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AWT1 is Constantly Expressed in Palmar Fascia Fibrosis and Promotes a Pro-Inflammatory Milieu

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Abstract

Palmar fascia fibrosis (Dupuytren's disease or DD), can cause permanent palmar-digital contractures. There are currently no effective treatments for DD. We have previously reported a DD-associated increase in the expression of *WT1*, encoding a transcription factor, Wilms Tumor 1 (WT1). We demonstrate that fibroblasts derived from fibrotic palmar fascia of DD patients constantly express an isoform of *WT1*, *AWT1*, unlike syngeneic pre-fibrotic (PF) or allogeneic normal control (CT) fibroblasts that only express *AWT1* in response to pro-inflammatory stimuli. Adenoviral transduction and constant expression of *AWT1* in PF and CT fibroblasts induced the expression of genes encoding pro-inflammatory cytokines and cytokine receptors. Co-culturing AWT1 expressing PF and CT fibroblasts with a monocyte cell line, THP-1, induced pro-inflammatory cytokine responses and the formation of a pro-inflammatory milieu. These data are consistent with the hypothesis that the constant expression of AWT1 in DD fibroblasts induces a local pro-inflammatory microenvironment that promotes fibrosis development.

Keywords

Dupuytren's Disease, Wilms' Tumor 1, Fibrosis, Inflammation, AWT1, Fibroblasts, Wound Healing

Summary for Lay Audience

The focus of this thesis is palmar fascia fibrosis, also known as Dupuytren's Disease (DD). The palmar fascia is a layer of connective tissue that lies below the skin of the palm where it protects the underlying nerves, blood vessels and bones of the hands. DD is characterized by the formation of abnormal scar tissue (fibrosis) in the palmar fascia that can cause permanent contracture of the affected digits, resulting in loss of dexterity and reduced quality of life. Unfortunately, there are no truly effective treatments for DD, as current therapies are associated with high rates of disease recurrence. Previous work in the O'Gorman laboratory has identified an increase in the expression of a transcription factor, Wilms' Tumor 1 (WT1), in contracture tissues from DD patients. Transcription factors are proteins that regulate the expression of genes, and abnormal transcription factor activity can lead to the development of diseases, such as cancers and fibroses. WT1 was first identified in a pediatric cancer but has since been identified in other cancers. Interestingly, WT1 appears to play multiple, even apparently contradictory, roles in the development of different cancers. This complexity may be explained by the existence of multiple versions (isoforms) of WT1, the results of alternative splicing in, and/or alternative start sites for, *WT1* mRNA transcripts.

Three major findings are described in this thesis: 1) hypercontractile cells that cause palmar fascia contractures, myofibroblasts, constantly express an isoform of *WT1*, *AWT1*, that can be induced in fibroblasts derived from visibly non-fibrotic palmar fascia by pro-inflammatory molecules, 2) constant expression of *AWT1* in fibroblasts derived from visibly non-fibrotic palmar fascia promotes the expression of pro-inflammatory cytokines and cytokine receptors, and 3) co-cultures of fibroblasts expressing AWT1 with THP-1 cells, which are derived from human immune system cells called monocytes, modify THP-1 cell gene expression and the secretion of pro-inflammatory molecules. These findings are consistent with the hypothesis that constant expression of *AWT1* in DD promotes chronic inflammation in the palmar fascia. As WT1 is currently a therapeutic target for various cancers, it may be possible to cross-purpose these drugs as a novel treatment for DD.

Co-Authorship Statement

All figures and data shown in this thesis were produced by Johnny Luo unless otherwise stated.

Figure 1.2 was by T. H. Trojian *et al*, Am Fam Physician (2007).

Figures 1.4 and 1.5 were produced by Justin Crawford (Crawford *et al*, JCCS 2015)

Figures 1.8 and 3.8 was produced by Ana Pena Diaz.

Figure 1.9 was produced by Trisiah Tugade prior to the commencement of this project.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

TNF	Tumor Necrosis Factor
IL-1β	Interleukin-1 Beta
IL-10	Interleukin-10
TGF- β	Transforming Growth Factor Beta
FGF	Fibroblast Growth Factor
VEGF	Vascular Endothelial Growth Factors
IFN-γ	Interferon Gamma
αSMA	Alpha Smooth Muscle Actin
ECM	Extracellular Matrix
PDGF	Platelet Derived Growth Factor
FN	Fibronectin
IL-8	Interleukin-8
DD	Dupuytren's Disease
MCP	Metacarpal Phalangeal
PIP	Proximal Interphalangeal
DIP	Distal Interphalangeal
IGF-II	Insulin-Like Growth Factor-II
TNFR2	Tumor Necrosis Factor Receptor 2
IL-33	Interleukin-33
PF	"Pre-Fibrotic" Syngeneic Controls
CT	"Healthy" Allogeneic Controls
WT1	Wilms' Tumor 1 Gene
WT1	Wilms' Tumor 1 Protein
CAF	Cancer Associated Fibroblast
KTS	Lysine-Threonine-Serine
Ex5	Exon 5
ExtWT1	Extended Wilms' Tumor 1
AWT1	Alternative Wilms' Tumor 1
TrWT1	Truncated Wilms' Tumor 1
IPF	Idiopathic Pulmonary Fibrosis
DMEM	Dulbecco Modified Eagle Medium

FBS	Fetal Bovine Serum
AdAWT1	Adenovirus Expressing Alternative Wilms' Tumor 1
AdGFP	Adenovirus Expressing Green Fluorescent Protein
GFP	Green Fluorescent Protein
FPCL	Fibroblast Populated Collagen Lattice
sFPCL	Stressed Fibroblast Populated Collagen Lattice
5'RACE	5 Prime Rapid Amplification of cDNA Ends
qPCR	Qualitative Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
cDNA	Complimentary Deoxyribonucleic Acid
RNA-seq	Ribonucleic Acid Sequencing
STAR	Spliced Transcript Alignment to a Reference
TMM	Trimmed Mean of M Values
GSA	Gene Specific Analysis
ELISA	Enzyme-Linked Immunosorbent Assay
BSA	Bovine Serum Albumin
TBS-T	1x Tris-Buffered Saline supplemented with 0.1% TWEEN-20
ATCC	American Type Culture Collection
RPMI	Roswell Park Memorial Institute
IHC	Immunohistochemistry
FDR	False Discovery Rate
OX-40L	OX-40 Ligand
PAI-1	Plasminogen Activator Inhibitor 1
MCP-1	Monocyte Chemoattractant Protein-1
M1	Pro-Inflammatory Phenotype
M2	Anti-Inflammatory/Pro-Resolving Phenotype
ChIP-seq	Chromatin-Immunoprecipitation Sequencing
SNP	Single Nucleotide Polymorphism
Veh	Vehicle control

Chapter 1

1 LITERATURE REVIEW

1.1 Wound Healing

All organisms that interact with their environment risk being damaged by those interactions. If damaged, an organism can either die or, if biologically capable, undergo repair. The molecular mechanisms that facilitate organ and tissue repair are complex and, remarkably, still very poorly understood. Normal tissue repair can be envisaged to occur in three orderly but overlapping stages: haemostasis and inflammation, proliferation and remodeling (Fig. 1.1).[1] The processes of haemostasis and coagulation occur immediately after tissue damage to cause the formation of provisional wound matrix. Fibrin forms a temporary wound closure to prevent blood loss. During this time, immune cells infiltrate the wound site.[2] Some of the first immune cells that arrive at the wound site, such as neutrophils, release hormone-like molecules known as cytokines, that signal and exert context dependent effects on other cells. Cytokines have numerous roles in regulating host immune response and homeostatic mechanisms. Cytokines are classified based on several factors including their overall signalling effects, their cellular sources, and the receptors to which they bind.[3]

In normal wound healing, pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1β (IL-1β) signal for the differentiation of monocytes into macrophages for clearing debris and pathogenic microorganisms.[4] Furthermore, these pro-inflammatory molecules continue to signal for additional neutrophil recruitment. The accumulated neutrophils contribute to the clearing of foreign material in addition to debridement of necrotic tissue.[5] The inflammatory phase begins to resolve when neutrophil infiltration stops and the accumulation of macrophages continues.[6] Inflammation eventually resolves by the release of anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). As inflammation resolves, this initiates the processes of angiogenesis and recruitment of fibroblasts to the wound site, indicating the start of the proliferation phase.

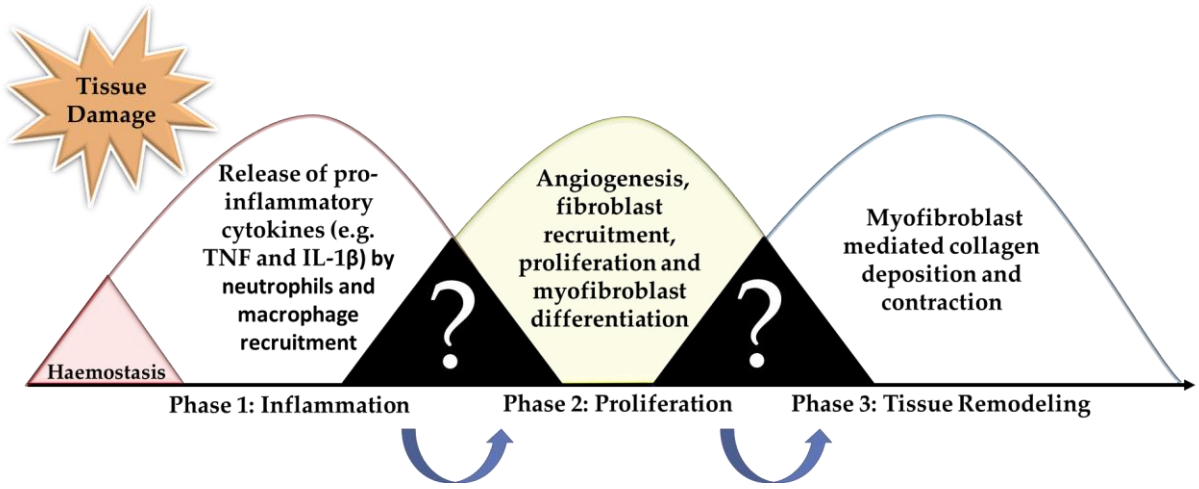

Figure 1.1. Phases of Wound Healing.

Wound healing can be envisaged as occurring in three overlapping phases, as shown above. The molecular mechanisms that promote transition from one phase to the next are tightly controlled and regulated by a number of known and unknown mediators. Orderly and efficient transition between phases in hypothesized to promote high quality repair that approaches regeneration.

Release of fibroblast growth factors (FGF) and vascular endothelial growth factors (VEGF) by fibroblasts and macrophages promote cell proliferation and differentiation for the formation of new blood vessels and recruitment of stromal cells.[7,8] In addition, fibroblasts and macrophages can promote re-epithelization in skin wounds.[5] Finally, macrophages secrete cytokines such as interferon-γ (IFN-γ) and TGF-β which activate fibroblasts into alpha smooth muscle actin (αSMA) expressing myofibroblasts to contract the wound while secreting extracellular matrix molecules (ECM), such as collagens, for tissue remodeling during the remodeling phase.[10] In addition to direct activation of fibroblasts to myofibroblasts through TGF- β signaling, myofibroblasts have been reported to be derived from many different cellular origins, including the differentiation of progenitor cells and through epithelial-mesenchymal transition. Once the remodeling of the wound site is complete, myofibroblasts and other immune cells are depleted by apoptosis, migration and other processes.

Unfortunately, the factors that mediate the transition between pro-inflammatory (or "classically activated", M1) and pro-resolving ("alternatively activated", M2) macrophages are very poorly understood. Additionally, the current dichotomous model of M1 (IFN-γ or LPS induced) and M2 (IL-4 induced) macrophages, both of which are largely based on *in vitro* models, have been challenged by recent findings.[11] Studies *in vivo* have shown that the transcriptomic profiles of macrophages are dynamic and often don't fall under M1 or M2 extremes.[12-14] The phases of wound healing are complex and intertwined, and the oversimplified M1/M2 model reflects how poorly we understand this dynamic process. While they may not conform to a simple biphasic model, it is nonetheless clear that macrophages play essential roles in normal wound repair. Studies by Lucas *et al*, have shown that depletion of wound macrophages during the initial inflammatory response resulted in impaired tissue healing by reduction in vascularization, whereas depletion of macrophages during the proliferative phase resulted in severe hemorrhage of the wounded tissue.[15] These data underline the importance of the normal sequential transition between overlapping phases and the maintenance of interactions between monocytes/macrophages and fibroblasts in promoting efficient repair that resembles tissue regeneration.

1.2 Abnormal Wound Healing: Fibrosis

In contrast to adults, wounds in embryos do not require αSMA expressing myofibroblasts to achieve closure and can heal without scar formation. However, this ability of embryos to heal scarlessly is lost by the third trimester.[16] In adults, dysregulation or delay in completing any of the three major phases of wound healing, such as chronic exposure to inflammatory cytokines, can result in chronic wounds or fibrosis.[17] Findings by Ferguson *et al* further suggest that the introduction of inflammation can over-ride scar-less fetal wound healing and induce scarring, known clinically as fibrosis.[18] Fibrosis is characterized by the excessive deposition of extracellular matrix molecules, such as type III and other fibrillar collagens, enhanced ECM contraction by αSMA expressing myofibroblasts and loss of tissue/organ function.[19,20] Major organs such as liver, kidney, lung and heart are all susceptible to fibrosis which, due to the current lack of effective treatments, often has fatal consequences.[21-24]

Persistent myofibroblasts are the major cellular cause of fibrotic tissue formation, whereas in normal healing conditions, these highly contractile cells are mostly depleted by apoptosis, de-differentiation or migration away from the wound site.[25] In embryos, the expression levels of TGF-β1 are generally lower relative to adults, inversely correlating with their capacity to achieve scar-less healing.[26,27] Targeting and neutralizing TGF-β at the margins of healing wounds in adult rats by injection with neutralizing antibodies attenuated fibrotic healing when compared to rats without treatment.[28] However, TGF-β in the myocardial muscle of mice was found to have protective effects against myocardial infarction. TGF-β was also found to prevent septic shock in the hearts, lung and liver of rats[29,30], revealing the importance of TGF-β during wound repair and the difficulties associated with targeting it as a fibrosis treatment. Although much is known about myofibroblasts and fibrosis development, it is still unclear how or why myofibroblasts persist and cause fibrotic tissue repair.

1.3 Inflammation and Fibrosis

Adequate intensity and duration of the early inflammatory phase of wound healing is crucial to eliminate pathogens at the wound site. However, persistent inflammatory responses of either excessive or inadequate intensity, and/or that fail to resolve (i.e. chronic inflammation) can result in fibrosis. Leukocytes, such as macrophages, that secrete cytokines are key contributors to inflammation.[31] Monocyte-derived macrophages express factors such as TGF-β and platelet derived growth factor (PDGF), which induce fibroblast activation into myofibroblasts, migration, and proliferation for contraction and collagen production.[32] Myofibroblasts also contribute to the inflammatory response by secreting cytokines in addition to ECM molecules, such as fibronectin (FN), which has been shown to upregulate key pro-inflammatory cytokines such as interleukin-8 (IL-8) and tumor necrosis factor (TNF).[33] The persistent expression of classical pro-inflammatory cytokines such as IFN-γ, TNF and IL-6 in the liver and peritoneum of mice were shown to be pro-fibrotic; however, inhibition of any one these cytokines led to decreased fibrotic responses, illustrating causal links between persistent inflammation and fibrosis development.[34,35]

Murine models of fibrosis also suggest that prolonged inflammation is key to fibrotic healing. The anti-cancer drug bleomycin, used to promote pulmonary fibrosis in mice, is pro-inflammatory and induces the expression of *IL-8* in human pulmonary microvascular endothelial cells.[36] Another pro-inflammatory cytokine, IL-1β, has also been shown to induce pulmonary fibrosis in mice.[37] Pirfenidone, a non-steroidal anti-inflammatory drug, inhibited bleomycin-induced pulmonary fibrosis in mice by decreasing levels of IL-1β.[38] Although these and other studies suggest that prolonged pro-inflammatory processes contribute to fibrosis development, there are still many gaps in our knowledge of the molecular mechanisms that link these processes.

1.4 Dupuytren's Disease

Dupuytren's Disease (DD), also known as Dupuytren's Contracture, palmar fibromatosis or palmar fascia fibrosis, was originally described by Felix Plater in 1614, but is named for the famous French surgeon who may have been the first to operate on patients with this condition, Baron Guillaume Dupuytren.[39] DD is a common, heritable and benign fibrosis of the palmar fascia characterized by excessive ECM deposition into fibrotic palmar nodules that often develop into palmar-digital contractures (Fig. 1.2). The ulnar digits and middle finger most often develop contracture, whereas the thumb and index fingers are less frequently affected.[40] Palmar-digital contractures, if left untreated, are permanent. Patients suffering from severe DD cannot extend their affected digits and this can severely impact their quality of life. DD is most prevalent in men over the age of 60 who are also of eastern European, Irish or Scandinavian descent, where it can affect more than 30% of this population.[41] The prevalence of DD in North America is believed to be as high as 7%.[42] The development of DD is hypothesized to be the result of abnormal palmar fascia repair after injury, where amplified cellular responses to inflammatory cytokines promote the persistence of hyper-contractile myofibroblasts.[43-46] In addition to the heritable (genomic) components of DD, lifestyle choices such as smoking and alcoholism have also been implicated in the development of this disease.[47]

1.5 DD Initiation and Progression

DD is hypothesized to be initiated by abnormal wound healing processes after palmar fascia injury and severity of the disease is classified in several stages or categories. Classifications are based on severity of the contracture relative to neutral (0°, extended): stage I (≤45°), stage II (46-90°), stage III (91-135°) and stage IV (>135°).[44] Histological analyses of these stages by Dr. Robert M. McFarlane, a world-recognized Canadian surgeon, co-founder of the Roth McFarlane Hand and Upper Limb Centre and expert in the treatment of DD, was the first to confirm that DD is a condition of the palmar fascia and not of the tendons or palmar skin. The earliest stage of DD is typically the formation of nodules in the palm containing proliferative spindle shaped cells. While there is no

evidence of excessive collagen deposition or contracture during the nodule stage, the palmar fascia around nodules can progressively thicken as the disease extends along the fascia, culminating in the formation of dense, multi-nodular, collagenous “cords” that induce palmar-digital contractures and loss of hand function[48].

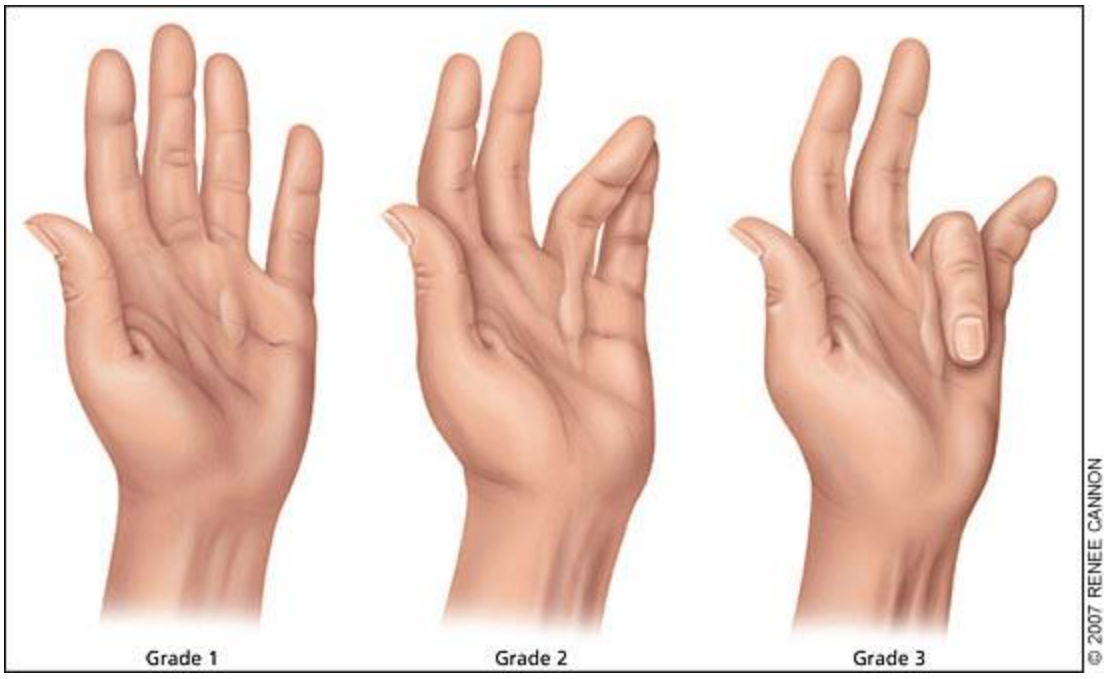

Figure 1.2. Progression of Dupuytren's Disease.

The earliest stage of Dupuytren's Disease is the formation of nodules. Nodules may be quiescent for years before extending along the palmar fascia into rope-like cords. Cords may extend across the metacarpal phalangeal (MCP), proximal interphalangeal (PIP) and distal interphalangeal (DIP) joints to induce debilitating palmar-digital contractures. Image by T. H. Trojian *et al*, Am Fam Physician (2007).

1.6 Treatment of DD

Fasciectomy, a form of invasive surgery to remove the contracted fascial tissues and restore palmar-digital extension, is often recommended to patients with debilitating contractures. Where operating room procedures are not warranted or are deemed too risky, other less invasive treatment modalities can be used. Two commonly utilized treatment options are percutaneous needle fasciotomy and collagenase clostridium histolyticum injections. Both involve the skilled use of a venipuncture needle to punch holes into the fibrotic palmar fascia tissue while avoiding adjacent nerves and blood vessels. Percutaneous needle fasciotomy physically dissects the contracture cord into sections, releasing the contracture tension and allowing extension of the digit. Collagenase clostridium histolyticum injections digest, instead of physically dissecting, the contracture cord to achieve contracture tension release and, typically 24 hours after treatment, extension of the affected digit.

Non-invasive treatment options include topical vitamin E or steroids to reduce inflammation, and physical therapy (splinting, massage or joint stretching) to increase joint maneuverability of the affected site.[49,50] Radiotherapy can also be employed, most effectively at the nodule stage, to reduce the development or activation of myofibroblasts.[51] Non-invasive treatment options are less frequently utilized than invasive treatments, potentially due to the lack of studies and evidence supporting their efficacy in "curing" or preventing disease recurrence.[52]

All of the currently available interventions fail to prevent disease recurrence in at least 30% of patients.[53,54] Non-invasive options are widely considered ineffective and therefore not recommended to patients with advance DD, further limiting treatment options for those suffering with disabling contractures.[52] Post-operative complications can include permanent joint stiffness and reflex sympathetic dystrophy which can result in lifelong pain in patients that have undergone invasive surgeries.[55] In severe cases, ray amputation, where the entire finger and the corresponding metacarpal bones are removed, can be employed as a last resort. This approach does not prevent the formation of contractures in the remaining digits. In summary, there is a clear need for more effective, evidence-based treatments for this debilitating disease.

1.7 Molecular Signatures of DD

Fibrotic palmar fascia can be differentiated from healthy tissue by histopathology. Firstly, collagen type III is highly abundant when compared to collagen type I which correlates with higher myofibroblast density in DD palmar fascia relative to healthy.[56] In addition to high cell density, these myofibroblasts persist and proliferate due to release of growth factors, such as PDGF, FGF [41] and possibly Insulin-like Growth Factor-II (IGF-II).[57] IGF-II promotes cellular proliferation in a variety of cancers, however Raykha *et al* have shown that IGF-II can also enhance collagen contraction by myofibroblasts derived from fibrotic palmar fascia. Some pro-inflammatory cytokines are also considered to be molecular signatures of DD. Izadi *et* al have reported abnormally high levels of tumor necrosis factor receptor 2 (TNFR2) and interleukin-33 (IL-33) in DD, both of which are associated with TNF signaling, a classic pro-inflammatory molecule.[58] As shown in Fig. 1.3, *TNF* expression is significantly higher in fibrosis derived DD fibroblasts, relative to "healthy" syngeneic (PF) and allogeneic (CT) control fibroblasts, further implicating inflammation in the development of DD.

Another molecular characteristic of DD is increased cytoplasmic and nuclear levels of ß-catenin, a well-known cancer associated trans-activator of gene transcription.[59] Chromatin immunoprecipitation analyses of ß-catenin interactions in fibroblasts derived from DD revealed that ß-catenin interacts with factors that bind the Wilms' Tumor 1 (*WT1*) gene, another known cancer associated transcription factor. While the roles of ß-catenin in regulating *WT1* expression levels are currently being investigated, Crawford *et al* reported that WT1 protein and *WT1* gene transcript expression are abnormally high in fibrotic palmar fascia tissue and fibroblasts derived from this tissue, respectively (Fig. 1.4 and 1.5).[60] It is currently unclear what roles these cancer associated molecules, including WT1, play in the development of DD, or if any of these molecules have potential as therapeutic targets to prevent DD progression and/or recurrence.

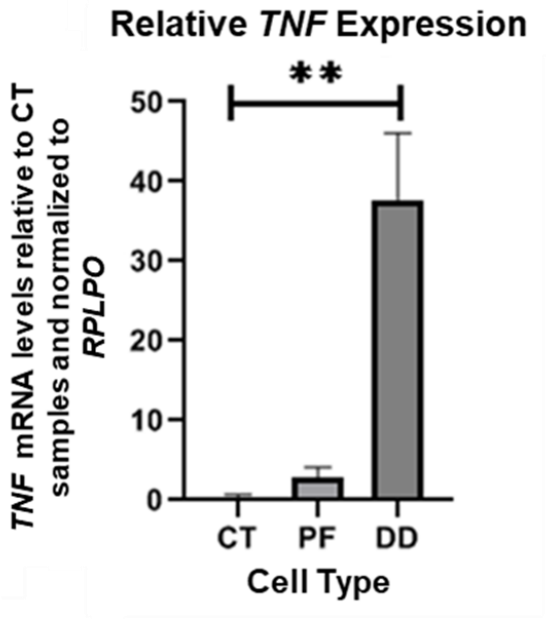

Figure 1.3. TNF mRNA expression is increased in primary fibroblasts derived from Dupuytren's Disease tissues.

TNF gene expression was assessed using Taqman primers (Thermofisher Scientific) in cDNAs derived from total RNA samples extracted from DD, PF and CT fibroblasts (each N=3, **p <0.001 as determined by ANOVA). Transcript levels were normalized to the expression of *RPLPO* as described in Chapter 2.

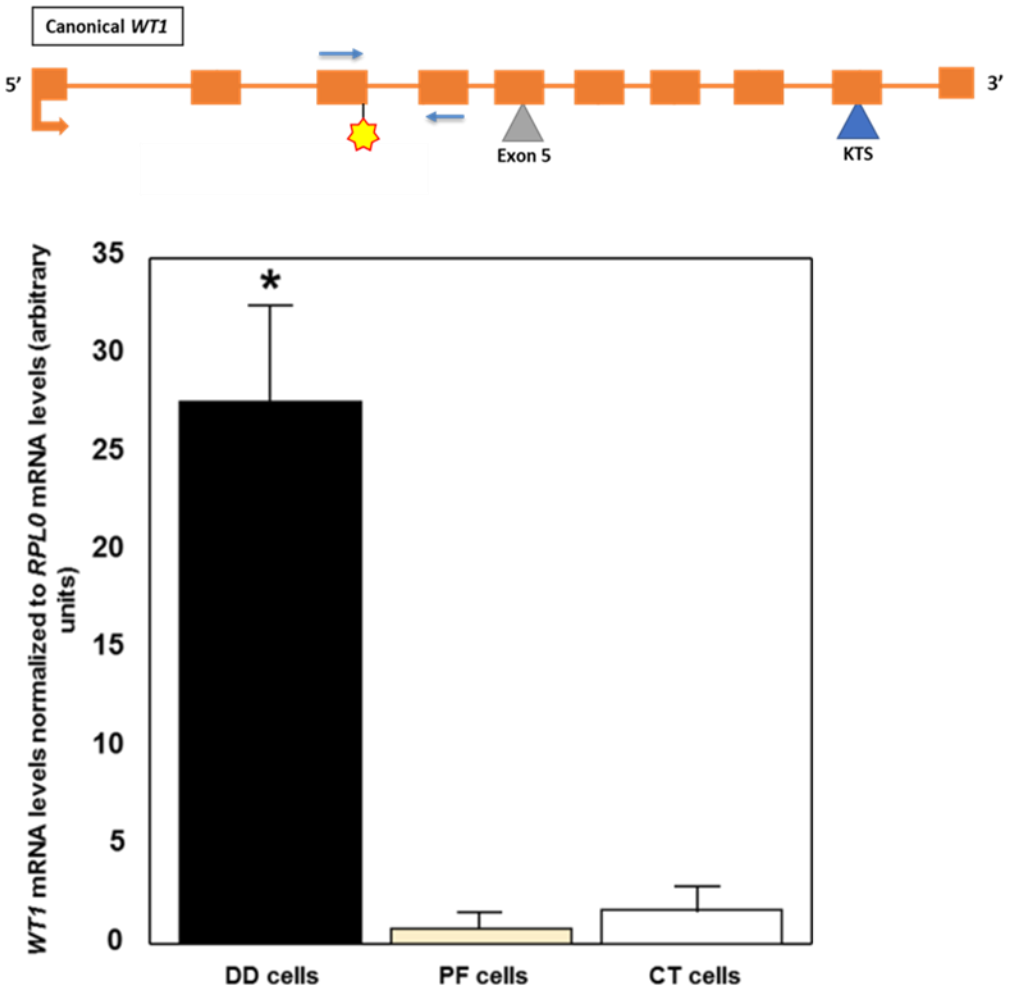

Figure 1.4. WT1 gene expression is increased in DD fibroblasts.

WT1 gene expression was assessed using Taqman primers (Thermofisher Scientific) to amplify a region in exons 3 and 4 (as shown as forward and reverse arrows) in cDNA derived from total RNA extracted from DD, PF and CT fibroblasts (N = 13, 13, and 6 respectively, * $p < 0.05$). From Crawford *et al*, JCCS 2015.

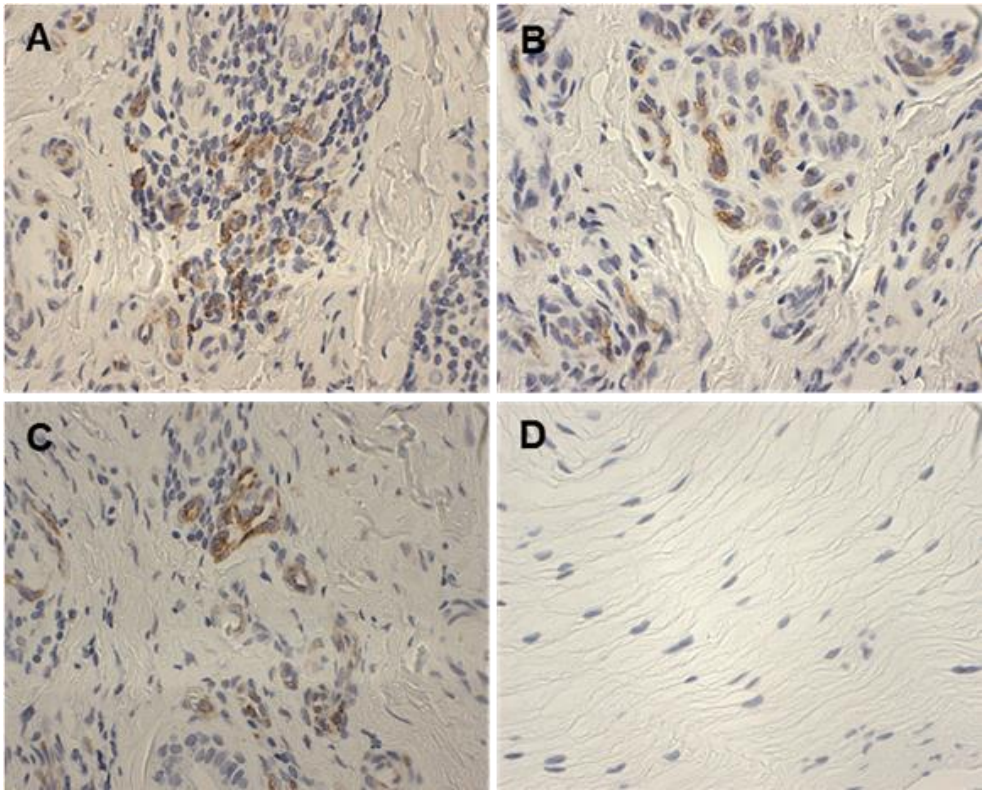

Figure 1.5. WT1 immunoreactivity in fibrotic and visibly unaffected palmar fascia tissues.

WT1 immunoreactivity in fibrotic and normal palmar fascia: Paraffin embedded fibrotic (A, B and C) and macroscopically unaffected palmar fascia (D) tissues were sectioned and assessed for WT1 immunoreactivity. The antibody clone 6F-H2 used for these analyses is reported to recognize an epitope in exon 1 of canonical WT1. Tissues were counterstained with Gills hematoxylin to distinguish cell nuclei from palmar fascia tissue matrix. From Crawford *et al*, JCCS 2015.

1.8 Wound Healing and Cancer

Potential connection and similarities between cancers and wound healing were noted as early as in 1974 by Sir. Alexander Haddow, famous for discovering the Haddow effect, however widespread interest in this idea gained traction when Dr. Harold Dvorak published "Tumors: wounds that do not heal" in 1986.[61,62] Comparisons by Dr. Dvorak and other researchers indicated that cancer development and wound healing relied on similar processes at the molecular level, such as the production of vascularized connective tissue stroma, a process in which VEGF plays an essential role.[63] In parallel with their roles in wound healing, inflammatory cells such as macrophages can contribute to an inflammatory microenvironment that promotes cancer cell growth and metastasis in osteosarcoma.[64] Additionally, IGFs promote cellular proliferation during wound remodelling[67] as well as promoting tumor invasion and chemoresistance.[65,66]

Fibroblasts play central roles in wound healing that include facilitating wound closure and tissue remodelling. These cells are also key contributors to cancer development and progression. Recent findings suggest that activated fibroblasts are multipotent and capable of differentiating into adipocytes or chondrocytes which can contribute to the growth of tumors.[67] Fibroblasts that are "cancer associated" (CAF) are capable of remodeling the extracellular matrix to promote resistance to chemotherapy, secreting growth factors that drive cancer growth and recruiting immune cells which are capable of modulating the tumor microenvironment.[67] Bissel *et al* have shown that cancer formation at sites of injury requires an inflammatory response.[68] These and other similarities between wound healing and cancer development are still being revealed as ongoing research provides additional evidence linking these processes.

1.9 WT1 in Cancers and Development

One novel potential contributor to both cancer and DD development is Wilms Tumor 1 (WT1). Originally identified in pediatric kidney (Wilms) tumors, the roles of WT1 in

cancer growth are context dependent, such that it is tumor growth suppressive or promoting in different cancers.[69] *WT1* transcripts are alternatively spliced and different splice variants encode regulatory proteins that can modify cancer cell growth and development.[70] Amongst 30+ potential *WT1* mRNA variants, there are four major splice variants which have been identified and well-studied in leukemia.[71] These splice variants are differentiated by the inclusion or exclusion of exon 5, encoding a 17 amino acid insert between the trans-regulatory and zinc finger domains of WT1, and by the alternative splicing of a region at the end of exon 9 encoding a Lysine-Threonine-Serine (KTS) motif within the zinc finger DNA binding domain (Fig. 1.6A).[72,73] Protein isoforms lacking the KTS motif (KTS-) are reported to bind DNA and act as transcription factors whereas KTS positive (KTS+) isoforms have diminished DNA binding and are proposed to have (as yet poorly defined) roles in RNA splicing.[74] The functional domains of WT1 include an RNA binding and repression domain located in regions of WT1 encoded by exon 1, an activation domain located in regions of WT1 encoded by exon 5, and zinc finger domains located in regions of WT1 encoded by exons 7 through 10 (Fig. 1.7).

In addition to alternative splicing, *WT1* variants can be transcribed from at least three alternative start sites. Relative to the transcriptional start site of "canonical" *WT1* transcripts, there is a 5' "extended" (*ExtWT1*) transcript that initiates at a CUG encoded upstream of the canonical sequence AUG start site, an "alternative" (*AWT1*) transcript that initiates at an AUG encoded within intron 1, and a truncated variant (*TrWT1*) that initiates from an AUG encoded within intron 5 (Fig. 1.6B).[75-80] *ExtWT1* transcripts are reportedly overexpressed in many different cancer cell lines and are causally linked to increased expression of several well-known cancer associated genes including *C-myc*, *Bcl-2* and *Egfr*.[76] Interestingly, *AWT1*, which encodes for a protein lacking the exon 1 repression domain of canonical WT1, has been reported to be overexpressed in leukemias and to result in increased expression of *CCNE1* and *IGF1*.[78] The functional properties of *TrWT1* transcripts are currently unknown.

In addition to cancers, *WT1* expression has also been implicated in organogenesis and homeostasis. Kreidberg *et al* demonstrated that *WT1*$^{-/-}$ null mice do not develop kidneys or gonads and die before birth, demonstrating the importance of *Wt1* expression in the

developing fetus.[117] *WT1* may also have roles in the developing heart. Mouse embryos lacking expression of *Wt1* in the epicardium lack coronary vasculature and also die before birth.[118] The lack of vasculature may be explained by the proposed roles for *WT1* in angiogenesis. Wagner *et al* reported that conditional deletion of *Wt1* in endothelial cells of mice and subsequent cancer cell injections resulted in reduced tumor vascularization.[119] Although it is becoming clear that *WT1*expression is required during the development of several organs, *WT1* expression in adulthood is not ubiquitous. In adults, *WT1* expression is found mostly in glomerular podocytes and mesothelium of some organs.[120] Conditional knock-out of *Wt1* expression in podocytes of mice resulted in stunted kidney function and glomerulosclerosis.[121] The roles of any specific WT1 isoforms in wound healing or fibrosis development are essentially unknown.

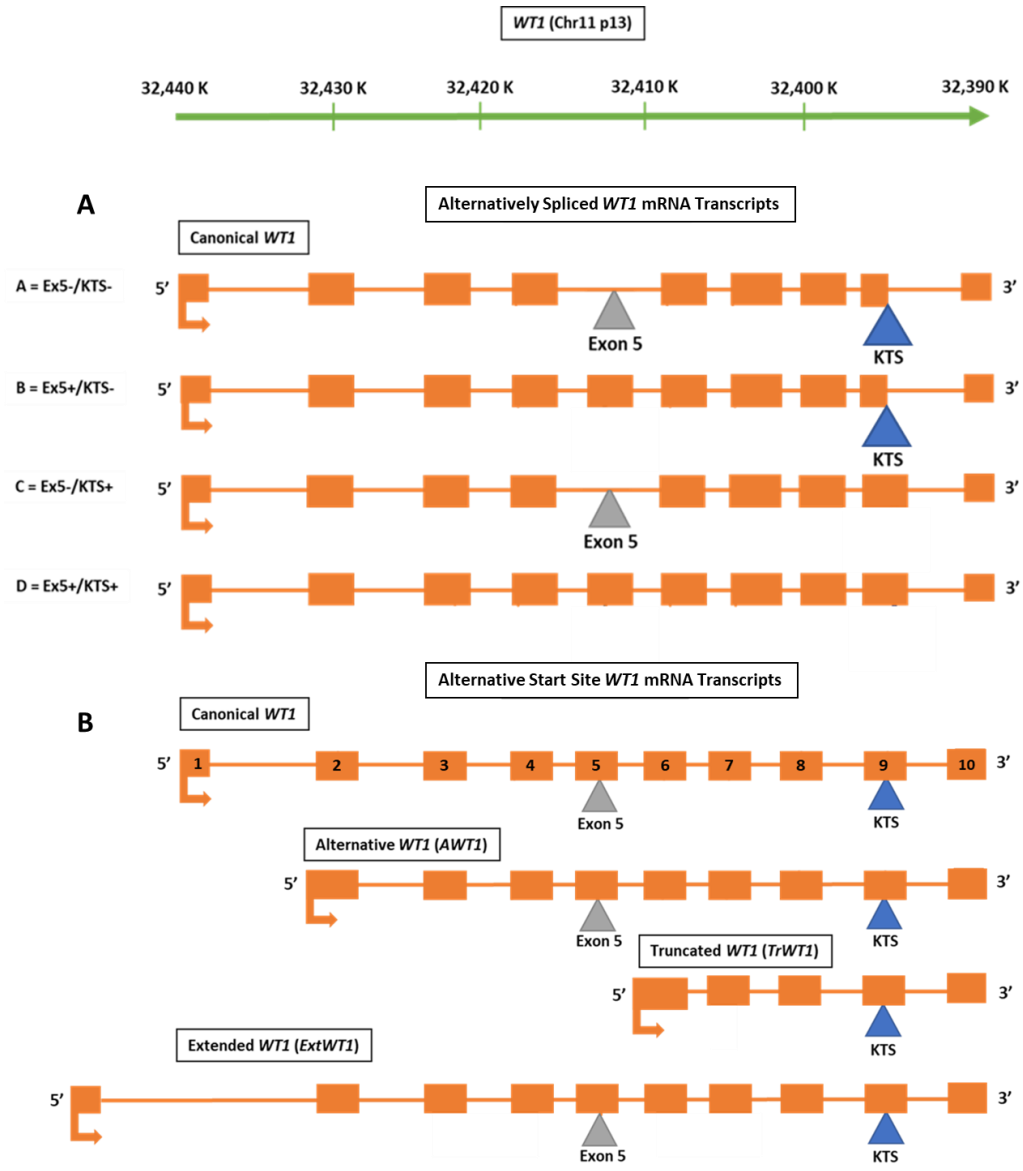

Figure 1.6. WT1 gene structure, alternatively spliced and alternative start site mRNA transcripts

The *WT1* gene is located on p arm of chromosome 11 and contains 10 major exons with multiple alternative transcriptional start sites. Major sites of alternative mRNA splicing are exon 5 (Ex5) and the end of exon 9 (KTS), denoting a sequence that encodes a KTS motif previously reported to impact the DNA binding and transcriptional capacity of the translated protein. **(A)** The four major alternative splice variants are shown as A: Ex5-/KTS-, B: Ex5+/KTS-, C: Ex5-/KTS+, and D: Ex5+/KTS-. **(B)** Previously reported alternative transcriptional start sites relative to canonical *WT1* mRNA transcripts include intron 1 (alternative *WT1*), intron 5 (truncated *WT1*) and the 5' untranslated region (extended *WT1*). Each of these four transcript variants can also be alternatively spliced, and thus there are four possible canonical WT1 variants, four possible AWT1 variants, two possible TrWT1 variants and four possible ExtWT1 variants, accounting for 14 different WT1 isoforms.

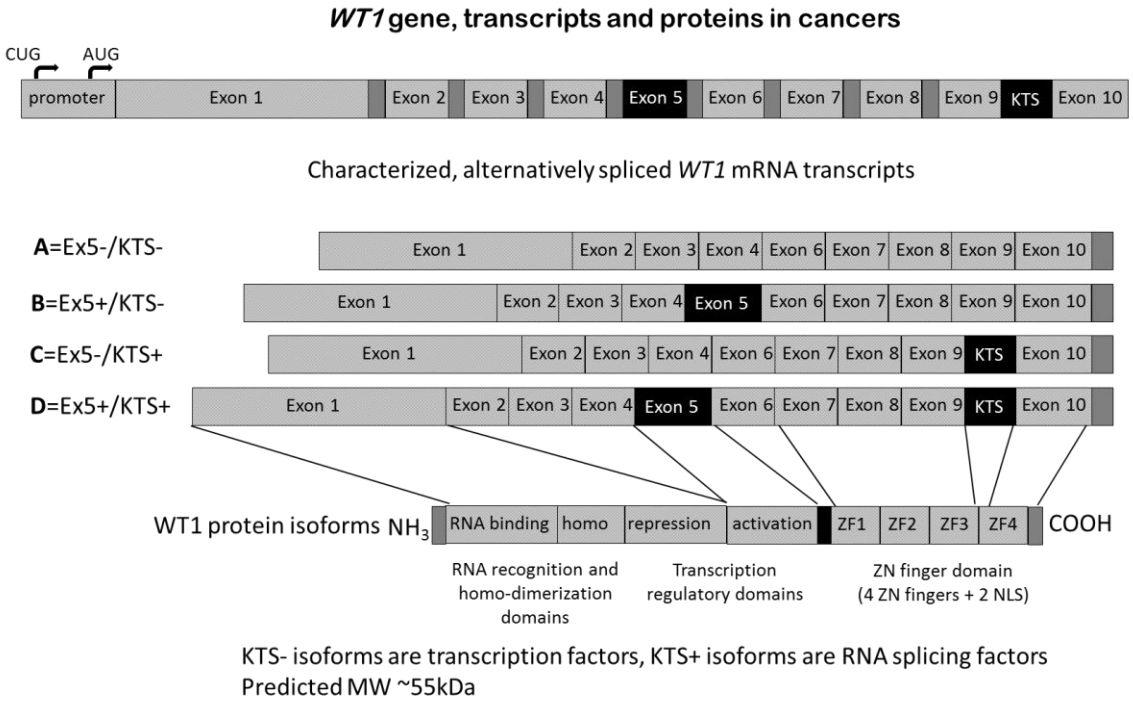

Figure 1.7. Functional domains encoded by *WT1* mRNA variants.

Diagram of the 4 major canonical *WT1* mRNA variants and the functional domains encoded. WT1 functional domains include RNA binding and repression domain encoded by exon 1, an activation domain encoded by exon 5, and zinc finger domains encoded by exons 7 through 10. Alternative splice sites are indicated by black boxes. This figure was adapted from K. Kramarzova *et al* Leukemia (2012).

1.10 WT1 in Fibroses

Crawford *et al* was the first report of increased *WT1* mRNA levels and WT1 immunoreactivity in DD.[60] Potential roles for *WT1* in the development of other fibroses have been proposed. Increased *WT1* transcript expression has been reported in lung and liver fibroses, indicating potential roles for WT1 in the development of fibrosis in different tissue and organs.[81,82] However, the roles of WT1 in lung fibrosis development are controversial and, currently, contradictory. Sontake *et al* have demonstrated that *WT1* expression was exclusive to idiopathic pulmonary fibrosis (IPF), and that no evidence of WT1 expression was evident in healthy lungs.[82] Consistent with pro-fibrotic roles for WT1, they showed that knock down of *WT1* in murine models of IPF attenuated the expression of extracellular matrix molecules and αSMA by fibroblasts. In contrast, Karki *et al* provided evidence that *WT1* expression was required for the integrity of healthy lungs and that the loss of *WT1* expression led to IPF development.[83] These discrepancies are currently unresolved. WT1 has recently been assessed (unpublished data) as a potential biomarker of fibrosis in murine models of Duchenne Muscular Dystrophy, a debilitating disease resulting in skeletal muscle atrophy and fibrosis. Conflicting reports and the inherent complexity of multiple WT1 isoforms with (potentially) different, context dependent roles in normal or abnormal tissue repair, make this a complex but intriguing research focus. Therefore, the objectives of this study were to investigate the roles of specific *WT1* isoforms in DD, and by extension, other connective tissue fibroses.

1.11 Rationale

To study DD, primary human fibroblasts were derived from resected DD tissue (DD fibroblasts), visibly non-fibrotic tissue from the adjacent palmar fascia (PF fibroblasts from the same DD patient) as genetically matched (syngeneic) controls and normal palmar fascia from patients with no prior history for Dupuytren's Disease (CT fibroblasts) as allogeneic controls. Where possible, these cells were cultured in 3D collagen-based hydrogels to more closely replicate tissue repair-associated processes such as 3D proliferation, migration and tissue remodeling/contraction *in vivo*. Surgical

resections to obtain palmar fascia tissues for cell derivation were performed by surgeons of the Roth McFarlane Hand and Upper Limb Clinic at St. Joseph's Hospital in London, Ontario in accordance with institutional ethics guidelines and approval (HSREB 104888).

Prior to the commencement of this project, Crawford *et al* has determined that *WT1* mRNA levels and WT1 immunoreactivity were abnormally high in primary DD fibroblasts and contracture tissues relative to syngeneic PF controls (Fig. 1.4, Fig.1.5).[60] Expanding on these studies, the O'Gorman laboratory have also investigated the expression of alternatively spliced WT1 mRNA transcripts using PCR primers that spanned exon 5 and the KTS-encoding section of exon 9. Splice-transcript-specific PCR analyses determined that DD fibroblasts expressed all four splice options (Ex5-/KTS-, Ex5+/KTS-, Ex5-/KTS+, and Ex5+/KTS-), unlike syngeneic controls, which did not express any detectible WT1 mRNA transcripts using this approach (Fig. 1.8).

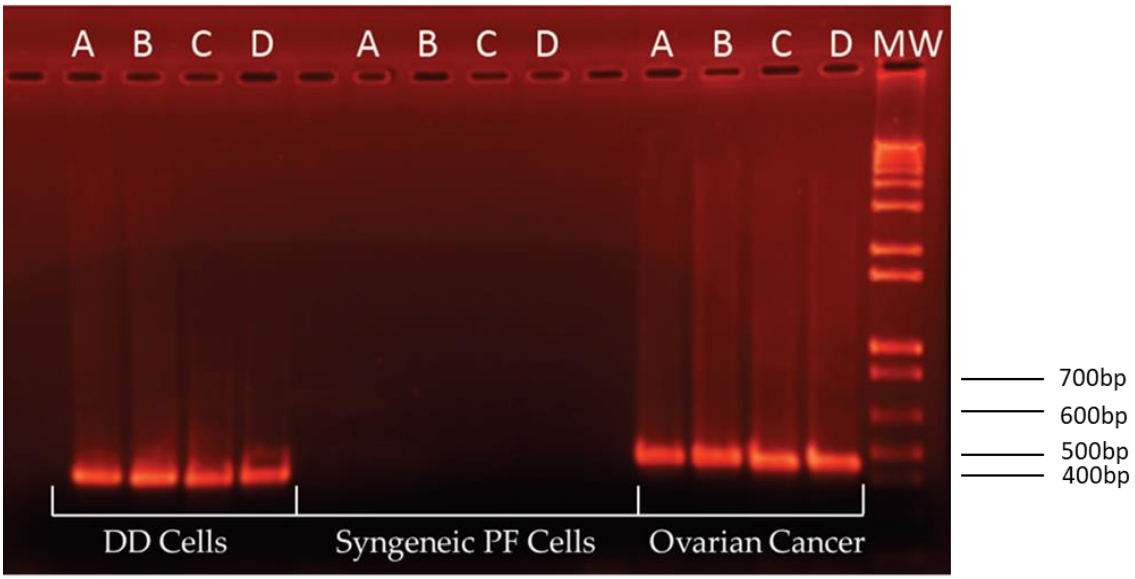

Figure 1.8. DD fibroblasts express alternatively spliced WT1 mRNA transcripts.

Representative image of transcript-specific PCR amplification of reverse transcribed *WT1* mRNAs with or without alternative splicing of exon 5 (Ex5) and/or the 3' end of exon 9 (KTS). Lanes A: Ex5-/KTS-, B: Ex5+/KTS-, C: Ex5-/KTS+, and D: Ex5+/KTS- were assessed in DD fibroblasts and in syngeneic (PF) controls. cDNA derived from the OVCAR3 ovarian cancer cell line was used as a positive control. Data produced by Ana Pena Diaz prior to this project. Primer sequences were adapted from Kramarzova *et al*, Leukemia 2012.

To determine if the expression of *WT1* transcripts in palmar fascia fibroblasts were regulated by the pro-inflammatory cytokines that characterize the early inflammation stage of tissue repair, DD, PF and CT fibroblasts were treated with a pro-inflammatory "cytomix" of TNF, IL-1ß and IFN-γ. DD, PF and CT fibroblasts treated with these cytokines for 24hrs (0.5 ng/ml) showed a significant (~10 fold) increase in *WT1* mRNA levels based on amplification of sequences encoded in exons 3 and 4 (Fig. 1.9). Furthermore, the *WT1* mRNA transcripts induced by pro-inflammatory cytokine stimuli in PF and CT fibroblast controls included the four major *WT1* splice variants shown in Fig. 1.8 to be expressed in DD fibroblasts (i.e., Ex5-/KTS-, Ex5+/KTS-, Ex5-/KTS+, and Ex5+/KTS-) (Trisiah Tugade 4th year thesis, data not shown). These data were interpreted to suggest that DD fibroblasts are in a chronically cytokine-activated state, where *WT1* transcript expression is constantly induced, whereas PF and CT fibroblasts are in a relatively quiescent state in the absence of cytokine stimulus. These data are consistent with the hypotheses that a transient increase in *WT1* transcript expression is a normal aspect of palmar fascia repair during the inflammation stage, and that sustained *WT1* expression in DD cells may be causally linked to fibrosis development.

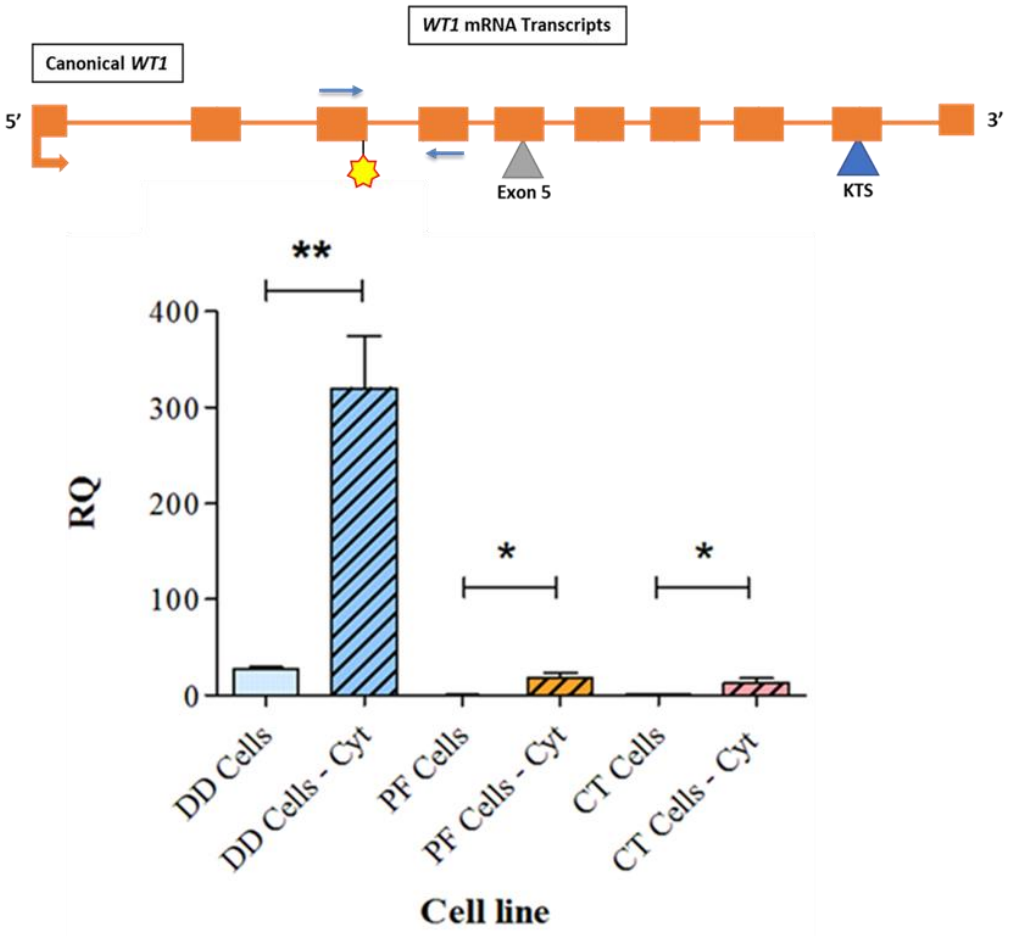

Figure 1.9. WT1 mRNA expression in DD, PF and CT fibroblasts is induced by pro-inflammatory cytokines.

WT1 mRNA level were assessed by qPCR in DD, PF and CT fibroblasts, with (-Cyt) or without stimulation with a cytomix of TNF, IL-1ß and IFN-γ (0.5ng/ml for 24 hours). N = 3, n = 3, * $p < 0.05$, ** $p < 0.01$ by ANOVA). Forward and reverse arrows represent Taqman primers recognizing and binding exons 3 and 4 of *WT1* mRNA respectively. Data produced by Trisiah Tugade (4th year student) prior to this project.

The central hypothesis of this thesis is that abnormally sustained expression of specific WT1 protein isoforms by DD myofibroblasts, induced by local chronic inflammation, promote fibrosis development. If this hypothesis is supported, these findings would implicate WT1 protein isoforms as potential therapeutic targets to prevent DD progression and recurrence.

The specific goals of this thesis were to:

1) Characterize the *WT1* mRNA start site variants that DD, PF and CT fibroblasts express, with and without pro-inflammatory cytokine stimuli, using 5'RACE, transcript specific PCR and cDNA sequencing.
2) Identify any DD fibroblasts specific *WT1* mRNA transcripts that may encode fibrosis specific WT1 transcription factor isoforms.
3) Express fibrosis specific *WT1* transcripts in syngeneic and allogeneic control cells and assess any changes in gene expression using RNA sequencing and bioinformatic analysis.
4) Determine if constant expression of DD-associated *WT1* transcripts conferred a fibrosis-like phenotype on PF or CT fibroblasts.
5) Determine if the constant expression of DD associated *WT1* transcripts in PF and CT fibroblasts induced the secretion of factors that modified the expression of cytokine-encoding genes in THP-1 monocytes as an *in vitro* model of paracrine interactions during tissue repair.

Chapter 2

2 METHODOLOGY

2.1 Derivation of Primary Fibroblasts and Cell Culture

Palmar fascia tissue samples were surgically resected from patients with Dupuytren's Disease (DD) and from patients undergoing hand surgery for unrelated conditions. Primary human fibroblasts were derived from visibly fibrotic palmar fascia tissue (DD fibroblasts) and from visibly non-fibrotic tissue from the same patient (PF fibroblasts) as syngeneic controls. Normal palmar fascia fibroblasts (CT fibroblasts) were derived from patients with no history of DD undergoing hand surgery for unrelated conditions, typically carpal tunnel release surgeries, and were utilized as allogeneic normal controls. Surgical resections to obtain palmar fascia tissues for cell derivation were performed by surgeons of the Roth McFarlane Hand and Upper Limb Clinic at St. Joseph's Hospital in London, Ontario in accordance with institutional ethics guidelines and approval (HSREB 104888).Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (Gibco) supplemented with 8% fetal bovine serum (FBS) (Gibco)**,** L-glutamine and antibiotic-antimycotic at 37 °C and under 5% CO_2. All cell cultures were used up to a maximum of 6 consecutive passages where possible to minimize any *in vitro* culture-induced changes in gene expression and/or phenotype.

2.2 RNA Extraction and First Strand Synthesis (cDNA)

Total RNA was isolated from DD, PF and CT fibroblasts in TRIzol (Thermofisher Scientific) using Direct-zol RNA extraction kit (Zymo Research, Cat# R2072) according to manufacturer's protocol. RNA isolates were DNase treated for 25 minutes to remove genomic or viral DNA contaminants and were assessed for quantity and quality ($A_{260//280}$ and $A_{260/230}$) using a DeNovix DS-11 Spectrophotometer. High quality RNA (2μg) was reversed transcribed into first strand cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturer's instructions. The following

thermocycle was utilized: 25 °C for 10 minutes, 37 °C for 2 hours, 85 °C for 5 minutes, 4 °C. All cDNA samples were stored at -20 °C.

2.3 Quantitative Taqman Polymerase Chain Reaction (PCR) analyses

One microlitre aliquots of first strand cDNA were assessed in triplicate for qPCR analyses using Taqman Primers (Thermofisher Scientific. See Appendix A) and Fast Advanced Taqman Master Mix **(**ThermoFisher Scientific**)** at a total volume of 10µl. qPCR reaction was set for initial denaturation at 95 °C for 20 seconds followed by 40 cycles of denaturation at 95 °C for 1 second and annealing/extension at 60 °C for 20 seconds using Quantstudio 5 Thermocycler (ThermoFisher Scientific)**.** The level of expression of target genes were calculated using ΔΔCt method[84], normalized to *RPLP0* or *GAPDH* housekeeping gene and relative to CT controls or THP-1 monocultures. Negative controls included minus reverse transcriptase and minus cDNA.

2.4 Qualitative PCR

First strand cDNA (100ng) was added into PCR master mix containing 10x PCR buffer (5µl), 50mM $MgCl_2$ (1.5µl), 25mM dNTP (0.5µl), forward primer (1µl), reverse primer (1µl), Platinum Taq Polymerase (0.4µl) and ddH_2O to a final volume of 50µl. Forward and reverse primers were designed in house, synthesized by Sigma Aldrich, and reconstituted to a concentration of 10µM in ddH_2O. Forward and reverse primer pairs were chosen to cross exon-exon boundaries to avoid amplification of genomic DNA. Touchdown PCR was carried out with initial denaturation at 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 seconds, annealing of 68 °C for 25 seconds and extension of 72 °C for ≤1 minute. Final extension was carried out at 72 °C for 10 minutes and held at 4 °C or frozen at -20 °C until needed. Amplified products were separated on 10% polyacrylamide gels at 90V for ~1.5 hours, stained with ethidium bromide and imaged using a gel imaging dock (Bio-Rad). Alternatively, amplified products were

separated in 1% agarose at 90V for ~1.5 hours, stained with ethidium bromide and bands were excised for cDNA extraction using QIAquick Gel Extraction Kit (Qiagen, Cat# 28704). Amplified DNAs samples were sent to the London Regional Genomics Center at Robarts Research Institute for DNA sequencing.

2.5 5’ Rapid Amplification of cDNA Ends (5’RACE)

DD fibroblasts were cultured as FPCLs and stimulated with TNF, IFN-γ and IL-1β (0.5ng/ml) for 24 hours. RNA was extracted from DD fibroblasts and 5μg of total RNA was used for 5’RACE in accordance with manufacturer’s protocol (ThermoFisher Scientific, Cat# 18374058). In brief, mRNA was copied into cDNA using GSP1 (Appendix 2) and superscript™ II reverse transcriptase and subsequently RNase treated to remove mRNA. TdT (terminal deoxynucleotidyl transferase) tailing of cDNA was then performed to create an abridged anchor primer binding site on the 3’-end of the cDNA for downstream amplification using abridged anchor primer (provided in kit) and GSP2 (Appendix 2). Amplified products from 5’RACE were reamplified using nested primers to determine amplified *WT1* transcripts (Appendix 2). Touchdown PCR was carried out with an initial denaturatio0n at 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 seconds, annealing of 68 °C for 25 seconds and extension of 72 °C for ≤1 minute.

2.6 Protein Isolation and Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

Fibroblasts were cultured in T75 flasks until 70% confluence. The cells were detached with trypsin (Gibco) for 5 mins at 37°C, and the detached cells were centrifuged at 700 x g. The cell pellets were isolated and resuspended in supplemented (0.1M NaF, 10mM PMSF and 10mM Na_3VO_4) RIPA buffer (Teknova), aspirated through a 27.5G needle to rupture the cells, and then centrifuged (12,000 x g) for 2 minutes. Supernatants containing protein lysate were quantified using the Bicinchoninic Acid (BCA) protein assay kit (Pierce BCA Protein Assay Kit, ThermoFisher Scientific, Cat# 23227)

according to manufacturer's protocol. 1μg of total protein lysate was used for WT1 quantification by human Wilms tumor protein ELISA kit (Mybiosource, Cat# MBS761090) according to manufacturer's protocol.

2.7 Adenoviral Amplification and Transduction

HEK293 cells were cultured in DMEM supplemented with 8% FBS, L-glutamine and antibiotic-antimycotic until 60-70% confluency. 1mL viral stock solutions of containing either cDNA encoding AWT1 (Ex5+, KTS-) (Vigene Biosciences VH801783) or cDNA encoding green fluorescent protein (GFP) (Vigene Biosciences CV10001) was added to infect cells (Appendix 6). Infected HEK293 cells were cultured until 95% of the cells detached from the dishes, collected and subjected to 3 freeze/thaw cycles in -80 °C freezer and 37 °C water bath. Cellular debris was removed by centrifugation for 10 minutes at 700 x g. Viral supernatant was collected, aliquoted and stored at -80 °C. CT and PF fibroblasts were transduced with adenovirus expressing either AWT1 (AdAWT1, Ex5+/KTS-) or GFP (AdGFP as viral control) at 1:3000 in DMEM in 30mm culture dishes for 24 hours. Exogenous expression of *WT1* was maintained for 72 hours prior to RNA extraction for qPCR or RNA sequencing analyses.

2.8 Western Immunoblotting for AWT1

Twenty micrograms of total protein lysate samples in RIPA buffer were derived from the OVCAR3 ovarian cancer cell line (positive control for WT1 protein)[85] and from CT fibroblasts transduced with either AdAWT1 or AdGFP and loaded onto Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, 4-15% SDS) for electrophoresis at 100V for ~1.5 hours. The polyacrylamide gels were transferred to an iBlot™ PVDF Transfer Stack (ThermoFisher Scientific, Cat# IB401002) and transferred to PVDF using an iBlot™ Dry Blotting System (ThermoFisher Scientific). After protein transfer, the PVDF membrane was incubated with blocking solution consisting of 1x Tris-buffered saline supplemented with 0.1% TWEEN-20 (TBS-T) and 5% w/v bovine serum albumin (BSA)

for 45 minutes at room temperature. Primary WT1 antibody (D6M6S Rabbit mAb, Cell Signalling, Cat# 13580) was diluted at 1:1000 in TBS-T (5% w/v BSA) and incubated with PVDF membranes at 4°C overnight. PVDF membranes were washed in TBS-T 3 times for 5 minutes each and incubated with anti-rabbit-HRP conjugated secondary antibody for 1.5 hours at room temperature, washed in TBS-T 3 times for 5 minutes each and incubated briefly in peroxidase: luminol solution at a 1:1 ratio of. Chemiluminescent images were captured using a Gel Doc XR+ System (Bio-Rad).

2.9 RNA Sequencing

Total RNA samples were further assessed for quantity and quality using Agilent 2100 Bioanalyzer. 1μg of high-quality RNA from each sample was submitted to London Genomics Sequencing Center at Robarts for RNA sequencing (RNA-seq) and library preparation using Illumina NextSeq Mid Output Kit (Vazyme VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina®). The raw data generated by library preparation was then analyzed using Partek Flow® data analysis software. Raw data was aligned to reference human genome (hg38) using Spliced Transcripts Alignment to a Reference (STAR) tool, normalized using Trimmed Mean of M values (TMM) and differential analysis was performed using Gene Specific Analysis (GSA). Inclusion criteria for downstream analysis were $p < 0.05$ and a fold change in gene expression of ≥ 1.5 or ≤ -1.5. Heat-map, gene set enrichment, and pathway enrichment analyses were performed and generated using Partek Flow® software.

2.10 Preparation of Fibroblast Populated Collagen Lattices (FPCL)

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 500μL. $1x10^3$ fibroblasts in 50μl volumes were added to 500μL of collagen mixture and cultured in triplicate in 24-well plates for 30 minutes until collagen polymerization was achieved at a total volume of 550μl. 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% CO_2 for 72 hours. FPCL cultures were then used for RNA isolation or for collagen contraction assays.

2.11 Collagen Contraction Assays

Modified versions of the collagen contraction assays as described by Bell *et al* were performed using CT and PF fibroblasts with and without transduction with adenoviral vector expressing *AWT1* variant or *GFP*.[86] Fibroblasts were cultured as FPCLs in 24-well culture trays (as described in the previous section) as quadruplicates and maintained in DMEM supplemented with 2% FBS, 1% L-glutamine and 1% antibiotic-antimycotic at 37 °C in 5% CO_2 for 72 hours. The 72-hour incubation period was performed to allow for fibroblasts to respond to the stressed collagen lattice for activation to a myofibroblast phenotype as described by Tomasek *et al*.[87] Collagen lattices were subsequently released from adhering to the culture wells. Floating lattices were digitally scanned using Canon scanner and observed over 0.5, 1, 2, 4, 6- and 24-hour time points. Surface area of the FPCL was measured using a free hand tool in Image J software, where a decrease in surface area over time was indicative of an increase in contraction. Data was represented as a measure of percent reduction in lattice area prior lattice release.

2.12 THP-1 Co-Cultures

Immortalized THP-1 monocytes were obtained from American Type Culture Collection (ATCC) which were derived from blood samples obtained from a patient diagnosed with acute monocytic leukemia.[88] THP-1 monocytes were maintained in Roswell Park Memorial Institute (RPMI) supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic-antimycotic at 37 °C in 5% CO_2. THP-1 cultures were kept below 10^6 cells per ml as recommended by ATCC. Prior to the addition of THP-1 monocytes, $1x10^5$ fibroblasts were seeded in 6-well trays in DMEM supplemented with 8% FBS, 1% L-glutamine and 1% antibiotic-antimycotic for 24 hours. Subsequently, to generate

conditioned media, fibroblasts were cultured for an additional 48 hours in serum free media consisting equal parts of DMEM and RPMI supplemented with 1% L-glutamine and 1% antibiotic-antimycotic. Co-cultures were maintained for 24 hours and RNA was subsequently isolated from monocytes in co-culture or monoculture in TRIzol (Thermofisher Scientific) using Direct-zol RNA extraction kit (Zymo Research, Cat# R2072) according to manufacturer's protocol. RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturer's instructions. Gene expression analyses were performed on THP-1 monocytes from co-culture and was relative to THP-1 monocultures

2.13 Statistical Analysis

Statistical analysis was conducted using Prism8 (GraphPad) software. For the FPCL collagen contraction data, one-way ANOVA was performed to identify significant changes in collagen contraction between PF and CT fibroblast cells without adenoviral transduction and with adenoviral transduction (AdAWT1 or AdGFP). One-way ANOVA was also performed to determine significant differences in WT1 concentration or target gene expression between DD, PF and CT fibroblasts cell lysate. Paired t-tests were used to determine significant changes in target gene expression between PF and CT fibroblasts with adenoviral transduction (AdAWT1 or AdGFP). Findings were considered statistically significant when $p < 0.05$.

Chapter 3

3 RESULTS

3.1 5’ Rapid Amplification of cDNA Ends (RACE) Analysis Reveals Alternative *WT1* Transcriptional Start Sites in DD Fibroblasts

Previous data from the O’Gorman laboratory had demonstrated that *WT1* mRNA levels were upregulated in DD fibroblasts relative to PF and CT fibroblasts[60], and that WT1 mRNA expression levels could be induced by pro-inflammatory cytokines (TNF, IL-1β, IFN-γ) in all three cell types (Fig. 1.4, Fig. 1.9). At the commencement of this thesis, it was unclear whether the WT1 mRNA transcripts expressed by DD, PF and CT fibroblasts had similar or differing transcriptional start sites. The qPCR (Taqman) primer set used to amplify the reverse transcribed *WT1* mRNA transcripts described in Crawford *et al* targeted to a region spanning exons 3 and 4 of the canonical WT1 mRNA sequence (Fig. 1.4). It was possible, therefore, that DD, PF or CT fibroblasts might express additional *WT1* mRNA alternative start site isoforms that did not include the sequences targeted by this qPCR (Taqman) primer set and were therefore yet to be detected.

To investigate this possibility, DD fibroblasts were treated with pro-inflammatory cytokine stimuli for 24 hours to maximize *WT1* transcript expression before isolating total RNA and performing *WT1* mRNA start site analyses using 5’ Rapid Amplification of cDNA ends (5’RACE). Using this approach, 5’RACE products were reamplified using nested primers (Appendix 2), designed for the detection of the *WT1* transcripts start site variants previously reported in cancers.[75-80] PCR amplicons consistent with *WT1* mRNA transcribed from the canonical start site (canonical *WT1*, Fig. 3.1A), intron 1 (*AWT1*, Fig. 3.1B) and 5’ untranslated region (*ExtWT1*, Fig. 3.1C), were all detected in DD fibroblasts after cytokine stimulus.

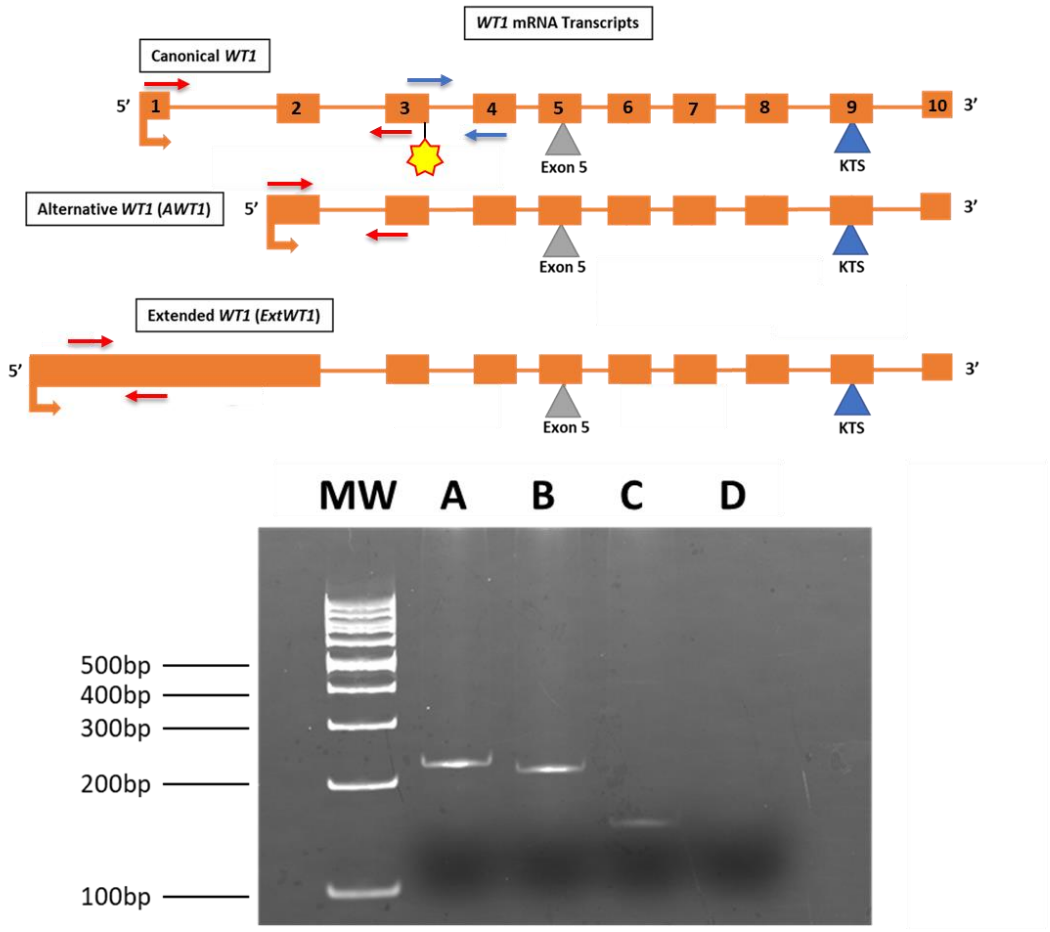

Figure 3.1. Cytokine stimulated DD fibroblasts express WT1 transcripts transcribed from alternative start sites.

PCR reamplification of 5'RACE enriched *WT1* transcripts. Lane A: Nested forward and reverse primers targeting exons 1 and 3 respectively of canonical WT1 sequence to enable the detection of *WT1* transcripts containing exon 1 (234bp amplicon). Lane B: Nested forward and reverse primers targeting intron 1 and exon 3 respectively of canonical WT1 sequence to enable *AWT1* transcript detection (219bp amplicon). Lane C: Nested forward and reverse primers targeting a region encoding the 5' untranslated region of canonical *WT1* and exon 1 respectively to detect *ExtWT1* transcript amplification (152bp amplicon). Lane D: No reverse transcriptase control amplified with primer pairs for detecting *ExtWT1*. Red forward and reverse arrows represent *WT1* exon/intron specific forward and reverse primer pairs for PCR reamplification. PCR amplification of canonical *WT1* and *AWT1* mRNA transcripts using in-house designed PCR primers resulted in amplicons that were 234bp and 219bp respectively. The qPCR (Taqman) primer set used to detect WT1 mRNA transcripts in Crawford *et al*, which amplify a region between exons 3 and 4 (shown as blue forward and reverse arrows) in the canonical WT1 mRNA sequence, are shown for comparison.[16]

3.2 *AWT1* is Constantly Expressed by DD Fibroblasts

In order to assess the expression of the *WT1* mRNA start site variants detected in the 5'RACE analyses, transcript specific PCR analyses of *WT1* were performed using custom designed primers. PCR amplified cDNA products were separated by electrophoresis in agarose gels and amplicons were extracted for DNA sequencing to confirm their identity. The analyses were initially performed using total RNA isolates from unstimulated DD, PF, and CT fibroblasts. Amplicons consistent with *AWT1* (Fig. 3.2B) were detected in unstimulated DD fibroblasts, whereas truncated *WT1* (*TrWT1*) transcripts, but not *AWT1* transcripts, were detected at low frequency in unstimulated PF and CT fibroblasts (Fig. 3.2C). Canonical *WT1* expression was not detected in any unstimulated DD (Fig. 3.2A), PF and CT fibroblast RNA samples assessed.

As mentioned previously, *WT1* expression was found to be upregulated and inducible in palmar fascia derived fibroblasts. Therefore, additional transcript specific PCR analyses were performed to determine if specific *WT1* transcript start site variants were induced in DD, PF, and CT fibroblasts after 24 hours of cytokine stimulus (TNF, IL-1β, IFN-γ). In contrast to unstimulated fibroblasts, amplicons consistent with canonical *WT1* and *AWT1* transcripts were detected in a subset of cytokine stimulated PF and CT fibroblasts (Fig. 3.3A-B). Canonical *WT1* and *AWT1* transcripts were readily detectable in all cytokine stimulated DD fibroblast RNA samples assessed (Fig. 3.3C).

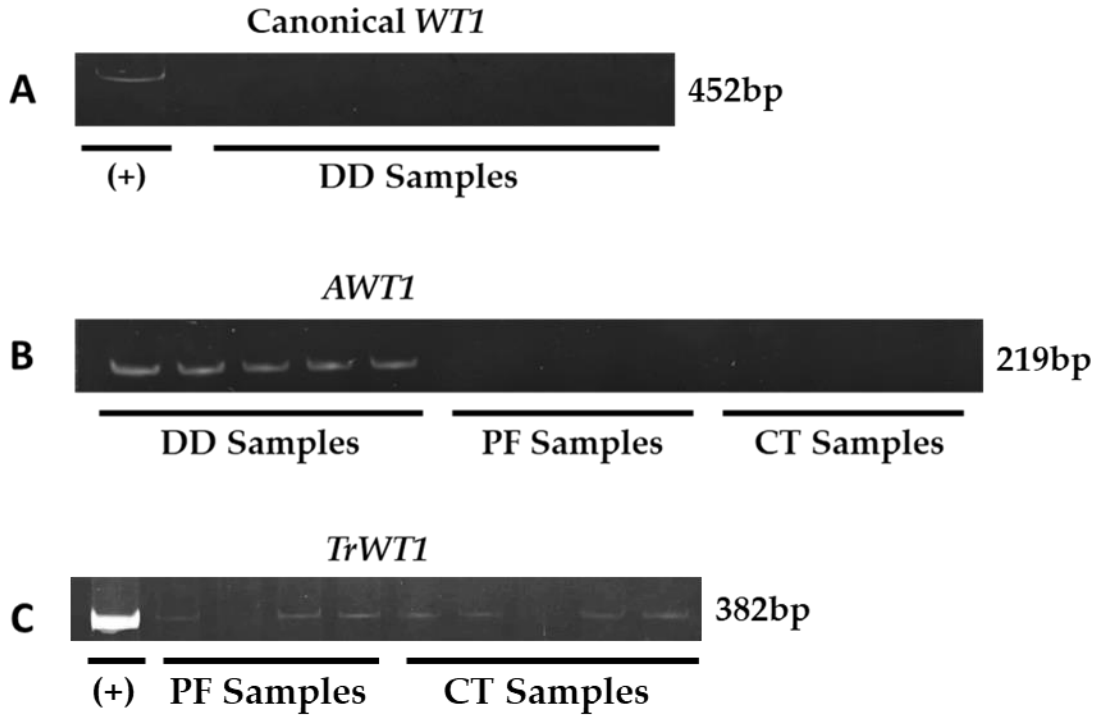

Figure 3.2. Unstimulated DD fibroblasts expressed AWT1 transcripts, whereas PF and CT fibroblasts express TrWT1 transcripts.

Representative images of RT-PCR amplification using intron/exon-specific primers of first strand cDNA for *WT1* variants in DD (N=5), PF (N=4) and CT (N=5) fibroblasts under basal culture conditions. Amplified PCR products were separated in 1% agarose gels, excised and purified for confirmatory DNA sequencing. **(A)** RT-PCR amplification of exons 1-3 (canonical *WT1*) in DD (N=5) samples (452bp). **(B)** RT-PCR amplification of intron 1 to exon 3 (*AWT1*) in DD, PF and CT samples (219bp). **(C)** RT-PCR amplification of exons 6 to exon 9 (KTS+) (Tr*WT1*) in PF and CT samples (382bp). RT-cDNA from OVCAR3 was included as a positive control (+).

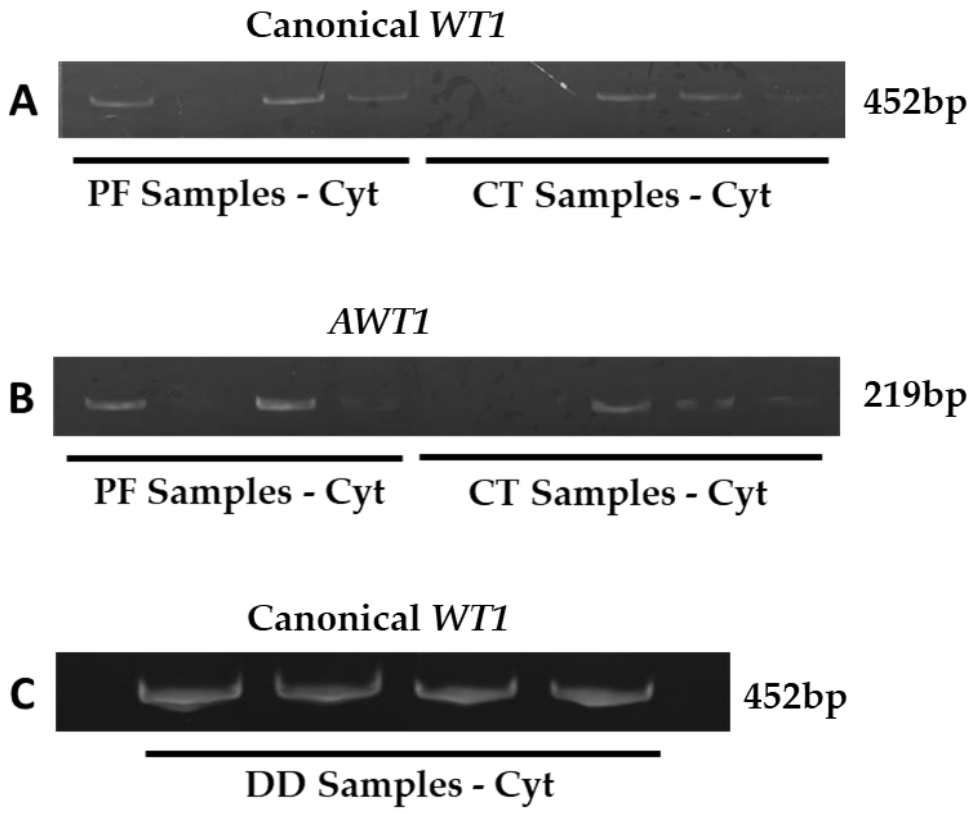

Figure 3.3. Canonical WT1 and AWT1 transcripts are cytokine inducible in palmar fascia fibroblasts.

Representative images of RT-PCR amplification using intron/exon-specific primers of first strand cDNA for *WT1* variants in DD (N=5), PF (N=4) and CT (N=5) fibroblasts after pro-inflammatory cytokine stimulus (TNF, IL1β, IFNγ) for 24 hours. Amplified PCR products were separated in 1% agarose gels, excised and purified for confirmatory DNA sequencing. **(A)** RT-PCR amplification of exons 1-3 (canonical *WT1*) in PF and CT samples (452bp). **(B)** RT-PCR amplification of intron 1 to exon 3 (*AWT1*) in PF and CT samples (219bp). **(C)** RT-PCR amplification of exons 1-3 (canonical *WT1*) in DD samples (452bp).

3.3 WT1 Protein Levels are Significantly Increased in DD Fibroblasts Relative to PF and CT Fibroblasts

The findings described in the previous sections indicated low level expression of *trWT1* mRNA transcripts in PF and CT fibroblasts, and moderate to high level expression of *AWT1* mRNA transcripts specifically in DD fibroblasts. It was unclear, however if any or all of these mRNA isoforms were translated into WT1 protein variants in these cells.

A previous report by Crawford *et al* had shown that WT1 immunoreactivity was evident in DD contracture tissues, but not in adjacent, visibly non-fibrotic palmar fascia controls (Fig. 1.4).[60] The antibody used for these analyses was reported (data provided by manufacturer, DAKO) to recognize an epitope encoded in exon 1 of the canonical *WT1* mRNA sequence. As this epitope was not predicted to be included in proteins encoded by *TrWT1* or *AWT1* mRNA transcripts (Fig 1.6B), these proteins, if present, would not have been detected in these IHC analyses. In the absence of WT1 variant-specific antibodies, a WT1 "sandwich" ELISA, which detects two different WT1 protein epitopes (details of which are proprietary information) was performed on cell lysates derived from DD, syngeneic PF and allogeneic CT fibroblasts. As shown in Fig. 3.4, WT1 immunoreactivity was detected in all three cell types, and a significant increase (~25%, $^{**}p < 0.01$) in WT1 immunoreactivity was evident in cell lysates derived from DD fibroblasts relative to CT controls. These data were interpreted to indicate that the *TrWT1* mRNA transcripts previously detected in PF and CT fibroblasts were translated into truncated WT1 proteins in these cells, and that the increase in WT1 immunoreactivity in DD fibroblasts was likely to represent translation of *AWT1* mRNA transcripts into AWT1 proteins.

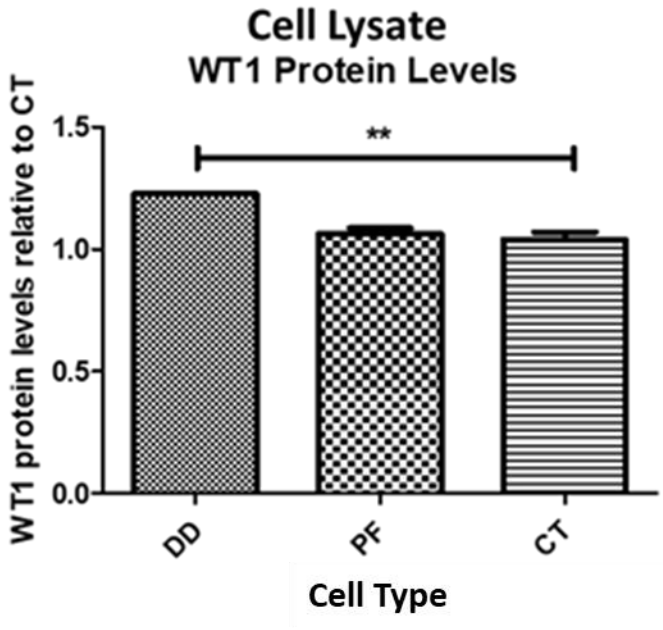

Figure 3.4. Enzyme Linked ImmunoSorbent Assay (ELISA) analyses indicate increased immunoreactivity to WT1 variants in DD fibroblast lysates.

WT1 protein levels were assessed by a "sandwich" ELISA, utilizing two antibodies detecting different WT1 epitopes, in DD, PF and CT fibroblast lysates (1 μg, N=3/group, ** $p < 0.01$ by ANOVA). WT1 protein levels are shown as normalized values relative to CT fibroblast (allogeneic normal) controls. Error bars for DD samples were too small to be shown.

3.4 Adenoviral Expression of *AWT1* Transcripts in Syngeneic and Allogeneic Control Fibroblasts

The data described in the previous sections revealed that *AWT1* was exclusively expressed by DD fibroblasts under basal culture conditions, was cytokine inducible in syngeneic PF and allogeneic CT control fibroblasts and was likely to be translated into functional protein(s). These findings implicated AWT1 as fibrosis-specific and inflammation inducible in palmar fascia fibroblasts, and therefore of interest for understanding inflammation-inducible palmar fascia fibrosis (DD). To study the potential roles of AWT1 in Dupuytren's Disease, adenoviral vector containing a cDNA encoding an *ATW1* variant predicted to function as a transcription factor (Ex5+/KTS-) was transduced into PF and CT fibroblasts (see 2.7 for details). As shown in Fig. 3.2B, DD fibroblasts express this AWT1 mRNA variant. The same adenoviral vector backbone containing a cDNA encoding *GFP* was used as a control to detect any non-specific effects from viral transduction. As neither PF or CT fibroblasts express detectable levels of AWT1 in the absence of cytokine stimuli, three PF fibroblast isolates from the visibly non-fibrotic palmar fascia of patients with DD (assumed to be predisposed to fibrosis and therefore "pre-fibrotic") and three normal allogeneic CT fibroblast isolates were transduced with these adenoviral constructs. Successful transductions were confirmed by qPCR (Fig. 3.5A) and Western immunoblotting with a WT1 antibody recognizing the C-terminal domain (region surrounding amino acid 306 as reported by manufacturer, Cell Signal) of WT1 (Fig. 3.5B).

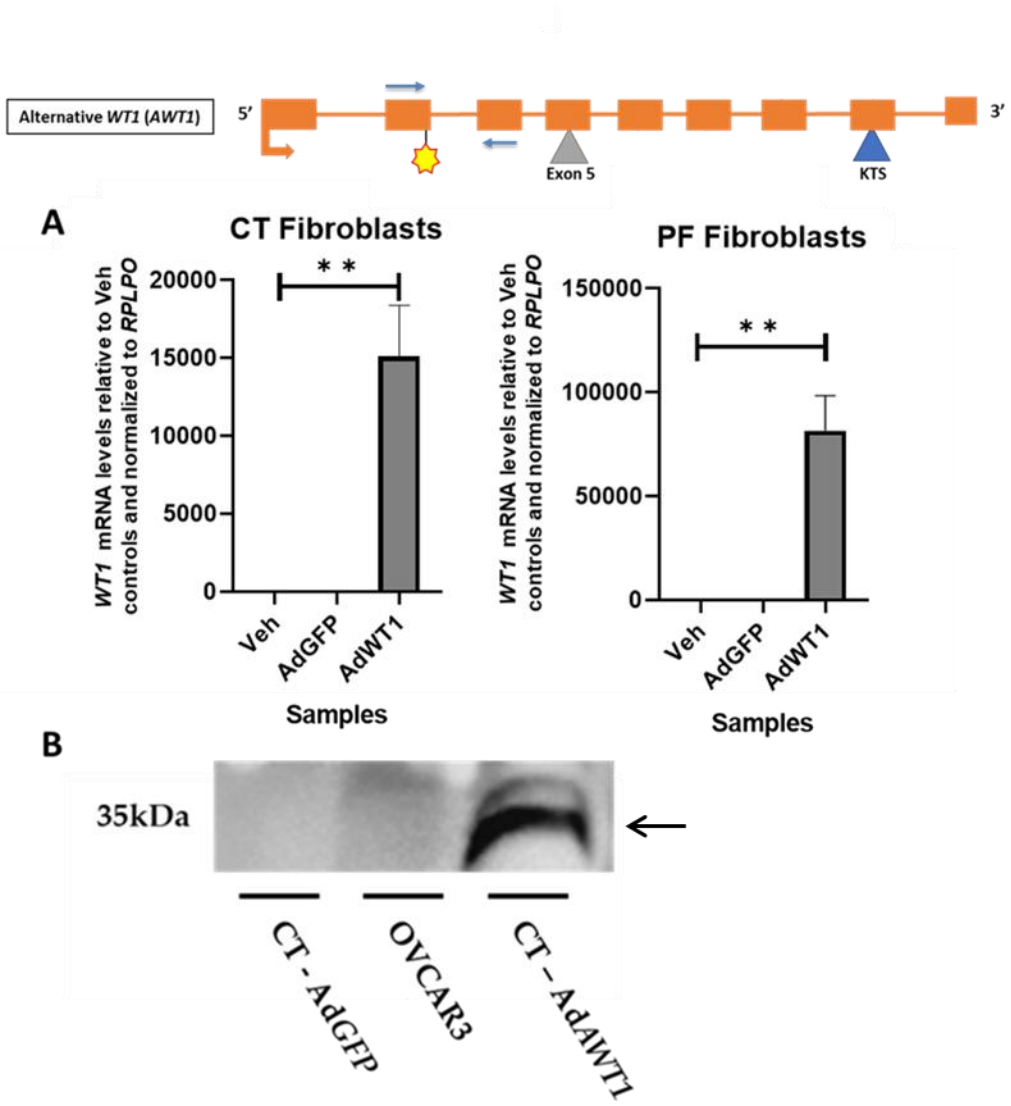

Figure 3.5. qPCR and Western immunoblotting to confirm successful adenoviral transduction of PF and CT fibroblasts.

(A) qPCR analyses of *WT1* gene expression in CT (N=3) and PF (N=3) fibroblasts transduced with DMEM only (Veh), adenoviral vector encoding green fluorescent protein (AdGFP) or adenoviral vector encoding *AWT1*. Forward and reverse arrows represent Taqman primers recognizing and binding exons 3 and 4 of *WT1* mRNA respectively. **(B)** Western immunoblotting with a WT1 antibody (D6M6S, Cell Signaling Technology) against total protein lysates of CT fibroblasts transduced with adenovirus encoding GFP (CT-AdGFP), OVCAR3 total protein lysate (positive control) and CT fibroblasts transduced with adenovirus encoding AWT1 (CT-AdWT1). The band representing predicted AWT1 isoform is highlighted by a black arrow. Based on *AWT1* cDNA sequence data, the predicted MW of AWT1 is 34,447 daltons (** $p < 0.01$ by ANOVA).

3.5 Transcriptome Wide Changes Associated with Patients with Dupuytren's Disease

Having confirmed constant *AWT1* expression and increased AWT1 levels in PF and CT fibroblasts, RNA-seq was utilized to assess transcriptome wide changes in AdAWT1 transduced fibroblasts relative to AdGFP transduced fibroblast controls (for details, see 2.2 and 2.9). Aligned RNA-seq data were normalized by TMM and differential analysis was performed by GSA. RNA-seq analysis identified 3017 gene transcripts that were differentially regulated in PF and CT fibroblasts (pooled, $p < 0.05$) expressing *AWT1* when compared to vector expressing fibroblasts (pooled, $p < 0.05$). Of the 3017 significantly up-or down-regulated gene transcripts, 961 differentially regulated gene transcripts were identified as unique to AWT1 expressing PF fibroblasts relative to AWT1-expressing CT fibroblasts. To generate visual representations of the data, a heatmap and clustering analyses of the RNA-seq data were performed. These approaches revealed that a subset of gene transcripts identified by RNA-seq were differentially expressed in PF (*AWT1* vs *GFP*) and in CT (*AWT1* vs *GFP*) groups and that each group clustered distinctly from the others (Fig. 3.6).

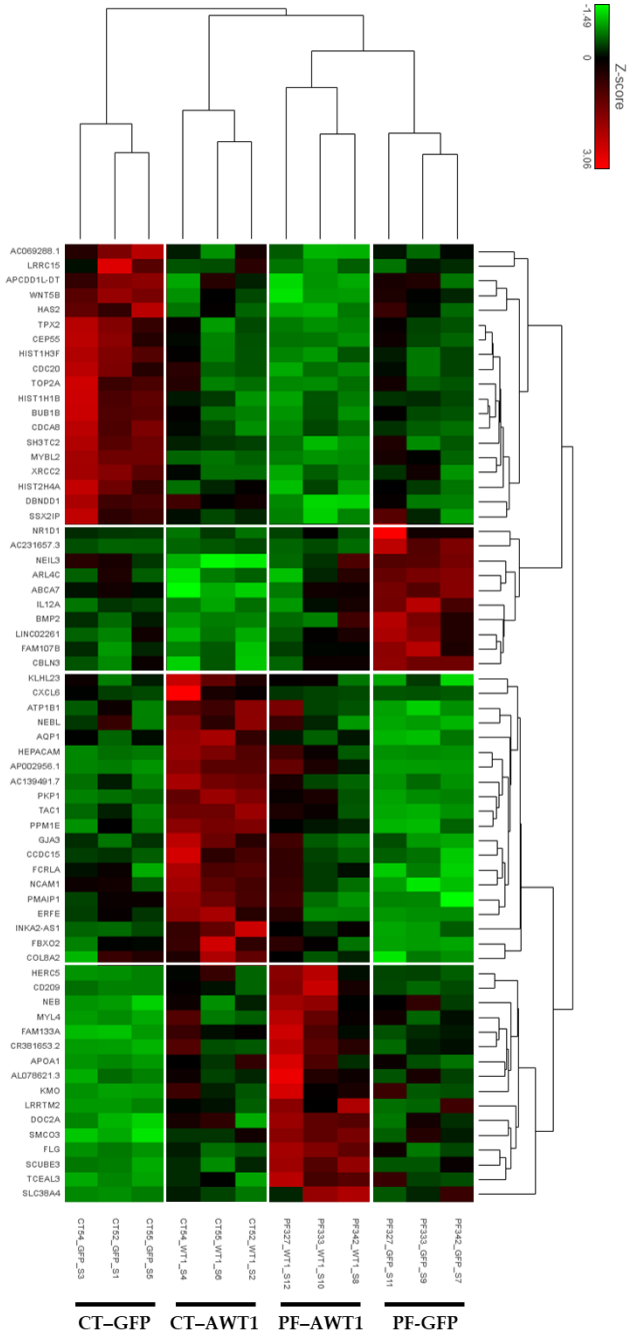

Figure 3.6. Differences in transcript abundance induced by *AWT1* expression in PF and CT fibroblasts.

Heatmap and Clustering analysis of PF (N=3) and CT (N=3) fibroblasts transduced with adenoviral vector control (PF-GFP and CT-GFP) or adenoviral vector encoding AWT1 (PF-AWT1 and CT-AWT1) respectively. Significant differential gene expression was indicated by green (negative fold-change) or red (positive fold-change) in *AWT1* expressing fibroblasts relative to vector controls. Inclusion criteria were p-values < 0.05 and fold changes in gene expression of ≥ 1.6 or <-1.6.

3.6 Constant Expression of *AWT1* Promotes a Pro-Inflammatory Milieu

To further evaluate the transcriptome wide changes associated with AWT1 expression, gene set enrichment and pathway enrichment analyses were performed to identify overrepresented biological processes or pathways. Enrichment scores indicate the negative natural logarithm of the p-value, thereby identifying significant processes and pathways. The highest enrichment score from the pathway enrichment analysis was for cytokine driven pathways (Table 1), specifically involving pro-inflammatory processes. Focus was placed on cytokine driven pathways, as these pathways are integral in modulating the wound healing phases.[4] As shown in Table 2, detailed analyses of the cytokine-cytokine receptor driven pathways revealed that ~69% of the modified gene transcripts, the majority of which exhibited increased expression, were categorized as contributing to pro-inflammatory, rather than inflammation resolving pathways. Additional top terms revealed that AWT1 play major roles in processes involved in response to stimuli, increased expression of cell surface receptors and in inflammation driven pathologies.

High-throughput analyses such as RNA-seq can be a powerful tool in analyzing thousands of genes in a single experiment. Unfortunately, with most computational analyses, there are some draw backs. Notably type I errors can occur when an analysis involve hundreds to thousands of genes. Therefore, to account for type I errors, Benjamin-Hochberg procedure was often used to correct p-values and account for false discovery rates (FDR). However, due to the number of biological replicates used in this study (PF N=3, CT N=3), along with the high variability of real patient samples, the Benjamin-Hochberg procedure was not utilized in order to reduce type II errors. Instead, independent confirmation of the changes in gene expression identified by RNA-seq was performed by qPCR analyses of 6 representative genes (*ICAM1*, *CXCL14*, *CXCR4*, *CXCL10*, *TNFSF4*, *IL-1B*), all of which have been shown to be associated with fibroses. The expression levels of all of these genes were found to be significantly upregulated in AWT1 expressing PF fibroblasts. The expression levels of *ICAM1*, *CXCR4*, and *TNFSF4* were all significantly upregulated in AWT1 expressing CT fibroblasts (Fig. 3.7). Where

significance was not achieved in AWT1 expressing CT fibroblasts, a strong trend towards an increase in expression was evident, consistent with highly variable gene expression levels and/or insufficient statistical power (N=3 samples) to achieve significance (Fig. 3.7). AWT1-induced *TNFSF4* expression was of particular interest, as parallel analyses in the O'Gorman laboratory had demonstrated that DD myofibroblasts in 3D collagen cultures exhibited a significant increase in *TNFSF4* expression relative to PF and CT myofibroblasts (Fig. 3.8).

Path term	Enrichment score
Cytokine-cytokine receptor interaction	21.47
MAPK signaling pathway	10.3
ABC transporters	7.35
Bile secretion	7.08
Gastric cancer	6.84
Neuroactive ligand-receptor interaction	6.65
Pathways in cancer	6.62
Transcriptional misregulation in cancer	6.43
Viral protein interaction with cytokine and cytokine receptor	6.34
MicroRNAs in cancer	6.29
Inflammatory bowel disease (IBD)	6.07
African trypanosomiasis	5.78
Systemic lupus erythematosus	5.74
Proximal tubule bicarbonate reclamation	5.69
Type I diabetes mellitus	5.68
Cell adhesion molecules (CAMs)	5.66
Toll-like receptor signaling pathway	5.59
Influenza A	5.34
Measles	5.31
NF -kappa B signaling pathway	5.21
RIG-I-like receptor signaling pathway	5.04
B cell receptor signaling pathway	4.85
Leishmaniasis	4.71
Hematopoietic cell lineage	4.58
Th17 cell differentiation	4.5
Rheumatoid arthritis	4.47
Aldosterone-regulated sodium reabsorption	4.42
Graft-versus-host disease	4.4
Carbohydrate digestion and absorption	4.23
Ras signaling pathway	4.22
Epstein-Barr virus infection	4.21
Fat digestion and absorption	4.19
Bladder cancer	4.13
JAK-STAT signaling pathway	3.95
Basal cell carcinoma	3.81
Protein digestion and absorption	3.78
cAMP signaling pathway	3.72
PI3K-Akt signaling pathway	3.71
Staphylococcus aureus infection	3.68
Allograft rejection	3.56
Arachidonic acid metabolism	3.54
IL-17 signaling pathway	3.53
ErbB signaling pathway	3.49
Apoptosis - multiple species	3.46
Intestinal immune network for IgA production	3.46
Mineral absorption	3.41
Hepatitis B	3.35
Steroid biosynthesis	3.32
p53 signaling pathway	3.28
EGFR tyrosine kinase inhibitor resistance	3.24

Table 1. AWT1 expression is associated with inflammation and inflammation driven pathologies.

Pathway enrichment analysis identifying overrepresented biological pathways in *AWT1* expressing PF and CT fibroblasts (pooled) relative to PF/CT fibroblasts GFP-expressing controls. Inclusion criteria were p-values < 0.05 and fold change in gene expression of > 1.5 or <-1.5. Enrichment scores indicate the negative natural logarithm of the p-value. As shown, the highest enrichment score was associated with cytokine-cytokine receptor interactions.

Gene symbol	P-value	Fold change
Pro-inflammatory Genes		
CCL1	4.34E-02	3.59E+00
CCL4	4.93E-02	2.56E+00
CCL11	3.63E-02	5.62E+00
CCL26	1.04E-02	-1.95E+00
CCL20	3.24E-03	1.75E+00
CXCL6	1.16E-03	5.38E+00
CXCL8	3.89E-02	2.58E+00
CXCL9	1.96E-03	8.75E+00
CXCL10	1.77E-08	1.27E+02
CXCL11	8.01E-04	4.27E+00
CXCL14	5.13E-05	5.93E+00
CXCR4	6.66E-04	6.17E+00
TSLP	1.02E-02	1.90E+00
IL12A	4.13E-03	-1.89E+00
IL12RB2	5.25E-05	5.89E+00
IL23A	2.57E+00	2.41E+00
IL19	1.13E-02	2.36E+00
IL20	1.81E-02	6.31E+00
IL1A	4.20E-03	2.37E+00
IL1B	7.66E-03	3.09E+00
FASLG	3.96E-02	2.33E+00
TNFSF15	1.20E-02	2.08E+00
TNFRSF21	3.60E-02	1.60E+00
TNFSFR8	5.45E-04	3.85E+00
TNFSF4	1.11E-04	2.88E+00
RELT	2.08E-02	1.53E+00
GDF15	1.66E-03	1.75E+00
ACVR1	2.16E-04	-1.81E+00
IL31RA	4.90E-02	1.82E+00
Anti-inflammatory Genes		
CLCF1	8.86E-04	-2.02E+00
CNTFR	2.76E-02	2.73E+00
TGFB1	2.77E-04	-1.66E+00
TGFBR1	7.32E-03	-1.52E+00
BMP4	7.17E-03	-1.57E+00
GDF6	1.81E-03	3.86E+00

Table 2. AWT1 enhances the expression of genes encoding pro-inflammatory cytokines and downregulates the expression of genes encoding anti-inflammatory cytokines.

The category with the highest enrichment score in Table 1, cytokine-cytokine receptor interaction, was reviewed at the level of individual gene expression and allocated to pro-inflammatory or anti-inflammatory categories based on literature review. As shown, 69% of the total number of gene transcripts could be readily categorized as classically pro- or anti-inflammatory, with ~82% of these (29/35) categorized as pro-inflammatory.

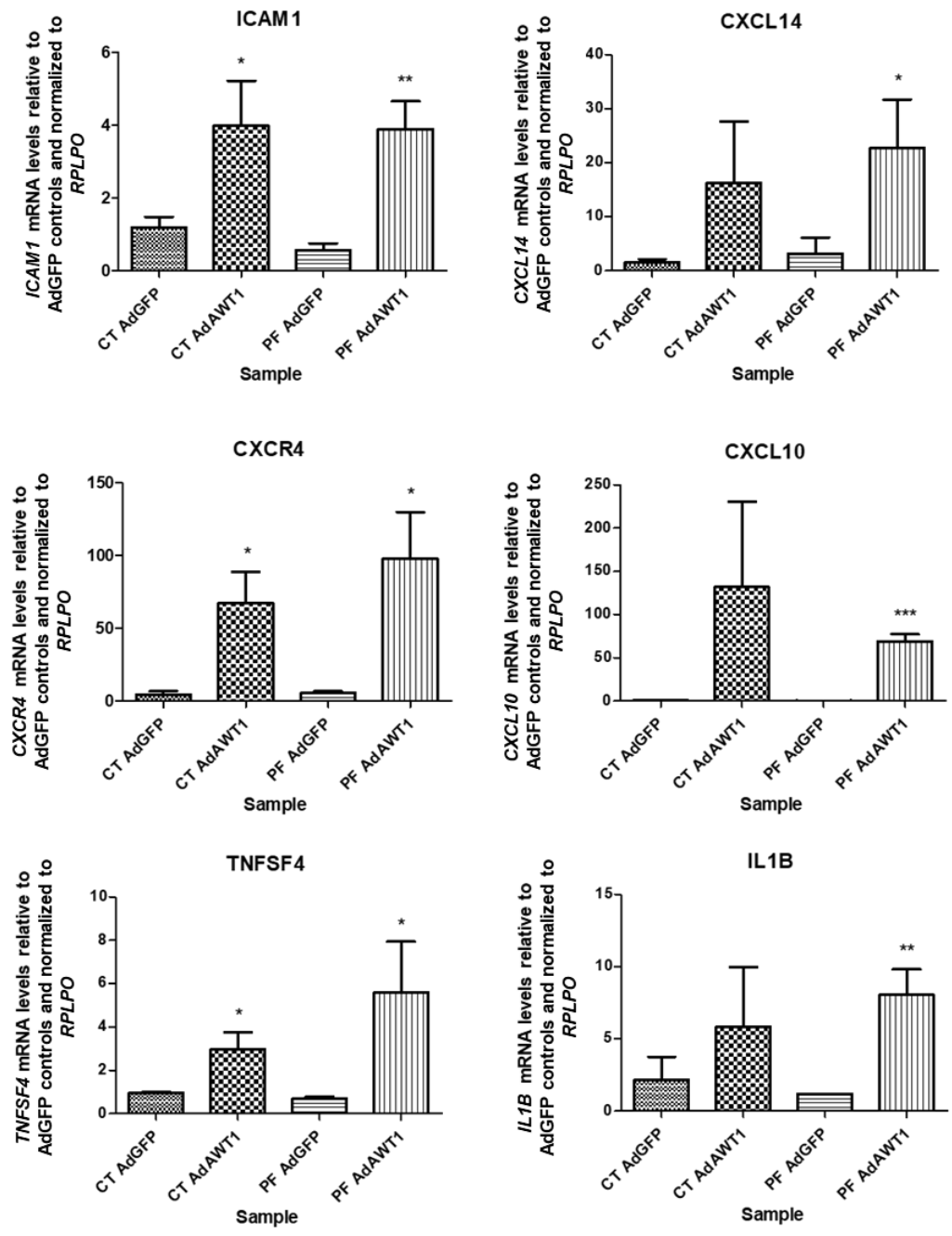

Figure 3.7. Confirmatory qPCR analyses of AWT1-mediated changes in the expression of fibrosis associated genes identified by RNA-seq.

qPCR analysis of *TNFSF4*, *CXCL10*, *CXCL14*, *IL-1B*, *CXCR4*, and *ICAM1* mRNA expression levels in PF and CT fibroblasts transduced with AdAWT1 or AdGFP (N=3/group, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by t-test).

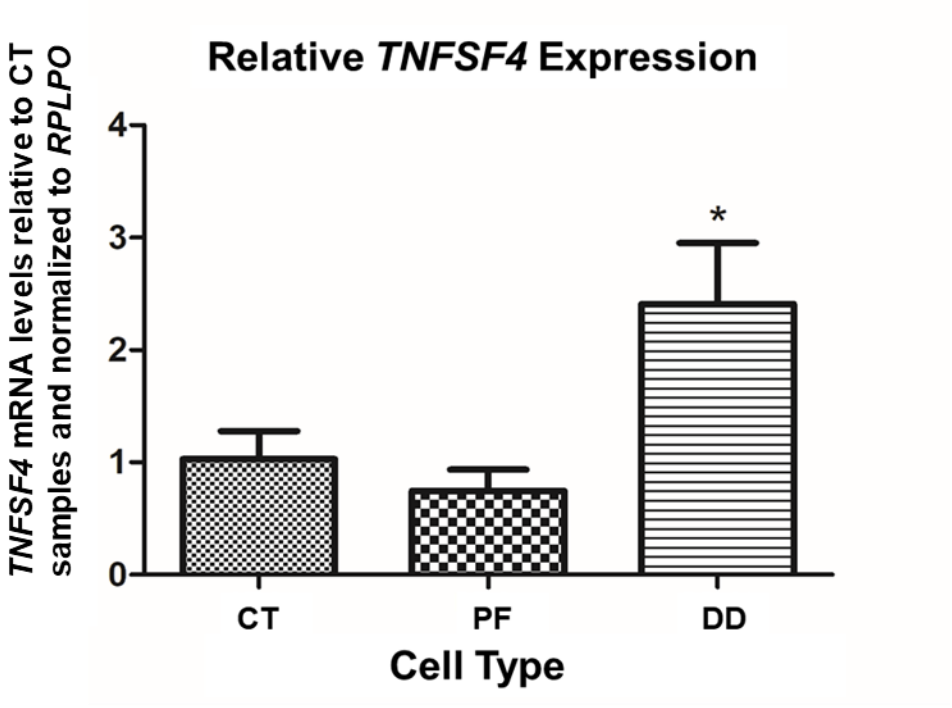

Figure 3.8. *TNFSF4* mRNA expression is increased in primary myofibroblasts derived from Dupuytren's Disease tissues.

TNFSF4 gene expression was assessed using Taqman primers (Thermofisher Scientific) in cDNAs derived from total RNA samples extracted from DD, PF and CT myofibroblasts (each N=3, **p <0.001 as determined by ANOVA). Data were normalized to CT myofibroblasts gene expression and produced by Ana Pena Diaz.

3.7 Functional analyses of *AWT1* Expressing Palmar Fascia Fibroblasts

Stressed fibroblast populated collagen lattice (sFPCL) contraction assays were performed to assess the impacts of constant AWT1 expression in PF or CT myofibroblasts. As detailed in 2.8, *AWT1* or vector expressing myofibroblasts were cultured in FPCLs for 72 hours prior to releasing the collagen lattices from the walls and bottom of the culture wells, after which collagen contraction was assessed by changes in collagen volume over time. A statistically non-significant trend towards increased collagen contraction by CT fibroblasts (N=3) expressing *AWT1* was evident over GFP-expressing and non-transduced vehicle controls (Veh) (Fig. 3.9A), whereas PF fibroblasts (N=3) expressing *AWT1* exhibited similar collagen contraction rates to controls (Fig. 3.9B). However, PF-Veh controls were significantly more contractile than CT-Veh controls at 24 hrs time point (p-value < 0.01). Additional analyses to increase the statistical power of these experiments were curtailed by COVID-19 associated research restrictions.

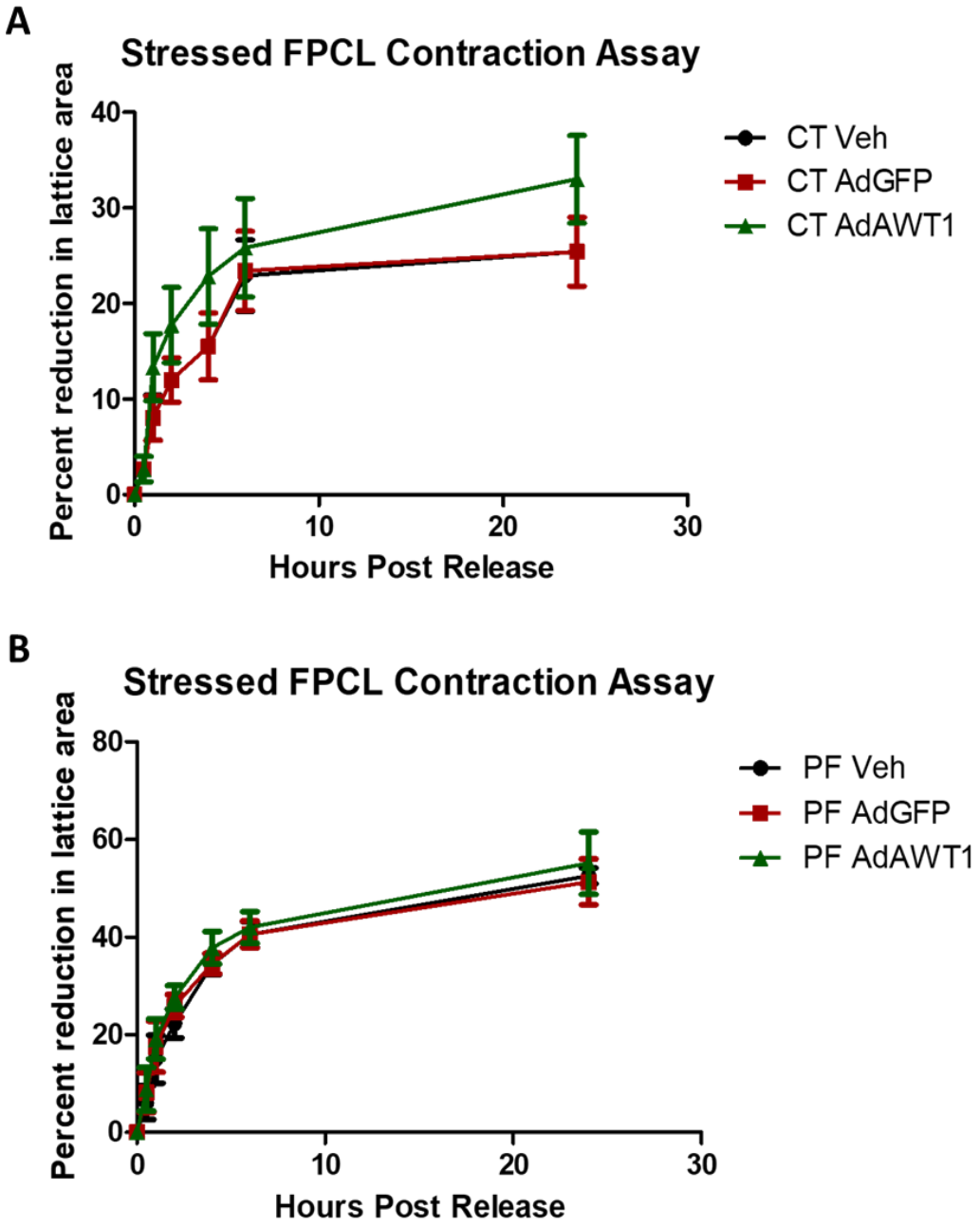

Figure 3.9. Collagen contraction analyses of AWT1-expressing CT and PF myofibroblasts.

Stressed Fibroblast populated Collagen Lattice (sFPCL) assays were performed using **(A)** CT myofibroblasts or **(B)** PF myofibroblasts. The collagen lattice areas of AWT1-expressing myofibroblasts are shown in green, GFP-expressing myofibroblast controls are shown in red and non-transduced myofibroblast controls are shown in black. No significant differences in contraction over time were detected within sample groups as determined by ANOVA. A significant difference in lattice area between PF-Veh and CT-Veh samples was found at 24 hrs time point ($p < 0.01$).

3.8 Palmar Fascia Fibroblast Secretomes Modify THP-1 Monocyte Cytokine Gene Expression Profiles

The RNA-seq and independent qPCR analyses of gene expression revealed that AWT1 promoted the expression of genes encoding pro-inflammatory cytokines and their receptors in PF and CT fibroblasts. To determine if these changes in gene expression induced corresponding changes in PF and/or CT fibroblast secretomes to induce a pro-inflammatory environment, adenovirally transduced PF (N=3) and CT (N=3) myofibroblasts expressing AWT1 were co-cultured with THP-1 cells, an immortalized human monocyte cell line. DD myofibroblasts (N=3) were also co-cultured with THP-1 monocytes to identify any impacts of their secretome on THP-1 monocyte gene expression that may correlate with the impacts of AWT1-expressing PF and CT myofibroblasts. Fibroblasts are adherent cells, whereas THP-1 monocytes are non-adherent. This facilitated the isolation of adherent and non-adherent cells after co-culture for separate gene expression analyses. Gene expression levels in THP-1 monocytes in co-culture with myofibroblasts were compared to THP-1 cells in monoculture THP-1 monocytes as described in section 2.12. Select target genes were chosen to detect changes in TGFß expression levels and/or signaling (*TGFB1, SERPINE1*), fibronectin expression levels (*FN1*), pro-inflammatory (*CXCL8, IL6, TNF, CCL2, IL1B*) and anti-inflammatory (IL10) cytokine gene expression levels in THP-1 monocytes. As shown in Fig. 3.10, co-cultures with myofibroblasts significantly increased the expression of *SERPINE1*, *FN1*, *IL6*, and *CCL2* in THP-1 cells. Highly variable and statistically non-significant, but trends toward increased expression levels of *SERPINE1, CCL2, IL1B, CXCL8, IL6, IL10*, and *FN1* were evident in THP-1 cells in co-culture with PF myofibroblasts expressing AWT1 relative to vector controls (Fig. 3.10). A trend toward an increase in *IL6* mRNA levels was also evident in THP-1 cells in co-culture with CT myofibroblasts expressing AWT1 relative to the corresponding vector controls.

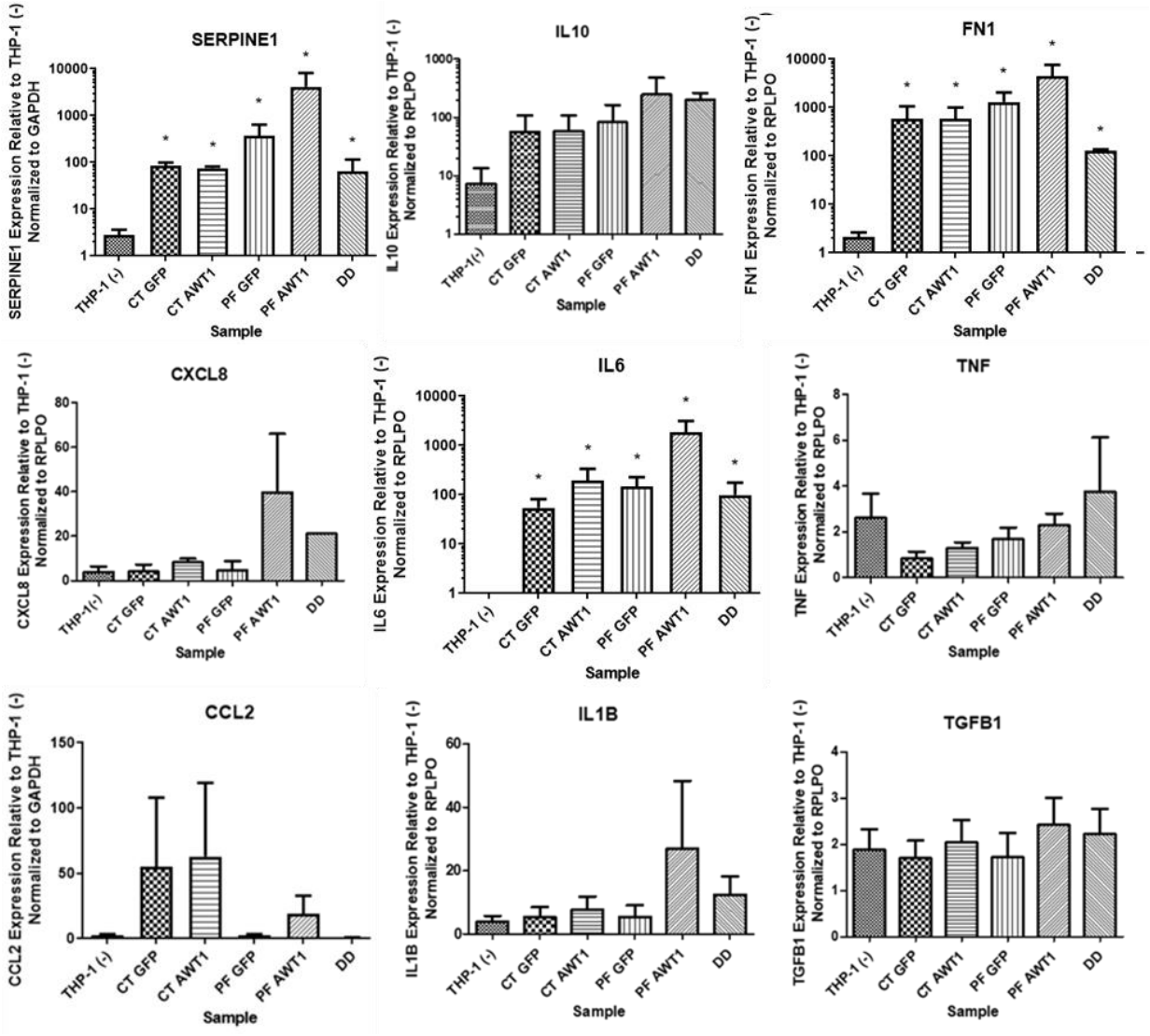

Figure 3.10. Palmar fascia myofibroblasts increase the expression of genes encoding cytokines and fibrosis-associated proteins in THP-1 cells in co-culture.

Expression of *SERPINE1*, *IL10*, *FN1*, *CXCL8*, *IL6*, *TNF*, *CCL2*, *IL1B* and *TGFB1* in THP-1 monocytes co-cultured with PF (N=3) or CT (N=3) myofibroblasts expressing *AWT1* or vector control (GFP), or DD (N=3) fibroblasts relative to THP-1 monoculture. (* $p < 0.05$ as determined by t-test). Additional analyses to assess N=6 THP-1 cultures/group were curtailed by COVID-19 associated restrictions.

3.9 Results Summary

The major findings described in this chapter can be summarized as follows:

1) The *WT1* mRNA start site variants expressed by DD fibroblasts differed from those expressed by PF and CT fibroblasts under the same basal culture conditions. In the absence of cytokine stimuli, DD fibroblasts expressed readily detectible levels of *AWT1* mRNA transcripts, whereas their syngeneic controls did not, identifying constant *AWT1* expression as a fibrosis-associated event. PF and CT fibroblasts (and potentially, DD fibroblasts) were found to express *trWT1* mRNA transcripts, and ELISA analyses were interpreted to suggest that both *AWT1* and *trWT1* mRNA transcripts were translated into proteins in these cells.

2) The *WT1* mRNA start site variants expressed by DD, PF and CT fibroblasts were modified by pro-inflammatory cytokine stimuli. In addition to *AWT1* mRNA transcripts, cytokine stimuli induced the expression of canonical and *ExtWT1* mRNA transcripts in DD fibroblasts. Cytokine stimuli induced *AWT1* and canonical *WT1* mRNA transcript expression in PF and CT fibroblasts.

3) Adenoviral expression and cellular translation of *AWT1* mRNA transcripts (Ex5+/KTS-) in PF and CT fibroblasts significantly modified the expression of genes encoding cytokines and cytokine receptors. The gene expression changes induced by AWT1 expression in PF fibroblasts differed significantly from those induced by AWT1 expression in CT fibroblasts, although there was also substantial overlap between these groups. No significant changes in the expression levels of established fibrosis-associated genes or fibrosis-associated function (collagen matrix contraction) were detected. The expression levels of *TNFSF4*, encoding a novel pro-fibrotic immunoregulatory protein, OX-40 ligand (OX-40L), were significantly increased in AWT1-expressing PF and CT fibroblasts, mimicking the increased expression levels of *TNFSF4* in DD myofibroblasts.

4) Co-cultures of AWT1-expressing PF fibroblasts with THP-1 monocytes resulted in strong trends toward increased cytokine gene expression, indicating AWT1-induced changes in the PF fibroblast secretome that were additional to those induced by the vector

controls. Modest effects on THP-1 gene expression were also evident in co-cultures with AWT1-expressing CT fibroblasts relative to their vector controls.

Chapter 4

4 DISCUSSION

4.1 Potential Roles for AWT1 in Palmar Fascia Repair

The findings in this thesis reveal for the first time that primary fibroblasts derived from syngeneic fibrotic and non-fibrotic palmar fascia can be consistently distinguished by the *WT1* mRNA transcript variants they express. Since these differences can be (at least partially) abolished by pro-inflammatory cytokine stimuli, these findings can be interpreted to suggest that DD-derived fibroblasts exhibit an epigenetic "memory" (epigenetic rather than genetic, since DD and PF fibroblasts are presumed to be syngeneic) of the chronic inflammatory environment from whence they were derived, and are "stuck" in this pro-inflammatory state. In a similar context, the RNA-seq data reported here can be interpreted to indicate the existence of a positive feedback loop, where a local pro-inflammatory environment induces AWT1 expression and translation in palmar fascia fibroblasts, resulting in the transcription of genes encoding pro-inflammatory cytokines and receptors that induce a pro-inflammatory environment.

Pro-inflammatory cytokine treatments were also shown to induce *AWT1* expression in fibroblasts derived from normal palmar fascia (CT fibroblasts), suggesting that a transient increase in AWT1 expression may be a normal component of the first phase of tissue repair (Fig. 1.1). Inflammatory cytokines have been previously shown to induce *WT1* expression in HaCat cells, an aneuploid immortalized keratinocyte cell line widely used to model normal human keratinocytes in re-epithelialization studies.[89] These findings suggest that cytokine-induced *WT1* expression is not limited to fibroblasts, but may be a feature of epithelial cells and, potentially, many other cell types. Inflammation is known to induce epigenetic modifications such as demethylation and histone acetylation.[64] As the region of the *WT1* gene which functions as a promoter for *AWT1* mRNA transcription is differentially methylated and genomically imprinted in normal human kidneys[90], it is possible that inflammatory cytokine stimuli induce *AWT1* mRNA expression in palmar fascia fibroblasts through an analogous mechanism. If this hypothesis proves to be

correct, it is also possible that cytokine-induced WT1 isoform expression is reversible in normal cells; i.e., that the expression of AWT1 and other cytokine-induced WT1 isoforms ceases once cytokine levels are depleted and the AWT1 promotor region returns to a transcription repressive state. This contrasts with the findings in DD fibroblasts, where AWT1 expression is constant and maintained without exogenous cytokine stimuli, again possibly the result of more permanent epigenetic modifications. It is currently unclear if any of the cytokines, growth factors or other molecules known to facilitate the transition from the inflammation to the proliferation phases of wound healing do so by actively attenuating WT1 isoform expression, and if any of these factors are down regulated in DD fibroblasts.

4.2 Potential Roles for *TrWT1* in Palmar Fascia Homeostasis

Unlike the mRNA transcripts encoding canonical, alternative and extended WT1 isoforms that were induced *de novo* by pro-inflammatory cytokine treatments, truncated *WT1* (*TrWT1*) mRNA transcripts were detectable in PF and CT fibroblasts under basal culture conditions. As the RT-qPCR (Taqman) assay used in this project amplifies a region upstream of trWT1 and therefore does not detect reverse transcribed *trWT1* mRNA transcripts, and qualitative RT-PCR analyses of exon sequences downstream of exon 6 cannot not distinguish between truncated and *AWT1* mRNA transcripts, it is possible that DD fibroblasts express both *TrWT1* and *AWT1* mRNA transcripts simultaneously (see Fig. 1.6B). The detection of WT1 immunoreactivity in PF and CT fibroblast lysates by ELISA (Fig. 3.4) is consistent with the hypothesis that *TrWT1* mRNA transcripts are translated into proteins. This finding, that despite barely detectible *TrWT1* transcript expression, PF and CT fibroblasts have readily detectible levels of WT1 proteins, may imply that TrWT1 proteins are stably produced by these cells and may have other, non-tissue repair associated functions, such as maintaining tissue homeostasis. While almost nothing is known about the specific functions of TrWT1 proteins, it is also tempting to speculate that at least some of the disease phenotypes associated with *WT1* mutation/depletion in developing tissues might be attributed to loss of TrWT1 functions.

4.3 *AWT1* Expression Upregulates Novel, Fibrosis Associated Genes in PF and CT Fibroblasts

Independent qPCR analyses were used to verify a subset of the AWT1-induced changes in PF and CT fibroblasts revealed by RNA-seq analyses. These genes were chosen from the 3017 gene transcripts that were significantly modified by AWT1 expression based on previous reports implicating their roles in inflammatory diseases associated with the development of fibroses. *TNFSF4* encodes OX-40 ligand (OX-40L), a recently identified driver of inflammation and fibrosis development in systemic sclerosis that is being actively assessed as a therapeutic target.[91] *IL1B* encodes interleukin-1β (IL1β), a pro-inflammatory cytokine and has been implicated in many inflammatory diseases and fibroses including DD.[92] *CXCL14* encodes a chemokine that attracts circulating monocytes, one of the two major sources of macrophages[93], to wound sites. *CXCL10*, another chemokine encoding gene, and *CXCR4* a gene encoding a chemokine receptor, are implicated in the development of pulmonary fibroses.[94,95] *ICAM1*, encoding intercellular adhesion molecule 1, is expressed at abnormally high levels in mouse models of idiopathic pulmonary fibrosis (IPF), in the serum of patients with IPF[96,97] and in mouse models of systemic sclerosis, where it has been proposed to mediate interactions between fibroblasts and immune cells.[98]

The qPCR analyses shown in Fig. 3.7 confirmed significant increases in the expression of the majority of these target genes, and where significance was not achieved, a strong trend toward an increase in expression was evident. These data are consistent with the gene list shown in Appendix 8, (FDR < 0.05, fold-change of > 1.5 or <-1.5) and the hypothesis that sustained AWT1 expression promotes a pro-inflammatory and pro-fibrotic response in palmar fascia fibroblasts.

4.4 THP-1 Monocyte Gene Expression is Modified by *AWT1* Expressing PF Fibroblasts

The RNA-seq and confirmatory qPCR analyses indicated that constant AWT1 expression by PF and CT fibroblasts induced a net increase in the expression of pro-inflammatory cytokines and cytokine receptors. To assess if these transcriptome wide changes had functional consequences on fibroblast-immune cell interactions, PF or CT fibroblasts expressing *AWT1* or *GFP* (viral transduction control), and DD fibroblasts were independently co-cultured with an immortalized monocyte cell line, THP-1. As shown in Fig. 3.10, relative to THP-1 cells in monoculture, the presence of palmar fascia fibroblasts of any subgroup potently and significantly induced the expression of many cytokine-encoding genes (e.g., *SERPINE1*, *FN1*, and *IL6*) in THP-1 cells in co-culture. While they are clearly extremely sensitive to paracrine signaling, THP-1 monocytes trend toward a pro-inflammatory phenotype and so are likely to provide more exaggerated "read-outs" than primary monocytes.[99] THP-1 monocytes were chosen for these co-culture analyses to provide a more consistent and simpler model of local immune cell infiltrate that could form the basis of additional, more physiologically relevant experiments using primary buffy coat isolates containing mixtures of neutrophils, monocytes, lymphocytes and other leucocytes.

As shown in Fig. 3.10, THP-1 monocytes co-cultured with AWT1- expressing PF fibroblasts exhibited clear but non-significant trends towards increased expression of *SERPINE1*, *CCL2*, *IL1B*, *CXCL8*, *IL6*, *IL10* and *FN1* genes. These trends were less, or not at all, evident in CT fibroblasts expressing *AWT1*. The changes in THP-1 monocyte gene expression induced by co-culture were widely variable, explaining the lack of statistical significance despite changes in mean values that, in some cases, approximated 100-fold. These included impacts on the expression levels of *SERPINE1*, encoding plasminogen activator inhibitor 1 (PAI-1), a phospho-SMAD3 activated gene that is routinely used as an indicator of TGFß1 signalling.[100] The apparent increase in *SERPINE1* expression induced by AWT1 expression in PF fibroblasts was over and above an already substantial background increase induced by all of the fibroblast subgroups tested, potentially indicating potent activation of this pathway. Widely

variable changes in the expression of *CCL2*, encoding monocyte chemoattractant protein-1 (MCP-1) with roles in immune cell attraction and fibrosis development[101], were also evident, as were apparent increases in the expression levels of *IL1B*, *CXCL8* and *IL6*. Apparent changes in the expression levels of *IL10* and *FN1* were also evident, a subset of genes that encode molecules that are classically considered to be anti-inflammatory. This mixture of pro-and anti-inflammatory gene expression responses may reflect the transitional roles played by some classically pro-inflammatory molecules. For example, *IL6*, a well-known pro-inflammatory cytokine, has been shown to play key roles in modulating the transition from a classical "M1" (pro-inflammatory) to an "M2 (anti-inflammatory, or pro-resolving) phenotype.[102-104]

4.5 Homeostasis, Fibrosis and "Pre"-Fibrosis

As described in detail in sections 3.6 and 3.8, adenoviral expression and cellular translation of *AWT1* in PF and CT fibroblasts induced significant transcriptome wide changes in these cells relative to their respective vector controls. Despite being derived from 6 randomly selected and presumably unrelated patients, the unbiased heatmap and clustering analyses of the RNA-seq data (Fig. 3.6, p-value < 0.05, fold-change > 1.6 or < -1.6) revealed that data from the three AWT1-expressing PF fibroblasts and data from the three AWT1-expressing CT fibroblasts clustered together into distinct groups. These findings are consistent with previous reports from the O'Gorman laboratory indicating that, despite being invariably derived from visibly non-fibrotic palmar fascia, PF fibroblasts and CT fibroblasts are transcriptionally and functionally distinct.[105,106] These differences can be interpreted to indicate that PF fibroblast are in a state of prodromal or "pre"-fibrosis, a poorly characterized, third state that is distinct from palmar fascia fibroblasts in homeostasis and fibroblasts derived from active fibrosis. The characteristics that identify fibroblasts in a pre-fibrotic state include some that can be interpreted as "intermediate" between normal homeostasis and fibrosis, and some characteristics that are unique to the pre-fibrosis category. [105,106] The latter are of particular interest clinically, as they may include useful biomarkers of a predisposition to fibrosis development that could indicate a need for additional immunoregulatory or other therapies to enhance post-

surgical outcomes for this patient subset. These biomarkers may include heritable genetic and/or epigenetic factors that can help us unravel some of the complexity associated with heritable fibroses such as DD.

Of the 3017 gene transcripts with significantly altered expression levels in AWT1-expressing palmar fascia fibroblasts, 961 (~ 1/3) were differentially expressed by AWT1 expressing PF fibroblasts. The co-factors that modify the impacts of AWT1 expression in PF fibroblasts relative to AWT1-expressing CT fibroblasts are currently unknown, but are presumed to include molecules that are dysregulated in PF fibroblasts relative to CT fibroblasts as a result of heritable single nucleotide polymorphisms (SNPs) and/or epigenetic modifications that, in combination, predispose individuals to developing DD.[107]

4.6 *AWT1*-Expressing CT fibroblasts Exhibit a Modest Trend Toward Increased Collagen Contractility

While the RNA-seq analyses strongly implicated roles for AWT1 in promoting inflammatory cytokine signalling, there was little or no evidence of changes in the expression of the "classical" fibrosis genes associated with myofibroblast activation and abnormal ECM remodeling. To confirm these findings, additional independent qPCR analyses were performed for an array of well-established myofibroblast associated genes (*FN1*, *SMAD2*, *TNF*, *ACTA2*, *COL3A1*, *TGFB1*, *POSTN* and *COL1A1*). *FN1* encodes for fibronectin, an extracellular matrix molecule, which is typically increased in fibrotic tissues and have roles in the assembly of collagen matrix and cellular proliferation.[108] *TGFB1* encodes for transforming growth factor 1, an anti-inflammatory cytokine and a classical pro-fibrotic gene that is regulated by SMAD2 proteins.[109] Extracellular matrix components such as collagen type III (encoded by *COL3A1*) and periostin (encoded by *POSTN*) are hallmark features of fibroses such as DD and IPF.[48,110] Finally, *ACTA2* encodes for alpha smooth muscle actin, the defining characteristic of myofibroblasts, the cell type that directly mediates contractile fibrosis development.[10]

Constant expression of *AWT1* in PF fibroblasts had no discernible impact on the expression of the majority of these genes (Appendix C), with the exception of *TGFB1*, where a modest decrease in transcript levels were evident, consistent with findings by RNA-seq and a previous report of transcriptional repression of *TGFB1* transcription by WT1.[111] Similarly, the RNA-seq analyses did not identify any significant changes in a majority of these target genes in AWT1-expressing CT fibroblasts. These findings were interpreted to indicate that that *AWT1* expression does not directly promote myofibroblast activation. To confirm this at a functional level, collagen contraction assays were performed on AWT1 or GFP-expressing palmar fascia myofibroblasts. Enhanced collagen contractility is a hallmark feature of fibrosis-associated myofibroblasts that can be assessed *in vitro* using stressed Fibroblast Populated Collagen Lattice assays (sFPCLs).[10,112]

Consistent with previous reports[113], PF myofibroblast controls exhibited a significant increase in collagen contraction over CT fibroblast controls at the 24hr time point after lattice release (Fig. 3.9). A modest but statistically non-significant trend towards increased collagen contraction by AWT1-expressing CT myofibroblasts was evident (Fig. 3.9A), whereas *AWT1* expressing PF fibroblasts exhibited unaltered FPCL contraction rates (Fig. 3.9B) relative to their respective controls.

Collagen lattice contraction by fibroblasts is primarily regulated by two cellular processes: myofibroblast activation and cellular migration. Myofibroblast activation is typically associated with increased *ACTA2* expression, encoding alpha smooth muscle actin, whereas increased cellular migration is often associated with increased expression of genes encoding migration-inducing factors such as platelet derived growth factor (*PDGF*) and epidermal growth factor (*EGF*). While the RNA-seq analyses did not identify any changes in *ACTA2* or *PDGF* mRNA levels in AWT1 expressing palmar fascia fibroblasts, a significant increase in *EGF* mRNA levels was evident. EGF has been previously demonstrated to increase the migration and collagen contractility of primary human skin fibroblasts.[114] Therefore, the modest effects on collagen contraction evident in AWT1 expression in CT fibroblasts were hypothesized to be due to a modest increase in 3D migration induced by EGF. It is plausible that similar changes in EGF expression

may have impacted AWT1-expressing PF fibroblasts, and that any additive effect of collagen contraction were too insignificant to be detected over the increased collagen contractility exhibited by these pre-fibrotic cells.

4.7 Limitations

Some limitations in the experimental design of this project were evident. Firstly, the number of biological replicates used for this study were lower than optimal. Primary fibroblasts derived from explant tissues are substantially more variable than immortalized cell lines, making modest effects on gene or protein expression or cellular function difficult to discern. There are currently no commercially available immortalized palmar fascia cells lines, and the inherent variability between primary cells can be considered an advantage, as it may reflect real variability between unrelated individuals with or without DD. This inherent variability also impacted the statistical analyses in this project. Previous power analyses had indicated that N=6 fibroblast isolates/group is required to reach a power of 80% (see appendix 9). N=3 fibroblast isolates/group were used for these analyses to accommodate the financial restraints in the laboratory. To accommodate this sub-optimal number of biological replicates for RNA-seq analyses, independent gene expression analyses were performed in additional primary cells. Additionally, although clear trends were observed in the collagen contraction assays and THP-1 co-cultures, statistical significance may have been achieved with the inclusion more biological replicates. Ideally, the latter analyses would have been complemented with multiplex analyses of the cytokines secreted by both cells types to more definitively determine if AWT1 expression induced a pro-inflammatory milieu. These analyses were not completed due to COVID-19 associated restrictions.

4.8 Future Studies Beyond the Scope of this Thesis

One of the more significantly increased gene transcripts induced by constant *AWT1* expression in PF and CT cells was *TNFSF4*, encoding OX40 ligand (OX-40L). OX-40L

is a newly recognized, potential therapeutic target for several different fibroses[91], and linking its expression to AWT1 may reveal a novel pro-fibrotic role for this transcription factor. Since the *TNFSF4* promoter contains a WT1 consensus binding site (GCGGGGGCG), chromatin immunoprecipitation and subsequent PCR amplification of the relevant section of the *TNFSF4* promoter sequence could confirm direct roles for *AWT1* in transcriptional regulation of this gene. Additionally, the RNA-seq data presented here were indicative of increased gene expression of pro-inflammatory cytokines. Therefore, confirmation of the RNA-seq data should be achieved by cytokine-multiplex assay to assess the levels of target cytokine production.

In order to identify the subset of gene transcripts identified in the RNA-seq data that were directly (rather than indirectly) regulated by AWT1, Chromatin-Immunoprecipitation Sequencing (ChIP-seq) of PF and CT fibroblasts transduced with AdAWT1 could be performed. AWT1 binding at WT1 consensus sites might imply transcriptional activation or repression by AWT1, and so these analyses would need to be performed in parallel with gene expression analyses. Finally, CRISPR-Cas9 or shRNA approaches could be used to silence all or specific *WT1* variants, such as *AWT1*, in DD fibroblasts to provide a complementary loss of function approach to the over-expression studies described in this thesis. Loss of function analyses using CRISPR-Cas9 could be challenging due to the need to use primary human cells which have a finite proliferative capacity and exhibit phenotypic changes when cultured at high passage numbers. Therefore, it may be necessary to generate representative immortalized DD cell lines for CRISPR-Cas9 manipulation, or to use shRNA-mediated (preferably inducible) approaches to feasibly achieve these studies.

4.9 Conclusions

The data presented here are consistent with dynamic and complex roles for increased and sustained *AWT1* expression in DD fibroblasts. The increase of pro-inflammatory cytokines and cytokine receptors in AWT1-expressing fibroblasts may increase their sensitivity to external cytokine and other molecular stimuli. *AWT1* may also promote the

attraction and accumulation of immune cells, including monocytes, macrophages and other cells that are major sources of TGFß1, to damaged palmar fascia tissue to promote myofibroblast activation and fibrosis. In summary, the findings presented here are consistent with the hypothesis that constant *AWT1* expression contributes to a pro-inflammatory milieu in the palmar fascia that is ultimately pro-fibrotic. As current treatments fail to prevent disease recurrence in about 30% of patients[53,54], new treatments available to target WT1 in cancers are promising and can potentially be cross purposed for use in treating DD patients.[115-116]

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Appendices

Gene ID	Cat #	Amplicon Length (bp)
SERPINE1	Hs00167155_m1	82
CCL2	Hs00234140_m1	101
RPLPO	Hs99999902_m1	105
FN1	Hs00356052_m1	82
GAPDH	Hs99999905_m1	122
TGFB1	Hs00998133_m1	57
IL6	Hs00174131_m1	95
IL1B	Hs01555410_m1	91
CXCL8	Hs00174103_m1	101
IL10	Hs00961622_m1	74
ACTA2	Hs00426835_g1	105
TNF	Hs00174128_m1	80
COL3A1	Hs00943785_g1	67
COL1A1	Hs00164004_m1	66
CXCL14	Hs01557413_m1	66
TNFSF4	Hs00182411_m1	72
CXCL6	Hs00605742_g1	125
CXCL9	Hs00171065_m1	60
CXCL10	Hs00171042_m1	98
ICAM1	Hs00164932_m1	87
CXCR4	Hs00237052_m1	78
WT1	Hs01103751_m1	72

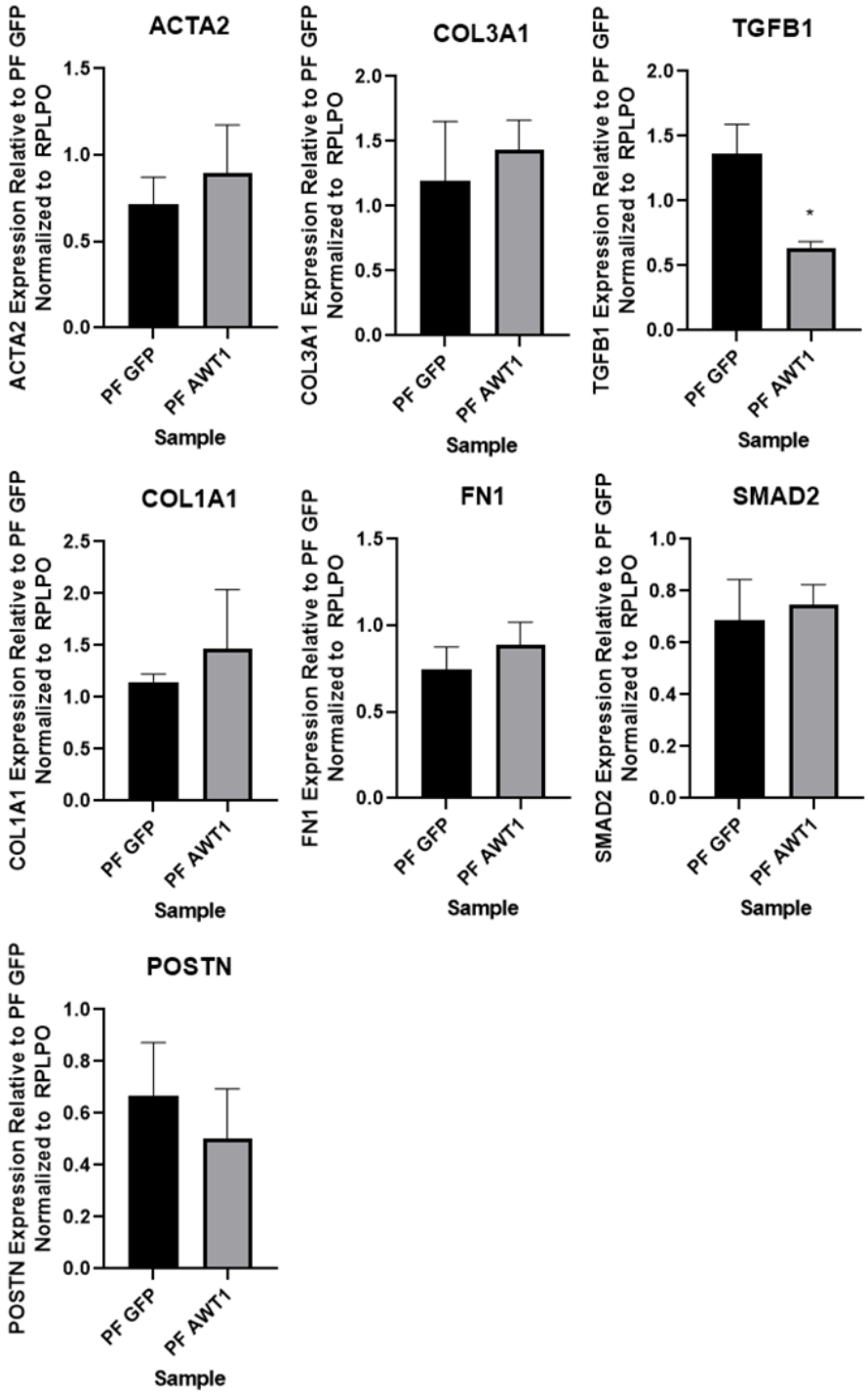
Appendix 1. Table listing qPCR primers used throughout the project

Table listing qPCR Taqman Primers (Thermofisher Scientific), gene ID, their corresponding catalog number and amplicon length (bp). All Taqman primers amplify gene products that cross exon-exon boundaries according to manufacturer.

Target Exon	Sequence 5'-3'
Exon 1 - Forward	AGCCCGCTATTCGCAATCAG
Exon 2 - Forward	TTACAGCACGGTCACCTTCG
Exon 3 - Reverse	TCCTCAGCAGCAAAGCCTGG
Intron 1 - Forward	GAGAAGGGTTACAGCACGGTC
Intron 5 - Forward	GACAGAAGGGCAGAGCAA
CUG - Forward	TACAGCAGCCAGAGCAGCAG
CUG - Reverse	GTCCCGCACGTCGGAGCCCAT
GSP1 - Reverse	GTGTGTATTCTGTATT
GSP2 - Reverse	CAACGCCCATCCTCTGCGGA

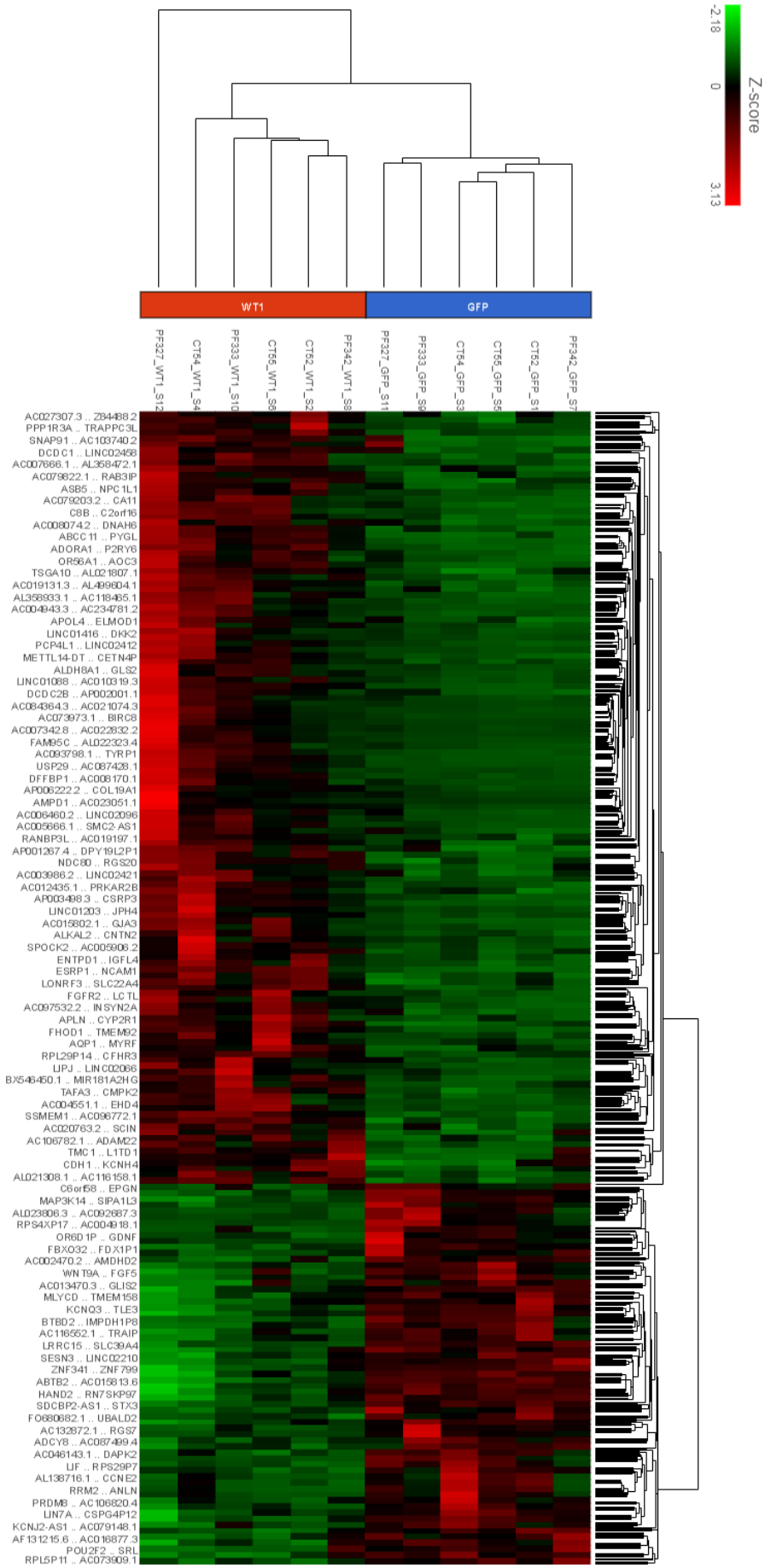
Appendix 2. Table listing the custom designed primers used for detection of *WT1* mRNA transcripts.

Table listing *WT1* Primers designed in-house and verified by DNA sequencing. CUG primers were designed to recognize *ExtWT1*, upstream of exon 1 (CUG start site), whereas intron 1 forward primers recognize ATG start site found within intron 1 of *WT1*. Primers designed to recognize specific *WT1* variants were also used as nested primers for 5'RACE. Gene specific primers 1/2 (GSP1/2) were designed in-house to recognize exon 7 and 6 respectively to generate 5'RACE products.



Appendix 3. Relative to vector controls, *AWT1* did not alter the gene expression of several "classical" pro-fibrotic genes.

Expression of *ACTA2, COL3A1, TGFB1, COL1A1, FN1, SMAD2*, and *POSTN* in PF (N=3) cells transduced with viral GFP control or adenoviral vector encoding *AWT1*. (* $p < 0.05$ as determined by t-test)


-2.18
0
3.13
Z-score
WT1
GFP
PF327_WT1_S12
CT54_WT1_S4
PF333_WT1_S10
CT55_WT1_S8
CT52_WT1_S2
PF342_WT1_S8
PF327_GFP_S11
PF333_GFP_S9
CT54_GFP_S3
CT55_GFP_S5
CT52_GFP_S1
PF342_GFP_S7
AC027307.3 .. Z84488.2
PPP1R3A .. TRAPPC3L
SNAP91 .. AC103740.2
DCDC1 .. LINC02458
AC007666.1 .. AL358472.1
AC079822.1 .. RAB3IP
ASB5 .. NPC1L1
AC079203.2 .. CA11
C8B .. C2orf16
AC008074.2 .. DNAH6
ABCC11 .. PYGL
ADORA1 .. P2RY6
OR56A1 .. AOC3
TSGA10 .. AL021807.1
AC019131.3 .. AL499604.1
AL358933.1 .. AC118465.1
AC004943.3 .. AC234781.2
APOL4 .. ELMOD1
LINC01416 .. DKK2
PCP4L1 .. LINC02412
METTL14-DT .. CETN4P
ALDH8A1 .. GLS2
LINC01088 .. AC010319.3
DCDC2B .. AP002001.1
AC084364.3 .. AC021074.3
AC073973.1 .. BIRC8
AC007342.8 .. AC022832.2
FAM95C .. AL022323.4
AC093798.1 .. TYRP1
USP29 .. AC087428.1
DFFBP1 .. AC008170.1
AP006222.2 .. COL19A1
AMPD1 .. AC023051.1
AC006460.2 .. LINC02096
AC005666.1 .. SMC2-AS1
RANBP3L .. AC019197.1
AP001267.4 .. DPY19L2P1
NDC80 .. RGS20
AC003986.2 .. LINC02421
AC012435.1 .. PRKAR2B
AP003498.3 .. CSRP3
LINC01203 .. JPH4
AC015802.1 .. GJA3
ALKAL2 .. CNTN2
SPOCK2 .. AC005906.2
ENTPD1 .. IGFL4
ESRP1 .. NCAM1
LONRF3 .. SLC22A4
FGFR2 .. LCTL
AC097532.2 .. INSYN2A
APLN .. CYP2R1
FHOD1 .. TMEM92
AQP1 .. MYRF
RPL29P14 .. CFHR3
LIPJ .. LINC02086
BX548450.1 .. MIR181A2HG
TAFA3 .. CMPK2
AC004551.1 .. EHD4
SSMEM1 .. AC096772.1
AC020763.2 .. SCIN
AC106782.1 .. ADAM22
TMC1 .. L1TD1
CDH1 .. KCNH4
AL021308.1 .. AC116158.1
C6orf58 .. EPGN
MAP3K14 .. SIPA1L3
AL023806.3 .. AC092687.3
RPS4XP17 .. AC004918.1
OR6D1P .. GDNF
FBXO32 .. FDX1P1
AC002470.2 .. AMDHD2
WNT9A .. FGF5
AC013470.3 .. GLIS2
MLYCD .. TMEM158
KCNQ3 .. TLE3
BTBD2 .. IMPDH1P8
AC116552.1 .. TRAIP
LRRC15 .. SLC39A4
SESN3 .. LINC02210
ZNF341 .. ZNF799
ABTB2 .. AC015813.6
HAND2 .. RN7SKP97
SDCBP2-AS1 .. STX3
FO680682.1 .. UBALD2
AC132872.1 .. RGS7
ADCY8 .. AC087499.4
AC046143.1 .. DAPK2
LIF .. RPS29P7
AL138716.1 .. CCNE2
RRM2 .. ANLN
PRDM8 .. AC106820.4
LIN7A .. CSPG4P12
KCNJ2-AS1 .. AC079148.1
AF131215.6 .. AC016877.3
POU2F2 .. SRL
RPL5P11 .. AC073609.1

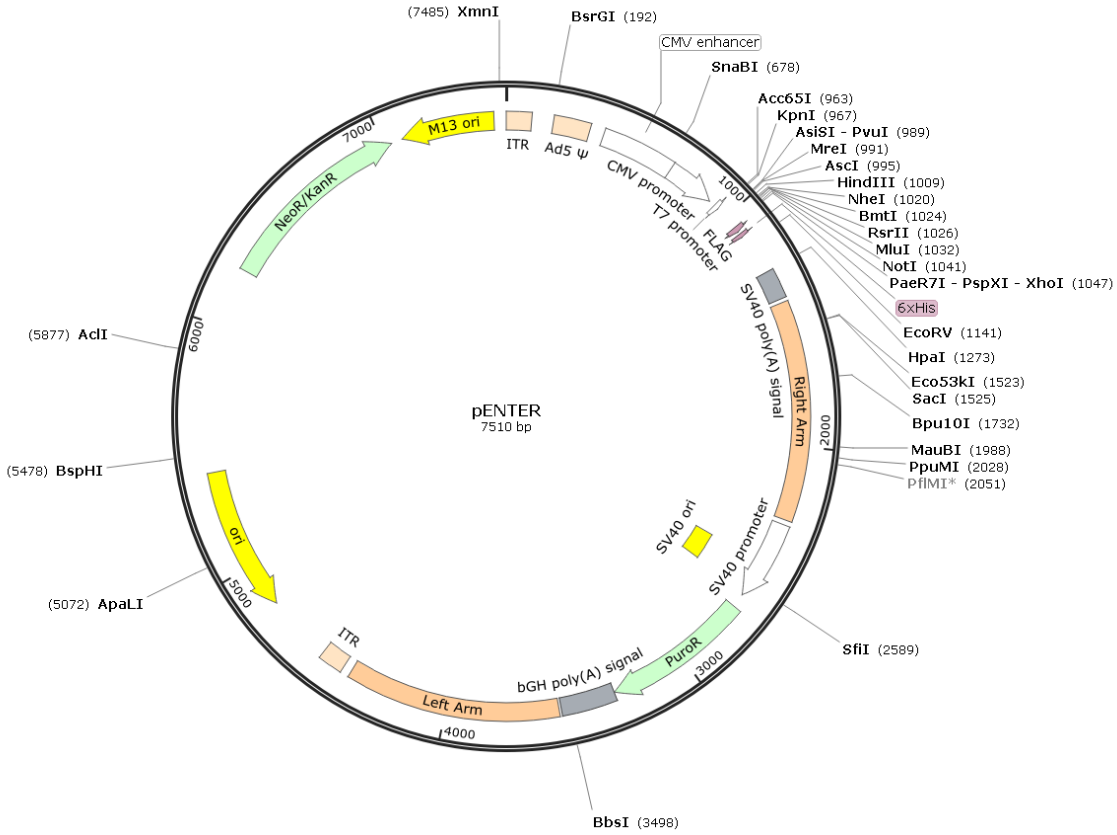
Appendix 4. Heatmap and clustering analysis demonstrating similarities between *GFP* expressing or *AWT1* expressing PF and CT fibroblasts.

Heatmap and Clustering analysis of PF (N=3) and CT (N=3) fibroblasts transduced with adenoviral vector control (PF-GFP and CT-GFP) or adenoviral vector encoding AWT1 (PF-AWT1 and CT-AWT1) respectively. Differential expression was indicated as green representing a negative fold-change and red representing positive fold-change in *AWT1* expressing fibroblasts relative to vector controls. Inclusion criteria were that p-value < 0.05 and fold change in gene expression of > 1.5 or <-1.5 for all significant genes as identified by RNA-seq.

Anti-WT1 Antibody	Specificity (amino acids)
Anti-WT1 (D6M6S)	Surrounding 306
Anti-WT1 (H-1)	123-164
Anti-WT1 (ab15249)	Surrounding 400
Anti-WT1 (6F-H2)	1-173

Appendix 5. List of anti-WT1 antibodies used throughout this project.

WT1 antibodies and their specificity. Canonical sequence of *WT1* encodes for a protein that is 449 amino acids in length (~50kDa). WT1 antibodies recognizing different epitopes of WT1 (specificity as reported by manufacturer) was utilized to identify/characterize different WT1 isoforms by Western immunoblotting and immunocytochemistry analyses on DD, PF and CT fibroblasts.



Appendix 6. Plasmid map for adenoviral vector encoding for *AWT1* or *GFP*.

First generation adenoviral vector (pAD) encoding for *AWT1* (Ex5+/KTS-) was used to transduce syngeneic (PF) and allogeneic (CT) fibroblasts for constant expression of *AWT1* or *GFP*. Adenoviral vector utilizes CMV promotor and encodes for AWT1 that is FLAG and His tagged at the C-terminus.

GO term	Enrichment score
plasma membrane	34.03
regulation of multicellular organismal process	26.81
plasma membrane part	26.34
integral component of plasma membrane	25.01
intrinsic component of plasma membrane	24.95
extracellular space	24.55
regulation of ion transport	24.49
signaling receptor activator activity	24.16
receptor ligand activity	23.92
regulation of localization	23.64
receptor regulator activity	23.54
regulation of response to stimulus	23.00
cell surface receptor signaling pathway	21.70
system process	21.48
intrinsic component of membrane	19.16
signal transduction	19.13
defense response	18.65
response to stimulus	18.58
integral component of membrane	18.34
ion transport	18.08
positive regulation of response to stimulus	17.48
cytokine-mediated signaling pathway	17.71
multicellular organismal process	17.49
negative regulation of multicellular organismal process	16.92
signaling receptor binding	16.60
nervous system process	16.58
immune response	16.18
positive regulation of MAPK cascade	16.15
regulation of transport	15.74
positive regulation of intracellular signal transduction	15.72
positive regulation of anion transport	15.59
positive regulation of multicellular organismal process	15.51
regulation of cellular component movement	15.46
transmembrane signaling receptor activity	15.40
cytokine activity	15.17
signaling receptor activity	15.11
positive regulation of ion transport	15.01
response to external biotic stimulus	14.94
signaling	14.89
anion transport	14.65
regulation of signaling	14.64
regulation of cell population proliferation	14.61
membrane part	14.49
regulation of anion transport	14.29
positive regulation of protein kinase B signaling	14.21
regulation of response to external stimulus	14.15
receptor complex	14.00
inflammatory response	14.00
response to biotic stimulus	13.82
defense response to virus	13.80

Appendix 7. AWT1 expression promotes cytokine activity and sensitivity to external signals.

Gene set enrichment analysis identifying overrepresented biological processes in *AWT1* expressing PF and CT fibroblasts (pooled). Inclusion criteria were that p-value < 0.05 and fold change in gene expression of >1.5 or <-1.5. Enrichment scores indicate the negative natural logarithm of the p-value.

Enrichment terms	False Discovery Rate
Cell surface receptor signalling pathway	1.30E-07
Regulation of multicellular organismal process	3.83E-07
Cellular response to chemical stimulus	2.87E-06
Cellular response to organic substance	8.65E-06
Response to stimulus	8.65E-06
Interferon alpha/beta signalling	1.24E-05
Type I interferon signalling pathway	2.98E-05
Interferon signalling	0.0014
Cytokine signalling	0.0019
Inflammatory response	0.0031
MAPK signalling pathway	0.0032

Appendix 8. AWT1 expression is associated with inflammation in palmar fascia derived fibroblasts.

Representative table for gene set and pathway enrichment analyses identifying overrepresented biological processes in *AWT1*-expressing PF and CT fibroblasts (pooled) relative to PF/CT fibroblasts GFP-expressing controls. Inclusion criteria were FDR < 0.05 and fold change in gene expression of > 1.5 or <-1.5. As shown, enrichment terms such as interferon signalling, cytokine signalling and inflammatory response are associated with a pro-inflammatory response.

Appendix 9. Sample size estimation for downstream statistical analyses.

Variability between samples is inherent to all analyses of primary cells derived from genetically distinct individuals. To achieve sufficient statistical power for analyses of primary cells, we use the following calculation:

$$N = \frac{(Z\alpha + Z\beta)^2 \{(Pe\,(1-Pe) + Pc\,(1-Pc)\}}{(Pe-Pc)^2}$$

for differences in proportions to analyze differences in expression or protein levels between disease and control samples. N is the sample size required and Z scores for α and β error represent level of significance testing (0.05) and power (80%) respectively. Pe is the proportion in the experimental group and Pc is the proportion in the control groups. We assume 80% expression in our disease group (Pe) with 20% expression in controls (Pc), resulting in a sample size requirement of N=6. All experiments are therefore performed on a minimum of 6 DD, 6 PF and/or 6 CT cell lines, each assessed in triplicate.

Appendix 10. Approved HSREB protocol letter.

Western Research

Date: 27 April 2020
To: Dr. David O'Gorman
Project ID: 104888
Study Title: Microenvironmental Regulation of Normal and Abnormal Connective Tissue Repair
Application Type: Continuing Ethics Review (CER) Form
Review Type: Delegated
REB Meeting Date: 04/May/2020
Date Approval Issued: 27/Apr/2020
REB Approval Expiry Date: 28/Apr/2021

Dear Dr. David O'Gorman,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the
expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for
Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5
of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario
Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services
under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Daniel Wyzynski, Research Ethics Coordinator, on behalf of Dr. Joseph Gilbert, HSREB Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Curriculum Vitae

Name:	Johnny Luo
Post-secondary Education and Degrees:	St. Clair College Windsor, Ontario, Canada 2011-2015 Degree in Chemical Laboratory Technologies The University of Windsor Windsor, Ontario, Canada 2015-2017 Honours BSc with Thesis in Biochemistry. Western University London, Ontario, Canada 2017-2020 MSc with Thesis in Biochemistry
Honours and Awards:	Wound Healing Society Travel Award 2018 Collaborative Specialization in Musculoskeletal Health Research Award 2017
Related Work Experience	Teaching Assistant Western University 2018-2019

Publications:

W. Luo, **J. Luo**, V. V. Popik, M. S. Workentin. (2019). A biorthogonal 'click and release' or 'double-click' strategy, based on strained-promoted cycloaddition and Staudinger ligation/reaction: a tool towards multifunctional nanomaterials. Bioconjugate Chem. Manuscript ID: bc-2019-00078y

W. Luo, L. Sydney, **J. Luo**, L. L. Francois, M. S. Workentin. (2019). Investigation of Au SAMs Photoclick Derivitization by PM-IRRAS. Langmuir. Manuscript ID: Ia-2019-03782v

Select Conferences:

Wound Healing Society (2020, Oral) - **Luo, J.;** Tugade, T.; Diaz, A.P.; Gan, B.S.; Suh, N.; O'Gorman, D.B. Wilms Tumor 1 isoforms transcriptionally regulate cytokine gene expression by myofibroblasts.

European Tissue Repair Society (2019, Oral) – **Luo, J.;** Tugade, T.; Diaz, A.P.; Gan, B.S.; Suh, N.; O'Gorman, D.B. Fibrosis-associated Wilms Tumor 1 isoforms promote *IL0* expression in palmar fascia myofibroblasts

Canadian Connective Tissue Conference (2019, Poster) - **Luo, J**.; Tugade, T.; Diaz, A.P.; Gan, B.S.; Suh, N.; O'Gorman, D.B. Characterizing Wilms' Tumor 1 Variants and their roles in Dupuytren's Disease Development.

London Health Research Day Conference (2019, Poster) - **Luo, J**.; Tugade, T.; Diaz, A.P.; Gan, B.S.; Suh, N.; O'Gorman, D.B. Characterizing Wilms' Tumor 1 Variants and their roles in Dupuytren's Disease Development.

Wound Healing Society Conference (2018, Oral) - **Luo, J**.; Sun. E.; Tugade, T.; Diaz, A.P.; Gan, B.S.; Suh, N.; O'Gorman, D.B. Characterizing Wilms' Tumor 1 Variants and their roles in Dupuytren's Disease Development.