylation status of the *FLI1* promoter was tested in cultured cells and in SSc and normal skin biopsy specimens.**

Results. **Increased levels of epigenetic mediators in SSc fibroblasts were noted. The addition of epigenetic inhibitors to cell cultures normalized collagen expression in SSc fibroblasts. The augmented collagen synthesis by SSc fibroblasts was linked to epigenetic repression of the collagen suppressor gene *FLI1*. Heavy methylation of the CpG islands in the *FLI1* promoter region was demonstrated in SSc fibroblasts and skin biopsy specimens.**

Conclusion. **The results of this study indicate that epigenetic mechanisms may mediate the fibrotic manifestations of SSc. The signal transduction leading to the SSc fibrotic phenotype appears to converge on DNA methylation and histone deacetylation at the *FLI1* gene.**

The etiology and pathogenesis of scleroderma (systemic sclerosis; SSc) remain unknown. However, the dominant clinical and pathologic features of SSc include progressive tissue fibrosis and widespread vascular disorder. It is generally believed that interstitial fibroblasts mediate tissue fibrosis in SSc, since these cells synthesize excessive amounts of collagen and other components of the extracellular matrix together with reduced expression of matrix metalloproteinases, leading to excessive matrix accumulation (1,2). The basis for the altered cellular phenotype in SSc has not been determined, but primary metabolic abnormalities, responses to abnormal environmental signals, and clonal selection have all been hypothesized to play a role (2). Scleroderma-associated cellular abnormalities have been shown to persist in multiple generations of SSc fibroblasts in vitro and the persistence of the profibrotic phenotype outside the disease environment suggests the possible in vivo imprinting of a profibrotic phenotype that is inherited and is transmitted from one generation of fibroblasts to the next. This inherited phenotype can ultimately lead to the development of clinical tissue fibrosis. In the current report, we present data indicating that an epigenetic alteration in SSc fibroblasts controls cellular events characteristic of these fibroblasts.

The term "epigenetics" describes all inherited changes in gene expression that are not coded in the DNA sequence itself. The 2 major mechanisms that are known to mediate epigenetic changes are DNA methylation and histone modification. Methylation of the CpG dinucleotides has long been recognized as a major epigenetic modification of the mammalian genome, and is implicated in imprinting, X chromosome inactivation, embryonic development, defense against retroviral se-

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quences, transcriptional repression of certain genes, and in carcinogenesis (3,4). CpG islands are stretches of DNA located within the promoter regions of ~40% of mammalian genes. When methylated, they cause a stable, heritable repression of transcription for the affected gene. Methylation is controlled by a number of DNA methyltransferases (Dnmts).

Histone modification has also been identified as an important epigenetic mechanism (5). Posttranslational modifications of histones, including acetylation and methylation of conserved lysine residues on the amino-terminal tail domains, have been studied closely over the last few years. Generally, the acetylation of histones marks active, transcriptionally competent regions, whereas deacetylated histones are found in transcriptionally inactive regions.

There is a direct causal relationship between methylation-dependent transcriptional repression and histone modifications. Thus, sites of DNA methylation are recognized by a family of proteins that contain a highly conserved methyl-CpG DNA binding domain (MBD). MBD-containing proteins recruit chromatin-modifying enzymes, such as histone deacetylase 1 (HDA-1) and HDA-6, which condense chromatin and repress transcription (6).

Given the stable cell dysregulation associated with SSc, we explored potential epigenetic influence on the expression of type I collagen in SSc fibroblasts, using the Dnmt inhibitor 2-deoxy-5-azacytidine (2-deoxy-5-azaC) and the HDA inhibitor trichostatin A (TSA). Examining the nuclear extracts of fibroblasts from SSc and healthy controls, we assessed the levels of Dnmt, MBD, and HDA. Levels of deacetylated histones in the *FLI1* promoter region in fibroblasts were measured by chromatin immunoprecipitation (ChIP) assay. Methylation of promoter region CpG islands was assessed by methylation-specific polymerase chain reaction (PCR) and by sequence analysis of DNA after bisulfite modification. Our results suggest that there are ongoing epigenetic modifications in SSc fibroblasts, which may explain the abnormal cell behavior and offer a new target for therapy.

MATERIALS AND METHODS

Fibroblast cultures. After informed consent was provided and in compliance with the Institutional Review Board of Human Studies, 5-mm skin biopsy specimens were obtained from affected areas (dorsal forearm) of 10 patients with diffuse cutaneous SSc. All patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for SSc (7). Dermal fibroblasts were obtained from the biopsy samples by enzymatically dissociating tissue specimens in 0.25% type I collagenase (Sigma, St. Louis, MO) and 0.05% DNase (Sigma) in Dulbecco's modified Eagle's medium with 20% fetal bovine serum. Normal control dermal fibroblasts were derived from 10 healthy adult donors who were matched with the SSc patients for age, sex, and race. SSc and normal fibroblast pairs were utilized at passages 4–6.

Western blotting analysis. For nuclear extraction, cells were harvested, washed with cold phosphate buffered saline, and lysed with hypotonic buffer (8). Nuclei were collected by centrifugation at 800*g* for 10 minutes at 4°C and resuspended in 200 μl buffer containing 20 m*M* HEPES, 25% glycerol, 0.4*M* NaCl, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, and 0.5 m*M* dithiothreitol (pH 7.9) containing 0.5 m*M* phenylmethylsulfonyl fluoride. The nuclei were extracted on ice for 30 minutes, followed by centrifugation for 30 minutes at 4°C. Supernatant was then collected. For whole cell lysates, cells were lysed in sodium dodecyl sulfate (SDS) sample buffer (62.5 m*M* Tris HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol [pH 6.8]) and heated at 95°C for 4 minutes. Protein concentrations in samples were assessed by RC DC protein assay according to the protocol recommended by the manufacturer (Bio-Rad, Hercules, CA.). Equal amounts of proteins for all of the samples were loaded, separated by SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes.

Standard Western blotting procedures were used (9). The membranes were blocked with 5% nonfat milk in Tris buffered saline with 0.5% Tween 20 and incubated with the primary antibodies for 1 hour at room temperature. Then the membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Specific bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to imaging films (Kodak, Rochester, NY). The following antibodies were used: anti–MBD-2 monoclonal antibody (mAb) (Imgenex, San Diego, CA), anti-Dnmt1 mAb (Novus Biologicals, Littleton, CO), anti–type I collagen (Southern Biotechnology, Birmingham, AL), anti-*FLI1* (sc-356), anti-Dnmt2 (sc-10227), anti-Dnmt3a (sc-10231), anti-Dnmt3b (sc-20274), anti–MBD-1 (sc-1075), anti–methyl-CpG binding protein 2 (anti–MeCP-2) (sc-5755), anti–HDA-1 (sc-8410), anti–HDA-6 (sc-11420), anti–histone H1 (sc-8030), and antitubulin (sc-5274) (all from Santa Cruz Biotechnology).

Reverse transcriptase–PCR (RT-PCR). Total RNA (1 μg) was isolated from either culture cells or biopsy samples using the RNeasy mini-kit (Qiagen, Valencia, CA), and was reverse-transcripted using Moloney murine leukemia virus (MMLV) RT (Promega, Madison, WI) and random hexamers. DNA fragments were amplified by PCR using the following primer pairs: procollagen I 5′-CTGCTGGTCCTAAGGGTGAGC-3′ (forward), 5′-CAGCTTCACCCTTAGCACCAAC-3′ (reverse); *FLI1* (fragment) 5′-GTAAGAATACAGAGCAACGGCCC-3′ (forward), 5′-GGAGGTCCAGTATTGTGATGCG-3′ (reverse); GAPDH (internal control) 5′-GTCCACCACCCTGTTGCTGC-3′ (forward), 5′-CTCTCCCATGACGAACATGG-3′ (reverse). PCR conditions were as follows: 94°C for 5 minutes, followed by 20–30 cycles of 94°C for 30 seconds, 53–60°C for 30 seconds, and 72°C for 30 seconds, and a final step at 72°C for 10 minutes.

Real-time quantitative PCR. SSc and control cells were examined before and after the addition of 2-deoxy-5-azaC (5

μM) for 5 days and TSA (100 ng/ml) for 24 hours. Cells were harvested and lysed in RLT buffer (proprietary lysis buffer; Qiagen). Total RNA was extracted using the RNeasy kit. The concentration of RNA in the samples was ascertained by measuring optical density at 260 nm. Total RNA from each sample was used to generate complementary DNA (cDNA) following treatment with MMLV RT. Briefly, 2 μg of RNA and 1 μg of random primers were mixed and heated to 70°C for 5 minutes, and tubes were immediately placed on ice. Then 5 μl of MMLV 5× reaction buffer, 6 μl of dNTP (40 m*M*), 1 μl of RNase in ribonuclease inhibitor, MMLV RT, and nuclease-free water were added to a final volume of 25 μl and reactions were carried out at 37°C for 60 minutes. The following primers were designed using the Quantitative PCR Primer Database (National Cancer Institute, Bethesda, MD): *FLI1* sense CCCACCAGCAGAAGGTGAAC, antisense ATGCGGCTCCAAAGAAGCT (amplicon size 81 bp, GenBank accession no. NM-002017); *p53* sense TCAACAAGATGTTTTGCCAACTG, antisense ATGTGCTGTGACTGCTTGTAGATG (amplicon size 118 bp, GenBank accession no. NM-000546); s*mad7* sense CCAACTGCAGACTGTCCAGA, antisense CAGGCTCCAGAAGAAGTTGG (amplicon size 106 bp, GenBank accession no. NM-005904); *Col1A1* sense GAACGCGTGTCATCCCTTGT, antisense GAACGAGGTAGTCTTTCAGCAACA (amplicon size 94 bp, GenBank accession no. NM-000088); *GAPDH* sense TGCCAAATATGATGACATCAAGAA, antisense GGAGTGGGTGTCGCTGTTG (amplicon size 121 bp, GenBank accession no. NM-002046).

Real-time PCR was performed with a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The RT-PCR conditions were as follows: 10 minutes at 95°C as an initial polymerase activation step, followed by 40 amplification cycles of 15 seconds at 95°C, and a cycle of 1 minute at 60°C. Individual real-time PCRs were carried out in 25-μl volumes in a 96-well plate (Applied Biosystems) containing 2× SYBR Green PCR Master Mix (25 μl; Applied Biosystems), 100 n*M* of forward and reverse primers, and 50 ng of cDNA template. Results were analyzed using 7500 System SDS software.

To calculate the relative amount of gene expression for both the target and housekeeping genes, the comparative delta C_t ($\Delta\Delta C_t$) method was used. The formulas used were as follows: $\Delta C_t = C_t$ target gene $- C_t$ GAPDH; $\Delta\Delta C_t = \Delta C_t$ treated $- \Delta C_t$ control, and fold $= 2 - \Delta\Delta C_t$. The level of gene expression in the control group was arbitrarily set at 1 to serve as a reference. Therefore, the expression of the target gene from experimental groups represents the fold difference expression relative to the reference control gene. Each sample was run in triplicate.

Transient transfection. The whole coding region of human *FLI1* from RNA of human normal fibroblasts was amplified by RT-PCR. It was cloned into pCR2.1 vector (Invitrogen, San Diego, CA) and then reinserted into the inducible expression vector pcDNA4/TO/myc-His (Invitrogen) between the polycloning sites *Eco* RV and *Xho* I. The fragment was sequenced using the cytomegalovirus forward and bovine growth hormone reverse primers provided with the vector. The pcDNA4/TO/*FLI1*AS (*FLI1* antisense construct) was obtained by reversing the insert using the 2 flanked *Fse* I sites.

The empty vector pcDNA4/TO or vectors containing fragments were cotransfected with the tetr-on regulatory vector pcDNA6/TR (Invitrogen) at a ratio of 6:1. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. After transfection, fresh medium was added for 24 hours to allow the cells to recover. Then tetracycline (Sigma) was added to induce expression. The cells were harvested for Western blotting.

ChIP assays. ChIP assays were performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). Cells (10^7) were treated with 1% formaldehyde for 60 minutes at 25°C and were then sonicated to fragment the genomic DNA. The crosslinked DNA–protein complexes were immunoprecipitated using anti–acetylated histone H3 and H4 antibodies. After precipitation with protein A–agarose beads, the complexes were unlinked by heating. Proteins were separated from the DNA by chloroform extraction; DNA fragments were detected by PCR. Normal rabbit IgG (Upstate Biotechnology) was used as a negative control. The following primers were used to amplify a 239-bp DNA fragment of the *FLI1* promoter region (−270 to −31): 5′-AGAGATAGGACTTCCTCCCCGATT-3′ (forward), 5′-TTCCACACATTGACCCGGTTAC-3′ (reverse).

Methylation-specific PCR and bisulfited DNA sequencing. To isolate genomic DNA from skin biopsy samples, the biopsy specimens were first frozen in liquid nitrogen and then ground into powder. Genomic DNA was isolated either from cells in culture or from tissue samples by digestion with RNase A and proteinase K. The sodium bisulfite modification was performed as previously reported (10). DNA was treated with sodium bisulfite to convert unmethylated cytosine to uracil (methylated cytosine is resistant to conversion by sodium bisulfite). Purified DNA was denatured in 22 ml of 0.3*M* NaOH at 37°C for 15 minutes. Then 208 ml of freshly prepared 2.2*M* sodium metabisulfite, containing 0.5*M* hydroquinone (pH 5.0), was added to the denatured DNA and incubated under mineral oil at 50°C for 12 hours. Bisulfite-modified DNA was purified on a Microcon Centrifugal Filter Device (Millipore, Bedford, MA). Modified DNA was desulfonated with 0.3*M* NaOH at 37°C for 15 minutes, followed by ethanol precipitation.

The primers for the *FLI1* methylation–specific PCR were as follows: unmethylated primers 5′-TATGAATGTGTTTGGGTATTTTTGT-3′ (forward), 5′-AAATAACTTCACTTTACAA ATCAAA-3′ (reverse); methylated primers 5′-GTATGAATGTGTTTGGGTATTTTC-3′ (forward), 5′-AAATAACTTCACTTTACGAATCGAA-3′ (reverse); wild-type primers 5′-CATGAATGTGTCTGGGCATCTCCGC-3′ (forward), 5′-GAAGTGACTTCACTTTGCGAATCGG-3′ (reverse). The amplified fragments were inserted into pCR2.1 vector. Sequencing was completed using ABI DNA sequencer, Model 310 (Perkin-Elmer, Emeryville, CA). The primers for the sequencing of bisulfited *FLI1* promoter were 5′-GAGAGAGAGAGATAGGATTTTTTTT-3′ (forward), 5′-AACTCCACTTTAAAAACTAAATTTC-3′ (reverse).

Intensity quantification of the images. The software Scion Image, beta 4.03 was downloaded from www.Scioncorp.com and used to quantify the specific bonds in Western blots and DNA gels.

Statistical analysis. Mean ± SD values were calculated, and the Mann-Whitney U test was used for analysis of statistical significance. *P* values less than 0.05 were considered significant.

RESULTS

Effects of 2-deoxy-5-azaC and TSA on type I procollagen (proα1) expression. Fibroblast cultures were established from clinically involved SSc skin and from skin of matched control subjects. Eight of 10 SSc fibroblast cell lines expressed significantly increased levels of proα1 at baseline, at both the message and the protein levels. Incubation of SSc fibroblasts in either 2-deoxy-5-azaC (inhibitor of Dnmt; 5 μM) for 5 days or TSA (inhibitor of histone deacetylases; 100 ng/ml) for 1 day resulted in significant decreases in proα1 expression and reduced proα1 expression levels to control values in all 8 SSc cell lines that had exhibited increased collagen expression levels at baseline. Results of a representative experiment are shown in Figure 1. The mean ± SD proα1 expression level determined by Western blotting in SSc fibroblasts, normalized to tubulin expression, was 2.5 ± 0.15 before and 1.04 ± 0.08 after treatment ($P < 0.05$) (Figure 1A), and the mean ± SD proα1 messenger RNA expression was 2.2 ± 0.3 before and 0.98 ± 0.2 after treatment ($P < 0.05$) (Figure 1B). Treatment had no effect on collagen expression levels in control fibroblasts (Figure 1).

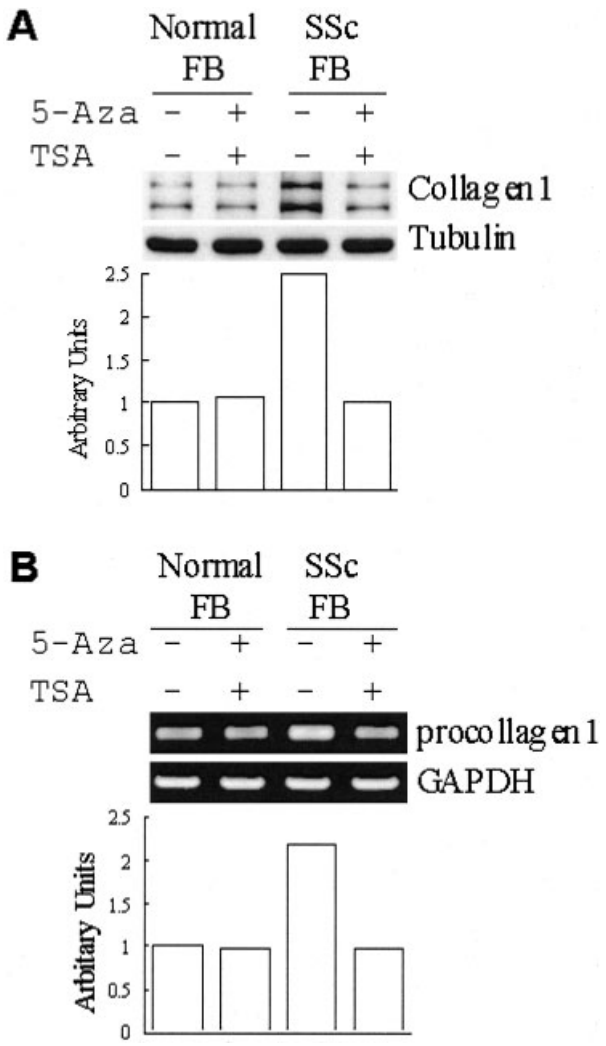

Figure 1. Modulation of type I collagen expression by epigenetic DNA modifications in systemic sclerosis (SSc) fibroblasts (FB). **A,** Normal and SSc fibroblasts were treated with 5 μM of 2-deoxy-5-azacytidine (5-Aza) for 5 days and 100 ng/ml of trichostatin A (TSA) for 1 day. Upper panel, Whole cell lysates were collected and analyzed by Western blotting for type I collagen expression. Untreated cells were used as controls, and the expression level of tubulin was used for normalization. Lower panel, Collagen expression levels were quantified using Scion Image analysis. Values are the mean from triplicate experiments. **B,** Reverse transcriptase–polymerase chain reaction was performed with total RNA extracted from treated and untreated cells. Upper panel, Amplified products of procollagen type I were separated on 1.5% agarose gels. The expression level of GAPDH was used for normalization. Lower panel, Expression levels were semiquantified. Values are the mean from triplicate experiments.

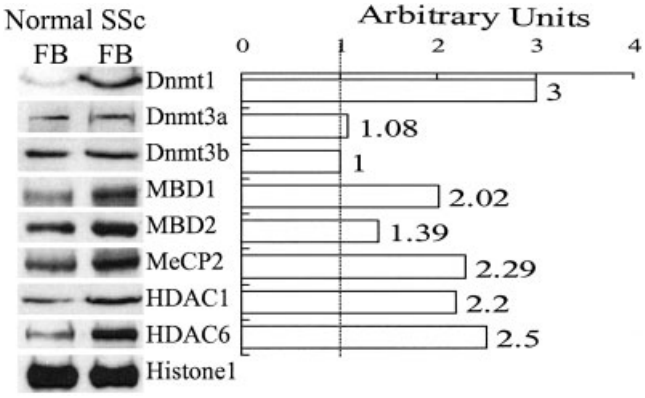

Figure 2. Increased expression levels of epigenetic maintenance mediators in systemic sclerosis (SSc) fibroblasts (FB). Nuclear extracts from SSc fibroblasts and their comparable normal pairs were used for Western blot analysis. The expression levels in comparison with levels in normal fibroblasts (ascribed an arbitrary value of 1) were quantified using histone 1 levels for normalization. Levels of DNA methyltransferase 1 (Dnmt1), methyl-CpG DNA binding protein 1 (MBD-1), MBD-2, methyl-CpG binding protein 2 (MeCP-2), histone deacetylase 1 (HDAC1), and HDAC6 were significantly increased in SSc fibroblasts. Values are the mean and SD of 4 SSc and 4 matched cell lines.

Levels of epigenetic mediators. Levels of Dnmt (Dnmt1, Dnmt3a, and Dnmt3b), MBD (MBD-1, MeCP-2, and MBD-2), and HDA (HDA-1 and HDA-6) were evaluated by Western blotting of nuclear extracts of fibroblasts, and findings in SSc versus control fibroblasts were compared. Compared with controls, SSc fibroblasts exhibited significantly higher levels ($P < 0.05$) of Dnmt1 (mean ± SD 3 ± 0.3), MBD-1 (2.02 ± 0.4), MeCP-2 (2.29 ± 0.3), HDA-1 (2.22 ± 0.4), and HDA-6 (2.57 ± 0.25) (Figure 2). These results are

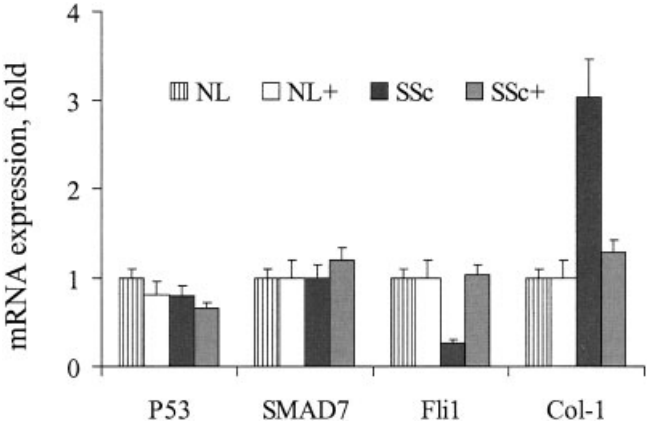

Figure 3. Levels of expression of the collagen suppressor genes *p53*, *smad7*, and *FLI1* in systemic sclerosis (SSc) and normal (NL) fibroblasts with (+) and without treatment with epigenetic modifiers. Expression levels were measured using real-time polymerase chain reaction before and after addition of 5 μM of 2-deoxy-5-azacytidine for 5 days and 100 ng/ml of trichostatin A for 1 day. Values are the fold increase/decrease compared with expression in the control group (ascribed an arbitrary value of 1) and are the mean and SD from quadruplicate experiments; findings were similar in 5 of 5 SSc cell lines.

indicative of sustained DNA methylation and chromatin deacetylation in SSc fibroblasts.

Role of the *FLI1* gene in collagen expression. The reduction in type I collagen expression after addition of 2-deoxy-5-azaC and TSA suggested the possibility that a collagen suppressor gene may be underexpressed in SSc

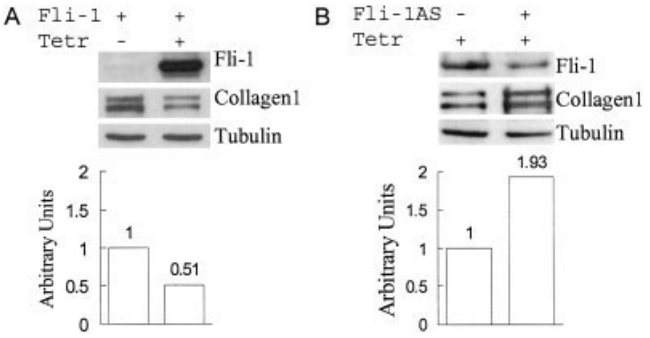

Figure 4. Repression of type I collagen expression by *FLI1* in both normal and systemic sclerosis (SSc) fibroblasts. **A,** SSc fibroblasts were transiently transfected with both pcDNA6/TR and pcDNA4/TO/*FLI1*, with or without tetracycline (Tetr). The levels of expression of *FLI1* and type I collagen were detected by Western blotting (upper panel), and tubulin was used as control for quantification of type I collagen (lower panel) (mean from triplicate experiments). **B,** Normal fibroblasts were transfected with the antisense construct pcDNA4/TO/*FLI1*AS and immunoblotted (upper panel), and expression levels of type I collagen were quantified (lower panel) (mean from triplicate experiments).

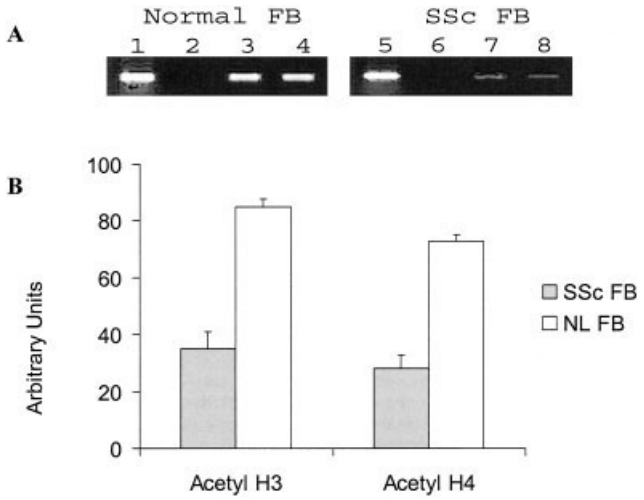

Figure 5. Control of the transcriptional level of *FLI1* by epigenetic modifications in systemic sclerosis (SSc) fibroblasts (FB). **A,** Chromatin immunoprecipitation analysis of acetylated histone 3 and histone 4 at the *FLI1* promoter region. Normal (NL) and SSc fibroblasts were studied in parallel. Lanes 1 and 5, Samples without antibody immunoprecipitation (positive controls); lanes 2 and 6, samples precipitated with normal rabbit IgG (negative controls); lanes 3 and 7, samples precipitated with anti–acetylated histone 3; lanes 4 and 8, samples precipitated with anti–acetylated histone 4. All samples were amplified by polymerase chain reaction. **B,** Quantification of the results as a ratio to positive control values (mean and SD from triplicate experiments).

fibroblasts due to an epigenetic mechanism. We tested the expression level of 3 collagen suppressor genes, s*mad7*, *p53*, and *FLI1*, before and after the addition of epigenetic modifiers. The s*mad7* gene was examined because of its known inhibitory effect on transforming growth factor β (TGFβ)–mediated augmentation of collagen expression and because findings of at least one study suggested that *smad7* deficiency occurs in scleroderma (11), although results of other studies did not support this finding (12). The gene *p53*, a potent and selective endogenous repressor of TGFβ-regulated collagen gene expression (13), is also a tumor suppressor gene that is a target for epigenetic repression. Reduced expression of *FLI1* gene, a suppressor of collagen transcription (14), in association with increased collagen expression in SSc fibroblasts and skin biopsy samples was recently described (15).

We examined the levels of expression of the s*mad7*, *p53*, and *FLI1* genes along with proα1 collagen, using a quantitative real-time PCR method (Figure 3). The proα1 collagen expression level was significantly elevated at baseline in SSc fibroblasts (mean $\pm$ SD fold increase over reference value 3.03 $\pm$ 0.2; $P < 0.05$) and decreased to 1.28 $\pm$ 0.03 after treatment. Expression

levels of *p53* in SSc fibroblasts were reduced both at baseline and after treatment (0.79 ± 0.06 and 0.65 ± 0.04, respectively, compared with reference value). A similar reduction in *p53* expression levels was noted in control fibroblasts following treatment. The data suggest that reduced *p53* expression in SSc fibroblasts may contribute to the augmented collagen expression; however, the reduction in expression after the addition of the epigenetic modifiers indicates that *p53* is not the collagen suppressor gene that is repressed by an epigenetic mechanism. The mechanism of reduced *p53* expression at baseline and after treatment was not investigated any further in this study. Levels of *smad7* expression were not significantly different between SSc and normal cells and did not change significantly with treatment, suggesting that this collagen suppressor gene is not involved in reduction of collagen expression after cell treatment. In contrast, *FLI1* expression levels were significantly reduced at baseline in SSc fibroblasts (0.27 ± 0.02; $P < 0.05$) and increased to 1.03 ± 0.06 after the addition of epigenetic modifiers.

Taken together, the data suggest that the *FLI1* gene is the target of epigenetic repression in SSc. Thus, we hypothesized that *FLI1* down-regulation is the reason for the augmented collagen expression observed in SSc fibroblasts. To test this hypothesis, we transiently transfected SSc fibroblasts with a tetracycline-regulated *FLI1* gene construct, which caused significant reduction in proα1 collagen expression, with the collagen:tubulin expression ratio decreasing to 0.51 ± 0.09 (mean ± SD) after addition of tetracycline ($P < 0.05$) (Figure 4A). In addition, we introduced an *FLI1* antisense construct into normal fibroblasts, which resulted in significant enhancement of proα1 collagen expression (expression increased to 1.93 ± 0.35) ($P < 0.05$) (Figure 4B). Addition of tetracycline alone or transfection with vector alone did not influence collagen expression (data not shown). These findings support the notion that the *FLI1* gene plays an important role in the regulation of collagen gene expression and resultant tissue fibrosis.

***FLI1* gene promoter deacetylation in SSc and normal fibroblasts.** Next we examined the deacetylation of the histones H3 and H4 in the promoter region of the *FLI1* gene in SSc and normal fibroblasts, by ChIP assay. Significant reduction in the acetylated forms of H3 and H4 was noted in SSc fibroblasts as compared with control cells. The mean ± SD levels of acetylated H3 and acetylated H4 (arbitrary units) were 36 ± 4 and 29 ± 3, respectively, both of which were significantly ($P < 0.05$) lower than normal values (85 ± 6 and 73 ± 7, respectively) (Figure 5). These data suggest that histone deacetylation is involved in epigenetic repression of *FLI1* expression.

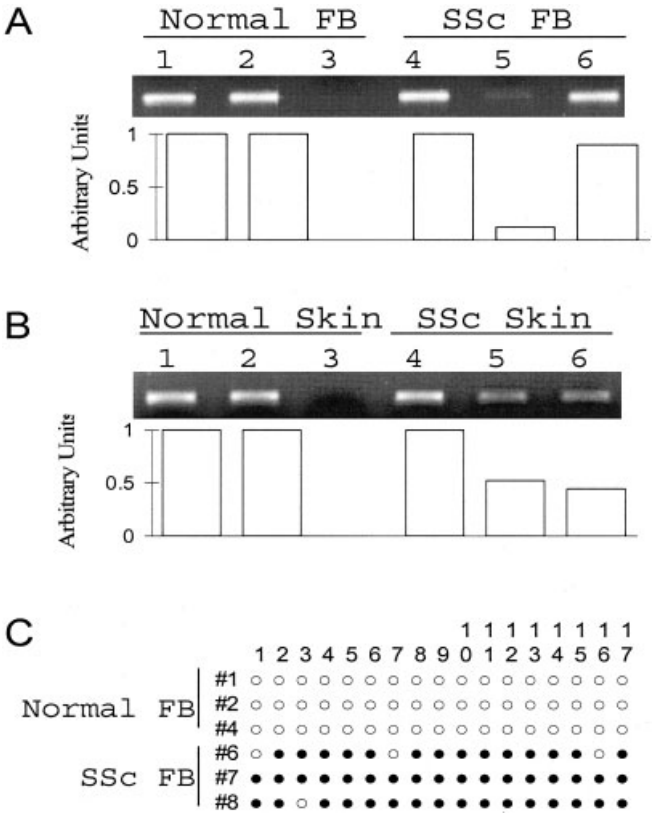

Figure 6. Methylation of the promoter region of *FLI1* in systemic sclerosis (SSc) fibroblasts (FB). **A,** Polymerase chain reaction (PCR) and methylation-specific PCR were performed using templates from normal and SSc fibroblasts. Lanes 1 and 4, PCR using unmodified genomic DNA and wild-type primers; lanes 2 and 5, methylation-specific PCR using bisulfited genomic DNA–unmethylated primers; lanes 3 and 6, methylation-specific PCR using bisulfited genomic DNA–methylated primers. The separated DNA fragments are shown (upper panel), and results were quantified using the normal PCR products as control (ascribed an arbitrary value of 1) (lower panel) (mean from quadruplicate experiments). **B,** Genomic DNA was prepared from either normal or SSc skin biopsy specimens and tested as described for cell lines in **A** (upper panel), and results were quantified (lower panel) (mean from quadruplicate experiments). **C,** Methylation patterns of the CpG island in the *FLI1* promoter region (−277 to 19). The cytosines in the CpG sequences −251, −246, −198, −181, −176, −161, −136, −132, −121, −103, −91, −89, −86, −76, −65, −48, and −19 are coded from 1 to 17, respectively. Open circles indicate no methylation; solid circles indicate CpG island methylation. Six genomic samples (3 from normal fibroblasts [numbers 1, 2, and 4] and 3 from SSc fibroblasts [numbers 6, 7, and 8]) were sequenced.

***FLI1* promoter CpG island methylation in SSc cells and skin biopsy samples.** The proportions of methylated and unmethylated CpG islands in the promoter region of *FLI1* gene were examined by

methylation-specific PCR after DNA bisulfite modification. We used the CpG island search software CpGIE (16) (http://bioinfo.hku.hk/cpgieintro) to define the location of the CpG islands in the promoter region of *FLI1*. The primers for methylation-specific PCR were designed using MethPrimer (17) (http://www.urogene.org/methprimer) to amplify a sequence from −462 to −211.

Increased methylation of the *FLI1* promoter was noted in SSc fibroblasts (mean ± SD ratio of methylated to wild-type amplicons 0.9 ± 0.025), with no detectable methylation products in control fibroblasts (Figure 6A). Since fibroblasts were cultured in vitro for multiple generations, we wondered whether similar findings could be observed in vivo. Thus, we used genomic DNA from freshly obtained 5-mm skin biopsy sections (3 SSc and 3 control) that were tested under conditions similar to those used with the cell lines. Increased methylation of *FLI1* promoter was noted in SSc skin samples, with a ratio of methylated to wild-type amplicons of 0.48 ± 0.2 (ratio of 0 in control skin) (Figure 6B). These results confirm the hypermethylation of the CpG islands in the *FLI1* promoter region of SSc skin and fibroblasts. To further confirm hypermethylation of the *FLI1* promoter, we cloned and sequenced the *FLI1* promoter region (−277 to 19) of bisulfited genomic DNA. Dense methylation in the predicted location of CpG islands in the SSc *FLI1* gene promoter was noted in DNA derived from 3 SSc cell lines, whereas no methylation was noted in 3 matched control cell lines (Figure 6C).

DISCUSSION

SSc is a systemic autoimmune disease of unknown etiology and pathogenesis. It is characterized by progressive vasculopathy and widespread tissue fibrosis. Activated fibroblasts are believed to play a key role in the development of tissue fibrosis and disease progression. Numerous in vivo and in vitro studies have helped to characterize the profibrotic phenotype of SSc fibroblasts and have shown that fibroblasts derived from involved SSc skin produce increased amounts of extracellular matrix proteins, including types I, III, VI, and VII collagen and fibronectin, and profibrotic cytokines, including TGFβ and connective tissue growth factor, in association with reduced synthesis of matrix metalloproteinases 1 and 3 (18–20).

This profibrotic phenotype persists in vitro for multiple generations, suggesting an inherited cellular trait that is transmitted from one generation to the next. One possible explanation for the persistence of the phenotype is in vivo clonal selection of a profibrotic fibroblast from a heterogeneous population (21). The clonal selection hypothesis has generated intense interest; however it has not been validated by experimental data. An alternative possibility is that of an acquired epigenetic alteration that is maintained during cell divisions. Epigenetics is defined as the heritable but potentially reversible changes in genetic material, including the DNA and chromatin, that lead to alterations in gene expression (22,23). Over the last decade, several disease conditions have been linked to epigenetic processes; diseases that have been suggested to be under epigenetic influence include several cancers (24), type 1 diabetes mellitus (25), and inflammatory bowel disease (26).

Our data suggest that an epigenetic mechanism may indeed lead to augmented collagen expression by SSc fibroblasts. First, the addition of inhibitors of DNA methyltransferases and histone deacetylases led to normalization of type I collagen expression levels in SSc fibroblasts, providing evidence of epigenetic influence on collagen gene expression. Second, the levels of factors involved in the maintenance of epigenetic mechanisms are clearly elevated in the nuclear extract from SSc fibroblasts, demonstrating the ability of the cells to sustain an epigenetic process.

We tested the possibility that the augmentation of collagen expression is related to epigenetic repression of a collagen suppressor gene. It is known that DNA methylation inhibitors boost the expression of repressed genes and reduce that of genes that are activated due to repression of a suppressor gene. For example, 2-deoxy-5-azaC has been shown to reduce aberrant p16INK4A RNA transcripts and restore the functional retinoblastoma protein pathway in hepatocellular carcinoma (27), and it can also reduce the expression of an up-regulated vascular endothelial growth factor gene in several leukemias and lymphomas by augmenting the expression of a suppressor gene (28). We examined 3 collagen suppressor genes, s*mad7*, *p53*, and *FLI1*, using quantitative real-time PCR along with type I collagen before and after addition of epigenetic modifiers. Another collagen suppressor factor is HDA-1 (29), and it was overexpressed in SSc fibroblasts.

Of the 3 genes investigated, only *FLI1* was significantly reduced in SSc fibroblasts and substantially increased after the addition of epigenetic modifiers. *FLI1* is a member of the Ets family of transcription factors that has been shown to have roles in hematopoiesis, embryonic development, and vasculogenesis (30). It is also a transcription factor that inhibits collagen gene via an Sp-1–dependent pathway (15). The role of *FLI1* as a

negative regulator of extracellular matrix genes is supported by the observations that FLI1 protein levels are inversely correlated with production of type I collagen in fibroblast cultures generated from *FLI1*$^{-/-}$, *FLI1*$^{+/-}$, and *FLI1*$^{+/+}$ mouse embryos (14). In the present report we describe an epigenetic regulation of *FLI1* gene expression in SSc that results in enhanced type I collagen gene expression. The transfection data in the current study support the notion of a link between levels of *FLI1* gene expression and type I collagen expression. Thus, the introduction of *FLI1* antisense constructs into normal cells led to a significant increase in type I collagen expression, while the transient transfection of SSc fibroblasts with *FLI1* gene led to a decrease in collagen gene expression.

Epigenetic regulation of a gene requires the presence of CpG islands in the promoter region. The promoter of *FLI1* contains CpG islands that can be methylated, as shown by binding of a genomic DNA fragment containing 206 nucleotides from positions −161 to 46 of the *FLI1* promoter with an MeCP-2 affinity column (31), indicating that the *FLI1* proximal promoter region can be methylated and bind to methylated nucleotide binding protein. In the present study we demonstrated epigenetic regulation of *FLI1* gene expression in SSc cells, which is related not only to CpG island promoter hypermethylation as shown by the methylation-specific PCR and promoter sequencing results, but also to chromatin deacetylation as shown in the chromatin immunoprecipitation experiments. Moreover, results of the methylation-specific PCR studies of skin biopsy samples demonstrated that almost half of *FLI1* promoters are methylated, suggesting that epigenetic repression of the *FLI1* gene occurs in vivo. The reported underexpression of *FLI1* in SSc skin supports our findings (15). The proportion of methylated *FLI1* promoters was clearly higher in SSc fibroblasts than in SSc skin specimens, possibly because of the mixed cellularity of the biopsy samples. Nonetheless, no significant methylation was noted in control cells or biopsy samples.

It is unclear whether *FLI1* expression is linked to the development of the full fibrotic phenotype of SSc fibroblasts, including the myofibroblast phenotype, or is related only to the level of type I collagen synthesis. Of interest, other collagen suppressor genes may be involved in different forms of fibrosis; for example, in liver cirrhosis, cytokine signaling 1 gene methylation is associated with hepatic fibrosis (32).

Finally, our observations may have significant implications with regard to therapy, because neither methylation of a gene nor alteration in chromatin structure is irreversible since the gene itself is not mutated in any way by methylation, and the chromatin is not irreversibly changed. Because of this reversibility, epigenetic gene regulation is theoretically amenable to intervention (33). Thus, a better understanding of the role of epigenetics in SSc tissue fibrosis may lead to the development of a novel therapy.

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