

Establishment and characterization of scleroderma fibroblast clonal cell lines by introduction of the hTERT gene

Bagrat Kapanadze[†], Erin Morris[†], Edwin Smith, Maria Trojanowska^{*}

Medical University of South Carolina, Division of Rheumatology, Charleston, SC, USA

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Abstract

Lack of an adequate experimental model has hindered the ability to fully understand scleroderma (SSc) pathogenesis. Current SSc research is based on the study of cultured fibroblasts from skin biopsies. In depth characterization of the SSc fibroblast phenotype is hindered by the limited lifespan and heterogeneity of these cells. The goal of this study was to isolate high collagen-producing fibroblasts from SSc biopsies and extend their lifespan with hTERT immortalization to enable characterization of their phenotype. Fibroblasts from two pairs of closely matched normal and SSc biopsies were infected with an hTERT lentivirus. Infected colonies were isolated, cultured into clonal cell lines and analysed with respect to profibrotic gene expression. The mRNA levels of nine profibrotic genes were measured by quantitative real-time PCR. Protein levels were assessed by Western blot. The hTERT SSc clones were heterogeneous with regards to expression of the profibrotic genes measured. A subset of the SSc clones showed elevated expression levels of collagen I, connective tissue growth factor and thrombospondin 1 mRNA, while expression of other genes was not significantly changed. Elevated expression of collagen I protein and mRNA was correlative with elevated expression of connective tissue growth factor. Several hTERT clones expressed high levels of pSmad1, Smad1 and TGF- β RI indicative of altered TGF- β signalling. A portion of SSc clones expressed several profibrotic genes. This study demonstrates that select characteristics of the SSc phenotype are expressed in a subset of activated fibroblasts in culture. The clonal SSc cell lines may present a new and useful model to investigate the mechanisms involved in SSc fibrosis.

Keywords: scleroderma • fibroblast • hTERT • fibrosis • collagen • TGF- β • Smad1

Introduction

Scleroderma (SSc) is an autoimmune disease characterized by excessive accumulation of components of the extracellular matrix (ECM) leading to skin and tissue fibrosis. Despite intense investigation, the factors leading to this abnormal ECM production and reduced ECM degradation are not fully elucidated. A recent gene array identified distinctive gene profiles for SSc patient skin with 1800 qualifier genes significantly distinguishing SSc skin from normal skin [1]. A second gene array by Milano *et al.* grouped SSc gene expression profiles into three groups of diffuse SSc and two

groups of localized SSc that each possessed a unique gene signature [2]. This demonstrated phenotypic variation between SSc patients presents a significant challenge in trying to understand biochemical pathways involved in the fibrotic process. Although fibroblast cultures isolated from SSc skin biopsies do not fully recapitulate the biopsy signature [1], nonetheless they have been the cornerstone of many landmark studies in SSc research [3, 4].

Distinct phenotypes with respect to collagen I mRNA production have been detected in normal and SSc fibroblast populations within the same tissue. Using *in situ* hybridization, low and high collagen α 1(I) mRNA-producing fibroblasts were detected in normal and SSc tissue sections with SSc skin having a higher distribution of high collagen α 1(I) mRNA-producing fibroblasts [5, 6]. A close relationship between the percentage of cells expressing high levels of collagen α 1(I) and collagen α 1(III) mRNA and histological findings in SSc skin has also been shown [7]. The activation of a select group of fibroblasts in SSc tissue is difficult to investigate without isolating these activated fibroblasts and characterizing their phenotype.

[†]These authors contributed equally to the manuscript.

^{*}Correspondence to: Maria TROJANOWSKA,
Medical University of South Carolina,
Division of Rheumatology, 96 Jonathon Lucas St.,
Clinical Sciences Building Suite 912,
Charleston, SC 29425, USA.
Tel.: 843 792-7921
Fax: 843 792-7121
E-mail: trojanme@musc.edu

The origin of high collagen-producing fibroblasts in SSc is a topic of debate in the field of research. SSc fibroblasts are likely to derive from multiple sources (resident fibroblast activation, bone marrow, endothelial-mesenchymal transition), which may contribute to their heterogeneity [8]. Gaining a better understanding of the signature fibrotic phenotype of these fibroblasts may help to elucidate where they originate from and how their activation occurs. In this work, we have used a new method to determine the gene profile of high collagen-producing, activated fibroblasts. hTERT lentiviral infection enabled us to isolate clonal fibroblast cell lines from NS and SSc biopsies and analyse their phenotype with qRT-PCR and Western blotting. With this method, we eliminated the heterogeneity that is present in primary fibroblast cultures. Investigation of several homogeneous, clonal cell lines provides information about individual fibroblasts from the original population. We can identify genes that are expressed in SSc fibroblasts simultaneously with collagen I and may contribute to the activated phenotype.

The hTERT gene was chosen for this study because of its potential ability to extend the lifespan of cultured fibroblasts [9]. It has been reported that the introduction and forced expression of hTERT can rescue cells from crisis and establish immortal cell lines [10]. A major limitation of studies with cultured fibroblasts is the finite lifespan of these cells. After 50–75 population doublings, adult skin fibroblasts will senesce by irreversibly arresting in the G1 phase of mitosis [11–13]. Senescence prevents excessive proliferation, which is a characteristic of cancer cells. To achieve unchecked proliferation, two mortality checkpoints must be overcome [14]. The first checkpoint, replicative senescence or M1, is regulated by the CDK inhibitors p21 and p16, which function in the p53 and Rb tumour suppressor pathways [15]. An important factor in M1 occurrence is the erosion of telomeres that occurs with each cell division in cells lacking telomerase activity [16]. Ectopic expression of hTERT, the catalytic component of telomerase, can reverse the effects of telomere loss and under appropriate conditions enables many types of human cells to bypass the M1 checkpoint and extend their lifespan [17–19]. The second checkpoint, crisis or M2, is described as a process in which continued proliferation is counteracted by extensive cell death [20]. Eventually, the culture declines as the rate of cell death overrides the rate of proliferation. This end-point differs from senescence where cells do not proliferate or undergo active cell death; senescent cells arrest in G1 and remain intact, viable and metabolically active [15]. Rarely, cells expressing exogenous hTERT may escape M2 and proliferate indefinitely. Human fibroblasts expressing the SV40 large T antigen escape crisis at a rate of 1 in 3×10^7 [14]. The majority of cells that escape crisis and become immortal express the enzyme telomerase, which maintains telomeric structure through continuous cell division [12, 16].

The goal of the present study was to investigate the expression patterns of individual SSc fibroblasts isolated from a whole cell population. The clonal cell lines produced after hTERT lentiviral infection are homogeneous and have a potentially extended lifespan to enable extensive mRNA and protein expression analysis. Our results demonstrate that: (i) the biopsy fibroblast population of SSc tissue is heterogeneous and (ii) a fraction of SSc fibroblasts express multiple profibrotic genes simultaneously.

Materials and methods

Fibroblast culture

Skin biopsy samples were taken from the affected areas of the dorsal forearm of two SSc patients who had diffuse cutaneous SSc. All patients fulfilled the American College of Rheumatology criteria for SSc [21] and had not undergone any treatment for SSc at the time of biopsy. The 'SSc 341' biopsy came from a 45-year-old, white female with a skin score of 16. The 'SSc 345' biopsy came from a 41-year-old black female with a skin score of 10. Normal adult control biopsies were derived from healthy donors matched with each SSc patient for age, gender and race. Informed consent was obtained from all study subjects prior to biopsy. Studies were performed in compliance with the Institutional Review Board of Human Studies, Medical University of South Carolina.

Biopsy tissue was rinsed several times with antibiotic-antimycotic solution (Life Technologies, Brooklyn, NY, USA). Tissue was then placed in 1 ml of 0.25% collagenase solution (Sigma, St. Louis, MO, USA) and incubated for 24 hrs at 37°C. The entire 1 ml was mixed together with 5 ml of media (DMEM + 20% FCS) then plated into a 25 cm² flask. The resulting confluent culture was then designated passage zero and was used for infection with hTERT lentivirus.

Construction of lentiviral vectors

An EcoRI fragment containing the Kozak consensus sequence and the coding sequence of hTERT were obtained from the recombinant plasmid pLPC-hTERT (Clontech, Mountain View, CA, USA). The Entry clone pENTR1A-hTERT was constructed by inserting the hTERT EcoRI fragment in EcoRI sites of pENTR1A vector (Invitrogen, Carlsbad, CA, USA). Using Gateway technology, we introduced the hTERT gene from pENTR1A-hTERT into pLenti4/V5-DEST vector to create the expression plasmid (Invitrogen).

Production of lentiviral stocks and titration

Lentivirus was made by transfecting 3 µg of pLenti4/V5-hTERT expression plasmid and 12 µg of viral packaging vectors (Packaging Mix, Invitrogen) into 293FT cells (Invitrogen) by lipid transfection (Transfectin, Bio-Rad, Hercules, CA, USA). 4×10^6 293FT cells were seeded into 10-cm tissue culture plates 8 hrs prior to transfection and the medium was replaced 16 hrs after the transfection. Vector conditioned medium was collected 24 hrs post-transfection, centrifuged at $2000 \times g$ to remove cellular debris and filtered through a 0.45-µm pore-size filter (Nalgene, Rochester, NY, USA) before being aliquoted and frozen at -80°C . High-titre vector stocks were prepared by ultracentrifugation ($45,000 \times g$, 90 min.). To determine vector titres, an aliquot of preparation was thawed and serial dilutions were added in the presence of 6 µg/ml polybrene (Sigma, St Louis, MO, USA) to 2×10^5 HeLa cells which had been seeded in 12-well plates 8 hrs earlier. Virus-containing media was replaced by complete culture medium the next day and replaced the day after by media with 100 µg/ml Zeocin. After 2–3 weeks, colonies were counted to determine the lentiviral stock titre (transducing units/ml; TU/ml).

Infection of cultured fibroblasts

The day before infection, 4×10^5 fibroblasts were seeded in 100-mm plates. Fibroblasts were incubated overnight with viral supernatant at a

multiplicity of infection of 0.5–1 and 6 $\mu\text{g/ml}$ polybrene. Virus-containing media was replaced by complete culture medium the next day and replaced the day after by media with 100 $\mu\text{g/ml}$ Zeocin. Media was replaced every 3–4 days until antibiotic-resistant colonies could be identified. Colonies were selectively trypsinized and grown into clonal cell lines.

Telomerase activity

Telomerase activity in each cell line was detected by telomeric repeat amplification protocol (TRAP) assay using the TRAPeze kit (Chemicon, Temecula, CA, USA) according to the manufacturer's protocol.

Quantitative real-time PCR

Total RNA was isolated from fibroblasts using Tri reagent (MRC Inc., Chicago, IL, USA) according to the manufacturer's instructions. Two μg of RNA was used for reverse transcription cDNA synthesis in 20 μl reaction volume using random primers and then diluted to 40 μl . Quantitative real-time PCR (qRT-PCR) was carried out using IQ Sybr green mix and an Icyler machine (Bio-Rad, Hercules, CA, USA) using 1 μl of diluted cDNA in triplicate with β -actin as the internal control. PCR conditions were 95°C for 3 min., followed by 40 cycles of 95°C for 30 sec., 58°C for 1 min. Melt curve analysis of the PCR products confirmed the absence of secondary products.

Western blotting

Confluent SSc and NS clones were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulphate [SDS], 1% Nonidet P40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride). Protein concentrations were quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked in 3% milk in Tris-Buffered Saline-Tween (TTBS) (Collagen I, TSP1, pSmad1, Smad1, TGF- β RI, β -actin) or 2% gelatin in TTBS (CCN2) for 1 hr at room temperature and probed with primary antibody overnight at 4°C. After TTBS washes, membranes were probed with horseradish peroxidase-conjugated secondary antibody against the appropriate species. Proteins levels were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and quantified using Image J densitometry software. The collagen antibody (Southern Biotechnology, Birmingham, AL, USA) was used at a 1:1000 dilution. The CCN2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a 1:3000 dilution. The TSP1 antibody was a gift from Murphy-Ulrich and was used at a 1:5000 dilution. pSmad1 and Smad1 (Cell Signaling, Danvers, MA, USA) and TGF- β RI (Santa Cruz, CA, USA) were used at 1:500 dilution. β -actin (Sigma) was used at a dilution of 1:5000.

Fibroblast treatments

hTERT clones were cultured to confluence and serum starved for 24 hrs before the addition of 2.5 ng/ml TGF- β (Peprotech, Rock Hill, NJ, USA), 0.5 μM sphingosine-1-phosphate (Avanti, Alabaster, AL, USA), 0.5 μM dihydroshingosine-1-phosphate (Avanti, Alabaster, AL, USA) or 10 ng/ml tumour necrosis factor α (Promega, Madison, WI, USA) for 48 hrs.

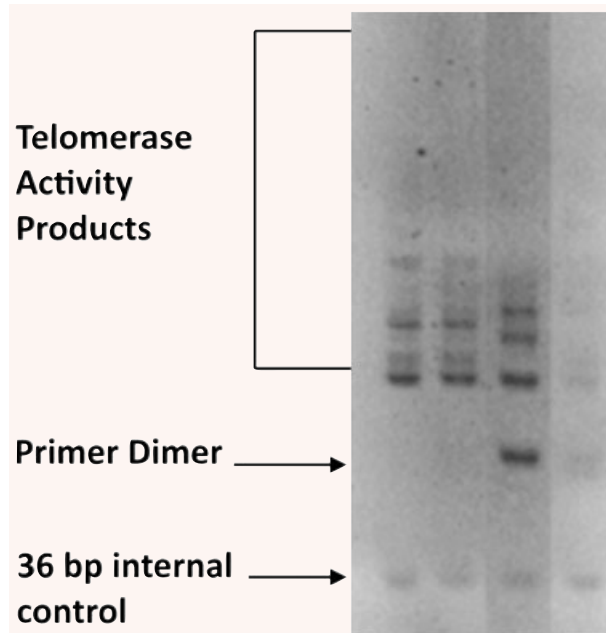


Fig. 1 hTERT clones maintain telomerase activity after repeated passaging. hTERT activity was assayed in each clone using the TRAPeze[®] Telomerase Detection Kit (Chemicon) by PCR amplification of the telomerase added repeats with primers specific for these telomeres. The resulting PCR product was resolved in an acrylamide gel. The presence of 6bp DNA steps demonstrates hTERT activity. Several NS/SSc clones show persistent hTERT activity after repeated passaging in culture. Representative hTERT clones NS342–3, SSc345–1 and SSc 345–3 are shown in lanes 1, 2 and 3. Lane 4 is a non-infected normal dermal fibroblast for a negative control.

Results

hTERT activity assay and clone lifespan

Fibroblasts were isolated and cultured from SSc and NS biopsies and transduced with hTERT DNA in passage zero. The hTERT gene was introduced into each culture using a lentivirus construct containing zeocin resistance. Several zeocin-resistant colonies were selected from each cell population for further characterization. We established 35 fibroblast clones that showed recombinant hTERT expression, 19 normal and 16 SSc, which may suggest that normal fibroblasts are easier to immortalize by this method. To confirm that hTERT overexpression was stably incorporated into the cellular DNA, hTERT activity was measured in several clones using the TRAP. This assay is not sensitive enough to detect levels of endogenous hTERT. The presence of six base pair DNA repeats indicates that hTERT is overexpressed in the clonal cell line. The PCR product of a representative clone is shown in Figure 1.

Most of the hTERT clones bypassed the first mortality checkpoint, M1 or senescence. Out of the 35 clones showing recombinant hTERT activity, 32 clones bypassed senescence and passed 90 population doublings (PDL). These clones showed no indication of the senescent-like phenotype of a flat shape, enlarged size and reduced growth rate. Control cells that were transfected with vector alone reached senescence before 50 PDL. Expression levels of collagen $\alpha 1(I)$ (COL1A1), thrombospondin 1 (TSP1), lysyl hydroxylase 2 (PLOD2) and connective tissue growth factor (CCN2) mRNA were analysed for NS342/SSc341 hTERT clones after 50 and 90 PDL. No significant change in the expression level of these genes was observed (data not shown), suggesting that the phenotype of the clones is stable. We continued growing five clones expressing high collagen levels from the NS346/SSc345 pair. After 110–150 PDL, four clones entered the second mortality checkpoint, M2 or crisis. Cells in crisis were contracted and loosely attached to the culture flask. Two clones passed crisis and continued proliferating for >200 PDL.

SSc and NS hTERT clones have similar morphology and growth rates

hTERT clones were passaged for greater than 90 population doublings without any apparent change in morphology. The morphology of hTERT clones NS342–7, SSc341–3, NS346–4, SSc345–1 and SSc345–3 is shown with phase contrast images in Figure 2A. The morphology of normal dermal fibroblasts in passage 6 is shown for comparison. hTERT clones maintain the appearance of normal fibroblasts demonstrating that hTERT lentiviral infection had no effect on clone morphology.

Six SSc341 and seven NS342 hTERT clones were used for analyses of growth rate. Two non-transfected normal SSc cell lines were included as controls. A total of 10,000 cells were plated in each well of a 24-well plate in DMEM + 10% FCS. Cells from three wells were trypsinized and counted every 2 days. Cell counts were plotted to determine the growth rate of each clone. Although some variation was observed between clones, the overall growth rate pattern of NS and SSc clones was similar (Fig. 2B). This indicates that altered growth rate is not an aspect of the SSc fibroblast phenotype in these hTERT clones. hTERT clone growth rates were also unchanged compared to non-transfected cells indicating that hTERT lentiviral infection does not affect growth.

mRNA and protein expression profiling of hTERT clones

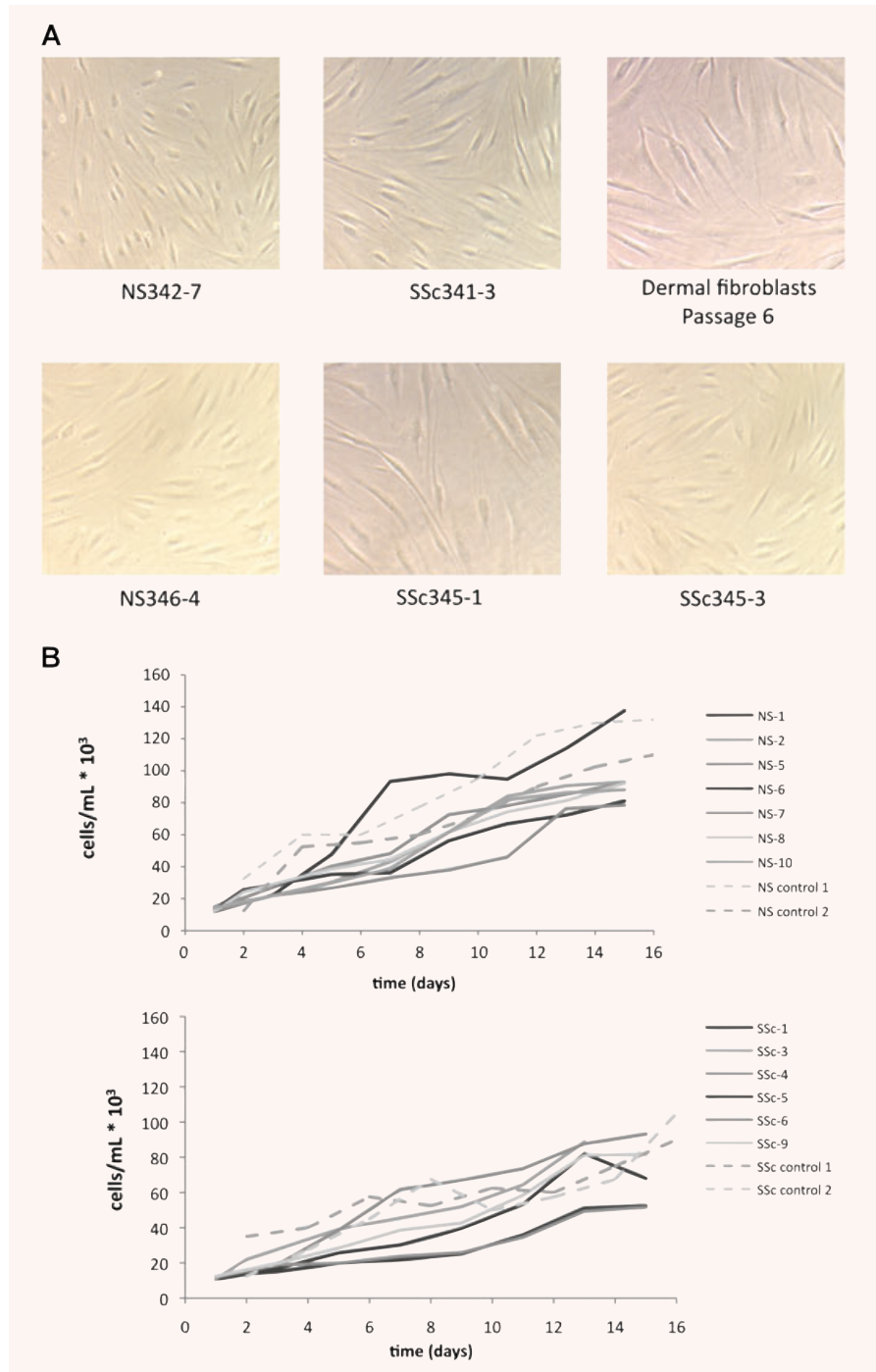
Seven SSc341 and eight NS342 clones were examined for the mRNA levels of nine profibrotic genes including COL1A1, TSP1, CCN2, Smad1, PLOD2, fibrillin 2 (FBN), collagen XI, desmin and tenascin C by qRT-PCR. An hTERT clone derived from each NS biopsy showing low levels of collagen expression was named normal clone 1 (NS1). mRNA levels for each hTERT clone were nor-

malized to NS1 so they could be compared between experiments. Average expression levels for each gene in NS and SSc clones are indicated with black bars (Fig. 3A). mRNA levels of COL1A1 and TSP1 were highly variable among SSc and NS clones measured. COL1A1 mRNA levels were significantly increased in SSc clones *versus* NS clones (6.2 ± 3.4 fold *versus* 2.87 ± 1.71 , $P < 0.01$). TSP1 mRNA levels were also significantly increased (5.01 ± 3.5 fold *versus* 1.67 ± 0.04 , $P < 0.01$). Expression of CCN2 and Smad1 was somewhat less variable, but the CCN2 mRNA level was also significantly increased in SSc clones (2.8 ± 0.9 fold *versus* 1.79 ± 0.69 , $P < 0.05$). Smad1 mRNA level was increased (2.08 ± 0.23 fold *versus* 1.27 ± 0.18), but the difference did not reach statistical significance. As shown in Figure 3A, PLOD2 and FBN2 average expression was similar in NS342 and SSc341. Other genes measured including desmin, collagen XI and tenascin C showed a similar expression pattern to FBN2 with only slight variation among hTERT clones from the NS and SSc populations (data not shown).

Consistent with the mRNA findings, the protein expression of collagen I, CCN2 and TSP1 displayed heterogeneous patterns among SSc and normal clones (Fig. 3B and C). Individual clonal expression of each protein reflected mRNA expression. The strongest agreement between mRNA and protein expression in SSc was observed in collagen I (Fig. 3B). Based on recent studies that demonstrated activation of the TGF- β receptor type I (TGF- β RI)/Smad1 pathway in a subset of SSc patients [22, 23], we also assessed protein expression of TGF- β RI, Smad1 and pSmad1 to detect the presence of altered TGF- β signalling in these clones. Only one clone, S3, showed elevated expression of TGF- β RI, Smad1, pSmad1 and ECM proteins suggesting altered TGF- β signalling. In other clones (S1, S4, and S9), ECM proteins were elevated, but altered TGF- β RI/Smad1 signalling was not observed suggesting activation of an alternative signalling pathway. Evidence that SSc fibroblasts from the same biopsy can be activated in different ways further underscores the complexity of SSc. Smad1 levels could not be measured in SSc 341 clone 6 because this clone reached crisis. NS342 clones also demonstrated heterogeneous expression of ECM proteins (Fig. 3C). Overall, NS342 clones showed lower levels of ECM proteins than SSc clones. N2 and N7 showed moderately elevated levels of collagen I. N2 also showed elevated levels of CCN2. Levels of TSP1 were increased in N5, N7 and N10. Although total Smad1 levels varied between individual clones, TGF- β RI and pSmad1 were not elevated in any NS342 clones demonstrating that TGF- β /Smad1 signalling was not activated.

Similar mRNA and protein analyses were performed for the SSc345 and NS346 hTERT clones (Fig. 4). A total of eight SSc and eight NS clones were analysed. Individual qRT-PCR runs were normalized to N1 in order to be compared. mRNA expression of COL1A1, TSP1, CCN2 and Smad1 were variable among NS346 and SSc345 clones (Fig. 4A). Average expression of these genes was significantly higher in SSc compared to NS, although differences between SSc and NS clones were less pronounced than in the previous pair. COL1A1 was increased in SSc *versus* NS (2.52 ± 0.09 fold *versus* 1.89 ± 0.14 , $P < 0.01$); TSP1 was increased

Fig. 2 hTERT clone morphology and growth kinetics are not altered. **(A)** Cells were cultured in DMEM with 10% fetal calf serum. hTERT clone morphology is similar to normal dermal fibroblasts. **(B)** NS342 and SSc342 clones were seeded at 10,000 cells/well in a 24-well plate. Normal and SSc non-telomerized cell lines were included in each growth curve (dotted lines). Cell counts were performed on three wells every 2 days for 15 days.



(3.23 ± 0.17 fold versus 2.05 ± 0.12 , $P < 0.05$); CCN2 was increased (2.88 ± 0.15 versus 1.86 ± 0.07 , $P < 0.05$); and Smad1 was increased (1.99 ± 0.12 versus 1.15 ± 0.09 , $P < 0.01$). Expression of PLOD2 and FBN2 mRNA was not variable among clones or elevated in SSc345. Other genes measured

including desmin, collagen XI and tenascin C showed a similar expression pattern to FBN2 with only slight variation among hTERT clones from the NS and SSc populations (data not shown).

Analyses of protein expression in SSc345 clones revealed that similar to the previous pair, protein levels of collagen I and CCN2

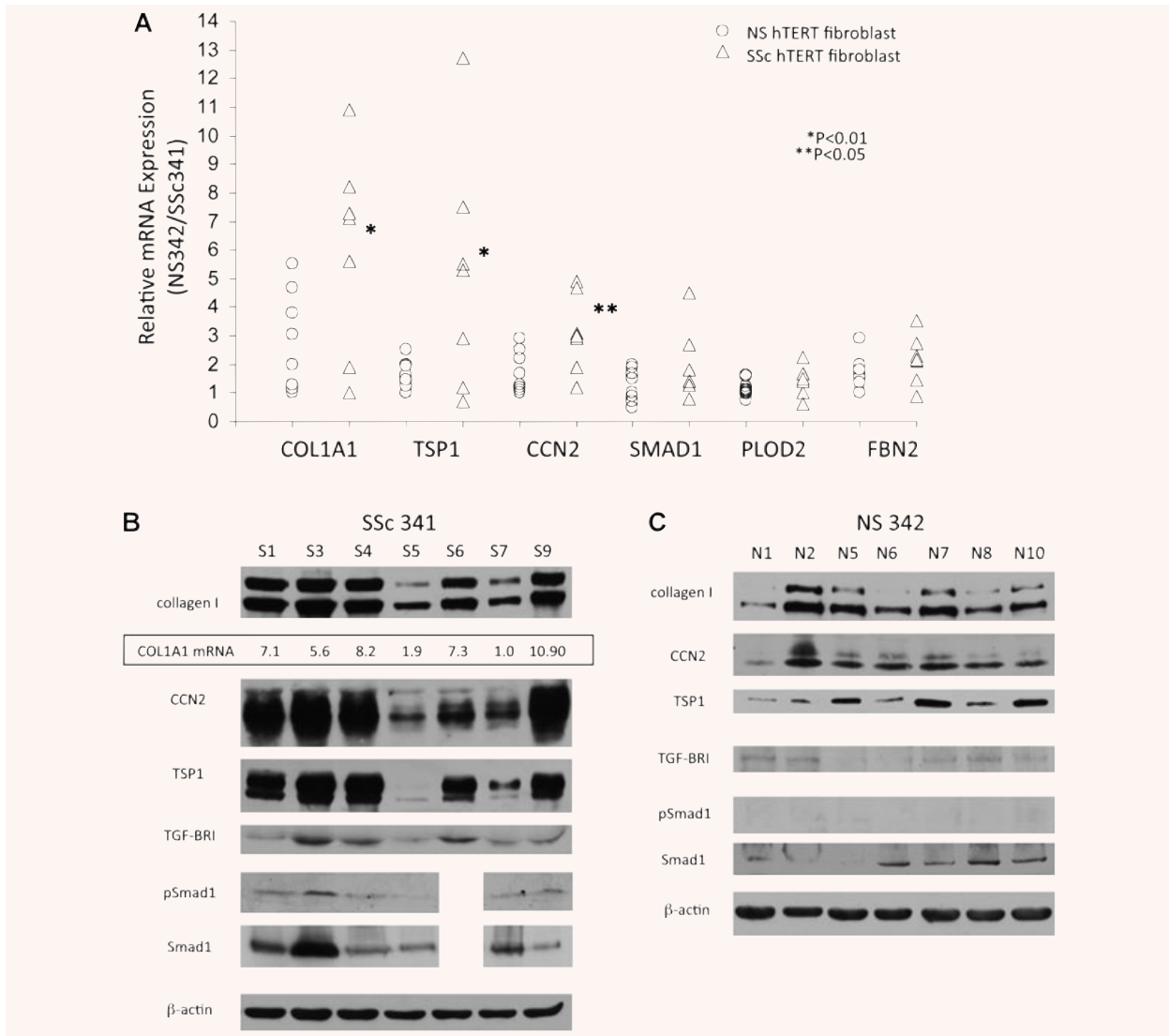
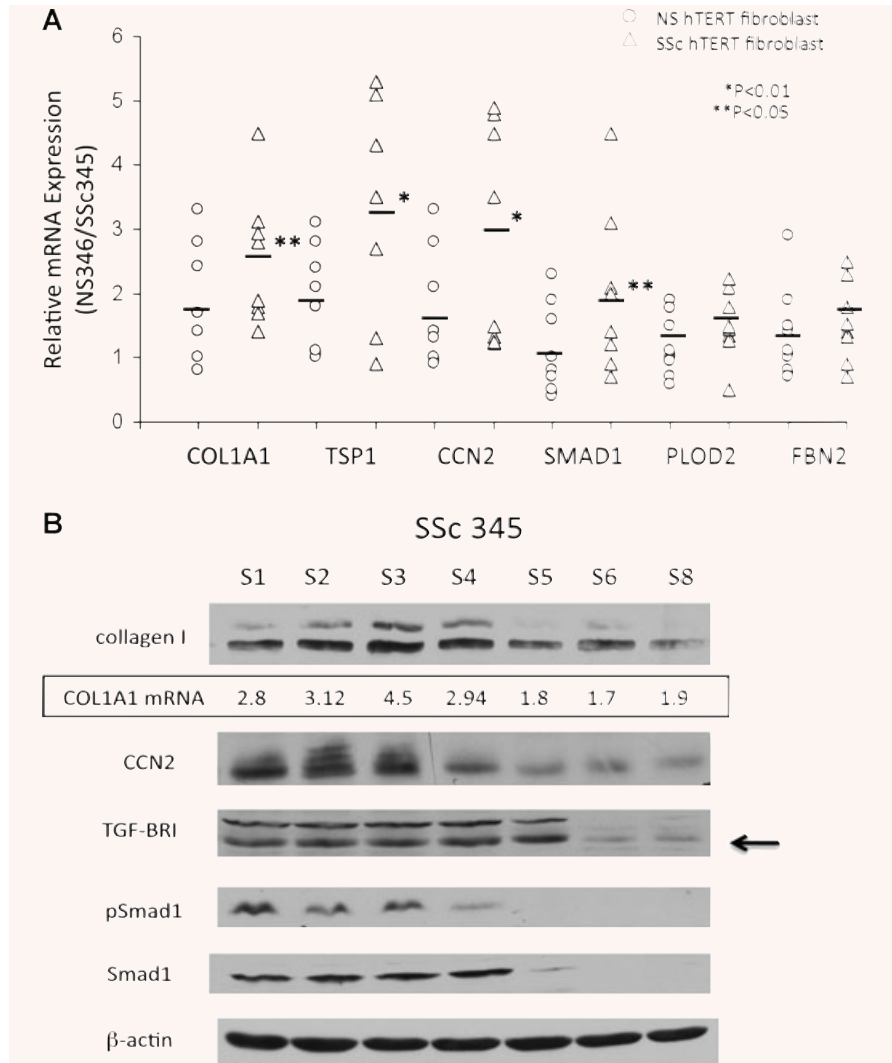


Fig. 3 mRNA and protein expression of SSc-related genes in NS342/SSc341 hTERT clonal fibroblasts. **(A)** Confluent dishes of hTERT clones were serum starved for 24 hrs before total RNA was isolated using Tri reagent. cDNA was reverse transcribed using 2 μ g of RNA. One μ l of cDNA was used for qRT-PCR. mRNA levels of COL1A1, TSP1, CCN2, Smad1, PLOD2 and FBN2 were measured in several NS and SSc clones from each pair and normalized to β -actin. Each experiment was repeated three times. SSc clonal expression levels are shown in circles and NS expression levels are shown in triangles. Mean mRNA expression levels for each gene are indicated with bars. A statistically significant difference between mean NS and SSc clone mRNA levels was detected for COL1A1, TSP1 and CCN2, * $P < 0.01$; ** $P < 0.05$. **(B)** SSc 341 clones were grown to confluence and serum starved for 24 hrs. Cells were harvested and lysates were subjected to SDS-PAGE. Membranes were probed with antibodies against specific proteins. Protein expression for seven SSc clones is shown to demonstrate the heterogeneity of clonal SSc phenotypes. Fold expression levels of COL1A1 mRNA (from Fig. 3A) are shown to demonstrate the correlation between collagen I protein and mRNA. **(C)** NS 342 clones were grown, harvested and lysed as described in part B. Protein expression for seven NS clones is shown.

were heterogeneous and showed a good correlation between mRNA and protein expression (Fig. 4B and data not shown). The relationship between collagen I mRNA and protein was the strongest, supporting the view that elevated expression of the collagen gene in SSc is mainly due to transcriptional activation. We

were not able to measure protein expression of TSP1 in SSc345 clones. In contrast to SSc341, the majority of the SSc345 clones showed altered TGF- β signalling. In clones S1-S4, elevated levels of TGF- β R1 correlated with elevated SMAD1 levels and in addition, phosphoSmad1 was detected in SSc 345 clones S1, S2 and S3.

Fig. 4 mRNA and protein expression of SSc-related genes in NS346/SSc345 hTERT clonal fibroblasts. **(A)** cDNA was made and qRT-PCR was performed as described in Figure 3A. The difference between mRNA levels in NS and SSc of COL1A1, TSP1, CCN2 and SMAD1 are statistically significant, * $P < 0.01$; ** $P < 0.05$. **(B)** SSc 345 clones were prepared as described in Figure 3B. Protein expression for seven SSc clones demonstrates the heterogeneity between clones. COL1A1 mRNA expression from Figure 4A is included.



This adds further complexity to SSc because the means of activation varies between individual fibroblasts from the same biopsy and also between biopsies from different patients.

observation provides further evidence for the existence of activated, profibrotic fibroblasts in the total population of fibroblasts.

Correlative expression of COL1A1 and CCN2 mRNA in NS/SSc clones

To further characterize the expression profile of the SSc hTERT clones, we tested each clone for correlative mRNA expression of the genes measured. In both pairs, we found that several clones expressed elevated levels of multiple pro-fibrotic genes. Conversely, many clones also demonstrated reduced expression of multiple genes. Correlative expression of CCN2 and COL1A1 was found in NS342/SSc341 and NS346/SSc345 with $R^2 = 0.845$ and $R^2 = 0.768$, respectively (Fig. 5). Interestingly, these activated fibroblasts were isolated as hTERT clones from both NS and SSc skin. This

TGF- β responsiveness is maintained in hTERT clones

SSc and normal clones from each pair of biopsies were treated with TGF- β , sphingosine-1 phosphate (S1P), dihydrosphingosine-1 phosphate (dhS1P) and tumour necrosis factor α (TNF α) in Figure 6. TGF- β and TNF α are classical mediators of the fibrotic response [24, 25], while S1P and dhS1P were recently shown to regulate collagen production in an opposite manner [26]. Clones were cultured for greater than 90 population doublings. Treatments were added for 48 hrs then Western blots for collagen I and CCN2 were performed to measure effects on ECM protein regulation. All clones retained the ability to respond to TGF- β with marked

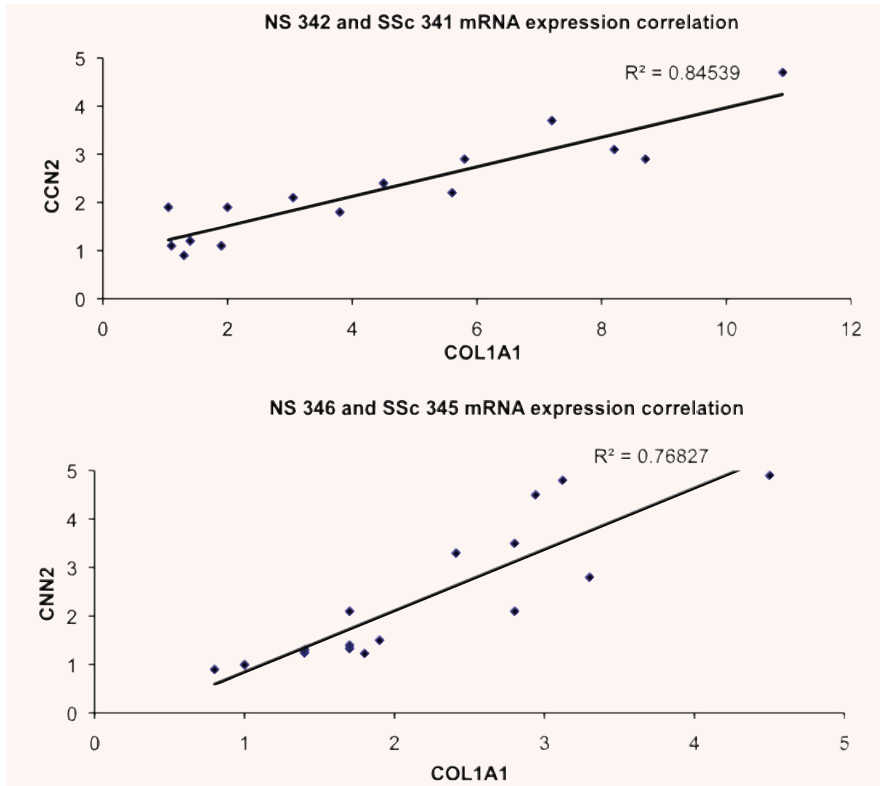


Fig. 5 Collagen and CCN2 mRNA expression correlates in NS and SSc hTERT clones. NS and SSc mRNA data from Figures 3A and 4A were used for correlation analysis between genes measured. A strong linear correlation was found in expression of CCN2 and COL1A1 mRNA for both pairs.

up-regulation in collagen I and CCN2. In comparison, S1P was a weak stimulator of either collagen or CCN2 only in selected clones (SSc345-1, NS342-7 and SSc341-3), and likewise, dhS1P was slightly inhibitory only in NS346-5, SSc 345-3 and SSc345-4. TNF α did not produce a consistent response. Despite continuous passaging for several months and greater than 90 PDL in culture, these clones do retain the ability to respond to treatment with TGF- β . Slight variations were observed in response to treatments between clonal cell lines.

Discussion

This study characterized clonal expression of profibrotic markers in fibroblasts isolated from SSc and healthy skin. hTERT overexpression in two SSc and normal fibroblast populations isolated distinct, heterogeneous phenotypes. As shown previously, SSc fibroblast populations are heterogeneous with regards to collagen I. This study has provided a more complete picture of the phenotype heterogeneity present in SSc fibroblast populations. qRT-PCR data show an average increase in collagen I, CCN2 and TSP1 in SSc and expression of these genes is very heterogeneous in SSc and NS fibroblast clones. SSc protein data are in agreement with mRNA expression levels. Several SSc clones show correlative

expression of multiple profibrotic proteins indicating expansion of select fibroblasts in SSc. In agreement with the principal role of CCN2 as a mediator of SSc fibrosis [27], we found a strong correlation between expression of collagen I and CCN2 in all fibroblasts. A matricellular protein, TSP1, was also consistently elevated. It has been suggested that TSP1 contributes to autocrine TGF- β signalling in SSc fibroblasts by promoting conversion of TGF- β from a latent to an active form and increasing availability of the ligand to bind to its receptors [28]. Interestingly, evidence for the activation of the TGF- β R1/Smad1 pathway was seen in SSc345, but not in SSc341, NS342 or NS345 clones. TGF- β signals through two distinct Smad pathways; receptor type I ALK5 and Smad2/3 or receptor type I ALK1 and Smad1/5/8 [29]. It was recently reported that activation of the Smad1 signalling pathway occurs in a subset of SSc patients and contributes to activation of SSc fibroblasts [23]. The presence of pSmad1 indicates activated TGF- β signalling through the profibrotic Smad1 pathway. Although our analyses were limited to clones derived from only two SSc patients, the results are consistent with the view that SSc is a highly heterogeneous disease [2].

The panel of profibrotic genes analysed in this study was selected based on a recent gene array analysis that compared the phenotype of SSc fibroblasts from fibrotic lesions to cultured fibroblasts isolated from the same lesions [1]. The mRNA levels of several genes including COL1A1, TSP1, CCN2, PLOD2, collagen XI, FBN2, desmin and tenascin C was shown to be elevated in SSc

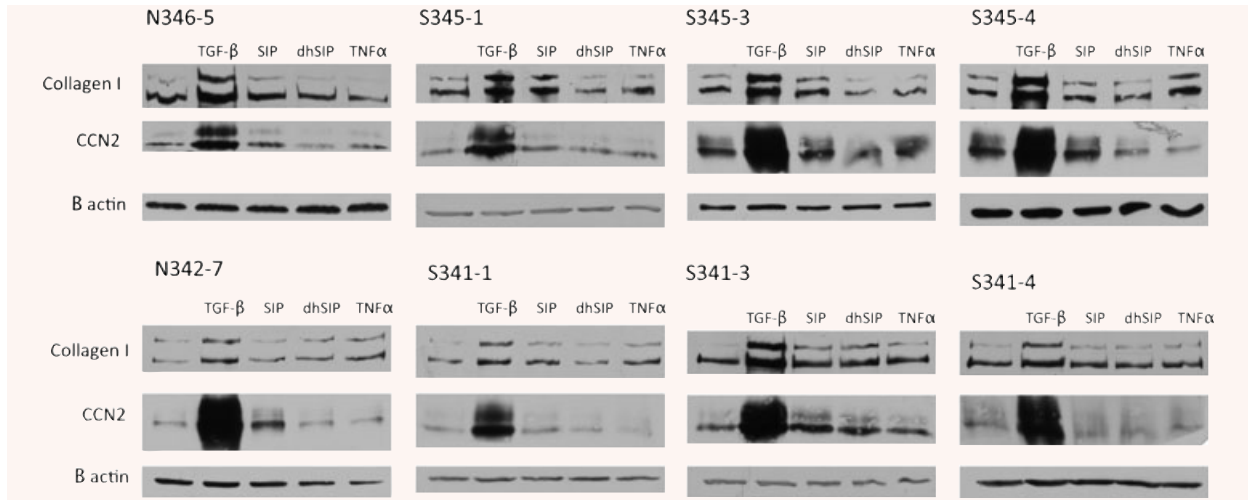


Fig. 6 NS342/SSc341 and NS346/SSc345 response to treatment with fibrotic mediators. hTERT clones were treated with TGF- β , SIP, dhSIP and TNF α after >90 population doublings in culture. Cell responses to TGF- β were the most pronounced and partial responses to other treatments were observed.

biopsies, but was not maintained in cultured fibroblasts. One possible explanation could be a loss of phenotype during passaging in culture. We show that average mRNA expression of COL1A1, TSP1 and CCN2 was significantly higher in SSc clones than NS in both pairs. However, the heterogeneous nature of individual clones might be the main reason why these differences are frequently lost during subpassaging. Surprisingly, PLOD2 and FBN2 were not altered in either NS/SSc pair suggesting that although these genes might be important in SSc *in vivo*, their expression pattern is not preserved *in vitro*. It is possible that *in vitro* culture conditions are artificial and cannot recapitulate the environment in which fibroblasts exist *in vivo*. In cell culture, fibroblasts are not exposed to other cell types and ECM components as they are *in vivo*. Furthermore, serum present in the culture media may activate fibroblasts and alter gene expression profiles and protein production. This suggests that only selected aspects of the fibrogenic pathway, *e.g.* CCN2/collagen axis, are accessible to mechanistic *in vitro* studies.

Previously, *in vitro* studies of SSc fibrosis have been based on whole fibroblast populations isolated from skin biopsies and cultured. hTERT clonal cell lines and whole fibroblast populations from biopsy represent distinct *in vitro* models of SSc. The hTERT model provides unique information about single fibroblast phenotypes, which can then be further investigated to delineate the mechanism involved in the activation process. The culture of whole fibroblast populations may represent a better model of the total disease phenotype and may be more useful when assessing trends in the SSc phenotype or in the initial characterization of potential drug therapies.

This study demonstrates that the lifespan of skin fibroblasts in SSc patients can be extended by introduction of the hTERT gene without alterations in growth rate or morphology. The majority of

clones successfully bypassed the initial mortality checkpoint, M1 or senescence. We have continuously passaged only five clones here. One clone (SSc 341 clone 6) stopped proliferating after this point. Two of our clones passed M2 or crisis for >200 PDL. One of these clones entered crisis after 115 PDL but recovered to continue proliferation. The second clone passed > 200 PDL without entering crisis and bypassed M2 entirely. Nakamura *et al.* described clones from the skin of normal and ataxia telangiectasia patients as immortalized after they passed 200 PDL without any crisis [30]. This group used an hTERT retrovirus, which infects only actively dividing cells while in these studies we have used lentivirus that infects both dividing and nondividing cells. Lentiviral selection may have contributed to our difficulty in immortalizing SSc fibroblasts. Other studies attempting immortalization with human embryonic fibroblasts described one-third of 26 clones as immortalized [31, 32]. The reasons behind successful immortalization of any cell type are not understood. While these SSc cell lines are not immortalized, merely the extension of their lifespan was useful for phenotype characterization. Extending the lifespan of clonal cell lines enabled us to characterize distinct, heterogeneous phenotypes within the same biopsy.

SSc is a complex disease and despite intense effort, effective antifibrotic treatments are currently not available. The molecular mechanisms involved in disease pathogenesis may differ in each patient making SSc a difficult disease to understand and cure. Extending the lifespan of SSc fibroblasts with hTERT expression allows detailed characterization of the selected fibrogenic pathways manifested in culture. From this model, we can begin a thorough investigation into the activated fibroblast population in SSc that is responsible for its phenotype. This study also underscores the great need for additional disease models that would recapitulate the heterogeneous disease characteristics of SSc.

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