Getting a Grip on CD109's Role in Dupuytren's Disease: A Novel Molecular Target to Attenuate Digito-Palmar Fibrosis

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science (MSc) in Experimental Surgery

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TABLE OF CONTENTS

ABSTRACT

INTRODUCTION: Dupuytren's disease (DD) is a common fibroproliferative condition of the digito-palmar fascia that can lead to debilitating contractures and complete loss of hand function, and aberrant transforming growth factor (TGF)- β signaling is thought to play a critical role in its pathogenesis. Despite its high prevalence, an effective treatment for DD is yet to identified and targeting (TGF)- β signaling may be a promising approach. Transforming growth factor (TGF)- β is a pleiotropic cytokine that regulates a broad range of biological processes including cell growth, differentiation, extracellular matrix (ECM) deposition, and immune modulation. TGF-β signaling is transduced by a pair of transmembrane serine/threonine kinases known as TGF-β receptor type I and type II receptors, and TGF- β co-receptors such as CD109 are known to strongly regulate TGF-β signaling in a variety of cell types. CD109 is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein that strongly inhibits TGF-β signaling and responses. Although aberrant regulation of TGF- β signaling is known to play a key role in Dupuytren's disease (DD), the expression of CD109 in DD myofibroblasts and adjacent palmar fascia is largely unknown.

METHODS: The current study examined whether CD109 expression in primary human DD fibroblasts is different from genetically-matched phenotypically normal fibroblasts from the adjacent palmar fascia (PF), or genetically different and anatomically-matched normal fibroblasts from carpal tunnel (CT) syndrome patients. Immunofluorescent staining was utilized to elucidate distinct staining patterns and subcellular locations of

CD109 and other GPI-anchored proteins. In addition, CD109 gene expression levels were measured by qPCR and western blotting. Treating with MG132 was adopted to evaluate the effects of proteasomal inhibition on CD109 levels.

RESULTS: The results obtained suggest that CD109 mRNA expression is similar in DD fibroblasts, adjacent PF fibroblasts, and normal CT fibroblasts. However, CD109 protein levels were found to be decreased while alpha-smooth muscle protein levels were increased in DD fibroblasts and adjacent normal PF fibroblasts when compared to normal CT fibroblasts. In addition, the results indicate that the decrease in CD109 protein levels in DD is unlikely due to a global defect in glycosylphosphatidylinositol anchor synthesis, as the expression of another GPI anchored protein, CD58, remained unchanged. Furthermore, the addition of a proteasomal inhibitor (MG123) led to an increase in CD109 levels in DD, suggesting that CD109 is degraded via the proteasomal pathway in fibroblasts.

CONCLUSIONS: The observation that CD109 protein levels are decreased in DD fibroblasts and adjacent PF fibroblasts as compared to normal CT fibroblasts, may potentially provide a mechanistic explanation for the increased fibrotic response in DD. Furthermore, the generated results suggesting that CD109 protein levels are decreased while the CD109 mRNA levels remain unchanged, raise the possibility that CD109 turnover/degradation may be enhanced in DD. Together, these observations suggest that decreased CD109 function may play a role in increased fibrosis in DD fibroblasts.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

1. I have demonstrated that CD109 mRNA levels are not significantly different across heterogenous primary human samples of Dupuytren's disease myofibroblasts compared to Palmar Fascia and Carpal Tunnel cells.

2. I have introduced a potential mechanistic explanation for the increased fibrotic responses in Dupuytren's disease.

3. Introduced a 2.5 and 3-dimensional analysis approach to evaluate CD109 protein levels in Dupuytren's disease myofibroblasts cells. If validated for CD109 levels, it could potentially account for the aberrant overactivation of canonical TGF-β signaling observed in Dupuytren's disease.

4. I have demonstrated that Dupuytren's disease myofibroblasts do not exhibit a global constitutive down-regulation of glycosylphosphatidylinositol (GPI)-anchored membrane proteins.

5. Confirmed that DD fibroblasts exhibit a myofibroblast phenotype, expressing increased alpha-smooth muscle actin and also expressing increased endoglin (CD105).

PRESENTATIONS

Oral Presentations:

1- "*Saving the Handshake: Uncovering CD109's Distinctive Role in Dupuytren's Disease*" - Experimental Surgery Research Day 'Frontiers in Surgery'. Montreal/QC (November 2019).
2- "*Getting a Grip on CD109's role in Dupuytren's Disease*"- The European Tissue Repair Society (ETRS) Annual Meeting. Munich/Germany. (September 2019).
3- "*Targeting CD109 in Dupuytren's Disease*"- Wound healing Society (WHS) 2019 Annual Meeting. San Antonio/USA. (May 2019).
4- *"Getting a Grip on CD109's role in Dupuytren's Disease*". Plastic Surgery Annual Visiting Professor Day at McGill University. Montreal/QC (June 2019).
5- *Targeting CD109 in Dupuytren's Disease*". Plastic Surgery Annual Basic Science Research Talks. Montreal/QC (May 2019).
6- Plastic Surgery Visiting Professor Day (Dr. Thomas Mustoe) "Targeting TGF-beta signaling in Dupuytren's Disease". Montreal/QC (June 2018).
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- September 2019: Invited International Young Investigator: The European Tissue Repair Society (ETRS) 2019 Annual Meeting Munich/Germany.

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LIST OF FIGURES

INTRODUCTION

METHODS

RESULTS

DISCUSSION

ABBREVIATIONS

α-SMA	alpha-smooth muscle actin
ALK	activin receptor-like kinase
BMP	bone morphogenenic proteins
CT	Carpal tunnel fascia
DD	Dupuytren’s disease
DMEM	Dulbecco's Modified Eagle's medium
ECM	extracellular matrix
ERK	extracellular Signal-Regulated Kinase
FDA	U.S. Food and Drug Administration
FGF	fibroblast growth factor
GDF	growth and differentiation factor

GPI	Glycosylphosphatidylinositol
IF	immunofluorescence
JNK	Jun N-terminal kinases
LAP	latency-associated protein
LLC	large latent complex
LTBP	latent binding protein
MAPK	mitogen-activated protein kinase K
MMP	matrix metalloproteinase
MIS	Mullerian inhibiting substance
TSP-1	thrombospondin 1
PF	Healthy appearing palmar fascia from Dupuytren's patients

P-SMAD	phosphorylated SMAD
PI3K	phosphatidylinositol-3-kinase
QoL	Quality of life
qPCR	quantitative polymerase chain reaction
mRNA	Messenger ribonucleic acid
TGF-β	transforming growth factor beta
TGF-βR	transforming growth factor beta receptor
TNF	tumor necrosis factor

CHAPTER 1:
INTRODUCTION

1.1 Overview of Dupuytren's disease:

Dupuytren's disease (DD) was First noted by Felix Plater in 1614 and later documented in greater detail by the famous French surgeon Guillaume Dupuytrens, which is a common benign fibroproliferative condition of the digito-palmar fascia [1,2]. This localized fibrotic condition represents a common presentation in hand and plastic surgery clinics with a 7.3% estimated prevalence among US adults, which notably increases with age, along with an observed discrepancy in male to female incidence approaching 6:1 that decreases to approach parity with increasing age [3,4]. Northern European or Nordic descent is reported to be strongly associated with DD, to the extent that some researchers estimated DD's prevalence in Iceland to be as high as 40% in males over the age of 70 [5]. Therefore, as populations age, the demand for novel therapeutics that could counter or minimize the fibrotic processes in DD will only continue to raise and heighten[6-8].

This fibroproliferative condition of the palmar fascia often leads to permanent debilitating hand contractures with a substantial deleterious impact on patients' quality of life (QoL) [9]. Despite growing interest in DD research, a single causative gene or transcription factor has not yet been identified. In addition, the molecular pathophysiology of DD is thought to be multifactorial, however, only limited progress has been made in understanding the molecular instigators, regulators and pathophysiological modulators of fibrosis in DD [10]. Therefore, significant investment in research aiming to unravel molecular signaling pathways in DD is warranted and could facilitate breakthroughs that may ultimately rescue and save tens of millions of hands -if not more- from this fibrotic condition.

Murrell et al hypothesized that local ischemia and oxygen radicals are the culprits behind DD and suggest that an up-regulation of collagen synthesis and downregulation of genes responsible for its enzymatic breakdown leads to the observed increase in type III/I collagen ratio [11]. Hand exposure to vibration and manual work have also been postulated to be involved in the pathogenesis of DD [12].

Gabbiani and Majno, in their ultrastructural study, identified the myofibroblast- a type of cell which shares morphological features with both fibroblasts and smooth muscle cells- as the "pathogenic cell" responsible for the digito-palmar contractures experienced in DD [13]. Proponents of cellular plasticity suggest that multiple cell types including resident fibroblasts and mesenchymal stem cells differentiate to become myofibroblasts in DD[14]. The percentages of myofibroblasts in DD versus control patients were reported to as high as 25 folds higher than the controls[15].

Dupuytren's disease presents as two phenotypes, first as nodules and then progresses to cords[16]. DD has histologically classified it into proliferative, involutional, and residual phases[17]. The nodules are a hallmark of the proliferative phase of the disease and are more pro-fibrotic, but express lower levels of collagen type I, however, cord tissue contains more heavily crossed linked collagen type I [18].

Despite previous efforts to expand our understanding of the fibrotic process in DD, there is a current lack of non-invasive, FDA-approved therapies for this recurrent fibrotic condition. Current surgical treatment options are not always effective, due to high recurrence rates in addition to difficulties that routinely arise post-intervention including potentially burdensome rehabilitation periods. The recurrence rate of DD after needle

aponeurotomy, collagenase injection or fasciectomy – the three mainstream surgical interventions- range between 21% to 84.9%[19].

1.2 Overview of TGF- β signaling:

TGF-β is a multifunctional cytokine that regulates a wide array of biological processes ranging from promoting cellular differentiation and growth to extracellular matrix (ECM) deposition and modulating immune responses[20-22].

TGF- β signaling is mediated by a pair of transmembrane serine-threonine kinase receptors known as type I Receptor (TβRI), and Type II receptor (TβRII). TGF- β first binds TβRII, a constitutively active kinase, which in turn phosphorylates TβRI, resulting in its activation [23-25]. TβRI, also known as activin receptor-like kinase (ALK5) subsequently phosphorylates intracellular Smad2 and Smad3 proteins which form a complex with Smad4, and accumulate in the nucleus leading to regulation of gene expression in concert with transcriptional co-activators and co-repressors [26,27].

In certain cell types, TGF- β can also signal via the non-canonical ALK1/Smad1/5 pathway, which opposes the ALK5-Smad2/3 signaling[28-33]. Furthermore, TGF-β is able to activate 'non-Smad' pathways including mitogen-activated protein (MAP) kinases (ERK, p38 and JNK), phosphatidylinositol-3-kinase (PI3K) and Rho-like GTPases in a cell-type-specific manner[34-36]. These other pathways can either synergize or antagonize TGF- β/Smad signaling.

TGF family superfamily and TGF-β isoforms:

The TGF- β superfamily contains many members. They are structurally and functionally related cytokines, that signal through a similar pair of serine/threonine kinase

receptors, and ultimately activate intracellular Smad transcription factors to mediate downstream signaling events.

This superfamily is divided into two subfamilies, the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF)/ Mullerian inhibiting substance (MIS) subfamily and the TGF- β /activin/Nodal subfamily. This classification and division are based on ALK binding and the activation of Smad transcription factors [37].

The mammalian TGF-β family includes three different subtypes or isoforms (TGF- β1, -β2, and -β3) that share high (~75%) sequence and structural similarity but perform distinct functions *in vivo*[38,39]. Each TGF- β isoform has relatively specific, functions *in vivo*.

Multiple cell types are involved in the production of TGF-β isoforms, but TGF-β1 is the most abundant isoform in adults[40 41]. TGF- β1 isoform is one of the most pro-fibrotic cytokines known and plays a central role in scar formation. TGF- β2 also shares those pro-scaring effects. On the other hand, TGF-β3 has anti-scarring effects. Exogeneous TGF-β3 has been shown to promote scar-free wound healing in rats. It is also believed to be responsible for scar-free wound healing for in utero [42,43]

Regulation of TGF- β:

Furin-Like enzymes cleave synthesized pro-TGF-β into a mature TGF- β dimer and a pro-peptide known as latency-associated peptide (LAP). The cleaved mature TGF-β and LAP remain non-covalently bound together forming the inactive small latent complex (SLC)[44]. This SLC is gets covalently linked to an ECM component, latent TGF-β binding protein (LTBP), forming the secreted large latent complex (LLC) which is

associated with ECM [45,46]. All three TGF- β isoforms exist in those latent complexes[47,48]. *In vivo* activation of Latent TGF- β is mediated by thrombospondin 1 (TSP-1), integrins, MMPs, and plasmin, and in vitro by acidic or alkali conditions, heat denaturation, or shear stress.[49-52]. After their activation, TGF- β isoforms exist as homodimers that are stabilized by a disulfide bridge and hydrophobic interactions.

Latent TGF- β activation may represent an important regulatory mechanism that controls TGF- β bioavailability and action in DD. This could be an interesting direction for future studies on signaling pathways in DD.

TGF- β co-receptors:

Betaglycan (TβRIII), endoglin (CD105), and CD109 are three TGF- β co-receptors. Their accessory role is important despite the fact that they lack any signaling or enzymatic activity. Structurally, Endoglin and betaglycan are very closely related[53,54], but despite the similarity, these co-receptors differ in their ligand-binding ability and expression.This is further elucidated by the fact that Betaglycan has ability to bind all three TGF-β isoforms with a high affinity [55].On the other hand, endoglin binds TGF- β1 and TGF- β3 in the presence of TβRII, but does not bind TGF- β 2 [56]. The differences between betaglycan and endoglin extend to their mechanism of signaling. Betaglycan will promote TGF- β signaling while endoglin inhibits TGF-β/ALK5/Smad signaling, among other mechanisms [57]

The TGF- β co-receptor, CD109, is a glycophosphatidylinositol-anchored protein with the capacity to bind TGF- β ligand and inhibit TGF- β signal transduction. CD109 can bind TGF- β1 with a high affinity and also forms a complex with TβRI, TβRII, and

betaglycan [58-60]. The inhibitory effect of CD109 has been shown to involve the internalization of TGF- β receptors and enhancing receptor degradation, especially TβRI degradation[61].

The role of various co-receptors in DD is largely uncharted. Given their important roles in modulating TGF- β signaling, further research is needed on the potential effect and role of various TGF- β co-receptors in DD.

1.3 TGF- β signaling in Dupuytren's disease:

The Central Role of the aberrant activation of TGF-β signaling in Dupuytren's disease:

Many growth factors and cytokines have been implicated in the pathogenesis of DD with TGF-β being the most well studied of them all. The preliminary demonstration of the presence of TGF- β1 in the nodules (early stage) but not in the cords (late-stage) of DD and the lack of TGF- β staining in normal fascia has identified TGF- β as a potential pathogenic factor in the development of DD [62]. Badalamente et al further illustrated the role of TGF- β in DD, where they demonstrated intense immunohistochemical staining for TGF- β1 in all 47 DD samples used[63]. However, the lack of normal control in this study to compare the staining patterns with DD samples limits the validity of their findings.

In a more recent study, it has been observed that the expression of α-SMA was significantly more expressed in Dupuytren's fibroblasts than in control fibroblasts[15]. Furthermore, TGF-β1 has been shown to promote the proliferation of Dupuytren's myofibroblasts from the same patients along with the increased expression of α-SMA [64]. TGF-β1 treatment has also been shown to lead to increased contraction of fibroblast-

populated collagen lattices (FPCL) in both Dupuytren's and normal fibroblasts[65]. Furthermore, TGF-β was shown to promote to increase Smad2 phosphorylation and expression of Alpha-actin-2, Collagen Type I, and Collagen Type III in Dupuytren's fibroblasts[66].

Other studies have demonstrated that TGF- β1 increased the expression ECM components, including fibronectin and collagens, and reduced their degradation by down-regulating the expression and activity of matrix-degrading enzymes and increasing the expression of protease inhibitors in normal keratinocytes and fibroblasts [67]. Thus, the upregulation of collagen synthesis and downregulation of its enzymatic breakdown has been proposed to be the mechanism underlying the development of DD[11], and suggest that TGF- β could represent a promising therapeutic target for this fibrotic condition.

Role of different TGF- β isoforms in Dupuytren's disease:

Given the distinct roles and effects of each TGF-β isoform, it would be interesting to know if their expression correlates with DD pathogenesis. Using mRNA expression in primary Dupuytren's fibroblasts, it was observed that TGF- β1 and TGF- β3 isoforms were significantly upregulated. However, TGF- β2 mRNA expression was found to be significantly decreased using real-time PCR analysis[15].

SMAD- dependent signaling in Dupuytren's disease:

The upregulation of TGF-β/Smad signaling is an important feature of DD [68]. The significantly upregulated level of Smad2, Smad3 and phospho-Smad2 in DD compared to the normal control tissue suggests the involvement of this signaling pathway. The expression levels of phospho-Smad3 and Smad1 were found to be comparable in both

DD and normal control samples, while phospho-Smad1 was undetectable[15]. The role of the Smad dependent signaling in DD is further underscored by the inhibition of the TGF-β1R1(ALK5)/Smad2/3 interaction by a small molecule activin receptor-like kinase (ALK)5 inhibitor SD208, leading to a decrease in α-SMA and collagen type I gene expression and α-SMA protein levels[69]. In addition SB-431542, a selective inhibitor of ALK4, ALK5 and ALK7 kinase activity [70], had a strong inhibitory effect on α -SMA expression and collagen contraction in Dupuytren's fibroblasts which further demonstrates the role of TGF- β signaling in DD [15]. Together, these results demonstrate that TGF-β/ Smad signaling is increased in this fibroproliferative disease, and that TGF-β/Smad- signaling may play an important role in the development of DD.

Role of other members of TGF- β superfamily in Dupuytren's disease:

Regarding the role of BMPs, one of the earlier studies pointed out the lack of expression of BMP-4 in Dupuytren's fibroblasts compared to normal fibroblasts. A decrease in the expression of BMP6 and BMP8 and the downstream mediator Smad1was also noted[71]. Krause et al further shed the light on the role of BMP6 by demonstrating the inhibitory effect of BMP6 on Dupuytren's fibroblasts, specifically on TGF- β1 and TGF-β3 mRNA expression along with a reduced Smad2 and Smad3 mRNA expression. Interestingly, BMP7 which was proposed as an antagonist of organ fibrosis [72] did not exhibit an inhibitory effect on Dupuytren's fibroblasts[15].

*SMAD-independent signaling in Dupuytren's disease (*P38 MAPK signaling pathway):

SMAD independent signaling pathways have also been shown to participate in TGF-β signaling[73]. The activation of MAPK kinase signaling pathway has been shown to be associated with DD[74]. Moreover, Increased levels of phosphorylated ERK1/2 were detected in Dupuytren's samples. However, It is not known if this a direct effect of TGF-β3 or through the induction of PDGF-B and/or PDGF-A. Interestingly, phosphorylated P38 and JNK were not detectable [15]. In addition, inhibition of this signaling pathway using SB203580, a p38α and p38β MAPK Inhibitor lead to a decrease in α-SMA expression which further illustrates a role for SMAD-independent signaling in DD[75].

Wnt signaling pathway in Dupuytren's disease:

The fibrotic signaling in ED has also been shown to be mediated through canonical Wnt signaling. In their genome-wide analysis, Dolmans et al, elucidated the role of Wnt signaling in DD. Out the nine loci that have been found to be associated with susceptibility to DD, Wnt signaling pathway genes were found in six of the nine loci[76]. In addition, it has been proposed that TNF- α activation of Wnt/β-catenin signaling pathway plays a role in DD[69]. TNF selectively converted normal fibroblasts from the palm of DD patients into myofibroblasts via activation of the Wnt signaling pathway, and blockade of TNF resulted in the reversal of the myofibroblast phenotype. Therefore, TNF inhibition may prevent the progression or recurrence of Dupuytren's disease.

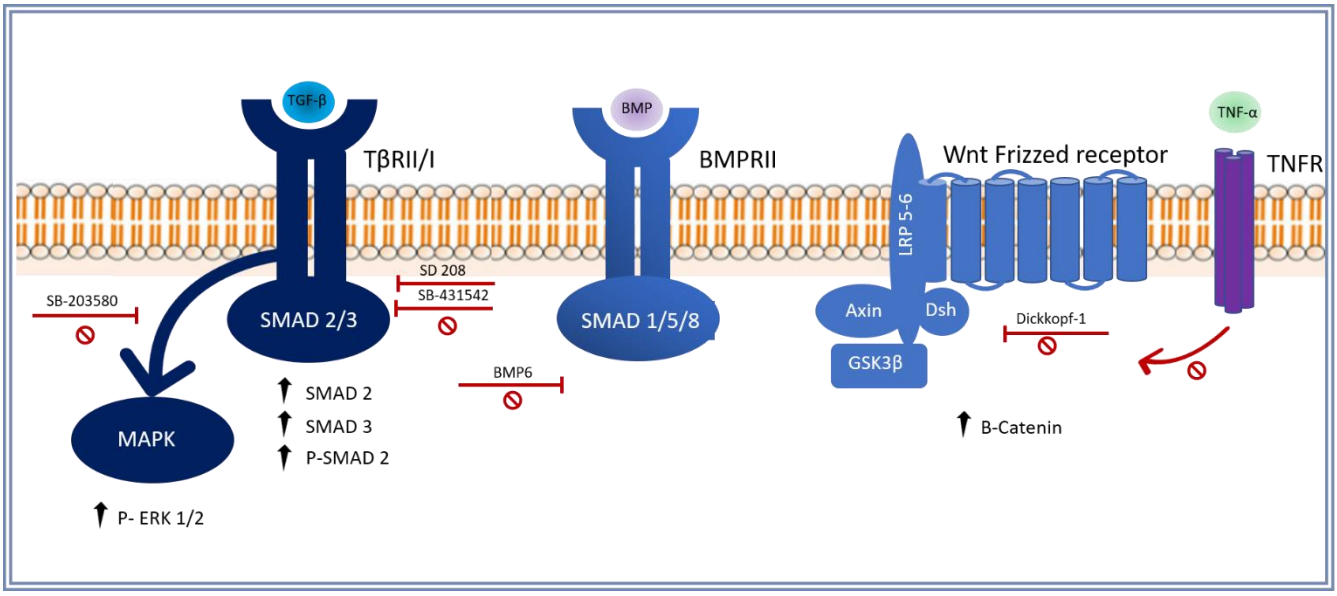


Figure 1. Summary of the various signaling pathways associated with Dupuytren's disease. TGF-β/Shad signaling is upregulated with significantly elevated levels of Smad2, Smad3 and P-Smad2 in DD compared to normal controls. ALK5 inhibitors, SB-431542 and SD208, *have a strong inhibitory effect on α -SMA,* collagen type I gene *expression and collagen contraction on Dupuytren's fibroblasts.P38 inhibitor, SB203580, leads to a decreased α-SMA expression.* TNF-α activation of Wnt/β-catenin signaling pathway will lead to the down regulation of Dupuytren's myofibroblasts leading to reduced expression of collagen type I and α-SMA. Despite this association, it appears that the myofibroblast phenotype is not dependent on the inhibition of this pathway. This is illustrated through the inhibition of Wnt ligand binding to its frizzled receptor utilizing Dickkopf-1.

A combinatorial transcriptional regulation analysis of α-SMA expression for myofibroblast differentiation, reveals that there is some evidence of convergence between Wnt/β-catenin and TGF-β1 signaling pathways[77]. More research is required to further shed the light on the interaction of the Wnt/β-catenin and TGF-β1 signaling pathways in DD.

1.4 Current preclinical therapeutic agents targeting TGF -β in Dupuytren's disease

There is a long list of experimental and non-FDA-approved medications proposed to potentially counteract the debilitating fibrotic process in DD. Many of these experimental medications are targeting TGF-β signaling. Pirfenidone- which is an active small molecule-is an experimental molecule targeting TGF-β1. An in vitro study using Pirfenidone showed down-regulation of TGF-β1-induced phosphorylation of Smad2/Smad3 as well as decreased expression of α-SMA, type I, type III collagens and

fibronectin in both Dupuytren's and normal control fibroblasts, but no *in vivo* study has been conducted on Pirfenidone yet [78]. In Addition, the previously mentioned small molecule activin receptor-like kinase (ALK)5 inhibitor SD208, and SB-431542, a selective inhibitor of ALK4, ALK5 and ALK7 kinase activity, have both been proposed as therapeutic agents in DD [79] [70] .

Another example of targeting TGF-β is through targeting TGF-β type 1 receptor (ALK 5) expression by antisense oligonucleotide-mediated exon skipping[80]. This in vivo study using antisense oligonucleotide showed a reduced overall protein expression Alpha-actin-2, Collagen Type I, and Collagen Type III and decreased activation downstream of phosphorylated Smad2 [80].

1.5 Future Directions in Dupuytren's Disease Research

Despite the presence of effective invasive therapeutic options for DD, it has been shown to recur and re-contract after all modalities of treatment[19]. Directly targeting the activated fibrotic signaling at the cellular and molecular level present a potential therapeutic option.

A deeper understanding of the signaling pathways involved in the development of DD could pave the way for new therapeutic strategies. Many of the current and ongoing preclinical studies are aimed at TGF-β signaling as a prime target to block fibroproliferative responses, in addition to reducing recurrences in this disease.

Despite the promising potential of targeting TGF- β signaling, the lack of a tested and reliable orthotopic animal model that exhibits DD phenotype has been a major challenge in evaluating interventions in DD. Recently, a promising experimental

orthotopic animal model utilizing orthotopic transplantation of human fibroblasts into athymic rats has been developed for DD [81]. This could contribute to the development of more therapeutic molecules for DD.

CHAPTER 2:

MATERIALS AND METHODS

MATERIALS & METHODS

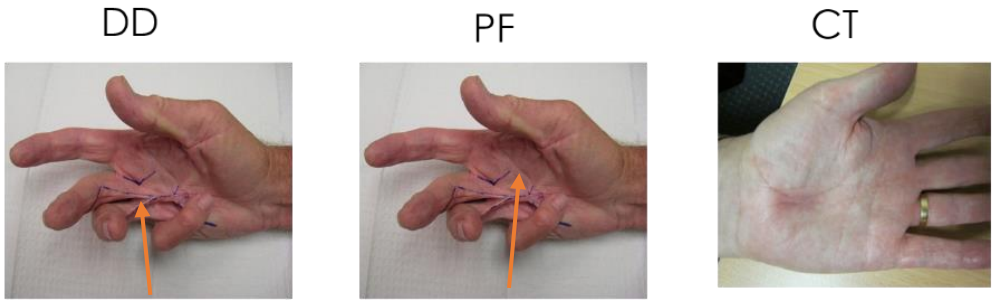
2.1 Cell cultures

Primary human cells form DD, PF and CT were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, USA Cat No. 11995-065) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Canada; Cat. No.12483-020) at 37°C in 5% CO2.

Dupuytren's disease was diagnosed clinically and intraoperative samples were harvested with informed consent and fibroblasts were isolated in DMEM Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, USA Cat No. 11995-065). Other collected specimens included healthy-appearing palmar fascia from Dupuytren's disease patients (PF) and fibroblasts extracted from the normal palmar fascia of carpal tunnel patients (CT) (Fig 1).

Briefly, specimens were incubated in 0.5% dispase (Invitrogen, Carlsbad, CA, USA) overnight at 4°C. Subsequently, fibroblasts were cultured in DMEM containing 10% fetal bovine serum (FBS) (FBS; Gibco Life Technologies, Canada; Cat. No.12483-020) and 100 U/ml penicillin-streptomycin at 37°C in 5% CO2. Experiments were performed using fibroblasts between passages 2 and 6. Once the cells reached 80% confluence in a T75 flask the cells were washed with PBS and trypsinized and a manual cell count was done to 1×10^5 cells. The cells were then plated to a six-well plate in 2ml of DMEM with penicillin and FBS in each well and were left for 24 hours. The medium was then discarded and after the cells were washed with PBS, 2ml of serum-free DMEM was added to each well and the cells were kept in the incubator.

For the conditioned media experiment, DD and CT cells were cultured in DMEM/10 % FBS to 90% confluence, and were switched to serum-free DMEM for an additional 48 h. Serum-free conditioned medium from DD and CT cells were centrifuged at 1200 rpm g at 4°C for 10 min and then at 2000 g at 4°C for another 20 min. The supernatants were filtered by 0.22-μm nylon filters and stored at -80°C until use. This was designated as DD-CM and CT- CM, respectively.



	Dupuytren's' disease	Palmar Fascia	Carpal Tunnel
Fibrotic Phenotype	+	-	-
Genetic Predisposition	+	+	-

Figure 1: Schematic representation of the various samples adopted in the study. DD signifies myofibroblasts that were cultured from samples extracted from fibrotic cords that inevitably carry a potential genetic predisposition. PF, indicated samples that were cultured from adjacent health appearing palmar fascia from Dupuytren's disease patients. CT, denotes normal fibroblast cultures from samples extracted from anatomically-matched disease-free carpal tunnel fascia.

2.2 Antibodies

Purified mouse monoclonal Anti-CD109 (sc-271085) was obtained from Santa Cruz (Santa Cruz Biotechnology). Anti-CD59 Antibody (H-7) was a mouse monoclonal antibody (sc-133170) obtained from Santa Cruz (Santa Cruz Biotechnology). Anti-rabbit IgG, HRP-linked Antibody #7074 and Anti-mouse IgG, HRP-linked Antibody #7076 were purchased from Cell Signaling Technology (Danvers, Massachusetts). Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 and Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 were bought from Thermo Fisher Scientific (Waltham, Massachusetts), Anti-Smad3 (phospho S423 + S425) antibody [EP823Y] (ab52903), Anti-Smad3 antibody (ab55480), Anti-Smad2 (phospho S255) antibody [EPR2856(N)] (ab188334), Anti-Smad2 antibody [7A5] (ab71109), Recombinant Anti-Vimentin antibody [EPR3776] - Cytoskeleton Marker (ab92547), were all obtained from Abcam (Cambridge, Massachusetts). β-Actin Antibody (C4) (sc-47778) and Endoglin Antibody (P3D1) (sc-18838) were obtained from (Santa Cruz Biotechnology), Anti-alpha smooth muscle Actin antibody (ab5694) was obtained from Abcam (Cambridge, Massachusetts).

2.3 Western blotting

Cell lysis was done by applying 100 µl of 1X Laemmli buffer in each well. The cell lysates samples were collected from each group and heated for 10 minutes at 100°C. The samples were then mixed by a spinner and by pipetting. An 8% SDS-polyacrylamide gel was made and 20 µl of cell lysate from each sample was loaded per well. After separation by electrophoresis, the gel was transferred to a nitrocellulose membrane (Fisher Scientific). This membrane was then blocked with Tris-buffered saline-Tween 20 (TBST) containing 5% nonfat dried milk diluted in TBST. for 1 hour at room temperature and incubated with primary antibodies at 4°C overnight. The membrane was washed with TBST 3 times for ten minutes each and was incubated with the secondary antibody using either Anti-rabbit IgG, HRP-linked Antibody or Anti-mouse IgG, HRP-linked Antibody (Cell Signaling) accordingly for 2 hours at room temperature. The primary antibodies were diluted in 5% milk with TBST. The signals on the membranes were detected using enhanced chemiluminescence (ECL) system (Millipore, Canada). Alpha Tubulin (Abcam ab4074) was detected afterward as a loading control.

2.4 Quantitative Polymerase Chain Reaction (qPCR)

Cells from six-well plates were extracted with TRIzol™ reagent (Invitrogen). Total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed using oligonucleotide primers made by Thermofisher for human CD109 – GAUCUAUCCAAAAUCAAGAtt and reverse primer

UCUUGAUUUUGGAUAGAUCtt ; GAPDH (forward primer, 5-GTCTCCTCTGACTTCAACAGCG -3; reverse primer, 5-ACCACCCTGTTGCTGTAGCCAA -3). Quantitative PCR reaction was performed using DyNAmo HS SYBR Green qPCR Kit(Thermo Fisher Scientific) with an initial denaturation step of 15 min at 95°C, followed by 40 cycles of denaturation at 94°C (10 s), annealing at 60°C (30 s), and extension at 72°C (30 s) and analyzed in the Applied Bioscience's Stepone real-time instrument. Quantification of target mRNA was carried out by comparison of the number of cycles required in order to reach the reference and target threshold values (ΔΔ CT method).

2.5 Immunofluorescence & Confocal microscopy

DD myofibroblasts were fixed in 4% paraformaldehyde (w/v) for 15 min, and permeabilized in PBS/0.3% Triton X-100 for another 15 min. Cells were washed with PBS, and blocked in 2% BSA for 1 h. Primary antibodies against, CD109 (sc-271085) was obtained from Santa Cruz (Santa Cruz Biotechnology), Anti-CD59 Antibody (H-7) was a mouse monoclonal antibody (sc-133170) obtained from Santa Cruz (Santa Cruz Biotechnology), recombinant Anti-Vimentin antibody [EPR3776] - Cytoskeleton Marker (ab92547), was obtained from Abcam (Cambridge, Massachusetts). β-Actin Antibody (C4) (sc-47778) and Endoglin Antibody (P3D1) (sc-18838) were obtained from (Santa Cruz Biotechnology), were added to cells at 1:250 dilution in 2% BSA and incubated overnight at 4˚C. Cells were washed with PBS and labeled for 1 h with fluorophore-conjugated secondary antibodies (1:500 dilution; Alexa Fluor 594 and Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 Thermo Fisher

Scientific (Waltham, Massachusetts)). Cells were washed with PBS and the slides were mounted on coverslips with DAPI-containing mounting medium

® Gold Antifade Mount ant with DAPI (P36935) (Thermofisher). Cells were visualized using a LSM780 confocal microscope and ZEN – ZEISS ® software (Blue edition) was utilized for analysis and quantification.

2.6 Three-Dimensional Collagen Cultures

Sterile rat tail collagen (1.8mg/ml) was mixed in 4:1 with a neutralizing solution containing 10x Waymouth media (Sigma, Cat# W1625) and 0.34M NaOH (Sigma, Cat# 221465) to a total volume of 500 uL $1x10^3$ fibroblasts in 50 ul volumes were added to 500 uL of collagen mixture and cultured in triplicate in 24-well plates for 30 minutes until collagen polymerization was achieved at a total volume of 550ul. DMEM supplemented with 2% FBS, 1% L-glutamine and 1% antibiotic-antimycotic was added to the top of the polymerized collagen and incubated at 37 °C and 5% CO2 for 72 hours 3D cultures were then used for RNA isolation. Fibroblasts were treated in DMEM 1% plus penicillin-streptomycin (PS) for 24 hours with a proinflammatory mixture containing recombinant human TNF-α (100 ng/mL), recombinant human IFN-γ (500 U/mL) and recombinant human IL-6 (10 ng/mL) [82].

CHAPTER 3:

RESULTS

RESULTS

3.1 qPCR analysis shows that there is no significant difference in CD109 mRNA levels between DD, adjacent PF versus CT fibroblasts

To evaluate for any potential differences in gene expression (mRNA) between DD, PF and CT fibroblasts, qPCR was utilized. Fibroblasts were first cultured in a traditional two-dimensional culture system. In addition, fibroblasts were cultured in a three-dimensional collagen matrix to better mimic the environment in the palmar fascia of the hand. Furthermore, to instigate an inflammatory process that is postulated to be involved in the pathogenesis of Dupuytren's disease, a pro-inflammatory mix of cytokines was added in a subsequent group (100 ng/mL TNF-α, 500 U/mL IFN- γ and 10 ng/mL IL-8) as previously described in the methods section [82].

qPCR analysis showed that when cells were cultured in a two-dimensional culture, DD fibroblasts did not exhibit any statistically significant difference in CD109 mRNA levels when compared to that of PF or CT fibroblasts. Similar results were obtained using three-dimensional cultures confirming that CD109 mRNA levels across the three study groups were similar. Furthermore, the addition of proinflammatory cytokines did not significantly alter CD109 mRNA levels (Fig. 1).

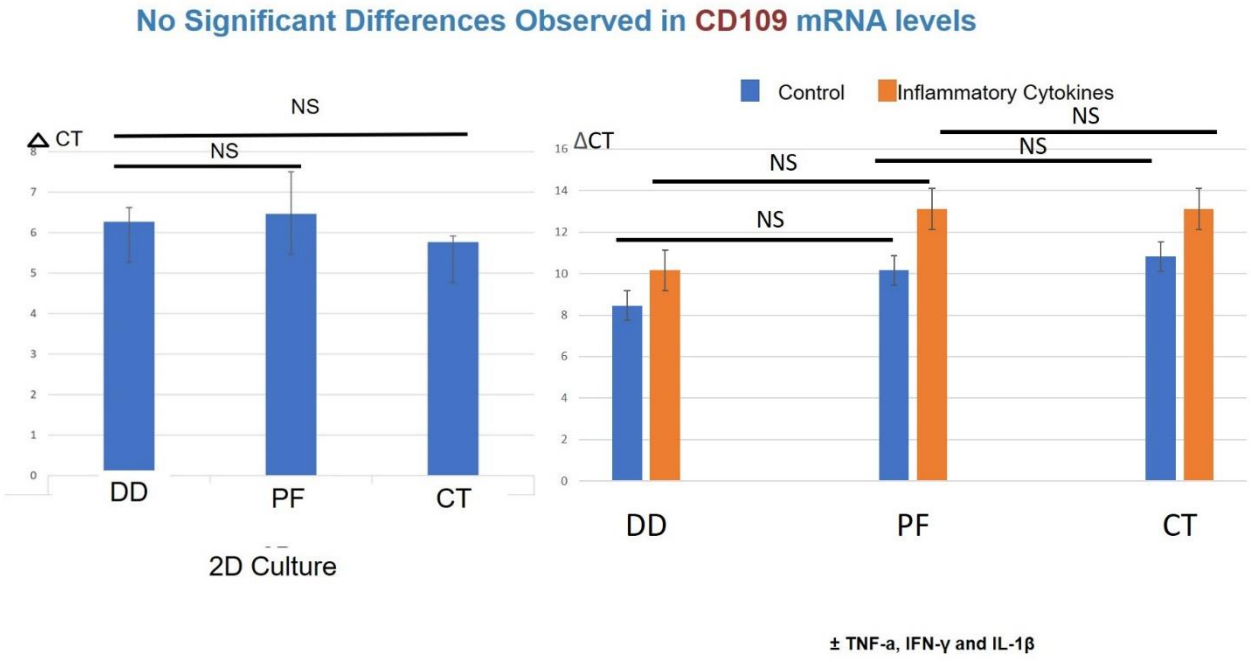


Figure 1: Fibroblasts cultured in a two-dimensional culture did not exhibit any statistically significant difference in CD109 levels when comparing DD vs. adjacent PF vs. CT fibroblasts. In addition, three-dimensional culturing did not significantly alter the CD109 mRNA levels across the three study groups. The addition of pro-inflammatory cytokines did not significantly alter CD109 mRNA levels. The figure is representative of 3 independent experiments with 3 patients in each group.

3.2 Levels of alpha-smooth muscle actin and endoglin are increased in DD and adjacent PF fibroblasts when compared to CT fibroblasts, as detected by immunofluorescent staining and confocal microscopy.

To examine levels of alpha-smooth muscle actin that represents a key marker for myofibroblasts immunofluorescent staining and confocal microscopy imaging were utilized [83]. Additionally, given the proliferative nature of DD fibroblasts, endoglin (CD105) levels were evaluated given its role as a TGF-β receptor that regulates TGF-β signaling [84,85].

Results shown in Fig 2 demonstrate that the levels of alpha-smooth muscle actin were significantly higher in DD and adjacent PF fibroblasts compared to CT fibroblasts, suggesting that DD and adjacent PF fibroblasts are likely myofibroblasts (Fig. 2). In addition, analysis of endoglin levels indicates that endoglin protein levels are significantly higher in DD and adjacent PF myofibroblasts when compared to that of CT fibroblasts (Fig. 3). This is the first report of endoglin expression in DD fibroblasts.

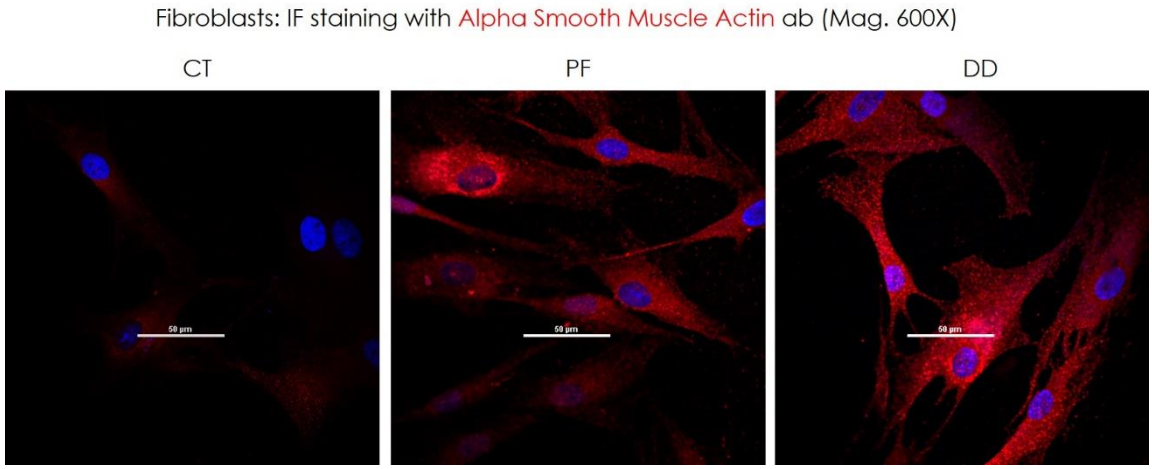


Figure 2: alpha smooth muscle actin levels are significantly higher in DD and adjacent PF fibroblasts compared to CT fibroblasts. The immunofluorescent staining was performed using an anti-smooth muscle actin antibody in technical replicates.

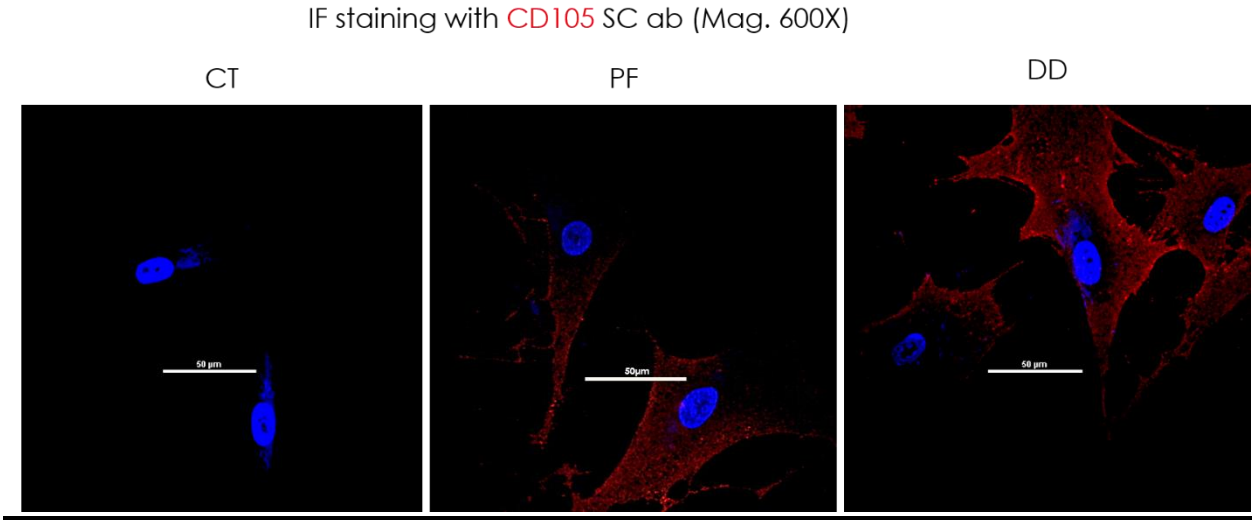


Figure 3: The levels of the TGF-β co-receptor endoglin are significantly higher in DD and adjacent PF fibroblasts when compared to CT fibroblasts. Immunofluorescent staining was performed using an anti-CD105 antibody in technical replicates.

3.3 CD109 levels are decreased in DD and adjacent PF fibroblasts, when compared to CT fibroblasts, as detected by immunofluorescent staining and confocal microscopy.

To assess levels of CD109 in DD and adjacent PF fibroblasts as compared to CT fibroblasts, immunofluorescent staining and confocal microscopy imaging were utilized. In both DD and adjacent PF, CD109 levels are lower than in CT fibroblasts (Figures 4 and 5). Immunofluorescent staining of DD myofibroblasts also exhibited enhanced vimentin expression compared to CT (normal) fibroblasts that exhibited less of this cytoskeleton protein.

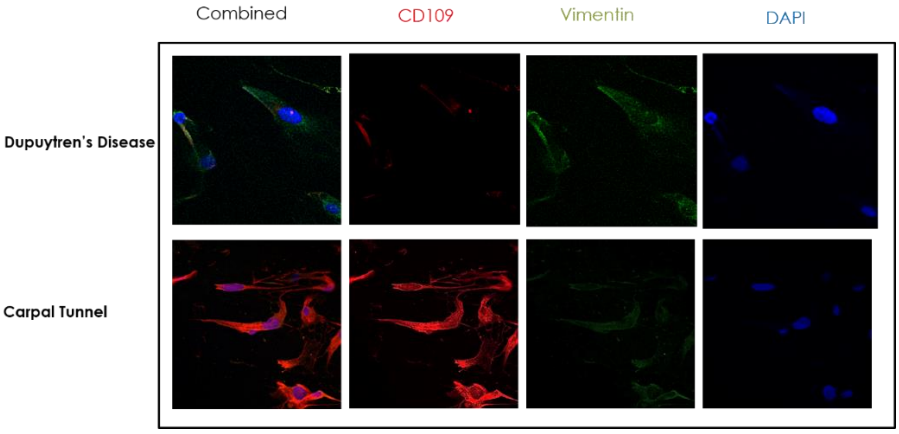


Figure 4 CD109 levels appear to be significantly lower in DD fibroblasts as compared to CT fibroblasts. The immunofluorescent staining was performed using an anti-CD109 or an anti-vimentin antibody. The figure is representative of 3 experiments using fibroblasts from 2 patients each.

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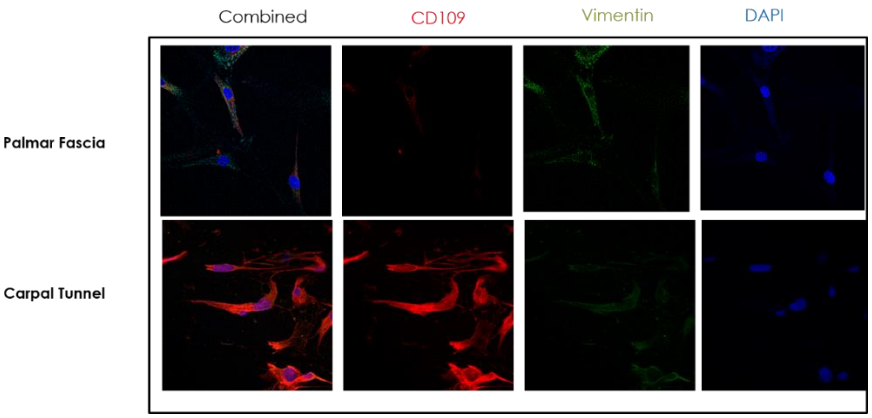

Figure 5 CD109 levels appear to be significantly lower in adjacent PF fibroblasts as compared to CT fibroblasts. The immunofluorescent staining was performed using an anti-CD109 or an anti-vimentin antibody. The figure is representative of 3 experiments using fibroblasts from 2 patients each.

3.4 Confirmation that CD109 protein levels are decreased in DD fibroblasts using immunofluorescent staining and 2.5-Dimensional analysis utilizing ZEN – ZEISS ® .

To further evaluate the levels of CD109 in DD fibroblasts, ZEN – ZEISS ® blue edition software was adopted to generate 2.5-dimensional renderings of completely scanned IF slides.

Analysis of the 2.5-dimensional renderings of CD109 levels demonstrates that CD109 levels are lower in DD fibroblasts compared to CT fibroblasts (Fig. 6).

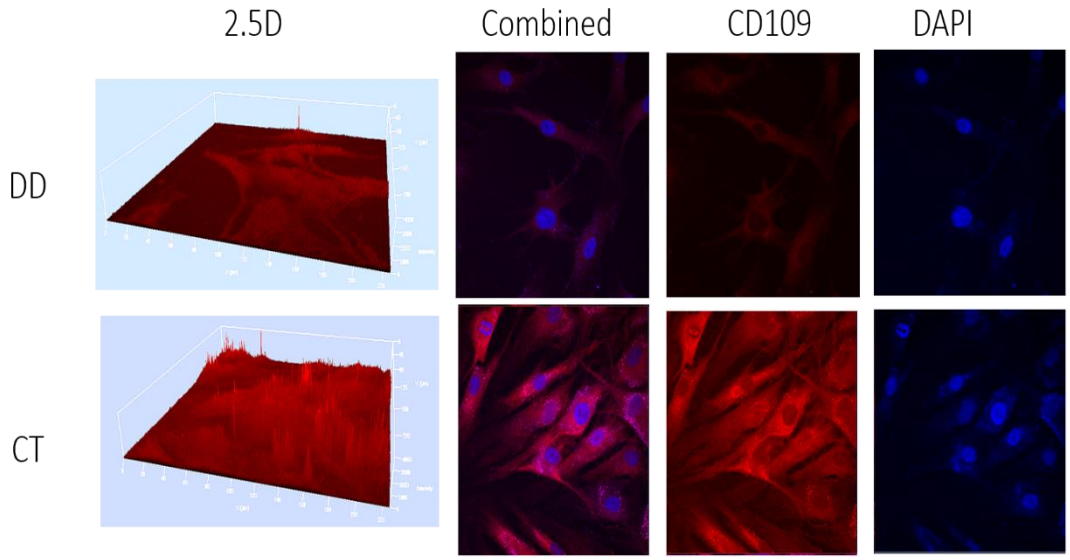

Figure 6: 2.5 dimensional renderings of CD109 levels in DD fibroblasts versus CT fibroblast showing that CD109 levels are lower in DD fibroblasts as compared to CT fibroblasts. The figure is representative of 3 experiments using fibroblasts from 2 patients each.

3.5 CD109 levels are decreased while the canonical TGF-β signaling is increased in DD fibroblasts, as detected by western blot analysis

Next protein levels of CD109 in DD and CT fibroblasts were evaluated using western blot analysis. Results shown in Fig 7 illustrate that CD109 levels in DD fibroblasts are lower than in CT fibroblasts. In addition, evaluation of the canonical TGF- β signaling pathway revealed that the levels of phospho-Smad2 and phospho-Smad3 levels are higher in DD fibroblasts as compared to CT fibroblasts, indicating an aberrant constitutive activation of the canonical TGF- β signaling pathway in DD (Fig. 7).

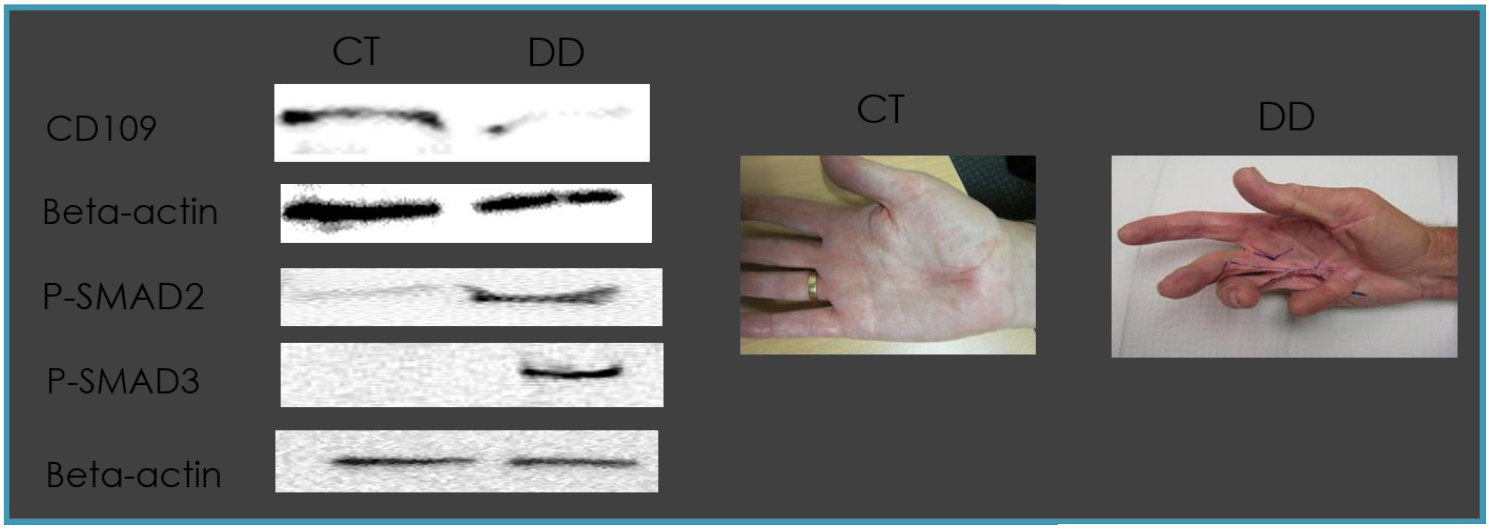

Figure 7: CD109 protein levels are lower in DD fibroblasts than in CT fibroblasts, as detected by Western blot. In addition, the levels of phopsho-Smad2 and phospho-Smad3 are higher in DD fibroblasts as compared to CT, suggesting an aberrant constitutive activation of the canonical TGF- β signaling pathway in DD fibroblasts in technical replicates.

3.6 Levels of CD58 in DD fibroblasts are similar to that of CT fibroblasts as detected by immunofluorescent staining and confocal microscopy.

To determine whether the decreased CD109 levels observed in DD fibroblasts is due to a global defect of GPI-anchored protein synthesis in DD, the levels of another GPI-anchored protein, CD58, were evaluated using immunofluorescent staining and confocal microscopy imaging.

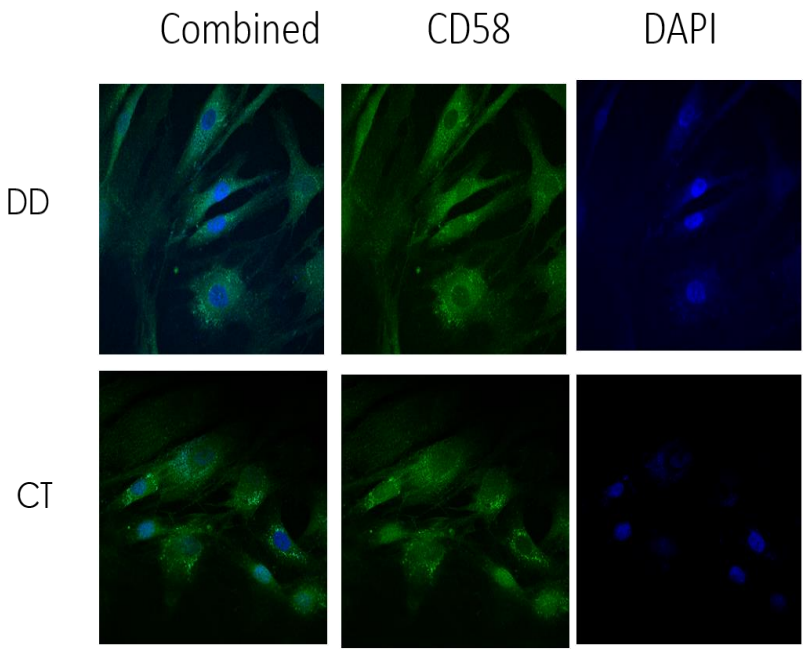

Figure 8: CD58 levels are similar in DD fibroblasts when compared to CT fibroblasts, suggesting that GPI anchor synthesis is intact in DD fibroblasts. The figure is representative of 3 experiments using fibroblasts from 2 patients each

Results shown in Fig. 8 indicate that the levels of CD58 protein expression are similar in DD fibroblasts when compared to CT fibroblasts, suggesting that GPI anchor synthesis pathway is not defective in DD fibroblasts.

3.7 Analysis of the cell surface expression of CD58 and CD109 in DD fibroblasts as compared to that in CT fibroblasts, as detected by immunofluorescent staining, confocal microscopy imaging and digital quantification:

To further quantify the levels of CD109 and CD58 protein expression, immunofluorescent staining, confocal microscopy imaging and digital quantification were utilized. Results shown in Fig 9 demonstrate that CD109 levels are lower in DD compared to CT while CD58 levels are similar in both cell types, as detected by digital quantification.

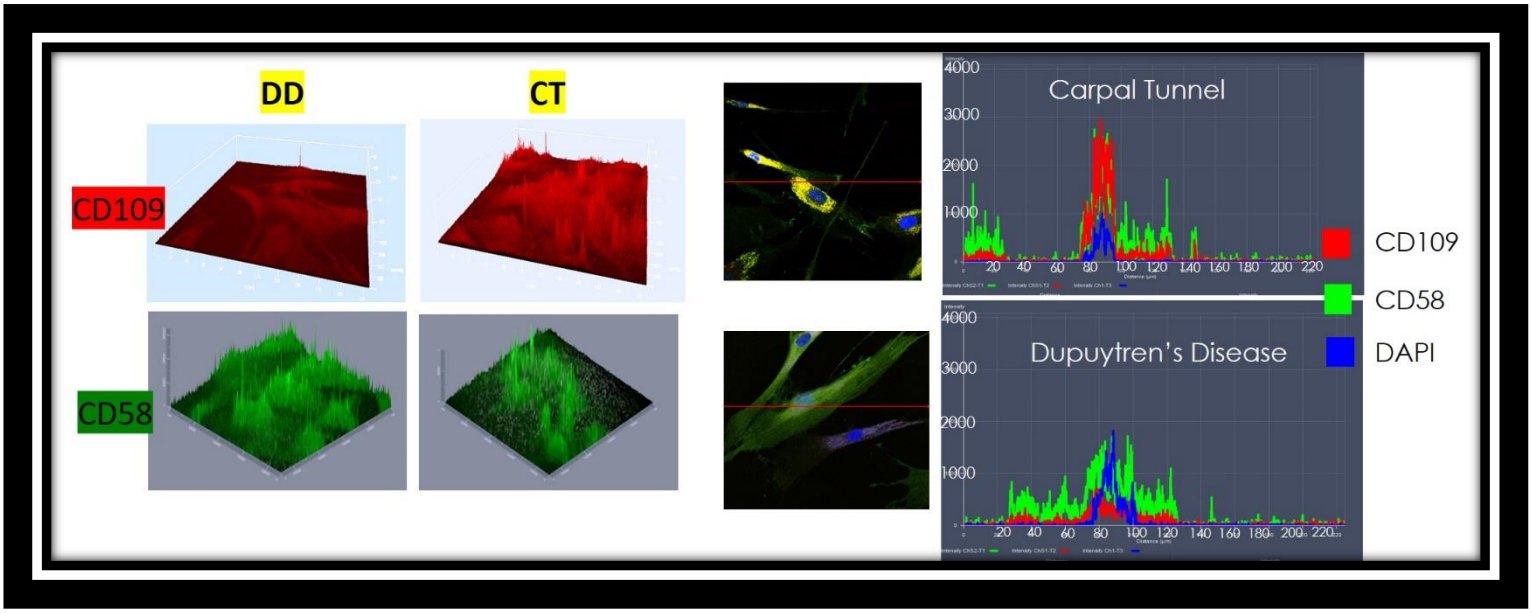

Figure 9: CD58 levels are similar in DD when compared to CT, while CD109 levels are significantly decreased in DD compared to CT. The figure is representative of 3 experiments using fibroblasts from 2 patients each

3.8 Immunofluorescent Z-stacking and 3D rendering indicate lower CD109 levels across all planes and sections.

To further examine the patterns of CD109 staining across all different planes (0.125um thick sections), a z-stacking video was generated. Results shown in Figure 10 demonstrate that CD109 levels are decreased across all different planes.

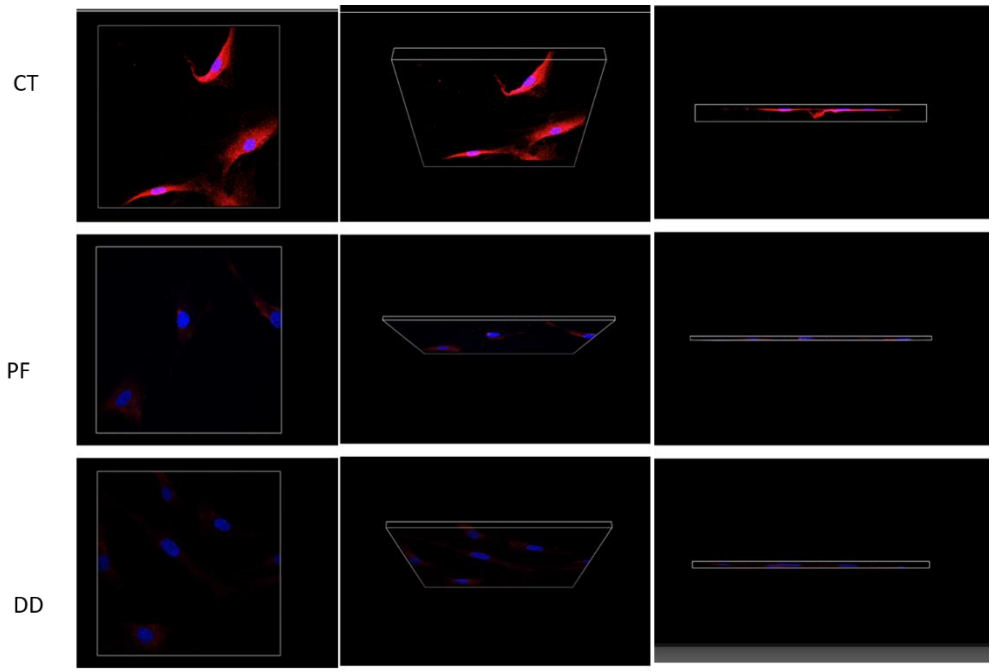

Figure 10: Three-dimensional reconstruction of immunofluorescent slides. The figure is representative of 3 experiments using fibroblasts from 2 patients each.

3.9 Blocking proteasomal degradation using MG132 leads to an increase in CD109 levels in DD fibroblasts:

To investigate the impact of blocking degradation, MG132 (a known proteasome-mediated lysis inhibitor [86]) was utilized to halt CD109's degradation in vitro.

The addition of MG132 led to a significant increase in CD109 levels in DD fibroblasts after 12 hours (Fig. 11), while the levels CD109 on CT fibroblasts are relatively unchanged at that time interval. These results demonstrate that CD109 is degraded via the proteasomal pathway and raise the possibility that the degradation of CD109 in DD fibroblasts might be higher than in CT fibroblasts. The decrease in CD109 levels observed at 14 hours probably reflects the cytotoxic effects of MG132 on DD fibroblasts, which are more fragile than CT fibroblasts under the culture conditions used.

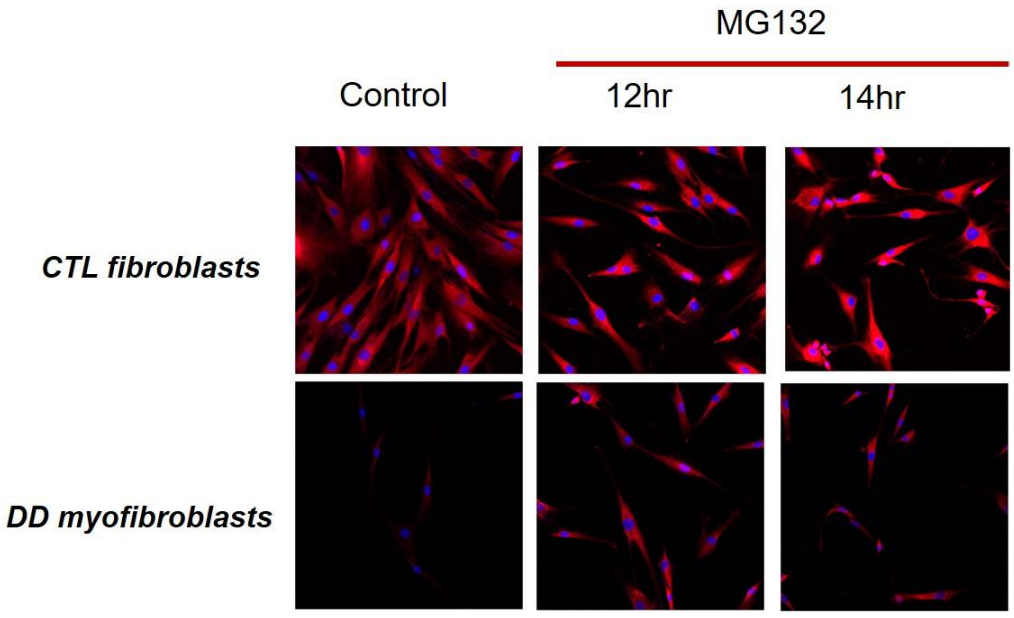

Figure 11: Treatment with MG132 leads to an increase in CD109 levels in DD fibroblasts in technical replicates.

CHAPTER 4:
DISCUSSION

DISCUSSION

Dupuytren disease (DD) is a common fibrotic disorder affecting the palmar fascia of the hand and can lead to the development of permanent debilitating contractures and ultimately loss of hand function [87]. An effective treatment and preventative strategies for this complex fibrotic condition is yet to be identified. This partially stems from the numerous challenges in studying DD, as it requires hard to acquire intraoperatively collected specimens, and lacks a reliable animal model, in addition to its enigmatic etiology.

The role of TGF-β in promoting fibrotic responses and sustaining the aberrant enhanced synthesis of extracellular matrix proteins is well documented within the context of fibrotic conditions [88-91]. In addition, associations between circulating levels of plasma

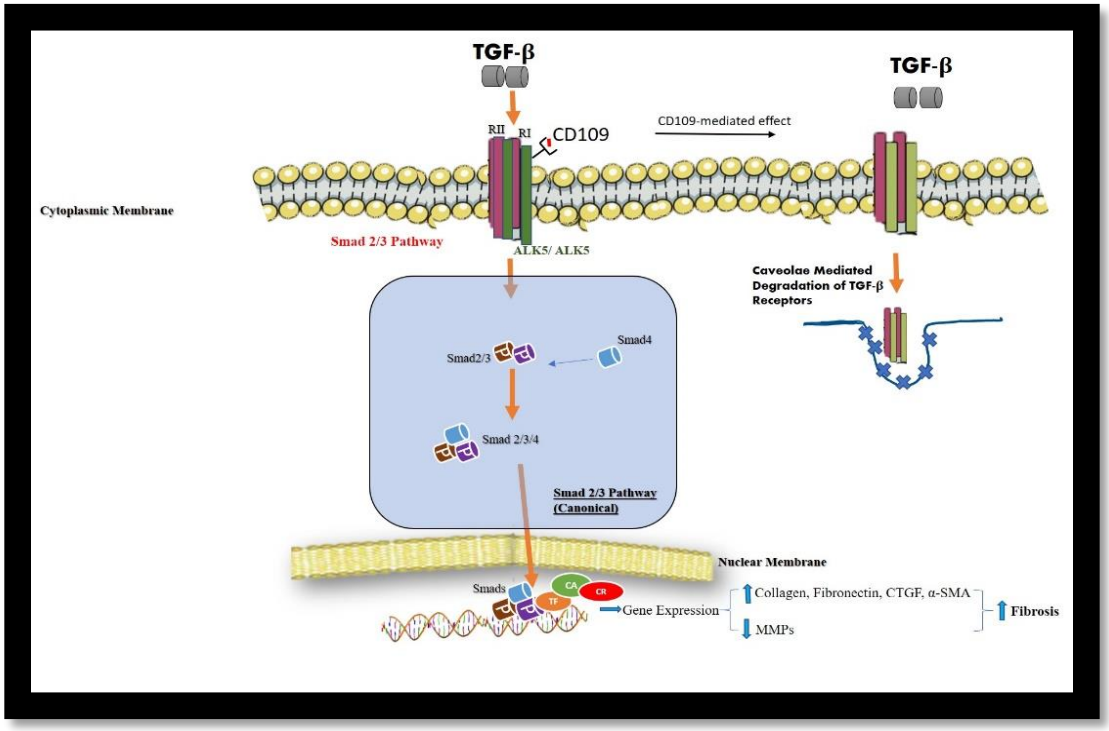

Figure 1: CD109 is a TGF-β coreceptor and a potent negative regulator of TGF- β signaling capable of binding TGF-β1 with a high affinity forming a complex with TβRI and TβRII leading to caveolae mediated receptor degradation.

TGFβ1 and enhanced cardiac fibrosis and dysfunction further emphasizes the critical role of this multifunctional cytokine in promoting fibrosis[92]. CD109 is a TGF-β coreceptor and a potent negative regulator of TGF- β signaling capable of binding TGF-β1 with high affinity forming a complex with TβRI and TβRII that could attenuate the fibrotic responses instigated by TGF- β (Fig. 1) [93 94 95].

Although the initiating pathophysiological mechanisms involved in the development of DD are not fully understood, many studies indicate that aberrant TGF-β signaling plays a key role in its etiology [96-98]. Studies of the role of TGF- β DD pathogenesis suggest that it's levels are increased by multiple folds within DD derived tissue samples [63]. In addition, TGF- β acts as a "mechano-transduction cytokine" significantly attenuating the force generation of the contractile cords of DD and enhancing DD cellular responses to external mechanical stimuli [65]. Immunofluorescent staining of DD myofibroblasts exhibited enhanced alpha-smooth muscle actin and vimentin expression. As previously reported, these molecules are involved in the formation of stress fibers (cytoskeleton). Additionally, these two proteins were previously proposed to be up-regulated during fibroblasts transformation to myofibroblasts [99,100].

CD109 has been shown to be a potent negative regulator of TGF- β signaling and to play a key role in keeping TGF-β's pro-fibrotic responses in check [101]. Therefore, it is possible that the decreased levels of CD109 observed in the current study may at least partially account for the uncurbed TGF- β-mediated fibrotic responses in DD fibroblasts. Such a notion is consistent with our data showing that the canonical TGF-β signaling is increased in DD fibroblasts when compared to CT fibroblasts, as demonstrated by enhanced phosphoSmad2 and phosphoSmad3 levels in DD fibroblasts. Whether

decreased CD109 levels represent a hallmark of DD fibroblasts cannot be ascertained from the current study, due to the small sample numbers used and in some cases depict technical replicates, representing a key limitation of the observations. As primary human fibroblasts cultured from patient specimens exhibit heterogeneity (unlike cell lines) multicentered studies involving large numbers of patients will be needed to overcome the limitations mentioned above. The current study provides a basis for future research by suggesting a potential mechanistic explanation of the aberrant activation of TGF-β signaling and fibrotic responses in DD fibroblasts. Thus the decreased levels of CD109, a critical negative regulator of TGF-β signaling may partially account for the enhanced fibrotic process in DD fibroblasts.

The addition of a Glycosylphosphatidylinositol (GPI) anchor represents a post-transcriptional modification of proteins to direct their localization to specific membrane locations or microdomains [102]. CD109 is one of the 150 GPI-anchored proteins synthesized within cells [103-105]. To rule out the possibility that the decreased CD109 levels in DD fibroblasts stem from a defect in the GPI anchor synthetic pathway, we have examined the levels of another GPI anchored protein CD58, and found that the decreased CD109 levels cannot be accounted for by a defect in GPI anchor synthesis in DD as has been noted for the psoriatic skin [106]. Other GPI-anchored proteins are expressed in DD fibroblasts making the possibility of global GPI-anchored protein synthesis less likely.

Another potential explanation for the decreased levels of CD109 observed in DD fibroblasts is an enhanced release from cells. [107 108]. Whether an enhanced release from DD fibroblasts may account for the diminished levels of CD109 in DD fibroblasts, remains to be determined. This will require a detailed analysis of CD109 protein released

into the conditioned medium as a function of time, and normalization to the number of cells in the culture plate. One caveat is that the secretion from cells does not always correlate with decreased intracellular protein levels[109] .

MG132 has been demonstrated to effectively inhibit the 26S proteasome complex. Our results using MG132 to inhibit the degradation of CD109[110], suggest that enhanced proteasomal degradation of CD109 may potentially play a role in regulating CD109's levels in DD. My results are, however, is nonquantitative and only serve as a possibility based on which further studies could be designed. This will involve determining CD109 turnover in DD fibroblasts when compared to CT fibroblasts, as a function of time.

Our finding that CD109 protein levels are potentially decreased in DD fibroblasts and adjacent PF fibroblasts as compared to normal CT fibroblasts, provides a potential mechanistic explanation for the increased fibrotic responses in DD fibroblasts. Furthermore, our results showing that CD109 protein levels are decreased while the CD109 mRNA levels and remain unchanged, suggest that CD109 turnover/degradation may be enhanced in DD fibroblasts. Together these findings suggest that decreased CD109 function may potentially play a critical role in increased fibrosis in DD fibroblasts and thus CD109 may represent the basis for a strategically tailored therapeutic approach to counter the deleterious effects of excessive fibrosis on numerous hands in DD.

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