Wnt pathway in Dupuytren disease: connecting profibrotic signals

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A role of Wnt signaling in Dupuytren disease, a fibroproliferative disease of the hand and fingers, has not been fully elucidated. We examined a large set of Wnt pathway components and signaling targets and found significant dysregulation of 41 Wnt-related genes in tissue from the Dupuytren nodules compared with patient-matched control tissue. A large proportion of genes coding for Wnt proteins themselves was downregulated. However, both canonical Wnt targets and components of the noncanonical signaling pathway were upregulated. Immunohistochemical analysis revealed that protein expression of Wnt1-inducible secreted protein 1 (WISP1), a known Wnt target, was increased in nodules compared with control tissue, but knockdown of WISP1 using small interfering RNA (siRNA) in the Dupuytren myofibroblasts did not confirm a functional role. The protein expression of noncanonical pathway components Wnt5A and VANGL2 as well as noncanonical coreceptors Ror2 and Ryk was increased in nodules. On the contrary, the strongest downregulated genes in this study were 4 antagonists of Wnt signaling (*DKK1, FRZB, SFRP1,* and *WIF1*). Downregulation of these genes in the Dupuytren tissue was mimicked in vitro by treating normal fibroblasts with transforming growth factor β1 (TGF-β1), suggesting cross talk between different profibrotic pathways. Furthermore, siRNA-mediated knockdown of these antagonists in normal fibroblasts led to increased nuclear translocation of Wnt target β-catenin in response to TGF-β1 treatment. In conclusion, we have shown extensive dysregulation of Wnt signaling in affected tissue from Dupuytren disease patients. Components of both the canonical and the noncanonical pathways are upregulated, whereas endogenous antagonists are downregulated, possibly via interaction with other profibrotic pathways. (Translational Research 2015;166:762–771)

Abbreviations: cDNA = complementary DNA; Fzd = Frizzled; HDF = human dermal fibroblasts; HPF = high power field; LRP = low-density lipoprotein receptor–related protein; siRNA = small interfering RNA; TGF = transforming growth factor; WISP1 = Wnt1-inducible secreted protein 1

INTRODUCTION

The Wnt pathway has been suggested as a primary cause of fibrosis in different organs.[1,2] In Dupuytren disease, a benign fibroproliferative disorder, several studies have attempted to elucidate its role in the progression of the disease.[3-6]

Dupuytren disease is characterized by the appearance of proliferative nodules in the palm of the hand,

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AT A GLANCE COMMENTARY

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Background

Dupuytren disease is a prevalent fibroproliferative disease of the hands and fingers that may cause serious disability. Current surgical treatment options are not always sufficient, because recurrence rates are high. Therefore, further knowledge on the pathways driving fibrosis is needed for new treatment options.

Translational Significance

This study compares affected and unaffected tissue from patients, providing insight into disease-specific changes. We demonstrate that the Wnt pathway is strongly dysregulated in Dupuytren disease and suggest that the downregulation of endogenous inhibitors and the upregulation of noncanonical signaling specifically provide new avenues of investigation.

followed by the development of cords.[7] These lead to flexion contractures and an inability to extend the fingers. The symptoms can be relieved either by surgery or local injection of collagenase, but both these treatments carry a high risk of recurrence. The primary cause of Dupuytren disease is still debated, but the occurrence of Dupuytren contractures has been shown to be associated with diabetes, smoking, the use of antiepileptic drugs, and a family history of Dupuytren disease. The prevalence of Dupuytren disease varies according to age and population sampled, and in a recent study, it was estimated to be 12% in those aged 55 years and 29% in those aged 75 years.[8]

The Wnt pathway can roughly be divided into a canonical and a noncanonical arm. Canonical Wnt signaling transmits signals via the Frizzled (Fzd) receptors and coreceptors low-density lipoprotein receptor–related protein (LRP)5 and LRP6. Activation of these receptors leads to the dissociation of the cytoplasmic destruction complex, which normally sequesters β-catenin. Free β-catenin translocates to the nucleus and initiates Wnt-driven transcription programs, which are important in the development, and mainly drive proliferation, but may be pathogenic as well.[9] Noncanonical Wnt signaling does not depend on β-catenin, but instead activates downstream mediators such as c-Jun N-terminal kinases, RhoA, and others. It signals partly through Fzd receptors, but generally uses different coreceptors, such as Ryk and Ror2.[10]

Previous studies have suggested the involvement of the Wnt pathway in Dupuytren disease (reviewed in Shih et al[6]). A genome-wide association study found 7 Wnt-related genes that were associated with Dupuytren patients in a large cohort.[11] Several studies reported an increased protein expression and nuclear translocation of β-catenin,[4,12] although there was no significant upregulation of messenger RNA (mRNA) coding for Wnt proteins themselves,[3] and no correlation was found between β-catenin levels and recurrence of Dupuytren disease after surgery.[4]

We propose that other components of the Wnt pathway may account for its activation and a possible role in Dupuytren disease and therefore examined a large set of Wnt pathway components and signaling targets in Dupuytren nodules and matched control tissue. Several parameters found dysregulated were subsequently studied on a protein level, and their mechanism of action was further elucidated in vitro.

MATERIALS AND METHODS

Ethics statement. Tissue samples were obtained after informed written consent and were approved by the Medical Ethics Committee of the University Medical Center Groningen (2007/067), in line with the Declaration of Helsinki.

Primary tissues. Dupuytren nodules and unaffected transverse ligaments of the palmar aponeurosis were obtained from patients undergoing limited fasciectomy or dermofasciectomy in the University Medical Center Groningen. Tissue from 28 patients (6 females, 22 males, average age 67 $\pm$ 10 years) in total was used; controls and nodules of 12 patients were analyzed in the Profiler array and by real-time polymerase chain reaction (qPCR). Because of changes in the Profiler platform, some of the genes were tested in 8 patients only. Controls and nodules of 12 other patients were analyzed using immunohistochemistry (Wnt1-inducible secreted protein 1 [WISP1] staining was analyzed in 9 patients). Tissue from 4 separate patients was used for isolation of myofibroblasts (see subsequently).

Cell isolation and culture. Primary Dupuytren myofibroblasts from 4 different patients were isolated by mincing nodule tissue with a scalpel and incubating the tissue with 1 mg/mL collagenase and 0.1 mg/mL deoxyribonuclease in Dulbecco's Modified Eagle Medium (DMEM; Lonza, Breda, the Netherlands) for 2 hours at 37°C. Cells were filtered through a cell strainer, and the resulting suspension was centrifuged at 300 $\times$ g for 10 minutes at 4°C. The cells were resuspended in DMEM with 10% fetal bovine serum (FBS; Thermo Scientific, Waltham, Mass), 1% penicillin, 1% streptomycin, and 2 mM of L-glutamine (both

Lonza) and cultured on normal tissue culture plastic. Cells between passages 3–5 were used for the experiments in this study.

Further experiments were performed in primary adult human dermal fibroblasts (HDF; ATCC, Manassas, VA). These were cultured in Eagle's Minimal Essential Medium (Lonza) with 10% FBS, 1% penicillin, 1% streptomycin, and 2 mM of L-glutamine. All fibroblasts were cultured at 37°C in a humidified atmosphere at 5% CO_2.

In vitro experiments. For small interfering RNA (siRNA) experiments, cells were plated at 15,000 cells/cm^2. MISSION esiRNA against *WISP1, DKK1, FRZB, SFRP1, WIF1* or control (enhanced green fluorescent protein [eGFP] or rLuciferase) was obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Cells were transfected with 125 ng of siRNA using Lipofectamine RNAiMAX (Life Technologies, Bleiswijk, the Netherlands) according to the manufacturer's instructions. When combinations of siRNAs were used, the total concentration was kept at 125 ng, consisting of equal amounts of each siRNA.

Stimulation with transforming growth factor β1 (TGF-β1) was performed by incubating HDF with TGF-β1 (PeproTech EC Ltd, London, UK) in DMEM containing 0.5% FBS, 1% penicillin/streptomycin, 2 mM of L-glutamine, and 0.17 mM of vitamin C (L-ascorbic acid 2-phosphate sesquimagnesium hydrate; Sigma-Aldrich).

Cell viability was determined by assessing mitochondrial activity 3 days after transfection with siRNA, using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay; Promega Benelux BV, Leiden, the Netherlands) according to the manufacturer's instructions. Briefly, cells were washed and MTS solution was added to each well and incubated for 2 hours at 37°C. Absorbance was read at 490 nm using a fluorescence microplate reader (Varioskan; Thermo Fisher Scientific Inc).

Gene expression analysis. The expression of Wnt-related genes was determined using an room temperature (RT)^2 Profiler PCR Array System (Qiagen, Hilden, Germany). RNA was isolated from tissue using the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions and quantified using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del). Complementary DNA (cDNA) was synthesized from RNA using the RT^2 First Strand Kit (Qiagen) and 400 ng of cDNA was used as input. Gene expression was calculated normalized to the geometric mean of 4 reference genes (β-*actin*, β2-*macroglobulin*, *GAPDH*, and *HPRT1*).

RNA from fibroblasts (Dupuytren myofibroblasts and HDF) was isolated using the Tissue Total RNA Purification Mini Kit (Favorgen, Vienna, Austria). cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, Ill). Primers were ordered from Sigma-Aldrich (sequences, Supplementary Table I). Gene expression was calculated relative to the geometric mean of the expression of the reference genes *GAPDH* and *YWHAZ*.

Plates were run using the ViiA 7 Real-Time PCR system (Applied Biosystems), and thermal cycling conditions were 10 minutes at 95°C, followed by 15 seconds at 95°C and 60 seconds at 60°C (40 cycles) for the Profiler system, and 15 seconds at 95°C and 30 seconds at 60°C and 30 seconds at 72°C (40 cycles) for other qPCR analyses. Threshold cycle numbers greater than 35 were set to 35 and considered not detectable.

Immunohistochemistry. Tissue for staining was stored at −80°C and cut into 5-μm cryosections. The sections were air-dried for 30 minutes and fixed in acetone for 10 minutes. Washing and blocking of aspecific binding sites and endogenous biotin were performed according to the standard procedures. Primary antibodies used were rabbit anti-VANGL2 (7 μg/mL, ab76174; Abcam, Cambridge, Mass), rabbit anti-Ryk (1:100, PAB3389; Abnova, Taipei, Taiwan), rabbit anti-WISP1 (10 μg/mL, ab10737; Abcam), and rabbit anti-Wnt5A (5 μg/mL, ab72583; Abcam). The sections were incubated for 60 minutes at RT with primary antibody, before incubation with biotinylated goat antirabbit polyclonal antibody (8.2 μg/mL; Dako), followed by streptavidin-alkaline phosphatase (1:400; SouthernBiotech, Birmingham, Ala). Stainings were visualized using a Vector Red kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, Calif). All immunohistochemical stainings were counterstained with hematoxylin (Merck, Darmstadt, Germany) and mounted in Kaiser's glycerol-gelatin (Merck).

Immunofluorescence. Cells were fixed 10 minutes at 4°C in 4% paraformaldehyde (WISP1) or 15 minutes at −20°C in methanol-acetone (smooth muscle 22 alpha [SM22α] and β-catenin). Paraformaldehyde-fixed cells were permeabilized 5 minutes in 0.2% Triton X-100 before staining. Washing and blocking of aspecific binding sites and endogenous biotin were performed according to the standard procedures. Primary antibodies used were rabbit anti-WISP1 (10 μg/mL, ab10737; Abcam), rabbit anti-SM22α (5 μg/mL, ab14106; Abcam), and mouse anti–β-catenin (2.5 μg/mL, 610153; BD Transduction Laboratories, San Diego, Calif). The cells were incubated for 60 minutes at RT with primary antibody, before incubation with biotinylated goat antirabbit polyclonal antibody (8.2 μg/mL; Dako), followed by streptavidin-Cy3 (1:400; SouthernBiotech).

Quantification of stainings. Immunohistochemical and immunofluorescent stainings were evaluated using a

Leica DM2000 microscope. For morphometric quantification of immunohistochemistry, 5 representative photomicrographs at ×40 magnification were taken per tissue section, using a Multispectral Imaging Camera (PerkinElmer, Cambridge, UK). Photomicrographs were analyzed using Nuance 3.0 software (PerkinElmer). Stained areas were quantified and expressed as square micrometer per high power field. Immunofluorescent stainings were performed 3 times on independent experiments, representative images of each set of experiments are shown.

Statistical analysis. Statistical analysis on the Profiler array was performed using SPSS 22, using a Wilcoxon paired rank test. For all other experiments statistical analysis was performed using GraphPad Prism 5.0. The quantifications of the immunohistochemical stainings were analyzed using a Wilcoxon paired rank test. Results of in vitro experiments are the average of 3 independent experiments (except WISP1 siRNA on HDF, n = 2). Results of all in vitro qPCR analyses were analyzed using a Student *t*-test. In all analyses, $P < 0.05$ was considered to be statistically significant.

RESULTS

Significant changes in Wnt signaling pathway in Dupuytren nodules. We examined the expression of 84 genes related to the Wnt pathway in Dupuytren nodules and unaffected control tissue from the same patients. Six genes had no detectable expression in control or affected tissue of more than half of the patients tested. These were removed from the analysis.

We found significant, but often small, changes in the expression of 41 of 78 of the remaining Wnt-related genes (Supplementary Table II). Of these, the expression of 10 genes was significantly increased 2-fold or more (Table I), and the expression of 14 genes was significantly decreased 2-fold or more (Table II). Notable among the upregulated genes were 2 transcription factors activated by the Wnt pathway (*LEF1* and *FOSL1*), Wnt receptors *FZD2* and *FZD3*, the canonical Wnt target *WISP1*, and components of the noncanonical pathway (*NKD1* and *VANGL2*). The factors that were markedly downregulated included 4 negative regulators of Wnt signaling (*DKK1*, *FRZB*, *SFRP1*, and *WIF1*). In addition, a large proportion of genes coding for the Wnt proteins specifically showed significant changes in expression, with the expression of 5 genes significantly downregulated (*WNT2*, *WNT4*, *WNT6*, *WNT10A*, *WNT11*) and the expression of 1 gene significantly upregulated (*WNT3*; Fig 1).

Changes in noncanonical Wnt pathway. Several components of the noncanonical pathway were significantly upregulated, such as *NKD1*, *PRICKLE1*, and *VANGL2* (Table II and Supplementary Table II). *WNT5A*, a Wnt protein usually associated with the noncanonical pathway, showed a trend toward upregulation, whereas *WNT11* was significantly downregulated (Fig 1). We verified the upregulation of Wnt5A and VANGL2 on protein level, using immunohistochemistry and found a significant increase in the expression of both proteins in nodule tissue compared with control tissue (Fig 2, *A* and *B*).

The noncanonical Wnt pathway can signal via Fzd receptors but also uses other specific coreceptors, such as Ryk and Ror2. The mRNA levels of these receptors were determined using qPCR, which showed a slight downregulation of *Ryk* and an upregulation of *Ror2*

Table I. Significantly 2-fold or more upregulated genes in Dupuytren nodules compared with matched controls

Gene	Median* control	Median* nodule	N	Fold	*P* value†
WISP1	0.021	0.109	12	6.62	0.002
LEF1	0.002	0.011	12	4.58	0.003
CHSY1	0.008	0.031	8	3.77	0.012
FOSL1	0.002	0.007	12	2.85	0.008
FZD2	0.002	0.004	12	2.63	0.003
KREMEN1	0.026	0.076	12	2.55	0.003
FZD3	0.002	0.004	12	2.54	0.004
VANGL2	0.006	0.022	8	2.53	0.050
DKK3	0.138	0.276	8	2.46	0.036
NKD1	0.005	0.008	12	2.42	0.019

*Median expression normalized to the geometric mean of 4 reference genes.
†*P* value as calculated using the Wilcoxon paired rank test.

Table II. Significantly 2-fold or more downregulated genes in Dupuytren nodules compared with matched controls

Gene	Median control*	Median nodule*	N	Fold	*P* value†
WIF1	0.008	8.9×10^{-5}	12	0.021	0.002
DKK1	0.004	0.001	12	0.100	0.002
FRZB	0.027	0.004	12	0.113	0.003
SFRP1	0.077	0.015	12	0.134	0.002
WNT11	0.004	0.001	12	0.137	0.003
CXADR	0.001	8.8×10^{-5}	8	0.145	0.012
WNT10A	3.0×10^{-4}	8.7×10^{-5}	12	0.272	0.012
FZD4	0.158	0.041	12	0.293	0.002
WNT6	0.001	1.9×10^{-4}	12	0.296	0.010
CYP4V2	0.056	0.019	8	0.359	0.017
WNT4	0.005	0.002	12	0.404	0.012
HSPA12A	0.040	0.016	8	0.411	0.025
NAV2	0.006	0.002	8	0.450	0.036
TCF7L1	0.031	0.016	12	0.473	0.003

*Median expression normalized to the geometric mean of 4 reference genes.
†*P* value as calculated using the Wilcoxon paired rank test.

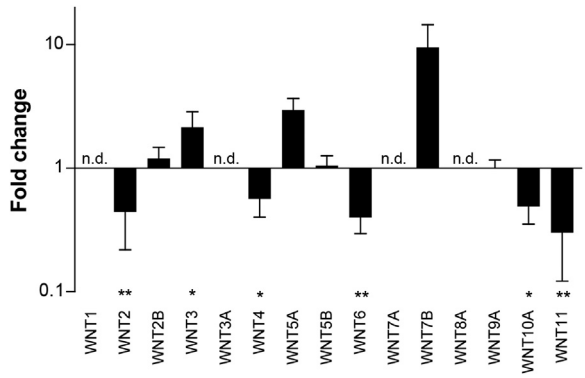

Fig 1. Expression of genes coding for Wnt proteins. Fold change of expression in nodule over patient-matched control tissue. $*P < 0.05$, $**P < 0.01$ as determined by the Wilcoxon paired rank test; n.d., no detectable expression in more than half of the patients tested.

(Fig 2, *C*). Immunohistochemistry showed an increased expression of Ryk protein in nodule tissue compared with matching controls (Fig 2, *D*).

Canonical Wnt target WISP1 is upregulated. *WISP1* is a known target of canonical Wnt signaling and was found to be upregulated in nodules (Table I). Immunohistochemical analysis showed that protein expression of WISP1 was also significantly increased in nodule compared with control (Fig 3).

To examine whether WISP1 plays a role in profibrotic signaling in the primary isolated Dupuytren myofibroblasts, we used siRNA against *WISP1*. qPCR analysis showed 85% knockdown of *WISP1* but no decrease in fibrotic markers *ACTA2* and *COL3A1*. Instead, an increase in *COL3A1* mRNA levels was seen (Fig 4, *A*) compared with myofibroblasts treated with siRNA against *eGFP*. Immunofluorescence images show strongly decreased protein expression of WISP1 after siRNA-mediated knockdown, but no effect was seen on fibroblast marker SM22α (Fig 4, *B*). Knockdown of *WISP1* in normal fibroblasts (HDF) also did not inhibit the expression of fibrotic markers in response to TGF-β1 (Supplementary Fig 1). *WISP1* knockdown did not influence cell proliferation (data not shown).

Wnt pathway negative regulators are downregulated in tissue of Dupuytren patients. Our study showed a strong downregulation of several negative regulators of the Wnt pathway, such as *DKK1*, *FRZB*, *SFRP1*, and *WIF1* (Table II). The downregulation of these negative regulators might influence overall Wnt signaling during Dupuytren disease, and thereby function as an additional profibrotic mechanism.

Wnt pathway negative regulators are downregulated by TGF-β1 signaling in normal fibroblasts. To find a possible endogenous mechanism that might be responsible for the decrease in negative regulators of the Wnt pathway in Dupuytren tissue, we performed experiments on HDF. We found that incubation with TGF-β1 led to a sharp decrease in the expression of all previously mentioned Wnt negative regulators (Fig 5, *A*).

To elucidate the effect of the decrease in the negative regulators of the Wnt pathway, we performed siRNA-mediated knockdown of the 4 negative regulators in normal fibroblasts. Simultaneous treatment with siRNA against all 4 antagonists led to 60%–80% knockdown of the individual antagonists (Supplementary Fig 2). On treatment of these cells with a low dose of TGF-β1, translocation of β-catenin to the nucleus was seen, whereas in control (siRNA against r-Luciferase [si-rLuc]–treated) cells, no or limited nuclear β-catenin expression was visible in response to low-dose TGF-β1 treatment (Fig 5, *B*). Cells not treated with TGF-β1 did not show nuclear β-catenin expression regardless of siRNA treatment (data not shown).

DISCUSSION

Wnt signaling has previously been implicated in various fibrotic diseases,[1,2] and studies have indicated that the Wnt pathway may play a role in Dupuytren disease.[5,11] In a complex, chronic, fibrotic disease such as Dupuytren disease, there is a high probability of interaction between a multitude of profibrotic pathways. This means that the Wnt pathway may either be involved in Dupuytren disease as a primary cause or as a secondary, additional, profibrotic pathway. In the present study, we found a complicated picture with many components of the Wnt pathway differently expressed in nodule tissue compared with control tissue of the Dupuytren patients. Components of the canonical and the noncanonical pathway, each of which have been shown to be profibrotic,[13,14] were activated in nodules.

One of the Wnt pathway targets found to be strongly upregulated in this study is WISP1. This is a member of the connective tissue growth factor cysteine-rich protein nephroblastoma overexpressed (CCN) family, which has been found increased in several forms of fibrosis.[15-17] Consistent with these studies, we found a strong upregulation of WISP1 both at the mRNA and protein level. However, on the knockdown of *WISP1* using siRNA in the Dupuytren myofibroblasts, no decrease in fibrotic markers was found, indicating that WISP1 is not necessary for the maintenance of profibrotic parameters in these cells. On the contrary, studies on the function of WISP1 in various other diseases have shown it to increase proliferation of fibroblasts and fibrosis in the lung.[15,16] Additional studies found that the use of anti-WISP1 antibodies was an effective treatment in an animal model of airway remodeling.[18] Thus far, we have not been able to clarify these contradictory results. Notably, in the study by Königshoff et al,[15] the

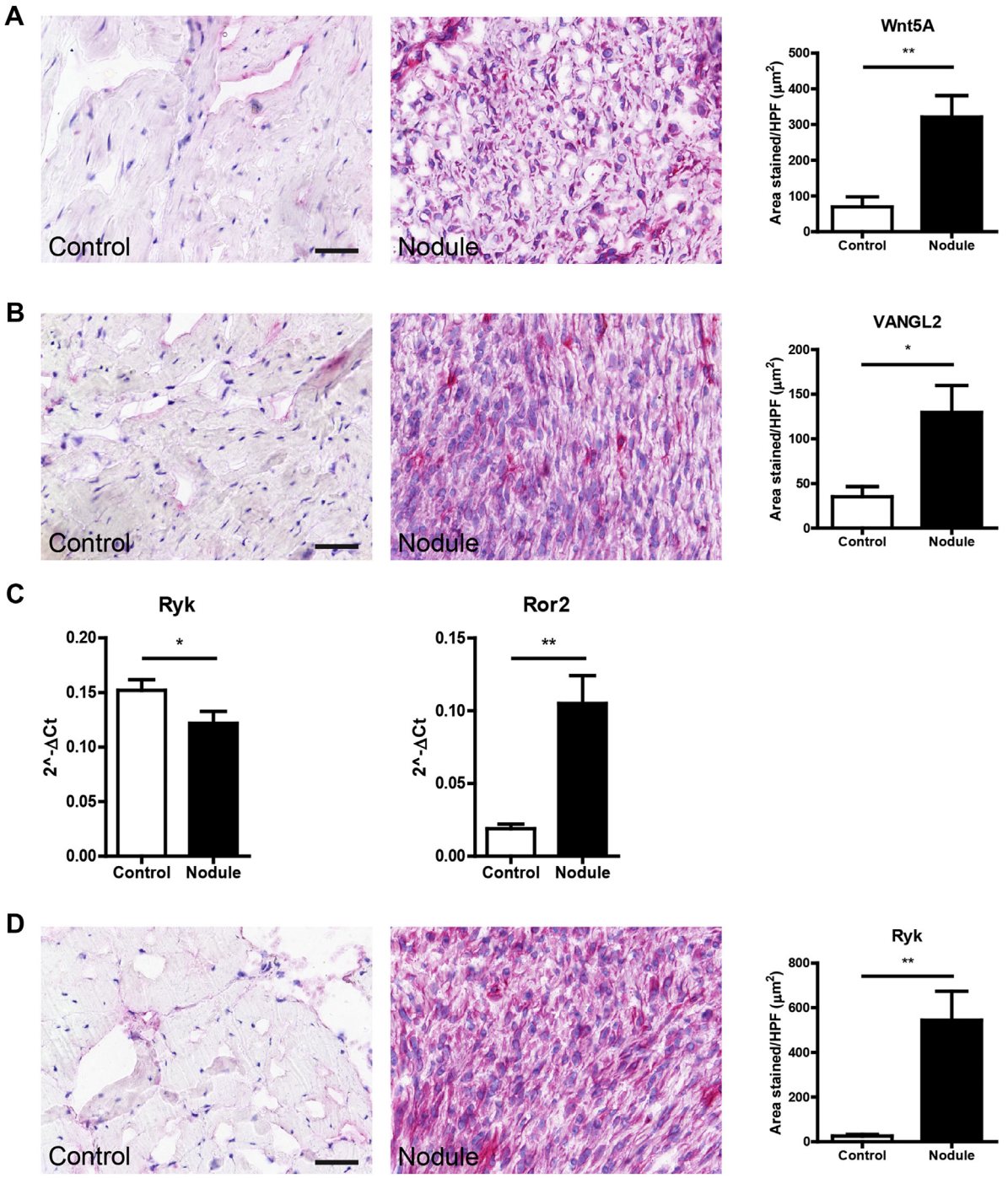

Fig 2. Noncanonical Wnt pathway mediators. (**A**) Representative pictures and quantification of Wnt5A immunohistochemical staining. Scale bar denotes 50 μm. (**B**) Representative pictures and quantification of VANGL2 immunohistochemical staining. Scale bar denotes 50 μm. (**C**) Expressions of Ryk and Ror2 in Dupuytren nodule and patient-matched control tissue as determined by qPCR. (**D**) Representative pictures and quantification of Ryk immunohistochemical staining. Scale bar denotes 50 μm. $^*P < 0.05$, $^{**}P < 0.01$ as determined by the Wilcoxon paired rank test. HPF, high power field.

strongest increase in WISP1 was found in airway epithelium, suggesting that the explanation might be in the interplay between cell types in vivo, which could not be replicated in our culture system. Therefore, we cannot rule out a role for WISP1 in the pathophysiology of Dupuytren disease in patients.

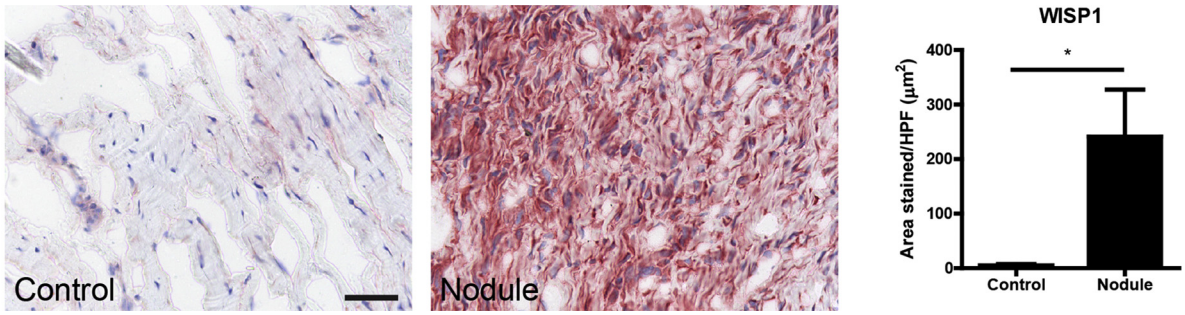

Fig 3. WISP1 expression in Dupuytren tissue. Representative pictures and quantification of WISP1 immunohistochemical staining in Dupuytren nodules and patient-matched control tissue. Scale bar denotes 50 μm. *$P < 0.05$ as determined by the Wilcoxon paired rank test. HPF, high power field; WISP1, Wnt1-inducible secreted protein 1.

Apart from the activation of the more commonly studied canonical pathway, we found that components of the noncanonical pathway were also increased in affected tissue derived from the Dupuytren patients. We confirmed increased protein expression of Wnt5A, noncanonical coreceptor Ryk, and PCP pathway component VANGL2. Previous studies had already shown the upregulation of noncanonical receptor Ror2 in tissue from the Dupuytren patients,[19] an upregulation of Wnt5A,[4] and the downregulation of microRNA targeting WNT5A.[20] The role of noncanonical Wnt signaling in fibrotic diseases has been the subject of several studies in recent years. The overall effect of the activation of this pathway is difficult to predict, because Wnt5A signaling may inhibit canonical Wnt signaling,[21] but high Wnt5A expression has also been associated with increased migration and proliferation of fibroblasts.[22,23] Interestingly, Wnt5A signaling has been shown to be strongly linked to the TGF pathway in lung diseases, a process which might be relevant for Dupuytren disease, where an upregulation of TGF has also been shown numerous times (reviewed in Shih and Bayat[24]).

Several studies have reported an upregulation of (nuclear) β-catenin in Dupuytren disease,[4,12] indicating the activation of the canonical Wnt pathway, although none have found an upregulation of a specific Wnt protein or other activator. We have now found evidence pointing to an alternative explanation for the reported activation, namely in the dramatic downregulation of negative regulators of the Wnt pathway. These include DKK1, which inhibits canonical Wnt signaling through the binding of coreceptors LRP5/6 together with Kremen, and FRZB, SFRP1, and WIF1, which bind both canonical and noncanonical Wnt proteins, and thus may inhibit both types of signaling.[25] Their downregulation could activate both canonical and noncanonical signaling.

The downregulation of endogenous negative regulators of the Wnt pathway suggests cross talk between profibrotic pathways. It has been reported that DKK1 may be downregulated by TGF-β signaling,[2] tenascin C,[26] and increased rigidity of the extracellular matrix.[27] These 3 factors have all been described to be present during Dupuytren disease[24] and might individually, or in combination, be the cause of the downregulation of *DKK1* found in this study. Factors influencing the expression of the other negative regulators decreased in the Dupuytren tissue have been studied less extensively. Interestingly, downregulation of *DKK1*, *SFRP1*, and *WIF1* has been described in systemic sclerosis.[28,29]

Our in vitro experiments confirmed a role for TGF-β1 in the downregulation of all the negative regulators that we examined, and they further show that the downregulation of Wnt pathway antagonists sensitizes cells to profibrotic signaling. Knockdown of the antagonists in normal fibroblasts increased β-catenin nuclear translocation in response to low-dose TGF-β1. Previously, knockdown of *SFRP1* has been shown to increase TGF-β1 signaling in mammary epithelial cells,[30] giving a similar example of cross talk between Wnt antagonists and TGF-β1 signaling. This in vitro experiment emphasizes that the observed downregulation of Wnt antagonists in tissue of Dupuytren patients may not only affect the sensitivity to Wnt signaling but also to other profibrotic pathways. In this respect, it is interesting to note that other studies[31] have shown cross talk between the tumour necrosis factor (TNF) pathway and Wnt signaling in Dupuytren myofibroblasts. Both TNF and TGF pathways are known to interconnect, for instance, they both can upregulate WISP1, which was found upregulated in this study as well.[32]

Through the use of matched control tissue, the present study design minimizes interpatient variation and increases the likelihood of finding strictly disease-related changes. However, all tissue examined came from patients in an advanced stage of Dupuytren disease, which made them eligible for surgery. This makes it difficult to determine which of the changes

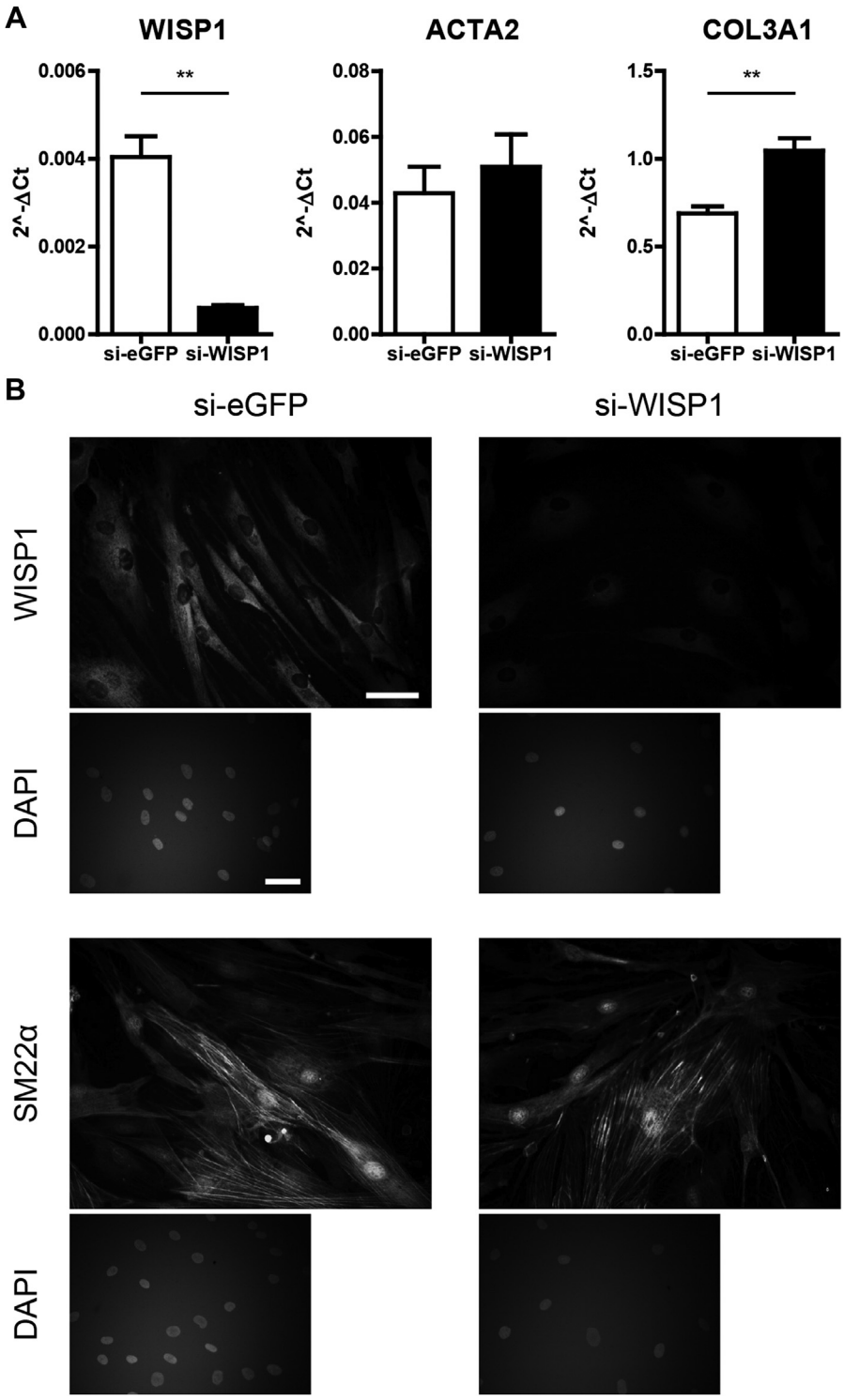

Fig 4. WISP1 function in Dupuytren fibroblasts. (**A**) Expressions of *WISP1*, *ACTA2*, and *COL3A1* in Dupuytren fibroblasts treated with siRNA against *eGFP* or *WISP1*. $^{**}P < 0.01$ as determined by the Student *t*-test. (**B**) Representative pictures of immunofluorescent staining for WISP1 (upper panel) and SM22α (lower panel) in the Dupuytren fibroblasts treated with siRNA against *eGFP* or *WISP1*. Scale bar denotes 50 μm, and smaller pictures show corresponding 4',6-diamidino-2-phenylindole (DAPI) staining. siRNA, small interfering RNA; WISP1, Wnt1-inducible secreted protein 1.

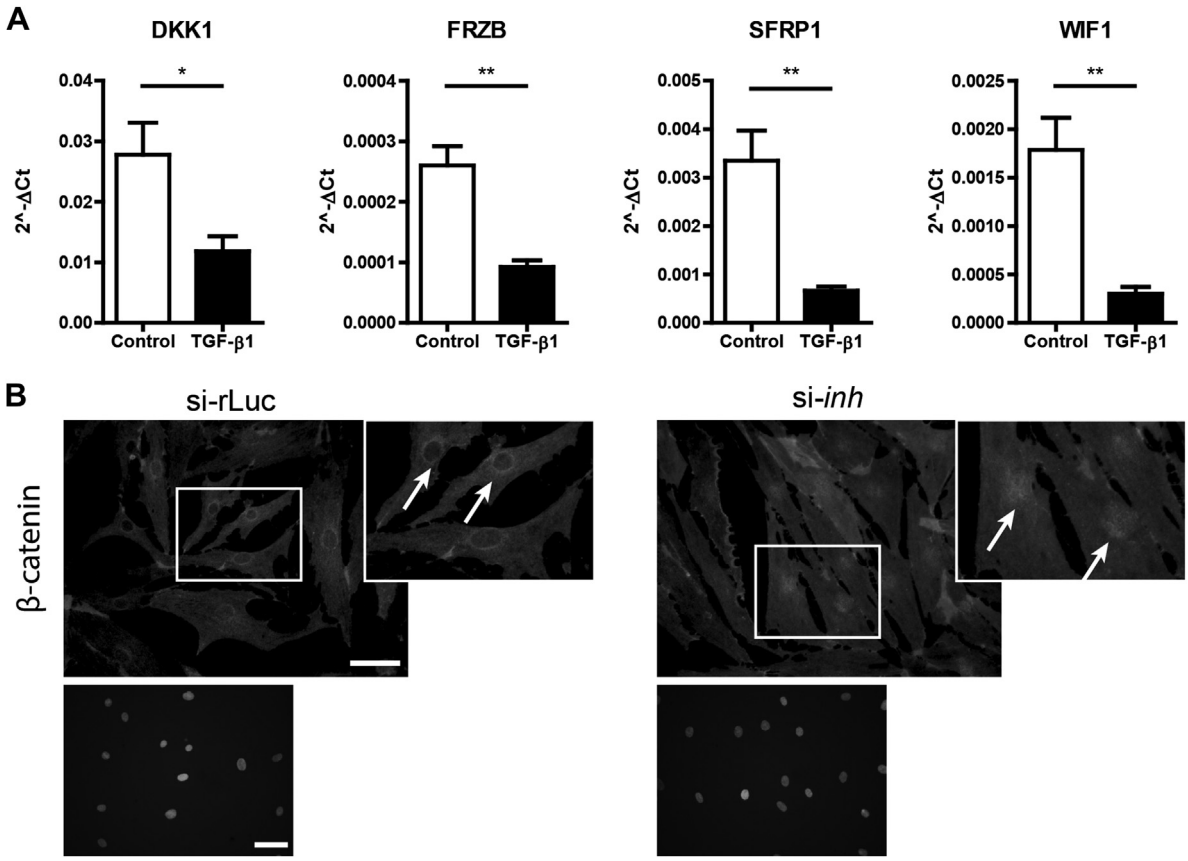

Fig 5. Endogenous antagonists of the Wnt pathway. (**A**) Expressions of *DKK1*, *FRZB*, *SFRP1*, and *WIF1* in HDF (N = 3) after treatment with 5 ng/mL of TGF-β1 for 72 hours. *$P < 0.05$, **$P < 0.01$ as determined by the Student *t*-test. (**B**) Representative pictures of immunofluorescent staining for β-catenin in HDF treated with siRNA against luciferase (left) and siRNA against *DKK1*, *FRZB*, *SFRP1*, and *WIF1* (right), after treatment with 1 ng/mL of TGF-β1 for 24 hours. Scale bar denotes 50 μm, arrows identify nuclei, and lower panel shows corresponding 4',6-diamidino-2-phenylindole (DAPI) staining. HDF, human dermal fibroblasts; TGF-β1, transforming growth factor β1.

found in this study are a cause of Dupuytren disease, and which are the consequence of extensive fibroproliferative changes induced by profibrotic pathways other than the Wnt pathway. Furthermore, although Dupuytren disease is restricted to the palm of the hand, the precursors of Dupuytren myofibroblasts may include cells from the local fat and dermis or circulating fibroblasts. Therefore, the control tissue in this study may behave differently than dermal fibroblast tissue from non-Dupuytren patients.

In view of the essential role of the Wnt pathway in development and homeostasis, it is a difficult target to modulate pharmacologically, because there are many possible adverse effects to such an approach. This study provides further insight into which parts of the Wnt pathway are dysregulated and as such can give us a better foundation toward a pharmacologic treatment for the Dupuytren disease, and possibly other fibrotic diseases, both by excluding targets and suggesting new. The present study implies that an attempt to inhibit Wnt signaling by inhibition of Wnt protein synthesis or secretion may not be the most effective treatment, because these are only moderately dysregulated, and in many cases even downregulated. Therefore, an attempt to normalize Wnt signaling by (locally) increasing its endogenous inhibitors, or influencing the noncanonical pathway, may well offer better therapeutic opportunities in the context of Dupuytren disease.

CONCLUSIONS

This study shows extensive dysregulation of the Wnt signaling pathway, both canonical and noncanonical, in nodules of Dupuytren patients compared with unaffected tissue of these patients. The study also raises the possibility of interaction between the canonical and noncanonical Wnt pathways, as well as cross talk with other profibrotic signaling pathways, such as the TGF-β pathway. Overall, the dysregulation leads to the activation of various profibrotic components of the

Wnt pathway, either by increased expression, such as components of the noncanonical pathway, or by a decrease in negative regulators, which could lead to excessive Wnt signaling.

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Conflicts of Interest: The authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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All authors have read the journal's authorship agreement and have reviewed and approved the manuscript.

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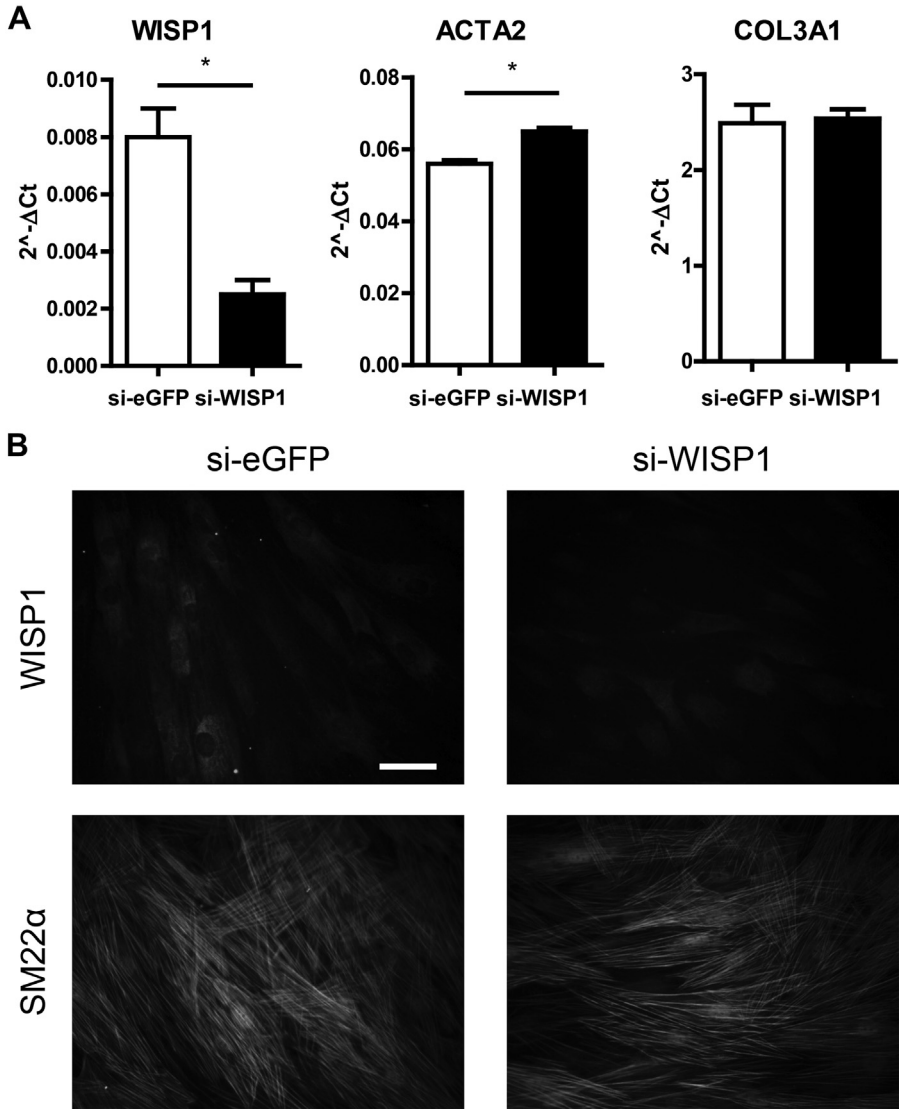
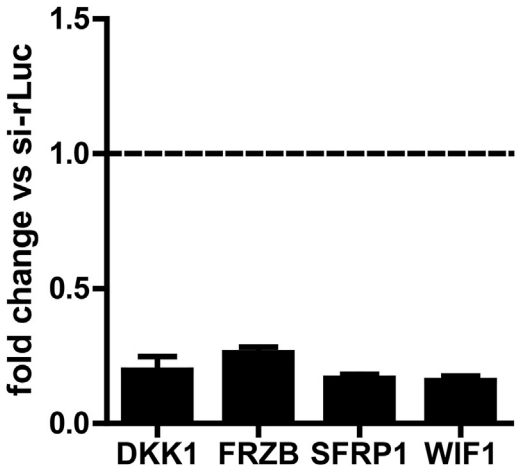
Appendix



Supplementary Fig 1. (**A**) Expressions of WISP1, ACTA2, and COL3A1 in HDF treated with siRNA against eGFP or WISP1. $*P < 0.05$, as determined by the Student *t*-test. (**B**) Representative pictures of immunofluorescent staining for WISP1 (upper panel) and SM22α (lower panel) on HDF treated with siRNA against eGFP or WISP1. HDF, human dermal fibroblasts; siRNA, small interfering RNA; WISP1, Wnt1-inducible secreted protein 1.



Supplementary Fig 2. Expressions of DKK1, FRZB, SFRP1, and WIF1 in HDF after treatment with combined siRNAs against DKK1, FRZB, SFRP1, and WIF1, normalized against siRNA against rLuciferase (control). HDF, human dermal fibroblasts; siRNA, small interfering RNA.

Supplementary Table I. Real-time qPCR primer sequences

Gene name	Forward primer	Reverse primer
ACTA2	ctgttccagccatccttcat	tcatgatgctgttgtaggtggt
COL3A1	ctggaccccagggtcttc	catctgatccagggtttcca
Daple	cgtagagctggccgacac	gctgctctgtcttatcctcca
DKK1	tgtttgtctccggtcatcag	tccatgagagccttttctcc
FRZB	aagtgccatgatgtgactgc	tgtcccgtggaatgtttacc
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc
Ror2	cccctcattaaccagcacaa	ttcccaaaccggtcctct
Ryk	cccaacaatgcaactcctatc	tcgttcttctctatccgcaag
SFRP1	gctggagcacgagaccat	tggcagttcttgttgagca
WIF1	ccagggagacctctgttcaa	ttgggttcatggcaggtt
WISP1	ctggcagcagtgacagca	ggagctggggtaaagtccat
YWHAZ	gatccccaatgcttcacaag	tgcttgttgtgactgatcgac

Supplementary Table II. Median expression levels and fold change of all genes analyzed in the Dupuytren nodule and matching control tissue

Gene name	Median control*	Median nodule*	N	Fold change	*P* value
APC	0.008	0.005	12	0.85	0.041†
AXIN1	0.019	0.015	12	0.98	0.814
AXIN2	0.022	0.026	8	1.19	0.327
BOD1	0.101	0.065	8	0.62	0.050†
BTRC	0.022	0.019	12	0.98	0.060
CALM1	0.033	0.018	8	0.72	0.123
CCND1	0.007	0.005	12	1.10	0.272
CCND2	0.140	0.150	12	1.10	0.695
CHSY1	0.008	0.031	8	3.77	0.012†
CSNK1A1	0.201	0.196	12	1.03	0.388
CTBP1	0.125	0.147	12	1.11	0.433
CTNNB1	0.084	0.094	12	1.27	0.010‡
CTNNBIP1	0.005	0.004	12	0.91	0.272
CXADR	0.001	8.8×10^{-5}	8	0.14	0.012†
CYP4V2	0.056	0.019	8	0.36	0.017†
DAAM1	0.079	0.053	12	0.71	0.028†
DAB2	0.055	0.065	8	1.13	0.208
DKK1	0.004	0.001	12	0.10	0.002‡
DKK3	0.138	0.276	8	2.46	0.036†
DVL1	0.049	0.039	12	0.80	0.034†
DVL2	0.008	0.010	12	1.13	0.433
EP300	0.042	0.042	12	1.20	0.182
FBXW11	0.040	0.046	12	0.98	0.875
FGF4	n.d.	n.d.	12		
FOSL1	0.002	0.007	12	2.85	0.008‡
FRAT1	0.004	0.003	12	0.63	0.028†
FRZB	0.027	0.004	12	0.11	0.003‡
FZD1	0.100	0.190	12	1.60	0.003‡
FZD2	0.002	0.004	12	2.63	0.003‡
FZD3	0.002	0.004	12	2.54	0.004‡
FZD4	0.158	0.041	12	0.29	0.002‡
FZD5	0.009	0.006	12	0.74	0.023†
FZD6	0.030	0.023	12	0.73	0.019†
FZD7	0.036	0.031	12	0.87	0.182
FZD8	0.046	0.049	12	1.32	0.117
FZD9	2.2×10^{-4}	3.3×10^{-4}	8	0.90	0.674
GSK3B	0.056	0.065	12	1.25	0.308
HSPA12A	0.040	0.016	8	0.41	0.025†
JUN	0.020	0.021	12	0.98	1.000
KREMEN1	0.026	0.076	12	2.55	0.003‡
LEF1	0.002	0.011	12	4.58	0.003‡
LRP5	0.012	0.007	12	0.54	0.019†
LRP6	0.066	0.033	12	0.54	0.003‡
MAPK8	0.050	0.050	8	1.02	1.000
MMP7	0.011	0.004	8	0.53	0.161
MT1A	0.019	0.011	8	0.60	0.093
MTFP1	0.001	0.004	8	2.55	0.123
MTSS1	0.015	0.008	8	0.55	0.017†
MYC	0.232	0.185	12	0.92	0.099
NAV2	0.006	0.002	8	0.45	0.036†
NFATC1	0.064	0.070	8	1.02	0.889
NKD1	0.005	0.008	12	2.42	0.019†
NLK	0.014	0.012	12	0.96	0.308
PITX2	n.d.	n.d.	12		
PORCN	0.017	0.017	12	1.04	0.530
PPARD	0.026	0.025	8	1.08	0.575
PRICKLE1	0.017	0.025	8	1.62	0.012†
PRMT6	0.003	0.002	8	0.69	0.069
RHOA	0.823	0.542	8	0.68	0.012†
RUVBL1	0.022	0.019	8	0.86	0.208
SFRP1	0.077	0.015	12	0.13	0.002‡
SFRP4	0.396	0.588	12	1.54	0.117
SLC9A3R1	0.016	0.010	8	0.58	0.012†
SOX17	0.003	0.002	12	0.57	0.272
TCF7	0.003	0.003	12	1.30	0.638
TCF7L1	0.031	0.016	12	0.47	0.003‡
VANGL2	0.006	0.022	8	2.53	0.050†
WIF1	0.008	8.9×10^{-5}	12	0.02	0.002‡
WISP1	0.021	0.109	12	6.62	0.002‡
WNT1	n.d.	n.d.	12		
WNT10A	3.0×10^{-4}	8.7×10^{-5}	12	0.27	0.012†
WNT11	0.004	0.001	12	0.14	0.003‡
WNT2	0.010	0.002	12	0.18	0.006‡
WNT2B	0.005	0.005	12	0.96	0.480
WNT3	0.002	0.002	12	1.55	0.034†
WNT3A	n.d.	n.d.	12		
WNT4	0.005	0.002	12	0.40	0.012†
WNT5A	0.005	0.010	12	1.78	0.117
WNT5B	0.014	0.010	12	0.86	0.433
WNT6	0.001	1.9×10^{-4}	12	0.30	0.010†
WNT7A	n.d.	n.d.	12		
WNT7B	2.0×10^{-4}	0.001	12	2.88	0.071
WNT8A	n.d.	n.d.	12		
WNT9A	0.008	0.007	12	0.89	0.182

n.d., no detectable expression in more than half of the patients tested.
*Median expression normalized to the geometric mean of 4 reference genes.
†$P < 0.05$ by the Wilcoxon paired rank test.
‡$P < 0.01$ by the Wilcoxon paired rank test.