Original Article

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Overexpression of hepatocyte growth factor receptor in scleroderma dermal fibroblasts is caused by autocrine transforming growth factor β signaling

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Summary **Cutaneous fibrosis seen in systemic sclerosis (SSc) is caused by fibroblast activation and abnormal collagen accumulation due to 'autocrine transforming growth factor (TGF)-β/Smad signaling'. Hepatocyte growth factor (HGF) may have therapeutic value against SSc, because of its inducible effect on the expression of matrix metalloproteinase (MMP)-1. Previous studies indicated SSc dermal fibroblasts overexpress HGF receptor c-met, which suggest specific and effective induction of MMP-1 in SSc fibroblasts caused by HGF treatment. However, the exact mechanism of c-met overexpression in SSc cells was hardly investigated. We hypothesized that such c-met overexpression is also caused by autocrine TGF-β/Smad signaling. Expression of c-met protein in cultured SSc dermal fibroblasts was significantly up-regulated compared with that in normal fibroblasts. Ectopic TGF-β stimulation induced c-met synthesis in normal fibroblasts, while a TGF-β knockdown normalized the up-regulated c-met levels in SSc fibroblasts. Furthermore, we found the c-met promoter contains a putative binding site for Smads, and the binding activity of Smad2/3 to the c-met promoter was constitutively up-regulated in SSc fibroblasts as well as in normal fibroblasts treated with exogenous TGF-β1. Taken together, c-met may be overexpressed due to autocrine TGF-β/Smad signaling in SSc. Considering that HGF has an antifibrotic effect, such c-met overexpression in SSc fibroblasts may be a negative feedback against cutaneous fibrosis. Clarifying the mechanisms of c-met overexpression and controlling the HGF/c-met pathway may lead to a new therapeutic approach for this disease.**

Keywords: Immunoblotting, fibrosis, extracellular matrix

1. Introduction

Systemic sclerosis (SSc) is one of the autoimmune disorders characterized by tissue fibrosis of the skin as well as internal organs. The activation of fibroblasts is thought to be responsible for the tissue fibrosis *via* the abnormal accumulation of extracellular matrix (ECM), mainly collagen (*1,2*). Although the mechanism of fibroblast activation is still unknown, many of the characteristics of SSc fibroblasts resemble those of transforming growth factor (TGF)-β1 stimulated normal fibroblasts (*3,4*). The principal effect of TGF-β1 on mesenchymal cells including fibroblasts is ECM production. Cultured SSc fibroblasts overexpress various ECM components, mainly type I collagen (*5,6*), as well as display constitutively up-regulated phosphorylation and promoter-binding activity of Smad, a mediator of TGF-β signaling (*7-9*). Also, our previous findings described below suggest that the activation of dermal fibroblasts in SSc is due to stimulation by 'autocrine TGF-β signaling'; (*i*) The blockade of TGF-β signaling with neutralizing antibody abolished the overexpression of collagen mRNA in cultured SSc fibroblasts (*10*), and (*ii*) the TGF-β-responsive element of α2(I) collagen promoter in normal fibroblasts and

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the sequence involved in the intrinsic up-regulation of α2(I) collagen promoter activity in SSc fibroblasts are both bp −376 ~ −108 sites (*11,12*). Thus, the intrinsic up-regulation of collagen genes seen in SSc fibroblasts utilizes a TGF-β1/Smad-dependent pathway.

Recently, hepatocyte growth factor (HGF) has attracted attention for its therapeutic value in treating various diseases. HGF regulates cell growth, motility and morphogenesis by binding to the receptor called c-met (*13*). HGF may also have an anti-fibrotic effect inducing the expression of matrix metalloproteinase (MMP)-1 in dermal fibroblasts (*14,15*). We previously compared the direct effect of HGF on the expression of type I collagen and MMP-1 in normal and SSc cultured dermal fibroblasts. HGF reduced type I collagen expression in SSc fibroblasts, but not in normal cells (*14*). On the other hand, MMP-1 expression was increased by HGF in both cells, but HGF had stronger effects in SSc fibroblasts than normal fibroblasts. We concluded that HGF reduces type I collagen accumulation only in SSc fibroblasts by the effective induction of MMP-1 in these cells, because of the overexpression of c-met. C-met overexpression in SSc fibroblasts was demonstrated by immunohistochemistry *in vivo* and immunoblotting *in vitro* (*14,16*). Thus, c-met overexpression in SSc fibroblasts is likely to be the key event to express the anti-fibrotic effect of HGF in this disease. However, its mechanism has not been investigated well. Clarifying the regulatory mechanisms of HGF/c-met signaling in SSc may contribute to further understanding of this disease and lead to a new therapeutic approach. We hypothesized that such c-met overexpression is also caused by autocrine TGF-β/Smad signaling. This study was undertaken to evaluate the hypothesis, and to clarify its mediators.

2. Materials and Methods

2.1. *Cell culture*

Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of 4 patients with diffuse cutaneous SSc and < 2 years of skin thickening as described previously (*17*). Control fibroblasts were obtained by skin biopsy from 4 healthy donors (*18*). Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. Primary explant cultures were established in 25-cm^2 culture flasks in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS) and an Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA, USA). Monolayer cultures independently isolated from different individuals were maintained at 37ºC in 5% CO_2 in air. Fibroblasts between the third and sixth subpassages were used for experiments. Before experiments, cells were serum-starved for 12-24 h.

2.2. *Cell Lysis and immunoblotting*

Fibroblasts were cultured until they were confluent, then cell lysates (normalized for protein concentration) were analyzed by immunoblotting as described previously (*19*). The antibodies for c-met and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. *RNA isolation and quantitative real-time polymerase chain reaction (PCR)*

Total RNA was extracted from culture cells with ISOGEN (Nippon Gene, Tokyo, Japan). First-strand cDNA synthesis and quantitative real-time PCR with Takara Thermal Cycler Dice (TP800) were performed as described previously (*19*).

Primer sets for c-met and GAPDH were purchased from SABiosciences (Frederick, MD). DNA was amplified for 40 cycles of denaturation for 5 sec at 95° C and annealing for 30 sec at 60°C. The relative fold changes of c-met and GAPDH were calculated by a standard curve method. For each gene of interest, we used at least 3 independent samples.

2.4. *Transient transfection*

The cells were transfected with TGF-β1 siRNA or control siRNA (Santa Cruz Biotechnology) mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) as a transfection reagent when cells were plated, and incubated for 96 h at 37°C in 5% CO_2 (*18*). Control experiments showed > 80% knockdown efficiency (data not shown).

2.5. *DNA affinity precipitation assay*

Three oligonucleotides containing biotin on the 5' nucleotide of the sense strand were prepared as described previously (*20*). The sequences of these oligonucleotides are as follows: (*i*) c-met promoter oligo, 5'-ACAGCACG CGAGGCAGACAGACACGTGCTGGGGCGG, which corresponds to bp −364 to −329 positions of the human c-met promoter; (*ii*) positive control CAGA oligo, 5'-TC GAGAGCCAGACAAGGAGCCAGACAAGGAGCC AGACACTCGAG, positive control with a trimer of the CAGA motif; (*iii*) negative control TATA oligo, 5'-ACA GCACGCGAGGTATATATATACGTGCTGGGGCGG, which has a mutated CAGA motif of the c-met promoter oligo. These oligonucleotides were annealed to their respective complementary oligonucleotides, and double-stranded oligonucleotides were gel-purified and used. Cell lysates were obtained using lysis buffer (*21*). Poly (dI-dC) competitor was incubated with the cell lysates, followed by incubation with each double-stranded oligonucleotide. After incubation, streptavidin-agarose (Sigma, Saint Louis, MS) was added to the reaction

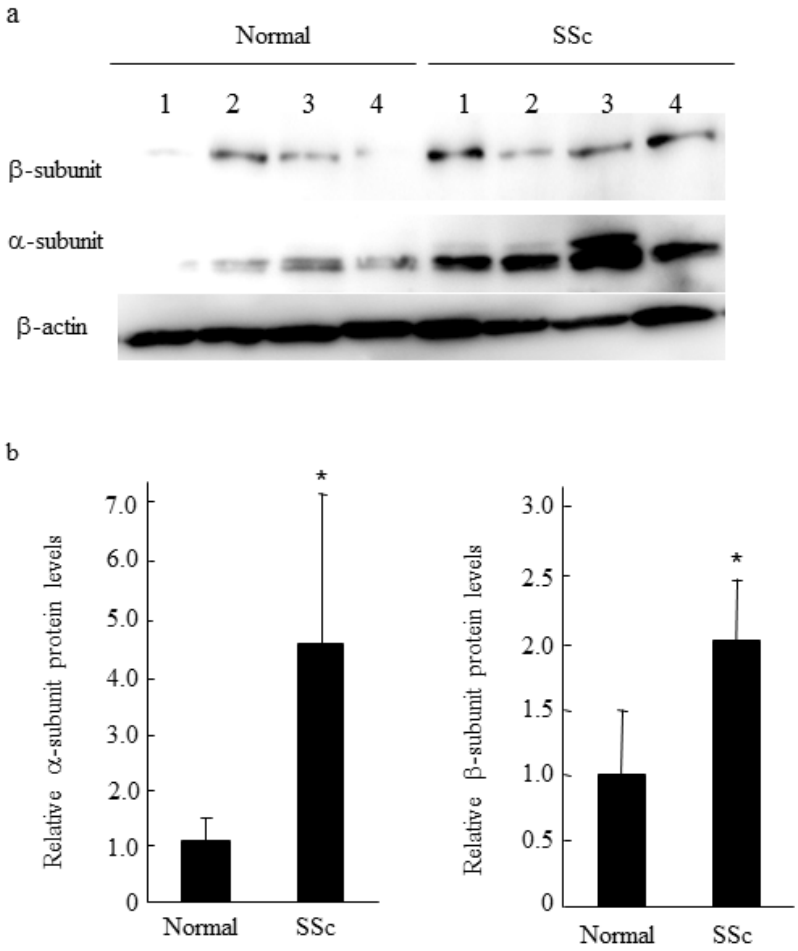

Figure 1. Levels of c-met protein synthesis in cultured dermal SSc fibroblasts. (a) The representative results of immunoblotting for lysates from 4 normal and 4 systemic sclerosis (SSc) fibroblasts. Cells were cultured until they were confluent, and then incubated for an additional 24 h under serum starvation conditions. Cell lysates were subjected to immunoblotting with antibodies against c-met or β-actin. The α- and β-subunits are indicated. **(b)** Expression of the α-subunit (left) or β-subunit (right) quantitated by scanning densitometry and corrected for levels of β-actin in the same samples are shown relative to those in normal fibroblasts (1.0). * $p < 0.05$ as compared with the values in samples from normal fibroblasts.

and incubated. The protein-DNA-streptavidin-agarose complex was washed and loaded onto a sodium dodecyl sulfate-polyacrylamide gel. Detection of Smad2/3 was performed by immunoblotting with monoclonal Smad2/3 antibody (BD Biosciences, Franklin Lakes, NJ, USA).

2.6. *Statistical analysis*

Data are expressed as the mean ± S.D. of at least three independent experiments. Statistical analysis was carried out with a Mann-Whitney *U*-test for comparison of medians. *p* values less than 0.05 were considered significant.

3. Results

3.1. *C-met protein synthesis in cultured SSc dermal fibroblasts*

As an initial experiment, we compared c-met protein expression levels between cultured normal and SSc fibroblasts by immunoblotting and confirmed the previous results (*14*). C-met is known to consist of an α- and β-subunit. The results showed that the amount of both subunits in the cell lysates from SSc fibroblasts was increased compared with that from normal cells (Figure 1a). When quantitated, the protein expression of α- or β-subunit was significantly increased about 2.0-fold or 4.3-fold in SSc fibroblasts compared to normal cells, respectively (Figure 1b). These data indicated that c-met protein synthesis was significantly and constitutively increased in cultured SSc dermal fibroblasts, as described previously (*14*).

3.2. *C-met protein expression induced by stimulation with TGF-β*

If autocrine TGF-β stimulation is the main cause of the constitutive up-regulation of c-met in SSc fibroblasts, exogenous TGF-β1 may increase c-met expression in normal fibroblasts. To test this possibility, we investigated the effect of exogenous TGF-β1 on the expression of

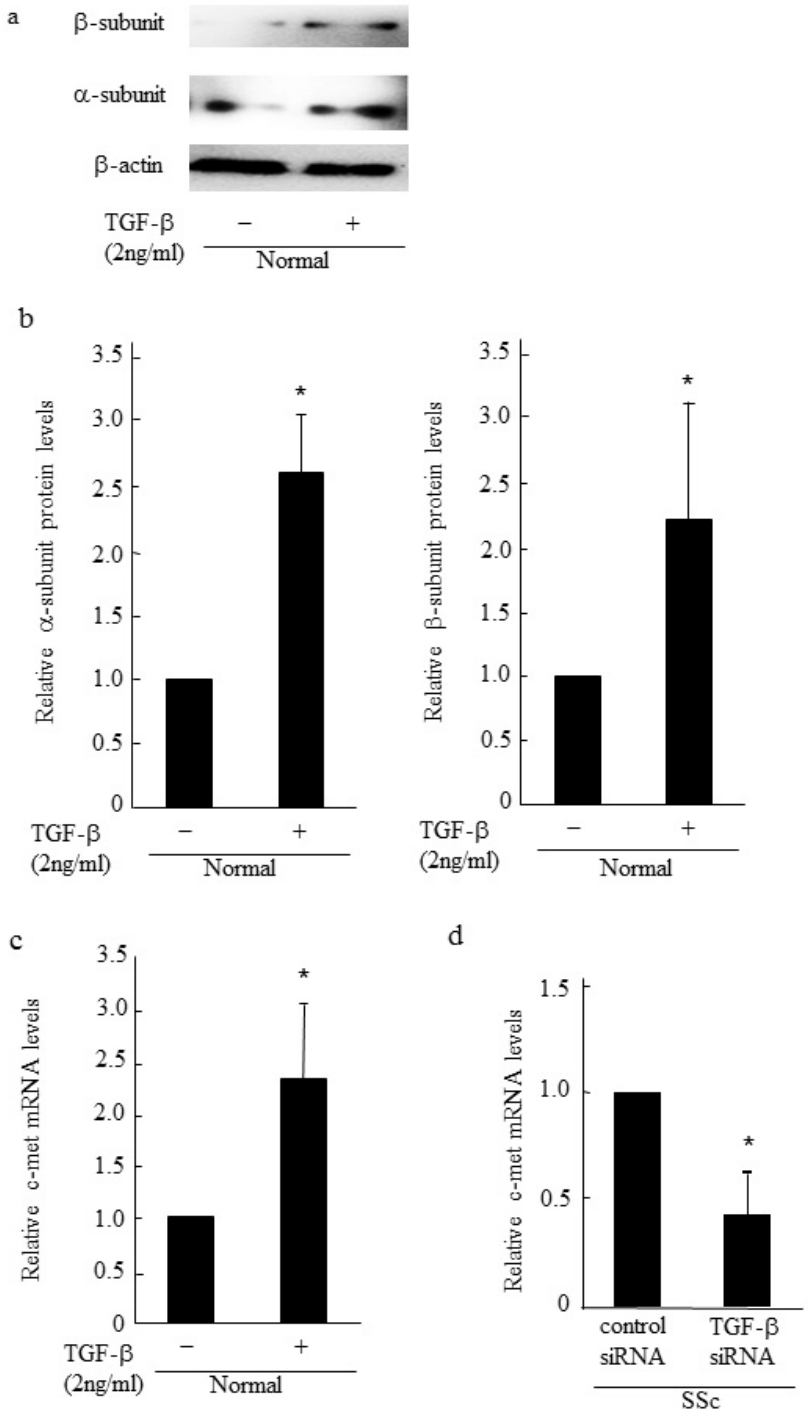

Figure 2. The effect of TGF-β1 on c-met expression in cultured dermal fibroblasts. (a) Cells were cultured until they were confluent, and then incubated for an additional 24 h under serum starvation conditions. Cells were incubated in the presence or absence of 2 ng/mL TGF-β1 for 24 h. Cell lysates were subjected to immunoblotting with antibodies against c-met or β-actin. **(b)** Expression of the α-subunit (left) or β-subunit (right) quantitated by scanning densitometry and corrected for levels of β-actin in the same samples are shown relative to those in untreated fibroblasts. Values in untreated fibroblasts were set at 1. * $p < 0.05$ as compared with the values in untreated fibroblasts. **(c)** Relative amounts of c-met transcripts (normalized with GAPDH) in fibroblasts stimulated with or without TGF-β1 (2 ng/mL) for 24 h were determined by real-time PCR. Values in untreated fibroblasts were set at 1. **(d)** SSc fibroblasts were transfected with control or TGF-β1 siRNA. After 96 h, c-met mRNA levels were determined by real-time PCR. * Values in fibroblasts transfected with control siRNA were set at 1. * $p < 0.05$ as compared with the value in cells transfected with control siRNA.

c-met protein and mRNA. Normal fibroblasts were cultured until they were confluent, and then incubated for an additional 24 h under serum starvation conditions. Cells were subsequently incubated for 24 h with or without 2 ng/mL TGF-β1 treatment. As shown in Figure 2a, the expression of c-met was increased by exogenous TGF-β1 stimulation in normal fibroblasts. In addition, the densitometric analysis revealed that overexpression of

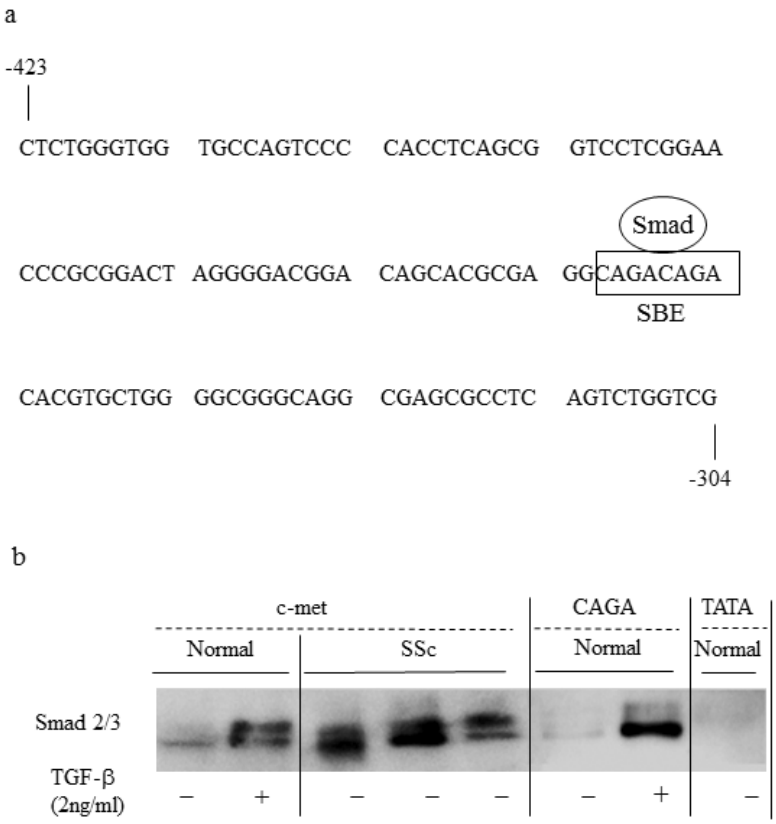

Figure 3. DNA-binding activity of Smad2/3 to c-met promoter in SSc fibroblasts. (a) Nucleotide sequence of c-met promoter region from bp −423 to −304 position. Putative Smad binding elements (SBE) which consisted of 2 CAGA motifs are boxed. **(b)** Comparison of the binding activity of Smad2/3 to the c-met promoter between normal and SSc fibroblasts by DNA affinity precipitation assay. Cells were incubated in presence or absence of 2 ng/mL TGF-β1 for 1 hour. Cell lysates were prepared and incubated with biotin-labeled oligonucleotides as described in Materials and Methods. Proteins bound to each nucleotide were isolated with streptavidin-agarose beads, and Smad2/3 was detected using immunoblotting. c-met; c-met promoter oligo, CAGA; positive control CAGA oligo containing CAGA motif, TATA; negative control TATA oligo with mutated putative SBE of c-met promoter.

the α- and β-subunit protein was statistically significant (Figure 2b). Also, quantitative real-time PCR showed c-met mRNA was elevated significantly by treatment with 2 ng/mL TGF-β1 (Figure 2c). Thus, stimulation of exogenous TGF-β increases the expression of c-met protein and mRNA in normal fibroblasts.

Moreover, to prove the involvement of TGF-β1 stimulation in c-met overexpression seen in SSc fibroblasts, we determined the effect of TGF-β1 siRNA. SSc fibroblasts treated with the siRNA showed a significant reduction in c-met mRNA expression (Figure 2d), which is consistent with normalization of abnormally increased collagen expression by TGF-β1 neutralizing antibody in SSc fibroblasts as described in the introduction (*10*). These results indicate that the overexpression of c-met as well as type I collagen in SSc fibroblasts is a result of stimulation by autocrine TGF-β activation.

3.3. *The promoter binding activity of Smad to c-met promoter in normal dermal fibroblasts treated with TGF-β1 and in SSc fibroblasts*

To further elucidate the detailed mechanisms involved in activation of c-met transcription by autocrine TGF-β signaling in SSc fibroblasts, we compared the DNA binding ability of endogenous Smad to the c-met promoter in normal and SSc fibroblasts using a DNA affinity precipitation assay.

We found that the c-met promoter has 2 tandem of CAGA sequences, known as the Smad binding element (SBE) (*22*), at the bp −351 ~ −344 position (Figure 3a). Fibroblasts were serum-starved for 24 h and treated with 2ng/mL TGF-β1 for 1 h. C-met promoter oligos were designed to correspond to bp −364 to −329 positions of the human c-met promoter, containing the putative SBE. As a positive control, we used the positive control CAGA oligo, which also contains the trimer of SBE (*8*). We also used a negative control (TATA oligo), in which the CAGA motif of c-met promoter oligo was mutated to TATA. As shown in Figure 3b, the results showed that Smad2/3 bound to the c-met promoter oligo strongly after TGF-β1 treatment in normal cells (lanes 1 and 2). SSc fibroblasts showed constitutive binding of Smad2/3 even without exogenous TGF-β stimulation (lanes 3-5). The positive control CAGA oligo could bind Smad2/3 as reported previously (*23*), whereas the negative control TATA oligo did not

show Smad2/3 binding. These results suggest that Smad2/3 bound to this site of the c-met promoter in an inducible and specific manner and supports the notion that TGF-β1/Smad mediates c-met overexpression in SSc fibroblasts.

4. Discussion

In this study, we have presented two major findings. First, as reported previously (*14*), SSc fibroblasts constitutively overexpress c-met. Exogenous TGF-β induces c-met expression in cultured human dermal fibroblasts at the mRNA level. Additionally, TGF-β knockdown by TGF-β1 siRNA decreased c-met mRNA in cultured SSc dermal fibroblasts which have autocrine TGF-β signaling. These results suggest that TGF-β plays a major role in c-met overexpression in SSc dermal fibroblasts.

Secondly, our report first indicated such overexpression of c-met in SSc fibroblasts is mediated by the Smad pathway. The DNA affinity precipitation assay revealed that c-met promoter contains a putative SBE, and the binding activity of Smad2/3 to the c-met promoter was constitutively increased in SSc fibroblasts, to a similar degree to that in normal fibroblasts treated with exogenous TGF-β1. As described above, the binding activity of endogenous Smad to α2(I) collagen promoter was also up-regulated remarkably in SSc fibroblasts compared with normal fibroblasts (*24*). Signals of TGF-β1 from the receptor to the nucleus are mediated by Smad proteins. The activated TGF-β receptor type I directly phosphorylates Smad2/3. Once activated, Smad2/3 associates with Smad4 and translocates to the nucleus, where the complex binds to the SBE in the promoter of target genes, resulting in modulation of their transcriptional activities. Thus, increased Smad binding to the c-met promoter in SSc fibroblasts without exogenous stimulation indicates that c-met overexpression in SSc fibroblasts results from stimulation of autocrine TGF-β signaling. Considering that HGF has an anti-fibrotic effect, such c-met overexpression in SSc fibroblasts may be a negative feedback against fibrosis of the skin.

Although a therapeutic effect of cyclophosphamide, prednisolone, or methotrexate therapy on the fibrosis of SSc (*25,26*) has been reported, various and considerable adverse effects of these treatments have to be a concern (*27*). On the other hand, as described above, the anti-fibrotic effect of HGF may be limited to fibrotic lesions of SSc because of c-met overexpression (*14*), indicating a less adverse effect. Clarifying the mechanisms of c-met overexpression and controlling the HGF/c-met pathway may lead to a novel therapeutic approach for this disease. The effect of other cytokines including TGF-β2 or -β3 on c-met expression should be examined in the future.

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