Mutual amplification of GLI2/Hedgehog and cJUN/AP1 signaling in fibroblast activation in SSc – potential implications for combined therapies

Supplementary Material

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Supplementary Material and Methods

Modulation of cJUN/AP-1 signaling and GLI2/Hedgehog-signaling *in vitro* and *in vivo*

The specific JUN/AP1- inhibitor T5224 (3-{5-[4-(Cyclopentyloxy)-2-hydroxybenzoyl]-2-[(3-hydroxy-1,2-benzisoxazol-6-yl)methoxy]phenyl}) propionic acid, ApexBio, Boston, MA, USA) and the GLI2 inhibitor GANT61 (2,2'-[[Dihydro-2-(4-pyridinyl)-1,3(2H,4H)-pyrimidinediyl]bis(methylene)]bis[N,N-dimethylbenzenamine, AdooQ, Irvine, CA, USA) were used to target AP1/cJUN- and GLI2/hedgehog-signaling *in vitro* and *in vivo*, respectively. For *in vitro* experiments, T5224 was applied at doses from 0.2 µM to 40µM [1]. In subsets of experiments, fibroblasts were stimulated with TGFβ (Peprotech, Hamburg, Germany) at a concentration of 10ng/ml or with recombinant Sonic Hedgehog (SHH) (Peprotech, Hamburg, Germany) at a dose of 5ng/ml. For *in vivo* experiments, T5224 was applied at 3mg/kgKG/d (referred to as "T5224 low") and 30mg/kgKG/d (referred to as "T5224 high") by oral gavage; GANT61 was applied at 2mg/kgKg/d (referred to as "GANT61 low") and 20mg/kgKg/d (referred to as "GANT61 high") by intraperitoneal injection [2] .

siRNA-mediated knockdown of *GLI2* and *cJUN* and overexpression of cJUN and GLI2

cJUN and *GLI2* were knocked down with siRNA using the following siRNA products: ON-TARGET plus Human JUN siRNA (Catalog ID: L-003268-00-0010, horizon discovery, Germany) and ON-TARGET plus Human GLI2 siRNA (Catalog ID: L-006468-00-0010, horizon discovery, Germany). Knockdown was performed using the nucleofection technique as previously described [3, 4]. Briefly, healthy human dermal fibroblasts were transfected with 2,5 µg of siRNA using an Amaxa 4D-Nucleofector Kit (Lonza, Cologne, Germany). Non-targeting siRNA duplexes (Catalog ID: D-001910-10-20, horizon discovery, Germany) served as controls. Effective knockdown of cJUN and GLI2 was confirmed by Western blot analysis.

To overexpress cJUN and GLI2 the following plasmids were used: c-JUN cDNA ORF Clone (HG11886-UT, Hölzel Diagnostika, Cologne, Germany) and GLI2 cDNA ORF Clone (HG18067-UT, Hölzel Diagnostika, Cologne, Germany). Plasmids were amplified in DH5α E.coli (C2987H, New England Biolabs, Frankfurt am Main, Germany). Positive clones were then selected with lysogeny broth medium supplemented with 1:1000 ampicillin. Single colonies were picked and amplified and the plasmids were isolated using a commercial kit (QIAGEN, Hamburg, Germany).

Healthy human dermal fibroblasts were transfected with 2,5 µg of plasmid using an Amaxa 4D-Nucleofector Kit (Amaxa, Cologne, Germany). Fibroblasts transfected with empty vectors served as controls (CV011, Sino biological, Cologne, Germany). The overexpression of cJUN- and GLI2-protein was confirmed by Western blot analysis, respectively.

Quantitative real time-PCR

Gene expression was quantified by SYBR-Green real-time-PCR using the MX3005P Detection System (Agilent Technologies, Santa Clara, USA) as described [3]. RNA was isolated using a Nucleospin RNA isolation kit (Macherey-Nagel, Düren, Germany). Reverse transcription was then performed and cDNA samples were mixed with SYBRGreen PCR Master Mix (Applied Biosystems, Darmstadt, Germany). Samples without reverse transcriptase-enzyme were used as negative controls. All primers are summarized in supplementary table 1. Gene expression levels were assessed through the comparative CT method using β-actin as a house keeping gene. The StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) was used to perform Real-time PCR. Unspecific signals caused by primer dimers were excluded by non-template controls and by dissociation curve analysis.

Western Blot analysis

Cell lysates were collected from cultured fibroblasts using Cell Lysis Buffer (Cell Signaling Technology, Frankfurt am Main, Germany). Protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes [4]. After blocking, membranes were incubated with primary antibodies at 4°C overnight (list of primary antibodies in supplementary table 2). Membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies and Amersham ECL Prime Western Blotting Detection Reagent (Cytiva, Freiburg, Germany). Blots were then visualized using a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Feldkirchen, Germany) and analyzed with the ImageJ software (NIH, version 1.46).

ChIP-Sequencing

Healthy human dermal fibroblasts were transfected with 2,5 µg of the following plasmids: c-JUN cDNA ORF Clone (HG11886-UT, Hölzel Diagnostika, Cologne, Germany) and GLI2 cDNA ORF Clone, (HG18067-UT, Hölzel Diagnostika, Cologne, Germany) using an Amaxa 4D-Nucleofector (Amaxa, Cologne, Germany). Fibroblasts transfected with empty vectors (CV011, Sino biological, Cologne, Germany) served as controls. Cells were selectively stimulated with TGFβ (10 ng/ml; PeproTech, Hamburg, Germany) for 24 hours. ChIP assays were performed using the ChIP-IT High Sensitivity Kit (Active Motif, Eching, Germany). After TGFβ stimulation, cells were fixated according to the manufacturer's instructions and chromatin was sonicated using an EpiShear™ Probe Sonicator (Active Motif, Eching, Germany). Sonicated chromatin was incubated with antibodies targeting cJUN (ab31419, Abcam, Cambridge, United Kingdom) and GLI2 (sc-271786 X, Santa Cruz Biotechnology, Heidelberg, Germany) or rabbit IgG antibody (#NI01; Millipore, Darmstadt, Germany). After immunoprecipitation, enriched DNA was sequenced by the Institute of Human Genetics at the University Hospital Friedrich-Alexander-University Erlangen-Nürnberg. ChIP-seq libraries

were generated using the NEBNext Ultra II DNA Library Prep Kit (Illumina, Frankfurt am Main, Germany) and subjected to paired-end sequencing (2x150 bp) on a NovaSeq-6000 platform (Illumina, Frankfurt am Main, Germany).

Analysis of ChIP-Sequencing data

Raw data (fastq files) were uploaded to the Galaxy web platform, and the public server at usegalaxy.eu was used for data analysis. Raw reads were trimmed out of the Illumina adapter by using the function TrimGalore with the default setting at usegalaxy.eu. Trimmed reads were aligned to the hg38 genome using Bowtie2, with the default setting for pair-end reads. MACS2 was used for peak calling with a bandwidth of 300, and peaks were filtered using an FDR-corrected q-value of 0.05 as well as removing peaks in the hg38 blacklist area. Peak annotation was done in R (version 4.3.1) using the "CHIPseeker" package. The g:Profiler web server was used to perform functional enrichment of Gene Ontology (Biological Process) with the Benjamini-Hochberg correction method to retrieve an adjusted-p value [11].

Immunofluorescence staining

Formalin-fixed, paraffin-embedded tissue sections were incubated with the primary antibodies listed in supplementary table 3 overnight at 4°C. Concentration-matched isotype antibodies were used as controls [3]. To perform immunofluorescence analysis of cultured fibroblasts, dermal fibroblasts were seeded into chamber slides in DMEM F12 (Thermofisher Scientific, Dreieich, Germany) with 10% FBS (Thermofisher Scientific, Dreieich, Germany) for 24h. After 24h of serum starvation with DMEMF12 with 0.1% FBS, cells were stimulated with TGFβ (Peprotech, Hamburg, Germany) or SHH (Peprotech, Hamburg, Germany) for 24h. Fixation and permeabilization was performed as previously described. Incubation with anti-αSMA specific antibodies (supplementary table 2) was performed over night at 4°C. Stress fibers were visualized with rhodamine-conjugated phalloidin (1:40, #R415, Sigma-Aldrich,

Germany). Nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI) (1:800, #sc-3598 Santa Cruz Biotechnology, Heidelberg, Germany). Staining analyses were performed using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and a Leica SP8 CLSM confocal microscope (Wetzlar, Germany). Images were quantified using ImageJ software (NIH, version 1.46).

Imaging Mass Cytometry (IMC)

Imaging Mass Cytometry (IMC) was conducted as previously described [5-7]. Antibodies were either obtained pre-conjugated (Standard Biotools, Munich, Germany) or in purified preparations, which were initially validated through standard immunofluorescence staining. Purified antibodies were then labeled with lanthanide metals using the Maxpar X8 antibody labeling kit (Standard Biotools, Munich, Germany) according to the manufacturer's instructions. The complete panel was re-validated for IMC, and antibody titration was performed. Deparaffinization and epitope retrieval were carried out on 5 µm paraffin-embedded skin sections. Subsequently, the sections were blocked with 2% BSA (Roth, Karlsruhe, Germany) in PBS (Thermofisher Scientific, Dreieich, Germany) and incubated overnight at 4°C with the antibody mix (in 0.5% BSA). The stained samples were then washed once with PBS-T [PBS (Thermofisher Scientific, Dreieich, Germany) and 0.2% Tween 20 (Roth, Karlsruhe, Germany)] and twice in PBS for 5 min. DNA was stained with Iridium-Intercalator (125 µM) (Standard Biotools, Munich, Germany) 1/400 during 5 min at room temperature. The samples were then washed in PBS and deionized water before being dried and stored at room temperature.

Ablated areas were preselected using hematoxylin-eosin stained sections. Data acquisition was performed with the Hyperion Imaging System (Standard Biotools, Munich, Germany) coupled to a Helios mass cytometer (Standard Biotools, Munich, Germany) with laser ablation

parameters set at a resolution of 1 μm² and a frequency of 200 Hz. IMC data was saved as MCD and .txt files.

IMC data analysis

After confirmation of good staining quality on the MCD viewer software (Standard Biotools, Munich, Germany), MCD files were converted to TIFF format. Cell segmentation was performed using a publicly available analysis pipeline (https://zenodo.org/record/3841961). Single cell data, including the mean expression of all pixels corresponding to each cell and spatial coordinates, were extracted as fcs and csv files, arcsinh normalized (cofactor 1) and rescaled from 0 to 1 using the R package Spectre [7]. Target populations were identified through manual gating and spatial referencing in the FlowJo software. Endothelial cells were defined as CD31+; immune cells were defined as CD45+; and the respective subsets were selected: cJUN+, cJUN-, GLI2+, GLI2-). Spatial distribution of distinct cell populations was visualized using the R package Cytomapper [8].

Evaluation of fibrosis *in vivo*

To analyze skin fibrosis, dermal thickening, the expression of myofibroblasts and the dermal hydroxyproline content were analyzed. Dermal thickness at the injection sites was analyzed measuring the largest distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction as described previously [4, 9]. The analysis was performed by an experienced assessor in a blinded manner. Sections were also stained with trichrome staining to directly visualize collagen. Myofibroblasts were identified as single, spindle shaped cells in the dermis positive for α-smooth muscle actin [3]. To determine the hydroxyproline content of skin tissue samples, skin biopsies from the upper back were digested in HCl 6M (Roth, Karlsruhe, Germany) for 3 hours, then supplemented with chloramine-T 0,06M (Sigma-Aldrich, Steinheim, Germany) and incubated at room temperature for 20 minutes.

Subsequently, perchloric acid 3.15M (Merck, Darmstadt, Germany) and p-dimethylaminobenzaldehyde 20% (Thermofisher Scientific, Dreieich, Germany) were added, and samples were further incubated at 60°C for 20 minutes. Absorbance was measured at 557 nm with a Spectra MAX 190 microplate spectrophotometer (Molecular Devices, Munich, Germany).

Reporter Assays

For the Gli2 reporter assay, NIH3T3-Light2 cells were kindly provided by the group of Dr Natalie Dye (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). Cells were cultured in high glucose medium (#11965092, Thermofisher Scientific, Dreieich, Germany) supplemented with Zeocin at 150 ug/mL (#R25001, Thermofisher Scientific, Dreieich, Germany) and Geneticin (#10131027, Thermofisher Scientific, Dreieich, Germany) at 400 ug/mL. Cells were then selectively stimulated with TGFβ (Peprotech, Hamburg, Germany) at 10ng/ml and treated with T5224 (ApexBio, Boston, MA, USA) at 10µM, GANT61 (AdooQ, Irvine, CA, USA) at 20µM, or recombinant Sonic Hedgehog (SHH) (Peprotech, Hamburg, Germany) for 24 hours. Following that, the cells were assayed using the Dual-Glo® Luciferase Assay System (Promega, Walldorf, Germany) according to the manufacturer's instructions, and luminescence levels were measured at 500ms using a GloMax® Discover Microplate Reader (Promega, Walldorf, Germany).

For the cJun reporter assay, healthy human dermal fibroblasts were transfected with 2,5 µg of the following plasmid: Flag-JunWT-Myc (#47443, Addgene, Watertown, MA, USA), using the Fugene HD Transfection Reagent (Promega, Walldorf, Germany) according to the manufacturer's instructions. The cells were then selectively stimulated with TGFβ (Peprotech, Hamburg, Germany) at 10ng/ml and treated with T5224 (ApexBio, Boston, MA, USA) at 10µM, GANT61 (AdooQ, Irvine, CA, USA) at 20µM, or recombinant Sonic Hedgehog (SHH) (Peprotech, Hamburg, Germany) for 24 hours. Following that, the cells were assayed using the

Dual-Glo® Luciferase Assay System (Promega, Walldorf, Germany) according to the manufacturer's instructions, and luminescence levels were measured at 500ms using a GloMax® Discover Microplate Reader (Promega, Walldorf, Germany).

Clinical monitoring of mice

Mice were monitored for adverse events on a daily basis using an established, standardized monitoring scheme that included assessment of the body weight, physical activity, posture, texture of the fur and behavior [10]. In addition, mice were analyzed for toxic effects on necropsy.

Statistics

Data are presented as median with interquartile range (IQR) with additional dots representing individual values. Differences between the groups were tested by non-parametric Mann-Whitney-U-test using GraphPad Prism 9. P-values less than 0.05 were considered as significant.

Supplementary tables

Supplementary table 1.

Target gene	Forward Primer Sequence	Reverse Primer Sequence
B-ACTIN	5'-AGA AAA TCT GGC ACC ACA CC-3'	5'-TAG CAC AGC CTG GAT AGC AA-3'
COL1A1	5'-ACG AAG ACA TCC CAC CAA TC-3'	5'-ATG GTA CCT GAG GCC GTT C-3'
b-actin	5'-TCT TTG ATG TCA CGC ACG AT-3'	5'-TAC AGC TTC ACC ACC ACA-3'
Ptch2	5'-CGG TGC TGA CAC TCT TGG GCC-3'	5'-AGA GCA GCA CAG GCA GCA GC-3'
Axin2	5'-GCC ACC AAG ACC TAC ATA CGA-3'	5'-GAG CCG ATC TGT TGC TTC TT-3'
c-Myc	5'-TGA AGG CTG GAT TTC CTT TGG GCG-3'	5'-ATC GTC GTG GCT GTC GGG GT-3'
cJun	5'-CGC CTG ATC ATC CAG TCC-3'	5'-GGG GTC GGT GTA GTG GTG-3'
JunB	5'-CAC AGG CGC ATC TCT GAA-3'	5'-CGA TCA AGC GCT CCA GTT-3'
JunD	5'-ACT ACCCCG ACC AGT ACG C-3'	5'-TCG CTA GCT GCC ACC TTC-3'
Fra2	5`-GAC CTG CAG TGG ATG GTA CA-3`	5`-GGA TTG GAC ATG GAG GTG AT-3`
FosB	CAT GCC AGG AAC CAG CTA CT	CAG TGC TGT AGG CAC TCA GG
FosC	5`-TCC TGT CAA CAC ACA GGA CTT T-3`	5`- GGC ACT AGA GAC GGA CAG AT-3`

Supplementary table 2: Antibodies used for immunofluorescence and Western Blot.

Target	Species/clonality	Cat. No.	Company	Application	Dilution
cJUN (h) cJun (m)	rb mab	ab32137	Abcam, Cambridge, UK	IF WB	1:100 1:2000
GLI2 (h)	ms mab	sc271786	Santa Cruz, Dallas, Texas, USA	IF	1:100
Gli2 (m)	gt pab	AF3635	R&D, Minneapolis, Minnesota, USA	IF	1:1000
P4Hβ (h)	gt pab	AF4236	R&D, Minneapolis, Minnesota, USA	IF	1:200
Vimentin (m)	rt pab	ab20346	Abcam, Cambridge, UK	IF	1:1000
pcJUN (h)	ms mab	sc-288	Santa Cruz, Dallas, Texas, USA	WB	1:1000
β-ACTIN (h)	ms mab	A5441	Sigma Aldrich, St. Louis, Missouri, USA	WB	1:10 000
αSMA (h)	ms mab	A5228	Sigma Aldrich, Germany	IF	1:1000

Supplementary table 3: IMC antibody panel

Metal	Antigen	Species/clonality	Company	Cat. No.	Dilution
145Nd	CD31	gt pab	Sigma	AF3628	1:200
152Sm	CD45	D9M8I	Standard Biotools	3152018D	1:200
149Sm	GLI2	ms mab	Santa Cruz	sc-271786	1:100
171Yb	cJUN	rb mab	abcam	ab32137	1:200
176Yb	Histone-3	D1H2	Standard Biotools	3176023D	1:1600

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