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*Elevated TGF- β and MAP kinase pathways
mediate fibrotic traits of Dupuytren's disease
fibroblasts*

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Chapter 8

Elevated TGF- β and MAP kinase pathways mediate fibrotic traits of Dupuytren's disease fibroblasts

8.1 Abstract

8.1.1 Background

Dupuytren's disease is a fibro-proliferative disorder of the fascia palmaris of the hand. Treatment to date is mostly surgical, but there is a high recurrence rate. Transforming growth factor beta (TGF- β) has been implicated as a key stimulator of myofibroblast activity and fascial contraction in Dupuytren's disease.

8.1.2 Results

We studied Dupuytren's fibroblasts in tissues *ex vivo* and in cells cultured *in vitro* and found increased TGF- β expression compared to control fibroblasts. This correlated not only with elevated expression and activation of downstream Smad effectors, but also with overactive ERK1/2 MAP kinase signaling. Treatment with the TGF- β type I receptor kinase inhibitor SB-431542 and bone morphogenetic protein-6 (BMP-6) led to inhibition of elevated Smad and ERK1/2 MAP kinase signaling, as well as inhibition of the increased contractility of Dupuytren's fibroblasts. BMP-6 attenuated TGF- β expression in Dupuytren's, but not in control, fibroblasts. PDGF expression was strongly promoted by TGF- β in Dupuytren's fibroblasts, and was curbed by SB-431542 or BMP-6 treatment. High basal expression of phosphorylated ERK1/2 MAP kinase and fibro-proliferative markers was attenuated in Dupuytren's fibroblasts by a selective PDGF receptor kinase inhibitor. Co-treatment of Dupuytren's fibroblasts with SB-431542 and the MEK-1 inhibitor PD98059 was sufficient to abrogate proliferation and contraction of Dupuytren's fibroblasts.

8.1.3 Conclusion

Both TGF- β and ERK/MAP kinase pathways cooperated in mediating the enhanced proliferation and high spontaneous contraction of Dupuytren's fibroblasts. Our data indicate that both signaling pathways are prime targets for the development of non-surgical intervention strategies to treat Dupuytren's disease. These findings provide possible new avenues for non-surgical interventions for Dupuytren's disease.

Keywords

disease, MAP kinase, myofibroblast, PD98059, signal transduction, SB-431542, Smad, STI571, TGF- β , BMP-6

8.2 Background

Dupuytren's disease (DD) is a common fibro-proliferative condition that only affects the hand. The characteristic feature is a progressive contracture of the palm and fingers. Patients commonly first display a nodule in the handpalm or on the volar (palmar) aspect of the finger(s) due to a thickened layer of tissue (fascia palmaris) between the skin and the tendons of the hand and the fingers; this is the key diagnostic feature and represents the early *proliferative* stage of the disease. The nodules contain mostly myofibroblasts [46, 44]; as the disease progresses, the nodules may disappear and give way to formation of cords. These cords represent characteristics of fibrosis within the emphynevolitional and emphyresidual stages of the disease and comprise mostly fibroblasts and extracellular matrix (ECM). Treatment of DD consists largely of surgical excision of the contracted tissue. Because of high recurrence rates following surgery, investigations are underway to determine the underlying causes of DD in order to optimize treatment strategies [46, 44]. The myofibroblast, a specialized fibroblast phenotype that expresses α -smooth muscle actin (α -SMA), provides the cell with contractile activity [26, 52, 18]. To date, many growth factors have been implicated in Dupuytren's contracture; transforming growth factor- β (TGF- β) in particular has been proposed to play a prominent role [30]. TGF- β is a member of a family that also includes activins, nodal, and bone morphogenetic proteins (BMPs). TGF- β family members signal through type I and type II serine/threonine kinase receptors [23]. Type I receptors are also called activin receptor-like kinases (ALKs); ALK-4, ALK-5, and ALK-7 are type I receptors of activin, TGF- β and nodal, respectively. SB-431542 is a selective inhibitor of ALK-4, ALK-5, and ALK-7 kinase activity [27]. Signaling from activated type I receptors are mainly transduced into the cytoplasm through phosphorylation of receptor-regulated Smads (R-Smads). Activated ALK-4, ALK-5, and ALK-7 induce phosphorylation of Smad-2 and Smad-3. BMPs mediate the activation of Smad-1, Smad-5, and Smad-8. Activated R-Smads form heteromeric complexes with Smad-4 that accumulate in the nucleus, where they regulate gene expression, including plasminogen activator inhibitor 1 (PAI-1, also known as SERPINE1; a TGF- β /ALK-5 target gene) and inhibitor of DNA binding 1 (ID1; a

BMP target gene) [23]. TGF- β can also activate non-Smad pathways, including the mitogen-activated protein (MAP) kinase/extracellular-signal-regulated kinase (ERK) pathway [17, 37]. TGF- β is a potent modulator of fibroblast and myofibroblast proliferation and differentiation [26, 24, 51, 55]. Previous studies in DD tissue found increased protein synthesis and expression of all three TGF- β isoforms and their receptors [31, 3, 4, 59, 7]. In vitro contraction assays revealed that TGF- β stimulation generates or increases contractile force in Dupuytren-derived cells [58, 9, 54, 10, 11]. In addition, TGF- β stimulation leads to upregulation of key matrix components, such as fibronectin (FN) and type I collagen (COL-1); this effect may be either direct or may occur indirectly via enhanced expression of matricellular protein connective tissue growth factor (CTGF/CCN2) [20, 34]. TGF- β stimulation can also induce the expression of growth factors, such as platelet-derived growth factor (PDGF) [6]. It is not known whether BMPs play a role in DD. Compared to normal fascia-derived cells, Dupuytren-derived cells do not express BMP-4 and exhibit decreased BMP-6 and BMP-8 expression [47]. A previous study found that there is decreased BMP receptor expression and, apparently, reduced BMP responsiveness in DD tissue; this has constrained research into BMPs as a potential antagonist of TGF- β -induced fibrosis in DD, as described in kidney and liver fibrosis [57]. In this study, we investigated the aberrant activation of TGF- β /Smad and PDGF/ERK1/2 MAP kinase pathways in DD tissue specimens and cell culture. Using BMP-6 and selective chemical inhibitors of the TGF- β receptors, the PDGF receptors, and the MAP kinase pathway, we attempted to counteract the fibrogenic characteristics of DD. Our insights may contribute to the development of new therapeutic strategies for sustained, non-surgical treatment of DD.

8.3 Methods

Clinical specimens

DD tissue specimens were obtained from four adult patients undergoing fasciectomy for DD. Patients who underwent carpal tunnel release and showed no evidence of DD contributed the control tissue from normal palmar fascia ($n = 3$) or carpal ligament ($n = 1$). All DD tissues used here were from primary releases. The tissue was separated macroscopically in nodule and cords. Only cords were used for the study. For details on how samples were prepared see supplemental information.

Clinical sample preparation

After excision, tissue was divided in three portions. One portion was placed in 10% formalin and further processed for immunohistochemistry. The second was immediately placed in liquid nitrogen for protein extraction. The third portion was used for primary cell culture. All of the patients underwent excision independent of this study and had not undergone previous surgery on their hands. Oral consent for removal of the tissue and storage in the tissue bank for research purposes was obtained from the patients. Individual consent for this specific project was waived by the ethics commit-

	Age	Gender	Tissue
Control 1	63	Female	fascia palmaris
Control 2	32	Female	carpal ligament
Control 3	67	Male	fascia palmaris
Control 4	38	Female	fascia palmaris
Dupuytren 1	56	Female	fascia palmaris
Dupuytren 2	43	Male	fascia palmaris
Dupuytren 3	71	Male	fascia palmaris
Dupuytren 4	47	Male	fascia palmaris

Table 1: Overview of patient's details.

tee, because the research was performed on 'waste' material, which was stored in a coded fashion.

Reagents

Recombinant human TGF- β 3 (OSI Pharmaceuticals) and recombinant human BMP-6 (Creative Biomolecules) was generously provided by K. Iwata and K. Sampath, respectively. SB-431542 compound, which targets ALK-4, ALK-5, and ALK-7, was purchased from Tocris Biosciences. PD98059 compound, which targets MEK1, was purchased from Cell Signaling. The vascular endothelial growth factor (VEGF) receptor inhibitor PTK787/ZK222584, the epidermal growth factor (EGF) receptor inhibitor PKI166, and the PDGF receptor inhibitor STI571 (also known as imatinib mesylate or Glivec®) were kindly provided by Novartis. The protein kinase C activator 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) was obtained from Sigma.

Cell culture

To obtain primary cells, tissues were minced under sterile conditions into pieces that measured approximately $1 \times 1 \times 2 \text{ mm}^3$. Ten to twenty pieces were placed as explants into the wells of six-well plates and stored in 37°C incubators in 5% CO_2 . Primary cells from passages 3-6 were used for the experiments. All of the cells were subcultured in 4.5 g/l Glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Integro), 100 IU/ml penicillin, and 100 IU/ml streptomycin (Invitrogen).

Reporter assays

Cells were seeded in 24-well plates (2×10^4 cells/well) and transiently transfected using polyethylenimine (PEI) transfection reagent (Polysciences), according to manufacturer's instructions, with $0.2 \mu\text{g}$ of the indicated BRE-luc and CAGA₁₂-luc firefly

luciferase transcriptional reporter reporter construct and 0.1 μ g of a CMV promoter-driven β -galactosidase expression construct. Cells were allowed to recover for 18 h and subsequently stimulated with the indicated ligands and inhibitors. Eighteen hours after stimulation, cells were washed with PBS and lysed. Luciferase assays were performed with the Luciferase Reporter Assay System according to the manufacturer's instructions (Promega), using β -galactosidase activity as an internal control. The transfection efficiency of control and Dupuytren's fibroblasts varied and was on average about 10%. In this transient transfection experiment cells we only examined the transcriptional reporter in the primary cells that are transfected and may therefore not necessarily be representative for all primary cells in the dish. Each transfection was carried out in triplicate and representative experiments are shown.

RNA isolation and quantitative real-time PCR

Total RNA was extracted with the RNeasy kit (Macherey-Nagel) according to the manufacturer's instructions. Reverse transcriptase PCR was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. All of the samples were plated in duplicate, and Taqman PCR reactions were performed using the StepOne Plus Real-Time PCR System (Applied Biosystems). Lack of DNA contamination was verified and gene expression levels were determined using the comparative $\delta \delta$ Ct method with *GAPDH* as the reference.

Quantitative PCR primers

Expression of human *TGF- β 1-3*, *α -SMA*, *PAI-1*, *c-myc*, *Col1A2*, *Fibronectin*, *Smad-1-3*, *CTGF*, *PDGFA*, *PDGFB* and *GAPDH* were analyzed using the following forward and reverse primers: *TGF- β 1*, 5'-CTCTCCGACCTGCCACAGA-3' and 5'-AACCTAGATGGGCGCGATCT-3'; *TGF- β 2*, 5'-CCGCCCACCTTTCTACAGACCC-3' and 5'-GCGCTGGGTGGGAGATGTAA-3'; *TGF- β 3*, 5'-CTGGCCCTGCTGAACTTTG-3' and 5'-AAGGTGGTGCAAGTGGACAGA-3'; *α -SMA*, 5'-CACCTTCCAGCAGATGTGGAT-3' and 5'-AAGCATTTGCGGTGGACAAT-3'; *PAI-1*, 5'-TCTTTGGTGAAGGGTCTGCT-3' and 5'-CTGGGTTTCTCCTCCTGTG-3'; *c-myc*, 5'-CGTCTCCACACATCAGCACAA-3' and 5'-CACTGTCCAACTTGACCTCTTG-3'; *Col1A2*, 5'-GATGTTGAACTTGTGCTGAGG-3' and 5'-TCTTTCCCCATTCATTTGTCTT-3'; *Fibronectin*, 5'-GAGGCCACCATCACTGGTT-3' and 5'-AGTGCGATGACATAGATGGTGTA-3'; *Smad-1*, 5'-TGAACCATGGATTTGAGACAGT-3' and 5'-CTGGCGGTGGTATTCTGC-3'; *Smad-2*, 5'-CGAAAAGGATTGCCACATGTT-3' and 5'-TTGAGTTCATGATGACTGTGAAGATC-3'; *Smad-3*, 5'-CGGTCAACCAGGGCTTTG-3' and 5'-CAGCCTTTGACGAAGCTCATG-3'; *CTGF*, 5'-TTGCGAAGCTGACCTGGAAGAGAA-3' and 5'-AGCTCGGTATGTCTTCATGCTGGT-3'; *PDGF-A*, 5'-CCTCACATCCGTGTCCTCTT-3' and 5'-ACACGAGCAGTGTCAAGTGC-3'; *PDGF-B*, 5'-TGCTGTTGAGGTGGCTGTAG-3' and 5'-GAAAATGCAGGGTGGAGGTA-3'; *TGF- α* , 5'-TAACCACGAGACCCTCAAC-3' and 5'-CCCAAGCCTTAGCTGTCTTG-3'; *GAPDH*, 5'-ATCACTGCCACCCAGAAGA-3' and 5'-ATGAGGTCCACCACCTGTT-3'.

MTS-based proliferation assay

Cells were seeded into 96-well plates at 7×10^3 cells/well and treated the day after with the indicated inhibitors or DMSO as a control. Increases in the numbers of viable cells after culture were measured daily for 4 d using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) according to the manufacturer's instructions (Cell Titer 96 Aqueous One Solution Cell Proliferation Kit; Promega) and using the measured absorbance at 490 nm on day zero as the reference.

Tissue lysate preparation and western blot analysis

For tissue lysates, biopsies were frozen in liquid nitrogen and pulverized using a mortar. Thereafter, the triturated tissues were incubated in ice-cold lysis buffer (150 mM NaCl, 20mM Tris-HCl [pH 7.5], 1% NP40, 5 mM sodium EDTA, and one Complete Protease Inhibitor Cocktail tablet [Roche] per 50 ml of solution) for 30 min. Prior to centrifugation at 4°C for 15 min at 14,000 rpm, the samples underwent extensive vortexing and sonification. The total protein content of the supernatant was determined using the DC Protein Assay (Bio-Rad). Equal amounts of total protein ($100 \mu\text{g}/\mu\text{l}$) were loaded onto a 10% gel, followed by SDS-PAGE and western Blotting. For cell-based assays, cells were plated onto six-well plates at a density of 4×10^5 cells/well, stimulated with the indicated reagents, and directly lysed in sample buffer (250mM Tris-HCl [pH 6.8], 8% SDS, 40% glycerol, 5% β -mercaptoethanol, and bromophenol blue) after 18 h. Antibodies specifically targeting Smad-1 (Zymed), Smad-2/-3 (Transduction Laboratories), phosphorylated ERK1/2 (Cell Signaling), PAI-1 (Santa Cruz Biotechnology), Col1A2 (Southern Biotech), α -SMA (Sigma), fibronectin/ED-A (Abcam), and c-myc (Santa Cruz Biotechnology) were purchased. Antibodies targeting phosphorylated Smad-1 and Smad-2 (P-Smad-1 and P-Smad-2, respectively) were described previously [15]. P-Smad-3 was obtained from E. Leof (Mayo Clinic, USA), and P-ERK1/2 antibodies were a gift from W. H. Moolenaar (Netherlands Cancer Institute, The Netherlands). Equal loading was confirmed using an anti- β -actin antibody (Sigma). Quantitative western blotting was performed using secondary goat anti-rabbit IRDye 680 and goat anti-mouse IRDye 800CW (LI-COR) with the Odyssey Scanner according to the manufacturer's instructions.

Immunofluorescence

For immunofluorescence staining, cells were grown on coverslips overnight. Cells were fixed with ice-cold methanol for 30 min, washed twice with PBS, quenched with 20 mM NH₄Cl, and permeabilized with 0.1% Triton X-100 the following day. Cells were then incubated in blocking solution (PBS containing 3.0% BSA) for 45 min followed by incubation for 1 h with anti-smooth muscle actin (α -SMA) antibody (Sigma) diluted 1:100 in blocking solution. After washing, the labeled secondary antibody AlexaFluor 488 goat anti-mouse IgG (Invitrogen) was used. Nuclei were stained

using Hoechst 33258 (Invitrogen) according to the manufacturer's instructions. Specimens were visualized with an Olympus IX51 microscope at 100 $\times$ magnification using the cell^F Soft Imaging System (Olympus).

Immunocytochemistry

Cells were cultured overnight on cover slips. Fixation in acetone followed by staining for α -SMA (α -SMA/1, M851, 1A4 clone; DAKO) at a 1:500 dilution was performed for 60 min the day after. Endogenous peroxidase was quenched with 0.1% sodium azide/0.3% hydrogen peroxide in PBS. After post-antibody blocking, goat poly-HRP anti-mouse IgG (Immunologic) was added for 30 min. The coloring reaction was developed with 3-amino-9-ethylcarbazole (AEC) and counterstaining was performed with haematoxylin.

Immunohistochemistry

Paraffin-embedded tissue samples of 5 μ m thickness were sequentially cut. Before blocking endogenous peroxidase activity with 1% hydrogen peroxide (Merck) in 2% PBS, sections were deparaffinized and rehydrated using xylene and a descending alcohol series. Blocking was performed with the following sequence: 2.5% periodic acid, 0.02% sodium borohydride, and Protein Block (DAKO). Detection of TGF- β 3: After deparaffinization antigen retrieval was done in citrate buffer. Blocking was done with Protein Block (DAKO, Carpinteria, CA, USA) for 20 min. TGF- β 3 antibody (Abcam, Cambridge, MA, USA) was applied overnight in a humid chamber at 4 $^{\circ}$ C. Slides were rinsed in PBS after which biotinylated link antibody was added (LSAB 2 System from DAKO, Carpinteria, CA, USA) for 60 min. After PBS washing streptavidin conjugate (LSAB 2 System from DAKO, Carpinteria, CA, USA) was applied for 60 min. Detection of phospho-Smad-2 (P-Smad-2): Prior the application of Protein Block (DAKO, Carpinteria, CA, USA) for 20 min, sections were pre-treated with Proteinase K (2 μ g/ml in PBS) at 37 $^{\circ}$ C for 30 min. Phospho-Smad-2 (Ser465/467, Cell Signaling Technology, Danvers, MA, USA) was added overnight in a humid chamber at 4 $^{\circ}$ C. Slides were rinsed in PBS after which biotinylated link antibody was added (LSAB 2 System from DAKO, Carpinteria, CA, USA) for 60 min. After PBS washing streptavidin conjugate (LSAB 2 System from DAKO, Carpinteria, CA, USA) was applied for 60 min. Detection of α -SMA: After quenching endogenous peroxidase activity with 0.3% H_2O_2 in Methanol, slides were heated in Tris-EDTA for 10 min at 100 $^{\circ}$ C for antigen retrieval. The α -SMA antibody (α -SMA/1, M851, 1A4 clone, DAKO, Glostrup, Denmark) was applied for 60 min followed by a post-antibody blocking (Immunologic, Duiven, the Netherlands) for 15 min. After rinsing, goat poly-HRP against mouse IgG (Immunologic, Duiven, the Netherlands) was added for 30 min followed by PBS. All coloring reaction were developed using 3-,3'-diaminobenzidine (DAB, Sigma Chemical Co, St. Louis, MO, USA) followed by a counterstaining with hematoxylin. Non-labeled samples were scored by an independent pathologist. Scoring was rated as follows: no staining (-) (except from staining in blood vessel walls), weak staining (+), moderate

(++) and intense staining (+++). Detection of phospho-ERK-1/2 (P-ERK1/2): Before blocking endogenous peroxidase activity with 40% methanol (Merck), 1% H_2O_2 (Merck) in PBS, sections were deparaffined and rehydrated using Xylene and a descending alcohol series. Antigen Retrieval using Proteinase K (2.5 μ l in 100mM Tris pH 9.0 and 50mM EDTA pH 8.0), for 10 min at 37°C was followed by three washes with 0.1M Tris buffered saline (pH 7.4) containing 0.02% Tween 20 (TNT). Thereafter slides were incubated in 0.5% Boehringer Blocking reagent (BMP) in TNT for 60 min at 37°C. Subsequently, the P-ERK1/2 antibody (1:100) (Cell Signaling) diluted in 0.5% BMP/TNT was applied overnight at 4°C. A species-specific biotinylated anti-IgG antibody (1:600 diluted in 0.5% BMP/TNT), followed for 45 min at 37°C. Incubation with streptavidin horseradish peroxidase (1:200 in 0.5% BMP/TNT) for 30min at 37°C preceded and followed an amplification step using Biotinyl Tyramine. Stainings were carried out using amino-9-ethyl-carbazole (AEC) (Sigma Chemicals, Zwijndrecht, The Netherlands) and Mayers Hematoxyline (Merck, Amsterdam, The Netherlands) according to the manufactures instructions. A water based mounting solution was applied and stainings were visualized with an Olympus IX51 microscope using the cell^F Soft Imagine System (Olympus). Non-labeled samples were scored by an independent researcher.

Fibroblast-populated collagen lattice contraction assay

Three-dimensional fibroblast-populated collagen lattice (FPCL) contraction assays were carried out with primary cell cultures from passages 4-6. The assay was performed as described before by others with some modifications [21, 25, 45]. The collagen lattices were prepared by mixing a neutralizing solution of type I collagen (eight parts collagen type I, 3 mg/ml (PurCol); one part 10 x α -MEM (Invitrogen); and one part HEPES buffer, pH 9.0). Final collagen and cell concentrations were adjusted to 2 mg/ml and 86×10^3 cells/ml using PBS, respectively. The cell-collagen mixture was aliquoted into phosphate buffered saline (PBS) + 2% bovine serum albumin (BSA) pretreated 24-well culture dishes (0.5 ml/well) and left to polymerize for 1 h at 37°C. In each well, to the top of the polymerized lattice, we added 0.5 ml/well of DMEM containing 10% FBS. After 2 d of incubation at 37°C, the attached FPCL were mechanically released from the sides of the culture plates and fresh media supplemented with 0.5% FBS and the indicated substances were added. Images were taken at various time points over a 5 d period using the Odyssey Scanner. Collagen lattice areas were measured with the corresponding software (Odyssey 2.1).

Statistical analysis

Values are expressed as the mean $\pm$ standard error (SE). For statistical comparisons of two samples, an unpaired two-tailed Student's t-test with distinction of equal and non equal variances in a group (Levene's test) was used to determine the significance of differences between means. In addition, a non-parametric Mann-Whitney *U* test under the null hypothesis that the distributions of both groups (control versus Dupuytren

derived fibroblasts) are equal was performed for the dataset of Figure 2C. All of the relevant comparisons were considered to be significantly different at $p \leq 0.05$. Experiments were performed at least three times and representative results are shown.

8.4 Results

8.4.1 TGF- β /Smad signaling is upregulated in DD

To evaluate the presence of TGF- β signaling in DD, nodules from the fascia palmaris of four DD patients were surgically removed and compared to normal fascia palmaris from four control patients who underwent carpal tunnel release surgery (Table 1). Previous studies had shown an increase in TGF- β 1 levels in DD; we extended these studies by examining TGF- β 3, and also examined phosphorylated Smad2 (P-Smad2) as a measure for active canonical TGF- β signalling, and α -SMA as a marker for myofibroblasts. Immunohistochemical staining of the normal fascia revealed weak TGF- β 3 and P-Smad2 signals and no α -SMA expression (except for pericytes in blood vessels). This was in contrast to the tissues derived from DD patients, which displayed strong staining for TGF- β 3, P-Smad2, and α -SMA. A high viable cell density, which is indicative of the proliferative stage of the chords, was confirmed with haematoxylin and eosin (H&E) staining (Figure 1A, Table 2).

Tissue samples were further investigated for active TGF- β signaling and for protein expression of key matrix components induced during fibrogenesis (Figure 1B). On average, Smad2 and Smad3 protein expression was significantly upregulated in DD patients when compared to β -actin protein expression levels. Furthermore, we detected an increase in P-Smad2, but not P-Smad3 when normalized to total Smad2 and Smad3, respectively, in DD patients versus controls (Figure 1B, Supplementary Figure 1). In contrast, Smad1 protein expression levels did not differ between control and DD patient material. P-Smad1 was not detected in control or DD samples (data not shown). Fibrogenesis matrix markers, such as type 1 collagen and fibronectin/ED-A, were detectable in DD tissue but not in control samples. The myofibroblast marker α -SMA was strongly upregulated in all four DD patients (Figure 1B). We next examined whether primary fibroblasts derived from the tissue samples described above had similar properties. We first investigated the presence of all three TGF- β isoforms. In particular, the mRNA expression of TGF- β 1 and TGF- β 3 isoforms were significantly upregulated in primary fibroblasts derived from DD tissue samples, whereas TGF- β 2 mRNA expression was barely detectable (Figure 2A). Consistent with the results of the immunohistochemistry performed on the tissue samples, cultured Dupuytren's fibroblasts stained positive for α -SMA protein expression, whereas the control-derived fibroblasts contained only very little; percentage of myofibroblast in DD versus control was 40-50% vs. 2-5% (Figure 2B).

We went on to quantitatively compare the mRNA expression levels of components involved in TGF- β signaling and fibrosis. On average, a non-parametric Mann-Whitney U (data not shown) followed by an unpaired Student's t -test revealed that *Smad-2* and *Smad-3* mRNA expression, as well as expression of the TGF- β target

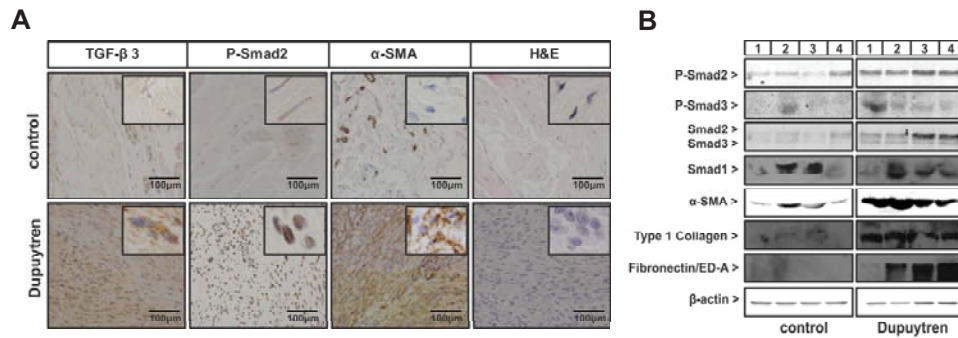


Figure 1: Characterization of DD and control tissue specimens. (A) Immunostaining of DD and control tissue specimens for TGF- β 3, phosphorylated Smad-2 (P-Smad-2), α -smooth muscle actin (α -SMA), and hematoxylin and eosin (H&E). Representative stainings from Dupuytren's patient 4 and control patient 1 are shown. Insets in each picture are higher magnification views. Evaluations of all four control and Dupuytren's tissue samples are described in Table 2. (B) Protein expression analysis for phosphorylated Smad-2 (P-Smad-2); phosphorylated Smad-3 (P-Smad-3); total Smad-2, Smad-3, and Smad-1; α -smooth muscle actin (α -SMA); type I collagen; and fibronectin/ED-A by western blotting of 100 μ g of total protein extracts isolated from four individual control and four individual Dupuytren's patient derived tissue samples. β -actin was used as a loading control.

	TGF- β 3	P-Smad2	α SMA
Control 1	+	++	-
Control 2	++	++	-
Control 3	+	++	-
Control 4	+	-	-
Dupuytren 1	+++	++	++
Dupuytren 2	+++	+++	+++
Dupuytren 3	+++	+++	+++
Dupuytren 4	+++	++	+++

Table 2: Evaluation of immunohistochemical stainings performed on four control and four Dupuytren tissue samples. Positivity was graded on a scale from (-) - (+++), with (-) representing no staining, (+) weak staining, (++) moderate staining and (+++) strong staining. Staining in blood vessels was not included. The scoring was performed on non-labeled samples by a pathologist not involved with the study. Representative pictures are shown in Figure 1A.

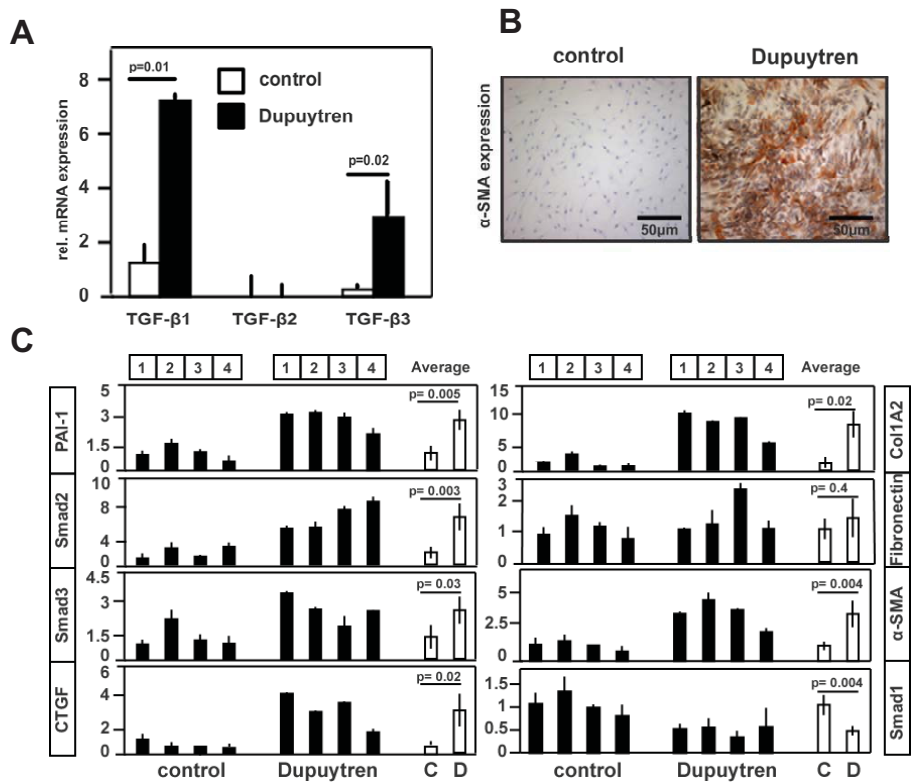


Figure 2: Characterization of primary Dupuytren's fibroblasts and control fibroblasts. (A) Expression of TGF-β1, TGF-β2, and TGF-β3 mRNA in Dupuytren's fibroblasts and control fibroblasts is shown relative to GAPDH mRNA expression. Analysis was done by quantitative PCR; RNA input for Dupuytren's fibroblasts and control cDNA synthesis consisted of an equal mixture of RNA derived from all four Dupuytren's fibroblasts (mixture 1-4) and all four control fibroblasts (mixture 1-4), respectively. Values are expressed relative to the average of control (mixture 1-4) TGF-β1 mRNA values. (B) Immunostaining of Dupuytren's and control fibroblasts for α-smooth muscle actin (α-SMA) expression. Representative staining is shown for fibroblasts derived from Dupuytren's patient 4 and control patient 1. (C) Expression of PAI-1, Smad-2, Smad-3, and CTGF mRNA in Dupuytren's and control fibroblasts derived from four individuals each, is shown relative to GAPDH mRNA expression. Analysis was done by quantitative PCR. The average of mRNA expression of the Dupuytren derived fibroblasts is stated relative to the average of the control values.

genes *PAI-1* and *CTGF*, were significantly upregulated in Dupuytren's fibroblasts compared to control fibroblasts. mRNA expression of the extracellular matrix component *COL1A2* and the cytoskeleton representative α -SMA were also significantly increased, whereas the expression of *fibronectin* mRNA did not differ from control cells. The BMP signaling component Smad-1 was present at lower levels in Dupuytren cells when compared to normal fascia-derived cells (Figure 2C). The fact that the null-hypothesis of the Mann-Whitney *U* test of equal distribution of control (1-4) and Dupuytren derived fibroblasts (1-4) was rejected in 87,5% tested samples, we concluded that both control and Dupuytren derived fibroblasts have an independent mRNA expression profile that also allows for statistical comparison which furthermore grants for statistical analysis of pooled cell samples. Taken together, these results suggest that TGF- β /Smad signaling is increased in this fibroproliferative disease.

8.4.2 SB-431542 inhibited fibrogenic properties of Dupuytren's fibroblasts

Because TGF- β signaling was proposed to play an important role in the etiopathogenesis of DD, we investigated the expression of TGF- β isoforms and the involvement of TGF- β -like signaling in the fibrogenic characteristics of the disease. We observed that TGF- β 1 and TGF- β 3 mRNA was expressed at much higher levels in Dupuytren's than in control fibroblasts (Figure 3A), and we noted a strong reduction in the elevated α -SMA expression in Dupuytren's fibroblasts upon treatment with SB-431542 (Figure 3B and Figure 3C). Importantly, SB-431542 had strong inhibitory effects in the collagen contraction assay on both control and Dupuytren's cells (Figure 3C and Supplementary Figure 2). Our data indicate that the self-induced basal contraction of Dupuytren's cells was caused by increased endogenous TGF- β -like Smad signaling, which enhanced α -SMA expression and promoted collagen contraction (Figure 3).

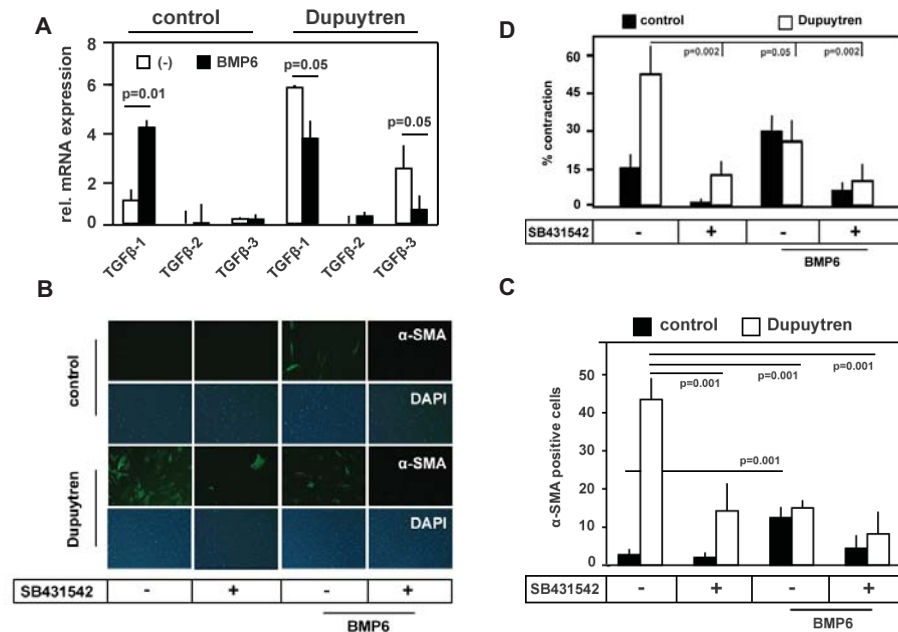


Figure 3: Effects of SB-431542 and BMP-6 on Dupuytren's and control fibroblasts. (A) Quantitative PCR was used to determine the average expression of TGF- β 1, TGF- β 2, and TGF- β 3 mRNA from control (mixture 1-4) and Dupuytren's (mixture 1-4) fibroblasts relative to *GAPDH* mRNA expression in the presence or absence of 100 ng/ml BMP-6 for 18 h. All values are expressed relative to the average of the control (1-4) TGF- β 1 mRNA values. (B) Immunofluorescence of α -smooth muscle actin (α -SMA) in control and Dupuytren's fibroblasts treated for 72 h with 20 μ M SB-431542 (+) in combination with or without 100 ng/ml rec. BMP-6. DMSO (-) treated cells were used as an internal control. Alexa 488 (green), α -SMA; DAPI (blue), DNA. Representative stainings are shown for Dupuytren's patient 4 and control patient 1. (C) Quantification of α -SMA expressing control (mixture 1-4) and Dupuytren (mixture 1-4) cells after treatment with SB-431542 (20 μ M) in the presence or absence of rec. BMP-6 (100 ng/ml) for 72 h as depicted in Figure 3B. (D) Fibroblast-populated collagen lattice (FPCL) of control (1-4) and Dupuytren's (1-4) fibroblasts treated with DMSO (-) or 20 μ M SB-431542 (+) in the presence or absence of 100 ng/ml rec. BMP-6. Quantifications of the average contractions of control (1-4) and Dupuytren's (1-4) fibroblasts were calculated after 72 h of treatment. Representative pictures are shown in the Supplementary Figure 2.

8.4.3 BMP-6 attenuated TGF- β signaling in Dupuytren's fibroblasts

Because it has been suggested that BMPs, particularly BMP7, can counteract TGF- β -induced fibrosis in the kidney, lung and liver, we investigated the effect of BMPs on Dupuytren's fibroblasts. BMP6, but not BMP7, attenuated endogenous TGF- β -like signaling. Quantitative PCR revealed that BMP6 strongly induced TGF- β 1 mRNA expression in control cells, while leaving the expression of TGF- β 2 and TGF- β 3 isoforms unaffected (Figure 3A). In contrast to the control cells, in Dupuytren's fibroblasts BMP6 counteracted TGF- β 1 and TGF- β 3 mRNA expression (Figure 3A) and reduced *SMAD2* and *SMAD3*, but not *SMAD1*, mRNA expression (data not shown). As predicted from its antagonistic effects on TGF- β -like signaling, BMP-6 (but not BMP-7) attenuated α -SMA expression and counteracted the spontaneous elevated contraction seen in Dupuytren's fibroblasts (Figure 3B–3D, Supplementary Figure 2 and data not shown). This inhibitory effect of BMP-6 was further potentiated by simultaneous treatment with SB-431542 (Figure 3C–3D, Supplementary Figure 2).

8.4.4 ERK1/2 MAP kinase signaling elevated in DD

It has been shown that TGF- β can activate non-Smad signaling pathways, such as MAP kinase signaling [17, 37]. In addition, MAP kinases are activated by growth factors such as PDGF that have been implicated in DD [2, 5].

We therefore investigated the phosphorylation of p38, JNK, and ERK in control and Dupuytren's tissue samples as well as in primary cells. While we did not detect phosphorylation of p38 and JNK (data not shown), phosphorylation of ERK1/2 was significantly increased in Dupuytren's tissue samples compared to control samples (Figure 4A and 4B). Similar results were obtained with fibroblasts isolated from Dupuytren's and control patients (Figure 4C and Figure 5A). We next determined the direct effects of TGF- β 3 on the phosphorylation of ERK1/2 in Dupuytren's fibroblasts. We found that 5 min of TGF- β 3 treatment induced a further increase in the phosphorylation of ERK1/2, which was inhibited by SB-431542 to a level lower than the basal level (Figure 4C). The presence of BMP-6, however, had only marginal effects on the direct TGF- β 3 induced phosphorylation of ERK1/2 (Figure 4C). In addition to its direct effect, TGF- β 3 also induced an increase in ERK1/2 phosphorylation after 18 h of stimulation. Interestingly, while SB-431542 showed only marginal effects on this sustained activation, BMP-6 strongly attenuated this effect after 18 h (Figure 4C). The sustained effect of TGF- β 3 on ERK1/2 was likely indirect and may have occurred via the TGF- β 3 mediated induction of growth factors. Indeed, in particular *PDGFB* (and *PDGFA*) mRNA expression was significantly upregulated in Dupuytren's fibroblasts and was strongly induced by TGF- β treatment (Figure 4D and data not shown). SB-431542 compound or BMP-6 counteracted the TGF- β induced increase in *PDGFB* mRNA expression (Figure 4D).

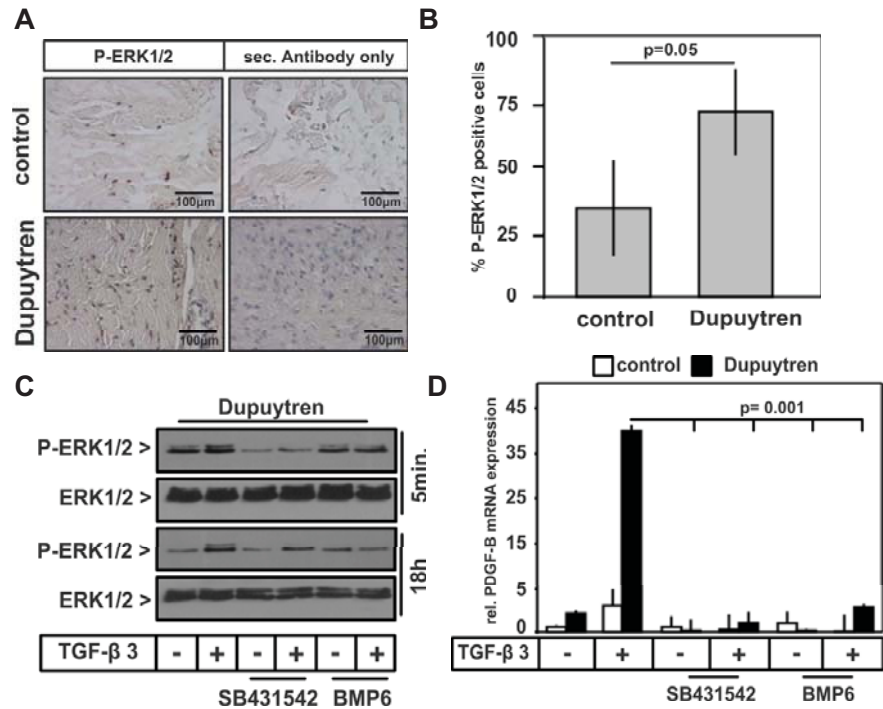


Figure 4: Elevated P-ERK1/2 expression in Dupuytren's tissue samples. (A) Left panel: immunostaining for phosphorylated ERK1/2 (P-ERK1/2) and secondary antibody control (P-ERK1/2 antibody omitted) of Dupuytren's and control tissue specimens. Representative stainings from Dupuytren patient 4 and control patient 1 are shown. (B) Quantitative analysis of P-ERK1/2 positive cells (as shown in Figure 4A) on three control and three Dupuytren's tissue samples expressed as percentages. (C) Western blot analysis of lysates from pooled Dupuytren's fibroblasts (mixture 1-4) to determine phosphorylation of ERK1/2 (P-ERK1/2) in untreated or rec. TGF- β 3 (0.1 ng/ml) treated cells for either 5 min or 18 h in the presence or absence of SB-431542 (20 μ M) or rec. BMP-6 (100 ng/ml). (D) Expression of *PDGFB* mRNA from four controls (mixture 1-4) and Dupuytren's (mixture 1-4) fibroblasts stimulated with 0.1 ng/ml rec. TGF- β 3 in the presence or absence of 20 μ M SB-431542 or 100 ng/ml rec. BMP-6 for 18 h. Analysis was performed by quantitative PCR and values are expressed relative to the average of the control (mixture 1-4) mRNA values using *GAPDH* as an internal reference.

8.4.5 Targeting of TGF- β type I receptor and ERK1/2 MAP kinase pathways in Dupuytren's fibroblasts

We next set out to determine whether the elevated TGF- β /Smad and MAP kinase signaling pathways were causally linked to an increase in the expression of key fibrotic and proliferation proteins by interfering with these pathways using the ALK-4, ALK-5, and ALK-7 inhibitor SB-431542 [27, 43] the MEK-1 inhibitor PD98059 [1] and BMP-6. Treatment of Dupuytren's fibroblasts with SB-431542 completely inhibited elevated basal P-Smad-2 levels and also attenuated P-ERK1/2 levels. This suggests that these increased basal activities are due to TGF- β or TGF- β -like ligands that are secreted by Dupuytren's fibroblasts themselves. PD98059 strongly inhibited elevated basal P-ERK1/2 levels, and had no significant effect on P-Smad-2 levels (Figure 5A). Both treatments were associated with decreased expression of fibrotic marker proteins such as type I collagen and α -SMA, and reduced expression of the proliferation marker c-myc proto-oncogene (Figure 5A). Both SB-431542 and PD98059 treatment also inhibited *COL1A2*, *CTGF*, and *PAI-1* gene expression (Supplementary Figure 5). The inhibitory effects of SB-431542 or PD98059 were potentiated by co-treatment with BMP-6 (Figure 5A). Co-treatment with SB-431542/BMP-6 and PD98059/BMP-6 combinations decreased the levels of P-ERK1/2, type I collagen, and α -SMA to non-detectable levels in Dupuytren's cells, the same as what was seen in non-treated control cells.

The c-myc level was significantly downregulated by PD98059/BMP-6, and reached the low levels observed in control cells (Figure 5A). We found that TGF- β 3 strongly induced *PDGF* (Figure 4D), which, via its receptor, can activate ERK1/2 MAP kinase signaling. To determine the role of PDGF signaling in the augmented ERK1/2 phosphorylation observed in DD, we treated Dupuytren's fibroblasts with a selective PDGF receptor tyrosine kinase inhibitor (STI571, also known as Glivec®) and compared its effect with the inhibitors SB-431542 and PD98059. EGF receptor and VEGF receptor tyrosine kinase inhibitors [48, 40] were used as specificity controls for the PDGF receptor kinase inhibitor. The PDGF receptor kinase inhibitor led to a strong but incomplete decrease in ERK1/2 phosphorylation and α -SMA and c-myc expression (Figure 5B). Its effect was weaker than co-treatment of Dupuytren's fibroblasts with SB-431542 and PD98059. The EGF- and VEGF-receptor kinase inhibitors showed only minor effects. We could however find no significant inhibition of the elevated α -SMA expression upon challenge of Dupuytren's fibroblasts with STI571 (Figure 5B), which is consistent with previous findings that link PDGF to proliferation and not to myofibroblast transdifferentiation response [42]. The inhibitory effects of PD98059 and STI571 suggest that PDGF and the ERK1/2 MAP kinase pathway play an important role in the increased fibrotic characteristics of Dupuytren's fibroblasts compared to control fibroblasts. When we stimulated Dupuytren's fibroblasts with TPA, which activates ERK1/2 MAP kinase pathways (as well as other pathways), we found elevated α -SMA expression and collagen contraction (Supplementary Figure 4A). Thus, ERK MAP kinase signaling may be sufficient to weakly mediate the fibroproliferative properties observed in Dupuytren's fibroblasts. Taken together, the results thus far indicated that

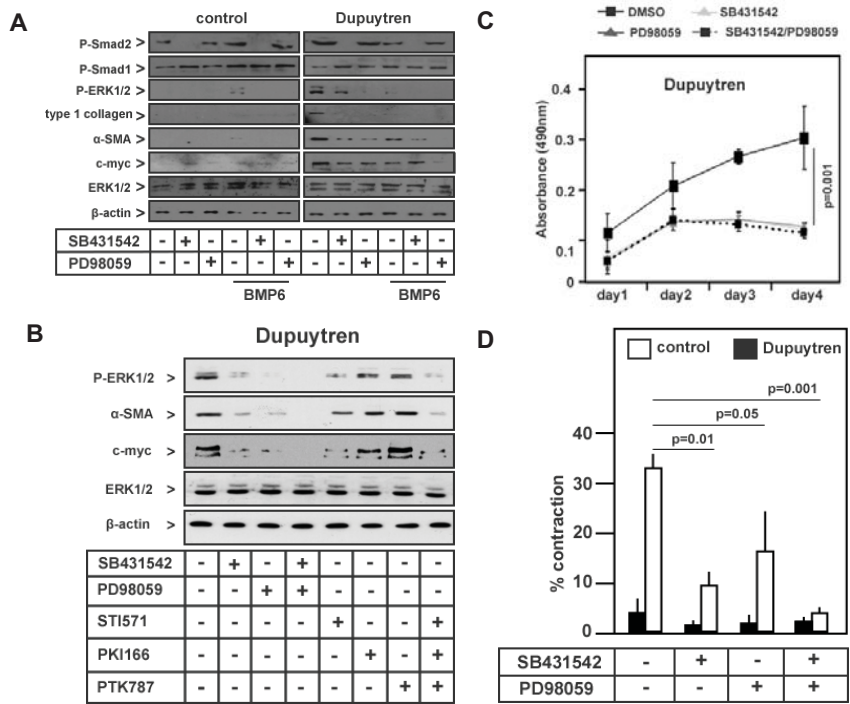


Figure 5: TGF- β induced ERK/MAPK signaling in DD is inhibited by selective small molecular weight kinase inhibitors and BMP-6. (A) Western blot analysis for phosphorylated Smad-2 (P-Smad-2), phosphorylated Smad-1 (P-Smad-1), phosphorylated ERK1/2 (P-ERK1/2), type I collagen, α -smooth muscle actin (α -SMA), c-myc, and total ERK1/2 using specific antibodies on pooled control (mixture 1-4) and Dupuytren's fibroblasts (mixture 1-4) treated with SB-431542 (20 μ M), MEK1 inhibitor PD98059 (10 μ M), and rec. BMP-6 (100 ng/ml) for 18 h. (B) Western blot analysis for phosphorylated ERK1/2 (P-ERK1/2), α -smooth muscle actin (α -SMA), c-myc, and total ERK1/2 using specific antibodies on pooled Dupuytren's fibroblasts (mixture 1-4) treated with SB-431542 (20 μ M), MEK1 inhibitor PD98059 (10 μ M), PDGF receptor (STI571, also called Glivec®), EGF receptor (PKI166), and VEGF receptor (PTK787/ZK 222584) kinase inhibitors (10 μ M) for 18 h. (C) MTS-based proliferation assay with pooled Dupuytren's fibroblasts (mixture 1-4) treated for 4 d with SB-431542 (20 μ M) and/or MEK1 inhibitor PD98059 (10 μ M). Absorbance at 490 nm was measured daily and the proliferation rate is stated relative to untreated cells at day zero. (D) FPCL on pooled control (mixture 1-4) and Dupuytren's fibroblasts (mixture 1-4) treated with SB-431542 (20 μ M) and/or PD98059 (10 μ M) for 72 h.

both TGF- β /Smad and ERK1/2 MAP kinase signaling pathways contribute to the fibrogenic responses of Dupuytren's fibroblasts. We therefore determined whether we could normalize the fibroproliferative characteristics of Dupuytren's fibroblasts by targeting TGF- β like signaling and ERK1/2 MAP kinase with SB-431542 and the MEK-1 inhibitor PD98059, respectively. Concurrent treatment of Dupuytren's fibroblasts with SB-431542 and PD98059 abrogated ERK1/2 phosphorylation and α -SMA and c-myc expression (Figure 5B). Consistent with this observation, we found that treatment with SB-431542 and/or PD98059 strongly inhibited the elevated basal proliferation of Dupuytren's fibroblasts, and had only minor effects on the proliferation rate of normal fibroblasts (Figure 5C and Supplementary Figure 4). The high spontaneous contraction rate in Dupuytren's fibroblasts was completely blocked by co-treatment with SB431542 and PD98059 (Figure 5D).

8.5 Discussion

DD is a chronic, fibroproliferative disorder that is most likely induced by overactive cytokines such as TGF- β , which is thought to play a prominent role by stimulating Dupuytren's fibroblasts to produce excessive levels of extracellular matrix proteins and by promoting their contractile phenotype [46]. In line with the results of previous studies, we found that biopsies and fibroblasts derived from primary cultures from affected areas in Dupuytren patients had elevated expression levels of TGF- β , and in particular TGF- β 1 and TGF- β 3 isoforms, and this correlated with increase in expression levels of smooth muscle actin, CTGF, fibronectin, and collagen in Dupuytren's fibroblasts compared to controls [7, 54].

TGF- β can signal via the Smad signaling pathways. We observed that DD patients showed elevated expression and activation of Smad-2 and Smad-3, but not Smad-1. Of note, whereas P-Smad-2 levels were found elevated, this was not clear for P-Smad-3 levels. Smad-2 and Smad-3 may have distinct roles; a recent paper has demonstrated that Smad-3 has a negative regulator of α -SMA expression and the activation of the myogenic program in the epithelium [36]. When we challenged Dupuytren's fibroblasts with SB-431542, which inhibits TGF- β -like signaling pathways, the expression of key fibrotic markers such as PAI-1, CTGF, α -SMA, and type 1 collagen, was decreased. Previous characterization of the promoters of these target genes showed that they are regulated in a Smad-dependent manner [56, 16]. Moreover, application of SB-431542 revealed that the high amount of spontaneous contraction of Dupuytren's fibroblasts when embedded in a collagen lattice was caused by overactive TGF- β -like signaling. TGF- β receptor kinase inhibitors have been shown to inhibit fibrotic responses in other cells *in vitro* [43, 1] and *in vivo* [43]. In recent years, a strong link has been established between TGF- β -induced fibrosis and BMP expression and signaling. Challenging the fibrogenic properties of Dupuytren's fibroblasts with BMP-6 inhibited the gene expression of TGF- β 1 and TGF- β 3 and their respective downstream Smad-2 and Smad-3 effectors. Whereas previous studies attributed antifibrotic effects to BMP-7, a close homolog of BMP-6 [57], we were unable to demonstrate this for Dupuytren's

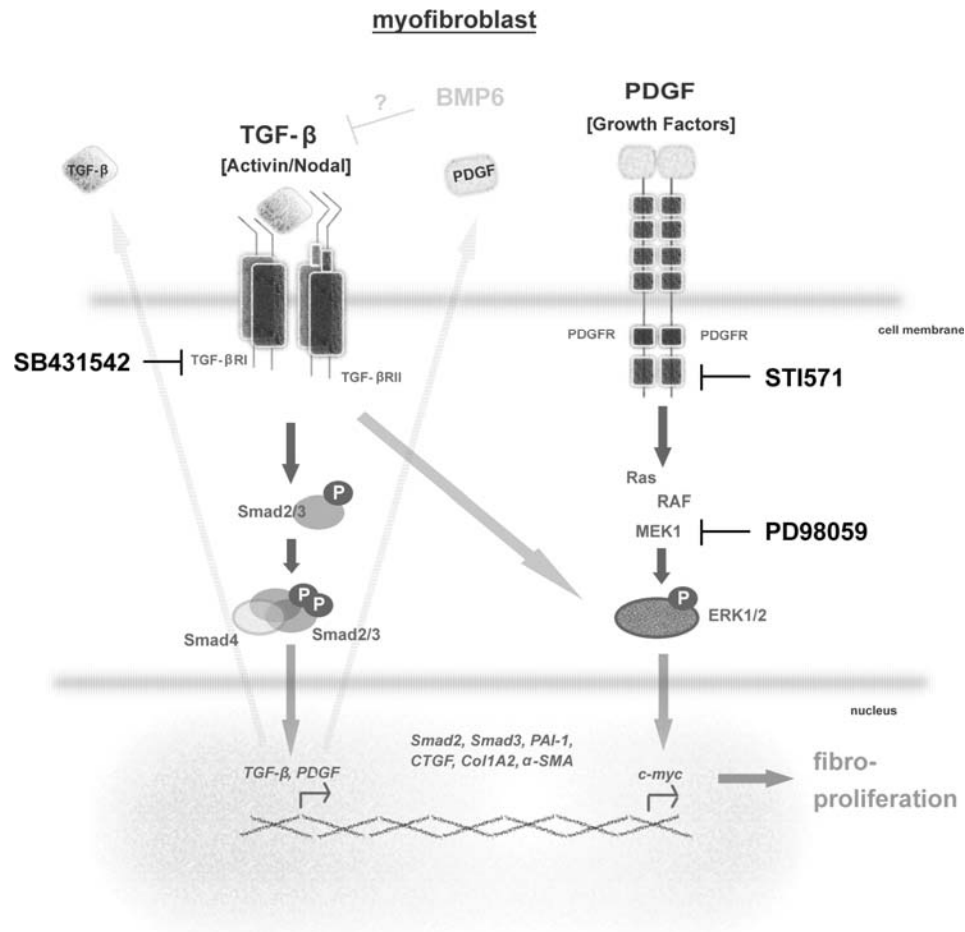


Figure 6: Schematic representation of perturbed TGF- β /Smad and MAP kinase signaling pathways in Dupuytren's fibroblasts. The TGF- β /Smad canonical Smad-2/-3 pathway and the non-canonical ERK1/2 MAP kinase signaling pathways are depicted, as well as the PDGF-induced Ras-RAF-MEK-ERK1/2 MAP kinase pathway. TGF- β induces the production of PDGF and other growth factors. The symbols of corresponding downstream regulated genes are in *italics*. Inhibition by STI571, SB-431542, and PD98059 of the aberrantly overactivated signaling pathways in Dupuytren's fibroblasts are indicated. The mechanism by which BMP-6 inhibits TGF- β expression and downstream responses is unclear. SB-431542 not only inhibits TGF- β /ALK-5 signaling, but also activin/ALK-4 and nodal/ALK-7 signaling; our experiments do not rule out a role for these signaling pathways in Dupuytren's fibroblasts. Our data suggests that the TGF- β /Smad and PDGF/ERK MAP kinase pathways are targets for the therapeutic intervention strategies to treat Dupuytren's disease.

fibroblasts. One could speculate whether BMP6 could compete with TGF- β for recruitment of distinct receptors, thereby limiting TGF- β activity. Our data suggests a novel level of crosstalk, as previous studies suggested that BMPs had an inhibitory effect on the TGF- β /Smad pathway through the formation of mixed Smad-1/5-Smad-2/-3 complexes [19, 14]. It is interesting that BMP-6 in particular had an antagonizing effect on TGF- β -driven DD, because it has been shown that myofibroblast progenitor cells derived from patients with diabetes are deficient in BMP-6 expression [38]; there is some evidence of a relationship between diabetes and DD [39]. In another study, BMP-6 and BMP-7 were found to have differential effects on chemotaxis via a Smad-4-independent, but PI3 kinase-dependent, pathway [41]. It would be worthwhile to explore whether similar mechanisms are of relevance in Dupuytren's fibroblasts. Although BMP-6 may inhibit fibrotic responses, when discussing it as a potential therapeutic agent, one needs to take into account BMP-6's action on normal fibroblasts and its strong osteoinductive properties [49]. We found that Dupuytren's fibroblasts displayed overactive ERK1/2 signaling, but neither the JNK nor the p38 MAP kinase signaling pathways showed increased activity. This could be due to both direct TGF- β -induced ERK1/2 phosphorylation, because it was observed within 5 min and inhibited by SB431542, and indirectly through the induction of PDGF expression, which can stimulate ERK1/2 phosphorylation (schematically represented in Figure 6). Consistent with the latter idea, we found that treatment with the PDGF receptor inhibitor STI571 strongly mitigated the expression of phosphorylated ERK1/2. The elevated ERK MAP kinase pathway could be linked to the elevated fibroproliferative characteristics of Dupuytren's fibroblasts. Treatment of cells with PD98059 inhibited the expression of fibrotic and proliferation markers. A role for MAP kinase signaling, also in cooperation with the Smad pathway, has been described for many TGF- β target genes [29, 13, 33, 22]. In line with its potent inhibitory effects on fibro-proliferative markers, spontaneous collagen contraction and elevated proliferation were inhibited by PD98059. Moreover, the finding that TPA induced ERK1/2 phosphorylation and collagen contraction suggests that activation of this pathway may be sufficient to induce contraction. BMP-6 was not able to counteract this TPA-induced ERK response, which is in line with its proposed inhibitory actions further upstream at the level of TGF- β and Smad expression. Consistent with our results, inhibition of ERK MAP kinase signaling has been shown to mitigate fibrotic responses in scleroderma [50, 8]. Our observations suggest a role for elevated PDGF signaling promoting the proliferation of Dupuytren's fibroblasts. Of note, overactive PDGF signaling has been implicated in fibrosis in multiple tissues [32, 53, 12]. and treatment with PDGF receptor kinase inhibitors has been shown to inhibit fibrosis [35, 28]. Importantly, when both TGF- β receptors and the ERK1/2 pathways were inhibited in Dupuytren's fibroblasts through simultaneous application of SB-431542 and PD98059, a complete block of the elevated basal proliferation and contraction was observed, which in turn commuted the Dupuytren's fibroblasts phenotype into 'normal' fibroblasts.

8.6 Conclusions

Both TGF- β and ERK/MAP kinase pathways cooperated in mediating the enhanced proliferation and high spontaneous contraction of Dupuytren's fibroblasts. Taken together, our data indicate that the TGF- β /Smad and ERK MAP kinase pathways are prime targets for the development of non-surgical intervention strategies to treat Dupuytren's disease. For example, concurrent topical application of inhibitors such as SB-431542, and PD98059 into the DD area could block fibro-proliferative responses and recurrence in DD while preventing the potential problems associated with systemic administration of such compounds (schematically represented in Figure 6).

8.7 Acknowledgments

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8.8 Supplementary Data

8.8.1 Supplementary Figures

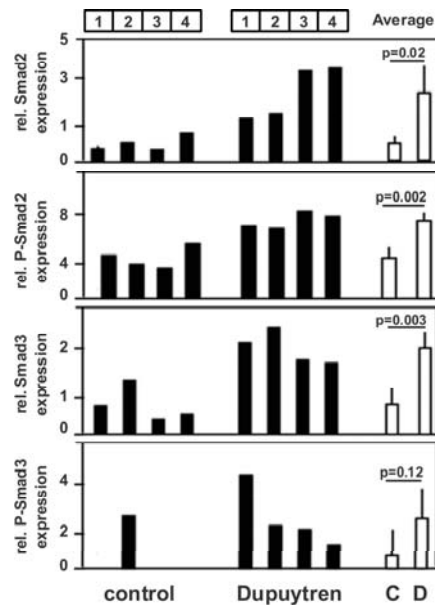


Figure S1: Quantification of total Smad and phosphorylated Smad (P-Smad) protein expression levels as depicted in Figure 1C. Quantification was performed by densitometric analysis using the Odyssey system. All values are expressed relative to β -actin protein expression levels (C = Control; D = Dupuytren).

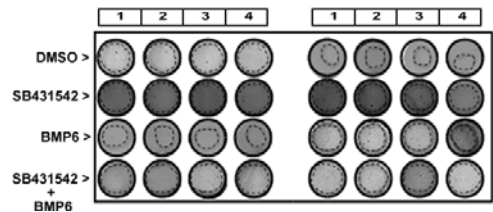


Figure S2: Fibroblast-populated collagen lattice (FPCL) of control (1–4) and Dupuytren's (1–4) fibroblasts treated with DMSO – or 20 μ M SB-431542 + in the presence or absence of 100 ng/ml rec. BMP6. Corresponding pictures are shown for the quantification of contraction in Figure 3D.

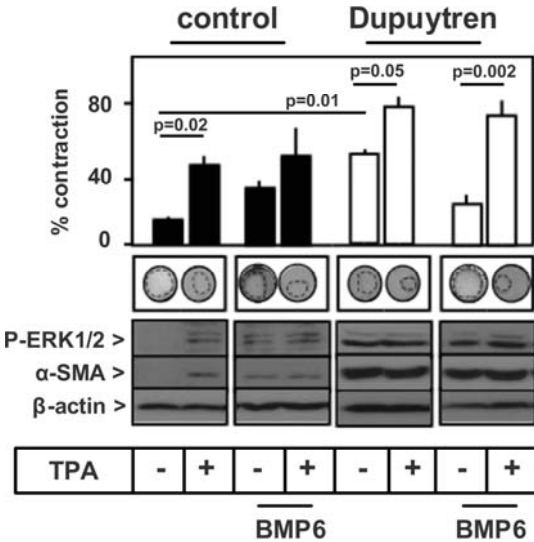


Figure S3: Top panel: FPLC on control (mixture 1-4) and Dupuytren's fibroblasts (mixture 1-4) treated with TPA (100nM) in the presence or absence of rec. BMP-6 (100ng/ml) for 72h. Middle panel: representative pictures are shown for each condition. Lower panel: western blot analysis for phosphorylated ERK1/2 (P-ERK1/2) and α -smooth muscle actin (α -SMA) on primary control (mixture 1-4) and Dupuytren's fibroblasts (mixture 1-4) treated with TPA (100nM) in the absence or presence of rec. BMP-6 (100ng/ml) for 18 h are depicted in the lower panel. β -actin was included as loading control.

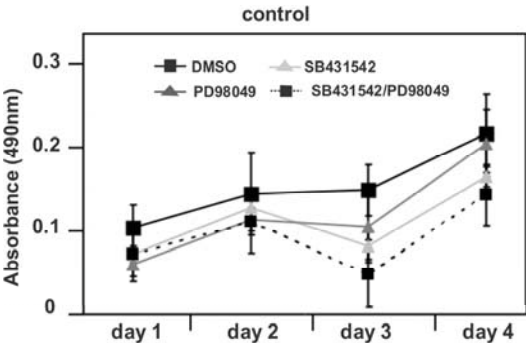


Figure S4: MTS based proliferation assay on pooled Control fibroblasts treated for 4 days with SB-431542 (20 μ M) and/or the MEK1 inhibitor PD98059 (10 μ M) where indicated. Absorbance at 490nm was measured daily and the proliferation rate is stated relative to untreated cells at day zero.

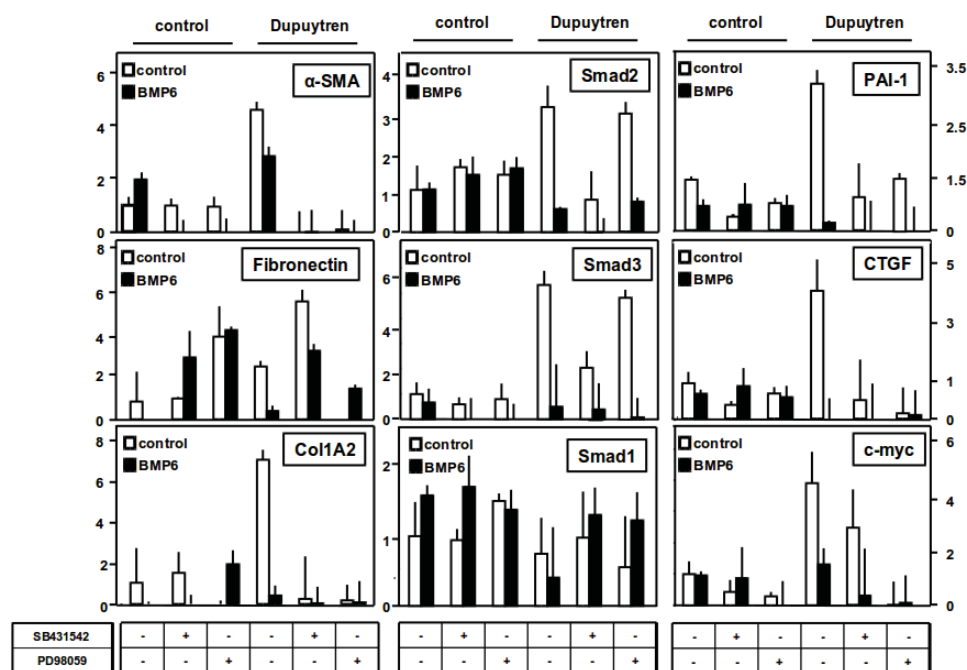


Figure S5: Quantitative PCR was used to determine the average expression of α -SMA, *Smad1*, *Smad2*, *Smad3*, *PAI-1*, *Fibronectin*, *CTGF*, *c-myc* and *COL1A2* mRNA from control (mixture 1–4) and Dupuytren's (mixture 1–4) fibroblasts relative to *GAPDH* mRNA expression in the presence or absence of SB-431542 (20 μ M) and/or the MEK1 inhibitor PD98059 (10 μ M) and/or 100 ng/ml BMP6 for 18 h. All values are expressed relative to the average of the untreated control (1–4) mRNA values.

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