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Targeting TGFβ signaling pathway in fibrosis and cancer

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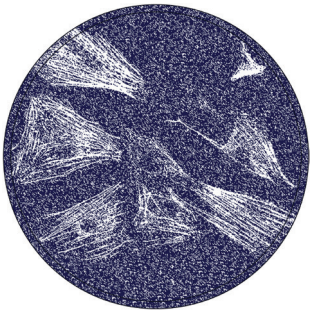

Chapter 5

Novel *ex vivo* culture method for the study of Dupuytren's disease: effects of TGFβ type I receptor modulation by antisense oligonucleotides

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Abstract

Dupuytren's disease is a benign fibro-proliferative disease of the hand. It is characterized by the excessive production of extracellular matrix proteins, which form a strong fibrous tissue between the handpalm and fingers, permanently disrupting the fine movement ability. The major contractile element in Dupuytren's disease is the myofibroblast. This cell has both fibroblast and smooth muscle cell-type characteristics and causes pathological collagen deposition. Myofibroblasts generate contractile forces that are transmitted to the surrounding collagen matrix. Major pro-fibrotic factors are members of the Transforming growth factor-β pathway which directly regulate the expression levels of several fibrous proteins such as collagen type I, collagen type III and alpha-smooth muscle actin. Molecular modulation of this signaling pathway could serve as a therapeutic approach. We, therefore, have developed an *ex vivo* "clinical trial" system to study the properties of intact, patient-derived resection specimens. In these culture conditions, Dupuytren's tissue retains its three-dimensional structure and viability. As a novel antifibrotic therapeutic approach, we targeted Transforming growth factor-β type I receptor (also termed Activin receptor-like kinase 5) expression in cultured Dupuytren's specimens by antisense oligonucleotide-mediated exon skipping. Antisense oligonucleotides targeting Activin receptor-like kinase 5 showed specific reduction of extracellular matrix and potential for clinical application.

Introduction

Dupuytren's disease (DD) is a common fibrotic disorder of the hand, found with high prevalence among Caucasians of Northern European descent[1]. This enigmatic benign fibro-proliferative disease affecting the connective tissue (**Fig.1A**) results from a complex interplay of genetic, anatomic and environmental factors[2] with main clinical manifestation being the excessive collagen deposition. A disturbance of the heterogeneous mix of static and dynamic contractile elements located throughout the fascia of the palm and digits can lead to the development of flexion deformities (contracture). Although not associated with high morbidity, the impact on movement ability and quality of life of the patients affected by DD is major. Currently, the common therapy for DD is palmar fasciectomy, which consists of surgical removal of fibrotic tissue and results in immediate improvement of disease. However, due to the high recurrence rate and re-manifestation of fibrotic bands, surgery is not a permanent solution.

Several studies have elucidated the aetiopathology of DD which is crucial for the design of novel therapies. Uncontrolled wound healing response leads to permanent extracellular matrix (ECM) deposition e.g. collagen. The cell responsible for ECM production in normal as well as in pathologic conditions is the myofibroblast (MFB), containing both fibroblast and smooth muscle cell-type characteristics[3]. MFBs generate contractile forces that are transmitted to the surrounding collagen matrix[4] and are distinguished by alpha-smooth muscle actin (ACTA2) expression. In pathological conditions ACTA2 expression is persistent. Major pro-fibrotic factors are members of the transforming growth factor-β (TGFβ) pathway which directly regulate the expression levels of several intracellular and extracellular fibrous proteins such as COL1A1, COL3A1 and ACTA2[3,5-7], fibronectin, matrix metalloproteases, integrins, all of which are aberrantly deregulated in DD[8,9]. TGFβ ligands interact with TGFβ type I receptor (also termed Activin receptor-like kinase 5 (ALK5)/ TGFβRI and type II (TGFβRII) receptor complexes which subsequently activate by phosphorylation the SMAD2/3 effectors,

which form heteromeric complexes with SMAD4 and act as downstream transcriptional effectors of the pathway. Activation and transdifferentiation of DD fibroblasts towards MFBs is mainly controlled by TGFβ signaling[10-13]. Other cytokines, such as platelet-derived growth factor (PDGF), are induced by TGFβ[14] and also enhance MFB differentiation. TGFβ and PDGF factors are aberrantly activated in DD[10,15,16]. In particular, in DD patient-derived MFB cultures, overactive TGFβ signaling causes spontaneous contraction and proliferation[13,15]. Contractility is attenuated by inhibiting TGFβ and TGFβ receptor (ALK5) function[15,17,18]. We have recently shown that the TGFβ/SMAD and PDGF/ERK1/2 MAP kinase pathways cooperate in mediating the enhanced proliferation and spontaneous contraction of DD fibroblasts[15]. Inhibiting the uncontrolled fibrotic mechanisms by directly targeting the overactivation of the TGFβ signaling, mediated via its ALK5 receptor, at the molecular level, could be an effective treatment.

A promising approach to deplete the cells from the function of key receptor of TGFβ signaling (ALK5) is by alternative splicing methodology. Particular exon(s) encoding protein domains crucial for protein function can become excluded from the mature messenger RNA (mRNA). Specific antisense oligonucleotides (AON) bind to sites involved in exon splicing to the splice sites of a targeted exon and interfere with the splice machinery; therefore the particular exon is not integrated as part of the mRNA[19]. The resulting mRNA has an intact open reading frame and is translated into a protein which lacks only the particular peptide sequence encoded by the skipped exon. The advantage of this system is that no genetic alterations are introduced, since interference is exclusive during pre-mRNA splicing process. AON methodology has broad therapeutic applicability in certain human diseases [20], particularly in the field of muscular dystrophies [20] with very promising results reported for clinical trials [21,22]. Based on this principle, we employed the AON-mediated exon skipping technology for disrupting the protein function of the ALK5, targeting in particular the extracellular ligand binding domain. AONs targeting splice sites of exon encoding extracellular ligand binding domain (exon 2) of the ALK5[23] have been developed and tested *in vivo* (D.U. Kemaladewi et al., manuscript submitted). This strategy ensures no loss of other important domains of ALK5, such as the transmembrane domain (encoded by exon 3) or serine-threonine kinase activity domain (exon 4- 9). ALK5 AON was administered directly to the DD patient-derived specimens by microinjecting it in the center of the tissue, and the effects on fibrosis and ECM deposition were assessed with various imaging and biochemical methods. In this study we show that DD resected specimens, which are discarded as waste material after surgery, can be maintained viable in defined culture conditions in our novel *ex vivo* model. Their study can provide us with useful information about the underlying patient-specific pathology and drug response.

Materials and Methods

Generation of 3D culture system

Specimens from DD surgeries are equally sliced and placed in transwell plates onto 0.4 um nitrocellulose membranes (Greiner Bio One) in defined culture conditions (Dulbecco's Modified Eagle's Medium (DMEM, with 1% fetal calf serum (FCS), 1% penicillin-streptomycin) and allowed to grow (seven days). Nutrient exchange occurs by diffusion from the medium through the membrane while DD tissue remains continuously in contact with the liquid but is not immerged. Tissue resection specimens (N=9 DD and N=4 normal fascia palmaris)

were treated with a combination of activators and inhibitors of the TGFβ signaling pathway (e.g. TGFβ, 5ng/ml; SB-431542, 10ng/ml, Tocris). After culture, tissues were processed for RNA isolation or were fixed in 4% paraformaldehyde, incubated in 30% sucrose buffer, embedded in Tissue Tek- O.C.T. compound and stored at -80°C.

Human tissue specimens

DD tissue was collected during a standard partial fasciectomy procedure. Indications for surgery were contracture(s) of the digit(s) with an inability to put the hand flat on the table (table top test). Only patients with first time occurrence of DD were included in this study. The range of age of patients was between 63- 88 years old, with 91% being males. Macroscopic identification (with surgical loupe magnification) of a nodule (representing most active disease) was done by the operating surgeon. Only nodules were used in our study. The nodules were defined as being the hard thick parts of the cord, mostly situated in the palm of the hand. This part was taken out of the cord after resection of the entire DD cord. The normal fascia palmaris tissue of the control group was collected during carpal tunnel release procedures. The included control patients did not suffer from DD. The operations were performed under local anaesthesia and under tourniquet control. After opening the skin through a longitudinal incision the fascia was identified and a small piece of fascia was harvested before incising the transverse carpal ligament. The tissue specimen was divided in two equal pieces, one of each was immediately processed for 3D culture and the other one was snap-frozen in liquid nitrogen and stored at -80°C. Oral consent for removal of the tissue for research purposes was obtained from the patients. Confirmation that the Medical Research Involving Human Subjects Act (WMO) does not apply to the present study was obtained by the local ethics committee (reference number W12_245 #12.17.0279) since the research was performed on "waste" material.

Antisense Oligonucleotides

The antisense oligonucleotides (AONs) used to target ALK5 were developed and recently described in another study, in which *in vitro* and *in vivo* efficiency of the different AONs was extensively tested in the context of muscular dystrophies (D.U. Kemaladewi et al., manuscript submitted). In short, the AONs targeting ALK5 specifically bind to and induce exon skipping of exon 2 of the ALK5 precursor mRNA transcript. Exon 2 encodes for the ligand binding domain, which is, together with the type II receptor, essential in capturing the ligand to initiate signaling. Exclusion of exon 2 generate a transcript with intact open reading frame, while the resulting protein will lack the ligand binding domain and is therefore functionally impaired. Vivo-morpholino AONs with a morpholino backbone and an octaguanidine moiety to enhance cellular uptake were used in this study since they have been shown to increase exon skipping efficiency in animal models[24]. ViM AONs (0.5 nmol and 1 nmol, Genetools) were diluted in 1% FBS-DMEM or PBS and were microinjected in the tissue. The sequences (5'-3') of the ViM AONs are the following: ALK5ViM: GCAGTGGTCCTGATTGCAGCAATAT, ScrViM: CCTCTTACCTCAGTTACAATTTATA. The 2'-O-methyl ribose AONs with phosphorothioate modifications (2'-O-Me) were obtained by Eurogentec.
ALK5 2'-O-Me: UGUACAGAGGUGGCAGAAACA, Scr 2'-O-Me: GCAAGAUGCCAGCAGA

RNA isolation, RT-PCR and Quantitative PCR

See Supplementary Materials and Methods for details.

Microscopy and Image analysis

See Supplementary Materials and Methods for details.

Immunofluorescence

See Supplementary Materials and Methods for details.

Results

Human derived DD tissue can be maintained under *ex vivo* culture conditions

Fibroblast derivation from DD specimens requires a long culture period during which cells adapt to culture conditions (plastic surface, high oxygen, removal of ECM). Such changes of the native microenvironment may result in partial recapitulation of the disease state or fibro-proliferative characteristics of the tissue in fibroblast two- dimensional cultures [25,26]. We have developed a three- dimensional (3D) culture system (**Fig.1A-B**), which allows human resection specimens to be grown *ex vivo* (up to seven days) in defined conditions. Longer culture periods (up to 12 days, data not shown) lead to increased cell death (cleaved caspase 3 positive cells) and absence of proliferation, suggesting non viability of tissue after a certain time point (day 7). We show that DD resection specimens in the *ex vivo* "clinical trial" system maintain viability, proliferation (phosphohistone-3, pH3) and apoptosis levels (TUNEL) (**Fig.1G**). As control tissue we have used normal fascia palmaris from carpal tunnel surgeries, which is not affected by DD. Control tissue was successfully maintained in culture for up to seven days and is characterized by low levels of proliferation (pH3) and apoptosis (TUNEL) (**Fig.S1**, upper panel). Histological characterization of the cultured DD biopsies showed that the high expression of fibrotic proteins: ACTA2, COL1A1 and COL3A1 is preserved (**Fig.1G**, representative images), therefore they recapitulate the *in vivo* properties. Similar data were obtained from a number of biopsies (normal fascia palmaris, N=7, DD non cultured tissue, N=4, and DD tissue after seven days 3D culture, N=9) indicating the reproducibility of the method. Quantification of immunofluorescence signal for COL1A1, COL3A1 and ACTA2 (**Fig.1D-F**), in multiple patient-derived specimens showed that biopsies cultured *ex vivo* retain the expression characteristics with regards to fibrosis.
Basal expression of ACTA2, COL1A1 and COL3A1 (**Fig.1C-G**), as well as *TGFβ1* and *PAI-1* mRNA levels (TGFβ target genes) (**Fig.1C**), are elevated in both cultured and non-cultured DD resection specimens compared to fascia palmaris (control, non- affected tissue). Moreover, the snap-frozen and the 3D cultured DD (matching) resection specimens similarly show areas of proliferating MFBs and low apoptosis (**Fig.1G**). All together, the above data indicate that DD tissue under culture conditions remains representative of the disease.

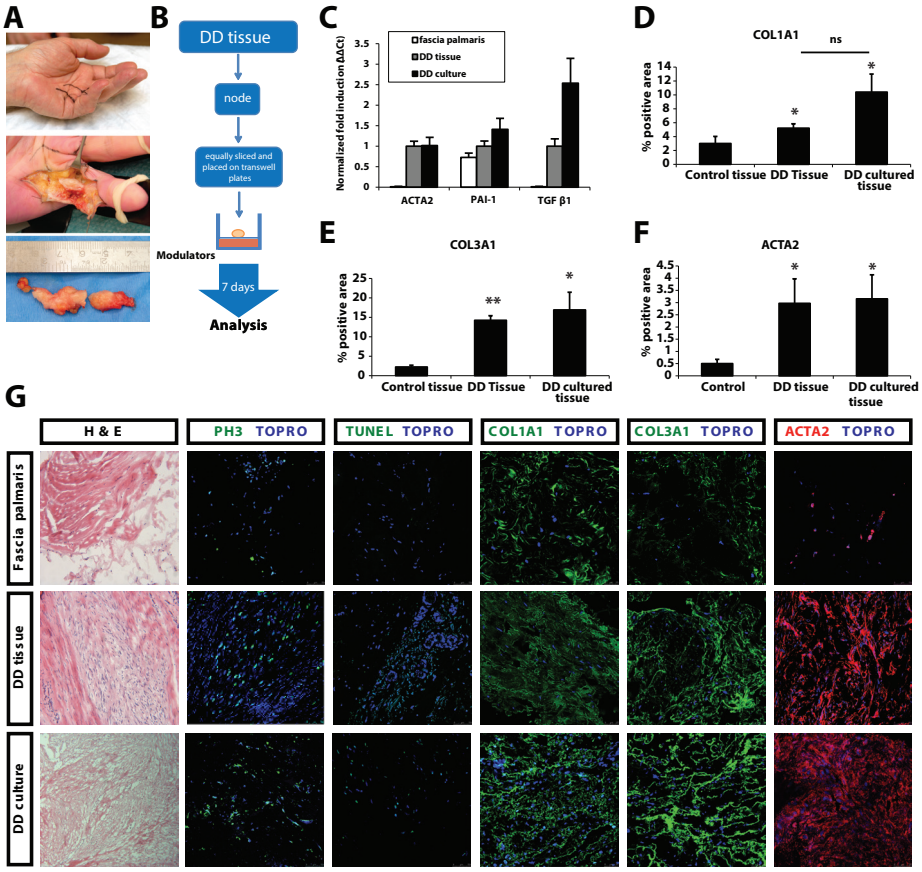

Fig.1. Characterization of DD contracture tissue in the 3D *ex vivo* "clinical trial" system
(A). DD contracture in the hand of a patient; example of DD tissue prior and after resection. (B). Cartoon describing 3D culture method: DD tissues are equally sliced (about 1mm), placed on a nitrocellulose membrane and cultured up to seven days. (C). Q-PCR on control normal fascia palmaris (N=7), DD non-cultured tissue (see DD tissue, N=4) and DD cultured tissue (see DD culture, N=9). Error bars represent ±S.E.M. *ACTA2*, *TGFβ1* and *PAI-1* mRNA levels have been quantified and normalized to *ACTRT1*. Fold induction values compared to DD non-cultured tissue are shown. (D-F). Quantification (described in methods) of immunofluorescence signal of COL1A1, COL3A1 and ACTA2 in normal fascia palmaris (N=7), DD non cultured tissue (see DD tissue, N=4) and DD tissue after seven days 3D culture (see DD culture, N=9). Multiple focal planes were quantified per sample, error bars represent ±S.E.M. Statistical significance was calculated by one-tailed paired t-test. *p<0.05, **p<0.01 (G). Immunohistochemical and immunofluorescent analysis of normal fascia palmaris, DD non-cultured tissue and DD cultured tissue. Hematoxylin and Eosin (H&E), proliferation marker phosphohistone 3 (PH3, green), apoptosis terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (green), Collagen type I (COL1A1, green), Collagen type III (COL3A1, green) and smooth muscle actin, alpha 2 (ACTA2, red). Nuclei were visualized with TO-PRO3 (TOPRO, blue). Scale bars, 25 um.

Small molecule inhibitor of TGFβ type I receptor kinase (SB-431542) decreases expression of fibrotic proteins in DD specimens

Our novel *ex vivo* culture method was further used to test the response of the DD tissue to stimulation with different factors directly after fasciectomy procedure. Main profibrogenic stimulus in DD is the TGFβ signaling; consequently we decided to interfere with the activation status of this particular pathway. Resection specimens (both control (**Fig.S1**) and DD (**Fig.2A**)) were treated with TGFβ ligand as well as a pharmacological TGFβ type I receptor (ALK4, ALK5, ALK7) kinase activity inhibitor (SB-431542). Addition of TGFβ to cultured DD specimens resulted in increased expression of target genes ACTA2, COL1A1 and COL3A1 in the majority of individual human samples tested or sustained the high levels (**Fig.2B-D**). This observation suggests high sensitivity of DD MFB cells to TGFβ, also confirmed by high expression of phosphorylated SMAD2 protein (pSMAD2) (**Fig.2A**). As expected, treatment with the SB-431542 inhibitor compound in our model suppressed the profibrogenic action of TGFβ and resulted in a trend reduction of the expression of fibrous proteins ACTA2, COL1A1 and COL3A1 (**Fig.2**). Differential expression levels among individual samples after TGFβ and/ or SB-431542 treatments were observed. Proliferation (pH3) and apoptosis (Caspase 3) were not significantly affected by the addition of either TGFβ or SB-431542 (**Fig.2A**). Treatment of control tissue with TGFβ cytokine caused an upregulation of ACTA2, COL1A1, COL3A1 expression (**Fig.S1**, middle panel), suggesting a responsiveness of the tissue to the treatment and underlining the profibrotic effect of TGFβ. The above observations may suggest that the 3D *ex vivo* culture system is suitable for chemical compound screening. Differences in the response of human specimens to growth factor or inhibitor SB-431542 most probably derives from variation among different individuals which can be effectively observed and represented using our *ex vivo* culture system.

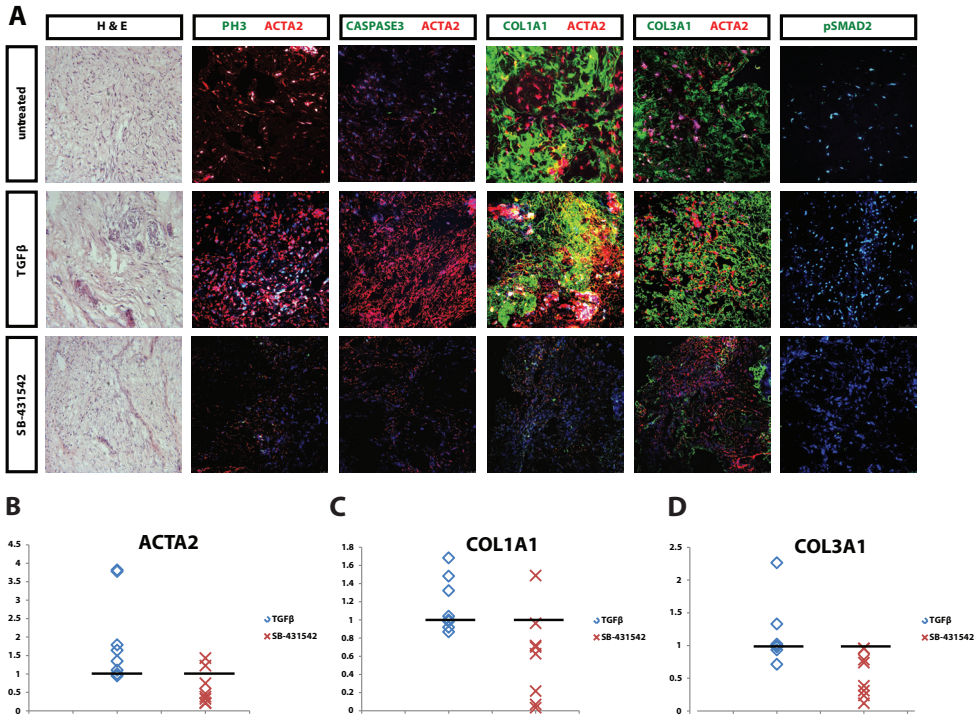

Fig.2. Inhibition and stimulation of TGFβ pathway in DD resection specimens cultured in 3D *ex vivo* "clinical trial" system

(A). Immunohistochemical and immunofluorescent analysis of 3D cultured DD resection specimens before treatment (untreated) and after seven-day treatment with TGFβ or ALK4/5/7 kinase inhibitor SB-431542. Hematoxylin and Eosin (H&E), proliferation marker phosphohistone 3 (PH3, green), apoptosis marker cleaved caspase 3 (CASPASE3, green), Collagen type I (COL1A1, green), Collagen type III (COL3A1, green), smooth muscle actin, alpha 2 (ACTA2, red) and phosphorylated SMAD2 (pSMAD2) (green). Nuclei were visualized with TO-PRO-3 (TOPRO, blue). Representative data from eight patient derived specimens (N=8). Scale bars, 25 um. (B-D) Distribution of fold quantitative values of ACTA2 (B), COL1A1 (C), and COL3A1 (D) among 8 patient-derived specimens after *ex vivo* culture. Quantification of fluorescent signal within certain area fraction was calculated in Image J software for every specimen in three different conditions; untreated (control, no exogenous factors), TGFβ cytokine and ALK4/5/7 kinase inhibitor SB-431542 compounds. Graph represents the values of positive signal of ACTA2, COL1A1, COL3A1, after treatment with TGFβ or SB-431542 compounds as a fold induction over the value of the control "untreated" sample (indicated by black line).

AON- mediated exon skipping of ALK5

While treatment with SB-431542 resulted in a promising downregulation of fibrotic pathways, this chemical inhibitor blocks the kinase activity of ALK4, ALK5 and ALK7 in a dose dependent way [27]. Thus, in order to ensure higher specificity and less interference with other signaling pathways we have tested a novel strategy to selectively inhibit the function of the ALK5. We used vivo-morpholinos (ViM) based on previously developed AON sequence (D.U. Kemaladewi et al., manuscript submitted) that selectively target and disrupt the ligand binding domain of ALK5 by inducing exon skipping of mRNA transcripts. Microinjection (**Fig.3A**) of resection specimens with fluorescently labelled AON demonstrated efficient uptake (>90%) and transport to the nucleus throughout the tissue (**Fig.3B-C**). Similarly, the AON targeting ALK5 (ALK5ViM) was microinjected in the centre of the tissues after placing them on the nitrocellular membrane of the transwell culture plates (**Fig.3A**). At day 3 we validated the skipping of exon 2 by PCR (**Fig.3D-E**) and verified reduction of full length ALK5 mRNA expression (**Fig.4B**) compared to tissues injected with control scrambled ViM (ScrViM). No effect on the proliferation rate and apoptosis was observed by the use of ALK5ViM (**Fig.4A**). We performed a time course experiment to monitor the levels of full length ALK5 mRNA expression versus the exon skipped mRNA. Full length ALK5 mRNA is decreased by 70-75% during the first 48 hours after AON administration (**Fig.S2A**). These data indicate that high rate of exon skipping is achieved at early time points and maintained in the tissue explant cultures. We also determined the collagen expression in different time points and observed a gradual decrease of COL1A1 expression (**Fig.S2B**).

ALK5 AON causes a reversal of fibrotic phenotype *ex vivo*

Constant collagen deposition is the main feature of DD, thus, clinical attempts have been focused on direct induction of collagen degradation *in vivo*, such as by injectable collagenase treatment [28]. Although very promising, this therapeutic approach is associated with several limitations (high morbidity), and cannot completely replace the surgical treatment[29].
Our objective was to interfere with the fibrogenic role of TGFβ in a clinically relevant manner. However, TGFβ is a regulator of many crucial processes such as inflammation and would healing in many organs and is secreted by many cell types including macrophages, endothelial cells, lymphocytes, epithelial cells. Thus, TGFβ should be tightly regulated and complete abolishment may lead to adverse effects. The process of exon skipping by AONs is advantageous because it results in partial and not complete blockage of the ALK5 receptor activity. Administration of the ALK5ViM directly to DD tissues (N=3) remarkably reduced the overall protein expression of ACTA2, COL1A1 and COL3A1 (**Fig.4A**) and activation of downstream pSMAD2 (**Fig.4A, S3**), rendering the tissue more similar to the control, normal fascia palmaris (**Fig.S1**). Quantification of the expression patterns among different specimens (N=3) confirmed a reproducible decrease observed after ALK5ViM treatment (**Fig.4C-E**). The spatiotemporal imaging of the endogenous extracellular distribution of collagen structure was determined by Second Harmonic Generation (SHG) on DD specimens during 3D culture, prior and after ALK5ViM application (**Fig.4F**). Reorganization/ degradation of collagen fibres, specifically at the site of injection with the ALK5ViM was observed with SHG (**Fig.4F**, white arrow) similarly to immunofluorescence signal (**Fig.4A**). In contrast, tissue injected with ScrViM retained the highly anisotropic collagen structures and did not exhibit signs of reorganization/degradation (**Fig.4F**). A partial reduction of available ALK5 molecules

appears already sufficient to reduce the fibro-proliferative effect mediated by TGFβ in fibroblasts/ MFBs. Importantly; mRNA molecules that escape exon skipping do produce functional ALK5 protein able for ligand binding (TGFβ) and protein complex formation with type II receptors. Thus, application of AONs does not cause complete abolishment of the TGFβ signaling, which is required at a basal level for tissue/ ECM maintenance.

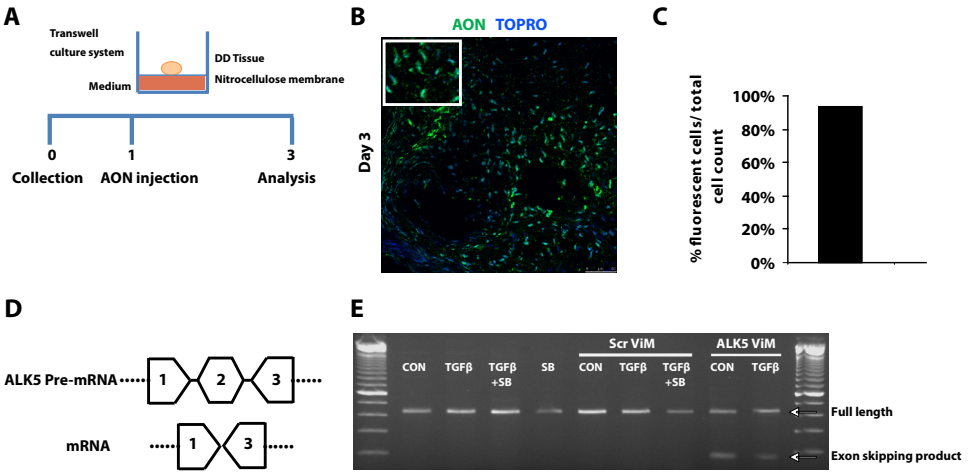

Fig.3. Microinjection of AONs in DD resection specimen maintained in 3D culture and ALK5 exon skipping (A). AONs coupled to a fluorochrome (AON-fluorescent) were delivered by microinjecting the center of the tissue on the nitrocellulose membrane as described in the cartoon. The tissue was then cultured for three days and sectioned in order to determine the presence of nuclei, which had taken up the AON. (B). Direct visualization of the AON-fluorescent (green) and nuclei (TOPRO, blue) in a DD tissue section. (C). Quantification of the percentage of fluorescent cells (AON) relative to total cell count. Scale bars, 50 um. (D). Description of exon skipping (exon 2) of the ALK5 pre-mRNA. Primer position (exon 1 and exon 3) of primers used for detecting the full length ALK5 and the exon skipped mRNA product are depicted here. (E). DD tissues were cultured and injected with either scrambled (ScrViM) or ALK5ViM. For comparison, treatment with TGFβ or SB-431542 compound was also combined with ViM administration. After three days of treatments, tissues were homogenized and used for RNA isolation and cDNA synthesis. Touchdown PCR was performed to validate the exon skipping and products were visualized by agarose gel electrophoresis. Full length ALK5 mRNA transcripts were detected in all conditions while exon2-skipped ALK5 mRNA transcripts were only detected in tissues injected with ALK5ViM. CON: untreated condition; TGFβ: treatment with TGFβ cytokine, SB: treatment with SB-431542 compound; TGFβ+SB: treatment with both TGFβ and SB-431542 compound.

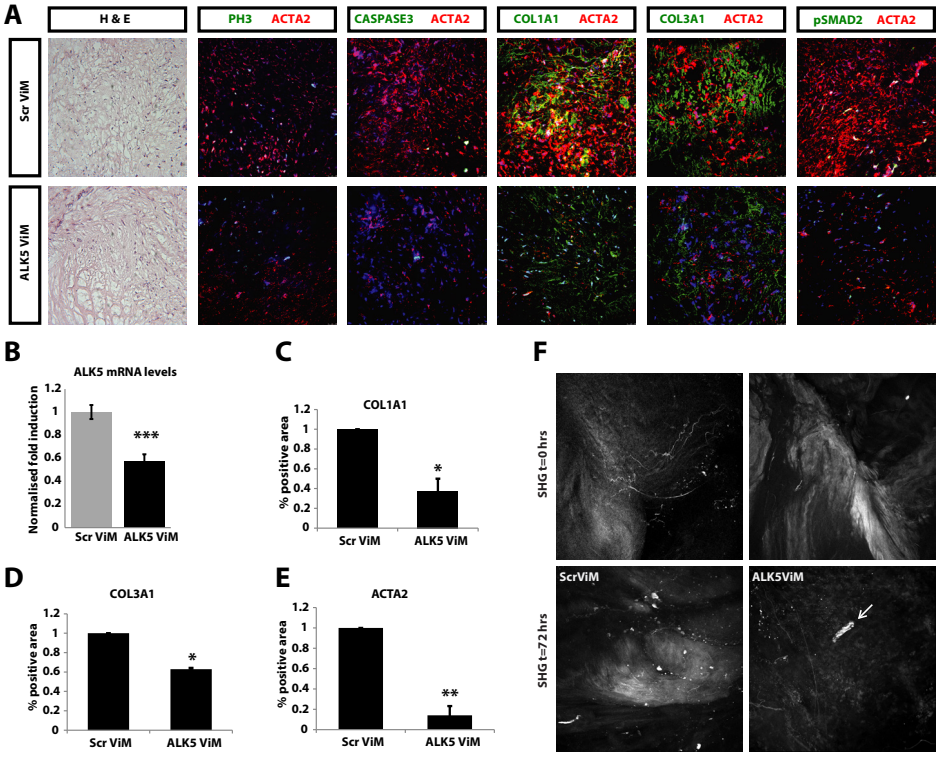

Fig.4. ALK5ViM treatment of DD resection specimens cultured in the 3D *ex vivo* "clinical trial" system
(A). Immunohistochemical and immunofluorescent analysis of 3D cultured DD resection specimens after three-day treatment with scrambled ViM (ScrViM) and ALK5ViM. Hematoxylin and Eosin (H&E), proliferation marker phosphohistone 3 (PH3, green), apoptosis marker cleaved caspase 3 (CASPASE3, green), Collagen type I (COL1A1, green), Collagen type III (COL3A1, green), phosphorylated SMAD2 (pSMAD2) (green) and smooth muscle actin, alpha 2 (ACTA2, red). Nuclei were visualized with TOPRO-3 (TOPRO, blue). (B). QPCR to detect expression levels of full length *ALK5* mRNA was performed on tissues injected with ScrViM and ALK5ViM (N=3). Values were normalized to *CAPNS1*. Fold induction values compared to ScrViM condition are shown. Statistical significance was calculated by one-tailed paired t-test. ***p<0.001. (C-E). Quantification (described in methods) of immunofluorescence signal of COL1A1, COL3A1, ACTA2 from different patient-derived specimens (N=3) after three-day treatment with scrambled ViM (ScrViM) and ALK5ViM. Fold induction values compared to ScrViM condition are shown. Multiple areas were quantified per sample, error bars represent ±S.E.M. Statistical significance was calculated by one-tailed paired t-test. *p<0.05, **p<0.01. Scale bars, 25 um. (F). SHG images of endogenous DD tissue in the 3D culture system. Collagen distribution was imaged at control time point (SHG, t=0, upper panel) in adjacent parts of the specimen. The exact tissue parts were imaged at 72 hrs (SHG, t=72 hrs, bottom panel) after injection of ScrViM or ALK5ViM. Arrow indicates site of injection.

Discussion

In the present study, we have developed a novel method for *ex vivo* analysis of human DD disease and we provide evidence of its suitability for molecular modulation by AONs. AONs were designed to target and inhibit a key profibrotic signaling pathway, which results in significant antifibrotic effects. Given the high risk of recurrence of DD, it would be therapeutically beneficial to reduce local collagen content in order to extend the symptom-free period after surgery, needle fasciotomy and/or collagenase injection. Our *ex vivo* "clinical trial" system allows the culture of DD specimens after surgical removal, without the need of fibroblast derivation, or grafting experiments[30], while preserving the pathological status of the disease by maintaining the complex organization of the ECM and the 3D tissue structure. The main challenges in *ex vivo* culture methods are viability and preservation of the *in vivo* normal or pathological traits of the tissue to be studied. Several organ culture and precision cut tissue slice methods have been developed such as the submerged system[31,34], the dynamic organ culture[32] and the gas exchange method [31]. Organ viability, functionality, metabolism and toxicity can be well studied in all these systems for complex organs such as liver, kidney, intestine, and lungs[33,34]. A limitation of these methods is the relatively short incubation time possible (approximately 24- 72 hours), depending on the tissue origin, as well as the challenge of organ/disease recapitulation. Our methodology is based on an enhanced setup where tissue parts are placed continuously and statically in contact with nutrients but are not fully immersed into medium, thus maintaining proper oxygenation and avoiding necrosis in the center of the tissue. Such setup appears suitable for culture of dense tissue such as DD fibrotic parts and facilitates viability for longer periods (up to seven days tested). Exposure of one side of the tissue to the medium is sufficient for diffusion and absorbance of nutrients throughout the tissue. Small tissue parts (<200um) are preferable in order to allow cell proliferation and longer viability[35]. Static incubation was performed, in contrast to most dynamic culture conditions, in order to maintain positional information and cellular sensing[36,37].

In addition, this particular setup allows for manipulation (e.g. AON injection) and direct visualisation of the effects on the ECM (SHG). Since DD tissue shows rapid production of ECM proteins, all tissues were cultured in absence of any exogenous matrix substrates. This is advantageous for the maintenance of native ECM turnover. Moreover, this culture setup is optimal for DD fibrotic tissue due to the content of highly proliferative MFBs and because the nodules and cords are *in vivo* quite isolated structures with autonomous characteristics (such as cell/ tissue growth and fibrosis). Due to these innate properties, it is likely that the tissues can be maintained *ex vivo* efficiently.

In this study, we have exclusively utilized the nodule parts, which are the firm thickenings and are considered pathologically very active due to the high content of MFBs. Cord parts are mainly fibrotic flexions and contain few fibroblasts, which are in a dormant state[38]. It has been proposed that active nodules may progress into cord structures at more advanced stage of the disease[39] therefore it is more clinically relevant to target the fibrotic characteristics of the node parts. TGFβ has been found to be expressed in both parts, as well as in the surrounding tissue[4] (appearing not affected by the disease), which may play a role in promoting recurrence of fibrosis as part of wound healing response due to tissue damage from the primary surgery. The majority of the resection specimens we have analysed using this system respond to TGFβ stimulation by upregulation or maintenance of the expression levels of fibrotic proteins (**Fig.2B-D**). Decrease in COL1A1 and COL3A1 but not of ACTA2 has

been detected in two biopsies after TGFβ stimulation which may suggest the function of a negative feedback loop due to high levels of TGFβ[40,41]. Upon treatment of DD biopsies with the SB-431542 inhibitor, expression of collagen and ACTA2 were decreased in the majority of biopsies or sustained the same levels as if untreated (**Fig.2B-D**).

Previous studies have attempted manipulation of TGFβ by neutralizing antibodies[30] and kinase inhibitors[15]. TGFβ has been also targeted in indirect ways such as by cyclic AMP[42], angiotensin inhibitors[43], tamoxifen[44], administration of Bone morphogenetic protein-6[15]. Given the pleiotropic effect of TGFβ signaling, the aim is to normalize and not completely abolish its function. Therefore, in order to restore the balance of pathway activation without fully disrupting its function, we have selectively inhibited the ALK5- mediated profibrotic pathway by exon skipping technology. It is worth noting that AON approach provides the advantage of high specificity exclusively for ALK5 mRNA (exon 2 encoding ligand binding domain), while the SB-431542 compound targets activity of three kinase receptors (ALK4, ALK5, ALK7), all implicated in the activin/ TGFβ pathway. Moreover, SB-431542 may not block TGFβ/ ALK5 induced non-SMAD signaling[45,46] whereas ALK5 AON will inhibit both pathways. TGFβ/ p38 and ERK MAP kinases have been shown to be involved in fibro-proliferative response in Dupuytren's disease[15]. Delivery of ALK5 AON by affecting SMAD and non-SMAD TGFβ signaling may thus achieve better inhibition than ALK5 kinase inhibitors by interfering with multiple pathways downstream of ALK5. Compared to regular oligonucleotides, small molecule inhibitors have better pharmacokinetic properties, due to the short half-life and inability to efficiently cross tissue membranes. However, currently there are many oligonucleotide modifications available that ensure improved stability, serum half-life and uptake of oligonucleotides. Our studies here use ViMs, which are antisense phosphorodiamidate morpholino oligomers covalently linked to a molecular scaffold that carries a guanidinium group at each of its eight tips to enhance delivery, to show proof-of-concept for this approach. Efficacy of ViMs has also been shown by others in animal models[47]. However, further clinical development of this particular compound is hampered by toxic effects. Nevertheless, there is a plethora of chemical modifications available that can be studied further for clinical development[48]. In light of this, it is encouraging that we were able to obtain similar results with ALK5 AONs of the 2'-O-methyl phosphorothioate AON chemistry (**Fig.S4**, which is very similar to the chemistry approved by FDA (mipomersen[49]) and identical to drisapersen, which is in phase III clinical trials for Duchenne muscular dystrophy). TGFβ secretion might also play a significant role in the recurrence of fibrosis after surgical removal. In this context, a hypothetical therapeutic setting would be the administration of AONs prior to or instead of the surgical intervention to counteract the TGFβ signaling in the remaining MFBs.

A challenge in the field of Dupuytren's is the lack of *in vivo* modelling of the disease. Here, we have developed a very robust and reproducible *ex vivo* 3D culture method with a simple setup (no growth factors or matrix protein support required). By using the ALK5ViM AON in this system, we have showed significant decrease in collagen protein expression and degradation/reorganisation of collagen structures. Excessive collagen production is the main clinical symptom in this disease and here we provide proof of decrease in collagen deposition *ex vivo*. The average reduction of full length ALK5 mRNA achieved was 70-75% within the first 48 hours (**Fig.S2A**) and about of 30-60% by day 3 (**Fig.4B, S2A**). Our data indicate the potential of MFBs to reverse into a less fibrotic phenotype and to respond to growth factor inhibition even after advanced disease progression. In addition, we show the feasibility of a well-established *ex vivo* imaging approach, such as the SHG[41,50], for the

study of ECM structure in native unstained tissue which to our knowledge has not been previously used for DD. The above observations may change the view of therapeutic approaches currently used for DD. Ultimately, the *ex vivo* "clinical trial" system can be applied for individualized therapy research after tissue resection as a drug screening method to test for specific responsiveness of DD tissues to a panel of growth factors and inhibitors and eventually lead to targeted therapy in case of recurrence.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary Material

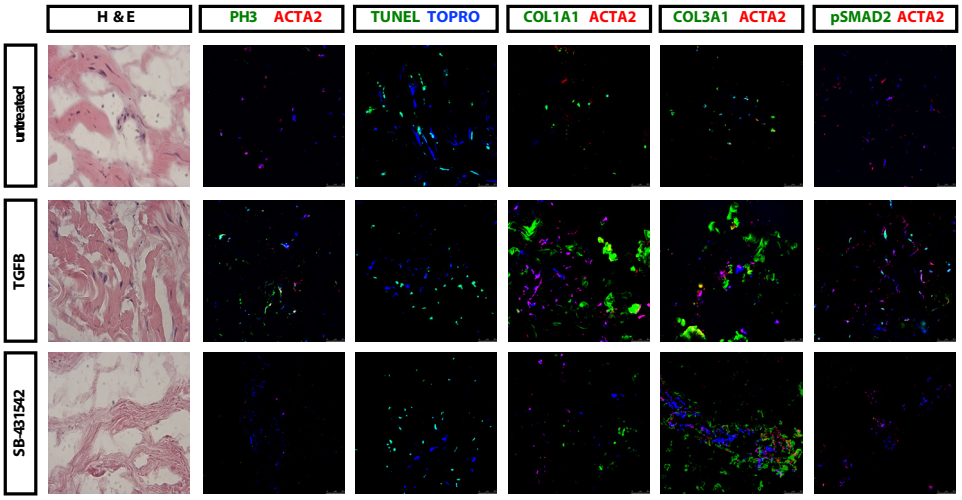

Fig.S1. Normal fascia palmaris tissue cultures in the 3D *ex vivo* "clinical trial" system
Representative immunohistochemical and immunofluorescent analysis of 3D cultured normal fascia palmaris specimens (N=4). Hematoxylin and Eosin (H&E), proliferation marker phosphohistone 3 (PH3, green), apoptosis terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL, green) in control normal fascia palmaris without any additional factors (untreated), after treatment with TGFβ and after treatment with ALK4/5/7 kinase inhibitor SB-431542. Collagen type I (COL1A1, green), Collagen type III (COL3A1, green), phosphorylated SMAD2 (pSMAD2, green) and smooth muscle actin, alpha 2 (ACTA2, red) protein expression in control normal fascia palmaris without any additional factors (untreated), after treatment with TGFβ and after treatment with ALK4/5/7 inhibitor (SB-431542). Nuclei were visualized with TO-PRO-3 (TOPRO, blue). Scale bars, 25 µm.

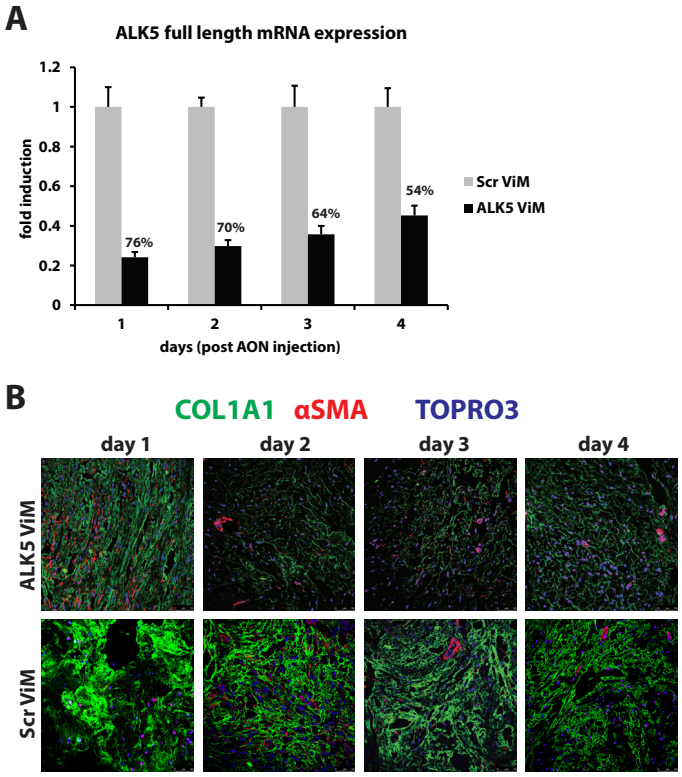

Fig.S2. Time course of *ex vivo* delivery of ALK5 ViM AON
Tissues were injected with ScrViM AON (scrambled sequence) or ALK5ViM AON and cultured for 1- 4 days. (A). Expression of full length ALK5 mRNA as measured by Q-PCR at different time points (day 1- day 4 after AON injection). Values were calculated as fold induction over the ScrViM (control) values for each time point. Error bars represent ±S.D. Percentages indicate the decrease of ALK5 full length mRNA expression. (B). Immunofluorescent analysis of DD tissue specimens cultured in the 3D *ex vivo* system. Expression of Collagen type I (COL1A1, green), smooth muscle actin, alpha 2 (ACTA2, red) is shown. Nuclei were visualized with TO-PRO-3 (TOPRO, blue). Scale bars, 50 µm.

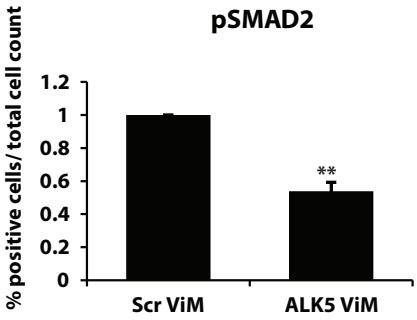

Fig.S3. Quantification of pSMAD2 immunofluorescence by image analysis.
Number of pSMAD2 positive nuclei over total cell count (TO-PRO-3) after three-day treatment with scrambled ViM (ScrViM) and ALK5ViM. Fold induction values compared to ScrViM condition are shown. Multiple areas were quantified per sample (N=3), error bars represent ±S.E.M. Statistical significance was calculated by one-tailed paired t-test. **p<0.01.

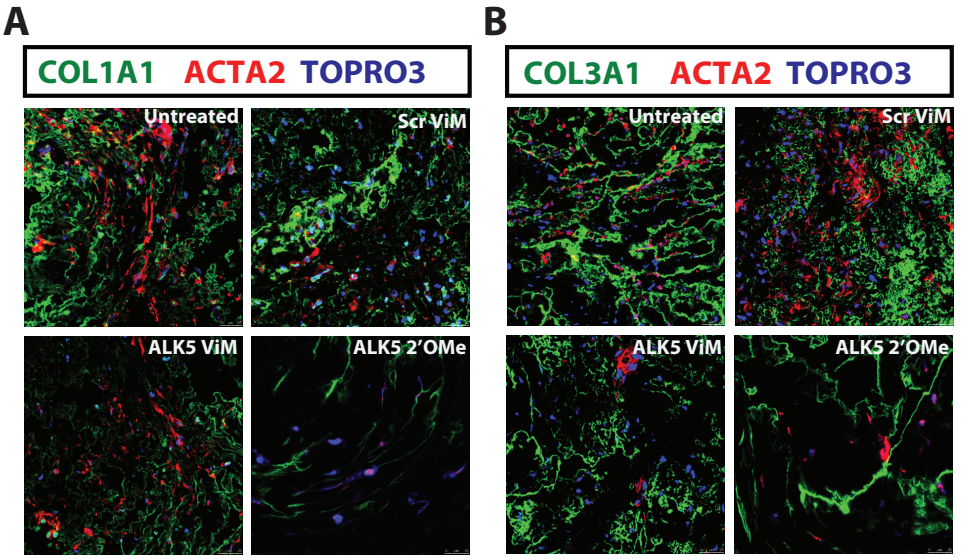

Figure S4. *Ex vivo* delivery of ALK5 AON with ViM and 2'OMe chemical backbones
Immunofluorescent analysis of DD tissue specimens cultured in the 3D *ex vivo* system. Tissues were injected with ScrViM AON (scrambled sequence), ALK5ViM AON or ALK5 2'OMe AON and cultured for 72 hours. (A). Expression of Collagen type I (COL1A1, green), smooth muscle actin, alpha 2 (ACTA2, red). (B). Collagen type III (COL3A1, green) and smooth muscle actin, alpha 2 (ACTA2, red) protein expression. Nuclei were visualized with TO-PRO-3 (TOPRO, blue). Scale bars, 25 µm.

Supplementary Materials and Methods

Generation of 3D culture system

In order to bypass the need for exogenous ECM and the derivation of fibroblasts, we have developed a patient- derived specimen culture system. This is the first tissue resection culture system that allows human tissue from DD patients to be grown *ex vivo* and functionally tested, recapitulating the *in vivo* situation. The system is based on a nitrocellulose membrane that allows contact of the tissue with the medium but not with the plastic, thus preventing the alteration of the tissue upon attachment, as previously observed when culturing DD fibroblasts. No collagen gel or other ECM protein substrate is required, since the DD tissue itself produces large amounts of these proteins. In brief: specimens from DD surgeries are equally sliced and placed in transwell plates onto nitrocellulose filters. Tissues remain continuously and statically in contact with defined culture conditions (Dulbecco's Modified Eagle's Medium (DMEM, with 1% fetal calf serum (FCS), 1% penicillin-streptomycin) but without being entirely submerged and are allowed to grow (seven days). Tissue resection specimens (N=9 DD and N=4 normal fascia palmaris) were treated with a combination of activators and inhibitors of the TGFβ signaling pathway (e.g. TGFβ, 5ng/ml; SB-431542, 10ng/ml, Tocris). After culture, tissues were processed for RNA isolation or they were fixed in 4% paraformaldehyde, incubated in 30% sucrose buffer, embedded in Tissue Tek- O.C.T. compound and stored at -80°C.

Immunofluorescence

Hematoxylin and Eosin (H&E) staining were performed using standard protocols on 10-µm cryosections. Immunofluorescence staining was performed on 10-µm cryosections. For antigen retrieval sections were boiled in antigen unmasking solution (Vector Labs) and were incubated in 3% H_2O_2 for endogenous peroxidase sequestering. Primary antibodies and dilutions used: anti-ACTA2 1:500 (Sigma), anti-COL1A1 1:500 (Southern Biotech), anti-COL3A1 1:500 (Southern Biotech), and anti-phospho-SMAD2 1:1000 (Cell Signaling). Sections were blocked with 1% bovine serum albumin (BSA)- PBS-0.1% v/v Tween 20) and incubated with primary antibodies diluted in the blocking solution, overnight at 4°C or room temperature. Sections then were incubated with secondary antibodies labeled with Alexa Fluor 488, 555, or 647 (Invitrogen/Molecular Probes, 1:250 in PBS-0.1% Tween 20). Detection of pSMAD2, was enhanced using tyramide amplification (Invitrogen/Molecular Probes) by incubation of slides with horseradish peroxidase (HRP)-conjugated secondary antibody (1:100 dilution) (Invitrogen/Molecular Probes), followed by incubation with tyramide-488 for 10 minutes. Sections were counterstained with TO-PRO3 (Invitrogen/Molecular Probes) at 1:1000 dilution in PBS-0.1% Tween-20 for nuclei visualization, and mounted with Prolong G mounting medium (Invitrogen/Molecular Probes), which contains DAPI.

RNA isolation, RT-PCR and Quantitative PCR

Nodule parts (100 µm) were homogenized using an Ultra Turrax homogenizer (T25 basic, IKA) in TRIpure reagent (Roche) and directly processed for total RNA isolation according to the TRIpure RNA extraction protocol. Total RNA (0.5 µg) was used for first strand cDNA synthesis using RevertAid H Minus first strand cDNA synthesis kit (Fermentas).
For quantitative PCR (Q-PCR) ten- fold diluted cDNA was amplified in a CFX Real Time Detection system (Bio-rad) using SYBR Green Supermix reagent (Bio-rad). To detect full length ALK5 mRNA levels, primers flanking exon 2 and exon 3 were used. Expression levels were normalized to housekeeping gene (*ACTRT1* or *CAPNS1*) and analyzed using the linear regression method. For exon skipping test, cDNA was amplified by touchdown PCR using ALK5 specific primers, which are designed to amplify both the full length and exon 2-skipped mRNA transcripts (forward primer in exon 1, reverse primer in exon 3). Primer sequences and detailed PCR protocol are available upon request.

Microscopy and Image analysis

Confocal microscopy of labelled specimens was performed on a Leica TC-SP5 microscope with a 40X 1.4 NA oil-immersion objective Z series were collected and reassembled in Image J software (rsbweb.nih.gov/ij). Mean area fraction fluorescence was calculated in Image J software using threshold to select the root boundary and measuring the percentage of positive surface inside the intensity defined by the threshold. Experiments were repeated three times and stained specimens in a given experiment were imaged using identical microscopic exposure and recording settings. Second-harmonic generation (SHG) was performed for imaging the organization of collagen fibers in native tissue. This type of two-photon microscopy was performed on a Zeiss 710 NLO upright confocal microscope (Jena, Germany) equipped with a femtosecond Spectra-Physics Deep See MP laser (Santa Clara, United States) using a Plan-Apochromat 20x/1.0 NA water-immersion objective. The images

were obtained with an excitation wavelength of 750 nm and emitted light was collected between 371–467 nm. Confocal stacks were processed with the Zeiss ZEN2009 software.

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