IL11 is elevated in systemic sclerosis and IL11-dependent ERK signaling underlies TGFβ-mediated activation of dermal fibroblasts

Running head: IL11 in dermal fibrosis

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Abstract

Objectives: Interleukin 11 (IL11) is highly upregulated in skin and lung fibroblasts from patients with systemic sclerosis (SSc). Here we tested whether IL11 is mechanistically linked with activation of human dermal fibroblasts (HDFs) from patients with SSc or controls.
Methods: We measured serum IL11 levels in volunteers and patients with early diffuse SSc and manipulated IL11 signalling in HDFs using gain- and loss-of-function approaches that we combined with molecular and cellular phenotyping.
Results: In patients with SSc, serum IL11 levels are elevated as compared to healthy controls. All transforming growth factor beta (TGFβ) isoforms induced IL11 secretion from HDFs, which highly express IL11RA and the gp130 co-receptor, suggestive of an autocrine loop of IL11 activity in HDFs. IL11 stimulated ERK activation in HDFs and resulted in HDF-to-myofibroblast transformation and extracellular matrix secretion. The pro-fibrotic action of IL11 in HDFs appeared unrelated to STAT3 activity, independent of TGFβ upregulation and was not associated with phosphorylation of SMAD2/3. Inhibition of IL11 signaling using either a neutralizing antibody against IL11 or siRNA against *IL11RA* reduced TGFβ-induced HDF proliferation, matrix production and cell migration, which was phenocopied by pharmacologic inhibition of ERK.
Conclusions: These data reveal that autocrine IL11-dependent ERK activity alone, or downstream of TGFβ stimulation, promotes fibrosis phenotypes in dermal fibroblasts and suggest IL11 as a potential therapeutic target in SSc.

Keywords
Systemic sclerosis, IL11, IL11RA, TGFβ2, antibody therapy, fibrosis, neutralizing antibody

Key messages

- Serum IL11 levels are elevated in patients with diffuse systemic sclerosis compared to healthy controls.
- IL11-dependent ERK signalling is required for TGFβ to elicit a profibrotic response in dermal fibroblasts.
- Neutralizing IL11 antibodies block TGFβ2-driven myofibroblast activation in systemic sclerosis fibroblasts.

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Introduction

Dermal fibrosis is a pathological hallmark of chronic inflammatory skin conditions such as systemic sclerosis (SSc), atopic dermatitis and psoriasis. In SSc, fibrosis also occurs in visceral organs, notably the lung, and is accompanied by proliferative vasculopathy (1,2) and inflammation (3).

Transforming growth factor beta (TGFβ) family proteins are the quintessential mediators of fibroblast activation across organs and diseases, including SSc (4). The TGFβ2 isoform is notably upregulated in fibroblasts and skin biopsies from patients with SSc as compared to healthy individuals, both at the RNA and protein levels (5,6). Thus, anti-fibrotic strategies for patients with SSc need to be carefully selected based on the ability to inhibit TGFβ2 signaling and those targeting integrin-mediated TGFβ activation may not be effective. Unfortunately, targeting TGFβ in general is associated with serious side effects, which have precluded the use of anti-TGFβ reagents in the clinic, to date (7,8).

Interleukin 11, recently identified as a TGFβ-responsive profibrotic cytokine (9,10), has been shown to be one of the most highly upregulated genes in fibroblasts from SSc lesional skin and in pulmonary fibroblasts of patients with interstitial lung disease due to SSc (6,11). Myofibroblasts are the major cell type that underlie fibrosis and secrete copious amounts of extracellular matrix while also being pro-inflammatory. Given that TGFβ-driven myofibroblast activation is IL11-dependent in fibroblasts from some tissues and stromal cells more generally (12), we set out to characterize IL11 as a potential fibrogenic factor in dermal fibroblasts from controls and patients with SSc.

Materials and Methods

Detailed methods are provided in the supplementary data file that accompanies this paper and includes detailed methods on the following: Patient cohort, cell culture, recombinant proteins and chemicals, sequencing of the *IL11RA* mutation, RNA sequencing, immunofluorescence staining, ELISA, immunoblotting, siRNA knockdown, RT-qPCR, high-content Imaging assays, half maximal inhibitory concentration measurement, migration assay and statistical analysis.

Results

IL11 levels are elevated in the serum of patients with early diffuse SSc

We measured circulating IL11 levels in serum from a cohort of 21 patients with early diffuse SSc (<2 years from onset of non-Raynaud's symptoms) by ELISA (Human Quantikine IL11, RnD Systems) and found that IL11 levels were significantly

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elevated in the serum of SSc patients compared to sex-matched healthy controls (Fig.1A, mean SSc: 27.2∓10.5pg/ml; mean controls: 9.6∓5.5pg/ml; P<0.001; further details in Supplementary Table S1 and Supplementary Fig.S1). Thus IL11 is not only highly expressed in skin and lung fibroblasts from patients with SSc (6,11) but is also systematically elevated.

IL11 signaling drives HDF-to-myofibroblast transformation

To begin to examine whether IL11 plays a role in dermal fibroblast biology, we performed RNA-sequencing (RNA-seq) of primary human dermal fibroblasts (HDFs). Analyses revealed that IL11 receptor alpha (IL11RA) is one of the most highly expressed members of the interleukin 6 (IL6) family of receptors (Fig.1B), which signal via glycoprotein 130 (gp130). TGFβR2 transcripts were also highly expressed, whereas TGFβR1 and TGFβR3 RNA levels were less abundant (Fig.1B). Immunofluorescence staining of HDFs revealed strong expression of IL11RA at the protein level (Fig.1C).

We stimulated HDFs with TGFβ1, TGFβ2, or TGFβ3 and found that all isoforms induced IL11 secretion (Fig.1D). These data provide evidence that HDFs are both a source and a target of IL11 and that IL11 signaling, perhaps in an autocrine mode, is related to TGFβ-stimulated effects in HDFs.

To assess whether IL11 secretion from HDFs following TGFβ stimulation was related to profibrotic phenotypes, we stimulated HDFs with IL11, TGFβ1, TGFβ2, or TGFβ3 in the presence of either non-targeting control siRNA or *IL11RA* siRNA (siIL11RA)(Fig.1E-F; Supplementary Fig.S2A-E). All stimuli induced HDF proliferation and secretion of the extracellular matrix (ECM) proteins Collagen 1 and Periostin. Inhibition of IL11 signaling by siIL11RA inhibited the profibrotic effects of not only IL11 but also of all TGFβ isoforms. These data show that IL11 is a profibrotic factor in HDFs and that TGFβ-driven HDF activation is dependent, at least in part, on autocrine IL11 activity.

Dissection of signaling events downstream of IL11 activation in dermal fibroblasts

To investigate the signaling mechanisms underlying the profibrotic effects of IL11 in skin fibroblasts, we employed the use of HDFs obtained from an individual with KREIBORG-PAKISTANI Syndrome (MIM:614188). This individual lacks IL11 signaling due to a homozygous p.Arg296Trp loss-of-function mutation in *IL11RA*, which we confirmed by sequencing (Supplementary Fig.S2F) (13). We compared these *IL11RA* mutant fibroblasts to a healthy age- and ethnically-matched control and treated both control and mutant HDFs with either low dose/physiological (10 ng/ml) or high dose/supra-physiological (1000 ng/ml) recombinant human IL11 (Fig.1G).

Consistent with data from fibroblasts from other organs, physiological concentrations (10 ng/ml) of IL11 maximally activated ERK in control human HDFs at an early time point (15 mins) and sustained this activation throughout the stimulation period (24h). While IL11 has been shown to stimulate STAT in some contexts, we

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observed negligible phosphorylation of STAT3 with physiological IL11 levels and limited phosphorylation with a supraphysiological dose in HDFs (Fig.1G). IL11-induced ERK and STAT3 phosphorylation were absent in IL11-stimulated HDFs from the IL11RA p.Arg296Trp mutant individual. IL11 stimulation of HDFs resulted in increased αSMA expression that was absent in IL11RA p.Arg296Trp HDFs (Fig.1G).

We next assessed the potential dependency of the pro-fibrotic effects of IL11 on canonical TGFβ2 and/or SMAD2/3 activity. We observed that following stimulation of HDFs with IL11 there was no increase in TGFβ levels (Supplementary Fig.S2G-H) and no detectable SMAD2/3 phosphorylation at either early or late time points (Fig.1H and I). These data are consistent with findings in cardiac fibroblasts (9) and rule out a substantive role for a TGFβ-dependent feed-forward loop downstream of IL11 activity in HDFs.

Inhibition of IL11 signaling blocks TGFβ2-stimulated dermal fibroblast- to-myofibroblast transformation.

Next, we sought to understand whether the use of a neutralizing IL11 antibody (X203)(14) could block activation of HDFs. We first tested the efficacy of X203 in inhibiting the secretion of MMP2 from TGFβ2-stimulated HDFs (14). X203 dose-dependently inhibited TGFβ2-induced MMP2 secretion by both primary HDFs (orange line) and SSc fibroblasts (purple line) with half-maximal inhibitory concentration (IC_{50}) of 0.34nM and 0.31nM, respectively (Fig.2A).

In migration assays, we found that IL11 or TGFβ2 promoted HDF migration and that X203 reduced the migratory capacity of TGFβ2-stimulated HDF as compared to control (Fig.2B-C and Supplementary Fig.S3A-B). Furthermore, inhibition of IL11 signaling with X203 in TGFβ-stimulated HDF markedly reduced cell proliferation and the expression of Collagen I and Periostin (Fig.2D-E and Supplementary Fig.S3C-E).

ERK signaling is important for IL11 effects in cardiac fibroblasts (9) and we found that TGFβ2 or IL11 stimulated ERK phosphorylation to a similar degree in HDFs (Supplementary Fig.S3F). We tested the mechanistic importance of ERK signaling and found that pharmacologic inhibition of ERK activity blocked the profibrotic effects of all TGFβ isoforms with the same effect as neutralizing IL11 antibody (Fig.2D-E and Supplementary Fig.S3C-E,G).

Finally and importantly, the use of IL11 neutralizing antibodies in SSc fibroblasts was shown to recapitulate the results seen in non-diseased HDFs. At baseline, SSc fibroblasts highly express IL11RA (Supplementary Fig.S3H) and stimulation of SSc fibroblasts with IL11 or TGFβ2 induced αSMA expression (Fig.2F-G), Collagen I, Periostin, and also increased cell proliferation (Supplementary Fig.S3I). Following TGFβ2 stimulation, inhibition of IL11 signalling with either X203 or ERK inhibition (U0126, 10 μM) resulted in a reduction in all profibrotic phenotypes tested (Supplementary Fig.S3I).

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Discussion

While many factors stimulate HDF activation, members of the TGFβ superfamily are regarded as the prototypical activators. Using fibroblasts explanted from healthy controls and patients with diffuse SSc treated with TGFβ, Sargent et al (15) identified IL6 and IL11, among others, as TGFβ-responsive genes *in vitro*. More recently, IL11 has been found to be highly upregulated in SSc skin fibroblasts (6) and in pulmonary fibroblasts of patients suffering from SSc-associated interstitial lung disease (11). Of all the IL6 family cytokines, IL11 is the only member whose expression correlates with lung fibrosis severity in patients with idiopathic pulmonary fibrosis (10). While targeting IL6 in SSc has proved unsuccessful for the primary endpoint of improvement in skin sclerosis (focuSSced trial, NCT02453256)(16), perhaps reflecting the low levels of IL6R on fibroblasts (9), the role of IL11 has remained unexplored.

In this study, we show that patients with early diffuse SSc have elevated serum levels of IL11 and that HDFs highly express IL11RA but not IL6R. In keeping with data on fibroblasts from other organs (12), we show that IL11 is required for the profibrotic effects of TGFβ in HDFs and that this is primarily driven via non-canonical ERK signaling. This is in agreement with previous work describing the central role for ERK1/2 cascade in relation to TGFβ-stimulated SSc fibroblast activation *in vitro* (17–19) and skin fibrosis *in vivo* (19). We conclude that non-canonical TGFβ and IL11 signaling act through ERK to cause fibrosis and that the effect downstream of TGFβ is IL11-dependent.

Some studies have suggested a role for STAT3 in the profibrotic effects of TGFβ (20–22). We found that IL11 consistently induced ERK phosphorylation concomitant with αSMA upregulation, while STAT3 phosphorylation was limited, not sustained and was only apparent at supraphysiological IL11 levels. Thus, we think it is unlikely that STAT3 activity is directly related to IL11 effects on fibrosis phenotypes although indirect mechanisms could play a role. Our data also rule out a dependency of IL11 on canonical TGFβ signaling as IL11-stimulated HDFs, while exhibiting pro-fibrotic phenotypes, do not upregulate TGFβ and show no evidence of SMAD2/3 activation. We point out that it is possible that IL11 induces reactive oxidative species (ROS) in HDFs, given that IL11 promotes NOX4 upregulation in hepatocytes and NOX4 is strongly upregulated in the skin of patients with SSc (6,23,24) and this requires further study.

SSc remains a disease for which no treatment has proven uniformly effective. Unfortunately, anti-TGFβ interventions have generic and recurrent concerns over efficacy and safety (7,8). More recently, molecules targeting integrin-mediated TGFβ activation have been developed to attempt to bypass toxicities. However, TGFβ2 is the most dysregulated TGFβ isoform in SSc patients and this isoform is refractory to

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integrin-mediated activation. Thus the utility of integrin targeting therapies in SSc is open to question and other approaches may be needed.

In conclusion, we present evidence for the involvement of IL11, a TGFβ responsive and pro-fibrotic cytokine that is highly upregulated in SSc fibroblasts, in HDF activation. If IL11 is shown to promote skin fibrosis in *in vivo* disease models, IL11 may represent a new and accessible therapeutic target for dermal fibrosis in SSc and perhaps other conditions.

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Author Contributions

SAC, EA and SV designed the study and planned experiments.
SV, EA, AAW, BN, JT, BLG carried out molecular biology experiments; SV, EA, AAW, BN analysed the data. LPM prepared RNA-seq libraries and SC and NZ analysed sequencing data. Human samples acquisition and preparation: SOR, ALHL, SA, BR, UA, KG, BSP. EA, AAW, SS and SAC prepared the manuscript with input from co-authors.

Conflict of interest statement

SAC, SS, AAW, BN are co-inventors on a number of patent applications relating to the role of IL11 in human diseases that include the published patents: WO2017103108, WO2017103108 A2, WO 2018/109174 A2, WO 2018/109170 A2. SAC and SS are co-founders and shareholders of Enleofen Bio PTE LTD, a company (which SAC is a director of) that developed anti-IL11 therapeutics, which was acquired for further development by Boehringer Ingelheim.

Data availability statement

Data are available under GEO accession number (GSE162966).

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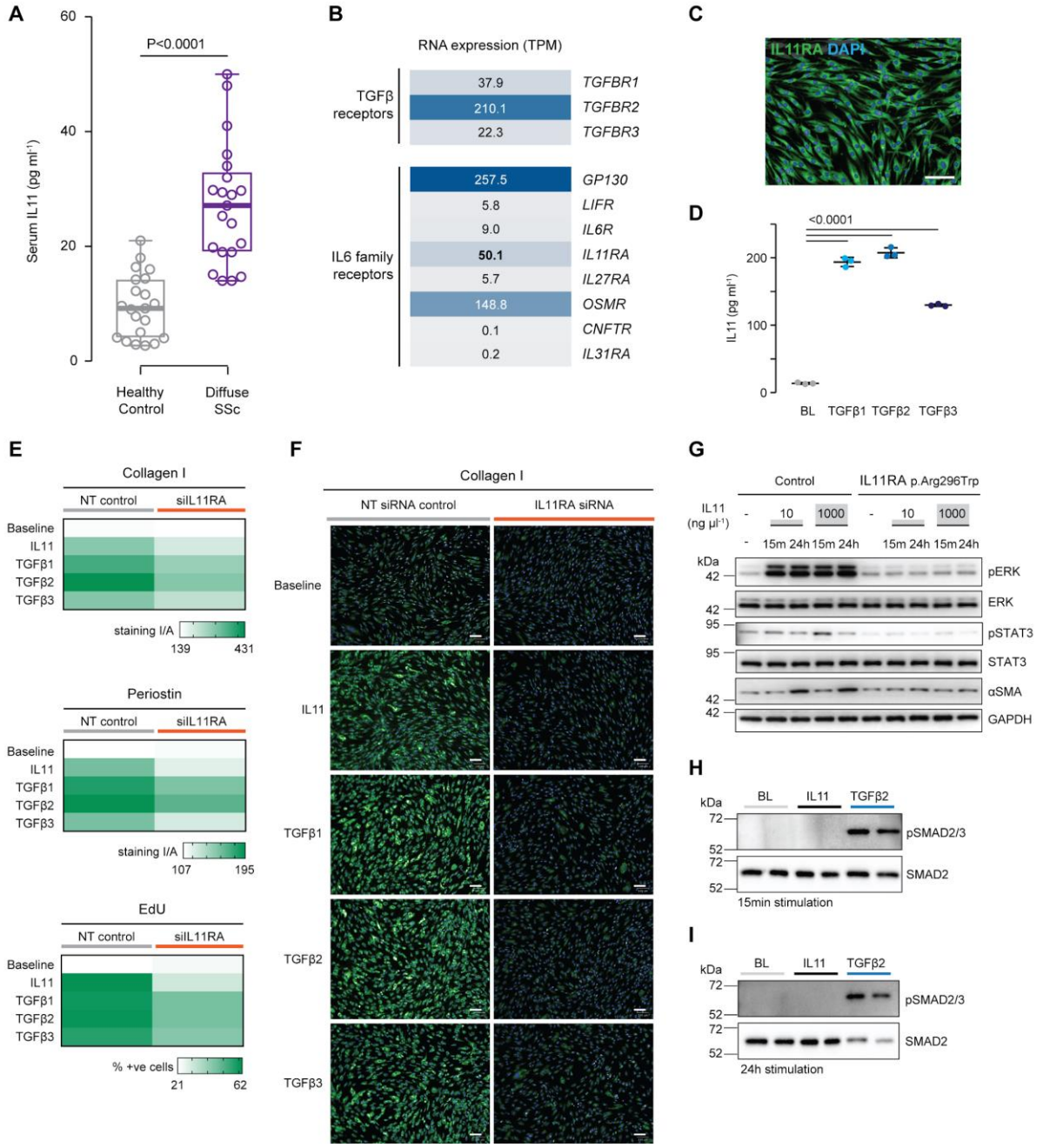


Figure 1. IL11 levels are increased in the serum of patients with systemic sclerosis and IL11 has SMAD-independent profibrotic activity in human dermal fibroblasts. (A) Serum IL11 levels in patients with diffuse systemic sclerosis (SSc) and sex-matched healthy controls. Data: n=21 per group (Supplementary Table S1), whiskers from min. to max. value, line at median. Statistics: two-tailed *t*-test. **(B)** RNA-seq of primary human dermal fibroblasts (n=4). RNA expression values are shown as transcripts per million (TPM) for receptors of the TGFβ and IL6 cytokine family. **(C)** IL11RA staining in unstimulated HDFs (scale bar, 100

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μm). **(D)** IL11 secretion by HDFs stimulated with three TGFβ isoforms (5 ng/ml) for 24h. Data (n=3): mean ±sd. Statistics: one-way ANOVA, Dunnett's multiple testing correction. **(E)** Quantification of signal intensity/area (Collagen I, Periostin) and percentage of EdU-staining positive HDFs subjected to siRNA knockdown for *IL11RA* and stimulation with TGFβ1, TGFβ2, TGFβ3 or IL11(5ng/ml) for 24h. NT: non targeting siRNA control. **(F)** Collagen I staining representative images (Periostin and EdU staining images are shown in Supplementary Fig.S2E) of HDFs that had been subjected to siRNA knockdown for *IL11RA* and stimulation with the indicated factors for 24h. **(G)** Western blots assessing protein levels of phospho(p)ERK, ERK, pSTAT3, STAT3, αSMA, GAPDH in primary dermal fibroblasts of control and from an individual carrying a homozygous germline loss-of-function p.Arg296Trp mutation in IL11RA. **(H-I)** Western blot of SMAD2 and pSMAD2/3 expression in HDF following stimulation with TGFβ2 or IL11 (5ng/ml; 15min or 24h).

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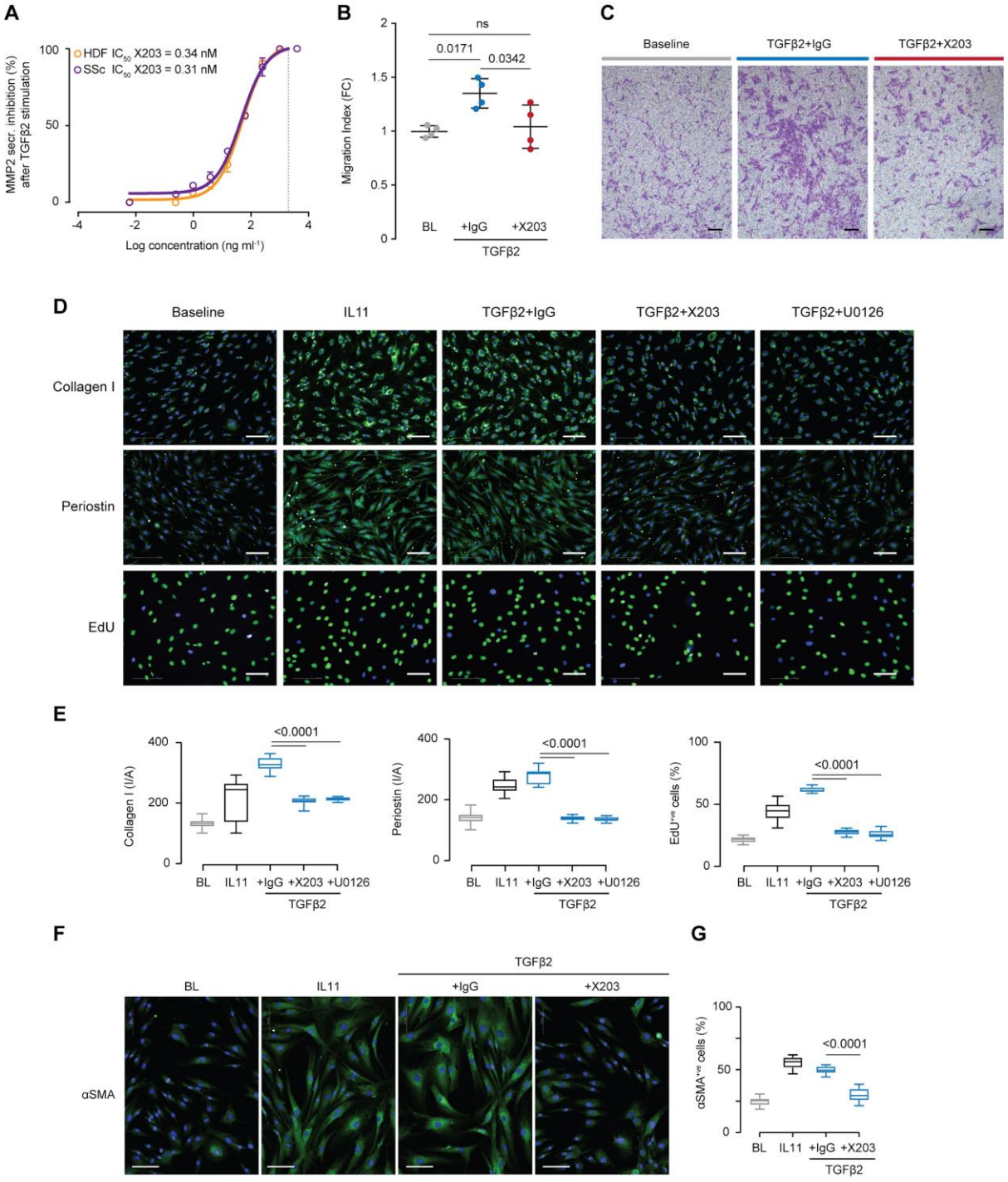


Figure 2. IL11 antibodies block TGFβ2-driven activation of skin fibroblasts from SSc patients and controls. (A) Dose-response curve and X203 IC_{50} value in inhibiting MMP2 secretion by HDFs and SSc dermal fibroblasts stimulated with TGFβ2. (X203 serial 4-fold dilution from 4 µg/ml to 61 pg/ml; line at x = 2 µg/ml; total n=2 per group). **(B)** Transwell migration indices of HDFs following 24h stimulation with TGFβ2 (5 ng/ml) and in the presence of IgG or X203 (2 µg/ml). Data: n=4, mean with s.d. Statistics: one-way ANOVA, Tukey's multiple testing correction. **(C)** Migration assay representative images; scale bar, 150 µm. **(D)** Representative images of Collagen I, Periostin and EdU stainings of HDFs, following a 24h stimulation with IL11 (5 ng/ml), or a combination of TGFβ2 (5 ng/ml) and IgG (2 µg/ml), IL11 neutralizing antibody (X203, 2 µg/ml) or ERK inhibitor (U0126, 10 µM); scale

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bar, 100µm. **(E)** Quantification of signal intensity/area (Collagen I, Periostin) and percentage of EdU-staining positive cells relative to experiment in **(D)**. Statistics: one-way ANOVA, Tukey's multiple testing correction. **(F-G)** Representative images and quantification of percentage of αSMA-staining positive cells following a 24h stimulation of SSc fibroblasts with the indicated factors. Data: whiskers from min. to max. value, line at median. Statistics: one-way ANOVA, Tukey's multiple testing correction.

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