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The Genetic Etiology Of Dupuytren’s Disease

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CHAPTER 8

Expression profiles of NEDD4 in Dupuytren's disease

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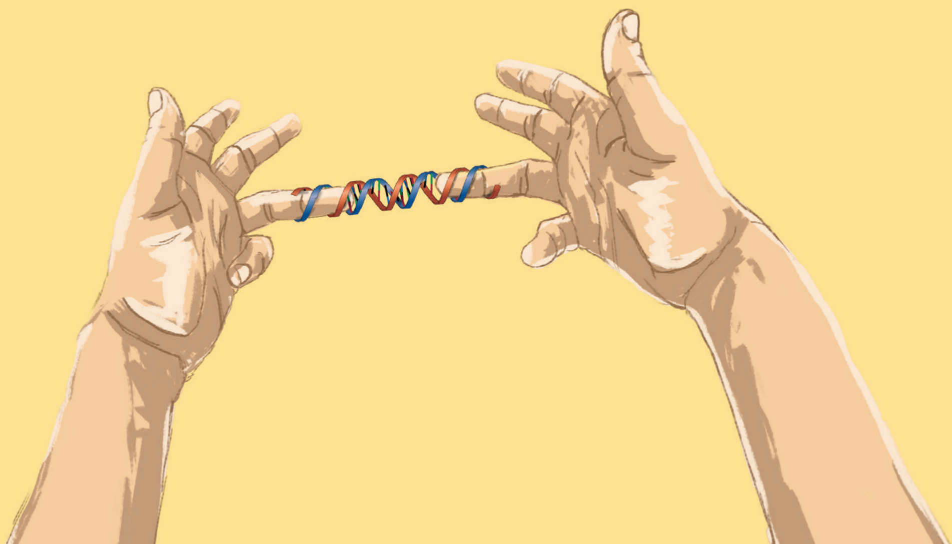
Preliminary work



ABSTRACT

Dupuytren's disease (DD) represents fibroproliferation of the palmar fascias. Similar to other fibrotic disorders, DD is characterized by myofibroblast proliferation and contraction, with excessive deposition of extracellular matrix components. Genome wide association studies for DD identified an intron variant (rs1509406) of the neural precursor cell expressed developmentally down-regulated protein 4 (*NEDD4*) gene. NEDD4 regulates cell proliferation and apoptosis in cancer and stem cells through multiple signaling pathways, including Hippo, Wnt, and TGF-β signaling, which are also involved in DD. Using Real-time (Taqman) qPCR we studied mRNA expression of six *NEDD4* protein-coding transcription variants (TV) and genes associated with fibrosis in myofibroblasts derived from DD tissue and normal skin (control), through means of TGF-β1 stimulation and *NEDD4* knock-down. We hypothesized that the intronic genetic variant in the *NEDD4* gene might cause differential expression of its TVs in myofibroblasts stimulated with TGF-β1. Although we indeed observed differential expression of *NEDD4* TVs in DD and control myofibroblasts, we were unable to draw conclusions concerning the function of *NEDD4* or its TVs in DD due to spurious results concerning the fibrosis-related genes in DD myofibroblasts. We furthermore newly determined basic expression of six *NEDD4* transcription variants in DD tissue (cords and nodules) and healthy palmar fascias, and found no significant differences in *NEDD4* TV-expression.

ABBREVIATIONS

α-SMA, α-smooth muscle actin; DD, Dupuytren's disease; ECM, extracellular matrix; EMEM, Eagle's minimum essential medium; esiRNA, endoribonuclease-prepared siRNA; FBS, fetal bovine serum; GWAS, genome-wide association study; NHDF, normal human dermal fibroblasts; TV, transcript variant; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; TGF-β1, transforming growth factor β1.

INTRODUCTION

Dupuytren's disease (DD) is a common inherited fibroproliferative disorder of the palmar fascias, affecting mainly individuals of Northern-European ancestry.(1) DD manifests as nodules or cords in the palm of the hand and fingers, and can considerably impair hand function. Treatment aims to dissect or excise diseased tissue; however, DD is incurable and recurrence rates are high.(2) Although the disease mechanism of DD is incompletely understood, it likely derives from a combination of genetic and environmental factors.(3) DD presumably originates with the pathologic activation status of myofibroblasts, a cell type that also plays a key role in other fibrotic disorders, including organ fibrosis.(4) Transforming growth factor β1 (TGF-β1) functions as one of the most potent activators of fibroblast differentiation into myofibroblasts.(5) Myofibroblasts express smooth muscle α-actin (α-SMA),(6) which enhances their contractile abilities. These cells synthesize excessive amounts of extracellular matrix (ECM) components, including collagens.(7)

The contribution of genetics to DD susceptibility (e.g. heritability) is large.(8) Genome wide association analysis (GWAS) have identified 26 single nucleotide polymorphisms (SNPs) associated with DD, but the biological mechanisms behind these signals remain elusive. Many lie within non-coding regions, but are presumably linked to variants in adjacent genes.(9) One SNP identified in GWASs, rs1509406 (chr15:55937562, GRCh38), is an intron variant of the *NEDD4* (neural precursor cell expressed, developmentally down-regulated protein 4) gene.(9) *NEDD4* encodes an E3 ubiquitin ligase that is involved in ubiquitin-mediated protein degradation.(10) *NEDD4* produces six protein-coding transcript variants (TV1-5 and 7), differing in the N-terminal C2 domain but being similar in the remaining protein (Supplementary Figure 1).(11) Ubiquitin-mediated proteasomal degradation is important in controlling post-translational expression of proteins.(12) It is a key regulatory node in proliferation, differentiation, and collagen secretion of skin fibroblast.(13) Literature on the function of *NEDD4* in fibrosis is scarce. NEDD4 is considered to be a potential mediator of chronic inflammation in keloids.(14) In cancer and intestinal stem cells, NEDD4 regulated cell proliferation and apoptosis through multiple signaling pathways, including Hippo and TGF-β signaling.(10,12,15,16) Recently, NEDD4 was unveiled to be a negative regulator of Wnt/β-catenin signaling: inactivation of NEDD4 increases Wnt activation,

enhancing tumor progression in the intestine.(17) The Hippo, Wnt, and TGF-β signaling pathways are all also involved in DD pathogenesis.(18–22)

Keloid, like DD, is a fibroproliferative disorder influenced by TGF-β1 production and Wnt-signaling.(18,23) Chung *et al.* demonstrated NEDD4 in keloid fibroblasts promoted fibroblast proliferation, upregulated type I collagen expression, and contributed to the excessive accumulation of extracellular matrix.(10) Nevertheless, the exact mechanism of NEDD4 in keloid remains unclear.(10,24) Since the SNP identified in the GWAS on keloid is an intron variant, Fujita *et al.* postulated this variant may influence alternative splicing of *NEDD4* mRNA and thus studied its six different TVs.(14,25) They found higher expression of TV3 in patients compared to controls, and inferred TV3 activates nuclear factor-κB (NF-kB), a protein complex that controls transcription of DNA, cytokine production, and cell survival.(14) A bioinformatic study, in turn, associated activation of NF-κB-inducing kinase activity with DD.(26) Several studies commend NEDD4 as a possible biomarker and therapeutic target for keloid.(10,14) Since DD and keloid are both fibrotic disorders, and GWAS studies of both diseases associated an intronic genetic variant located in *NEDD4* (9,25), we aimed to explore the expression and results of inhibition of *NEDD4* in DD, in particular the expression of its different mRNA TVs.

MATERIALS AND METHODS

Primary Dupuytren's tissue collection

DD tissues were obtained from patients during primary (initial) limited fasciectomy at the department of Plastic Surgery of the University Medical Center Groningen, the Netherlands. Informed written consent for this use of residual materials was obtained and tissues were processed anonymously.

Tissue samples

To asses baseline *NEDD4* RNA levels of unprocessed tissues, we snap froze DD nodules, cords, and unaffected palmar fascias in liquid nitrogen after resection. In preparation of RNA extraction, tissue chunks were first disrupted using glass beads in a Mini-BeadBeater-24 (BioSpec products, Bartlesville, USA), five cycles of 45 seconds.

Cell culture

All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. Fresh DD nodules were divided into pieces of approximately 5x5 mm and incubated on 6-wells plates for 45 minutes, followed by culture in Eagle's minimum essential medium (EMEM, BE12-662F; LONZA, Basel, Switzerland) containing 1% penicillin/streptomycin (ThermoFischer Scientific, Carlsbad, CA), and 2 mmol/L L-glutamine (Lonza), supplemented with 20% fetal bovine serum (FBS, Sigma Aldrich, Waltham, MA). Tissues were cultured until sufficient outgrowth of myofibroblasts was observed (approximately 50%). Subcultures were maintained in EMEM containing 1% penicillin/streptomycin, and 2 mmol/L L-glutamine, supplemented with 10% FBS until reaching 80% to 90% confluence, before starting experiments. Cells from all DD donors were used up to passage 9.

Normal Human Dermal Fibroblasts (NHDF) were purchased from Promocell (C12302, LOT# 41270292) and subcultures were maintained in EMEM containing 1% penicillin/streptomycin, and 2 mmol/L L-glutamine, supplemented with 10% FBS.

Transfections and treatments

For all experiments, unless specified otherwise, 10,000 cells/cm² were seeded on TCPS and kept in starvation medium (EMEM containing 1% penicillin/streptomycin, and 2 mmol/L L-glutamine, supplemented with 0.1% FBS and 0.17 mmol/L vitamin C (L-ascorbic acid 2-phosphate sesquimagnesium hydrate; Sigma-Aldrich) for 16 hours. As DD myofibroblast revert to a quiescent state during *in vitro* culture, subcultured cell lines were stimulated with 5 ng/mL TGF-β1 (PeproTech EC Ltd, London, United Kingdom) in starvation medium for 72 h. (27) NHDF were stimulated with 5 ng/mL TGF-β1 for the generation of myofibroblasts.

Sixteen hours after seeding, cells were transfected with endoribonuclease-prepared siRNA (esiRNA) against *NEDD4* (62.5 ng/mL; Sigma-Aldrich, St. Louis, MO) and a nonsense sequence was used (62.5 ng/mL). Transfections were carried out with Lipofectamine RNAiMAX (Invitrogen Thermo fisher) in complete growth medium free from antibiotics as per the manufacturer's instructions. Medium was refreshed 8 hours after the transfections and cells were starved overnight with starvation medium. Subcultures were stimulated with 5 ng/mL TGF-β1 as described above for 72 hours.

RNA extraction and Taqman qPCR

RNA was isolated for gene expression analysis using silica column technology (Favorgen Biotech Corp.). RNA quantity and purity were determined through UV spectrophotometry (NanoDrop Technologies, Wilmington, DE). RNA was reverse transcribed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time Taqman qPCR was performed with a set of primers (Thermo Scientific) and a FAM-MGB NFQ probe specific for each transcription variant of *NEDD4* (Supplementary Table 1). Real-time qPCR was performed using the Taqman Fast Advanced Mastermix containing 1.8 µM of each primer and 500 nM of the probe. The thermal cycling conditions were 2 minutes at 50°C, 20 seconds at 95°C (enzyme activation), followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C. The mRNA expression levels were normalized to the reference genes *GAPDH* and *YWHAZ*.

Real-time SYBR Green qPCR was performed to quantify mRNA levels of several genes involved in fibrosis (Supplementary Table 1). The thermal cycling conditions were 15 seconds at 95°C, 30 second at 60°C and 30 seconds at 72°C, for 40 cycles, followed by a melting curve analysis protocol. The mRNA expression levels were normalized to the reference gene *YWHAZ*.

Statistical analysis

All data were analyzed with GraphPad Prism version 8.03 (GraphPad Software, La Jolla, CA), using paired t-tests and two-way ANOVAs, and α-levels were Bonferroni-corrected for multiple testing.

RESULTS

Baseline RNA expression in tissues

As, to our knowledge, expression of *NEDD4* TVs has not been studied in DD tissue before, we aimed to quantify the baseline expression of the *NEDD4* TVs in freshly excised, unprocessed tissue. Patients genotypes for the GWAS SNP located in *NEDD4* were not known. Basic expression of the six *NEDD4* transcription variants was measured in cord, nodule, and control tissue of seven donors, others than the donors of the cultured cells. No significant differences in expression of any of the *NEDD4* TVs could be observed between cords, nodules, and control tissue (Figure 1). For only four of these patients, enough RNA could be extracted from

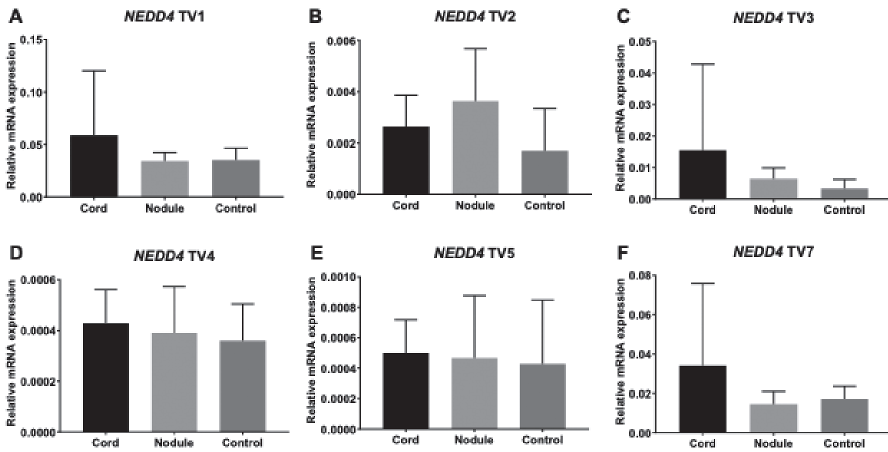


Figure 1. Expression of *NEDD4* TVs, in unprocessed cord, nodule, and control tissue from Dupuytren's patients. **A)** *NEDD4* TV1 is higher in cord than nodule or control. **B)** *NEDD4* TV2 is higher in nodule than cord or control. **C)** *NEDD4* TV3 is higher in cord than nodule or control. **D)** *NEDD4* TV4 has the same expression in cord, nodule and control. **E)** *NEDD4* TV5 has the same expression in cord, nodule and control. **F)** *NEDD4* TV7 is higher in cord than nodule or control.

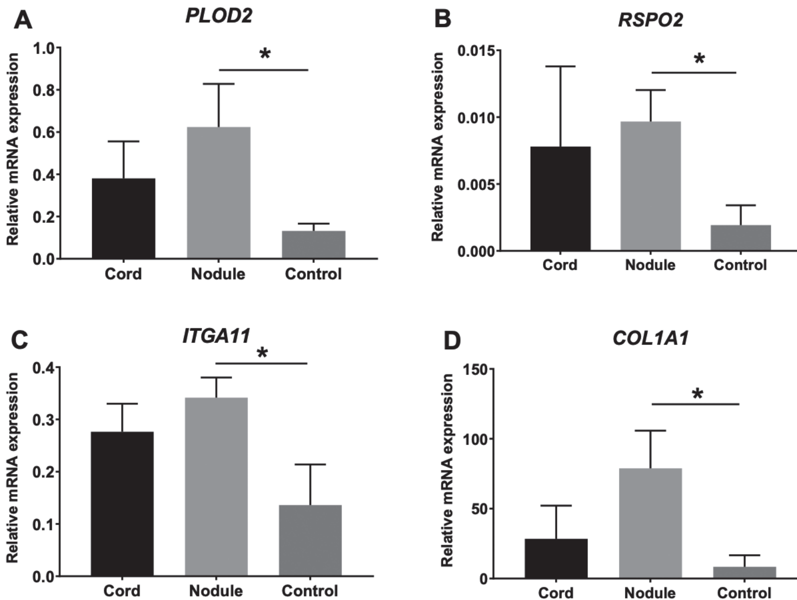


Figure 2. Expression of genes associated with fibrosis, relative to *YWHAZ*, significantly different between unprocessed cord, nodule, and control tissue from Dupuytren's patients. **A)** *PLOD2* is significantly higher expressed in nodule than control tissue. **B)** *RSPO2* is significantly higher expressed in nodule than control tissue **C)** *ITGA11* is significantly higher expressed in nodule than control tissue. **D)** *COL1A1* is significantly higher expressed in nodule than control tissue.

cord, nodule and control per patient, to determine expression of genes associated with fibrosis (Supplementary Table 1). For nodules vs. control tissue, significantly different expression of *PLOD2*, *RSPO2*, *ITGA11* and *COL1A1* was observed (Figure 2). Other genes did not significantly differ in expression.

RNA expression in cultured cells

To establish whether NEDD4 is involved in myofibroblast differentiation, DD myofibroblasts from four different donors were cultured on TCPS in the presence and absence of TGF-β1, after transfection with either esiRNA against *NEDD4* (siNEDD4) or nonsense esiRNA (siControl). Patients' genotypes for the genetic variant located in the *NEDD4* gene were available from our previous studies (Supplementary Table 2). (18)

TGF-β1 modulates NEDD4 transcript variant expression

First, the DD cell lines were cultured for 72 hours in presence and absence of TGF-β1. Taqman qPCR showed RNA levels of TV5 significantly increased, whereas TV7 significantly decreased (p=0.034 and p=0.024 respectively, Figure 3). Of the genes associated with fibrosis (Supplementary Table 1), SYBR Green qPCR showed *ACTA2* and *ELN* increased after stimulation with TGF-β1, and *WNT2* was significantly decreased (p=0.012, p=0.014 and p=0.007 respectively, Figure 4).

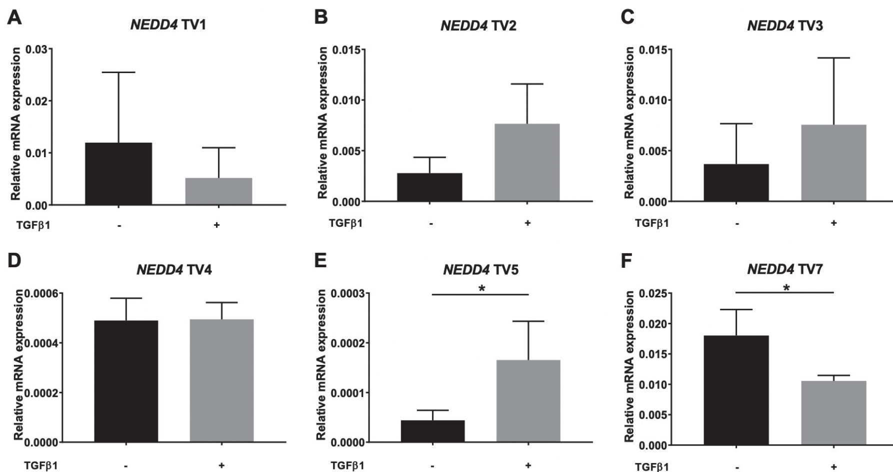


Figure 3. Expression of *NEDD4* TVs, relative to *YWHAZ*, in Dupuytren's cells cultured for 72 hours in the presence and absence of TGF-β1. **A)** Expression of *NEDD4* TV1. **B)** Expression of *NEDD4* TV2. **C)** Expression of *NEDD4* TV3. **D)** Expression of *NEDD4* TV4 **E)** Expression of *NEDD4* TV5. **F)** Expression of *NEDD4* TV7.

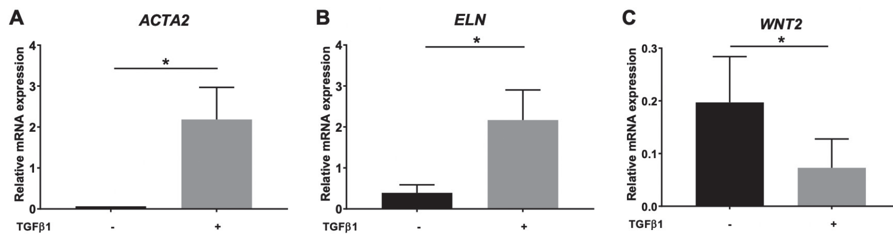


Figure 4. Expression of fibrosis-associated genes in Dupuytren's cells cultured for 72 hours in the presence and absence of TGF-β1, relative to *YWHAZ* expression. **A)** Expression of *ACTA2*. **B)** Expression of *ELN*. **C)** Expression of *WNT2*.

In NHDF, TGF-β1 stimulation significantly increased *NEDD4* TV2, TV3 and TV4 expression, but in contrast to DD cells, had no effect on TV5 or TV7 (Supplementary Figure 2). Similar to DD cells, *ACTA* and *ELN* expression increased after stimulation (Supplementary Figure 3). The decreased expression of *WNT2* in DD cells was not observed in NHDF. In addition, *COL1A1, COL3A1, FN, ITGA11* and *PLOD2* increased after stimulation (Supplementary Figure 3).

Combined transfection with siRNA and treatment with TGF-β1 was inconclusive

Taqman qPCR of in DD myofibroblasts treated with siRNA against *NEDD4* showed a decreased expression of all *NEDD4* TVs compared to nonsense RNA treatment. TV2 and TV4 expression were both significantly decreased in cells treated with siNEDD4 and stimulated with TGF-β1 (p=0.013 and p=0.036 respectively, Supplementary Figure 4B and 4D), whilst no significant differences were observed for TV1, TV3, and TV5 (Supplementary Figure 4A, 4C and 4E). TV7 showed a significant decreased expression after *NEDD4* knock down in unstimulated cells (p=0.007, Supplementary Figure 4D).

Considering the donors individually, in general, higher expression of *NEDD4* TV2-5,7 (but not TV1) were observed in DD cells from the donor homozygous for the risk allele of SNP rs1509406 located in the *NEDD4* gene (patient 3, Figure 5). The two donors who were heterozygous for that SNP (patient 2 and 4), showed intermediate expression of TV2-4,7, compared to patient 2 and the donor homozygous for the non-risk alleles of the SNP (patient 1). Patient 2 showed a higher expression for TV1 and TV5 than the other TVs. Patient 1 showed high expression of TV1, relative to the other donors, but lower for other TVs.

Expression of genes associated with fibrosis (Supplementary Table 1) was also measured with SYBR Green qPCR, but showed no significant differences in expression levels between cells treated with and without TGF-β1.

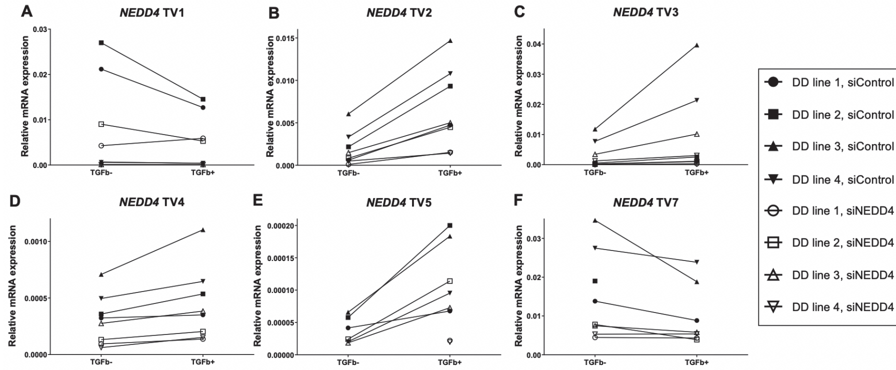


Figure 5. Difference in relative expression of the *NEDD4* TVs without and with TGF-β1 stimulation in cells transfected with siControl (filled shapes) and siNEDD4 (blank shapes), for DD line 1 (circle), DD line 2 (square), DD line 3 (pyramid), and DD line 4 (cone). **A)** *NEDD4* TV1. **B)** *NEDD4* TV2. **C)** *NEDD4* TV3. **D)** *NEDD4* TV4. **E)** *NEDD4* TV5. **F)** *NEDD4* TV7.

Interestingly, in DD cells only treated with TGF-β1, *SFRP4* expression increased, while DD cells transfected with siNEDD4 and treated with TGF-β1, *SFRP4* expression decreased, though both not statistically significant (Figure 6A and 6B). For *ELN*, with solely TGF-β1 stimulation, cells showed an increased expression (Figure 2B), but with simultaneous knock-down of *NEDD4* cells did not show a difference in expression (Figure 6C).

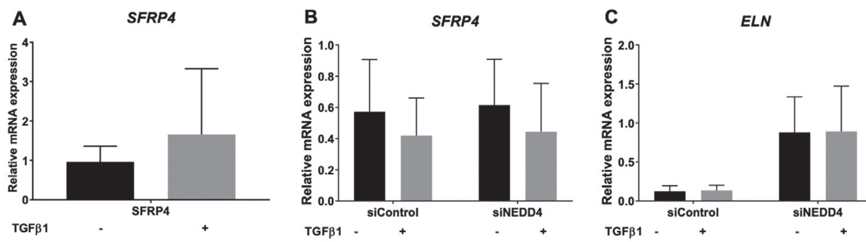


Figure 6. A) Expression of *SFRP4*, relative to YWHAZ, in cells only stimulated with TGF-β1. **B)** Expression of *SFRP4*, relative to YWHAZ, in cells transfected with siControl and siNEDD4 and stimulated with TGF-β1. **C)** Expression of *ELN*, relative to YWHAZ, in cells only stimulated with TGF-β1.

In NHDF, siNEDD4 transfection lead to significantly decreased expression of all *NEDD4* TVs (Supplementary Figure 5). Whilst none fibrosis-associated genes showed a significantly different expression in DD cells transfected with siControl or siNEDD4 stimulated with TGF-β1, NHDF showed significantly decreased expressions of *COL1A1*, *COL3A1*, and *SFRP4* between cells transfected with siControl and siNEDD4. *RSPO2* showed a significant increase in expression between siControl and siNEDD4, without TGF-β1 stimulation (Supplementary Figure 6).

DISCUSSION

In this work we set out to study *NEDD4* expression in DD, since a genetic variant within *NEDD4* (rs1509406) was associated to DD in a genome-wide association study.(9) As associated genetic variants are not necessarily causal themselves, but may be correlated to (in linkage disequilibrium with) causal genetic loci, functional investigations are necessary to study the relevance of the *NEDD4* gene in DD. Here we studied mRNA expression levels of the protein-coding transcription variants (TV) of the *NEDD4* gene in DD and control tissues and cultured cells. First, we assessed the baseline expression of *NEDD4* TVs in DD cords, DD nodules, and unaffected palmar fascias of seven donors. We found no significant differences in expression of any of the *NEDD4* TVs between tissues, but observed a wide variation in TV-expression. A possible reason for this non-significant finding is that the SNP in the *NEDD4* gene does not influence its mRNA levels but rather protein levels or function in DD. Another explanation is that due to the polygenic nature of DD, *NEDD4* potentially has multiplicative effects with other susceptibility genes not captured in this study design with its modest sample size. In addition, since cord, nodule, and control tissues were obtained from the same surgical sample, one could argue that the control tissue (i.e. macroscopically unaffected palmar fascia) adjacent to the affected tissue was in fact affected by DD microscopically, leading to non-significant *NEDD4* TV-expression differences. The significantly higher expression of type I collagen in nodule versus control tissue however indicates that the control tissue was indeed unaffected.(28) However, for keloids no significant difference in *NEDD4* mRNA expression was observed compared to normal skin, thus this might still be the case for DD.(29) To infer whether *NEDD4* expression does indeed not differ between DD and control tissues, as for keloids, the experiment sample size needs to be expanded.

In cultured fibroblasts, lower protein expression levels of NEDD4 have been observed for keloid than for normal skin.(10) However, a higher frequency of the high-risk allele of the keloid *NEDD4* SNP has been related to the selectively higher expression of TV3 in keloid fibroblasts.(25,30,31) In our study, we expected to see an increase in expression of *NEDD4* TVs in cells treated with TGF-β1. We observed an increased expression of TV5 an decreased expression of TV7 in DD myofibroblasts. In NHDF, we observed an increased expression of *NEDD4* TV2, TV3 and TV4. As expected after TGF-β1 stimulation, in both DD myofibroblasts and NHDF fibroblasts we observed a significantly increased expression of *ACTA2* (encoding *α-SMA*, is a known marker of myofibroblasts)(32–35). In contrast to NHDF fibroblasts, DD myofibroblasts did not show increased expression of type 1 and 3 collagens. This is not in line with previous work on cultured DD myofibroblasts, since collagen production is part of the hallmark features of myofibroblasts and TGF-β1 is a known regulator of collagen expression.(19,20,22) In addition, Chung *et al.* demonstrated that NEDD4 upregulated type I collagen in keloid fibroblasts, and Liu *et al.* observed that NEDD4 may promote the expression of type I collagen in mice fibroblasts.(36) These findings raise questions as to the validity of this DD myofibroblast experiment.

In the experiments including siNEDD4 knock-down in DD myofibroblasts, we observed a significant decrease in expression of only *NEDD4* TV2 and TV4 after knock-down. However, the increased expression of *ACTA2* and *ELN*, and decreased expression of *WNT2* that were observed in DD cells treated with TGF-β1 stimulation alone, was not similarly observed. Consequently, we were unable to draw substantiated conclusions from the experiments concerning DD cells treated with siRNA. We seemingly observed differential expression of certain TVs among the donors with different haplotypes for the risk allele of SNP rs1509406. Additional experiments with new cell lines (from other donors) are needed to infer conclusions concerning the functions of *NEDD4* and expression levels in relation to risk allele haplotypes. In NHDF, significant knock-down of all *NEDD4* TVs was observed. Stimulated myofibroblasts treated with siNEDD4 showed and significant decrease in mRNA expression of type 1 and 3 collagens, but not for *ACTA2*. This might indicate that knock-down of *NEDD4* may partially decrease fibrosis, but protein levels should also be studied for changes in expression, since these can differ largely from expression from their coding mRNA.(22) Although in

this work we attempted to study protein levels and cellular localization, the failure of acquiring a functioning antibody against NEDD4 prevented produce of results.

In this study, we postulated that the SNP in the *NEDD4* gene (rs1509406) was associated with increased expression of *NEDD4* in DD, and that as it is an intronic variant, it could affect expression of tis TVs via alternative splicing. A SNP can, aside from being causal themselves or correlated to causal SNPs, also be associated to gene expression.(37) After performing the work described in this chapter, we carried out a meta-analysis of genome-wide association studies and performed a follow-up transcriptome-wide association analysis (Chapter 5), which affirmed the association of *NEDD4* to DD (via SNP rs8032158) and indeed indicated that increased expression *NEDD4* is associated with DD. This further implicates the importance of understanding the mechanistical function of *NEDD4* in DD, but additional investigations are required to draw conclusions concerning the expression levels and functions of *NEDD4* TVs in DD.

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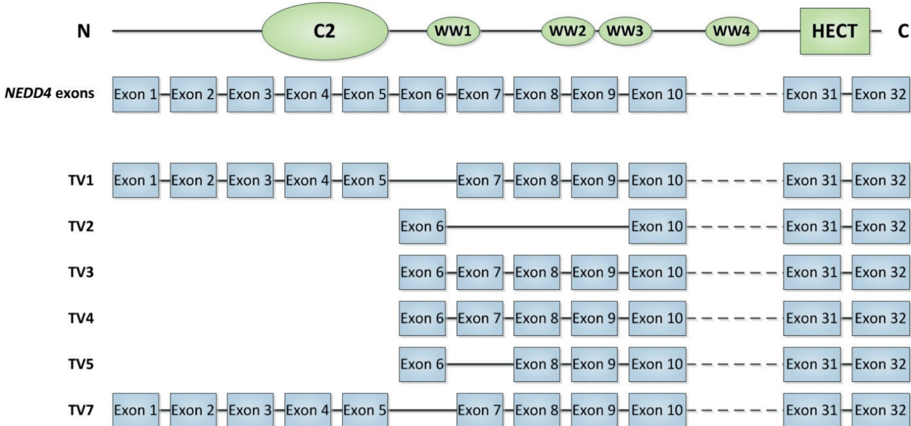
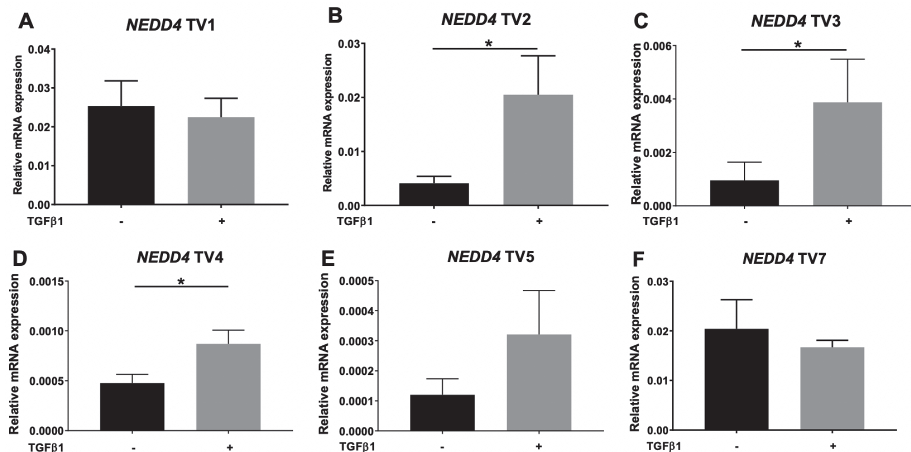
SUPPLEMENTS

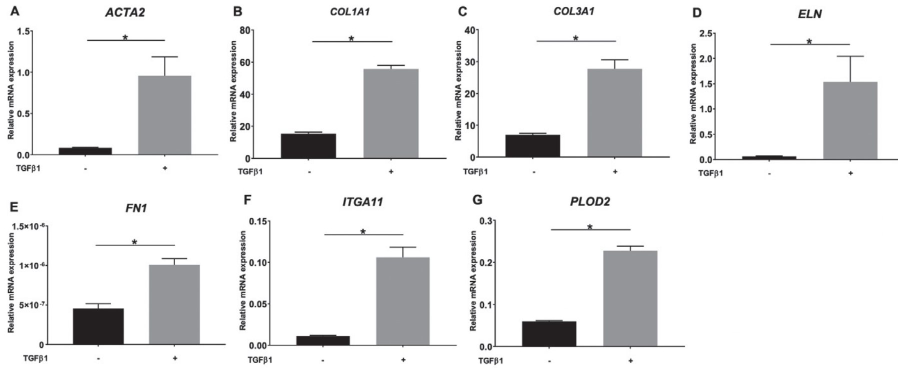
Supplementary figures



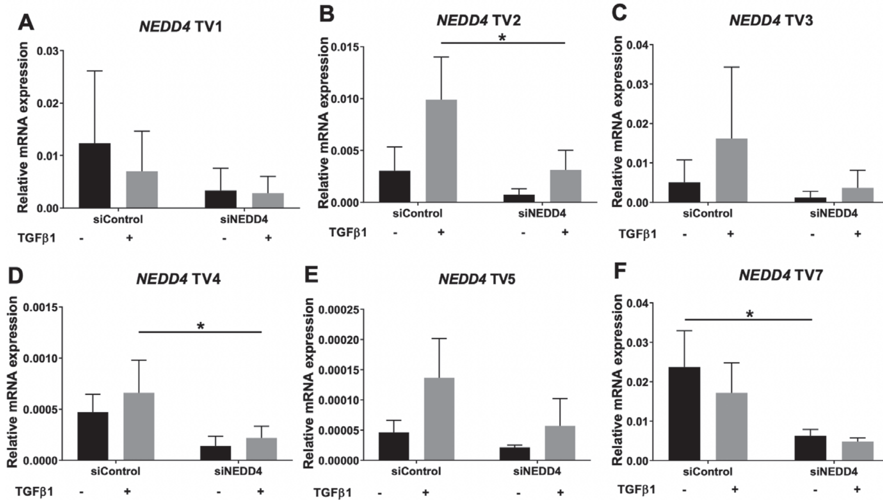
Supplementary Figure 1. Schematic overview of human NEDD4 protein-coding transcript variants (NCBI build 38, 2022). C2: Calcium/phospholipid biding domain, WW: Protein/protein interaction domains, HECT: Catalytic ubiquitin ligase domain.



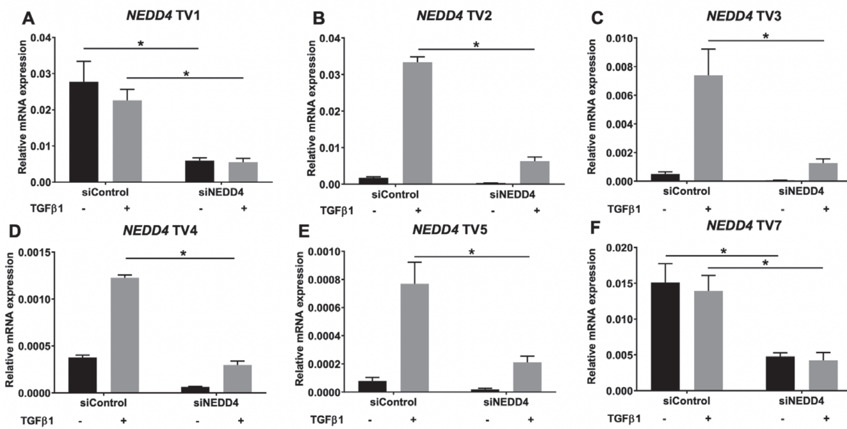
Supplementary Figure 2. Expression of *NEDD4* TVs, relative to *YWHAZ*, in NHDF cultured for 72 hours in the presence and absence of TGF-β1. **A)** Expression of *NEDD4* TV1. **B)** Expression of *NEDD4* TV2. **C)** Expression of *NEDD4* TV3. **D)** Expression of *NEDD4* TV4. **E)** Expression of *NEDD4* TV5. **F)** Expression of *NEDD4* TV7.



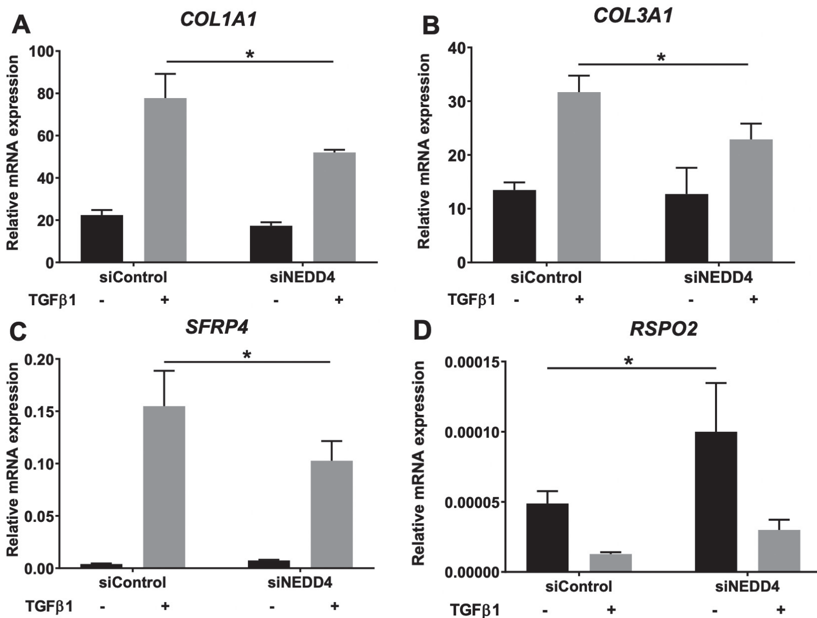
Supplementary Figure 3. Expression of fibrosis-associated genes in NHDF cultured for 72 hours in the presence and absence of TGF-β1, relative to *YWHAZ* expression. **A)** Expression of *ACTA2*. **B)** Expression of *COL1A1*. **C)** Expression of *COL3A1*. D) Expression of *ELN*. **E)** Expression of *FN1*. **F)** Expression of *ITGA11*. **G)** Expression of *PLOD2*.



Supplementary Figure 4. Expression of *NEDD4* TVs, relative to *YWHAZ*, in Dupuytren cells transfected with siControl or siNEDD4, cultured for 72 hours in the presence and absence of TGF-β1. **A)** Expression of *NEDD4* TV1. **B)** Expression of *NEDD4* TV2. **C)** Expression of *NEDD4* TV3. **D)** Expression of *NEDD4* TV4. **E)** Expression of *NEDD4* TV5. **F)** Expression of *NEDD4* TV7.



Supplementary Figure 5. Expression of *NEDD4* TVs, relative to *YWHAZ*, in NHDF transfected with siControl or siNEDD4, cultured for 72 hours in the presence and absence of TGF-β1. **A)** Expression of *NEDD4* TV1. **B)** Expression of *NEDD4* TV2. **C)** Expression of *NEDD4* TV3. **D)** Expression of *NEDD4* TV4 **E)** Expression of *NEDD4* TV5. **F)** Expression of *NEDD4* TV7.



Supplementary Figure 6. Expression of fibrosis-associated genes in NHDF transfected with siControl or siNEDD4, cultured for 72 hours in the presence and absence of TGF-β1. **A)** Expression of *COL1A1*. **B)** Expression of *COL3A1*. **C)** Expression of *SFRP4*. **D)** Expression of *RSPO2*.

Supplementary tables

Supplementary Table 1. Primer and probe sequences used in RT TaqMan and SYBR Green qPCR.

	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Probe (5′ to 3′)	PCR type
GAPDH	CTCCTCTGACTTCAACAGCGA	CCAAATTCGTTGTCATACCAGGA	Pre-designed	Taqman
YWHAZ	GATCCCCAATGCTTCACAAG	TGCTTGTTGTGACTGATCGAC	Pre-designed	Taqman
NEDD4 TV1	ATAGGCCTTGCCAAGAAGGATA	ACTTTGGATTCAAACTCTTTTTAATGG	CGTCACTCTCACGTAAGGATCACTAGCTCC	Taqman
NEDD4 TV2	GCATCTGGAAATGAAGGTAAAGTGG	TGGCAAGCAGCATCTGGTTG	GTCTAATTCTTGCAAGCCTGGCTGGGTTGT	Taqman
NEDD4 TV3	GGATAATTTATCAAGAGACAGCAACA	CAAAACAACCCAGCCAGGCT	ATCCATTACCGACAGAAAATCCAAGATTGGAGAGA	Taqman
NEDD4 TV4	TGAGGAATTAGAGCAACAACAAGAA	CATGGTTTACATAATAGGTCCTTCCA	ATATCCTGCCTCTCTTCCCACCCTGGAG	Taqman
NEDD4 TV5	TCTGGAAATGAAGGTAAAGTGGATAA	CTCCAATCTTGGATTTTCTGTCTTG	ACAGCAACAGAGATTGCACAAATGAACTGT	Taqman
NEDD4 TV7	ATAGGCCTTGCCAAGAAGGATA	AGTATAACTTTTTAATGGTTTTTGTTTGC	CGTCACTCTCACGTAAGGATCACTAGCTCC	Taqman
ACTA2	CTGTTCCAGCCATCCTTCAT	TCATGATGCTGTTGTAGGTGGT	-	SybrGreen
COL1A1	GCCTCAAGGTATTGCTGGAC	ACCTTGTTTGCCAGGTTCAC	-	SybrGreen
COL3A1	CTGGACCCCAGGGTCTTC	CATCTGATCCAGGGTTTCCA	-	SybrGreen
DDR2	ATTCCAGTCACCGACCACTC	ATTCCAGTCACCGACCACTC	-	SybrGreen
ELN	CGCTAAGGCAGCCAAGTATG	GGGACACCAACACCTGGA	-	SybrGreen
FN1	CT GGCCGAAAATACATTGTAAA	CCACAGTCGGTCAGGAG	-	SybrGreen
ITGA11	CTTTTCCTCGCACGTGGT	GCTCCATTCCAGTCATAGGC	-	SybrGreen
PLOD2	GGGAGTTCATTGCACCAGTT	GAGGACGAAGAGAACGC	-	SybrGreen
RSPO2	CCCACGTGCTAACCAAGC	CATCTCTCCGCCACGAAC	-	SybrGreen
SFRP4	GCCTGAAGCCATCGTCAC	CCATCATGTCTGGTGTGATGT	-	SybrGreen
WNT2	TTTGGCAGGGTCCTACTCC	CCTGGTGATGGCAAATACAA	-	SybrGreen
WNT4	GCAGAGCCCTCATGAACCT	CACCCGCATGTGTGTCAG	-	SybrGreen
WNT7B	CACAGAAACTTTCGCAAGTGG	GGCTAGGCCAGGAATCTTGTT	-	SybrGreen
YAP1	AATCCCACTCCCGACAGG	GACTACTCCAGTGGGGGTCA	-	SybrGreen
YWHAZ	GATCCCCAATGCTTCACAAG	TGCTTGTTGTGACTGATCGAC	-	SybrGreen

Supplementary Table 2. Patient genotypes for the *NEDD4* variant.

	Allele 1	Allele 2	Number of risk alleles for DD
Patient 1	A	A	0
Patient 2	G	A	1
Patient 3	G	G	2
Patient 4	G	A	1