

# Transforming growth factor beta 1 induces endogenous expression of Interferon gamma in palmar connective tissues

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# Abstract

Fibrosis is characterized by transdifferentiation of quiescent fibroblasts to myofibroblasts that results in overexpression and deposition of extracellular matrix proteins that subsequently leads to organ impairment or dysfunction. Dupuytren's disease, a benign fibroproliferative disorder of the palmar fascia, represents an ideal model to study tissue fibrosis. Fibroblasts isolated from nodules and cords of Dupuytren's disease (DF) served as model of fibrosis. Here we report that DF and control fibroblasts (CF) derived from tendon pulleys of the hand express endogenous Interferon gamma (IFNG). Application of recombinant transforming growth factor beta 1 (TGFB1) resulted in upregulation of profibrotic proteins and subsequent elevated expression levels of SMAD7. Surprisingly, TGFB1 additionally induced transcription of IFNG in stimulated DF and CF. As a consequence, IFNG signaling is presumably enhanced by an autocrine mechanism leading to upregulation of SMAD7, thereby indicating a new negative feedback mechanism of TGFB1 signaling. On the other hand cell infection with adSMAD7, an adenovirus coding for SMAD7, leads to downregulation of IFNG and subsequent signaling, indicating a further novel negative feedback mechanism of IFNG signaling. AdIFNG, an adenovirus coding for human IFNG, in combination with recombinant TGFB1 initiate higher levels of IFNG than separate stimulation, thus indicating a co-inducing effect of TGFB1 on IFNG transcription. Therefore, our results point to new perspectives concerning IFNG-TGFB1-crosstalking and its possible relevance in a standard model of fibrosis.

## Background

Interferons (IFNs) are widely expressed cytokines with potent antiviral and growth-inhibitory effects. These cytokines are the first line of defence against viral infections and have important roles in immunosurveillance of malignant cells. The IFN family includes two main classes of related cytokines: type I IFNs and type II IFN (1). Unlike the many different types of type I IFNs there is only one type II IFN, Interferon gamma (IFNG). After binding of IFNG to the two-parted IFNG cell membrane receptor janus tyrosine kinase 1 (JAK1) is rapidly associated to the receptor and reveals after phosphorylation a docking site for the latent cytoplasmatic signal transducer and activator of transcription 1 (STAT1). Upon phosphorylation, STAT1 homodimerizes, translocates in the nucleus and regulates IFNG dependent transcription by binding to IFNG activated sequences (GAS) in IFNG inducible genes (2).

In many clinical trials and in-vitro-studies IFNG has been reported as a possible agent to affect fibroproliferative diseases in an antifibrotic manner, e.g. IFNG has been demonstrated to inhibit collagen synthesis in human fibroblasts (3), to modulate galactosaminoglycans produced of human skin fibroblasts (4) and to block induction of myofibroblasts (5).

Fibrosis is characterized by transdifferentiation of quiescent fibroblasts to myofibroblasts that results in overexpression and deposition of extracellular matrix proteins that subsequently leads to organ impairment or dysfunction(6–11). On the molecular level, profibrotic changes and regulatory mechanisms in fibroblasts are induced and controlled by members of the transforming growth factor

beta (TGFB) family that transduce their signals via receptor regulated Smads (R-SMADs), common mediator Smads (Co-SMADs), and inhibitory Smads (I-SMADs) following specific receptor activation(12, 13). During signaling phosphorylated R-SMADs form oligomeric complexes with the common mediator SMAD4 (14, 15). These complexes translocate into the nucleus and regulate transcription of target genes(15).

Dupuytren's disease (DD) is a fibroproliferative disorder of the hand, characterized by formation of nodules and cords that appear in the palmar and digital fascia, leading to disfigurement and functional impairment of the hand. Recently, DD was characterized as a useful model of fibrosis, since it displays the entire temporal and histological architecture of cells, cytokines and extracellular matrix involved in fibroproliferative processes(16). Like other fibroproliferative disorders, TGFB1 is a pivotal factor during pathogenesis of palmar fibrosis, where inhibitory SMAD7 acts to oppose signal(17) transducing R- and Co-SMADs by forming stable associations with activated type I receptors, thereby preventing phosphorylation of R-SMADs, thus acting as a negative feedback regulator(18).

Since this model provides a reasonable explanation for induction and progression of fibroproliferative phenomena, it does not explain clinical findings in patients suffering from DD, where sudden arrest of the disease through all stages can be observed.

During an immunohistochemical study we surprisingly found high levels of intra- and extracellular INFG. Since other groups have demonstrated that INFG leads to SMAD7 upregulation and thereby blocking TGFB1 signaling our study addressed the question whether endogenous expression of INFG is influenced by TGFB1, thus indicating a bidirectional crosstalk of signaling pathways.

## **Experimental Procedures**

Cells – Tissues from Dupuytren's disease (DF) and Control Fibroblasts (CF) derived from normal tendon pulleys were obtained, isolated and cultured as described previously (21). All individuals suffered from contractures of the fingers rated as second or third degree deformities according to the Tubiana score (22).

Immunohistochemistry/-fluorescence – DF and CF were incubated on 8-well-Chamber Slides (Nunc) and fixed in ice-cold ethanol at the end of each experiment. Subsequently, cells were rehydrated in PBS, blocked with fetal calf serum (Biochrom) and incubated with primary antibodies at 4 °C diluted in antibody diluent (DAKO) overnight. The next day, slides were washed 3 times with PBS and incubated for 1 hour with secondary antibodies conjugated to Biotin. After another 3 washing steps with PBS slides were incubated with StreptABComplex/HRP (DAKO) for 30 minutes and stained with AEC + Substrat-Chromogen (DAKO) for 5 minutes. Mayers-Hämalaun (Merck) was used for nucleus-staining. Slides were mounted with Faramount Mounting Medium (DAKO) for microscopy. For Immunofluorescence specimen were rehydrated in PBS, blocked with donkey-serum (Jackson ImmunoResearch) and incubated with primary antibodies diluted in antibody diluent overnight at 4 °C. The following day, slides were washed 3 times with PBS and incubated with multi-labeling secondary antibodies conjugated to either cy2 or cy3 or

cy5 (Jackson ImmunoResearch) at room temperature for 1 hour avoiding light exposure. After secondary labeling, slides were washed 3 times in PBS and rinsed in DAPI (Sigma-Aldrich) for nucleus staining and mounted in fluorescent mounting medium (DAKO). Imaging of stained slides was performed using an Axioplan2 microscope (Zeiss). All slides were treated identically and scanned using the same settings in each experiment. A list of used antibodies is provided in Supplemental Table 1.

Preparation of whole cell lysates and immunoblot analysis – Total lysates from DF and CF were prepared by solubilization in RIPA lysis buffer (Santa Cruz) according to manufacturer's instructions. The amount of protein in lysates was estimated by BCA protein quantification assay (Pierce). Twenty micrograms per lane of protein were loaded onto NuPAGE Novex Bis-Tris or NuPAGE Novex Tris-Acetate Gels (Invitrogen) for electrophoresis in a Xcell SureLock Electrophoresis cell (Invitrogen). Separated proteins were transferred to polyvinylidene difluoride membranes (Roth) using an Xcell II Blot Module (Invitrogen). Primary antibody and horseradish-peroxidase-secondary antibody labeling was performed using guidelines proposed by blot module manufacturer followed by incubation with western blotting luminol reagent (Santa Cruz). Chemoluminescence signal was detected by using a Lumi-Imager LAS 1000 (FujiFilm). Quantitation of bands in immunoblot results was performed by using Syngene GeneTools 3.08 software (SynGene Ltd). A list of used antibodies is provided in Supplemental Table 1.

Qualitative and quantitative RT-PCR – Total RNA was purified from DF and CF monolayer cell cultures with an RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. During purification RNA was treated with DNase1 (Qiagen) to avoid contamination with genomic DNA. To generate cDNA 2 micrograms of total RNA were reverse transcribed using Omniscript RT Kit (Qiagen) according to manufacturer's instructions. Qualitative PCR was performed using Hot Star Taq Plus DNA Polymerase Kit (Qiagen) following manufacturer's guidelines. Real time quantitative PCR was conducted using an iQ iCycler Real-Time PCR Detection System (BioRad) with SYBR green fluorophore (ABgene). Quantification was performed by using the  $\Delta\Delta CT$  method. All used primers were QuantiTect Primer Assay primers (Qiagen). PCR conditions are provided in Supplemental Table 2.

siRNA – siRNA for JAK1 and STAT1 was synthesized by Qiagen (Qiagen) and was transfected by HiPerfect Transfection reagent (Qiagen) according to manufacturer's instructions.

Adenoviral construction and purification – Smad7 expressing adenovirus has been described previously (23). Human IFN $\gamma$  coding adenovirus was constructed using the Transpose-Ad Adenoviral Vector System (Qbiogene). Briefly, human IFN $\gamma$  was excised from pORF hINF $\gamma$  (Invitrogen) using SgrAI and NheI and ligated into transfecting vector pCR259. Subsequently, pCR259 was transposed in Transpose-Ad 294 vector. Transpose-Ad 294 vector was linearized using PacI and transfected in HEK 293 cells (Biochrom) using Superfect Transfection Kit (Qiagen). Adenoviruses were amplified using AdenoX Virus Purification Kit (Clontech) corresponding to Manufacturer's instructions. Infections of DF and CF were performed according to other reports (24). Routine infections were accomplished at 50 m.o.i. with single virus clones of the same virus stock preparation. Infection efficiency was proven by adenoviral constructs expressing

$\beta$ -Gal and, each experiment, about 90% of the cells were infected. Staining of infected cells was performed using an X-Gal staining kit (Roche).

TGFB1 responsive reporter assay – For TGFB1 responsive reporter assay DF and CF were infected with (CAGA)<sub>9</sub>-MLP-Luc (25) as described previously (21). Cell lysis and luciferase assays were performed using Steady-Glo® Luciferase Assay System (Promega). Luciferase activity was measured in a CENTRO LB 960 Luminometer (Berthold Technologies).

Human subjects – Written informed consent to perform research on surgically excised human tissues was obtained from all patients. The research protocol applied during the experiments was approved by the Ethics Committee of the Medical Faculty of the University of Erlangen.

Statistical analysis – Results were given mean  $\pm$  standard deviation. Statistical analysis was performed by using GraphPad Prism 4.02. According to data 1- or 2-way ANOVA with Bonferroni post hoc tests was applied. P-values < 0.05 were considered significant.

## Results

To investigate the role of the IFNG pathway in patients with skin fibrosis, paraffin-embedded sections from third degree Dupuytren's cords were stained for IFNG and JAK1 (Fig. 1a). Surprisingly, 95% of the fibroblasts express both, IFNG and JAK1. The result was confirmed by staining cultured Dupuytren's fibroblasts isolated from 2nd degree (DF II°) and 3rd degree (DF III°) contractures for IFNG (Fig. 1b), demonstrating IFNG expression in every fibroblast. To show the de-novo-synthesis of IFNG in fibroblasts and myofibroblasts (MFB), we conducted PCR for IFNG from total RNA lysates (Fig. 1d). The IFNG-amount of whole cell lysates determined by immunoblot was increased for DF II° and significantly increased for DF III° in comparison to control fibroblasts (CF) (Fig. 1c), suggesting that IFNG is expressed at higher levels in MFB than in fibroblasts. We proved this at the mRNA level by qPCR of CF, DF II° and DF III°, and found 5.9-fold increased (DF II°), respective 3.8-fold increased (DF III°) IFNG-mRNA values in comparison to CF (Fig. 1e). By immunofluorescence we demonstrated that serum starved DF II° express alpha 2 smooth muscle actin (ACTA2) and IFNG simultaneously (Fig. 1f).

To demonstrate the dynamics of SMAD7 expression upon IFNG stimulation of DF II°, we used triple-immunostaining. Following stimulation with recombinant IFNG, serum starved DF II° were fixed on slides at 10-minute-steps. After 10 minutes JAK1 expression started to increase, after 60 minutes a maximum expression level was reached that subsequently decreased to initial levels. STAT1 began to boost after 75 minutes, reaching a maximum about 120 minutes post stimulation, which was kept until 240 minutes post stimulation, when monitoring ended. Finally, SMAD7 started to show up 150 minutes post stimulation, reaching its maximum after 180 minutes, which was also steady state until the end of monitoring (Fig. 2a).

We infected CF and DF III° with Ad-(CAGA)<sub>9</sub>-MLP-Luc, a luciferase-linked reporter construct comprising nine copies of a PAI1-specific TGFB response element. 6 hours post stimulation with recombinant TGFB1,

reporter gene activity was induced 2.8-fold for CF and 5.9-fold for DF III°, after 12 hours 7.9-fold for CF and 11.8-fold for DF III°. When cells were co-stimulated with recombinant TGFB1 and IFNG, reporter gene activity was reduced about 20% (6 and 12 hours post stimulation) for CF and between 10% (6 hours post stimulation) and 20% (12 hours post stimulation) for DF III° in comparison to solely TGFB-induced reporter gene activity. Recombinant IFNG treatment alone had no significant effect (Fig. 2b).

SMAD2 is an intracellular mediator of TGFB1 signaling that is activated by phosphorylation(15). ACTA2, PAI1, Procollagen type1  $\alpha_1$  (ProCOL1A1) and Fibronectin (FN1) are effector-proteins during fibrogenesis of DD that are upregulated following stimulation with TGFB1. To demonstrate the TGFB directed antifibrotic effect of IFNG, we infected DF III° with adIFNG, an adenovirus overexpressing IFNG and then tested TGFB1 effects. We find that (1) TGFB induces endogenous IFNG expression, (2) ectopic expression of IFNG decreases fibrogenic gene expression in untreated and TGFB1 treated cells after 24 hours. More specifically, phosphorylation of SMAD2, expression of ACTA2, ProCOL1A1 and FN1 is significantly decreased after 24 hours, whereas expression of PAI1 remains unchanged (Fig. 2c).

SMAD7 is an inhibitory protein of intracellular TGFB1 signaling that is transiently upregulated by TGFB1 thereby providing a negative feedback regulation(12, 19). To investigate a possible cross-effect of SMAD7 on IFNG expression and signaling, we infected DF II°, DF III° and CF with adSMAD7, an adenovirus overexpressing SMAD7. Both, DF II° and DF III° show reduced expression of endogenous IFNG and decreased IFNG induced JAK1 and STAT1 expression, as shown by immunostaining (Fig. 3a). This is further supported by a significant decrease of IFNG, phosphorylated JAK1, JAK1, phosphorylated STAT1 and STAT1 in western blots and qPCR of adSMAD7 infected CF, DF II° and DF III° (Fig. 3b,c), indicating bidirectional IFNG-TGFB-crosstalking. Y-box binding protein 1 (YBX1) is supposedly the crucial mediator of antifibrotic IFNG-effects by interference with TGFB1 signaling via upregulation of SMAD7(20). In fibroblasts overexpressing SMAD7 we detected a 2.8-fold decrease of YBX1 in CF and a 3.7-fold decrease in DF II° (Fig. 3c), suggesting that SMAD7 levels have impact on availability of its transcription regulator downstream of IFNG signaling.

To prove the supposed activating effect of TGFB1 on IFNG and subsequent phosphorylation and expression of JAK1, STAT1, YBX1 and SMAD7, we stimulated serum starved CF, DF II° and DF III° with recombinant TGFB1. 24 hours later, cells were fixed on slides and stained for TGF beta type 1 receptor (TGFB1R), SMAD7 and ACTA2 as commonly known members of the TGFB1 signaling pathway and specific protein response. In addition, immunodetection for IFNG, pJAK1 and pSTAT1 was performed, demonstrating increased expression levels of these proteins (Fig. 4a). To compare the levels of expression we stimulated CF, DF II° and DF III° either with recombinant TGFB1 or recombinant IFNG and probed whole cell lysates by western blotting. The analysis revealed significantly increased expression levels of IFNG and YBX1, as well as upregulated pJAK1 and pSTAT1 levels upon TGFB1 treatment, although protein-levels were lower in comparison to stimulation with recombinant IFNG. The data indicate that TGFB1 acts as a costimulating effector of IFNG signaling (Fig. 4b).

To show IFNG dependence of SMAD7 expression, we knocked down IFNG signaling in DF III° by transducing siRNA targeting JAK1 (siJAK1) or STAT1 (siSTAT1). Following stimulation with recombinant IFNG, SMAD7 expression is significantly decreased in DF III° transfected with siJAK1. Surprisingly, when stimulated with recombinant TGFB1, SMAD7 induction in JAK/STAT depleted cells is comparably decreased as upon stimulation with IFNG (Fig. 4c).

## Discussion

The discovery that IFNG is expressed in fibroblasts elucidates a new facet of fibrotic remodelling processes with implications for the proposed crosstalk between TGFB1- and IFNG-signaling. The data presented here demonstrate, to our knowledge, for the first time endogenous expression of IFNG in quiescent fibroblasts as well as myofibroblasts of hand connective tissues. In addition, we show de-novo synthesis of IFNG in CF and DF with elevated IFNG amounts in activated fibroblasts, whereas other cell types like NK cells lose IFNG production when they are stimulated with TGFB1(21–23).

SMAD7 acts as antagonistic signal protein in the feedback mechanism of TGFB1 signaling(24, 25). Overexpression of SMAD7 blocks TGFB1 signaling at the receptor level through binding to type 1 receptors, thereby inhibiting phosphorylation of R-SMADs(19). Moreover, SMAD7 can lead to degradation of receptor (R)-SMADs by E3 ubiquitin ligases after binding of SMURF1-SMAD7(26) complexes to TGFB1 receptors(27). This results in downregulation of functional proteins(19, 28), as we verified for pSMAD2, COL1A1, FN1 and ACTA2 in adSMAD7 infected CF and DF (data not shown). Other data suggest that SMAD7 may act independently of type 1 receptors in the nucleus by repressing SMAD2, SMAD3 and SMAD4 transcription through binding to a SMAD-responsive element via its MH2 domain(29). Consistently, we demonstrated high levels of SMAD7 in the nucleus of DF.

The regulation of IFNG gene transcription and the interaction between TGFB1 and IFNG signaling is well controlled and has been a subject for some time. The most important sources for IFNG are, amongst others, T-Cells and NK-cells. It has been demonstrated that TGFB1 inhibits expression of IFNG by NK-cells(21). This was explained via downregulation of T-box 21 (TBX21), a gene shown to be required for IFNG-expression in CD4<sup>+</sup>T-Cells(30), mediated through SMAD3 protein interaction with the TBX21 promoter(31, 32). However, in T-Cells TBX21 is not required for IFNG expression, as TBX21 decreases GATA binding protein 3 (GATA3), an inhibitory transcription factor of IFNG expression(33). Interestingly, we offer a new regulatory mechanism of IFNG expression deciphering an additional inhibitory effect of SMAD7 on IFNG transcription in DF and even in CF. Expression of JAK1 and STAT1 is blunted Smad7 dependently, thereby indicating a new negative feedback mechanism of IFNG signaling through upregulation of the I-SMAD SMAD7 in fibroblasts and myofibroblasts in DD.

IFNG is known to reduce ACTA2 expression and inhibit transdifferentiation of fibroblasts to myofibroblasts(5, 34–36). The molecular mechanism was demonstrated via an assumed IFNG-TGFB1-crosstalk (18, 19), e.g. in hepatic stellate cells(20, 37). Consistent with these findings, transient adIFNG infection or recombinant IFNG stimulation of CF and DF provide antifibrotic effects via upregulation of

SMAD7. Subsequently, TGFB1 signal transduction is blunted as measured by reporter gene activity and immunoblotting for pSMAD2, PAI1, ProCOL1A1 and FN1. Unexpectedly, ACTA2 was elevated, indicating that IFNG has the opposite effect on differentiated respective quiescent myofibroblasts(34). When IFNG treated cells are activated by recombinant TGFB1, ACTA2 levels decrease significantly in comparison to uninfected but TGFB1 stimulated DF III°.

YBX1 is a negative regulator of collagen expression, which relies on two different mechanisms, either acting directly through binding to an interferon-gamma response element within the COL1A2 promoter or by binding to a recognition site within the SMAD7 promoter followed by SMAD7 transcription(20). Our experimental data demonstrate the assumed crosstalk with YBX1 as binding protein and suggest time dependence of this interaction *in vitro*. Following stimulation of CF and DF with recombinant IFNG, JAK1, STAT1 and SMAD7 are upregulated within 3 hours as shown by immunostaining and YBX1 immunoblotting. Consistently, in adIFNG infected myofibroblasts, we found stable but slightly reduced levels of pSMAD2, ProCOL1A1, PAI1 or FN1. By combining adIFNG with recombinant TGFB1, the cellular response is significantly reduced for pSMAD2, ACTA2, PAI1, ProCOL1A1 and FN1. A new finding is that there are significantly higher levels of IFNG in TGFB1 stimulated DF III° and also in adIFNG infected DF °III, when co-stimulated with recombinant TGFB1, as compared to adIFNG infected DF °III. This suggests a co-activating effect of TGFB1 on IFNG expression in fibroblasts and myofibroblasts, which was neither expected nor described before and has to be studied in future.

TGFB1 is a strong inhibitor of keratinocyte proliferation and an activator of extracellular-matrix production in fibroblasts(38). Further it is a potent chemoattractant for monocytes(38). Besides, TGFB1 has an essential role in regulation and strict control of T-cell homeostasis, to permit normal immune responses and prevent undesirable self-targeted responses(39). Therefore, TGFB1 is fine tuning fibrotic remodeling processes and immune responses according to requirements.

## Conclusion

The fact that fibroblasts are able to produce IFNG and, moreover, that TGFB1 stimulation results in IFNG upregulation establishes a new field in the understanding of fibrosis with implications for cutaneous wound healing and immune defense *in vitro*.

The TGFB1 cytokine signaling pathway leads through SMAD dependent transcription factors into programs of gene activation and repression(25). Since our results indicate that knocking down IFNG signaling by transducing specific siRNA results in downregulation of the TGFB1 signaling pathway, it can be implied that both pathways are more associated than presumed. Additionally, our findings suggest that IFNG-TGFB1-crosstalking interacts at least in activated fibroblasts bidirectionally. As the intracellular link for bidirectional cross-talking has not been determined yet, the identification of the regulatory mechanisms responsible for these phenomena requires further investigation.

## Abbreviations

DD, Dupuytren's disease; DF, fibroblasts derived by Dupuytren's disease; DF II°, Dupuytren's fibroblast derived by individuals suffering from contractures of the fingers rated as second degree; DF III°, Dupuytren's fibroblast derived by individuals suffering from contractures of the fingers rated as third degree; CF, fibroblasts derived by normal tendon pulley; R-SMAD, receptor regulated Smads; Co-SMAD, common mediator SMAD; I-SMAD, inhibitory SMAD.

## Declarations

Human subjects – Written informed consent to perform research on surgically excised human tissues was obtained from all patients. The research protocol applied during the experiments was approved by the Ethics Committee of the Medical Faculty of the University of Erlangen.

All data generated or analysed during this study are included in this published article.

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The authors declare that they have no competing interests.

Contributors: H.S. and O.A. performed cell isolations, adenoviral infections, rtPCR, histologies, reporter assays, western blots, IFG adenoviruses virus amplifications, created figures and contributed to the design of the study and the writing of the manuscript. A.A. and P.F. performed statistic evaluation. .E.H. and S.D. contributed to the design of the study and supply of tissue specimen and adenoviral constructs.

J.K, SSM and H.S. designed and coordinated the study, contributed to experimental data analysis and interpretation, and drafted and edited the manuscript.

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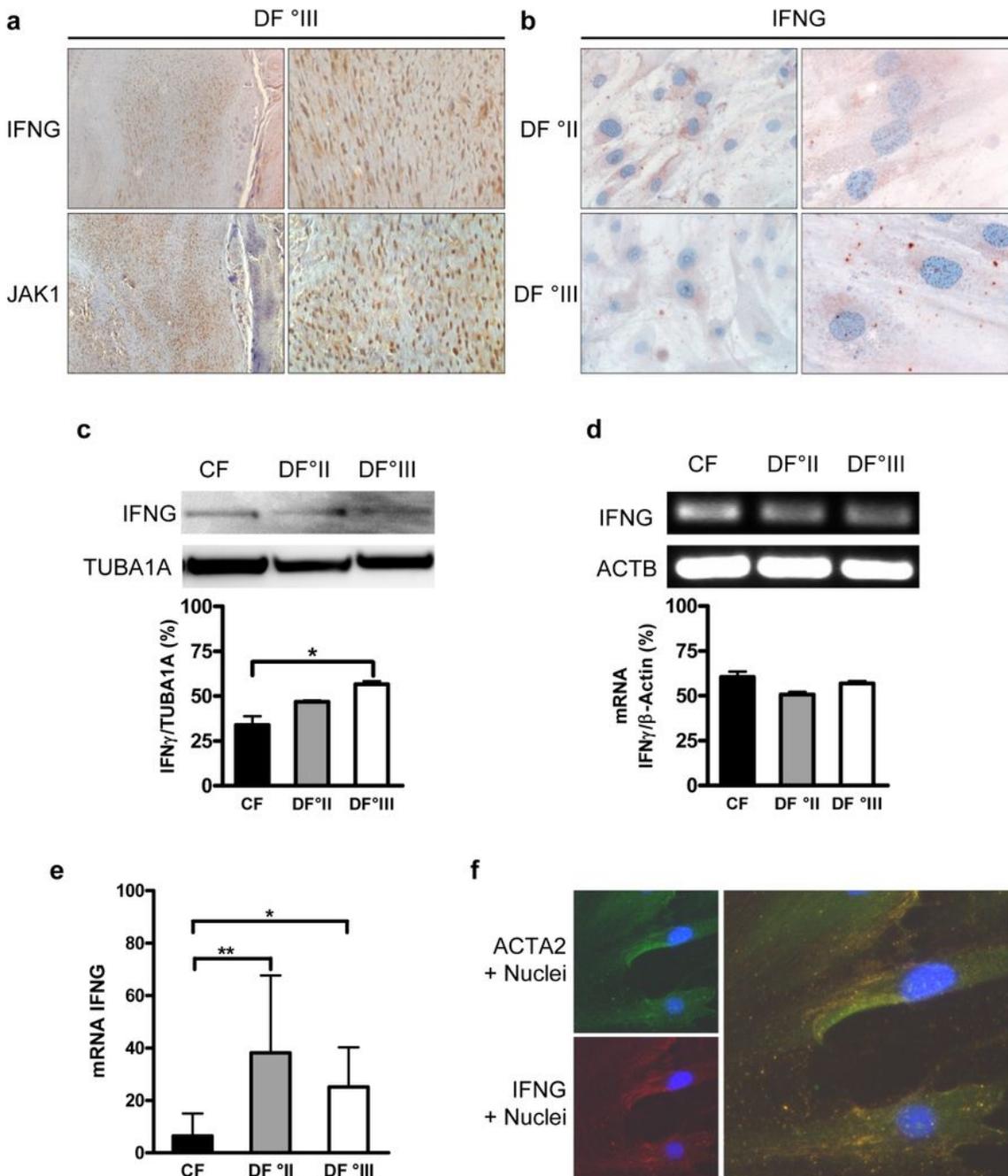
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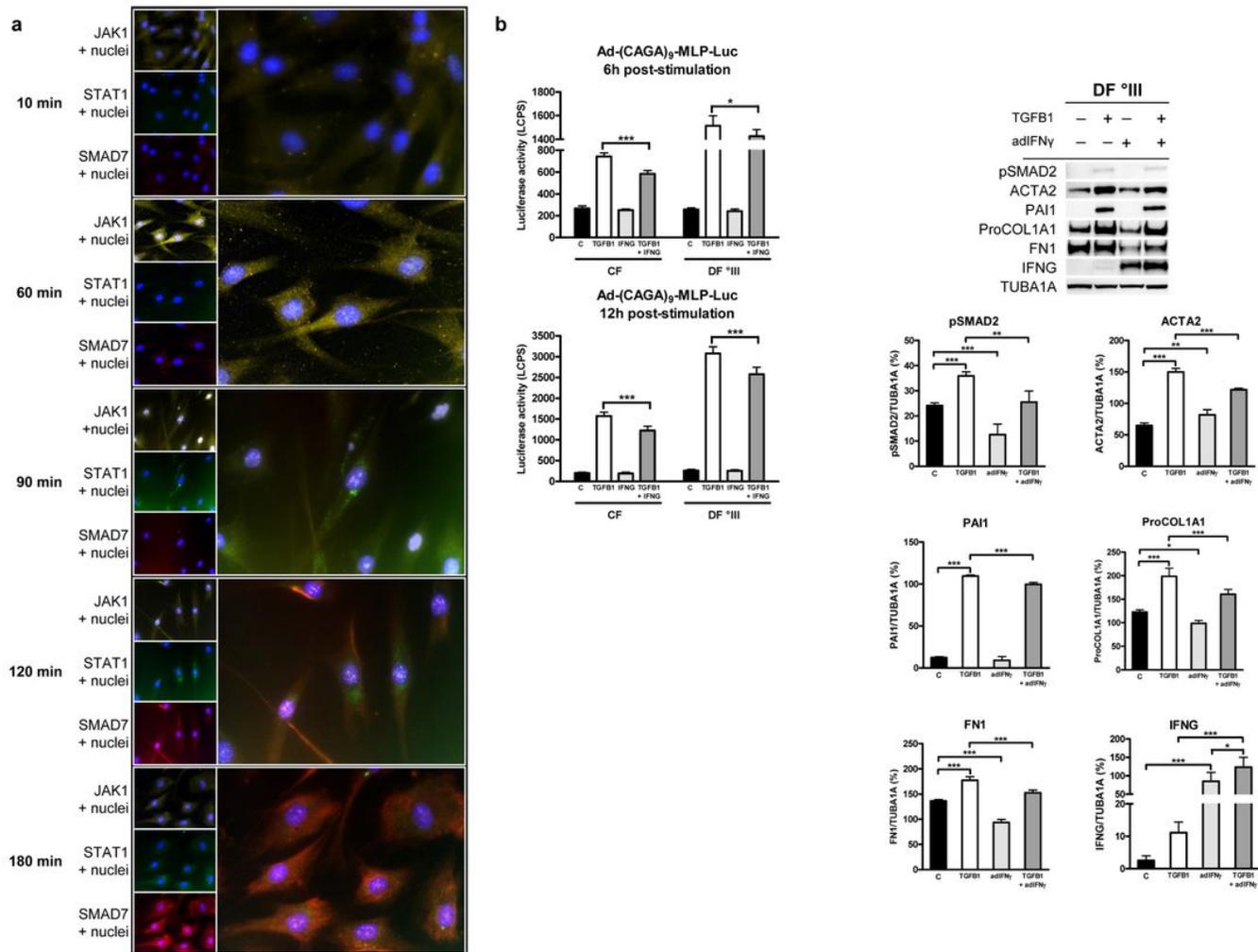
## Figures



**Figure 1**

IFNG is expressed in DF II°, DF III° and CF. (a) Representative micrographs of Paraffin-embedded sections from Dupuytren's cords of third degree contractures stained for IFNG or JAK1 at 10-fold (left column) and 40-fold (right column) magnification. (b) Representative Micrographs of cultured DF stained for IFNG at 40-fold (left column) and 100-fold (right column) magnification. Each cell of 5 randomly chosen visual fields at 40-fold magnification was positively stained for IFNG. (c,d,e) DF II°, DF III° and CF were serum

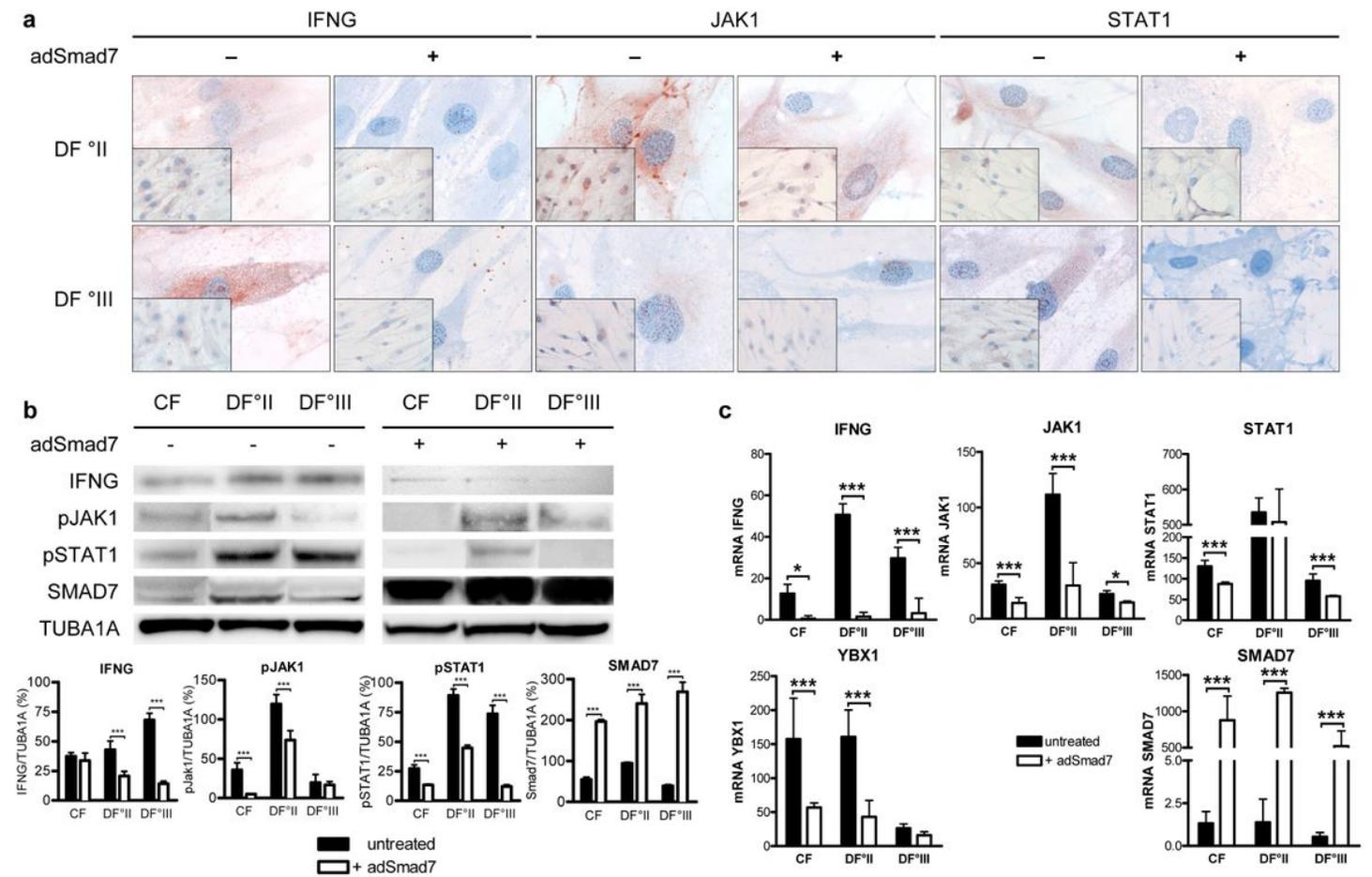
starved overnight and whole cell lysates were prepared for western blotting and mRNA analysis. (c) Lysates were probed with an antibody against IFNG in western blots. A representative immunoblot is shown and quantification of 4 individual experiments normalized to TUBA1A is presented below ( $*P \leq 0.05$ ). (d,e) mRNA levels of IFNG, as determined by PCR (d) and qPCR (e). Results are shown as mean  $\pm$  standard deviation normalized to ACTB of 4 individual experiments (d,e  $*P \leq 0.001$ ,  $**P \leq 0.01$ ). (f) To demonstrate simultaneous expression of ACTA2 (green) and IFNG (red) in cultured DF II<sup>o</sup>, cells were serum starved for 12 hours and investigated by immunofluorescence. Nuclei were stained blue. A representative micrograph of 3 individual experiments is shown at 63-fold magnification.



**Figure 2**

IFNG decreases phosphorylated SMAD2 (pSMAD2) and expression of ACTA2, PAI1, procollagen type 1  $\alpha$ 1 (ProCOLT1A1) and FN1 via JAK1 dependent signaling, STAT1 nuclear translocation and SMAD7 expression. (a) DF II<sup>o</sup> were serum starved overnight and treated with 1000 U/ml IFNG. Subsequently, DF II<sup>o</sup> were fixed in 10-minute-steps until 4 hours post stimulation and stained for JAK1 (yellow), STAT1 (green), SMAD7 (red) and nuclei (blue). The experiment was performed in quadruplicate and representative micrographs are presented at 63-fold magnification depicting a dynamic time course. Small micrographs

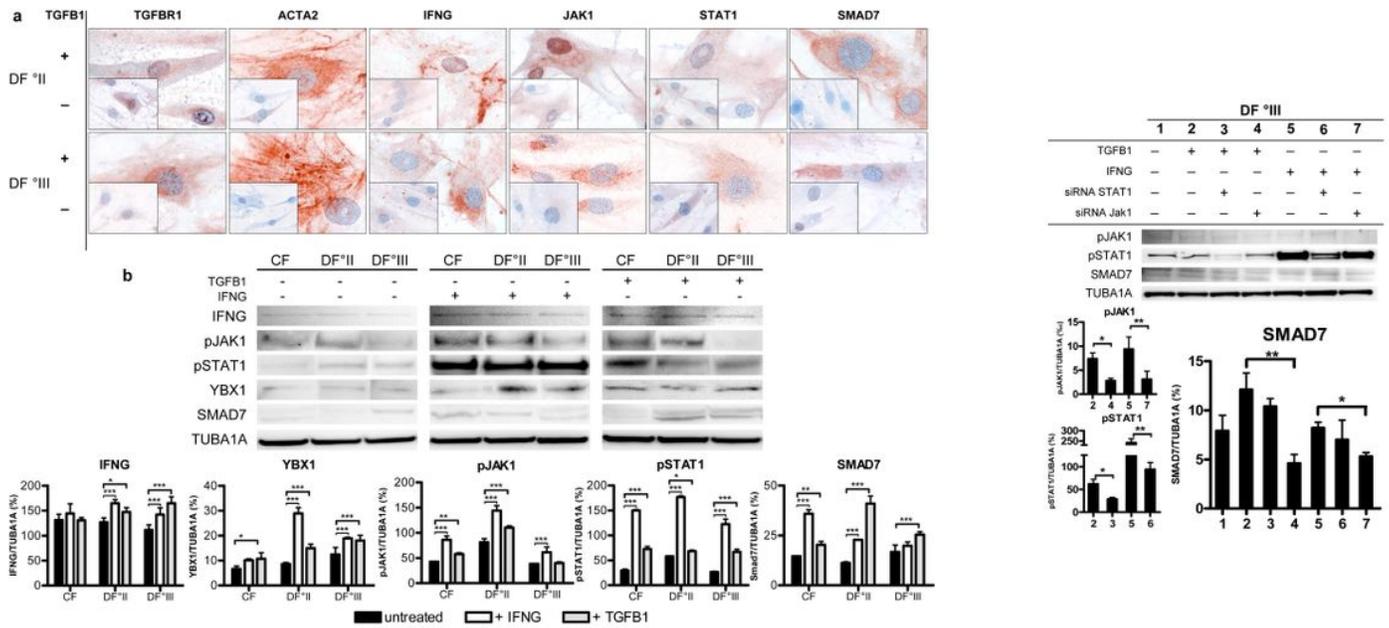
show each individual protein with nuclei, large micrographs are arranged as overlay. (b) CF and DF II° were infected with ad-(CAGA)9-MLP-Luc and, subsequently, after 12 hours of serum starvation stimulated with 5ng/ml recombinant TGFB1 or 1000 U/ml recombinant IFNG for 6 hours or 12 hours, as indicated. Luciferase activity was determined and expressed as means  $\pm$  standard deviation of three independent experiments and triple values for each measuring point were analysed (\* $P \leq 0.05$  \*\*\* $P \leq 0.001$ ). (c) 50 percent of cultured DF III° were infected with adIFN $\gamma$ , followed by 24 hours incubation, 12 hours serum starvation and treatment with 5 ng/ml recombinant TGFB1 for 24 hours, as indicated. Whole cell lysates were prepared and probed by western blotting applying antibodies against pSMAD2, ACTA2, PAI1, ProCOL1A1, FN1 or IFNG. TUBA1A served as control. Representative immunoblots of four independent experiments are demonstrated and quantification of band values normalized to TUBA1A is provided below (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ ).



**Figure 3**

SMAD7 downregulates expression of IFNG and IFNG mediated signal transduction. (a,b,c) Cultured DF II°, DF III° and CF were serum starved overnight. Subsequently, 50% of DF were infected with adSMAD7 and 24 hours later, cells were lysed. (a) DF were fixed and stained for IFNG, JAK1 or STAT1, as indicated. Representative micrographs are shown at 40-fold (inset) and 100-fold magnification. (b) Whole cell lysates were probed with antibodies against IFNG, phosphorylated JAK1 (pJAK1), phosphorylated STAT1

(pSTAT1) or SMAD7 and detected by western blotting, whereby TUBA1A served as loading control. Immunoblot quantification normalized to TUBA1A of 4 individual experiments is provided below. (\*\* $P \leq 0.001$ ). (c) mRNA levels of IFNG, JAK1, STAT1, YBX1 and SMAD7 were determined by qPCR. Results are shown as mean  $\pm$  standard deviation normalized to ACTB of 4 individual experiments and triple values for each measuring point (\* $P \leq 0.05$ ; \*\* $P \leq 0.001$ ).



**Figure 4**

IFNG and TGFβ1 induce endogenous production of IFNG followed by IFNG dependent upregulation of pJAK1, pSTAT1 and YBX1. (a) DF°II and DF°III were serum starved overnight and treated with 5ng/ml recombinant TGFβ1 for 24 hours. Subsequently, cells were fixed and stained for TGFβ receptor1 (TGFBR1), ACTA2, IFNG, JAK1, STAT1 or SMAD7, as indicated. Representative micrographs are shown at 100-fold magnification. Untreated controls are provided as small inserts. (b) DF°II, DF°III and CF were serum starved overnight and treated with 5ng/ml recombinant TGFβ1 or 1000U/ml recombinant IFNG for 24 hours. Whole cell lysates were analyzed by western blots with antibodies against IFNG, pJAK1, pSTAT1, YBX1 or SMAD7 with TUBA1A as loading control. Immunoblot quantification normalized to TUBA1A of 4 individual experiments is provided below. (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ ). (c) DF°III were serum starved overnight and treated with either 5ng/ml recombinant TGFβ1 or 1000 U/ml recombinant IFNG for 24 hours. Beforehand, samples 3 and 6 were transfected with siRNA targeting STAT1 and accordingly samples 4 and 7 with siRNA targeting for JAK1. Whole cell lysates were analyzed by western blotting for pJAK1, pSTAT1 and SMAD7 with TUBA1A as loading control. Immunoblot quantification normalized to TUBA1A from 3 individual experiments is shown (some samples for pJAK1 and pSTAT1 and all samples for SMAD7; \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ ).

## Supplementary Files

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