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Acid Sphingomyelinase Deficiency Contributes to Resistance of Scleroderma fibroblasts to Fas-mediated Apoptosis

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Abstract

Background—Scleroderma (SSc) is characterized by excess production and deposition of extracellular matrix (ECM) proteins. Activated fibroblasts play a key role in fibrosis in SSc and are resistant to Fas-mediated apoptosis. Acid Sphingomyelinase (ASMase), a major sphingolipid enzyme, plays an important role in the Fas mediated apoptosis.

Objective—We investigated whether dysregulation of ASMase contributes to Fas-mediated apoptosis resistance in SSc fibroblasts.

Methods—Fibroblasts were isolated from SSc patients and healthy controls. Western blot was performed to analyze protein levels and quantitative real time RT-PCR was used to determine mRNA expression. Cells were transiently transfected with siRNA oligos against ASMase or transduced with adenoviruses overexpressing ASMase. Apoptosis was induced using anti-Fas antibody (1µg/mL) and analyzed using Caspase-3 Antibody or Cell Death Detection ELISA.

Results—SSc fibroblasts showed increased resistance to Fas mediated apoptosis. ASMase expression was decreased in SSc fibroblasts and Transforming Growth Factor beta (TGFβ), the major fibrogenic cytokine involved in the pathogenesis of SSc, downregulated ASMase in normal fibroblasts. Forced expression of ASMase in SSc fibroblasts restored sensitivity of these cells to Fas mediated apoptosis while blockade of ASMase was sufficient to induce partial resistance to Fas-induced apoptosis in normal fibroblasts. In addition, ASMase blockade decreased activity of Protein Phosphatase 2A (PP2A) through phosphorylation on Tyr³⁰⁷ and resulted in activation of extracellular regulated kinase 1/2 (Erk1/2) and acutely transforming retrovirus AKT8 in rodent T-cell lymphoma (Akt).

Conclusion—In conclusion, this study suggests that ASMase deficiency promotes apoptosis resistance and contributes to activation of profibrotic signaling in SSc fibroblasts.

INTRODUCTION

Wound healing or the repair of damaged tissue is a fundamental process that restores injured tissue to maintain normal tissue architecture and function [1,2]. This process is critical for survival, however in the presence of chronic stimulus or inflammation the wound repair

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process becomes dysregulated and results in fibrosis. During normal wound healing, activation of the immune system and release of cytokines results in recruitment, activation or differentiation of myofibroblasts which play a key role in wound closure [3]. These myofibroblasts then undergo apoptosis, however in the context of fibrosis, activation of fibroblasts is persistent due to chronic secretion of proinflammatory cytokines such as Interleukin 4 (IL-4), Interleukin 13 (IL-13), Tumor Necrosis Factor alpha (TNF α) and profibrotic cytokines such as TGF β and Platelet Derived Growth Factor (PDGF) [4–6]. In contrast to normal wound healing, during pathological fibrosis, myofibroblasts persist and continue to produce and deposit ECM components [3, 7].

SSc is an autoimmune connective tissue disease that is characterized by excess production and deposition of extracellular matrix proteins by activated fibroblasts (myofibroblasts) resulting in extensive fibrosis of skin and internal organs [8]. Persistent TGF β signaling is considered to be the major factor contributing to chronic fibrosis [9]. SSc patients express elevated TGF β levels in the early lesions, but not in established fibrotic tissue [10]. Additionally, fibroblasts from SSc patients also express higher levels of TGF β receptors suggesting a role for TGF β signaling in initiating as well as maintaining the fibrotic response [11].

Dermal fibroblasts express the Fas-receptor and can be induced to undergo Fas-mediated apoptosis upon stimulation with Fas ligand. Fas (CD-95/ APO-1) belongs to the TNF receptor superfamily and is a potent inducer of apoptosis. Fas induced apoptosis helps terminate the fibroproliferative response in experimental models of liver and lung fibrosis [12]. In liver fibrosis, injury to hepatic stellate cells results in upregulation of Fas receptor and induction of Fas-mediated apoptosis [13]. However, the failure of the normal wound healing response to terminate results in fibrosis of skin, and internal organs such as lung, heart, kidney and GI tract. Several studies have shown that SSc fibroblasts are particularly resistant to Fas-induced apoptosis despite similar levels of Fas receptor [14,15]. Interestingly, chronic exposure of normal dermal fibroblasts to TGF β , the major profibrogenic cytokine involved in SSc pathogenesis, enhances their resistance to apoptosis [15]. Synovial fibroblasts treated with TGF β also show increased resistance to apoptosis in conjunction with decreased Fas expression and increased B-cell CLL/lymphoma 2 (Bcl2) expression [16]. However studies with SSc fibroblasts do not show a significant change in expression Fas receptor suggesting that other mechanisms may be involved [14].

Recent studies have implicated a major sphingolipid enzyme ASMase in the process of Fas mediated apoptosis. ASMase is involved in the conversion of sphingomyelinase to ceramide and studies using ASMase $^{-/-}$ cells from patients with NPDA (Niemann Pick Disease A), who have an inherent lack of ASMase and from ASMase knock-out mice show that ASMase is crucial for the induction of apoptosis through external stimuli including CD95/Fas, tumor necrosis factor receptor (TNF-R) and other stress stimuli [17]. Upon an external stimulus such as Fas ligand, ASMase is rapidly translocated to the outer leaflet of the lipid bilayer where it is activated and hydrolyses sphingomyelin to ceramide, resulting in formation of ceramide rafts and further clustering of Fas receptors, initiating the apoptotic process [18–20]. Although there are conflicting reports with regard to the role of ASMase in Fas signaling, evidence predominantly suggests that ASMase plays a key role in this process. Other relevant molecules involved in apoptosis have also been shown to be dysregulated in SSc fibroblasts and have been described in the ASMase mediated apoptotic pathway. Akt and ERK1/2 are two major survival pathways that are hyperphosphorylated in SSc fibroblasts and contribute to fibrosis. A chief Akt and ERK1/2 phosphatase PP2A is also dysregulated in SSc fibroblasts. In particular, previously published data from our laboratory suggests that PP2A is downregulated in SSc in response to TGF β and contributes to aberrant ERK1/2 phosphorylation [21]. Interestingly, PP2A is activated by ceramide, the product of

ASMase activity suggesting a role for PP2A and the Akt/ERK1/2 pathway as potential downstream mediators of ASMase dysregulation.

The goal of this study was to delineate the role of ASMase in resistance of SSc dermal fibroblasts to Fas-mediated apoptosis. Our study demonstrates that ASMase is constitutively down-regulated in SSc fibroblasts and in response to TGF β signaling in healthy fibroblasts. Overexpression of ASMase in SSc fibroblasts reversed the resistance of these fibroblasts to Fas mediated apoptosis whereas blockade of ASMase in normal fibroblasts partially induced resistance to Fas-mediated apoptosis. Furthermore, our study also shows that ASMase regulates other important mediators implicated in fibrosis, PP2A, and ERK1/2 suggesting multiple mechanisms by which ASMase could contribute to the SSc phenotype.

Materials and Methods

Reagents

The following antibodies were used: anti-SMPD1 (ASMase), anti-phospho ERK1/2, anti-phospho Akt, anti-ERK1/2, anti-Akt, anti-cleaved caspase 3 (Cell Signaling, Beverly, MA), monoclonal β -actin (Sigma, St Louis, MO), anti-PP2A (Millipore, Billerica, MA) and anti-phospho PP2A Y307 (Epitomics, Burlingame, CA). Recombinant human TGF- β 1 was obtained from Peprotech (Rocky Hill, NJ). Imipramine, Annexin V and Propidium Iodide were purchased from Sigma Aldrich (St Louis, MO). Tissue culture reagents, Dulbecco's Modified Eagle Medium (DMEM) and 100x antibiotic antimycotic solution (penicillin streptomycin and amphotericin B) were purchased from Gibco BRL (Grand Island, NY) and fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). Enhanced chemiluminescence reagent (ECL) and BCA protein assay reagent were purchased from Pierce Chemical Co. (Rockford, IL). TriReagent was obtained from the Molecular Research Center Inc. (Cincinnati, OH). Primers were purchased from Operon (Huntsville, AL). The Cell Death Detection ELISA Plus Kit was purchased from Roche Applied Sciences (Indianapolis, IN).

Cell Culture

Human dermal fibroblast cultures were established from biopsy specimens obtained from the dorsal forearm of SSc patients with diffuse cutaneous disease and from age, race and gender matched healthy donors, upon informed consent and in compliance with the Institutional Review Board. Dermal fibroblasts were cultured from the biopsy specimens as described previously [22]. Normal and SSc skin fibroblasts were cultured in DMEM supplemented with 10% FBS and 1% antibiotic antimycotic solution.

Real-Time PCR

Total RNA was isolated from dermal fibroblasts using TriReagent according to the manufacturer's instructions. 2 μ g of RNA was reverse transcribed in a 20- μ l reaction using random primers and Transcriptor First Strand synthesis kit (Roche Applied Sciences). qPCR was carried out using IQ SYBR Green mixture (Bio-Rad) on an iCycler PCR machine (Bio-Rad) using 1 μ l of cDNA in triplicate with β -actin as the internal control. The primers used are as follows: PP2A C-subunit: forward, 5'-GCACTTGATCGCCTACAAGA-3' and reverse, 5'-GAAATATCTTGCCCAAAGGTGT-3'. β -actin: forward 5'-AATGTCGCGGAGGACCTTTGATTGC-3' and reverse 5'-AGGATGGCAAGGGACTTCCTGTAA-3'. Ad5: forward 5'-GCACACGTGCATACACTTCC-3' and reverse 5'-TGTTCCCTGGGATATGGTTC-3'. ASMase: forward 5'-CTATGAAGCGATGGCCAAG-3' and reverse 5'-TGGGGAAAGAGCATAGAACC-3'.

Immunoblotting

Whole cell protein extracts were prepared in RIPA buffer and protein quantified by BCA protein assay kit according to the manufacturer's recommendations (ThermoScientific, Rockford, IL). Equal amounts of total protein from each sample were separated via SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were then blocked in milk in Tris-buffered saline with Tween (TBST) and probed overnight with primary antibody at 4°C. Following washes with TBST, the membranes were probed with HRP-conjugated secondary antibody against the appropriate species for 1–2 h at room temperature. Protein levels were visualized using ECL reagents.

RNA interference

Smartpool siRNA against SMPD1 (ASMase) was purchased from Dharmacon RNA technologies (Lafayette, CO), and negative-control siRNA from Qiagen (Germantown, MD). HiPerFect siRNA transfection reagent (Qiagen) was used for transfection of dermal fibroblasts according to the manufacturer's recommendations.

Immunofluorescence

For immunocytochemistry experiments, dermal fibroblasts were grown on chamber slides and following various treatments were washed 3X with phosphate buffered saline (PBS). Cells were fixed in 4% paraformaldehyde for 10 min and washed 3X with PBS. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, followed by blocking in 1% BSA for 1 hour. Cells were then stained with ASMase (rabbit, 1:200) antibody in 1% BSA in PBS. The bound antibody was detected by rabbit secondary antibody to either AlexaFluor 488 or 546 and used at 1:200 dilutions. Ten random blinded fields were examined using an Olympus microscope attached to a digital camera.

Cell Death Detection ELISA

The cell Death Detection ELISA kit that uses mouse monoclonal antibodies against DNA and histones was used to determine percentage of apoptosis. Cell lysates from treated and control cells were pipetted onto streptavidin coated plates and relative rate of apoptosis was determined spectrophotometrically according to the manufacturer's protocol.

Overexpression of Adenoviral ASMase

An adenoviral vector expressing ASMase or green fluorescent protein (GFP) was generated using the AdEasy method described by He et al [23]. cDNA encoding ASMase was cloned in the shuttle vector pAdTRACK-CMV, which contains a GFP expression cassette driven by a separate CMV promoter, and was used to generate recombinant adenoviruses. A control adenovirus expressing GFP alone was generated via the same method for use as a control vector. The ASMase construct contains a V-5 tag, which can be used to visualize the levels of adenoviral ASMase expression using western blot analysis. SSc fibroblasts were transduced with adenoviral ASMase (AdASM) or adenovirus expressing GFP (AdG0) in serum free media for 48 h and cells were analyzed for mRNA and protein expression and induction of apoptosis.

RESULTS

SSc dermal fibroblasts are resistant to Fas-mediated apoptosis

We first investigated the sensitivity of SSc and normal fibroblasts to Fas-mediated apoptosis. Following stimulation with anti-Fas antibody, apoptosis was measured in SSc and normal fibroblasts using the Cell Death Detection ELISA from Roche and cleaved caspase-3 western blot analysis. During Fas mediated apoptosis, Caspase-3, a critical executioner of

apoptosis is activated and cleaved into a large 17/19kDa fragment and a smaller 12kDa fragment. The antibody used in these experiments detects the larger 17 and 19 kDa fragments. Using the Cell Death Detection ELISA, the relative rate of apoptosis was significantly decreased in SSc fibroblasts compared to healthy fibroblasts in response to anti-Fas antibody (Fig 1a). These results were confirmed using Caspase-3 western blot analysis. SSc fibroblasts showed decreased levels of cleaved Caspase-3 when compared to healthy dermal fibroblasts (Fig 1b). These experiments are consistent with previous reports demonstrating increased resistance of SSc fibroblasts to Fas mediated apoptosis.

Acid Sphingomyelinase is decreased in SSc fibroblasts

To determine whether Fas mediated apoptosis resistance of SSc fibroblasts could be a result of altered ASMase expression the mRNA and protein levels of ASMase were analyzed. In the majority of SSc fibroblasts, basal expression of ASMase was decreased in comparison to normal control fibroblasts (Fig 2a). Due to a lack of availability of commercial antibodies suitable to detect ASMase by western blot, protein levels were analyzed by immunofluorescence. Antibody specificity was confirmed by siRNA experiments (See Fig 5b). Consistent with the mRNA expression, a decrease in the protein levels was also observed by immunostaining (Figure 2b). These results indicate that ASMase levels are decreased in SSc fibroblasts suggesting a possible role for ASMase in mediating apoptosis resistance in these cells. Our attempts to verify these results in vivo using sections from SSc patient skin and normal controls were unsuccessful, because we were unable to obtain optimal staining with currently available antibodies.

TGF β regulates ASMase expression in normal dermal fibroblasts

TGF β , the major profibrogenic cytokine involved in SSc pathogenesis, regulates many of the phenotypic features of SSc fibroblasts. To determine if TGF β signaling regulates ASMase expression, normal fibroblasts were treated with 2.5 ng/ml of TGF β for 24 hours. ASMase mRNA and protein expression were measured in untreated and treated fibroblasts using real time PCR and immunostaining. A significant decrease in mRNA and protein levels of ASMase was observed after TGF β treatment, suggesting that TGF β is involved in the regulation of ASMase gene expression (Fig 3a, b). Further, blockade of autocrine TGF β signaling in SSc fibroblasts using TGF β neutralizing antibody (TGF β NA) restored mRNA expression of ASMase further confirming the role of TGF β in regulation of ASMase (Fig 3c).

Overexpression of ASMase restores susceptibility of SSc fibroblasts to Fas mediated apoptosis

Based on the known role for ASMase in Fas mediated apoptosis, we analyzed whether restoration of ASMase in SSc fibroblasts could reverse the apoptosis resistance of these fibroblasts. An adenovirus vector expressing ASMase (AdASM) was used restore ASMase levels within the cells. To establish experimental conditions that mimic the expression levels of ASMase in normal cells, adult dermal fibroblasts were transduced with increasing multiplicity of infection (MOI) of AdASM. The expression levels of ASMase were monitored by real time PCR (Fig 4a) and Western blotting using the V-5 tag antibody (Fig 4b) and normalized to β -actin control. Adenovirus expressing GFP alone (AdGo) was used as a control. For further experiments, the lowest dose of adenovirus (10 MOI) was used. The adenovirus specific Ad5 gene was used to titrate equal amounts of AdASM and AdGo (Fig 4c). As expected, ASMase overexpression induced increased apoptosis in SSc fibroblasts in response to anti-Fas antibody as measured by levels of cleaved Caspase-3 by western blot (Fig 4d) and further confirmed by the cell death detection ELISA (Fig 4e).

Blockade of ASMase induces apoptosis resistance in normal fibroblasts

To determine if ASMase is important for Fas mediated apoptosis signaling in normal dermal fibroblasts, ASMase was inhibited using siRNA. Effective inhibition of ASMase mRNA and protein was observed after 72-hour treatment with 30nM siRNA (Figure 5a, b). ASMase inhibition in dermal fibroblasts resulted in a decrease in Fas mediated apoptosis as measured by Caspase-3 western blot and Cell death detection ELISA (Fig 5c, d). Following ASMase inhibition a slight increase in apoptosis was observed in some normal fibroblasts prior to addition of Fas although overall there was no significant change at the basal level. These results suggest that inhibition of ASMase in normal fibroblasts is sufficient to induce at least partial resistance to Fas-mediated apoptosis.

ASMase depletion decreases PP2A activity and enhances Akt and ERK1/2 phosphorylation

Next, we wanted to determine whether downregulation of ASMase could affect other signaling pathways that are dysregulated in SSc. Previous data also link PP2A activity to ceramide, the product of ASMase activity [24]. Phosphorylation of the catalytic subunit of PP2A at tyrosine residue 307 has been shown to inactivate PP2A [25, 26]. To determine whether dysregulation of ASMase could affect expression or activity of PP2A, and other signaling molecules under its regulation ASMase was depleted in normal fibroblasts. Blockade of ASMase modestly stimulated PP2A phosphorylation although total PP2A levels remained unchanged (Fig 6a, b). Furthermore, ASMase blockade induced Akt and ERK1/2 phosphorylation, both of which have been previously described as targets of PP2A and have been implicated in apoptosis as well as fibrosis (Fig 6c). To further confirm the role of ASMase in regulation of Akt and ERK1/2 phosphorylation we overexpressed adenoviral ASMase in SSc fibroblasts for western blot analysis. ERK1/2 phosphorylation was decreased upon ASMase overexpression as expected, however Akt phosphorylation did not change.

DISCUSSION

Previous studies have shown that induction of Fas-mediated apoptosis is impaired in SSc fibroblasts when compared to normal fibroblasts. This study describes a novel role for acid sphingomyelinase in Fas-mediated apoptosis resistance in SSc fibroblasts. We observed that ASMase expression was decreased at the mRNA and protein levels in SSc fibroblasts and in normal fibroblasts treated with TGF β , suggesting transcriptional regulation of ASMase by TGF β . Overexpression of ASMase in SSc fibroblasts restored sensitivity of SSc fibroblasts to Fas-mediated apoptosis, while blockade of ASMase in normal fibroblasts partially induced resistance to Fas mediated apoptosis. In addition, blockade of ASMase led to activation of other prosurvival/profibrotic pathways, Akt and ERK1/2. Together, these results suggest that ASMase is a critical mediator of the anti-apoptotic effects of TGF β in dermal fibroblasts and its deficiency plays a central role in apoptosis resistance in SSc fibroblasts.

ASMase is one of the major enzymes involved in generation of ceramide, a key signaling molecule involved in various physiological and pathological aspects of cell signaling, growth and development. Ceramide has been widely studied for its role in inducing apoptosis in various cell types. Interestingly, ceramide also plays a role in activation of major phosphatases known as Ceramide Activated Protein Phosphatases (CAPPs), including PP2A and phosphoprotein phosphatase 1 (PP1) [27]. Several studies have shown that inhibition of PP2A prevents apoptosis in various tissues in response to ceramide, suggesting a role for PP2A in ceramide-mediated apoptosis [28, 29]. Ceramide mediated activation of PP2A promotes inactivation of prosurvival/anti-apoptotic mediators such as Akt, Bcl2 and CL2-associated agonist of cell death (Bad) [28, 30, 31]. Our data show that depletion of

ASMase inactivates PP2A via phosphorylation at the Tyr³⁰⁷ and this correlates with increased phosphorylation of Akt and ERK1/2. We have previously shown that PP2A is also constitutively downregulated in SSc fibroblasts as a result of chronic autocrine TGF β signaling [21]. TGF β transcriptionally regulates both ASMase and PP2A, suggesting that the ASMase-PP2A pathway is important in maintaining the SSc phenotype. Furthermore, this study demonstrates that PP2A can also be inactivated via ASMase blockade, suggesting a dual role for TGF β in regulation of PP2A, directly through transcriptional modulation and indirectly via posttranslational modification.

The importance of myofibroblast apoptosis in wound healing and normal repair is demonstrated by the persistence of these cells in pathogenic conditions, leading to excess fibrosis and scarring. Defects in fibroblast apoptosis have been implicated in a variety of fibrotic diseases including SSc. Several studies have suggested that the persistence of myofibroblasts in fibrosis is a key reason for the chronic production and deposition of ECM proteins. Our study describes a novel role for ASMase in SSc pathogenesis through its contribution to apoptosis resistance in SSc fibroblasts. Targeting the high collagen producing SSc fibroblasts to undergo apoptosis could be a novel therapeutic approach to alleviate fibrosis and restore normal cell function.

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Fig 1. SSc fibroblasts are resistant to Fas-mediated apoptosis

Normal and SSc dermal fibroblasts were treated with anti-Fas antibody (1 ug/mL) for 18h and induction of apoptosis was assessed by a) Cell Death Detection ELISA which was measured spectrophotometrically and represented as relative rate of apoptosis (* $p < 0.01$, $n = 3$, normal fibroblasts, $n = 3$, SSc fibroblasts). b) Cleaved Caspase-3 western blot, ($n = 3$, representative blot), β -actin was used as a loading control.

Fig 2. ASMase expression is decreased in SSc fibroblasts

Normal and SSc dermal fibroblasts were analyzed for a) ASMase mRNA expression by real time PCR (* $p < 0.05$, $n = 5$ SSc and 5 normal cell lines) and b) ASMase protein expression by immunofluorescence microscopy using a specific antibody against ASMase (Red) and nuclei counterstained with Dapi (Blue) ($n = 3$, representative image is shown).

Fig 3. TGFβ signaling regulates ASMase expression in normal fibroblasts

Normal dermal fibroblasts treated with TGFβ (2.5ng/mL) for 24 h were analyzed for a) ASMase mRNA expression by real time PCR (*p<0.05, n=3) and b) ASMase (red) protein expression by immunofluorescence (n=3, representative image is shown). Dapi was used for counterstaining of nuclei (blue). c) ASMase mRNA expression after treatment of SSc fibroblasts with TGFβNA (10ug/mL) for 24 and 48h (*p<0.05, n=5).

Fig 4. ASMase overexpression reverses apoptosis resistance of SSc fibroblasts

Dose response of ASMase adenovirus to increasing MOI at the a) mRNA level using real time PCR (n=3, p< 0.05) and b) protein level using V-5 tag antibody western blot (n=3). c) Equal amounts of AdASM and AdG0 (10 MOI) were titrated using adenoviral Ad5 gene by real time PCR, (n=3, p<0.05). d) SSc fibroblasts treated with ASMase adenovirus (48h) and then with anti-Fas antibody (18h) were analyzed for apoptosis by cleaved caspase-3 western blot (n=3, β-actin was used as the loading control) and e) relative rate of apoptosis as measured spectrophotometrically by the Cell Death Detection ELISA (n=3, *p< 0.05).

Fig 5. ASMase depletion contributes to apoptosis resistance in normal fibroblasts

a) mRNA levels ($*p < 0.001$) and b) protein levels of ASMase (green) after depletion by specific siRNA. Dapi (blue) was used as a nuclear counterstain. c) Normal fibroblasts treated with control siRNA (Scr) or ASMase siRNA (ASMsi) and then treated with 1 μ g/mL anti-Fas antibody for 18h and analyzed for apoptosis by cleaved caspase-3 western blot (n=3, β -actin was used as the loading control). d) Cells from c) were analyzed using the Cell death detection ELISA and results represented as relative rate of apoptosis (n=3, $*p < 0.05$).

Fig 6. Blockade of ASMase inactivates PP2A and increases phosphorylation of Akt and ERK1/2 Cell extracts of normal dermal fibroblasts treated with 30nM of Scr or ASMsi for 48h were analyzed for a) PP2A mRNA expression by real time PCR, b) protein expression of phospho-PP2A and total PP2A by western blot analysis, (n=3, β -actin was the loading control, representative blot is shown) c) Cell extracts of normal dermal fibroblasts treated with 30nM of Scr or ASMsi for 48h were analyzed for phosphorylation of Akt and ERK1/2 by western blot analysis (n=3, β -actin was used as the loading control, representative blot is shown). d) SSc fibroblasts treated with AdG0 or AdASM were analyzed by western blot for phosphorylation of Akt and ERK1/2 (n=3, β -actin was used as the loading control, representative blot is shown).