RESEARCH HIGHLIGHT

Fibrosis: a novel approach for an old problem

Sofia Karkampouna[1], Marianna Kruithof-de Julio[2]

[1]*Department of Molecular Cell Biology, Cancer Genomics Centre and Centre for Biomedical Genetics, Leiden University Medical Center, Einthovenweg 20, 2333 ZC Leiden, The Netherlands*
[2]*Department of Dermatology, Leiden University Medical Center, Einthovenweg 20, 2333 ZC Leiden, The Netherlands*

Correspondence: Marianna Kruithof-de Julio
E-mail: m.de_julio@lumc.nl
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Dupuytren's disease is a fibroproliferative disease of the hand palm that results in permanent finger flexion and is the most common inherited condition to affect the body's connective tissue with a global presence of 3-6%. It was originally known as the Viking's disease and thought to be originated in the seventh or early eight century AD. In 1832 Baron Guillaume Dupuytren named and gave the first anatomo-clinical description of the disease. To date the causes underlying Dupuytren's disease are unknown and surgery remains the standard of care. Multiple recurrences are a common feature of this disease, following surgical removal of the primary fibrotic formations. Our research focuses on understanding of the molecular mechanisms that are at the basis of the progression of fibrosis. In our recently published manuscript we described how modulation of the TGFβ signaling pathway results in "reduction" of the fibrotic content of the disease. Dupuytren's resection specimens can now be maintained *ex vivo* providing a suitable model for preclinical screening of different classes of potential antifibrotic drugs; antisense oligonucleotides, chemical inhibitors, and miRNA- based therapies. Our findings indicate that decrease of collagen deposition, which is pathological characteristic of Dupuytren's, can be achieved by blocking the main regulatory pathway of collagen expression and remodeling. Short term gene expression modulation could have prolonged antifibrotic effects, even upon withdrawal of the modulatory factor (e.g. pharmacological inhibitor). Dupuytren's field of research is shifting direction from symptom-oriented studies towards molecular pathogenesis and gene expression "correction" attempts. Future studies shall focus on the molecular, epigenetic or immune modulation of key fibrotic stimuli in combination with current treatments with ultimate goal, to not only relief the primary symptoms, but mainly to prevent recurrence of the disease. Here, we discuss the applicability of *ex vivo* screening of potential pre-drugs on Dupuytren's-derived tissue for the treatment of various fibrotic diseases.

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Introduction

Fibrosis is a pathological state characterized by the excessive deposition of connective tissue commonly occurring during wound healing and tissue regeneration. It can affect most organ systems and lead to a variety of diseases including liver cirrhosis, pulmonary hypertension, systemic sclerosis and congestive heart failure, representing one of the major medical challenges of our time. To treat fibrotic disease, it is necessary to control the improper wound healing

mechanism and redirect it towards a pure regenerative/repair process.

Dupuytren's disease (DD) is one of the most common connective tissue disorders among Caucasians of northern Europe, with a higher prevalence in males [1, 2]. DD it was known also as the Viking disease on the assumption that the disease spread to Scandinavia and Europe during the Viking Age, although it has been proposed that the disease existed much earlier[3]. DD is a fibroproliferative disease affecting the palmar fascia, and may lead to permanent flexion contracture of the digits[4]. In affected individuals, DD-like fibrosis can also be found over the knuckles (Garrod's pads), feet (Ledderhose's disease) and penis (Peyronie's disease) [4]. Current treatment of DD is symptomatic; surgical removal of the fibrotic nodules and cords leads to immediate relief of the contractured digits. Injection of collagenase enzyme obtained from Clostridium Histolyticum, has been approved by FDA (Xiaflex, Pfizer) as alternative treatment for DD[5]. However, the recurrence rate of the disease using the current therapeutic approaches remains high.

Several environmental and genetic risks have been linked to DD supported by studies of familial cases, ethnicity and sex prevalence, occurrence in twins and postoperative recurrence[6]. However, the genetic mechanism is not fully understood and there is no evidence of a single genetic deregulation as a cause of DD. Thus, the aetiopathogenesis of DD remains unknown.

Design of novel anti-fibrotic therapies can only be achieved after thorough characterization of the disease profile. Our aim was to improve the current methodology available for DD fibrosis in order to better study the molecular pathogenesis. We have developed a method that allows *ex vivo* maintenance of human fibrotic tissue and characterization of patient-derived waste material in a cell-matrix orientated way. Fibroblast cell derivation remains the main method to study fibrosis; however it can only provide one-sided information excluding the influence of the microenvironment on cell behavior. The advantage of this system is that we circumvent the need for fibroblast cell derivation and instead we study the three-dimensional context of cells and surrounding extracellular matrix.

A complex set of genetic, immune response, epigenetic factors may lead to fibrosis by triggering constant activation of quiescent tissue fibroblasts to myofibroblasts (MFBs). MFBs are the key pathogenic cells in fibrosis. The cellular and molecular phenotype of MFBs is highly dependent on Transforming growth factor-β (TGFβ) signaling pathway [7, 8]. The canonical branch of TGFβ pathway involves interaction of TGFβ cytokines (TGFβ1, β2, β3) with the Activin receptor-like kinase 5 (ALK5) membrane receptor in fibroblasts and via ALK1 receptor in endothelial cells[9]. TGFβ pathway is known to be involved in fibrosis of many organ systems and anti-fibrotic strategies against TGFβ signaling-related molecules have been explored. Small molecule kinase inhibitors have proved very effective; however, they can target the kinase domain of multiple receptor kinases. For instance, SB-431542 blocks effectively the activity of ALK4, ALK5, ALK7 type I receptors activity [10], which could lead to adverse effects since all these receptors control TGFβ, BMP, Nodal signaling involved in many cellular processes. To limit unpredictable consequences of TGFβ inhibition we targeted exclusively the ALK5 receptor availability by antisense oligonucleotides (AON). AONs targeting the mRNA region encoding the ligand binding domain of the ALK5 receptor were successfully introduced in the *ex vivo* culture system.

The mechanism of TGFβ signaling

At the molecular level TGFβ signaling orchestrates wound healing response in most organ systems. If aberrantly regulated, it may lead to excess scar tissue formation, accumulation of collagen-producing cells and matrix and eventually disrupt normal tissue structure and physiology.

Extracellular TGFβ-family ligands (TGFβ1, β2, β3) internalize their signaling by binding to type II receptor (TβRII), which form heterodimers with type I receptor TβRI/ ALK5. This interaction activates the receptor's serine/threonine kinase activity to phosphorylate and activate SMAD transcription factors. Phosphorylation of receptor-activated SMADs (R-SMADs) by the receptor complex allows the R-SMADs to form heterodimers with partner SMADs (co-SMADs) and translocate to the nucleus where, in collaboration with transcription factor complexes, they activate or inhibit the transcription of target genes.

High TGFβ1, TGFβ2 mRNA and protein levels have been associated with DD fibrosis[7, 11, 12]. TGFβ2 shows intracellular localization within MFBs in the proliferative and involution stages of the disease[13, 14]. TGFβ target genes include extracellular matrix and cytoskeleton proteins that are often deregulated in DD

Table 1. Anti-fibrotic strategies targeting TGFβ signaling

Drug	Type	Target	Disease	Stage	Refs/Identifier
Trabedersen	Antisense oligo	TGFβ2	Glioblastoma, Pancreatic cancer	Phase II	[22]
Belagen-pumatucel-L (Lucanix)	Antisense oligo-mediated tumor cell vaccine	TGFβ2	Non-small-cell-lung-carcinoma	Phase III	[23, 24]
LY2382770	Small molecule	TGFβ1	Diabetic kidney fibrosis	Phase II	NCT01113801
Pirfenidone	Small molecule	TGFβ activity	Idiopathic pulmonary fibrosis	Clinic	[53]
P144	peptide	TGFβ1 binding to TGFβ type III receptor (β-glycan)	Skin fibrosis Systemic sclerosis	Phase II	[20]
CAT-192	Neutralizing antibody	TGFβ1	Systemic sclerosis	Phase II	[54]
GC-1008/ Fresolimumab	Neutralizing antibody	TGFβ1,2 ,3	glomerulosclerosis Melanoma, Renal c, glaucoma	Phase I	[18, 19], NCT01472731
Lerdelimumab / CAT-152	Neutralizing antibody	TGFβ2	Post-operative glaucoma	PhaseIII	[16, 17]

and other fibrotic diseases, such as PAI-1, COL1A1, COL1A2, COL4A2, COL5A1, COL5A2, ACTA2 [12, 15].

Targeting TGFβ receptors in fibrosis

Several components of the TGFβ pathway have been investigated for drug development. In the cancer field inhibition of TGFβ type I and type II receptors has been accomplished. However, in the fibrosis field most of the anti-fibrotic drugs are designed to interfere at the ligand level of pathway transduction, therefore preventing their binding to the receptors, thus interfering indirectly with receptor function. The most successful TGFβ inhibitory strategies used in experimental and clinical studies are the AONs targeting TGFβ ligand mRNA (Antisense Pharma), the competitive peptides against ligands (Digna

Biotech), the neutralizing antibodies against receptors and the small molecule inhibitors of receptor kinase activity (table 1). In particular, Lerdelimumab is a humanized antibody against TGFβ2, used in reducing fibrosis as postoperative treatment of glaucoma[16, 17]. Fresolimumab, also a neutralizing antibody, targets all three TGFβ ligands and is being tested for its anti-fibrotic and anti-cancinogenic potential. Single-shot treatment with Fresolimumab against glomeruloschelosis is in phase I [18], or in malignant melanoma and renal cell carcinoma [19]. A promising drug against renal fibrosis is LY2382770 antibody targeting TGFβ1 ligand, and it has successfully progressed to phase II of clinical trials. P144 TGFβ1-inhibitor has been specifically designed to block the interaction between TGFβ1 and TGFβ1 type III receptor, thus blocking its biological effects. It has shown significant anti-fibrotic activity in mice receiving repeated subcutaneous injections of bleomycin, a widely accepted animal model of human scleroderma [20].

Inhibition of TGFβ by AON approach

Antisense methodology has showed promising results in several disease applications, particularly in cancer. Interference with TGFβ1 production at the mRNA level by AON AP11014, developed by Antisense Pharma, significantly reduces TGFβ1 in prostate, lung and colon cancer cell lines[21]. TGFβ2 cytokine plays a key role in glioblastoma and pancreatic cancer. Trabedersen, interferes with TGFβ2 mRNA translation and has reached the phase III of clinical trials for glioblastoma treatment [22]. Another antisense TGFβ2 strategy has been developed for tumor vaccines

(Lucanix, NovaRx) [23, 24]. TGFβ2 AON sequence is transfected into lung cancer cells, which are used for anti-tumor vaccination. The vaccine has progressed into phase III clinical trials. Thus far, TGFβ receptors have been targeted for drug design by small molecular weight inhibitors of the kinase activity of the receptor or by monoclonal antibodies. As a potential anti-fibrotic therapeutic approach, key receptor ALK5 mRNA expression was depleted in cultured DD specimens by AON-mediated alternative splicing methodology [25].

Particular exon(s) encoding protein domains crucial for protein function can become excluded from the mature mRNA. Specific AONs bind to sites involved in exon splicing of a targeted exon and interfere with the splice machinery; therefore the particular exon is not integrated as part of the mRNA[26]. The resulting mRNA has an intact open reading frame and is translated into a protein that 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 occurs exclusively at the pre-mRNA splicing process. AON methodology has broad therapeutic applicability in many human diseases particularly in the field of muscular dystrophies[27] with very promising results reported for clinical trials[28, 29]. 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[30] have been developed and tested *in vivo*[31]. 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).

Ex vivo application of ALK5 AON showed efficient cell delivery in DD culture tissue and exon skipping. Expression of TGFβ-regulated fibrotic proteins such as COL1A1, COL3A1 and α-smooth muscle actin was down regulated in all patient-derived materials tested [25]. A hypothetical therapeutic setting for DD could be the administration of AONs prior to the surgical intervention or postoperatively, in order to counteract the TGFβ activity in the remaining MFBs. Early application of ALK5 AON could prevent destructive TGFβ action triggered by tissue damage during surgery, with consideration of the long term fibrotic effect that results from even brief exposure to TGFβ [32].

Novel *ex vivo* culture method of Dupuytren's fibrosis: preclinical translation for human anti-fibrotic drug screening

Although much work has attempted to unravel the complex mechanisms underlying fibrosis, the current state of the art in organ fibrosis research fails to meet the demanding need of treatment while organ fibrosis remains one of the major causes of death[33]. Cell culture models for studying fibrosis currently include primary cells and/or cell lines as well as the use of different culture matrices and co-cultures. For experimental reasons, connective tissue obtained from carpal tunnel operations is used for comparison to DD palmar fascia, and arbitrarily considered "healthy control" while it might be molecularly very similar to DD [34]. All these have led to one of the biggest limitations of the field, i.e. the lack of an *in vitro*/ *ex vivo* model that recapitulates the human disease and can be manipulated molecularly and genetically in a temporal manner. It is now clear that two-dimensional (2D) culture of fibroblasts has distinctly different properties and gene expression profile then the intact tissue[35, 36]. This can be, in part, attributed to the *in vitro* protocols and adaptation to the growth conditions. In our recently published DD study[25] we show that resected specimens, which are discarded as waste material after surgery, can be maintained viable in defined culture conditions *ex vivo*, in a model that recapitulates the human disease. This can provide us with useful information about the underlying patient-specific pathology and drug response and circumvent the use of carpal tunnel connective tissue that is currently used as a control; in our system the experimental comparison is done between different parts of tissue derived from the same patient (e.g. nodule versus cord).

DD tissue composition consists mainly of fibroblasts, MFBs, with fewer subset of endothelial, smooth muscle cells and immune cells[37]. ALK5 is expressed mainly in MFBs in DD, and not in endothelial cells, which express ALK1 [38]. In our system, the fibroproliferative properties of the disease have been hampered by ALK5 AON treatment, suggesting that partial reduction of the TGFβ signaling is sufficient to "reduce" the fibrotic content of the disease. One possible explanation of the strong anti-fibrotic effect of ALK5 AON observed in DD tissue may be attributed to the occurrence of "flywheel effect" upon downstream proteins. Although, the initial stimulus is removed (ALK5 exon skipping) there seems to be a continuous oscillation of downstream effects, such as protein expression of target genes (collagens, alpha smooth muscle actin). Another consideration is that full skipping is not the goal for the

treatment of DD especially since partial skipping already has beneficial effects of collagen deposition. Complete abrogation of ALK5 receptor activity might elicit compensatory mechanisms by other signaling pathways with no therapeutic benefit. Therefore a more subtle and titrable regulation is required, which is one of the major advantages of the AON approach.

A great advantage of this culture system is the candidate anti-fibrotic compounds screen approach; compounds can be tested for efficiency in human tissue at early stages of drug development. The tissue derived from Dupuytren's not only is ideal for the study of fibrosis, but also could serve as a more informative alternative to screens performed in human-derived, cell-based screens. We have tested two compounds Pirfenidone and Cis-4 hydroxy-L-proline [39]. The mechanism of action of Pirfenidone is not fully understood, it is thought to exert its effects by suppressing fibroblast proliferation, reducing the production of fibroblast-associated proteins and decreasing the response to growth factors such as TGFβ and platelet-derived growth factor. In our *ex vivo* model, treatment with increasing dose of Pirfenidone reduces overall, proliferation, αSMA and collagen type I expression. Higher doses induce apoptosis. Cis-4 hydroxy-L-proline is a compound that targets the production of L-proline rich proteins and critical L-proline residues. This compound has been shown to have no adverse effects on liver parameters such as ALAT, ASAT, γ-glutamyltransferase (γ-GT) and alkaline phosphatase (AP). However, in our *ex vivo* model treatment with Cis-4 hydroxy-L-proline does not reduce proliferation or have an effect on the expression of αSMA, at the different concentrations tested, suggesting it might not be as efficient in ameliorating the fibroproliferative disease profile. Performing drug screens on surgical waste, patient-derived tissue, instead of e.g. rodent models, could be an effective and quick way of drug categorization for further experimentation or dismiss, and thus it could reduce the number of animal studies.

Our current research is focused on understanding the role of the immune system in DD contracture and ways of altering the immune response to trigger matrix degradation. (Pre)clinical studies involving DD *ex vivo* culture method could potentially reveal immunological response triggered by candidate substances, and could be further studied in a different therapeutic context (e.g. not fibrosis necessarily) or focused in patient-specific immune responses. In this way, the selection of patients participating in clinical trials can be better monitored based on the immunological and fibrotic profile.

Use of *ex vivo* culture method of DD fibrosis for characterization of molecular pathogenesis

DD follows an epidemiological and familial spread typical of a genetic-driven disease, such as its high prevalence in north Europeans, particularly Norway, Iceland, and Scotland and in males over females [1, 2]. However, no single gene mutations have been found as disease determinants, thus the common perception is that DD is a multifactorial disease, driven by a combination of genetic and environmental stimuli. Our method can be used for studying the pathological factors driving excessive fibrosis in the nodular versus cord formation in fascia palmaris, focusing on chromosomal abnormalities and mutations in key fibrogenic pathways. For instance, chromosome 8 trisomy has been found in a subset of patients in the nodular DD tissue, however there are contradictory data obtained from patient derived fibroblast cultures [40]. Such events could be the leading cause of fibrosis or a late outcome of the MFBs, e.g. aberrant cell proliferation resulting in genetic abnormalities.

Although there is no evidence regarding DD being an X-linked inherited disorder, the incidence of disease is about 40% higher in males. Future studies may focus on sex-specific expression patterns or extracellular matrix composition that is differential between males and females and how this contributes to connective tissue fibrosis (DD, hypertrophic scar formation). For example, in the *ex vivo* cultured DD specimens we could investigate the response of male-female derived tissue to sex-specific hormones (testosterone, progesterone etc.) and assess whether the levels of these hormones is sufficient to influence fibrosis. Similar studies have shown a role of androgen receptor in the MFBs of the DD fascia palmaris suggesting that androgens stimulate the transition of fibroblasts to MFBs *in vitro* and the induction of αSMA protein expression [41]. It would be worthwhile to assess tissue response from a 3D angle in the *ex vivo* system that allows manipulation of the environment (nutrients, mechanical tension, cytokines and hormones) and is able to distinguish molecular differences among recurrent/ non recurrent or sporadic/ hereditary DD.

Future prospective on the field of fibrosis

During the last decades fibrosis accounts for up to 45% of deaths [42] in the developed world and yet there are no approved anti-fibrotic therapies available.

Extensive studies have led to the identification of many novel candidate genes that are being tested *in vivo* in single-species single-organ model of fibrosis. These limitations could be bypassed if we approach the fibrotic research from another angle, in a multi-organ manner, keeping in mind that the regulatory pathways that play a role in fibrosis may have lesser or greater effect between the organs, the species and the individuals [43]. Mehal *et al* suggest dividing the signaling pathways in those that are essential for conversion of an initial stimulus in the development of fibrosis, the "core" pathways, and ones that influence these pathways but cannot initiate the fibrosis itself, "regulatory" pathways [43]. The idea is that the core pathways are essential and common among fibrosis whereas the regulatory ones are organ specific.

Although, an increasing number of studies link fibrosis with inflammatory response there are several molecular pathways contributing to fibrosis, which might be distinct from the factors initiating the inflammation. A combinatory approach targeting inflammation, core and regulatory/ organ specific mechanisms is most likely to be clinically relevant for the treatment of fibrosis. In this regard, the use of the *ex vivo* DD fibrotic model as a drug screening method during early clinical development of anti-fibrotic and anti-inflammatory drugs is an appealing approach. Compounds targeting "core" fibrotic pathways implicated in multiple organs may have application, not exclusively for DD therapeutics, but also for other fibrotic diseases such as in hypertrophic scar formation, liver, lung, renal or skin fibrosis.

A novel class of anti-fibrotic targets is miRNA-based therapeutics. Multiple miRNAs are promising therapeutic targets and their potential use in fibrosis has given rise to a new research field exploring the role of fibromiRs [44]. miRNAs are short RNA oligonucleotides encoded by non-coding sequences (introns) or by intergenic sequences with their own promoter element. A single miRNA can have multiple targets in one or several signaling pathways and a single 3' UTR of an mRNA is targeted by several distinct miRNAs. FibromiRs are miRNAs implicated in gene pathways crucial for the progression of fibrosis, wound healing response or anti-fibrotic pathways. Deregulation of expression of certain miRNAs can have pro-fibrotic or anti-fibrotic effects depending on the molecular pathways that are affected.

One of the first studies attributing a role for miRNAs in fibrosis was performed few years ago, implicating deregulation of miR-29 and miR-108 in cardiac fibrosis after myocardial infarction [45, 46]. In addition, SMAD transcription factors of the profibrogenic TGFβ pathway have been found to control expression of several fibromiRs[47, 48]. During cardiac and skeletal muscle differentiation and development, miR-133a plays a crucial role. Because miR-133a levels decrease during cardiac hypertrophy, restoring miR-133a levels could reverse pathologic hypertrophy in the adult heart tissue [49, 50] as well as in liver fibrosis[51]. Similarly, in liver fibrosis, down-regulation of two over-expressed miRNAs, miR-27a and 27b allows culture-activated rat MFBs to switch to a more quiescent hepatic stellate cell phenotype [52]. Several miRNAs were uniquely identified in DD contracture samples, including miR-29c, miR-130b, miR-101, miR-30b, and miR-140-3p, and were linked to gene regulation of the Wnt signaling pathway components (WNT5A, ZIC1)[36].

"Correction" of fibromiR aberrant expression can be achieved by either miRNA inhibition (antagomiR) in case of an overexpressed miRNA with profibrotic effects or replacement therapy to stimulate the expression of a certain anti-fibrotic miRNA using synthetic miRNAs with the same sequence (miRNA mimics). Drugs targeting fibromiRs are under rapid development due to their advantageous, multi-target and multi-pathway effects. An appealing approach is the use of the DD *ex vivo* culture method for effectivity tests of fibromiR targeting drugs. Tissue and cell delivery of miRNA-based drugs, potential off target effects or toxicity can be investigated in a preclinical or early clinical stage in the DD model. We have showed that AONs with two different chemical modifications, Vivo morpholinos and 2'-O-methyl (2'-O-Me), are well delivered in tissue and do not cause cell death or toxicity. Common chemical modifications used for AONs are also applied in miRNA-based therapeutics (morpholinos, phosphor- rothiorate backbone, 2'-O-methyl sugar modifications). Since DD is a well characterized human fibrotic tissue with several molecular pathways contributing to its pathogenesis, *ex vivo* research on fibromiRs-based drugs in the DD may shed light not only on intracellular molecular signaling pathways but also on the changes or contribution of the extracellular space and fibrotic microenvironment.

Nevertheless, the pathogenesis of fibrosis can be triggered by a plethora of distinct genetic alterations which all lead to the same end point, thus it is crucial that the individual disease profile of each patient is not overlooked. The field of fibrosis could benefit from

involving a pharmacogenetic approach of drug screens during clinical or preclinical studies.

Conflicting interests

The authors have declared that no competing interests exist.

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