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Distler, O ; Schett, G ; Distler, J H W

Abstract: **BACKGROUND:** Dermal fibroblasts from patients with systemic sclerosis (SSc) release excessive amounts of collagen resulting in tissue fibrosis. The molecular mechanisms underlying this pathological activation are incompletely understood. **OBJECTIVE:** To investigate whether Notch signalling contributes to the uncontrolled activation of fibroblasts in SSc. **METHODS:** Activation of the Notch pathway was assessed by immunohistochemistry or Western blot for the Notch intracellular domain and the Notch ligand Jagged-1 (Jag-1) and real-time PCR for the target gene *hes-1*. Differentiation of resting dermal fibroblasts into myofibroblasts was assessed by staining for α -smooth muscle actin. The synthesis of collagen was quantified by real-time PCR and Sircol assays. **RESULTS:** Notch signalling was activated in lesional skin of patients with SSc. The activation persisted in cultured dermal SSc fibroblasts. Stimulation of healthy dermal fibroblasts with recombinant human Jag-1-Fc chimera resulted in an SSc-like phenotype with increased release of collagen and differentiation of resting fibroblasts into myofibroblasts. Consistent with the selective activation of the Notch pathway in dermal SSc fibroblasts, DAPT or siRNA against Notch strongly reduced the basal collagen expression in SSc fibroblasts, but not in fibroblasts from healthy volunteers. **CONCLUSION:** It was shown that Notch signalling is activated in SSc and plays an important role in fibroblast activation and collagen release. Inhibition of Notch signalling might be an effective strategy to selectively prevent the aberrant activation of SSc fibroblasts.

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Notch signaling regulates fibroblast activation and collagen release in systemic sclerosis

Clara Dees¹, Michal Tomcik^{1,2}, Pawel Zerr¹, Alfiya Akhmetshina¹, Angelika Horn¹, Katrin Palumbo¹, Christian Beyer¹, Jochen Zwerina¹, Oliver Distler³, Georg Schett¹, Jörg H.W. Distler¹

¹ Department of Internal Medicine III and Institute for Clinical Immunology, University of Erlangen-Nuremberg, Germany; ² Institute of Rheumatology and Connective Tissue Research Laboratory, Department of Rheumatology of the First Faculty of Medicine, Charles University Prague, Czech Republic; ³ Center of Experimental Rheumatology and Zurich Center of Integrative Human Physiology, University Hospital Zurich, Switzerland

Corresponding author and reprint requests: Jörg H. W. Distler, MD; Department of Internal Medicine 3 and Institute for Clinical Immunology, University of Erlangen-Nuremberg, Germany, Tel.: +49 9131 8543008, FAX: +49 9131 8539226, Email: joerg.distler@uk-erlangen.de

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Abstract

Objectives: Dermal fibroblasts from patients with systemic sclerosis (SSc) release excessive amounts of collagen resulting in tissue fibrosis. The molecular mechanisms underlying this pathologic activation are incompletely understood. This study aimed to investigate whether Notch signaling contributes to the uncontrolled activation of fibroblasts in SSc.

Methods: Activation of the Notch pathway was assessed by immunohistochemistry or Western Blot for the Notch intracellular domain (NICD) and the Notch ligand Jagged-1 (Jag-1) and real-time PCR for the target gene *hes-1*. Differentiation of resting dermal fibroblasts into myofibroblasts was assessed by staining for α -smooth muscle actin and stress fibers. The synthesis of collagen was quantified by real-time PCR and SirCol assays.

Results: Notch signaling was activated in lesional skin of SSc patients. The activation persisted in cultured dermal SSc fibroblasts. Stimulation of healthy dermal fibroblasts with recombinant human Jag-1-Fc chimera resulted in an SSc-like phenotype with increased release of collagen and differentiation of resting fibroblasts into myofibroblasts. Consistent with the selective activation of the Notch pathway in dermal SSc fibroblasts, DAPT or siRNA against Notch strongly reduced the basal collagen expression in SSc fibroblasts, but not in fibroblasts from healthy volunteers.

Conclusion: We demonstrated that Notch signaling is activated in SSc and plays an important role in fibroblast activation and collagen release. Inhibition of Notch signaling might be an effective strategy to selectively prevent the aberrant activation of SSc fibroblasts.

Key words: Systemic sclerosis, Scleroderma

Introduction

The major histopathologic hallmark of SSc is an excessive accumulation of extracellular matrix proteins.[1, 2] The molecular mechanisms underlying the pathologic activation of SSc fibroblasts have only partially been elucidated.

Notch signaling, first discovered in *Drosophila*, is essential for cell fate decision and development.[3, 4] Activation of the Notch pathway by binding of ligands such as Jagged-1 (Jag-1) results in cleavage of Notch receptors by the γ -secretase complex with subsequent release of the active Notch intracellular domain (NICD).[3] Two families of canonical Notch ligands have been described (Jagged and Delta-like), which seem to have distinct function.[5, 6] As described for T helper cell differentiation, Jagged induces Th2 response, whereas Delta-like, mainly Dll-4, is responsible for Th1 differentiation.[5] These ligands can interact with Notch receptors on both the surface of another cell (*trans*-interaction) and of the same cell (*cis*-interaction).[6] In contrast to the *trans*-interaction, *cis*-interactions seem to inhibit Notch signaling. The mechanisms and effects of *cis*-interactions, however, are still unknown.[6] In addition to the canonical ligands, several non-canonical ligands such as CCN3, MAGP-1 and MAGP-2 have been described.[6] Translocation of the NICD into the nucleus activates the transcription of target genes such as the Hairy/Enhancer of Split (Hes).[7] Alterations in Notch signaling are implicated in the pathogenesis of several human diseases such as T cell acute lymphoblastic leukemia (T-ALL), melanoma and Alagille syndrome.[8-11] Clinical trials with γ -secretase inhibitors in patients with T-ALL already yielded promising results.[12]

The present study aimed to investigate whether Notch signaling may promote fibroblast activation and persistently increased release of collagen in SSc.

Material and Methods

Fibroblast cultures

Dermal fibroblast cultures were obtained from skin biopsies of SSc patients. All patients fulfilled the criteria for SSc as suggested by LeRoy et al.[13] Biopsies from SSc patients (n = 10) were taken from involved skin of the forearm (supplementary Table 1). Control dermal fibroblasts (n = 10) were obtained from forearm skin biopsies of healthy age- and sex-matched volunteers. Dermal fibroblasts from passages 4-8 were used for the experiments. All patients and controls signed a consent form approved by the local institutional review boards.

Stimulation of dermal fibroblasts with Jag-1 and inhibition of Notch signaling

Confluent dermal fibroblasts were cultured in DMEM containing 0.1 % FCS for 24 h before the experiments. Dermal fibroblasts were stimulated with recombinant human Jag-1 Fc Chimera (R&D Systems, Minneapolis, MN, USA) dissolved in PBS in concentrations from 0.04 to 5.0 µg/ml for 24 h.

To assess the effects of Notch signaling inhibition, dermal fibroblasts were incubated with the γ -secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Sigma-Aldrich, Steinheim, Germany) at concentrations of 1.0 and 10 µM for 24 h. Jag-1 was added one hour after DAPT at a concentration of 5.0 µg/ml.

In addition to DAPT, dermal SSc fibroblasts were transfected with 1.5 µg siRNA against Notch-1 using the human dermal fibroblast Nucleofector Kit (Amaza, Cologne, Germany) (supplementary Table 2).[14] Fibroblasts transfected with control siRNA (Ambion, Darmstadt, Germany) served as controls. Cells were harvested after 48 h.

mRNA-stability assay

To investigate whether Jag-1 stabilizes collagen mRNA, mRNA-stability assay was performed as previously described.[15]

Luciferase reporter assay

The -353 col1a2 / luciferase construct was kindly provided by M. Trojanowska.[15] HEK293T cells were transfected in serum-free medium with 0.5 µg reporter construct mixed with polyethyleneimine (PEI). A common lacZ reporter vector was used as control. Cells were stimulated with Jag-1 directly after the transfection. The luciferase activity was analyzed after 24 h.

Quantitative real time PCR

Total RNA was isolated with the NucleoSpin RNA II extraction system (Machery-Nagel, Düren, Germany) and converted into cDNA as described.[16] In skin samples, the epidermal and dermal layers were separated for RNA isolation. The primer pairs used for real-time PCR are given in Supplementary Table 2. A pre-developed β-actin assay (Applied Biosystems) was used to normalize for the amounts of loaded cDNA. All samples were measured in duplicates.

Quantification of soluble collagen protein

Total soluble collagen in cell culture supernatants was quantified using the SirCol collagen assay (Biocolor, Belfast, Northern Ireland) as described.[17]

Immunohistochemistry for Jag-1, CD3 and the NICD

Fresh frozen sections of forearm skin from SSc patients (n = 13; supplementary Table 3) and healthy individuals (n = 10) were used for immunohistochemical analyses (see Supplementary Methods).

Immunofluorescence for α -smooth muscle actin and the NICD

Fibroblasts stimulated with Jag-1-Fc (5 μ g/ml) were incubated with monoclonal anti- α SMA antibodies (clone 1A4, Sigma-Aldrich, Steinheim, Germany) and polyclonal rabbit-anti-human NICD (Abcam, Cambridge, UK). TRITC conjugated goat anti-mouse antibodies and FITC conjugated goat anti-rabbit antibodies (Invitrogen, Karlsruhe, Germany) served as secondary antibodies. Counterstaining was performed with DAPI (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) for ten minutes at room temperature.

Western Blot

Whole cell lysates were prepared as previously described.[18] PVDF membranes were incubated with polyclonal rabbit-anti Notch-1 (Abcam, Cambridge, UK) or polyclonal rabbit-anti activated Notch-1 (Abcam, Cambridge, UK). Horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibodies (DAKO, Hamburg, Germany) were used as secondary antibodies. Equal loading of proteins was confirmed by visualization of α -tubulin (Invitrogen).

Statistics

Data are expressed as mean $\pm$ standard error of the mean. The Wilcoxon signed rank tests for related samples and the Mann-Whitney-U-test for non-related samples were used for statistical analyses. A p-value of less than 0.05 was considered statistically significant.

Results

Notch signaling is activated in lesional skin of SSc patients

We first analyzed the expression of the four Notch receptors in dermal fibroblasts. The levels of Notch-1 mRNA were significantly increased in SSc fibroblasts as compared to controls (Supplementary Figure 1). Notch-2 was detectable, but in contrast to Notch-1, its levels did not differ between SSc and controls. Notch-3 and Notch-4 were not detectable by real-time PCR in dermal fibroblasts as these receptors are predominantly expressed in vascular smooth muscle cells and vascular endothelial cells.[19]

To investigate whether Notch signaling is activated in lesional skin of SSc patients, we stained for the NICD, which is only released upon ligand binding to Notch and therefore is a marker for activation of the Notch pathway.[3] The NICD was almost undetectable in healthy individuals (Figure 1a). In contrast, we observed an intensive staining in all SSc patients, particularly in fibroblasts of the papillary dermis (Figure 1a). Staining for the NICD, however, was not restricted to fibroblasts and was also prominent in keratinocytes, endothelial cells, pericytes, vascular smooth muscle cells and T cells as described before (Figure 1a).[20] Consistent with increased Notch signaling, the mRNA levels of the Notch target gene *hes-1* were elevated by 392 ± 33 % in skin biopsies of SSc patients as compared to skin biopsies from healthy individuals ($p < 0.05$) (Figure 1b). To investigate the mechanisms that activate Notch signaling in SSc, we analyzed the expression of Notch ligands and observed an upregulation of Jag-1 mRNA levels by 608 ± 86 % in SSc skin compared to control skin samples ($p < 0.05$) (Figure 1c). Expression of Jag-1 protein was detected in **epidermis and dermis** of all SSc patients (Figure 1d). A prominent expression of Jag-1 was observed in inflammatory infiltrates in fibrotic skin. Double-staining for Jag-1 and CD3 showed elevated Jag-1 expression particularly in T cells (Figure 1e). In contrast to SSc patients, no significant expression of Jag-1 was detected in healthy individuals.

Persistent activation of the Notch pathway in cultured SSc fibroblasts

Of particular interest, the increased activation of the Notch pathway in dermal SSc fibroblasts persisted *in vitro*. A significant accumulation of the NICD occurred in SSc fibroblasts, whereas the NICD was barely detectable in fibroblasts from healthy individuals (Figure 2a). Consistently, the mRNA levels of *hes-1* were upregulated in cultured SSc fibroblasts in passages 4 to 8 with increases of 375 ± 50 % compared to fibroblasts from healthy individuals ($p < 0.05$) (Figure 2b).

Jag-1 stimulates the release of extracellular matrix

To investigate whether the overexpression of Jag-1 and the increased activation of the Notch pathway contribute to the aberrant production of extracellular matrix, healthy dermal fibroblasts were stimulated with recombinant Jag-1.[21, 22] Activation of the Notch pathway upon stimulation with Jag-1-Fc was confirmed by a dose-dependent increase in *hes-1* mRNA levels (Figure 3a). Jag-1 upregulated dose-dependently the expression of extracellular matrix components reaching a maximum at concentrations of $5 \mu\text{g} / \text{ml}$ (Figures 3b-d). Stimulation with Jag-1-Fc increased the mRNA levels of *col 1a1* and *col 1a2* to 294 ± 37 % and 217 ± 28 %, respectively ($p < 0.05$) (Figure 3b and 3c). Consistent with the results on the mRNA level, collagen protein in cell culture supernatants increased to 259 ± 32 % upon stimulation with Jag-1-Fc as assessed by the SirCol assay ($p < 0.05$) (Figure 3d).

To discriminate between increased gene transcription and altered mRNA stability in response to stimulation with Jag-1-Fc, we performed mRNA stability and reporter assays. The relative changes in mRNA levels of extracellular matrix proteins did not differ between Jag-1-Fc stimulated fibroblasts and controls over time upon incubation with actinomycin D, suggesting that Jag-1-Fc does not increase mRNA stability (supplementary Figure 2a). In contrast, Jag-1-Fc stimulated collagen transcription by 173 ± 12 % as analyzed by a luciferase

expression vector under the control of the col 1a2 promoter ($p < 0.05$) (supplementary Figure 2b).

Jag-1 induces differentiation of resting fibroblasts into myofibroblasts

Myofibroblasts play an important role in the pathogenesis of fibrotic diseases. They are identified by the expression of contractile proteins such as α SMA. Upon stimulation with Jag-1-Fc, the mRNA levels of α SMA increased dose-dependently by $239 \pm 37 \%$ ($p < 0.05$) in normal dermal fibroblasts (Figure 4a). Intensive staining for α SMA protein and for the NICD was observed in Jag-1-Fc stimulated healthy fibroblasts as analyzed by immunofluorescence (Figure 4b). The percentage of double positive cells increased from 19 % in unstimulated control cells to 82 % in Jag-1-Fc stimulated fibroblasts, whereas only 3 % of the cells were positive for the NICD but negative for α SMA, and 6 % of the cells were negative for the NICD but positive for α SMA. Together, these data suggested that Jag-1-Fc stimulates differentiation of resting fibroblasts into myofibroblasts.

DAPT effectively reduces the stimulatory effects of Jag-1

The activity of γ -secretase is essential for the activation of Notch signaling. To evaluate the effects of the γ -secretase inhibitor DAPT on the release of extracellular matrix, we incubated Jag-1 stimulated healthy dermal fibroblasts with DAPT at pharmacologically relevant concentrations.[23, 24] DAPT inhibited the mRNA expression of col 1a1 in healthy dermal fibroblasts stimulated with Jag-1-Fc dose-dependently by up to $99 \pm 23 \%$ ($p < 0.05$) (supplementary Figure 3a). Similar effects were obtained for col 1a2 ($p < 0.05$) (supplementary Figure 3b). Consistent with reduced mRNA levels, the release of collagen protein was reduced by $94 \pm 34 \%$ upon pretreatment with DAPT ($p < 0.05$) (supplementary Figure 3c).

Inhibition of Notch signaling reduces the basal collagen synthesis specifically in SSc fibroblasts

We demonstrated a persistent activation of the Notch pathway in cultured fibroblasts from SSc patients. To assess whether inhibition of the Notch pathway can inhibit the basal release of collagen, SSc fibroblasts were incubated with DAPT in the absence of Jag-1-Fc. Treatment of unstimulated SSc fibroblasts with DAPT reduced the mRNA levels of col 1a1 and col 1a2 by up to $49 \pm 9 \%$ and $41 \pm 6 \%$, respectively ($p < 0.05$ for both) (Figure 5a and b). The collagen content in the supernatants was reduced by up to $59 \pm 11 \%$ upon incubation with DAPT ($p < 0.05$) (Figure 5c). Consistent with the persistent activation of the Notch pathway in SSc fibroblasts, no effects of DAPT on collagen expression were detected in cultured fibroblasts from healthy individuals in the absence of exogenous Jag-1 (Figures 5a-c).

To exclude off-target effects, we confirmed the anti-fibrotic effects of DAPT signaling by transfection of SSc fibroblasts with siRNA against Notch-1 which resulted in significantly decreased mRNA and protein levels of Notch-1 ($p < 0.05$) (Figure 6a). Knockdown of Notch-1 efficiently reduced mRNA levels of col 1a1 and col 1a2 by $58 \pm 11 \%$ and $48 \pm 9 \%$ compared to mock transfected SSc fibroblasts (Figure 6b and c). Consistently, the release of collagen protein was reduced by $42 \pm 8 \%$ ($p < 0.05$) upon silencing of Notch-1 (Figure 6d).

Discussion

We demonstrate in the present study that Notch signaling is activated in SSc with a prominent expression of the ligand Jag-1 in infiltrating T cells and active Notch signaling with accumulation of the NICD in fibroblasts. We further showed that the activation of Notch signaling has major implications on the activation of fibroblasts. The expression patterns of Jag-1 and the NICD suggest that infiltrating T cells expressing Jag-1 might activate Notch signaling in resident fibroblasts. Jag-1 is prominently expressed in T cells in fibrotic skin. Direct cell-cell contact between T cells expressing the ligand Jag-1 and fibroblasts expressing high levels of Notch-1 may be the major mechanism for the activation of Notch signaling in SSc fibroblasts with accumulation of the NICD and increased transcription of target genes such as *hes-1* in fibroblasts. Considering the potent stimulatory effects of Jag-1-Fc on cultured fibroblasts, T cell mediated activation of Notch signaling in fibroblasts might be an important mechanism for the aberrant activation of fibroblasts in SSc. However, further studies are needed to confirm this hypothesis and to further elucidate the molecular mechanism of this activation. Consistent with our findings of an increased activation of Notch signaling in SSc, Kavian et al recently demonstrated an activation of Notch signaling in SSc as well as in mouse models of dermal fibrosis. [25]

We demonstrate that activation of Notch signaling induces a pro-fibrotic phenotype in resting dermal fibroblasts. Jag-1-Fc stimulates the release of collagen protein and induces differentiation of resting dermal fibroblasts into myofibroblasts. Consistent with the increased expression of contractile proteins observed in our study, Notch may also regulate migration of fibroblasts.[23] Thus, pathologic activation of Notch signaling stimulates resting dermal fibroblasts to express contractile proteins, differentiate into myofibroblasts and release excessive amounts of extracellular matrix proteins.

Although evidence for the important role of Notch signaling is growing, it remains unclear how Notch signaling induces myofibroblast differentiation and stimulates the release

of collagen in fibrotic diseases on a molecular level. In this context, it is still obscure if hes-1 induction is essential for these stimulatory effects. Notch signaling may also be induced under hypoxic conditions or by TGF β . [26-28] Consistently, we have preliminary evidence that Notch-1 and hes-1 are increased in dermal fibroblasts upon stimulation with TGF β or under hypoxia. In addition, NICD and pSmad3 seem to be co-expressed in skin of SSc patients. Moreover, recent work indicated that local production of reactive oxygen species might induce ADAM17, a protease involved in the activation of Notch signaling. [25]

Finally, we demonstrated that inhibition of the Notch pathway by either pharmacological blockade of the γ -secretase complex or siRNA against Notch-1 exerted potent anti-fibrotic effects selectively in dermal SSc fibroblasts, whereas the basal collagen synthesis in healthy dermal fibroblasts was not affected by inhibition of Notch signaling. Thus, targeting Notch signaling might have the potential to selectively reduce the activation of dermal SSc fibroblasts without affecting the collagen synthesis in resting normal dermal fibroblasts. In addition, pharmacologic blockade of the γ -secretase complex might decrease the proliferation of SSc fibroblasts. Our in vitro findings are further extended by recent work from Kaviani and coworkers. [25] Treatment with the γ -secretase inhibitor DAPT prevented development of experimental dermal as well as pulmonary fibrosis. In mice, DAPT significantly ameliorated the development of dermal and pulmonary fibrosis, decreased proliferation of fibroblasts, reduced the production of H₂O₂ and abrogated the production of anti-DNA topoisomerase-1 antibodies. [25] These findings might have translational implications as different inhibitors of the γ -secretase complex are in clinical trials for different types of cancer (www.clinicaltrials.gov). However, given the activation of Notch signaling in endothelial cells in our study and the role of Notch signaling in vascular homeostasis (reviewed in [29]), inhibition of Notch signaling might be hindered by anti-angiogenic effects in SSc patients suffering from extensive small-vessel vasculopathy with capillary rarefactions. By contrast, inhibitors of the γ -secretase complex might be beneficial in patients

with proliferative vasculopathies such as pulmonary arterial hypertension, because Notch-3 is activated in vascular smooth muscle cells (vSMCs) from PAH tissue and contributes to the increased proliferation of vSMCs.[30]

In summary, Notch signaling is activated in lesional skin of SSc patients. Considering its potent stimulatory effects on dermal fibroblasts, Notch signaling might contribute to the pathologic activation of SSc fibroblasts.

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Figure legends

Figure 1: Notch signaling is activated in lesional skin of SSc patients. **Figure 1a:** An intensive staining for the Notch intracellular domain was observed in fibroblasts in skin sections of SSc patients, whereas the NICD was almost undetectable in skin sections of healthy individuals. **Figure 1b:** The Notch target gene *hes-1* is overexpressed in lesional skin of SSc patients. **Figure 1c:** Increased mRNA levels of the Notch ligand Jag-1 in skin of SSc patients. **Figure 1d:** Jag-1 is overexpressed in lesional skin of SSc patients. **Figure 1e:** Jag-1 expression is elevated in T cells of inflammatory infiltrates of fibrotic skin.

n = 13 for SSc samples and n = 10 for healthy skin samples. Representative skin sections are shown at 200 fold (Figure 1a and d) or 400 fold (Figure 1e) magnification, fibroblasts are shown at 1000 fold magnification.

* indicates statistical significant differences compared to skin samples from healthy controls.

Figures 2: Persistent activation of the Notch pathway in SSc fibroblasts after long-term culture. **Figure 2a:** Elevated levels of the NICD in SSc fibroblasts as determined by Western blot (n = 3 each). **Figure 2b:** Increased mRNA levels of the target gene *hes-1* (n = 8 for healthy fibroblasts, n = 6 for SSc fibroblasts). * indicates statistical significant differences compared to healthy dermal fibroblasts.

Figure 3: Jag-1 stimulates the synthesis of collagen in fibroblasts. Stimulation of healthy dermal fibroblasts with Jag-1 at concentrations from 0.04 µg/ml to 5 µg/ml increased the mRNA levels of *hes-1* (**Figure 3a**), *col 1a1* (**Figure 3b**) and *col 1a2* (**Figure 3c**) in a dose-dependent manner. Consistently, Jag-1 stimulated the release of collagen protein (**Figure 3d**) (n = 8 each). * indicates statistical significant differences compared to unstimulated fibroblasts.

Figure 4: Jag-1 induces differentiation of resting fibroblasts into myofibroblasts. **Figure 4a:** Jag-1 increased the mRNA levels of α SMA in a dose-dependent manner (n = 8). * indicates statistical significant differences compared to unstimulated fibroblasts. **Figure 4b:** Jag-1 induced the release of NICD and the expression of α SMA protein in resting fibroblasts (n = 5). Representative examples are shown at 200 fold magnification.

Figure 5: The γ -secretase inhibitor DAPT reduces the basal synthesis of extracellular matrix only in SSc fibroblasts. DAPT reduced the basal mRNA levels of col 1a1 (**Figure 5a**) and col 1a2 (**Figure 5b**) and decreased the release of collagen protein (**Figure 5c**) in SSc fibroblasts (n = 7). In contrast, no effect of DAPT on the basal collagen expression was observed in healthy fibroblasts in the absence of exogenous Jag-1 (**Figure 5a-c**) (n = 6). * indicates statistical significant differences compared to untreated fibroblasts.

Figure 6: Knockdown of Notch-1 by siRNA decreases collagen expression in SSc fibroblasts. Nucleofection of SSc fibroblasts with 1.5 μ g of siRNA against Notch-1 efficiently reduced Notch-1 mRNA and protein (**Figure 6a**). Knockdown of Notch-1 decreased the mRNA levels of col 1a1 (**Figure 6b**) and col 1a2 (**Figure 6c**) and reduced the release of collagen (**Figure 6d**) (n = 6 each). * indicates statistical significant differences compared to mock transfected fibroblasts.

Supplementary Figure legends

Supplementary Figure 1: Expression of Notch receptors in dermal fibroblasts. Notch-1 and Notch-2 were expressed in dermal fibroblast, whereas Notch-3 and Notch-4 were not detectable by real-time PCR. However, only Notch-1, but not Notch-2 is overexpressed in dermal fibroblasts from SSc patients (n = 8 each). * indicates statistical significant differences compared to dermal fibroblasts from healthy volunteers.

Supplementary Figure 2: Jag-1 stimulates the transcription of col1a2, but does not affect the half-life of col1a2 mRNA. **Supplementary Figure 2a:** The mRNA half-life of col 1a2 is not altered by Jag-1 (n = 3). **Supplementary Figure 2b:** Stimulation with Jag-1 increased the transcription of col1a2 in reporter assays (n = 5). * indicates statistical significant differences compared to unstimulated controls.

Supplementary Figure 3: The γ -secretase inhibitor DAPT reduces the stimulatory effects of Jag-1 on the expression of extracellular matrix proteins. Pre-incubation with DAPT reduced the mRNA levels of col 1a1 (**supplementary Figure 3a**) and col 1a2 (**supplementary Figure 3b**) in healthy dermal fibroblasts stimulated with Jag-1. Similar results were obtained for the release of collagen protein (**supplementary Figure 3c**). (n = 8 each) * indicates statistical significant differences compared to fibroblasts stimulated with Jag-1.

Tables

Gender (F/M)	Age (median/range)	Disease subset (limited/diffuse)	Disease duration (median/range)	Medications
11/3	49 years (33-74)	9/5	6 years (1 – 16)	No DMARDs, Corticosteroids or NSAIDs

Supplementary Table 1: Characteristics of SSc patients at date of biopsy for generation of dermal fibroblast cultures. F = female, M = male. The disease subset was determined according to the criteria proposed by LeRoy et al. Disease duration was measured from the onset of the first non-Raynaud symptoms attributable to SSc. DMARDS = (potentially) disease-modifying anti-rheumatic drugs, NSAIDS = non-steroidal anti-inflammatory drugs

Supplementary Fig. 1

Supplementary Fig. 2