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Investigation of β-Catenin-Mediated Regulation of IGFBP-6 and the Roles of IGFBP-6 and IGF-II in Dupuytren's Disease

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biochemistry

INVESTIGATION OF β-CATENIN-MEDIATED REGULATION OF IGFBP-6 AND THE ROLES OF IGFBP-6 AND IGF-II IN DUPUYTREN'S DISEASE

(Thesis format: Integrated Article)

by

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Abstract

Dupuytren's Disease (DD) is a benign and heritable connective tissue fibrosis that affects the palmar fascia and typically results in permanent finger contracture(s). Similar to other fibroses, DD is characterized by increased fibroblast proliferation, myofibroblast differentiation and excess collagen deposition. Currently, there are no truly effective treatment options for connective tissue fibroses.

Increased levels of β-catenin, an intracellular trans-regulator of gene transcription, have been previously reported in DD. Genes that are associated with, and therefore potentially transcriptionally regulated by, β-catenin during DD development were identified in this thesis. One of these gene targets, *IGFBP6*, was shown to consistently be associated with β-catenin in fibroblasts derived from phenotypically normal palmar fascia (PF cells) but not fibroblasts derived from diseased tissues (DD cells). β-catenin association with the *IGFBP6* promoter in these cells was directly correlated with *IGFBP6* expression levels and with the secretion of its protein product, Insulin-like Growth Factor Binding Protein-6 (IGFBP-6). In addition, 1438 unique genes were shown to associate with β-catenin in DD cells but not PF cells derived from the same patients.

The functional consequences of IGFBP-6 repression, and the increased availability of its primary ligand, IGF-II were also elucidated. Exogenous addition of IGFBP-6 attenuated the proliferation of DD and control fibroblasts, and inhibited IGF-II induced contraction of DD cells. IGF-II stimulated the proliferation of normal fibroblasts but not fibroblasts derived from patients with DD. The gene encoding IGF-II, *IGF2*, was found to be up regulated in DD cells, and potential mechanisms facilitating *IGF2* overexpression were investigated. Loss of imprinted expression of *IGF2* was detected in a subset of patients and a corresponding loss of *H19* expression, a non-coding RNA that is reciprocally expressed relative to *IGF2*, was observed. Aberrant *IGF2* promoter usage was also identified in a subset of DD and PF cells. In combination, these disease-associated changes may explain the increased *IGF2* expression in DD. Identification of novel gene targets of β-catenin and the factors that regulate the expression of *IGFBP6* or *IGF2*

during the development of this debilitating fibrosis may allow us to identify novel therapeutic targets.

Keywords

Dupuytren's Disease, IGFBP-6, β-catenin, IGF-II, Fibrosis, Fibroproliferation, Myofibroblast differentiation, ChIP Sequencing, Loss of Imprinting, *IGF2* Promoter Usage

Co-Authorship Statement

This thesis was written by Christina Raykha and edited by Dr. David O'Gorman.

The ChIP Sequencing data in chapter 2 is in preparation for a manuscript. I cultured and collected all the cells required for analysis, conducted the ChIP experiments, prepared the libraries and then sent these samples to the Centre for Applied Genomics at Sick Kids Hospital in Toronto, ON. Once the raw data was received, Dr. Greg Gloor helped process the raw data, wrote programs as needed and guided me with the ChIP Seq data analysis. Dr. O'Gorman coordinated the experiments and aided in study design.

Chapter 3 is published in BBA: Molecular basis of disease. The immunohistochemistry analysis was completed at Victoria Hospital in London, ON and the *IGFBP6* expression and secretion analyses were performed by Justin Crawford. The IGFBP-6 mutant was supplied by Drs. Ping Fu and Leon Bach, and they conceptually aided in study design. I performed all other experiments and assisted in the experimental design with Dr. O'Gorman.

I performed all of the experiments in chapter 4, and aided in study design with Dr. O'Gorman.

Dr. Gan provided the lab with clinical isolates for derivation of primary cells for all the analyses.

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List of Abbreviations

Abbreviation	Meaning
α-MEM	α-minimum essential medium
α-SMA	α-smooth muscle actin
ADAM	A disintegrin and metalloprotease
APC	Adenomatous polyposis coli
BCA	Bicinchoninic acid
CAFs	Cancer-associated fibroblasts
ChIP	Chromatin immunoprecipitation
ChIP Seq	Chromatin immunoprecipitation sequencing
COX-2	Cyclooxygenase-2
CTCF	CCCTC binding factor
DD	Dupuytren's disease
DMR	Differentially methylated region
DNMT	DNA methyltransferase
Dvl	Dishevelled
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EGR1	Early growth response-1
EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOXO	Forkhead box O

FPCLs	Fibroblast populated collagen lattices
GAG	Glycosaminoglycans
GSK-3β	Glycogen synthase kinase-3β
HDACs	Histone deacetylases
HRP	Horseradish peroxidase
HSCs	Hepatic stellate cells
HSREB	Health Sciences Research Ethics Board
ICR	Imprinting control region
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IGFIIR	Type II Insulin-like Growth Factor receptor
IGFIR	Type I Insulin-like Growth Factor receptor
InsR	Insulin receptor
IPA	Ingenuity pathway analysis
jMOSAiCS	Joint model-based one- and two-sample analysis and inference for chip
LAP	Latency associated peptide
Lef	Lymphoid enhancer factor
LOI	Loss of imprinting
LRP	Lipoprotein receptor-related protein
LTBP	Latent TGF-β Binding Protein
MACS	Model-based Analysis of ChIP Seq
MAP	Mitogen associated protein
MFOLD	Model-fold
MSCs	Mesenchymal stem cells

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PMSF	Phenylmethylsulfonyl fluoride
QPCR	Quantitative real-time polymerase chain reaction
RACK1	Receptor for activated C kinases
RFLP	Restriction fragment length polymorphism
rFPCLs	"Relaxed" fibroblast populated collagen lattices
sFPCLs	"Stressed" fibroblast populated collagen lattices
SMCs	Smooth muscle cells
SNPs	Single nucleotide polymorphisms
TCF	T cell factor
TGF	Transforming growth factor
TSA	Trichostatin A
UTR	Untranslated region
WST-1	Water soluble tetrazolium -1

Chapter 1

1 General Introduction

1.1 Connective Tissue

The tissues of the human body can be divided into the four broad categories of nervous, epithelial, muscle, and connective tissues [1,2]. Of these, connective tissues are the most abundant and widely distributed, and they have essential structural and supportive functions to hold and connect the other tissues of the body together. Connective tissue itself can be subdivided into the four main classes of blood, bone, cartilage and connective tissue proper [2,3], the latter encompassing loose (e.g. adipose, areolar, reticular) and dense (e.g. regular, irregular and elastic) tissues [2].

Many connective tissues can be envisaged as relatively small numbers of cells embedded in a "sea" of extracellular matrix (ECM). While the constituents of the ECM vary considerably between the different classes, most consist of various fibres and components of "ground substance", such as proteoglycans and hyaluronic acid, that allow these tissues to maintain a high water content [2]. The fibres secreted by connective tissue cells include collagens, reticular and elastic fibres [1–3]. While many different cell types make up the different classes of connective tissue, connective tissue proper is primarily populated by fibroblasts [3]. This poorly characterized and heterogeneous group of pleomorphic cells are of mesenchymal origin [3,4] and play central roles in secreting the ground substance, fibres and many other molecules that make up the ECM of these tissues [2,3,5].

1.2 Fibrosis

The term "fibrosis" refers to a disease state of connective or other tissues that is characterized by abnormal remodelling of the ECM, which results in thickening and hardening of tissues [6]. Major organ fibroses, such as those of the kidney [7], liver [8], lungs [9,10], and heart [11] can result in catastrophic organ failure and death. Fibrosis has

a significant etiological role in nearly 45% of deaths in the Western world that are the result of loss of major organ function [12].

While the majority of connective tissue fibroses are not life threatening, they often cause long term and debilitating loss of function of the affected area(s) and decreased quality of life [13,14]. Fibroses can have variable etiologies and characteristics that are dependent on the affected tissues, however many similarities between different fibroses exist at the basic cellular and molecular levels. These similarities include increased Transforming Growth Factor (TGF)-β1 signalling, excessive fibroproliferation, collagen deposition into the ECM, and the formation and persistence of contractile myofibroblasts that contract and remodel the ECM to increase tissue density [6,15–19].

The primary effector cell type in virtually all fibroses is the myofibroblast [17,20–22]. This cell type was initially characterized in granulation tissue as an "activated" contractile form of dermal fibroblast that expresses α-smooth muscle actin (α-SMA) within stress fibres [17,23–25]. Stress fibres allow myofibroblasts to generate the contractile forces required to induce wound closure and tissue contraction [23]. These fibroblasts can be resident in, or derived from tissues adjacent to, any tissue undergoing fibrosis and their differentiation into myofibroblasts is central to fibrosis development [20]. The conversion from fibroblast to myofibroblast can be initiated by changes in mechanical tension, various growth factors such as TGF-β1, or a combination of these factors [23–25]. Generally, highly proliferative fibroblasts do not express α-SMA or have abundant stress fibres, whereas myofibroblasts invariably display α-SMA positive stress fibres and often display a reduced proliferative potential [26]. There is an intermediate form between the fibroblast and myofibroblast, the "protomyofibroblast", that is thought to retain the enhanced proliferative potential of a fibroblast, express cytoplasmic β and γ, but not α-smooth muscle, actins in their stress fibres, and can generate an intermediate level of contractile force on the ECM [27,28]. However, fully differentiated myofibroblasts are the major source of the excessive collagen deposition, ECM contraction and remodelling that are causal for the development of the dense collagenous tissue that characterizes fibroses [27]. Under non-pathological circumstances, myofibroblasts undergo apoptosis once wound contraction has been achieved. In fibroses,

hyper-contractile, collagen-depositing myofibroblasts can persist for months or years [29], and can continually activate ECM-associated latent TGF-β1 through a tension-dependent mechanism [30]. As TGF-β1 signalling can inhibit myofibroblast apoptosis [31], these factors can act in combination to promote further fibrosis development.

1.3 TGF-β1 activation and signalling

TGF-β1 signalling is considered to be central to the development of virtually all fibroses [20] as it can induce the production of α-SMA-containing stress fibres and enhanced cellular contractility of myofibroblasts [23,32,33]. This cytokine is synthesized in an inactive (latent) form and must first be activated to signal through its cognate receptors [34,35]. Disulfide-linked dimers of Latency Associated Peptide (LAP) and TGF-β1 associate non-covalently to form the small latent complex, and if Latent TGF-β Binding Protein (LTBP) is present in the ECM, it covalently binds LAP through two disulfide bonds to form the large latent complex [34]. Active TGF-β1 can be released from this complex by the actions of proteases [36] and, in the context of myofibroblast contraction, increased tension or tissue stiffness through integrin binding to ECM-associated LAP [30].

Once TGF-β1 has been released from the small- or large latent complex, it can transduce signals from the ECM. TGF-β1 signals through two membrane associated receptors, TGF-β Receptors I and II, to activate canonical SMAD (Sma/MAD) signalling [37,38]. The receptor-associated SMADs, SMAD2 and 3, are phosphorylated by receptor activation and form a complex with SMAD4, allowing SMAD2 and SMAD3 to enter the nucleus and elicit effects on the transcription of target genes that feature SMAD binding elements, or they can interact with other transcription factors and trans-regulate gene expression [37–40]. TGF-β1 can also initiate many parallel downstream pathways which are not dependent on SMAD phosphorylation, such as the MAP kinase pathway [41].

1.4 Other pathways that cross-talk with TGF-β1

TGF-β1 signalling can induce diverse and, in some cell types, mutually exclusive outcomes that include fibroblast proliferation and myofibroblast differentiation. For

example, combined epidermal growth factor and TGF-β1 signalling can induce normal, quiescent murine fibroblasts to proliferate faster than either factor in isolation, while Insulin-like Growth Factor (IGF)-II and TGF-β1 signalling can induce the same fibroblast cell line to become non-proliferative and take on features of contractile myofibroblasts [42]. Both TGF-β1 and IGF signalling can induce β-catenin accumulation in cells, and this intracellular signalling molecule can induce fibroblast proliferation or migration [43–47]. As increased myofibroblast differentiation and fibroblast proliferation are characteristics of all fibroses, and TGF-β1 signalling can have direct or indirect roles in promoting these effects, many groups have attempted to target this cytokine to prevent fibrosis development [11,48,49]. Unfortunately, TGF-β1 has integral and essential roles in normal wound healing and the inflammatory response [50–52], which greatly complicates its usefulness for therapeutic interventions. More recently, researchers have opted for identifying and targeting molecules that are downstream of TGF-β1 signalling or activate parallel signalling pathways that cross-talk with TGF-β1 signalling, to promote pathological rather than normal tissue repair [53]. Further insights into the molecular mechanisms that drive fibrosis development are required in order to identify optimal targets for these approaches.

1.5 Dupuytren's Disease

Our laboratory has focused on identifying these targets in Dupuytren's Disease (DD). DD is a fibrosis of the palmar fascia, which is a thin layer of connective tissue below the skin in the palm of the hand. Like virtually all fibroses [15,21,54,55], DD is characterized by increased TGF-β1 signalling, fibroblast proliferation, myofibroblast differentiation and excess collagen deposition [56–59]. Patients with DD usually present to the clinic with permanent and irreversible finger contractures [60–62]. Clinically, this disease is initially evident as a nodule in the palm of the hand [63] consisting of proliferating myofibroblasts [64] (Figure 1-1). Over time, nodules can progress to the involutional phase of DD where a collagenous and contractile disease cord forms along the palmar fascia. This process is associated with an increase in myofibroblasts that impose significant contractile forces on the palmar fascia [23] and induce permanent contracture of the associated finger(s). Once finger contracture has occurred, the disease typically enters the final residual phase

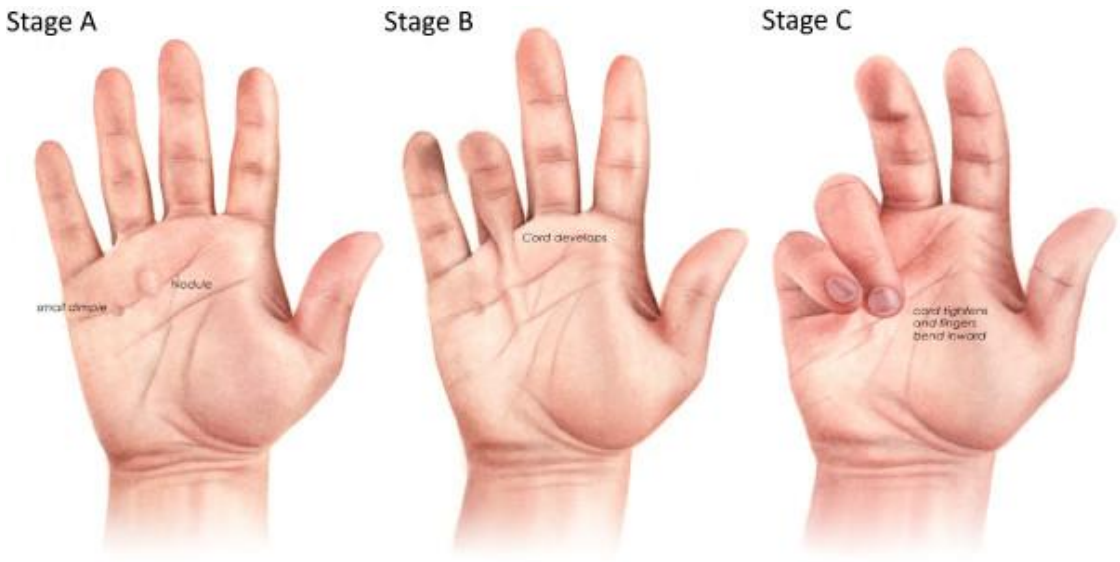

Figure 1-1: Clinical presentation of Dupuytren's Disease.

In the proliferative stage of DD, a nodule is formed in the palm (Stage A), which then progresses to a collagenous disease cord in the involutional stage (Stage B). Finally, in the residual phase of disease (Stage C), the cord tightens which results in permanent contracture of the affected finger(s) and onset of the residual phase. (Reprinted with permission from Rehman *et al.* [178])

[64] where the contractile cord becomes a heterogeneous structure with regions of acellular tissue, which are very collagenous, interspersed with cell-rich nodules, referred to as the "nodule-cord unit" [64]. The abundance of apoptosis-resistant myofibroblasts in nodules and disease cords is considered both a distinguishing feature and functionally important component of DD development [65,66], and new therapeutic treatment strategies are aimed at inhibiting the proliferation and differentiation of myofibroblasts and/or inducing their apoptosis.

DD is also known as Dupuytren's Contracture or palmar fibromatosis, and is the most common inherited connective tissue disorder of Caucasian males over 60 years of age [67,68]. In Europe, DD prevalence can be as high as 20% over a broader age range [67,69], while in Western countries, the prevalence of the disease increases with age and has been estimated to be approximately 15% at 60 years of age and up to 30% at 75 years [70]. The incidence of DD in the general US population was estimated to be 3 new diagnosed cases per 10, 000 in 2007 [71]. While males and females have similar global disease prevalence up to age 45, thereafter males are afflicted 3 to 4 times more often than females [72]. Almost 40% of patients with DD report that a family member is also affected, suggesting a strong genetic predisposition to its development [73,74].

Despite being first described in 1831 by Guillaume Dupuytren, more than 150 years later the etiology of DD remains poorly understood and a cure remains elusive. Both genetic and environmental factors have a role in determining susceptibility to this disease, as evidenced by the occurrence of both familial and apparently sporadic cases [73,75,76]. Risk factors for DD have been described, including manual labour, trauma, smoking, alcohol, diabetes and epilepsy [74,77–80]. While the conclusions drawn from many of these studies are controversial, the association with diabetes appears to hold true [77], as the prevalence of DD can be as high as 40% when assessed within a diabetic population [81,82]. The Dupuytren's "diathesis" i.e. the list of characteristics that identify an individual as likely to develop DD, includes palmar bilateral lesions, positive family history, lesions over the knuckle (Garrod's pads), Caucasian ethnicity, male gender, and age at onset of less than 50 years. These factors predict the risk of DD recurrence after treatment to be more than 70% if the individual satisfies all of these criteria [74]. The

genetic basis of the Dupuytren's diathesis remains unclear, and there is an obvious need to identify the genetic links in DD in order to develop effective treatment strategies for these patients.

1.6 Treatments for DD

Surgical resection of the disease cord is the current "gold standard" treatment option for DD patients [71,83], despite this approach resulting in disease recurrence in at least one of every three patients [74,84,85]. This unsatisfactory outcome occurs despite extensive and expensive [86] post-operative rehabilitation that negatively impacts patient quality of life [13]. For some patients, continual recurrence of finger contractures after surgeries leads them to consider amputation as a final option to eliminate the risk of relapse [87]. Alternative non-surgical interventions have been gaining popularity in recent years, however these treatments are associated with disease recurrence rates of 50% or higher [88–90]. These treatments include needle aponeurotomy, where a needle is used to manually sever and dissect the cord within the hand, and injections of Clostridial collagenase, an enzyme that breaks down the type I and type III collagen in the cord [88,91]. In the context of the high disease recurrence rates, there are currently no truly effective treatment options for DD [92]. A detailed understanding of the molecular processes that regulate fibroblast proliferation and their subsequent differentiation into myofibroblasts, as well as the genetic basis of DD, is required to design rational and more effective molecular therapies.

1.7 Genetics of DD

The increased incidence of DD in the Northern European population and the disease heritability within families strongly implicates a genetic component to Dupuytren's Disease [72,73]. This concept has gained considerable support following a recent study demonstrating that one twin in a monozygotic male pair is 37% more likely to be affected by DD if the other has this disease versus just 7% in fraternal male twins [93]. Inheritance of DD is suggested to follow an autosomal dominant inheritance pattern with variable penetrance [94] and, while numerous studies have sought to identify the specifics of these genetic links, relatively few insights have been achieved. Chromosome

instability was suggested to be part of the genetic susceptibility for DD [95] and comparative genomic hybridization studies were completed to assess the possibilities of gene copy number variations, however subsequent findings did not support this hypothesis [96]. Another study reported DNA copy number variations in chromosome 6 near the HLA locus, and chromosome 7 and 14 near the T-cell receptors - γ and -α, respectively, but the sample size was limited in both studies [97,98] and the general applicability of the findings are unclear. There has been one genetic linkage study performed thus far in a Swedish family which identified an association in 16q [94], but these results are yet to be confirmed. A genome wide association study performed on European DD patients identified 11 associated single nucleotide polymorphisms (SNPs), 6 of which were located near or within Wnt-related genes [99]. However the effects of these SNPs on Wnt regulation have not been reported, nor have consistent changes in the expression of these Wnt genes been detected in some expression analyses of DD cells [100]. Dysregulated Wnt gene expression was implicated in an exon array study that compared fibroblasts derived from DD palmar fascia from mostly male patients to fibroblasts derived from the thigh of female patients unaffected by DD [101]. However, most of the dysregulated genes that are consistently identified in previous microarray analyses of DD cells and tissues [98,102–104] were not identified in this exon array analysis, making it difficult to interpret these results. In summary, while some of the most recent genetic and transcriptional analyses are consistent in identifying genes that encode Wnts and/or molecules associated with Wnt signalling as potential contributors to DD development, their contributions remain controversial and very poorly understood.

1.8 Molecular mechanisms of DD development

TGF-β1 signalling is increased in DD and has been shown to induce myofibroblast differentiation [58,105] and fibroproliferation [106] of fibroblasts derived from fibrotic DD tissues (DD cells). As described in 1.4, the concurrent activation of parallel signalling pathways can direct TGF-β1 signalling intermediates to induce either of these outcomes in other systems [42]. The Wnt/β-catenin signalling pathway implicated in recent genetic and transcriptional analyses of DD tissues and cells is one of these parallel signalling pathways, and cross-talk with TGF-β1 signalling has been shown to elicit effects on

proliferation and myofibroblast differentiation in other systems [45,107]. Total cellular β-catenin levels are increased in fibrotic DD tissues relative to the adjacent, non-fibrotic palmar fascia [108] and β-catenin levels in primary DD cells are sensitive to fibrosis-associated changes in ECM tension *in vitro* [44]. These findings suggest that a detailed understanding of interactions between the TGF-β1 and Wnt/β-catenin signalling pathways may provide valuable insights into the molecular mechanisms of DD development.

1.9 β-catenin

β-catenin is best recognized as a 90 kDa [109] protein intermediate in the canonical Wnt signalling pathway that has important and mutually exclusive cell signalling and structural roles [110,111]. Structurally, β-catenin is essential for adherens junction formation, where it can interact with γ-, α-catenins and E-cadherin to promote the cell-to-cell contacts [112,113] required during embryonic differentiation [110]. In addition, nuclear localization of β-catenin can induce interactions with T cell factor/Lymphoid enhancer factors (TCF/Lef) or other transcription factors to regulate gene transcription [114,115]. β-catenin-mediated trans-regulation of gene transcription is required for mesoderm formation in embryonic stem cells and in mice [110,116], illustrating its central roles in development. In *Homo sapiens*, β-catenin consists of 781 amino acids and can be functionally divided into the N-terminal, central and C-terminal domains [109]. The N-terminal domain is required for β-catenin signalling, but it is the central domain, which contains 12 armadillo repeats in a rigid scaffold, that is most important for β-catenin function [109]. Many β-catenin binding partners interact with the armadillo repeat region in β-catenin. E-cadherin interacts with all 12 repeats [117] and TCF/Lef interacts with 8 of these repeats [118], thereby precluding the simultaneous binding of both partners [119]. Whether the same pool of β-catenin that is involved in cell-cell contacts is released to trans-regulate gene transcription in the presence of Wnt signalling is somewhat controversial, however recent evidence in other signalling pathways is supportive of this concept [120,121].

Canonical Wnt signalling (Figure 1-2) is integral to cellular proliferation and differentiation during embryonic development (reviewed in [122,123]). β-catenin is a key

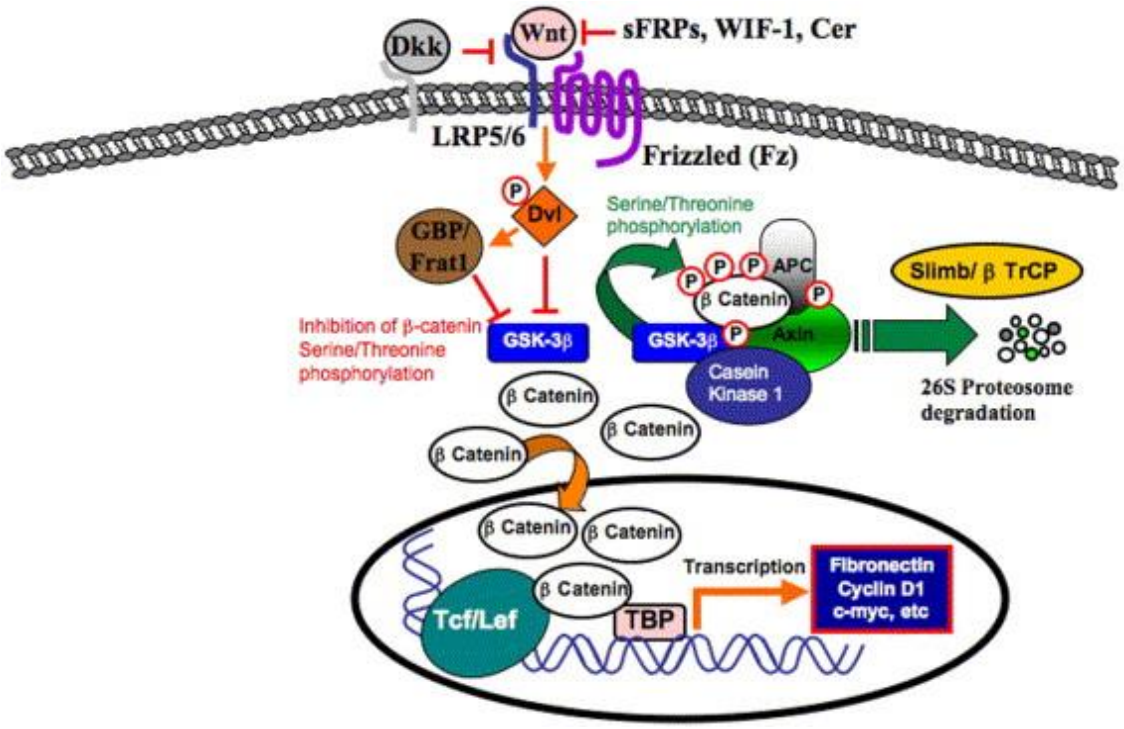

Figure 1-2: Canonical Wnt Signalling Pathway.

Stimulation of the Frizzled receptor/LRP5/6 by Wnts results in phosphorylation of Dishevelled (Dvl), which in turn inactivates GSK-3β. Inactivation of GSK-3β allows β-catenin to be released from the destruction complex (which facilitates its degradation in the absence of Wnts in an ubiquitin-mediated mechanism) and to accumulate within the cytoplasm, translocate to the nucleus and transactivate the Tcf/Lef transcription complex. (Reprinted with permission from Bowley *et al.* [139]**)**

effector molecule in these processes under the regulation of glycogen synthase kinase (GSK)-3β. In the absence of a Wnt signal, GSK-3β phosphorylates β-catenin on serine residues 33 and 37 [124–126] and, with other components of the "destruction complex" including axin, adenomatous polyposis coli (APC) and casein-kinase 1, targets phospho-β-catenin to the E3 ubiquitin ligase complex for degradation by the 26S proteasome [127,128]. Wnt activation of the Frizzled receptor and its co-receptor, low density lipoprotein receptor-related protein (LRP5/6), facilitates binding with the cytoplasmic protein Dishevelled [129], phosphorylation of LRP and subsequent inactivation of GSK-3β [130]. When the destruction complex is destabilized by the loss of axin and GSK-3β activity [130] in the presence of Wnts, β-catenin accumulates in the cytoplasm and translocates to the nucleus to regulate its target genes. Consistent with its central role in β-catenin signalling, GSK-3β is believed to be the major point of convergence for the many of the signalling pathways that cross-talk with the Wnt signalling pathway [123].

1.10 β-catenin-mediated transcription

The mechanisms that regulate the nuclear translocation of β-catenin are not clearly understood, as it does not contain a nuclear localization signal and the signals that induce nuclear localization appear to vary between different cell types and systems. β-catenin does not bind DNA, but rather interacts with DNA binding transcription factors in order to elicit repressive or activating effects on transcription [109,119]. While Wnt-dependent β-catenin transcription is mainly transduced through TCF/Lef transcription factors, β-catenin can also interact with SMADs or c-Jun/AP-1, or other complexes to regulate target genes [131–133]. Numerous β-catenin target genes have been documented, including c-myc [134], cyclin D1 [135], axin2 [136] and epidermal growth factor receptor [137]. To our knowledge, the majority of the genes that are regulated by or associated with β-catenin have been identified in cancer cells, and there are currently very few reports of β-catenin regulated genes in non-malignant cells.

1.11 β-catenin in fibrosis and DD

Increased β-catenin levels and signalling have been reported in many diseases [138–140], with or without obvious changes in Wnt signalling [43], and activation of this signalling

pathway is thought to contribute to disease progression by regulating cellular proliferation. Mutations in the gene encoding β-catenin, *CTNNB1*, have been reported in numerous cancers [141–144]. These mutations typically delete or inhibit β-catenin phosphorylation sites, allowing β-catenin to avoid degradation, translocate to the nucleus and dysregulate gene transcription. Very little is known about the identities of the genes that are regulated by β-catenin or the consequences of β-catenin-mediated gene transcription in fibrosis. One of the few β-catenin gene targets that has been identified is *IGFBP6*, encoding Insulin-like Growth Factor Binding Protein (IGFBP)-6 [145]. *IGFBP6* expression is reported to be transcriptionally downregulated by β-catenin in aggressive fibromatosis, and by unknown interactions in DD [145,146]. As β-catenin levels are increased in DD [44,108], these reports led us to hypothesize that β-catenin has a similar role in attenuating *IGFBP6* expression in DD.

1.12 IGFBP-6

IGFBP6 encodes IGFBP-6, one of the six Insulin-like Growth Factor Binding Proteins (IGFBP-1 to -6) that bind the peptide growth factors IGF-I and IGF-II with high affinity [147–149]. While many IGFBPs also have IGF-independent roles [150–153], a major function of these proteins is to regulate the availability of the IGFs to their primary signalling receptor, the type I IGF receptor (IGFIR) [147]. IGFBP-6 is the only IGFBP which displays a 20-100X preference for IGF-II over IGF-I [154–156], making it a relatively specific inhibitor of IGF-II signalling. Originally isolated from cerebrospinal fluid, where its levels are highest [157–159], IGFBP-6 is a 23 kDa protein in its native state, but 28-34 kDa when O-glycosylated *in vivo* [157]. IGFBP-6, like the other IGFBPs, consists of 3 distinct N, C and L domains, however it differs from other IGFBPs in lacking two otherwise conserved cysteine residues in the N-domain. The N and C domains are highly conserved between all IGFBPs, and the N domain contains the primary IGF binding site. However, the strong preferential binding affinity for IGF-II by IGFBP-6 is conferred by its C domain [154,155], a region that is also involved in binding glycosaminoglycans (GAGs) [160]. IGF-II binding affinity is decreased upon GAG binding due to overlap between the binding sites in the C domain. There are 5 O-glycosylation sites within the L domain of IGFBP-6 and its glycosylation confers stability

and protection from proteolysis [161]. Glycosylation also increases IGF-II binding affinity and inhibits GAG binding, rendering IGFBP-6 a very stable IGF-II signalling inhibitor [160,162].

1.13 *IGFBP6* gene characterization and regulation

The *IGFBP6* gene contains four exons spanning 4.7 kb and is found on the long arm of chromosome 12. The gene does not contain a TATA box or CAAT motifs, however basal promoter activity is evident within 158 bp of the transcriptional start site [163]. The *IGFBP6* promoter region has a high GC ratio, and its expression can be epigenetically downregulated by methylation of CpG islands [164]. Retinoic acid is a potent inducer of *IGFBP6* expression and secretion [157,165] and *IGFBP6* is considered a p53 responsive, pro-apoptotic gene in some cell types [166]. Cyclic AMP has been shown to decrease *IGFBP6* expression, and TGF-β [157,167], glucocorticoids, and estradiol have all been shown to affect IGFBP-6 secretion [157]. Other than a single report in aggressive fibromatosis implicating roles for β-catenin in the transcriptional downregulation of *IGFBP6* [145], very little is known about trans-regulation of its transcription.

1.14 IGFBP-6 in disease and fibrosis

IGFBP-6 has well documented roles as an inhibitor of cellular proliferation in numerous systems and cell types [168,169]. It has also been reported to induce apoptosis through various mechanisms including Ku80 binding [170] and activating early growth response-1 (*EGR1*) expression [168]. Additionally, IGFBP-6 has been shown to inhibit osteoblast and myoblast differentiation [156,171]. While most reports of IGFBP-6 actions are IGF-II dependent, IGF independent effects in promoting cell migration [172] and inhibiting angiogenesis [173] have been recently reported.

IGFBP-6 levels are dysregulated in many cancer systems [174–176] and increased in the cerebrospinal fluid of Alzheimer's patients [158]. The role of IGFBP-6 in fibrosis is not well characterized to date. *IGFBP6* mRNA has been detected in hepatic stellate cells, the precursors of myofibroblasts in liver fibrosis, however IGFBP-6 protein levels were undetectable based on an IGF-I ligand blot [177]. Aside from this study, all that appears

to be known about IGFBP-6 in fibrosis is that *IGFBP6* expression in aggressive fibromatosis and Dupuytren's Disease is suppressed [145,146]. As IGFBP-6 consistently inhibits cell proliferation and/or viability in other systems, downregulated *IGFBP6* expression and IGFBP-6 levels in fibroses would be predicted to enhance fibroblast proliferation through IGF-II dependent and/or IGF-II independent processes.

1.15 IGF-II and the *IGF2* gene

IGF-II is a 7.5 kDa peptide consisting of 67 amino acids [178], which signals through the Type I IGF Receptor to elicit effects on apoptosis, proliferation, differentiation, survival, invasion, migration and a host of other cell behaviours in a variety of systems [179–182]. The *IGF2* gene spans 30 kb on chromosome region 11p15, and contains 10 exons with the functional peptide only encoded by the last 3 exons [183]. *IGF2* transcription can be initiated from 4 distinct promoters, all encoding the same mature IGF-II peptide [183] (Figure 1-3). Additionally, *IGF2* is genomically imprinted and expressed only from the paternally-derived allele in most mammalian tissues [184].

While the role of IGF-II in fibrosis is poorly understood, IGF-II has been shown to induce collagen and fibronectin production in pulmonary fibrosis [185], and to induce myofibroblast differentiation in combination with TGF-β in murine fibroblasts [42]. Thus, increased IGF-II bioavailability would be predicted to contribute to the increased myofibroblast differentiation and collagen deposition established in Dupuytren's Disease. The possible role(s) of IGF-II in DD have not yet been elucidated.

1.16 Summary and rationale

Dupuytren's Disease is characterized by excessive TGF-β1 signalling, fibroblast proliferation, collagen deposition and an abundance of apoptosis-resistant myofibroblasts [61]. TGF-β1 signalling has been shown to increase β-catenin levels in DD [44] and to repress *IGFBP6* expression [157,167]. Additionally, β-catenin levels are upregulated while IGFBP-6 levels are downregulated in DD and aggressive fibromatoses [108,145,146]. β-catenin is reported to repress *IGFBP6* expression [145] and to enhance fibroblast proliferation by activating the transcription of proliferation-inducing target

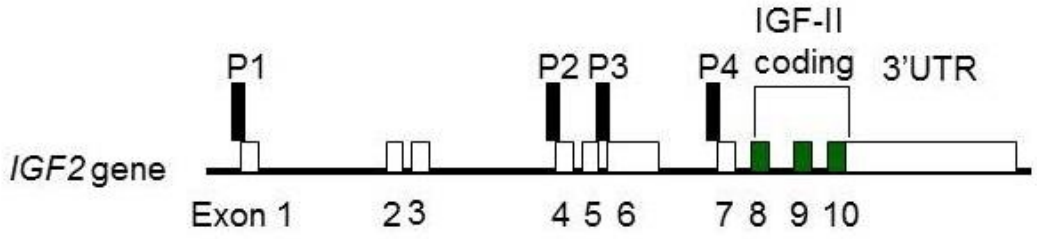

Figure 1-3: Simplified map of the human *IGF2* gene.

The human IGF2 gene consists of 10 exons with exons 8, 9 and 10 (shown in green) encoding mature IGF-II. Initiation of transcription can occur from any of 4 distinct promoters (P1-P4), the activity of which are both developmental stage- and tissue-specific.

genes [135]. Decreased IGFBP-6 levels are predicted to increase IGF-II signalling, and IGF-II signalling can cross-talk with TGF-β1 signalling to induce myofibroblast differentiation [42]. Based on these data, the central hypothesis of this thesis is that increased levels of endogenous TGF-β1 signalling induce β-catenin accumulation and signalling to repress the expression of *IGFBP6* and other genes, the dysregulation of which, promote DD development. The resulting decrease in IGFBP-6 levels will increase fibroblast proliferation and/or the differentiation of myofibroblasts through IGF-II dependent or independent mechanisms in DD.

1.17 Specific objectives of this thesis

The specific objectives of this thesis were:

1) To investigate the interactions between β-catenin, the promoter of *IGFBP6* and *IGFBP6* transcription in *in vitro* models of DD. These studies were subsequently expanded to identify all of the genes that interact with β-catenin during DD development.

2) To determine the functional consequences of restoring IGFBP-6 to normal physiological levels and of increasing the levels of IGF-II, the primary ligand of IGFBP-6, in *in vitro* models of DD.

3) To elucidate the mechanisms that dysregulate the expression of *IGF2*, encoding IGF-II, in *in vitro* models of DD.

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Chapter 2

2 Identification of β-catenin gene targets in Dupuytren's Disease patients

2.1 Introduction

β-catenin is a central mediator of the canonical Wnt signalling pathway that plays essential roles in embryonic development [1–3] and disease progression [4–8]. In the absence of Wnt stimulus, β-catenin is phosphorylated by glycogen synthase kinase (GSK)-3β, ubiquitinated and degraded by the 26S proteasome. This process prevents translocation of β-catenin to the nucleus, and the activity of T cell factor/ lymphoid enhancer factor (TCF/Lef) transcription factors are inhibited by Groucho/TLE co-repressors and the recruitment of histone deacetylases [9]. In the presence of Wnts, GSK-3β is phosphorylated and inactivated, allowing β-catenin to accumulate in the cytoplasm and subsequently translocate to the nucleus. Nuclear β-catenin can displace Groucho/TLE co-repressors, interact with TCF/Lef transcription factors and recruit transcriptional activators to its target genes [10].

β-catenin levels and associated trans-regulation of gene transcription can also be increased by factors other than Wnts. One of these factors is Transforming Growth Factor (TGF)-β [11–13], which can activate pathways that intersect with the canonical Wnt signalling pathway to stabilize β-catenin levels, promote its nuclear translocation, and elicit effects on gene transcription to enhance proliferation [13]. TGF-β-induced fibroblast proliferation in wound healing and fibrosis is dependent on cytoplasmic stabilization, accumulation and nuclear transport of β-catenin [14]. Increased β-catenin signalling and/or levels are reported in aggressive fibromatosis [12], Dupuytren's Disease [11,15], lung, kidney and liver fibroses [6,16,17], supporting the hypothesis that β-catenin plays a central role in the hyper-proliferation of fibroblasts that subsequently differentiate into myofibroblasts in these conditions.

Dupuytren's Disease (DD) is a benign and heritable fibrosis that is characterized by the formation of collagenous and contractile disease cords that result in permanent finger

contracture(s) [18,19]. At a cellular level, excessive TGF-β signalling and the abundance of apoptosis-resistant myofibroblasts are considered distinguishing features of DD, as they are of other fibroses [20–22]. The proliferation and differentiation of fibroblasts within, or in close proximity to, the tissue undergoing fibrosis is considered to be a major source of myofibroblasts in these conditions, and these processes are potently induced by TGF-β [23–25]. TGF-β has been shown to increase β-catenin accumulation in Dupuytren's Disease [15]; however the transcriptional consequences of the increased β-catenin levels are yet to be reported. TGF-β typically signals through SMADs to elicit its effects through the TGF-β receptors I and II. SMAD2 and 3 are phosphorylated upon stimulation with TGF-β, allowing their interaction with SMAD4 to facilitate the entry of SMADs 2 and 3 into the nucleus to regulate gene transcription. SMAD3 and β-catenin have been reported to act in concert to induce gene expression [26] and in chondrocytes, SMAD3 and SMAD4 can act to stabilize β-catenin levels to prevent its degradation and facilitate nuclear translocation [27]. β-catenin does not bind DNA [28,29], so it must interact with transcription factors to elicit its actions. In addition to TCF/Lef, β-catenin can interact with Forkhead box O (FOXO) transcription factors, which prevents transcription of β-catenin-TCF-Lef target genes [30]. TGF-β signalling can phosphorylate FOXO3 [31], which leads to its exclusion from the nucleus and the enhanced transcription of β-catenin-TCF/Lef target genes [30].

The β-catenin target genes identified to date have mostly been reported on an individual basis in cancer cell lines [32–35]. These gene targets include, amongst others, c-myc, cyclin D1 and epidermal growth factor receptor (EGFR) [32,34,36–38]. Advances in bioinformatics have made it feasible to simultaneously identify all of the genes in the human genome that associate with β-catenin by chromatin immunoprecipitation sequencing analyses (ChIP Seq). This approach has been used to identify genome-wide β-catenin associations in two independent studies on colon cancer cells, which were limited to a single tumour cell line in each case [38,39]. Approximately 2 200 β-catenin gene targets that could be regulated by interaction with either TCF/Lef or AP-1 sites were identified in one study, while the other study identified ~ 2 800 gene targets potentially regulated by TCF/Lef only [38,39]. The relevance of these findings to benign diseases is currently unclear.

Very few β-catenin gene targets have been previously identified in any fibrosis. One of them is *IGFBP6,* encoding Insulin-like Growth Factor Binding Protein (IGFBP)-6. *IGFBP6* expression was reported to be decreased in aggressive fibromatosis and to be correlated with β-catenin interactions with TCF3 [12]. Interestingly, *IGFBP6* expression is also significantly decreased in DD, and like aggressive fibromatosis, β-catenin levels are increased in DD [7,12,15,40]. These findings led to the hypothesis that β-catenin association with the *IGFBP6* promoter region is inversely correlated with its expression, and that *IGFBP6* was one of many other genes that might be differentially regulated by β-catenin associations during DD development. The aim of this study was to use *IGFBP6* as a model β-catenin target gene to gain a more detailed understanding of its transcriptional regulation in fibroblasts derived from fibrotic DD tissues (DD cells) and fibroblast from normal palmar fascia (CT cells). In addition, a third category of fibroblasts were derived from the phenotypically unaffected palmar fascia in a digit adjacent to DD. These fibroblasts (PF cells) are predicted to be genetically identical to the DD cells derived from the same patient, and to provide a uniquely powerful set of controls to identify β-catenin interactions with *IGFBP6* and other genes in patients that may be pre-disposed to DD development. Using these cells as comparators, multi-patient genome-wide ChIP Seq analyses were performed to identify all of the genes that are differentially associated with β-catenin in each group. The identification and characterization of the genes regulated by β-catenin is predicted to yield potential molecular targets to inhibit fibroblast proliferation and differentiation in DD and conditions with similar molecular characteristics.

2.2 Materials and Methods

2.2.1 Derivation of primary fibroblasts

Small palmar fascia tissue samples were resected from patients with Dupuytren's Disease (DD) and from patients undergoing carpal tunnel release (CT) during surgery at the Roth | McFarlane Hand and Upper Limb clinic with the approval from the University of Western Ontario Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB protocol # 08222E, Appendix C). Patient de-identification and confidentiality were achieved by assigning numbers to these samples prior to processing.

All patients received a letter of information and signed consent forms for their tissues to be used for research purposes. Primary fibroblasts were derived from the disease cord (DD cells), and from phenotypically normal adjacent palmar fascia of the same DD patient (PF cells), as genetically matched controls. Lastly, normal CT fibroblasts were derived from the palmar fascia of patients undergoing carpal tunnel release, with no prior history of Dupuytren's Disease (CT cells). Patient demographics for the cell lines used in these analyses are listed in Table 2-1.

Table 2-1: Patient demographics for cell lines used in the ChIP and ChIP Seq analyses

Patient	Cell Line	Gender	Age	Application
1	244PF/DD	M	30	ChIP, ChIP Seq
2	271PF/DD	M	58	ChIP, ChIP Seq
3	275PF/DD	M	91	ChIP, ChIP Seq
4	276PF/DD	M	45	ChIP
5	CT24	M	66	ChIP
6	CT25	F	44	ChIP
7	CT26	F	35	ChIP, ChIP Seq
8	CT27	F	48	ChIP, ChIP Seq
9	CT30	F	59	ChIP
10	CT31	M	78	ChIP, ChIP Seq

2.2.2 Chromatin Extraction

DD, PF and CT cells (N = 4) were grown to confluence in 15 – T175 flasks up to passage 3 in 20 ml α-minimal essential media (α-MEM). Chromatin immunoprecipitation was performed using the SimpleChIP Enzymatic Chromatin IP Kit as per the manufacturer's protocol and with minor optimization (Cell Signalling Technologies, Beverly, MA). Media was aspirated and cells were collected into a 50 ml conical tube and incubated with 1% formaldehyde in PBS solution for 10 min with rotation. Glycine was added to the solution to inactivate the formaldehyde crosslinking reaction for 5 min with rotation, after which it was centrifuged for 5 min at 2000 rpm (Hettich Zentrifugen Universal 32). The cell solution was washed twice with cold PBS for 5 min with rotation and then centrifuged. To lyse the membranes, cells were incubated in 10 ml Buffer A (proprietary

buffer included in the SimpleChIP Kit) for 10 min on ice with inversion every 3 min. The nuclei were pelleted at 3000rpm for 5 min at 4°C and resuspended in 10 ml Buffer B (proprietary buffer included in the SimpleChIP Kit) for 10 min on ice for lysis. The centrifugation was repeated and the pellet was then resuspended in 1 ml Buffer B prior to nuclease digestion and sonication.

The samples were incubated with 10 µl Micrococcal Nuclease (2000 gel units/µl) for 20 min at 37°C with frequent mixing to digest DNA to ~150-200 bp. The enzymatic reaction was halted by placing the sample on ice and adding 100 µl 0.05 M EDTA. The nuclei were pelleted at 13 000 rpm for 1 min at 4°C and the pellet was resuspended into 1 ml ChIP Buffer (proprietary buffer included in the SimpleChIP Kit) supplemented with protease inhibitors and phenylmethylsulfonyl fluoride (PMSF). The nuclear suspension was split into 2 x 500 µl samples and incubated on ice for 10 min. The crude chromatin extract was sonicated on ice twice per sample with the following settings: 1 sec pulse on, 1 sec pulse off, amplitude = 30, duration = 30 sec. Lysates were centrifuged at 10 000 rpm for 10 min at 4°C to obtain the chromatin preparation and pellets were discarded.

A small sample from the chromatin extraction was used to confirm DNA concentration and fragment sizes of 150-200 bp, after the DNA was purified on the spin columns included with the SimpleChIP Kit as per the manufacturer's protocol. The purified ChIP DNA was loaded in a 1% agarose gel to determine fragment size by electrophoresis. To determine DNA concentration, 1 µl of each sample was loaded onto the NanoDrop Spectrophotometer ND-1000 and quantified.

2.2.3 Immunoprecipitation

For each immunoprecipitation, 15-20 µg of chromatin was diluted into 500 µl samples in 1X ChIP Buffer supplemented with Protease Inhibitor Cocktail. Ten µg of rabbit monoclonal Histone H3 antibody, 10 µg of rabbit polyclonal β-catenin antibody, 10 µg rabbit polyclonal SMAD2/3 antibody (all from Cell Signalling Technologies, Beverly, MA), 4 µg mouse monoclonal RNA Polymerase II antibody (Upstate Biotechnology, Etobicoke, ON Canada), and 2.5 µg rabbit or mouse IgG were added to each tube and rotated overnight at 4°C. Thirty µl of ChIP grade Protein G Magnetic Beads were added

to each sample and rotated at 4°C for 2 hours. Immunoprecipitated (IP) samples were washed according to the manufacturer's protocol 3X in a low salt buffer wash, and once in a high salt buffer containing 5M NaCl. The supernatant was carefully removed and 1X ChIP Elution buffer was added to each IP sample prior to elution at 65°C for 30 minutes in a water bath with gentle mixing every 5 minutes. The magnetic beads were discarded and the supernatant was transferred to a new tube. Proteinase K and NaCl were added to each eluted chromatin sample and the inputs (containing no antibody) and incubated for 2 hours at 65°C. ChIP DNA was isolated according to the manufacturer's protocol in the supplied spin columns. Briefly, DNA Binding Buffer was added to each sample and transferred to a DNA spin column. Samples were centrifuged for 30 sec at 14 000 rpm and washed in DNA Wash Buffer and spun down again, discarding the liquid in the collection tube each time. Fifty µl of DNA Elution Buffer was added and spun at 14 000 rpm for 30 sec. Samples were stored at -20°C until further analysis. For ChIP Seq analysis specifically, the DNA from 3 individual IPs were isolated in one DNA spin column in order to achieve enough DNA for the library preparation.

2.2.4 Real Time PCR Analysis

Primers were designed to amplify regions of the *IGFBP6* promoter surrounding each of the TCF sites by real-time PCR after DNA isolation from the ChIP experiments. TCF1_F 5'-TGCTGACAATGAGGTTCGTAT-3' and TCF1_R 5'-GTTATGCAACAGGGACCATC-3' were used for the TCF1 site located at -1210 to -1204 from the start site in the *IGFBP6* promoter. For the TCF2 site, at -155 to -148, the following primers were used: TCF2_F 5'-CATACACACTAAGTGGATTGC-3', and TCF2_R 5'-CTCCCTTTCCAGTTTCTGTT-3'. The PCR procedure employed iQ SYBR Green Supermix chemistry (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and the following program was run: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, and 60°C for 1 min on the Bio-Rad CFX384 Real Time System.

2.2.5 Library Preparation

ChIP DNA was quantified using the Qubit Fluorometer High Sensitivity DNA assay (Invitrogen Corporation, Carlsbad, CA). Ten ng of DNA was required for the library

preparation using the NEBNext ChIP-Seq Library Prep Master Mix Set for Illumina (New England Biolabs) and the manufacturer's protocol was followed with size selection using AmpureXP Beads. Briefly, ChIP DNA was end-repaired, after which a series of deoxy-adenosines were added to the DNA. Adaptors compatible with the Illumina platform were purchased in a NEBNext Multiplex Oligos for Illumina kit (New England Biolabs) and ligated to the dA-tailed DNA. This ligated DNA-adaptor was enriched by PCR using the following program: Initial denaturation at 98°C for 30 sec, 15 cycles of denaturation at 98°C for 10 sec, annealing at 65°C for 30 sec, and extension at 72°C for 30 sec, and lastly, final extension at 72°C for 5 min. These library preparations were then sent to The Centre for Applied Genomics at the Sick Children's Hospital in Toronto for quality control and sequencing. One patient sample did not pass quality control analyses on the Bioanalyzer, and thus it was excluded from the sequencing run. Thus sequencing was performed once on 3 different primary cell lines per group (N = 3, n = 1, CT, PF and DD), corresponding to 6 different patients.

2.2.6 ChIP Sequencing Analysis

All libraries were sequenced on the Illumina HiSeq 2500 across 3 lanes, using v3 chemistry in a multiplex paired end protocol (2x100-bases). Base calling was performed with the Illumina pipeline 1.8.1. Raw sequence data files (referred to as reads) were merged and aligned to the hg19 human genome using a PERL script written in-house by Dr. Greg Gloor (See Appendix D). The aligned reads data were uploaded to the Galaxy Browser (http://usegalaxy.org) [41–43] where further downstream analysis was performed. Peaks were called using Model-based Analysis of ChIP Seq (MACS) v1.0.1 [44] using an MFOLD enrichment factor of 5, tag size = 100, bandwidth = 150 and $p < 0.05$ for forward and reverse read files separately for each sample. The intersect tool in the BEDtools package [45] available on Galaxy was used to retain only peaks common between forward and reverse datasets (ie. overlapping). From these, only those peaks that were common to all 3 patient samples within each group were retained as consistent β-catenin associations within the human genomes of that group. These peaks were then intersected with RefSeq files to determine their location within the genome. Peaks that fell within a gene region were mapped back to the RefSeq file using another PERL script

(See Appendix E) which isolated the RefSeq accession numbers from each association in order to obtain a gene identity using NCBI Batch Entrez.

2.2.7 Ingenuity Pathway Analysis

Networks, and disease and biological function associations between the genes isolated from the ChIP Seq analysis were generated with QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, www.qiagen.com/ingenuity). P values were determined using a right-tailed Fisher Exact test, which calculates the probability that the observed gene associations to each category are due to random chance. In general, $p < 0.05$ indicates a non-random association.

2.3 Results

2.3.1 Chromatin Immunoprecipitation (ChIP) Experiments

Increased β-catenin and decreased *IGFBP6* levels have been independently reported in Dupuytren's Disease (DD) and aggressive fibromatosis [12,15,40]. Denys *et al.* reported that the downregulated expression of *IGFBP6* in aggressive fibromatosis was due to β-catenin interactions with TCF3, a member of the TCF/Lef transcription factor family, in the *IGFBP6* promoter [12]. To determine whether β-catenin had a role in repressing *IGFBP6* transcription in DD, ChIP analyses were performed on untreated DD, PF and CT cells as described in the methods. Histone H3 and IgG antibodies were included as positive and negative controls, respectively, in addition to a no-antibody (input) control. DNA was isolated from the pull-downs and primers specific to each TCF binding site within the *IGFBP6* promoter were used in the real-time PCR analysis. Two consensus TCF binding sites have been reported within the *IGFBP6* promoter region; the site designated "TCF1" is located at -1210 to -1204bp and the "TCF2 site" is located from -155 to -148bp from the transcriptional start site of *IGFBP6* (Figure 2-1) [12]. As shown in Figure 2-2A, ChIP analyses revealed similar levels of β-catenin association with the TCF1 and TCF2 sites in the *IGFBP6* promoter of PF cells; however these differences were not significant ($p > 0.05$). While β-catenin interaction at the TCF2 site was detected in CT and PF cells, (compared to the TCF1 site where β-catenin association with *IGFBP6* was detected only in PF cells), there were no significant differences in the levels of

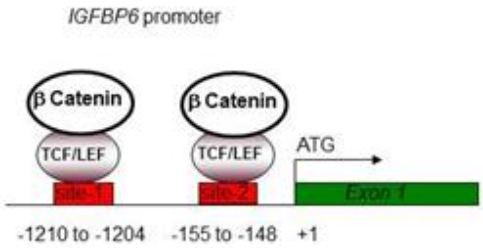

Figure 2-1: Locations of the TCF sites in the *IGFBP6* promoter region.

There are 2 TCF sites located within the *IGFBP6* promoter region as identified previously [12]. TCF1 is located at -1210 to -1204 and TCF2 is located at -155 to -148 from the ATG start site.

associations. β-catenin association with the TCF sites in the *IGFBP6* promoter was variable across the 4 PF/DD patients (Figure 2-3A & B). Assessed overall, β-catenin association with the *IGFBP6* promoter was lowest in DD cells relative to PF cells and CT cells, directly correlating with *IGFBP6* expression levels in these cells. Histone H3 was utilized as a positive control for these analyses and found to be highly associated with exon 3 of *RPL30* in DD, PF and CT cells (Figure 2-2B).

TGF-β1 treatment simultaneously represses *IGFBP6* levels and enhances cytoplasmic β-catenin accumulation in both DD and aggressive fibromatosis [11,12]. TGF-β signalling through SMADs can negatively regulate gene transcription [46,47] by recruiting histone methyltransferases or histone deacetylases to target genes [48,49]. As the *IGFBP6* promoter contains multiple SMAD binding elements (CAGA sequences) [26], it was of interest to determine if SMAD interactions with the *IGFBP6* promoter correlated with β-catenin interactions with this gene. Additional ChIP experiments were performed using a SMAD2/3 antibody to detect either SMAD2 or SMAD3 associations with the *IGFBP6* promoter. As shown in Figure 2-2C, little to no SMAD2/3 was shown to associate with the *IGFBP6* promoters of untreated DD cells at the TCF2 site and CT cells at the TCF1 site. SMAD2/3 association with the *IGFBP6* promoter was more detectable at the TCF1 site in PF/DD cells and in contrast, more detectable in the control PF and CT cells at the TCF2 site. These differences in the levels of association did not reach statistical significance. ChIP experiments were also performed with an RNA Polymerase II antibody to correlate RNA Polymerase II association with *IGFBP6* and *IGFBP6* expression in DD and PF cells. As *IGFBP6* lacks an obvious TATA box, primers were designed to span 250 bp upstream of the transcriptional start site based on a report of basal promoter activity being maintained within 153 bp from the ATG [50]. RNA Polymerase II was found to be more highly associated with the *IGFBP6* promoter of PF cells than with DD cells (Figure 2-2D), correlating with *IGFBP6* expression levels in these cells. PF cells, which are genetically identical to the DD cells, appear to have β-catenin, SMAD2/3 and RNA Polymerase II located within the *IGFBP6* promoter region, while little or no interactions of these proteins were evident in genetically identical, patient-matched DD cells.

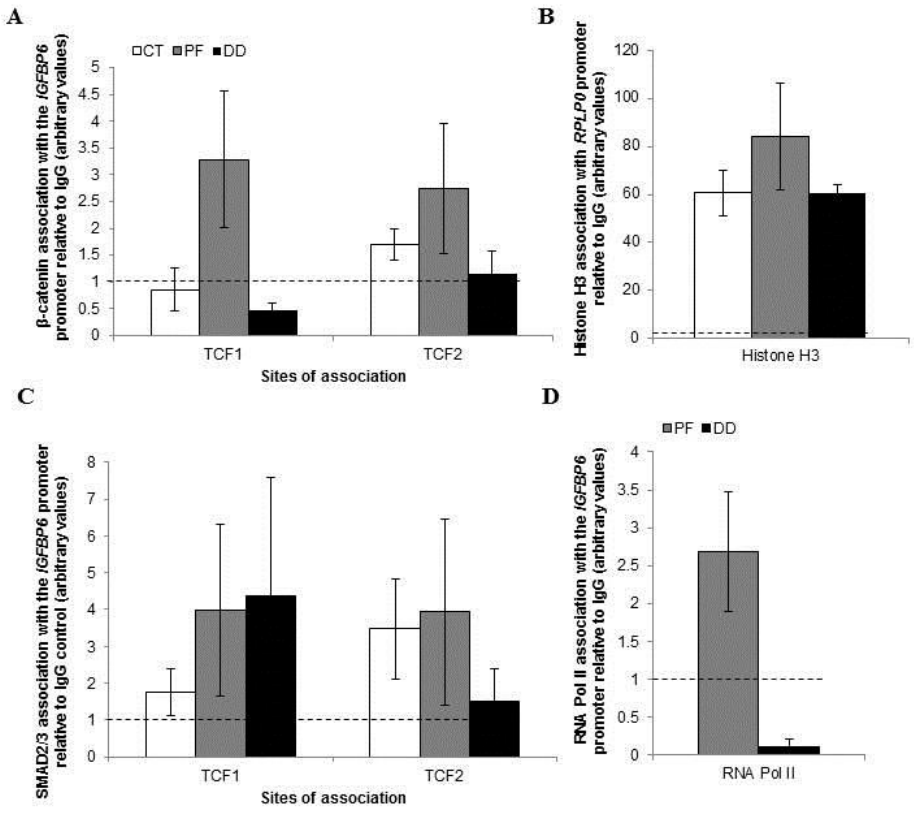

Figure 2-2: Association of A) β-catenin, B) Histone H3, C) SMAD2/3, and D) RNA Polymerase II in CT, PF and DD cells by chromatin immunoprecipitation.

Chromatin was extracted from primary CT (white bars), PF (grey bars) and DD (black bars) fibroblasts. Immunoprecipitations were performed using β-catenin (N = 4, n = 1), SMAD2/3 (N = 4, n = 1), and RNA Polymerase II (N = 2, n = 1) antibodies with Histone H3 (N = 2, n = 1) and species-specific IgG as a control. β-catenin and SMAD2/3 association with the *IGFBP6* promoter was examined at the 2 TCF sites (Figure 2-1). Histone H3 association was assessed within exon 3 of Human *RPL30*. Mean association was plotted normalized to IgG (dotted line, below 1 signifies background noise) with standard error of the mean.

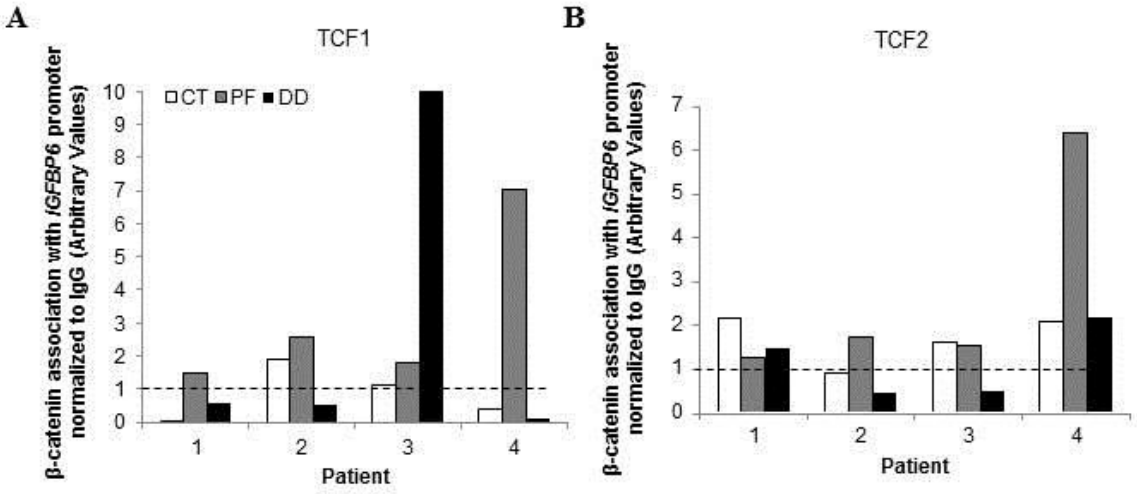

Figure 2-3: β-catenin association with the A) TCF1 and B) TCF2 sites in the *IGFBP6* promoter by patient.

ChIP experiments were performed to assess β-catenin association with the *IGFBP6* promoter region in CT (white bars), PF (grey bars) and DD (black bars) cells. β-catenin association with the *IGFBP6* promoter in each patient is plotted relative to rabbit IgG (dotted line) background at 1.

2.3.2 ChIP Seq preparation

Having confirmed that the levels of β-catenin association with the *IGFBP6* promoter varied between DD, PF and CT cells, ChIP Sequencing (ChIP Seq) studies were performed to identify all of the other β-catenin gene targets that varied between these groups. β-catenin and input ChIP samples were used in this analysis as recommended by technical reviews of this procedure [51]. In order to prepare the ChIP samples for sequencing, separate libraries were created for each DD, PF and CT sample (β-catenin IP and input samples) using commercially available kits compatible with the Illumina platform. Briefly, 10 ng of DNA for each sample was end repaired, dA-tailed and ligated to Illumina adaptors to facilitate complementarity for downstream sequencing and for PCR amplification. The quality of each DNA library was confirmed on a Bioanalyzer (N = 3 per group, done once n = 1) prior to paired-end multiplex (2x101 bp) sequencing on the Illumina HiSeq 2500. Approximately 40 million reads were obtained per sample in the forward and reverse directions, with approximately 80% of these reads aligning exactly once to the genome. These reads were aligned with the human hg19 genome and peaks were called using Model-based Analysis of ChIP Seq (MACS) [44].

2.3.3 Selecting peak calling parameters

To our knowledge, there are no previous reports of ChIP Seq analyses of trans-activators of transcription in samples derived from multiple patients. For this reason, it was necessary to carefully design *de novo* parameters for the peak calling portion of the workflow. As β-catenin association with *IGFBP6* in PF cells was previously confirmed by ChIP, these interactions were used as a baseline parameter in the ChIP Seq analysis. Peaks were identified in the *IGFBP6* promoter in 2 out of 3 PF samples from the forward analysis, and 1 out of 3 from the reverse analysis (Table 2-2) using a model-fold (MFOLD) enrichment factor of 5 and a p-value < 0.05, demonstrating a low overall enrichment in the study. Using these interactions as a baseline measure, these parameters were stringently applied in the final study to call only those overlapping peaks that appeared in all 3 patients and in both forward and reverse analyses.

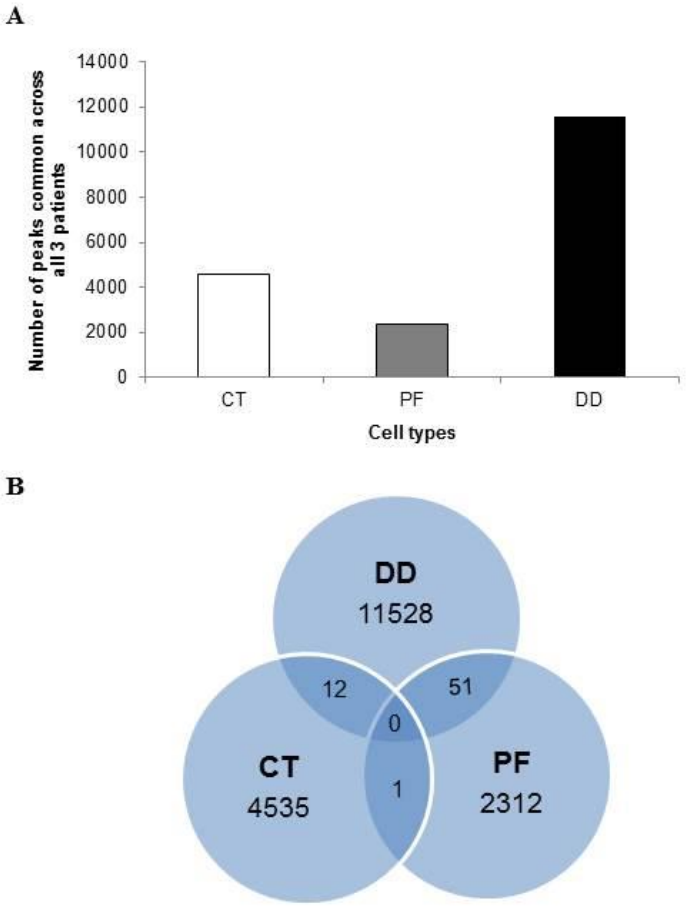

Figure 2-4: A) Peak numbers and B) overlaps identified during MACS analysis in CT, PF and DD cells.

Peak calling was performed using MACS, with an MFOLD enrichment = 5 and $p < 0.05$. A) Only peaks or β-catenin associations common between forward and reverse analyses and across all 3 primary cell lines per group (CT, PF and DD), corresponding to 6 different patients (PF and DD are from the same patient) were used in downstream ChIP Seq analysis. B) Peak overlaps were assessed between cell groups using the Intersect tool available with the BEDtools package in Galaxy.

Table 2-2: Peaks located within the IGFBP6 promoter region that correspond to the TCF2 site where β-catenin associates as identified in ChIP analysis

Patient	Forward or Reverse	Peak Start	Peak End	Position from *IGFBP6* gene*	P-value
244 PF	F	53 490 823	53 491 223	-397	0.0109
275 PF	F	53 490 730	53 491 112	-490	0.0365
244 PF	R	53 490 735	53 491 096	-485	0.0158

*Relative to *IGFBP6* gene position at chr12: 53 491 220

2.3.4 Peaks identified by MACS

MACS analysis identified an average of ~400 000 peaks in each CT sample, ~350 000 peaks in each PF sample and ~630 000 peaks in each DD sample. However, when both the forward and reverse analyses were assessed across all 3 patients in each group, only 1.13%, 0.68% and 1.84% of CT, PF and DD peaks respectively were retained for further analysis (Figure 2-4A). There were almost 5 times as many β-catenin associations with chromosomal regions of DD cells than with PF cells, and almost 2.5 times more associations with DD cells than with CT cells. No peaks were identified at this level of stringency that overlapped between all three cell types, in all 3 patients, in both forward and reverse analyses (Figure 2-4B). There was minimal peak overlap between the three cells types: 51 overlapping peaks were identified between DD and PF cells, 12 between CT and DD and only 1 between CT and PF.

Interestingly, only 32.4% of the peaks of β-catenin associations with the genomes of the DD samples, 33.5% of the PF samples, and 43.6% in CT samples fell within a gene or within 10 kb of a gene (Figure 2-5). The identities of the genes within 10 kb of the regions of β catenin association were obtained by mapping the peak locations back to the RefSeq Genebase and a selection are listed in Tables 2-3 (CT cells), 2-4 (PF cells) and 2-5 (DD cells). These analyses can be interpreted to indicate that more than 65% of the β-catenin associations in DD and PF cells and 55% of the β-catenin associations in CT cells were with regions of the genome where there are no known genes. The majority of the β-catenin associations with regions of the genome that contain protein encoding genes were

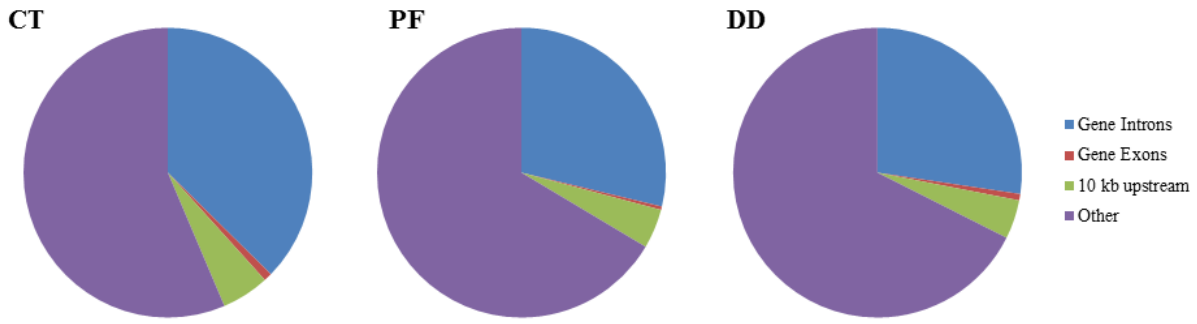

Figure 2-5: Peak locations within the human hg19 genome by cell type.

Overlapping peaks common across all patients were mapped back to the RefSeq genome to determine locations of peaks using the Intersect tool in BEDtools. β-catenin associations within 10 kb, introns or exons of a gene were identified. Most of the β-catenin associations are within regions with no known RefSeq genes.

Table 2-3: Select β-catenin associations within 10 kb of a gene in CT cells.

RefSeq Accession Number	Gene Symbol	Gene Description	Chromosomal Location
NC_000012.12	ABCD2	ATP-binding cassette, sub-family D (ALD), member 2	12q12
NC_000008.11	ADAMDEC1	ADAM-like, decysin 1	8p21.2
NC_000002.12	ADCY3	adenylate cyclase 3	2p23.3
NC_000005.10	ARHGEF28	Rho guanine nucleotide exchange factor (GEF) 28	5q13.2
NC_000017.11	CCL1	chemokine (C-C motif) ligand 1	17q12
NC_000003.12	CCR2	chemokine (C-C motif) receptor 2	3p21.31
NC_000012.12	CD163	CD163 molecule	12p13.3
NC_000003.12	COL6A5	collagen, type VI, alpha 5	3q22.1
NC_000007.14	EGFR	epidermal growth factor receptor	7p12
NC_000007.14	ELFN1	extracellular leucine-rich repeat and fibronectin type III domain containing 1	7p22.3
NC_000008.11	EPHX2	epoxide hydrolase 2, cytoplasmic	8p21
NC_000012.12	EPS8	epidermal growth factor receptor pathway substrate 8	12p12.3
NC_000001.11	FAM110D	family with sequence similarity 110, member D	1p36.11
NC_000011.10	FAT3	FAT atypical cadherin 3	11q14.3
NC_000014.9	FBLN5	fibulin 5	14q32.1
NC_000009.12	FCN1	ficolin (collagen/fibrinogen domain containing) 1	9q34
NC_000004.12	FNIP2	folliculin interacting protein 2	4q32.1
NC_000016.10	FOXL1	forkhead box L1	16q24
NC_000006.12	GPR110	G protein-coupled receptor 110	6p12.3
NC_000013.11	GPR183	G protein-coupled receptor 183	13q32.3
NC_000014.9	GSKIP	GSK3B interacting protein	14q32.2
NC_000001.11	HHAT	hedgehog acyltransferase	1q32
NC_000006.12	HIST1H1C	histone cluster 1, H1c	6p21.3

NC_000006.12	HLA-DMA	major histocompatibility complex, class II, DM alpha	6p21.3
NC_000006.12	HLA-DOB	major histocompatibility complex, class II, DO beta	6p21.3
NC_000001.11	IFI16	interferon, gamma-inducible protein 16	1q22
NC_000001.11	MACF1	microtubule-actin crosslinking factor 1	1p32-p31
NC_000015.10	MAP2K1	mitogen-activated protein kinase kinase 1	15q22.1-q22.33
NC_000001.11	MIR197	microRNA 197	1p13.3
NC_000004.12	MIR577	microRNA 577	4q26
NC_000017.11	MIR634	microRNA 634	17q24.2
NC_000019.10	MIR643	microRNA 643	19q13.41
NC_000009.12	MIR873	microRNA 873	9p21.1
NC_000020.11	MMP24	matrix metallopeptidase 24 (membrane-inserted)	20q11.2
NC_000006.12	NKAPL	NFKB activating protein-like	6p22.1
NC_000008.11	NRG1	neuregulin 1	8p12
NC_000005.10	PCDHGA4	protocadherin gamma subfamily A, 4	5q31
NC_000002.12	PDE11A	phosphodiesterase 11A	2q31.2
NC_000005.10	PDE4D	phosphodiesterase 4D, cAMP-specific	5q12
NC_000011.10	PGR	progesterone receptor	11q22-q23
NC_000014.9	PRKCH	protein kinase C, eta	14q23.1
NC_000001.11	PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	1p13.2
NC_000015.10	RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	15q14
NC_000014.9	RNASE9	ribonuclease, RNase A family, 9 (non-active)	14q11.2
NC_000001.11	S1PR1	sphingosine-1-phosphate receptor 1	1p21
NC_000018.10	TCEB3B	transcription elongation factor B polypeptide 3B (elongin A2)	18q21.1
NC_000002.12	TCF7L1	transcription factor 7-like 1 (T-cell specific, HMG-box)	2p11.2
NC_000006.12	TJAP1	tight junction associated protein 1 (peripheral)	6p21.1

NC_000022.11	TTLL12	tubulin tyrosine ligase-like family, member 12	22q13.31
NC_000013.11	TUBGCP3	tubulin, gamma complex associated protein 3	13q34
NC_000017.11	USP32	ubiquitin specific peptidase 32	17q23.3
NC_000017.11	VTN	Vitronectin	17q11
NC_000001.11	WNT3A	wingless-type MMTV integration site family, member 3A	1q42
NC_000022.11	WNT7B	wingless-type MMTV integration site family, member 7B	22q13

Table 2-4: Select β-catenin associations within 10 kb of a gene in PF cells.

RefSeq Accession Number	Gene Symbol	Gene Description	Chromosomal Location
NC_000001.11	ACTN2	actinin, alpha 2	1q42-q43
NC_000010.11	ANXA2P3	annexin A2 pseudogene 3	10q21.3
NC_000004.12	ARAP2	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	4p14
NC_000001.11	ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	1q31
NC_000020.11	CDH4	cadherin 4, type 1, R-cadherin (retinal)	20q13.3
NC_000020.11	DEFB119	defensin, beta 119	20q11.21
NC_000007.14	EGFR-AS1	EGFR antisense RNA 1	
NC_000001.11	ELAVL4	ELAV like neuron-specific RNA binding protein 4	1p34
NC_000022.11	FAM19A5	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5	22q13.32
NC_000001.11	FCRL5	Fc receptor-like 5	1q21
NC_000011.10	GAS2	growth arrest-specific 2	11p14.3
NC_000004.12	GNRHR	gonadotropin-releasing hormone receptor	4q21.2
NC_000003.12	GPR15	G protein-coupled receptor 15	3q11.2-q13.1
NC_000006.12	GPR63	G protein-coupled receptor 63	6q16.1-q16.3

NC_000011.10	GRIK4	glutamate receptor, ionotropic, kainate 4	11q22.3
NC_000003.12	GRM7	glutamate receptor, metabotropic 7	3p26.1-p25.1
NC_000003.12	KCNH8	potassium voltage-gated channel, subfamily H (eag-related), member 8	3p24.3
NC_000003.12	LINC00879	long intergenic non-protein coding RNA 879	3q11.2
NC_000004.12	LINC01098	long intergenic non-protein coding RNA 1098	4q34.3
NC_000016.10	LOC390705	protein phosphatase 2, regulatory subunit B", beta pseudogene	16p11.2
NC_000010.11	MBL2	mannose-binding lectin (protein C) 2, soluble	10q11.2
NC_000018.10	METTL4	methyltransferase like 4	18p11.32
NC_000003.12	MIR1324	microRNA 1324	
NC_000021.9	MIR155HG	MIR155 host gene (non-protein coding)	
NC_000012.12	MIR3612	microRNA 3612	
NC_000019.10	MIR371A	microRNA 371a	19q13.42
NC_000019.10	MIR372	microRNA 372	19q13.42
NC_000003.12	MIR3919	microRNA 3919	
NC_000009.12	MIR4290	microRNA 4290	
NC_000011.10	MRGPRX1	MAS-related GPR, member X1	11
NC_000002.12	NRXN1	neurexin 1	2p16.3
NC_000011.10	OR10G7	olfactory receptor, family 10, subfamily G, member 7	11q24.2
NC_000012.12	PDE1B	phosphodiesterase 1B, calmodulin-dependent	12q13
NC_000004.12	PGRMC2	progesterone receptor membrane component 2	4q26
NC_000001.11	PPIH	peptidylprolyl isomerase H (cyclophilin H)	1p34.1
NC_000013.11	PRR20A	proline rich 20A	13q21.1
NC_000010.11	PTPRE	protein tyrosine phosphatase, receptor type, E	10q26
NC_000008.11	REXO1L2P	REX1, RNA exonuclease 1 homolog (S. cerevisiae)-like 2 (pseudogene)	8q21.2
NC_000017.11	RHOT1	ras homolog family member T1	17q11.2

NC_000008.11	RIMS2	regulating synaptic membrane exocytosis 2	8q22.3
NC_000001.11	S100A9	S100 calcium binding protein A9	1q21
NC_000011.10	SCN3B	sodium channel, voltage-gated, type III, beta subunit	11q23.3
NC_000015.10	SEMA7A	semaphorin 7A, GPI membrane anchor (John Milton Hagen blood group)	15q22.3-q23
NC_000011.10	SPON1	spondin 1, extracellular matrix protein	11p15.2
NC_000018.10	TCEB3CL	transcription elongation factor B polypeptide 3C-like	18q21.1
NC_000018.10	TCEB3CL2	transcription elongation factor B polypeptide 3C-like 2	18q21.1
NC_000013.11	TPTE2	transmembrane phosphoinositide 3-phosphatase and tensin homolog 2	13q12.11
NC_000004.12	USP17L30	ubiquitin specific peptidase 17-like family member 30	4p16.1
NC_000015.10	WDR72	WD repeat domain 72	15q21.3
NC_000019.10	ZNF285	zinc finger protein 285	

Table 2-5: Select β-catenin associations within 10 kb of a gene in DD cells.

RefSeq Accession Number	Gene Symbol	Gene Description	Chromosomal Location
NC_000007.14	ADAM22	ADAM metallopeptidase domain 22	7q21
NC_000002.12	APOB	apolipoprotein B	2p24-p23
NC_000017.11	CCL23	chemokine (C-C motif) ligand 23	17q12
NC_000001.11	CD1C	CD1c molecule	1q23.1
NC_000019.10	CD33	CD33 molecule	19q13.3
NC_000001.11	CD58	CD58 molecule	1p13
NC_000001.11	CD84	CD84 molecule	1q24

NC_000003.12	CD86	CD86 molecule	3q21
NC_000003.12	CNTN4	contactin 4	3p26.3
NC_000004.12	CXCL13	chemokine (C-X-C motif) ligand 13	4q21
NC_000008.11	DEFB105A	defensin, beta 105A	8p23.1
NC_000019.10	FCGBP	Fc fragment of IgG binding protein	19q13.1
NC_000012.12	FGF6	fibroblast growth factor 6	12p13
NC_000005.10	FGFR4	fibroblast growth factor receptor 4	5q35.2
NC_000002.10	FSIP2	fibrous sheath interacting protein 2	2q32.1
NC_000004.12	GABRG1	gamma-aminobutyric acid (GABA) A receptor, gamma 1	4p12
NC_000001.11	GBP7	guanylate binding protein 7	1p22.2
NC_000005.10	GHR	growth hormone receptor	5p13-p12
NC_000020.11	GHRH	growth hormone releasing hormone	20q11.2
NC_000001.11	GJA5	gap junction protein, alpha 5, 40kDa	1q21.1
NC_000001.11	GJA8	gap junction protein, alpha 8, 50kDa	1q21.1
NC_000001.11	GJB5	gap junction protein, beta 5, 31.1kDa	1p35.1
NC_000006.12	GPR63	G protein-coupled receptor 63	6q16.1-q16.3
NC_000006.12	GPRC6A	G protein-coupled receptor, class C, group 6, member A	6q22.1
NC_000021.9	GRIK1	glutamate receptor, ionotropic, kainate 1	21q22.11
NC_000002.12	ICA1L	islet cell autoantigen 1,69kDa-like	2q33.2
NC_000009.12	IFNA10	interferon, alpha 10	9p22

NC_000009.12	IFNA7	interferon, alpha 7	9p22
NC_000004.12	IL2	interleukin 2	4q26-q27
NC_000016.10	ITGAX	integrin, alpha X (complement component 3 receptor 4 subunit)	16p11.2
NC_000009.12	LOC642929	general transcription factor II, i pseudogene	9p11.2
NC_000018.10	MIR187	microRNA 187	18q12.2
NC_000013.11	MIR320D1	microRNA 320d-1	13q14.11
NC_000002.12	MIR548F2	microRNA 548f-2	2q34
NC_000020.11	MIR646HG	MIR646 host gene (non-protein coding)	20q13.33
NC_000013.11	MTUS2	microtubule associated tumor suppressor candidate 2	13q12.3
NC_000006.12	NKAIN2	Na+/K+ transporting ATPase interacting 2	6q21
NC_000011.10	OR4C45	olfactory receptor, family 4, subfamily C, member 45	11p11.12
NC_000011.10	OR51I1	olfactory receptor, family 51, subfamily I, member 1	11p15.4
NC_000003.12	OR5H1	olfactory receptor, family 5, subfamily H, member 1	3q11.2
NC_000006.12	PKIB	protein kinase (cAMP-dependent, catalytic) inhibitor beta	6q22.31
NC_000008.11	POTEA	POTE ankyrin domain family, member A	8p11.1
NC_000005.10	PPP2R2B	protein phosphatase 2, regulatory subunit B, beta	5q32
NC_000013.11	PRR20A	proline rich 20A	13q21.1
NC_000001.11	S100A7A	S100 calcium binding protein A7A	1q21.3

NC_000015.10	SNORD109A	small nucleolar RNA, C/D box 109A	15q11.2
NC_000015.10	SNORD116-17	small nucleolar RNA, C/D box 116-17	15q11.2
NC_000002.12	SOX11	SRY (sex determining region Y)-box 11	2p25
NC_000018.10	TCEB3CL	transcription elongation factor B polypeptide 3C-like	18q21.1
NC_000018.10	TCEB3CL2	transcription elongation factor B polypeptide 3C-like 2	18q21.1
NC_000019.10	TPM4	tropomyosin 4	19p13.1
NC_000004.12	USP17L13	ubiquitin specific peptidase 17-like family member 13	4p16.1
NC_000004.12	USP17L15	ubiquitin specific peptidase 17-like family member 15	4p16.1
NC_000004.12	USP17L21	ubiquitin specific peptidase 17-like family member 21	4p16.1
NC_000004.12	USP17L30	ubiquitin specific peptidase 17-like family member 30	4p16.1

located within the intronic regions of those genes rather than their promoters or other locations.

2.3.5 Vertical analyses: β-catenin associations within each patient

Each set of PF and DD cells derived from the same patient are predicted to be genetically identical, as Dupuytren's Disease is a benign fibromatosis and any germline mutations that predispose patients to DD are predicted to be evident in fibroblasts irrespective of their disease state. Therefore it was appropriate to perform vertical analyses of the peaks isolated from each matching PF and DD sample for each of the 3 patients. The amount of

overlap within each patient cell line differed, further complicating the ability to derive common features from the analyses. Specifically, patients # 244, 271 and 275 displayed 4.3%, 16.1% and 8.7% overlap between β-catenin associations in PF and DD cells respectively. When all 3 DD cell lines were assessed relative to their corresponding PF cells, there were 8 805 regions of β-catenin association in all 3 DD patients, but not their corresponding PF samples, which translated to 2 792 gene associations within genes or within 10 kb upstream of a gene. There were 1 544 regions or 547 genes where β-catenin associated within a gene or within 10 kb upstream of a gene that were specific to all PF cells assessed relative to all DD cells assessed. From these, 1 438 and 497 unique genes were identified in DD and PF cells, respectively. Only 51 regions of β-catenin association were common between PF and DD cells. A selection of the genes identified from the β-catenin associations within intronic regions from these analyses are listed in Appendices F and G.

Table 2-6: Separating β-catenin associations according to presence within PF cells, DD cells or both, within each patient.

Patient#	Both DD and PF	PF only	DD only
#244	48 388	250 000	810 000
#271	250 000	500 000	800 000
#275	59 753	310 000	320 000

2.3.6 Ingenuity Pathway Analysis of β-catenin associations within 10 kb of a gene

To determine the networks, diseases and signalling pathways associated with the genes that had β-catenin associations within 10 kb of their location, Ingenuity Core Analyses were conducted on the complete lists of genes used to generate Tables 2-3, 2-4 and 2-5. The top disease and biological function association for all 3 cell types was identified as “cancer” (Figure 2-6). Other prominent disease and biological function associations in PF and CT cells were “cell growth”, “proliferation”, “morphology” and “development”.

Interestingly, the genes identified in DD cells were mostly categorized as contributing to pathological, rather than normal or developmental, processes.

2.4 Discussion

β-catenin was shown to associate with the *IGFBP6* promoter in both PF and CT cells, while little to no association of β-catenin with *IGFBP6* was evident in DD cells. These data suggest that there is a direct correlation between β-catenin association with the *IGFBP6* promoter and *IGFBP6* mRNA levels in DD, PF and CT cells, and that loss of β-catenin at the *IGFBP6* promoter leads to its transcriptional downregulation. This interpretation is consistent with RNA Polymerase II association with the *IGFBP6* promoters in PF cells, but not DD cells, despite these cells being derived from the same hand of the same patient. Additional patients would have to be assessed to confirm β-catenin association levels with the *IGFBP6* promoter, as the results in this study did not achieve statistical significance, likely due to the small patient sample size. Experiments confirming whether β-catenin is an absolute requirement for *IGFBP6* expression in DD will be a focus of future studies.

These findings are in direct contrast to a previous report in aggressive fibromatosis indicating an inverse correlation between β-catenin association and *IGFBP6* transcription [12]. As PF cells are derived from the non-fibrotic palmar fascia of patients with DD, they might represent "pre-DD" fibroblasts that are somewhere between normal and diseased at the level of β-catenin interactions with target genes. Changes in β-catenin association with *IGFBP6* or other genes between CT, PF and DD cells may provide insights into the processes that lead to the eventual downregulation of *IGFBP6* transcription in DD cells. TGF-β can further repress *IGFBP6* expression in DD and PF cells, and future studies will focus on elucidating its ability to regulate β-catenin and RNA Polymerase II association with the *IGFBP6* promoter.

The association of SMAD2/3 with the *IGFBP6* promoter region demonstrated in this report may provide some insight into the possible roles of TGF-β in this process. SMADs may repress gene transcription by recruiting histone methyltransferases or histone

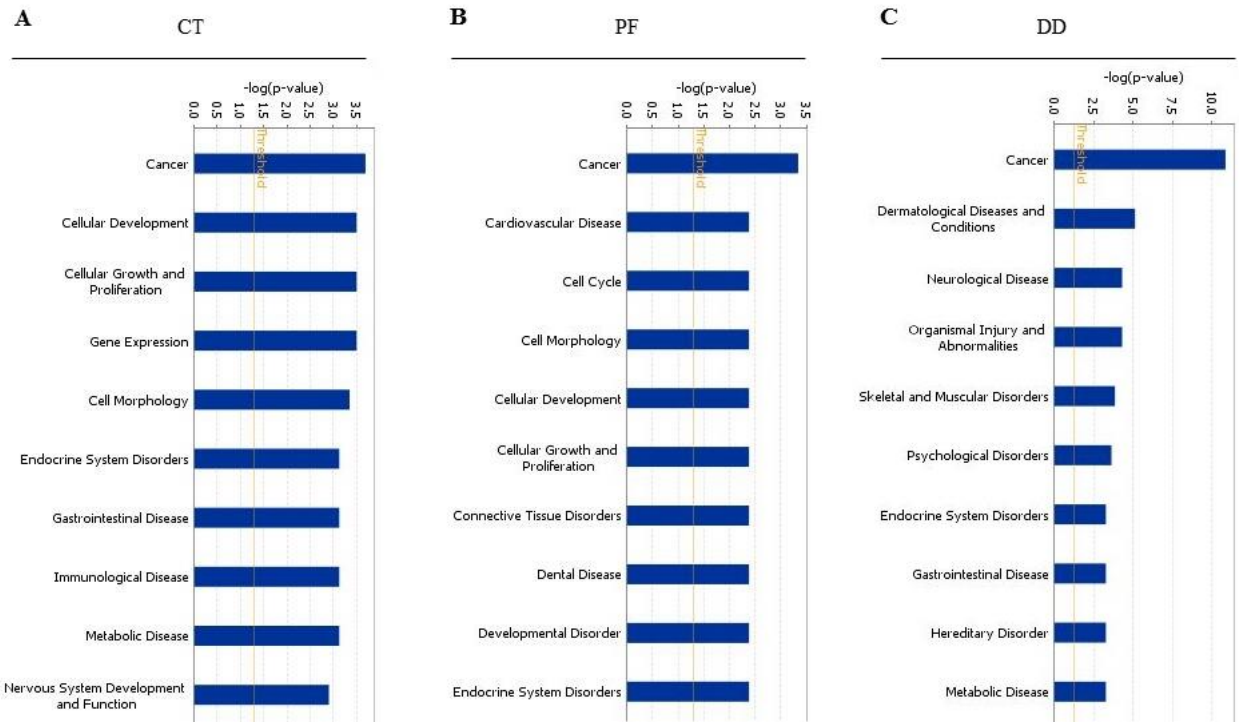

Figure 2-6: Disease and biological function categories determined by Ingenuity Pathway Core Analysis of β-catenin associated genes of CT, PF and DD cells.

Genes identified from the β-catenin associations within 10 kb of a gene were input into IPA (QIAGEN, Redwood City, www.qiagen.com/ingenuity) to determine indirect and direct relationships between the genes. These genes were then allocated to specific biological function or disease categories using a right-tailed Fisher's Exact statistical test to determine a threshold for significance.

deacetylases to target genes, and the recruitment of histone deacetylases by TCF/Lef factors to inhibit transcription has been previously documented [9,48,49]. Preliminary studies in our laboratory using chaetocin, a histone methyltransferase inhibitor [52], indicated that treatment markedly induced *IGFBP6* expression in DD and PF cells, but only mildly in CT cells, consistent with differing levels of histone methylation at the *IGFBP6* locus in these cells.

Genome-wide associations of β-catenin were identified in this study by ChIP sequencing across the genomes of 9 different cell lines derived from 6 different patients. This is the first study, to our knowledge, to report β-catenin associations with genes in human cells derived from multiple patients with any fibrosis, or indeed for any disease of any kind. The β-catenin gene associations identified in the genomes of PF and DD cells may provide insight into the processes that drive Dupuytren's Disease progression. During the vertical analyses, the overlapping regions between PF and DD cells were removed, such that the peaks that remained were specifically associated in PF cells or in DD cells. Identical genes within these DD and PF lists are present because β-catenin is associating with different regions of the gene in these 2 cell types, which could be due to β-catenin transactivation of different transcription factors or the same transcription factor binding another area. In addition, overlap was evident between β-catenin association with genes encoding related, but different factors in DD, PF and CT cells. For example, while *FGF14* was identified as a β-catenin associated gene in all 3 cell types, *FGF12* was specifically identified in DD cells and CT cells, while *FGF10* was identified only in CT cells. Interestingly, *FGF10* expression has been shown to attenuate fibrosis in mice [53], which would suggest that β-catenin is required for its expression in CT cells, and that it may be transcriptionally downregulated in DD in a similar manner to *IGFBP6*. Different fibroblast growth factors may have distinct temporal roles in the fibroproliferation in DD and each may contribute uniquely to disease progression. The potential for different FGFs to perform distinct functions is yet to be explored in DD. Interestingly, β-catenin associated with many different genes encoding cadherins in DD cells, while β-catenin associated more often with genes encoding protocadherins in PF and CT cells (See Appendices F and G). Additionally, β-catenin associations with numerous collagen genes were identified in these analyses, supporting previous suggestions that β-catenin may

have a role in regulating collagen expression [54–56]. As excess collagen deposition is characteristic of essentially all fibroses including Dupuytren's Disease, it is feasible that β-catenin may transactivate expression of these genes. Previous microarray data reports for DD indicate that *COL18A1* and *COL6A1* are upregulated in DD nodule tissue relative to external control tissue [40], consistent with the hypothesis that β-catenin might promote the transcriptional activation of these collagen genes.

β-catenin has been reported to associate with a variety of genes in many cell types, the majority of which were derived from cancers or other diseased tissues. This is the first study to our knowledge to identify the genes that β-catenin associates with in normal (in this case, palmar fascia-derived) fibroblasts. CT cells were shown to exhibit intermediate levels of β-catenin association with various genes relative to the levels of interactions evident in DD and PF cells. Remarkably, no overlapping peaks of β-catenin association were identified in all 3 cell types. This implies that β-catenin association with genes is distinct in normal palmar fascia fibroblasts derived from patients without DD, and normal palmar fascia fibroblasts derived from patients with DD in an adjacent digit. These findings may suggest that the development of DD is incremental, and that normal fibroblasts (CT cells) progress to fibroblasts that are "primed" for disease development (PF cells) to diseased fibroblasts (DD cells). β-catenin interactions may contribute to this intricate process by trans-regulating gene expression in a specific manner. The single peak overlap between PF and CT cells suggests that it might be possible to identify genetic biomarkers that distinguish an individual who is genetically predisposed to disease development from an individual who is not.

The majority of β-catenin gene associations identified in this study were localized within intronic regions. Introns have been shown to regulate gene expression in a variety of systems [57], and in some cases may have more influence on gene expression than the promoter [58]. This has been definitively demonstrated for *Arabidopsis* profilin genes, where swapping intron 1 of vegetative profilin-2 with intron 1 of reproductive profilin-5 led profilin-5 to express a more vegetative profilin-2 pattern [58]. These findings have contributed to the acceptance of introns playing more important roles in mediating gene expression in humans than was previously thought [57]. For example, *IGFBP6*

expression can be induced by retinoid X receptor activation of an AP-1 site within its first intron [59] and Ubiquitin C expression can be enhanced by Yin Yang 1 trans-activation within its first intron [60]. Interestingly, when this intronic region is moved upstream of the promoter, Yin Yang 1 can no longer affect Ubiquitin C expression, indicating a spatial dependence for intron-mediated enhancement of transcription. Additionally, removal of regulatory regions for splicing can diminish gene expression, demonstrating that splicing is also required for optimal gene expression [60]. As such, intronic regions also function as areas where splicing is regulated, and this may be another reason for β-catenin association within these regions. While most of the β-catenin gene associations were within intronic regions, more than 50% were located in regions of the genome with no known genes, the significance of which is currently unknown.

The Ingenuity pathway analysis provided further insight into the types of genes being regulated by DD, PF and CT cells. In all 3 cell types, cancer was the top disease/biological function category which was identified based on the β-catenin gene associations. While these gene associations may truly reflect a similar disease mechanism between DD and cancer, this can also be the result of the bias in the literature towards cancer research and development, as IPA creates its categories based on the research available in multiple databases. Nonetheless, associations between DD and cancer development have been identified in a few studies [61,62]. Interestingly, despite the minimal overlap in peaks between PF and CT cells, the biological function categories determined from the gene lists were similar, identifying cell proliferation, morphology and development as significant, which confirms previous reports listing β-catenin as a mediator of cell proliferation [7,30,63]. In contrast, the top 10 categories in DD cells were all pathological processes, supporting the concept that molecular therapies must achieve normal homeostasis in the palmar fascia, rather than just the absence of disease, to prevent DD progression.

While many previous studies have identified specific TCF/Lef targets, only 2 studies in human colon cancer cells have looked specifically for β-catenin gene targets throughout the human genome [38,39]. In HCT116 cells, 2 168 β-catenin gene targets were identified, while 2 794 were identified in SW480 cells, both of which are comparable to

the 2 792 β-catenin gene targets identified in this study of DD cells [38,39]. While there was little overlap between the genes identified in SW480 and DD cells, this could be due to the adenomatous polyposis coli mutation in SW480 cells which stabilizes β-catenin, and may in turn affect β-catenin-mediated gene transcription. Surprisingly, there were only 168 genes which overlapped between the two studies on HCT116 and SW480 cells [39], which may be the result of the additional transcription factor, AP-1 possibly playing a role in mediating β-catenin effects on transcription in HCT116 cells [38], but not SW480 cells. Many Wnt genes were identified as β-catenin gene targets in SW480 cells [39]. In the current study, Wnt-associated genes were identified mostly in CT cells, which might suggest that the Wnt pathway is turned off in DD cells, since our lab was unable to detect any consistent changes in Wnt gene expression in DD cells relative to non-fibrotic cells [64]. Lack of Wnt involvement in DD may also be the result of SNPs located within Wnt genes, as identified in a genome wide association study of DD cells where 6/11 SNPs were reportedly within Wnt genes. Based on these data, it was hypothesized that SNPs in Wnt genes may pre-dispose patients to DD [65].

In contrast, considerable overlap was evident between the HCT116 study and this study in terms of the gene families that were identified, although the specific members of those gene families differed. For example, *ADAM 12, ADAM19* and *ADAMTS16* were identified as β-catenin gene targets in colon cancer while *ADAMs 2, 5, 18, 19, 22, 29, 32, TS16, TS18, TS19* and *TS20* were identified as β-catenin gene targets in DD. ADAM12 is a highly up-regulated gene in DD [40,66] where it is believed to function as an adhesion molecule and an IGFBP protease [67]. It is currently unclear if the up-regulated expression of *ADAM12* in colon cancer and DD is the result of distinct mechanisms, or if the association between β-catenin and *ADAM12* in DD was excluded due to the stringency of peak calling parameters implemented that required peaks to be identified in all 3 patients in forward and reverse analyses.

Currently, there is a lack of standards and guidelines for assessing ChIP sequencing data derived from multiple patients. Many algorithms for analyzing multiple datasets focus on integrating ChIP Seq studies performed by various groups [68–70]. Unfortunately, peak calling can be achieved through multiple approaches that can vary between analyses,

adding an additional layer of complexity to an integrative analysis of multiple studies. In this study, each patient was assessed individually and the data was compared after peak calling was complete. Between-patient variability made this a very difficult and complex process, as each patient displayed different levels of β-catenin association within their genome despite beginning the analyses with similar numbers of reads. β-catenin association was much higher in the DD cells derived from two of the three DD patients assessed, while the levels of β-catenin association was comparable between the PF and DD cells derived from the third patient (Table 2-6). A recent publication identified jMOSAiCS (joint model-based one- and two-sample analysis and inference for ChIP) as a new ChIP Seq software specifically designed to analyze multiple datasets by controlling for the different amount of enrichment between samples [71]. It has also been optimized to work for trans-acting transcriptional regulators, such as β-catenin. Current algorithms are optimized either for *cis*-acting transcription factors or for histone modifications, as the nature of the peaks derived from each analysis differ (sharp peaks for transcription factor interactions versus broad peaks for histone marks) [72]. It is unclear if a *trans*-acting factor like β-catenin would be expected to generate sharp or broad peaks, making it difficult to select an optimal program. While jMOSAiCs does seem to address many issues that are faced by researchers analyzing ChIP Seq data derived from clinical isolates, it is best equipped to handle two different datasets, making it less than ideal for this study.

A limitation of the current study is that β-catenin enrichment was low, which made it difficult to identify significant peaks over background input "noise". Increasing DNA concentration, and/or testing more β-catenin antibodies for use with ChIP Seq would be required to optimize the enrichment [73]. It is important to note that this study was limited to identifying significant levels of association between β-catenin and the human genomes derived from the various cell types. These analyses did not address the consequences of these interactions in terms of gene transcription. Additional studies will be required to independently confirm the associations reported in this analysis and to test the effects of β-catenin association with the expression of specific candidate genes of

interest. Confirmatory ChIP and the corresponding PCR analyses for a selection of the genes identified in this study will be the focus of future studies.

In summary, β-catenin-mediated trans-regulation of gene transcription is likely to contribute to the development of Dupuytren's Disease, as it does many other diseases [7,74]. *IGFBP6* was confirmed as a target of β-catenin interactions in PF and CT cells, which display relatively robust *IGFBP6* expression, whereas this association was attenuated in DD cells. The lack of β-catenin interactions with the *IGFBP6* promoter in DD cells is likely to contribute to the down-regulated *IGFBP6* expression in these cells. A large number of additional β-catenin target genes were identified that were specific to DD cells, and detailed analyses of their transcriptional regulation are likely to provide novel insights into their contribution to fibrosis development. These genes may also have potential as biomarkers to distinguish individuals predisposed to developing DD from the non-predisposed population. Chapter 3 will focus on elucidating the functional consequences of IGFBP-6 downregulation and subsequent increased IGF-II signalling in DD.

2.5 References

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Chapter 3

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3 IGF-II and IGFBP-6 regulate cellular contractility and proliferation in Dupuytren's Disease

3.1 Introduction

Dupuytren's Disease (DD) is a debilitating condition of the hand [1,2] characterized by the formation of collagenous cords in the palmar and/or digital fascia and permanent finger contractures. Despite being amongst the most common inherited connective tissue diseases [1–4], the etiopathology of DD has remained elusive since its description in the 1830s [5]. While microsurgical excision [6] of the contractile cord tissue can temporarily restore dexterity, this and other treatment approaches fail to prevent disease recurrence in more than 30% of patients [7–10]. There is a clear need for a better understanding of the molecular pathology of DD to achieve more effective therapeutic approaches.

Due to the unique physiology of the palmar fascia and the lack of understanding of DD at a molecular level, there are no established animal or immortalized cell models in which to study DD development. We take the approach of comparing primary fibroblasts derived from surgically resected DD contracture (cord) tissue (DD cells) to fibroblasts derived from the palmar fascia of the adjacent, phenotypically unaffected digit exposed during surgery (PF cells). While DD may be associated with increased cancer mortality in some populations [11,12], patients with a family history of finger contractures do not display any major chromosomal rearrangements or deletions [13] and DD is considered a benign, heritable fibrosis [4]. Single nucleotide polymorphisms (SNPs) have been identified as potential markers of this heritability [14], and these polymorphisms are predicted to be present in all somatic tissues in these patients rather than limited to diseased tissues. PF cells can therefore be viewed as genetically matched "latent disease" cells that carry the same predisposing SNPs as DD cells, making them the ideal controls

for studies of the molecular mechanisms that promote disease development in this population. In addition, we derive a second control group of palmar fascia fibroblasts (CT cells) from patients with no history of DD who are undergoing surgeries for unrelated reasons, such as carpal tunnel release. We consider CT cells to be normal controls and useful comparators to patient matched PF/DD cells in studies designed to identify characteristics that are specific to cells predisposed to DD development [15,16].

Using these unique cell models, we have focused on identifying molecules that regulate the proliferation and differentiation of palmar fascia fibroblasts into myofibroblasts, the hyper-contractile cell type that remodels the palmar fascia to induce finger contractures in DD. Several research groups have previously reported gene expression analyses of DD tissues or cells with the aim of identifying dysregulated genes with potential roles in myofibroblast development [13,16–20]. Our previous studies have focused on TGF-β1, encoding transforming growth factor-β1 (TGF-β1) and TGF-β-induced genes such as periostin [21]. In parallel, we and others [19] have identified *IGFBP6* as a significantly downregulated transcript in DD tissue. The relevance of downregulated *IGFBP6* expression to the proliferation and differentiation of palmar fascia fibroblasts into DD myofibroblasts has not been previously explored.

IGFBP6 encodes insulin-like growth factor binding protein (IGFBP)-6, one of a family of six secreted proteins that bind insulin-like growth factors (IGFs)-I and -II with high affinity and regulate their bioavailability [22]. While most IGFBPs can regulate the availability of both IGF-I and IGF-II under normal physiological conditions, IGFBP-6 is unique in displaying a 50 fold higher affinity for IGF-II [23,24], identifying it as an IGF-II-specific binding protein under normal physiological conditions [23].

In this study, we demonstrate that *IGFBP6* mRNA and IGFBP-6 protein levels are downregulated components of DD cells, and that *IGF2* mRNA and IGF-II peptide levels are upregulated in DD cells relative to controls. Recombinant IGFBP-6 was found to attenuate the proliferation of DD, PF and CT cells, and co-treatment with IGF-II was ineffective at neutralizing these effects. IGF-II significantly enhanced DD cell contractility and this effect could be abrogated by IGFBP-6. Overall, these findings

implicate IGFBP-6 and IGF-II as previously unrecognized regulators of DD cell proliferation and contractility that may have potential as therapeutic targets in this and related fibrocontractile diseases.

3.2 Materials and Methods

3.2.1 Clinical specimen collection

Surgically resected Dupuytren's disease cords were collected from patients undergoing primary surgical resections at St Joseph's Hospital, London, Ontario. Samples of phenotypically normal palmar fascia tissue exposed during surgery were collected from an adjacent, visually unaffected digit for comparative analyses. None of these patients were being treated for recurrent disease. In addition, small samples of palmar fascia were collected from patients without Dupuytren's disease who are undergoing hand surgery for unrelated reasons, such as carpal tunnel release, in which the palmar fascia was phenotypically unaffected. All subjects provided written informed consent and specimens were collected with the approval of the University of Western Ontario Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB protocol # 08222E).

3.2.2 Immunohistochemistry

Surgically resected DD cord and patient matched, phenotypically normal palmar fascia samples were fixed in 10% formalin prior to dehydration, paraffin embedding and microtome sectioning. Paraffin-embedded specimens were sectioned (5 μm), dewaxed, rehydrated and treated with a 3% hydrogen peroxide solution to quench endogenous peroxidase activity. Slides were treated with a serum-free blocking reagent (Background Sniper, Biocare Medical, Concord, CA) for 10 min and rinsed in PBS prior to incubation with rabbit polyclonal IGFBP-6 (Austral Biologicals, San Ramon, CA) overnight at 4 °C. After a wash in PBS, the slides were incubated (30 min, 22 °C) with a biotinylated secondary anti-rabbit antibody (Vector Labs, Burlington ON), washed briefly in PBS, and incubated (30 min, 22 °C) with avidin/biotin/HRP complex (Vector elite PK-6100, Vector Labs, Burlington ON). Finally, the slides were washed with PBS, and incubated (1 min) in an enhanced diaminobenzidine (Cardassian DAB; Biocare Medical, Concord,

CA). Sections were counterstained with methyl green (10 min), dehydrated, cleared, and mounted with Permount (Fisher Scientific, Ottawa, ON).

3.2.3 Primary cell culture

Primary fibroblasts were isolated from surgically resected DD cord tissues (DD cells), phenotypically normal (non-fibrotic) palmar fascia tissue from an adjacent, visually unaffected digit (PF cells) and normal palmar fascia (CT cells) as previously described [25]. The cultures were maintained in α-MEM-medium supplemented with 10% fetal bovine serum (FBS, Invitrogen Corporation, Carlsbad, CA) and 1% antibiotic–antimycotic solution (Sigma-Aldrich, St. Louis, MO). All primary cell lines (DD, PF and CT cells) were assessed at the lowest passage number achievable up to a maximum of 6 passages, after which the cells were discarded. No changes in cell morphology, growth/viability or contractility attributable to serial passage were evident in these cells.

3.2.4 Real time PCR

Total RNA samples from primary DD, PF and CT cells were assessed for quality on an Agilent 2100 Bioanalyzer. 2 μg of high quality total RNA was reverse transcribed into cDNA first strand using the High-Capacity cDNA Archive Kit (Applied Biosystems) in accordance with the manufacturer's instructions. TaqMan gene expression assays were used to measure *IGFBP6*, *IGF2* and *IGF1* expressions. *IGFBP6* (Hs00181853_m1) and *IGF2* (Hs01005963_m1) expressions were measured relative to the RPLP0 endogenous control (Hs99999902_m1), and *IGF1* (Hs01547656_m1) was measured relative to *GAPDH* (Hs99999905_m1) using the $\Delta\Delta C_T$ method after confirmation of parallel PCR amplification efficiencies on a Real-Time PCR ABI Prism 7500. PCR reactions were carried out under the following conditions: Initial denaturation at 95 °C for 5 min followed by cycles of denaturation (95 °C for 15 s), primer annealing (60 °C for 1 min) and transcript extension (50 °C for 2 min) for 45 cycles.

3.2.5 Western immunoblotting

Surgically resected tissues were snap-frozen in liquid nitrogen and protein extracts were prepared using a tissue bio-pulverizer and PhosphoSafe Protein Extraction Buffer (VWR,

Mississauga, ON). Cultured cells were lysed in RIPA Cell Lysis Buffer (Teknova Inc., Hollister, CA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), 0.1 M NaF, 10 mM PMSF and 10 mM sodium orthovanadate and placed on ice for 30 min after needle aspiration. After centrifugation to remove insoluble material, total protein concentrations were determined by BCA analysis. Gel electrophoresis on a 15% polyacrylamide gel and Western immunoblotting were performed using standard procedures, and proteins were visualized using enhanced chemiluminescence (ECL). The primary antibodies utilized were mouse monoclonal IGF-II (Upstate Biotechnology, Etobicoke, ON) and mouse monoclonal β-actin (Santa Cruz Biotechnology, Dallas, TX). Total protein levels were assessed by Ponceau S staining (Sigma Aldrich, Oakville, ON, Canada).

3.2.6 Quantitative IGFBP-6 immunoassay

The levels of secreted IGFBP-6 in DD and PF cell conditioned media were assessed using the Luminex xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX) and the Bio-Plex 200 readout system according to the manufacturer's instructions (Bio-Rad Laboratories Inc., Hercules, CA). IGFBP-6 levels were calculated from standard curves of recombinant IGFBP-6 in a solution using Bio-Plex Manager software (v.4.1.1, Bio-Rad).

3.2.7 WST-1 cell proliferation assay

The WST-1 assay (Roche, Mississauga, ON, Canada) was adapted to measure changes in the proliferation of primary fibroblasts grown on type-1 collagen, the most abundant protein component of palmar fascia. We have included type-1 collagen substrates in all our assays to more closely replicate in vivo conditions. In brief, 2×10^3 cells were plated in α-MEM/2% FBS in 4 × 96 well trays pre-coated with 60 µl of type-1 collagen (1.8 mg/ml), treatments or vehicles were added, and the trays were incubated at 37 °C for 7 days. WST-1 reagent was added to the wells on days 1, 3, 5 and 7 to allow cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenases. Equal volumes of supernatant were transferred to additional 96 well trays and absorbance measurements were taken at 450 nm and 650 nm (reference wavelength). This assay

measures electron transport across the plasma membrane of dividing cells, and thus functions as an indirect measure of viable cell proliferation. We interpret the outputs as net proliferation (total cell number changes due to the combined effects of mitogenesis and apoptosis). All experiments were performed at least three times on a minimum of 4 DD, PF and CT cell lines, each assessed in triplicate.

3.2.8 Fibroblast populated collagen lattice assays

Collagen contraction assays were carried out using modified versions of Bell *et al*. [26] and Tomasek and Rayan [27]. In brief, the contractility of DD, PF and CT cells at low passage (≤6) was assessed in three-dimensional fibroblast populated collagen lattices (FPCLs). Collagen lattices were cast in 24-well tissue culture trays with each well containing 400 μl collagen (final collagen concentration of 1.8 mg/ml), 100 μl neutralization solution, treatment or vehicle, and 1×10^5 cells. FPCLs were maintained in α-MEM supplemented with 2% FBS and 1% antibiotic–antimycotic solution at 37 °C in 5% CO_2. For "relaxed" FPCLs (rFPCLs), lattices were allowed to polymerize for 1 h before being gently released from the sides and bottoms of the wells using a metal rod. Relaxed FPCLs typically undergo gradual lattice contraction over 24 h in tissue culture media. Floating lattices for rFPCLs were digitally scanned at 24.0 h only. For "stressed" FPCLs (sFPCLs), the collagen lattices remained attached to the wells for 72 h to allow the cells to induce (and respond to) stress within the lattice and differentiate toward a contractile myofibroblast phenotype [28]. Lattices were released after 72 h, typically resulting in rapid contraction over 6 h. Floating lattices for sFPCLs were digitally scanned at release (0 h), 0.5 h, 1.0 h, 2.0 h and 6.0 h. The areas of individual lattices in rFPCLs and sFPCLs were determined using the freehand tool in ImageJ software. Sequential area calculations were then normalized to the area of the lattice (i.e. the area of the well in which the lattice was cast) prior to release. All experiments were performed on a minimum of 3 and a maximum of 10 DD, PF and CT cell lines, each assessed in 3 separate experiments, in triplicate.

3.2.9 Statistical analyses

Statistical analysis was conducted using SPSS v. 17 and Microsoft Excel 2007 statistical software. For sFPCL data, repeated measures analysis of variance analyses were used to assess the significance of treatment effects and treatment/time interactions to distinguish overall treatment-induced changes in contractility from treatment induced changes in contractility that became significant over time. Significant treatment/time interactions were further assessed with simple main effects analyses to determine at which time point a treatment had a significant effect. Paired t-tests were used to determine significant changes in contractility at 24 h in rFPCLs and in proliferation by change score analysis between day 7 and day 1. Results were deemed significant when $p < 0.05$.

3.3 Results

3.3.1 *IGFBP6* mRNA and IGFBP-6 levels are attenuated in Dupuytren's disease tissues and in DD cells

While *IGFBP6* has been identified as a significantly downregulated gene transcript in gene array studies comparing DD tissues to phenotypically unaffected palmar fascia [19,20], corresponding changes in IGFBP-6 levels have not been reported. As shown in Figure 3-1A, decreased IGFBP-6 immunoreactivity was evident in paraffin-embedded sections of DD cord relative to visibly unaffected palmar fascia tissue from an adjacent digit in the same patient (Figure 3-1B). This decrease in IGFBP-6 immunoreactivity in DD tissue was despite an inverse disparity in the number of palmar fascia cells (stained with methyl green in Figure 3-1A and B) in DD cord tissues and the adjacent palmar fascia. To confirm the cellular source of IGFBP-6 in DD tissues, we assessed *IGFBP6* expression in primary fibroblasts derived from diseased palmar fascia (DD cells) relative to primary fibroblasts derived from the adjacent, phenotypically unaffected palmar fascia of the same patients (PF cells). As shown in Figure 3-1C, *IGFBP6* mRNA levels were significantly lower in DD cells than in genetically matched PF cells. We also assessed IGFBP-6 secretion in DD and PF cells, and compared these levels to primary fibroblasts derived from normal palmar fascia from unrelated patients undergoing treatment for unrelated conditions (CT cells). IGFBP-6 secretion was significantly reduced in DD cells

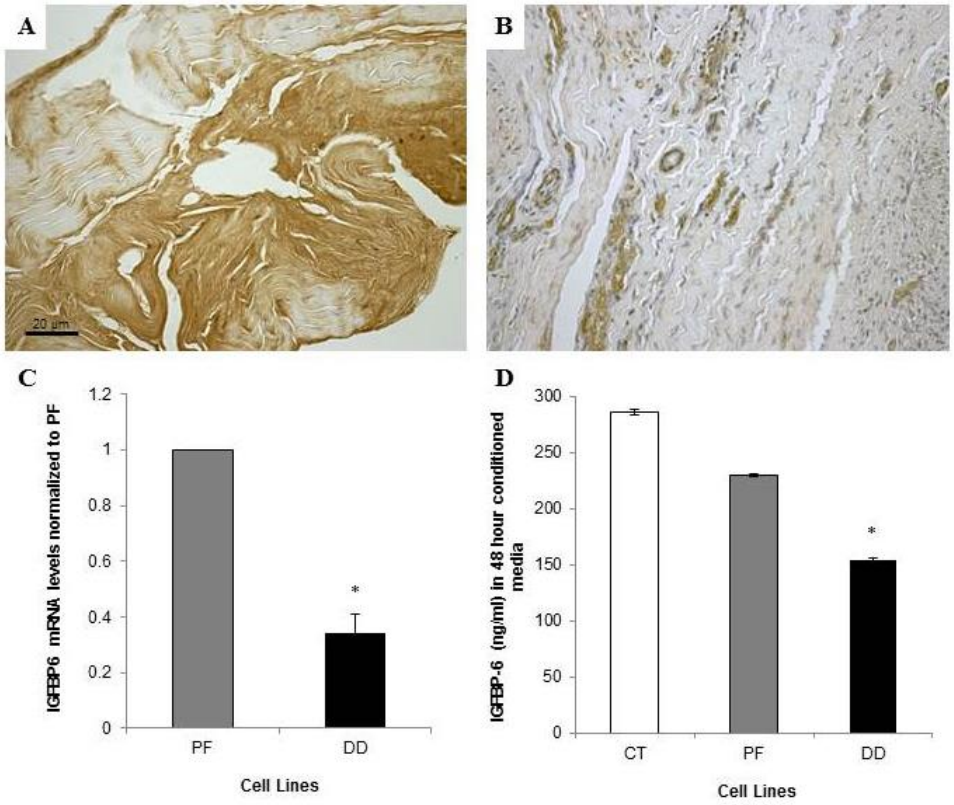

Figure 3-1: *IGFBP6* expression and IGFBP-6 secretion are downregulated in Dupuytren's disease (DD).

Sections of paraffin embedded phenotypically normal, unaffected palmar fascia tissue (A) and DD "cord" tissue (B) from the same patient were assessed for IGFBP-6 immunoreactivity, evident as brown (diaminobenzidine, DAB) staining. Palmar fascia fibroblasts were counter-stained with methyl green. C) QPCR analysis of *IGFBP6* mRNA levels in primary fibroblasts derived from phenotypically unaffected palmar fascia (PF cells) and DD cord tissue (DD cells) from the same patients (* = $p < 0.05$ for DD vs PF samples, N = 3, n = 3). D) IGFBP-6 secretion by normal palmar fascia fibroblasts (CT cells), PF cells and DD cells in serum free media over 48 h (* = $p < 0.05$ for DD vs CT samples, N = 3, n = 1).

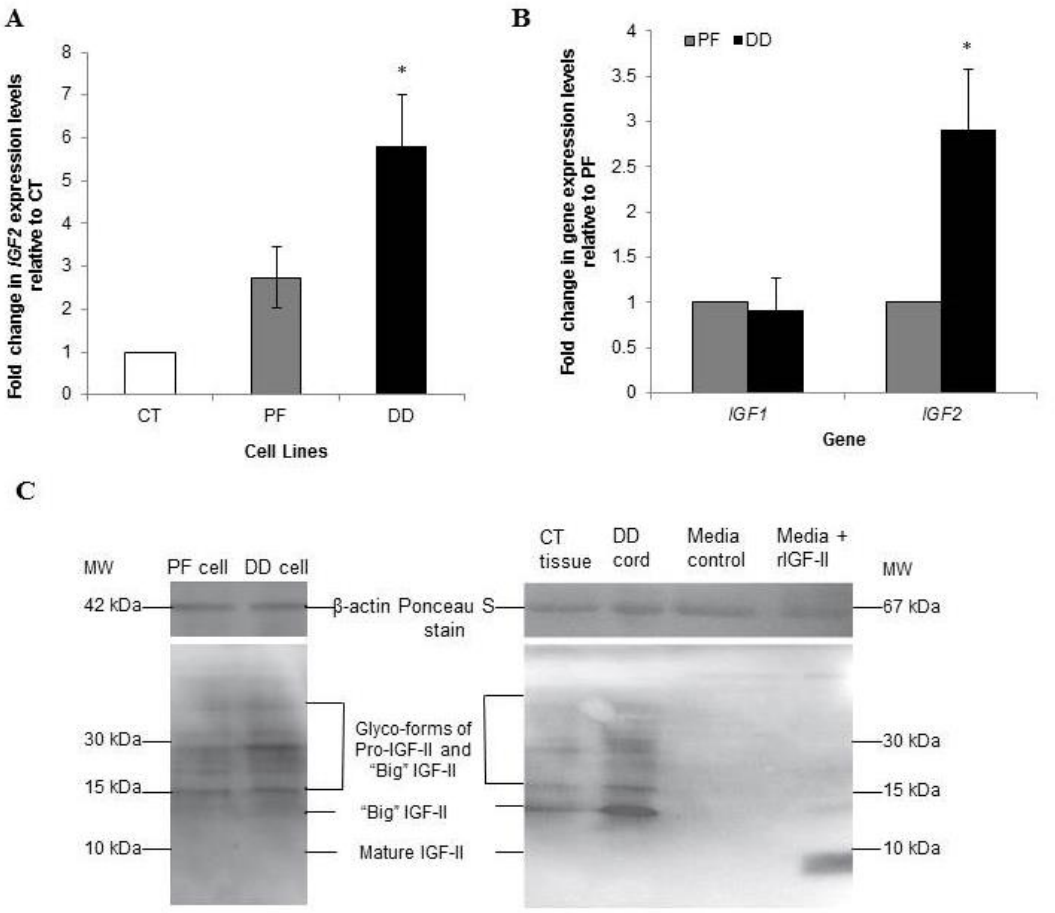

Figure 3-2: *IGF2* expression and IGF-II levels are upregulated in DD.

A) QPCR analysis of *IGF2* mRNA levels in CT, PF and DD cells (* = $p < 0.05$ for DD vs CT samples, N = 6, n = 1). B) QPCR analyses of *IGF1* and *IGF2* mRNA levels in primary PF cells (grey bars) and DD cells (black bars) (* = $p < 0.05$ for DD vs PF samples, N = 6, n = 1). C) Representative Western immunoblotting analyses of DD and PF cell lysates and normal palmar fascia tissue (CT) and DD cord tissue lysates with an IGF-II antibody. Molecular weight (MW) markers are indicated in kDa. Immunoblotting for β-actin and Ponceau S staining for total protein (the major protein band at 67 kDa, albumin, is shown) confirmed equal protein loading. Tissue culture media samples with or without recombinant IGF-II (rIGF-II) were included as negative and positive controls.

relative to PF cells and CT cells over 48 h when cultured under identical serum free conditions (Figure 3-1D).

3.3.2 *IGF2* mRNA and IGF-II levels are increased in DD cells

As IGFBP-6 has been reported to elicit effects by sequestering IGF-II, we assessed basal *IGF2* expression in DD and PF cells. *IGF2* mRNA levels were consistently ($n = 6$ patients) and significantly ($p < 0.05$) increased in DD cells relative to patient-matched PF cell controls and non-patient-matched CT cells (Figure 3-2A). In contrast to *IGF2*, the expression of *IGF1*, encoding Insulin-like Growth Factor-I, was unchanged between DD and PF cells (Figure 3-2B). To determine if increased *IGF2* expression resulted in increased IGF-II secretion, we assessed IGF-II levels in DD cord tissue and DD cell lysates. As shown in Figure 3-2C, IGF-II immunoreactivity was increased in DD tissue and cell lysates relative to normal palmar fascia tissue and PF cell lysates. Multiple bands correlating with precursor forms of IGF-II, including "big" IGF-II and variably glycosylated pro-IGF-II previously reported in human serum [29] were evident in all cell lysates. As our findings indicated reciprocal changes in *IGFBP6* and *IGF2* mRNA levels and IGFBP-6 and IGF-II protein levels in DD cells relative to controls, we compared the effects of recombinant IGFBP-6 and recombinant IGF-II, individually and in combination, with or without TGF-β1, on the net proliferation and contractility of DD and PF cells.

3.3.3 IGFBP-6 attenuates DD cell proliferation in an IGF-II independent manner

Based on our analyses of IGFBP-6 secretion by CT cells (Figure 3-1D) and previous studies in tumor cells [30], we chose to assess the effects of 400 ng/ml of recombinant IGFBP-6 on net cellular proliferation. As shown in Figure 3-3A, a single treatment with 400 ng/ml of recombinant IGFBP-6 on day 0 induced a significant reduction in DD ($p < 0.01$), PF and CT (each $p < 0.05$) cell proliferation. As TGF-β1 is an established component of DD [31], and has been shown to deplete IGFBP-6 secretion in human fibroblasts [32], the effects of TGF-β1 on proliferation of CT, PF and DD cells were also

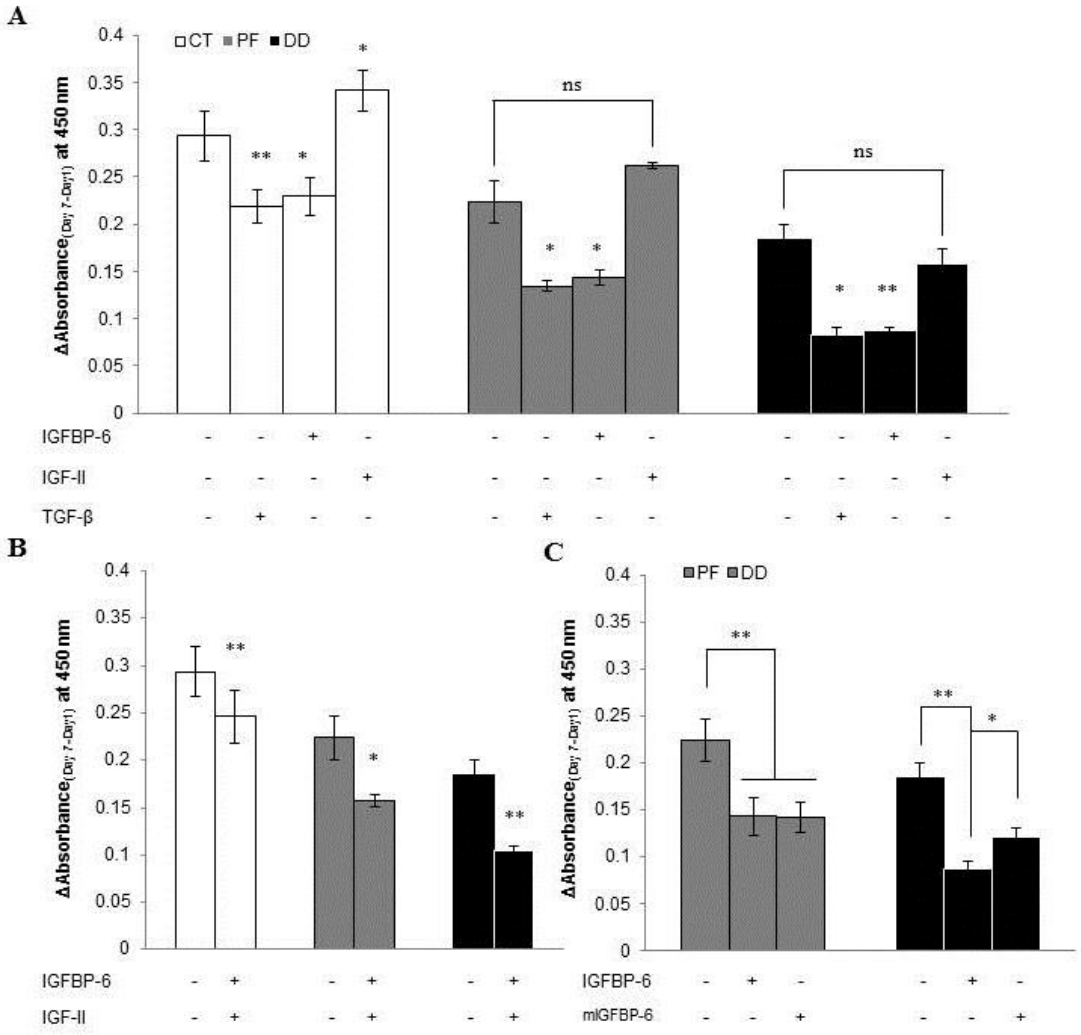

Figure 3-3: IGFBP-6 attenuates the proliferation of DD, PF and CT cells.

Net proliferation (growth and/or apoptosis) of CT (white bars, N = 3 patients, n = 3), PF (grey bars, N = 4 patients, n = 3) and DD (black bars, N = 4 patients, n = 3) cells cultured on type-I collagen substrates was assessed using the WST-1 assay. Data are shown as change scores (absorbance on day 7 minus absorbance on day 1). Significant effects of treatment vs vehicle are indicated as * $p < 0.05$ and ** $p < 0.01$, ns = not significant. Treatments were the following: A) IGFBP-6 treatment (400 ng/ml) or IGF-II treatment (100 ng/ml) or TGF-β1 (12.5 ng/ml) vs vehicle, B) IGF-II + IGFBP-6 treatment (100 ng/ml and 400 ng/ml respectively) vs vehicle, and C) mIGFBP-6 (400 ng/ml) or IGFBP-6 (400 ng/ml) vs vehicle.

assessed. A single treatment with 12.5 ng/ml of recombinant TGF-β1, as previously demonstrated [33] in DD and PF (each $p < 0.05$), and CT ($p < 0.01$), attenuated proliferation over the subsequent 7 days relative to vehicle treated cells. To assess the effects of IGF-II on cellular proliferation, DD, PF and CT cells were treated with a single dose of recombinant IGF-II at 100 ng/ml, as previously described [34,35]. While treatment with recombinant IGF-II at 100 ng/ml significantly enhanced CT cell proliferation over 7 days ($p < 0.05$), no significant effects of IGF-II treatment on the proliferation of DD or PF cells were detected (Figure 3-3A). As shown in Figure 3-3B, co-incubation of cells with IGFBP-6 and IGF-II at equimolar concentrations did not rescue the inhibitory effects of IGFBP-6 treatment. To confirm IGF-II independence of these effects on cell proliferation, DD, PF and CT cells were treated with a single dose of a non-IGF-II binding IGFBP-6 analog (mIGFBP-6) [36] at 400 ng/ml. IGFBP-6 and mIGFBP-6 had similar inhibitory effects on the proliferation of PF cells ($p < 0.05$, Figure 3-3C) and CT cells ($p < 0.01$, data not shown). Unlike in PF and CT cells, mIGFBP-6 was significantly ($p < 0.05$) less effective at inhibiting DD cell proliferation than IGFBP-6, although both treatments had statistically significant negative effects relative to vehicle treated cells ($p < 0.01$).

3.3.4 IGF-II enhances DD cell contraction of collagen lattices

To assess the effects of IGF-II and IGFBP-6 on DD and PF cell contractility, we utilized two types of fibroblast populated collagen lattice assays, relaxed FPCLs (rFPCLs) [28,37–39] and stressed FPCLs (sFPCLs) [21,40,41]. The 72 h incubation period in the presence of treatments in the sFPCL protocol allowed us to distinguish the effects of treatment-induced myofibroblast differentiation [42] from the immediate effects of treatment that are measured in rFPCLs [38].

DD cells were more contractile than PF cells in rFPCL assays in the absence of treatment (Figure 3-4A). IGFBP-6 (400 ng/ml) did not significantly affect the contractility of DD, PF or CT cells in rFPCLs (data not shown). IGF-II (100 ng/ml) significantly enhanced rFPCL contraction by DD cells ($p < 0.05$) but did not significantly affect PF (Figure 3-4A) or CT cell (data not shown) contractility. Platelet derived growth factor (PDGF), an

established inducer of fibroblast contractility in rFPCLs [38], enhanced both DD and PF cell contractility in these assays. IGFBP-6 (400 ng/ml) did not significantly affect the contractility of DD, PF or CT cells in sFPCLs (data not shown). IGF-II treatment significantly enhanced both PF ($p < 0.05$) and DD ($p < 0.001$) cell contractility over 6 h after lattice release (Figure 3-4C and D respectively). In addition to treatment effects, significant IGF-II treatment/time interactions were evident for both cell types ($p < 0.01$). While IGF-II significantly induced DD cell contraction at all time points after release, the level of significance increased from $p < 0.01$ at release to $p < 0.001$ 6 h after release. Significant effects of IGF-II treatment were only evident 2 h after release in PF cells ($p < 0.05$). TGF-β1 treatment (12.5 ng/ml) significantly enhanced DD cell ($p < 0.01$) contractility in these assays, whereas a significant TGF-β1 treatment/time interaction was evident in PF cells ($p < 0.01$) 6 h after lattice release ($p < 0.01$) (Figure 3-4B). IGF-II did not significantly affect sFPCL contraction by CT cells (data not shown). Co-treatments with equimolar IGFBP-6 and IGF-II abrogated the IGF-II induced contractility of both DD and PF cells (Figure 3-5A).

As TGF-β1 signaling intermediates and IGF-II have been reported to elicit combinatorial effects on myofibroblast differentiation in murine fibroblasts [43], we assessed the effects of treating PF and DD cells with both growth factors in combination (Figure 3-5B). While TGF-β1 and IGF-II treatments significantly enhanced the contractility of PF and DD cells relative to vehicle treated controls, no significant combinatorial treatment effects were detected over treatment with either factor in isolation.

3.4 Discussion

In this study, we have identified *IGFBP6* and *IGF2* as reciprocally dysregulated genes in DD cells relative to PF cells. Previous studies have reported increased TGF-β1 expression and sensitivity, enhanced contractility, α smooth muscle actin (αSMA) expression and myofibroblast differentiation in DD cells [44–46], features shared with primary myofibroblast-like cells derived from other fibroses [47–49]. Other than TGF-β1, very few of the dysregulated genes in DD cells have been functionally linked to the main characteristics of DD development, namely fibroblast proliferation and palmar fascia contraction by myofibroblasts. Based on our findings, we hypothesize that downregulated

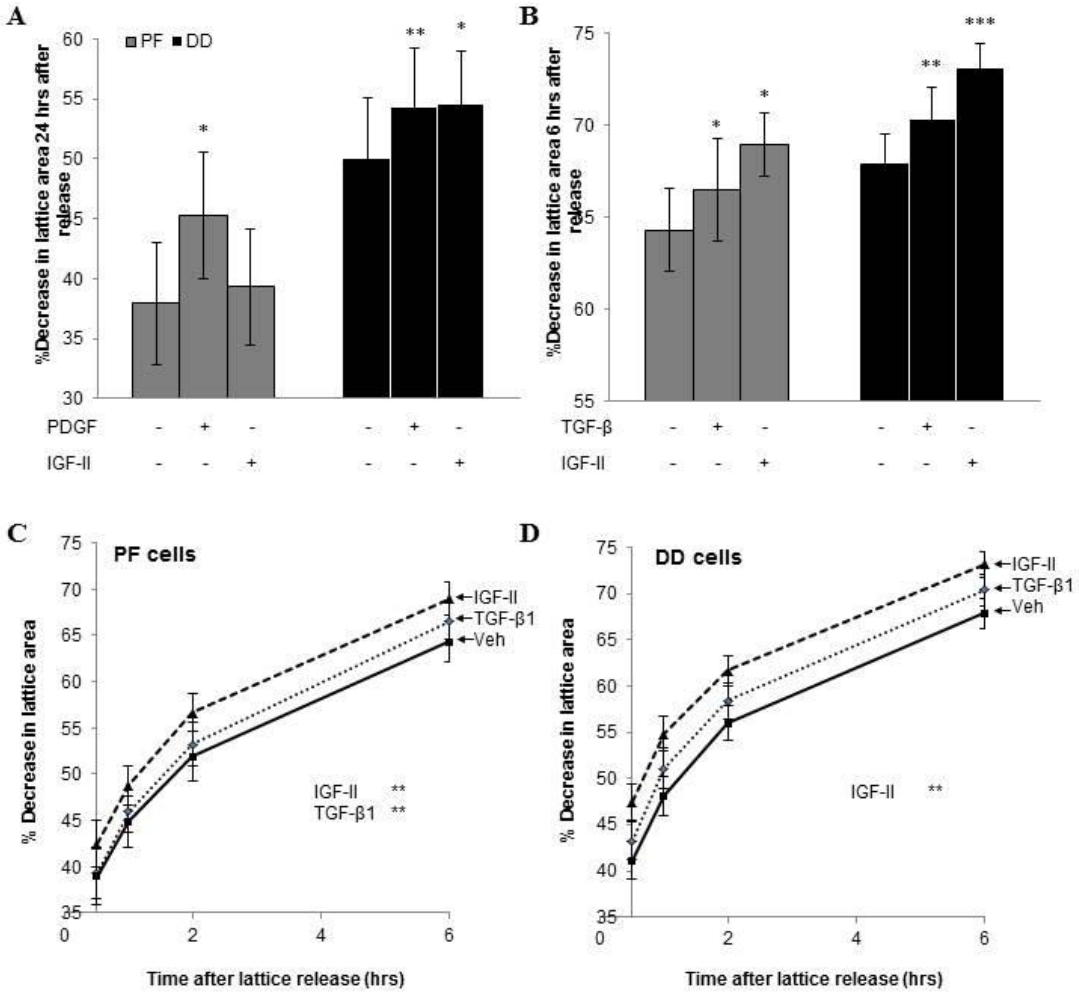

Figure 3-4: IGF-II enhances DD cell contraction of collagen lattices.

The effects of IGF-II on PF (grey bars) and DD (black bars) cell contractility were assessed in relaxed fibroblast populated collagen lattice assays (rFPCLs) over 24 h (A) and stressed FPCLs (sFPCLs) over 6 h (B, C and D) as described in the Methods section. Significant treatment effects are denoted as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. The effects of treatment vs vehicle on rFPCL contraction were assessed by t-test analyses at 24 h (N = 4 patients, n = 3). The effects of treatment vs vehicle on sFPCL contraction were assessed by ANOVA of repeated measures analyses and, where treatment/time interactions were detected, by simple main effects analyses (N = 10 patients, n = 3). Treatments were the following: A) IGF-II (100 ng/ml) or PDGF (100 ng/ml) in rFPCLs and B) IGF-II (100 ng/ml) or TGF-β1 (12.5 ng/ml) in sFPCLs. C) Contraction curves for PF cells from 0.5 h to 6 h after lattice release with vehicle, TGF-β1 or IGF-II treatment. D) Contraction curves for DD cells from 0.5 h to 6 h after lattice release with vehicle, TGF-β1 or IGF-II treatment. Significant treatment/time interactions are denoted as * $p < 0.05$ and ** $p < 0.01$.

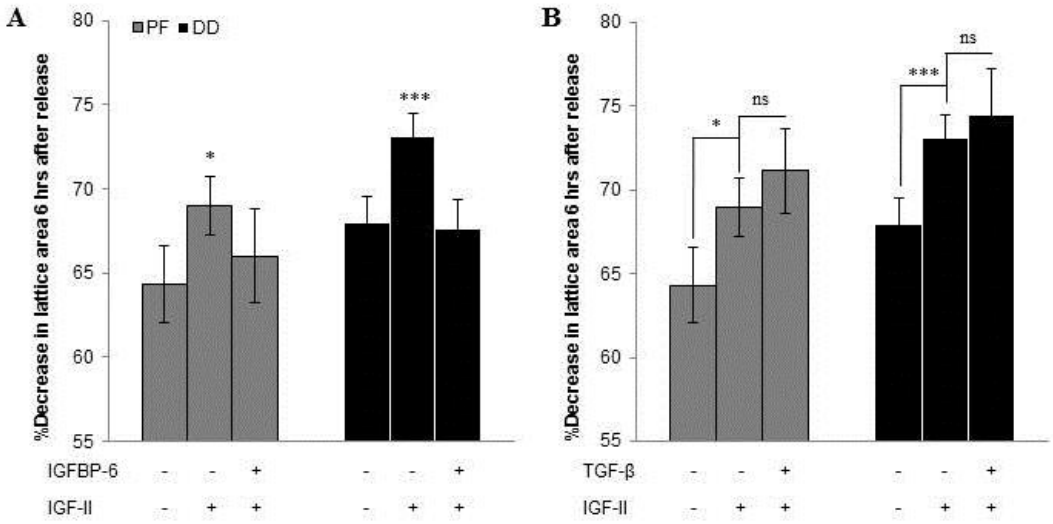

Figure 3-5: IGF-II-induced PF and DD cell contractility is attenuated by IGFBP-6, but not enhanced by TGF-β1.

The effects of co-treatment with IGF-II and IGFBP-6 (N = 3 patients, n = 3), and IGF-II and TGF-β1 (N = 6 patients, n = 3) were assessed on PF (grey bars) and DD (black bars) cell contractility in stressed fibroblast populated collagen lattice assays (sFPCLs) over 6 h. Significant effects of treatment vs vehicle on sFPCL contraction were detected by ANOVA of repeated measures analyses. Statistically significant treatment effects are denoted as * $p < 0.05$ and *** $p < 0.001$, ns = not significant. Treatments were the following: A) IGFBP-6 (400 ng/ml) and IGF-II (100 ng/ml) and B) TGF-β1 (12.5 ng/ml) and IGF-II (100 ng/ml).

IGFBP6 expression and upregulated *IGF2* expression jointly contribute to DD progression by promoting fibroblast proliferation, contractility and palmar fascia contraction in DD.

As IGFBP-6 is a high affinity IGF-II binding protein [23], we hypothesized that attenuation of *IGFBP6* expression and IGFBP-6 protein levels in DD would enhance IGF-II availability and signaling. IGF-II signaling can promote mitogenesis [50–53] and inhibit apoptosis [54,55], and IGFBP-6 can antagonize these effects by sequestering and inhibiting IGF-II signaling [30,56–58]. However, co-treatment with equimolar IGFBP-6 and IGF-II elicited similar negative effects on the net proliferation compared to cells treated with IGFBP-6 alone. Consistent with these findings, an IGFBP-6 analog with attenuated IGF-II binding capacity (mIGFBP-6) exhibited similar effects on PF cell proliferation to native IGFBP-6. These data suggest that IGFBP-6 attenuates PF cell proliferation through mechanisms that are independent of IGF-II sequestration, consistent with a subset of reports of IGFBP-6 actions in other diseases [36,59,60]. In contrast to PF and CT cells, mIGFBP-6 was significantly less effective at decreasing DD cell proliferation than native IGFBP-6. *IGF2* expression is enhanced in DD cells relative to PF and CT cells, and exogenous IGF-II treatment did not promote DD cell proliferation (Figure 3-3A). We speculate that increased endogenous IGF-II expression in DD may be one of the (potentially several) factors that enhance DD cell survival (rather than proliferation) in a low IGFBP-6 environment. The identification and elucidation of the IGF-II dependent and independent mechanisms activated by IGFBP-6 in DD cells will be a focus of future studies.

In contrast to its inhibitory effects on cellular proliferation, IGFBP-6 treatment did not significantly inhibit cellular contractility. However, IGFBP-6 was effective at attenuating the increase in PF and DD cell contractility induced by IGF-II treatment (Figure 3-5A). Overall, our data imply two distinct consequences of IGFBP-6 depletion in DD that are likely to enhance disease progression; the induction of IGF-II dependent and/or independent processes that increase the net proliferation of palmar fascia cells, and the enhancement of IGF-II induced cellular contractility.

In contrast to a previous study in nodule-derived DD cells cultured on tissue culture plastic where TGF-β1 was shown to be growth-stimulatory [44], TGF-β1 consistently inhibited the proliferation of DD, PF and CT cells cultured on type-1 collagen culture substrates (Figure 3-3A). This may reflect the effects of differing culture substrates (tissue culture plastic vs type-1 collagen in this study) and/or a biphasic effect of TGF-β1 on fibroblast proliferation that is dose-dependent (0.025 ng/ml vs 12.5 ng/ml in this study). TGF-β1 concentrations used in this study were based on a previous report in DD identifying 12.5 ng/ml as the maximum concentration required to induce myofibroblast formation without having negative inhibitory effects [33]. As enhanced fibroblast proliferation and myofibroblast differentiation have been previously described as mutually exclusive outcomes in some fibroblast models [43], and TGF-β1 is an established inducer of myofibroblast differentiation [42,48], myofibroblast differentiation may be the dominant phenotype under these culture conditions. The increased TGF-β1 expression and enhanced basal contractility of DD cells [44–46] suggest that DD cells have differentiated further toward a myofibroblast phenotype than PF cells.

Our analyses of DD and PF cells treated with TGF-β1 or IGF-II revealed some interesting and distinct behaviors in these genetically matched cells at differing stages of differentiation. TGF-β1 treatment significantly enhanced DD cell contractility ($p < 0.01$) but no significant treatment/time interactions were evident, suggesting that the procontractile effects of TGF-β1 treatment were induced prior to, and not after, lattice release. In contrast, no significant TGF-β1 treatment effects on PF cells were detected, and a significant ($p < 0.01$) time/treatment interaction was evident, suggesting that most of the contractility induced by TGF-β1 in PF cells occurred during the lattice contraction phase after release. These differences are visually evident in the parallel versus divergent contraction curves in Figure 3-4C and D. In contrast, IGF-II induced treatment and treatment/time interactions in DD cells, indicating effects of treatment both pre- and post-release. IGF-II also induced treatment and treatment/time interactions in PF cells; however simple main effects analyses revealed that IGF-II treatment did not significantly affect lattice contraction until 2 h after release. These data suggest that IGF-II can act both pre- and post-release to enhance DD contraction, while acting mostly post-release to increase PF cell contraction. Thus, IGF-II and TGF-β1 may activate or potentiate distinct

pro-contractile signaling pathways in DD and PF cells. We were unable to detect significant additive effects of TGF-β1 and IGF-II treatment in our analyses; however it is unclear if that is because the pro-contractility pathway(s) were fully activated by each factor in isolation or if our assays were too insensitive to detect modest additive effects of treatment in cells with enhanced levels of contractility.

The molecular mechanisms activated by IGF-II to enhance the contractility of DD and PF cells are currently unclear. Consistent with their contractile myofibroblast-like phenotype, untreated DD cells contracted rFPCLs to a greater extent than PF cells over 24 h. DD cell contraction of rFPCLs was significantly ($p < 0.05$) enhanced by IGF-II, whereas PF cells were insensitive to IGF-II treatment under identical conditions. These data suggest that IGF-II can induce lattice contraction by DD cells without modifying the differentiation state of these cells, a process that typically requires a 48–72 h pre-incubation period under isometric tension. We speculate that IGF-II enhances the three-dimensional migration of DD cells in these rFPCLs, an effect previously shown for normal fibroblasts treated with PDGF [33]. PDGF treatment significantly enhanced rFPCL contraction by both DD ($p < 0.01$) and PF ($p < 0.05$) cells (Figure 3-4A), whereas TGF-β1, which significantly enhanced DD cell contraction in sFPCLs (Figure 3-4B), had no discernible effects on the contractility of DD or PF cells in rFPCLs (data not shown). That IGF-II enhanced rFPCL contraction by DD cells but not PF cells suggests that DD cells have enhanced sensitivity to IGF-II signaling, and that there may be a functional link between IGF-II sensitivity and the differentiation state of these cells. If future studies are able to substantiate this link, local administration of IGF signaling inhibitors [61–63] may have utility as novel treatment approaches to attenuate the contractility of myofibroblasts in DD patients post-surgery. Alternatively, novel therapies that restore IGFBP-6 levels to those evident in normal palmar fascia may be sufficient to attenuate IGF-II signaling and myofibroblast contractility.

IGFBP-6 attenuated DD proliferation, while exogenous IGF-II potently enhanced the contractility of DD cells. Future studies will focus on elucidating the IGF-II-independent mechanisms regulated by IGFBP-6 and the mechanisms by which IGF-II signaling enhances DD cell contractility. These findings implicate IGFBP-6 and IGF-II as novel

regulators of DD cell proliferation and contractility that may have utility as targets to inhibit DD progression and recurrence. Additionally, these results support our hypothesis that the attenuation of *IGFBP6* expression and enhancement of *IGF2* expression promote DD development. Investigation into the molecular mechanisms that increase *IGF2* expression in DD will be the subject of chapter 4.

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Chapter 4

4 An analysis of transcriptional dysregulation of *IGF2* expression in Dupuytren's Disease

4.1 Introduction

The Insulin-like Growth Factor (IGF) family is comprised of 2 ligands, IGF-I and –II, 6 high affinity binding proteins (IGFBP-1 to -6) and 2 receptors, the Type I and Type II IGF receptors [1]. IGFBPs regulate the bioavailability of their ligands to their signalling receptor, the Type I IGF receptor (IGFIR) [1–3]. The Type II IGF Receptor (IGFIIR), which is also a cation-independent mannose-6-phosphate receptor, is a negative regulator of IGF-II signalling in most tissues. The IGFIIR can internalize IGF-II, and molecules with a mannose-6-phosphate moiety, for lysosomal degradation [4].

IGF signalling through the IGFIR can regulate proliferation, differentiation, and survival in a variety of systems (reviewed in [5,6]). As their names suggest, IGFs can also signal through the insulin receptor (InsR) to elicit metabolic or proliferative effects (reviewed in [7]). While isoform B of InsR has much higher affinity for insulin than for either IGF, IGF-II can interact with the isoform A of the InsR, (which lacks exon 11) with high affinity at physiologically relevant concentrations [8]. Additionally, subunits of IGFIR and InsR can create heterodimers and act as an additional set of signalling receptors for IGFs and insulin [9]. A key difference between the two IGFs is that IGF-I is largely regulated by growth hormone [10,11] while IGF-II is largely growth hormone independent and subject to multiple layers of complex genomic regulation and processing instead [12]. IGF-II levels are at their highest in the fetus, where it is believed to play an integral role in embryonic development [13–16]. Following birth, IGF-II levels decline and then increase again in adult humans until IGF-II becomes the predominant IGF in the circulation [14].

The gene that encodes IGF-II, *IGF2*, is located within a 30 kb DNA region on chromosome 11p15.5. This gene contains 10 exons and can be transcribed from 4 distinct promoters; however the functional peptide is only encoded by the last 3 exons [17].

Unlike most autosomal genes, which are expressed or transcriptionally silent irrespective of the parent from which they were derived, *IGF2* is genomically imprinted and expressed only from the paternally-derived allele in most mammalian tissues [18]. Like many imprinted genes, *IGF2* is located within a "cluster" of other genes that are also imprinted in various tissues. *IGF2* is located adjacent to the insulin gene, *INS*, which is imprinted in yolk sack [19], and *H19* [20]. *H19* encodes a non-coding RNA that is reciprocally expressed with *IGF2* i.e. *H19* is only expressed from the maternally derived allele in most mammalian tissues [20]. *IGF2* and *H19* are separated by a CG-rich motif known as the H19 imprinting control region (ICR). In tissues where *IGF2* is imprinted, the H19 ICR is methylated on the paternally derived allele and unmethylated on the maternally derived allele. ICR methylation prevents CCCTC binding factor (CTCF) interactions and maintains transcriptional silencing of *H19* by allowing transcriptional enhancers to drive *IGF2* expression [21,22]. In addition to being regulated by the H19 ICR, *IGF2* contains another differentially methylated region (DMR) that is unmethylated on the paternally derived allele where *IGF2* is expressed. The H19 ICR is unmethylated on the maternally derived allele, allowing CTCF to bind and prevent transcriptional enhancers from activating *IGF2* expression [21,23]. The *IGF2* DMR is methylated on the maternally derived allele and is believed to act as an additional mechanism to prevent *IGF2* transcription from this allele (Figure 4-1).

In Wilm's tumour [24] or other cancers [25], the imprinting control mechanism can be disrupted and *IGF2* can be expressed from both alleles. This is usually, but not exclusively, associated with loss of *H19* expression from both alleles [26]. Unlike most tissues, *IGF2* is biallelically expressed in adult human liver and in some areas of the brain [27,28]. The biological significance of genomic imprinting of genes in some tissues and not in others is still being elucidated.

In addition to its transcriptional regulation by imprinting, tissue-specific regulation of *IGF2* transcription can be imposed by the use of any or all of 4 distinct promoter regions designated P1, P2, P3, and P4 [17]. Transcription from these promoters can result in up to 5 distinct *IGF2* mRNA transcripts, each of which encodes the same peptide [17]. In adult humans, transcription from P2 – P4 varies between subsets of tissue or organs, and

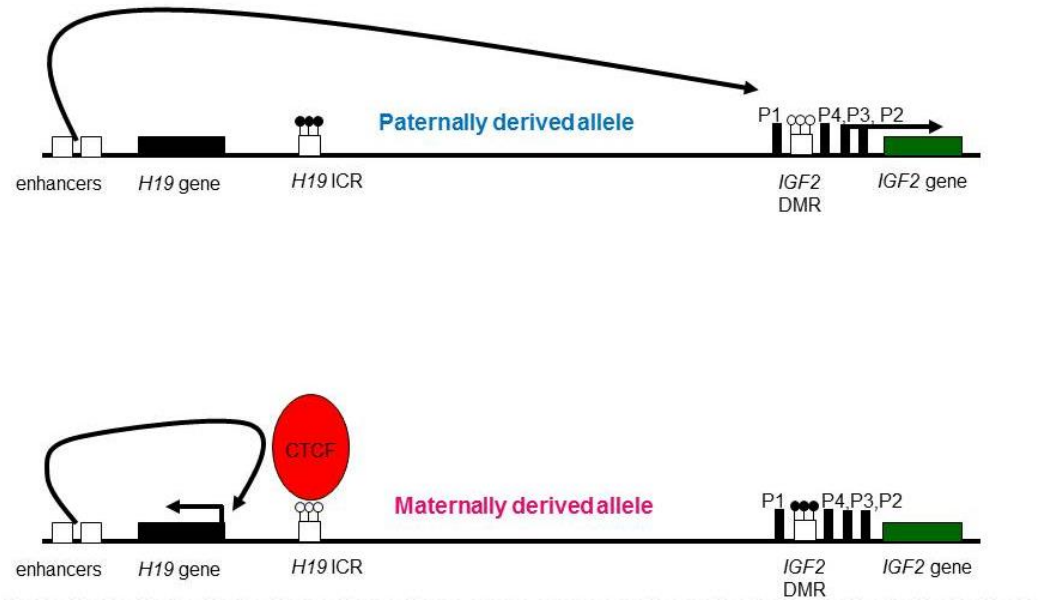

Figure 4-1: Allele-specific human *IGF2* and *H19* gene map

The human *IGF2* gene is genomically imprinted and reciprocally expressed with the maternally expressed *H19* gene, which encodes a non-coding RNA. On the paternal allele, the *IGF2* differentially methylated region (*IGF2* DMR) is unmethylated while the H19 imprinting control region (H19 ICR) is methylated. Methylation of the H19 ICR prevents CTCF from binding, and allows enhancer elements to initiate *IGF2* expression. On the maternal allele, the H19 ICR is unmethylated allowing CTCF to bind and block enhancer activity. This prevents *IGF2* expression from the maternally-derived allele.

P3 and P4 appear to be the most active [17,29,30]. P1 transcripts are usually associated with the subset of tissues that biallelically express *IGF2*, such as the liver and some parts of the brain [27,31], while P2 transcripts have been shown to be associated with transformed or neoplastic cells [30]. The increased expression of *IGF2* in some cancers can be the result of multiple promoter usage that results in multiple *IGF2* transcripts, or the enhanced activity of individual promoters. For example, the P3 promoter is hypomethylated in a subset of human osteosarcomas and results in increased *IGF2* transcripts from P3 [32], while imprinting is lost (LOI) in other osteosarcoma cells [24] and *IGF2* is expressed from several promoters on both alleles [29]. The potential for additional and/or enhanced *IGF2* promoter usage contributing to increased *IGF2* mRNA levels in benign fibrosis such as Dupuytren's disease (DD) has not been reported.

4.1.1 Dupuytren's Disease

DD is a benign fibromatosis affecting the palmar fascia that is characterized by the development of permanent finger contractures. The palmar fascia thickens and hardens as the result of excess collagen deposition by fibroblasts and contractile myofibroblasts within the disease cord [33–35]. This fibroproliferative disorder is one of the most common inherited connective tissue disorders affecting Caucasian males over 60 years [36,37] and there is a lack of truly effective treatment options. The "gold standard" treatment option, surgical resection of the disease cord [38,39], is associated with extensive post-operative rehabilitation and at least 1 in 3 patients will experience recurrence of contractures [40]. There is an obvious need for a better understanding of the molecular mechanisms that drive Dupuytren's Disease development so that more effective and non-surgical therapeutic alternatives can be made available to these patients.

Like virtually all fibroses, DD is characterized by excessive fibroproliferation, the increased presence and persistence of contractile myofibroblasts, and excess collagen deposition [41]. While relatively little is known about the roles of IGF-II in fibrosis development, it has been shown to induce collagen expression in systemic sclerosis-associated pulmonary fibrosis [42] and to increase the contractility of DD cells [43]. *IGF2* expression is also increased in DD cells relative to fibroblasts derived from non-

fibrotic palmar fascia whereas *IGF1* expression is unaffected [43]. The mechanisms that induce increased *IGF2* expression in DD have not yet been investigated.

4.2 Materials and Methods

4.2.1 Sample Collection: tissue and cells

Surgeons at the Roth | McFarlane Hand and Upper Limb clinic resected small palmar fascia tissue samples from patients with Dupuytren's Disease (DD) and from patients undergoing carpal tunnel release (CT) with the approval from the University of Western Ontario Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB protocol # 08222E, Appendix C). An additional tissue sample was taken from Dupuytren's Disease patients as adjacent, phenotypically normal palmar fascia (PF). All three samples were also cultured for primary fibroblasts. Patients signed consent forms, received a letter of information prior to surgery, and their samples were de-identified to ensure patient confidentiality.

4.2.2 Primary cell culture

Primary fibroblasts were grown for a week in alpha-minimum essential media (α-MEM) containing 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic and 1% L-Glutamine (all from Invitrogen Corporation, Carlsbad, CA) (growth media). Cells were passaged by first rinsing with 5 ml of phosphate buffered saline (PBS), and then incubating them with 2 ml 0.05% trypsin-EDTA (Invitrogen Corporation, Carlsbad, CA) at 37°C for 5 minutes to allow the cells to detach from the surface. Inactivation of the trypsin was accomplished by adding 2 ml of growth media with FBS. Cells, media and trypsin were then collected into a 15 ml conical tube by centrifugation (Hettich Zentrifugen Universal 32) at 2000 rpm for 5 minutes. The supernatant was then aspirated and the pellet was resuspended in 6 ml of media, split, and fresh growth media was added. The dishes were then incubated for 3-5 days. Fibroblasts were assessed up to a maximum of 6 passages.

4.2.3 RNA isolation and Real Time PCR

Total RNA samples were isolated from primary DD, PF and CT cells using the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol. The quality and concentration of RNA were assessed on a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA). cDNA was made from 2 μg of high quality total RNA using the High-Capacity cDNA Archive Kit (Life Technologies, Burlington, ON, Canada) in accordance with the manufacturer's instructions. TaqMan gene expression assays were used to measure *IGF2* (Hs01005963_m1) and *H19* (Hs00262142_g1) expressions relative to the *RPLP0* endogenous control (Hs99999902_m1) after validation of parallel PCR amplification efficiencies. PCR reactions employing the $\Delta\Delta C_T$ method were carried out under the following conditions: Initial denaturation at 95 °C for 5 min followed by cycles of denaturation (95 °C for 15 s), primer annealing (60 °C for 1 min) and transcript extension (50 °C for 2 min) for 45 cycles on a Real-Time PCR ABI Prism 7500.

4.2.4 Loss of imprinted expression

Loss of imprinting (LOI) was assessed using nested PCR as described previously [24,44]. Genomic DNA was isolated from patient-derived fibroblasts using the DNeasy Blood and Tissue Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. The DNA samples were screened for heterozygosity of a restriction fragment length polymorphism (RFLP) in the 3' untranslated region of the *IGF2* gene which creates an *Apa*I restriction enzyme site. The first round of PCR with primers PA and PC was performed on 20-50 ng of genomic DNA and the amplified products were used as the template for a second round of PCR using primers PB and PC. The PCR amplicon from the second PCR round was subjected to restriction digestion with *Apa*I (New England Biolabs, Whitby, ON, Canada) at room temperature overnight. Primer sequences were: PA: 5' TCCTGGAGACGTACTGTGCTA-3', PB: 5'-CTTGGACTTTGAGTCAAATTGG-3' and PC: 5'-GGTCGTGCCAATTACATTTCA-3' based on previous studies [24,44]. The positions of the primers are depicted in Figure 4-2. Cells were deemed "informative" if they were heterozygous for the *Apa*I polymorphism and their alleles could be distinguished by agarose gel electrophoresis.

Total RNA was isolated from informative patient-derived cells using the RNase-free DNase set with the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) in order to avoid genomic DNA contamination. First-strand cDNA was synthesized using Superscript II RT (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol with 500 ng of total RNA using the PC primer. The nested PCR protocol and restriction enzyme digest analyses described from genomic DNA samples was repeated on ~50 ng cDNA from these cells and a reverse transcriptase negative control was included to detect any chromosomal DNA contamination.

4.2.5 *IGF2* Promoter Usage

The protocol for identifying specific *IGF2* promoter usage was adapted from Grbesa *et al.* [29]. Promoter-specific primers were used to amplify reverse-transcribed RNA (cDNA) immediately downstream of specific *IGF2* promoters in DD, PF and CT cells. Primers are listed in Table 4-1 and locations in Figure 4-2. The reverse primer (PR) was common to all promoter-specific forward primers. Touchdown PCR was carried out with initial denaturation at 95°C for 5 min followed by 4 cycles of 95°C for 30 s, 68°C for 1 min and 72°C for 1 min, then again for 13 cycles except the annealing temperature decreased by 1°C each cycle from 68°C, followed by 25 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1 min, with the final extension at 72°C for 10 min in the Px2 Thermal Cycler (Thermo Scientific, Waltham, MA).

Table 4-1: Primers used during touchdown PCR to determine *IGF2* promoter usage in DD, PF and CT cells. The reverse primer was common to all 4 promoter-specific PCR reactions.

Promoter	Primer (5'to 3')	Expected Band Size
P1	CGAATTCTGGGCACCAGTGACTCCCCG	376 bp
P2	ACCGGGCATTGCCCCCAGTCTCC	254 bp
P3	CGTCGCACATTCGGCCCCCGCGACT	186 bp
P4	TCCTCCTCCTCCTGCCCCAGCG	134 bp
PR (reverse)	CAAGAAGGTGAGAAGCACCAGCAT	--

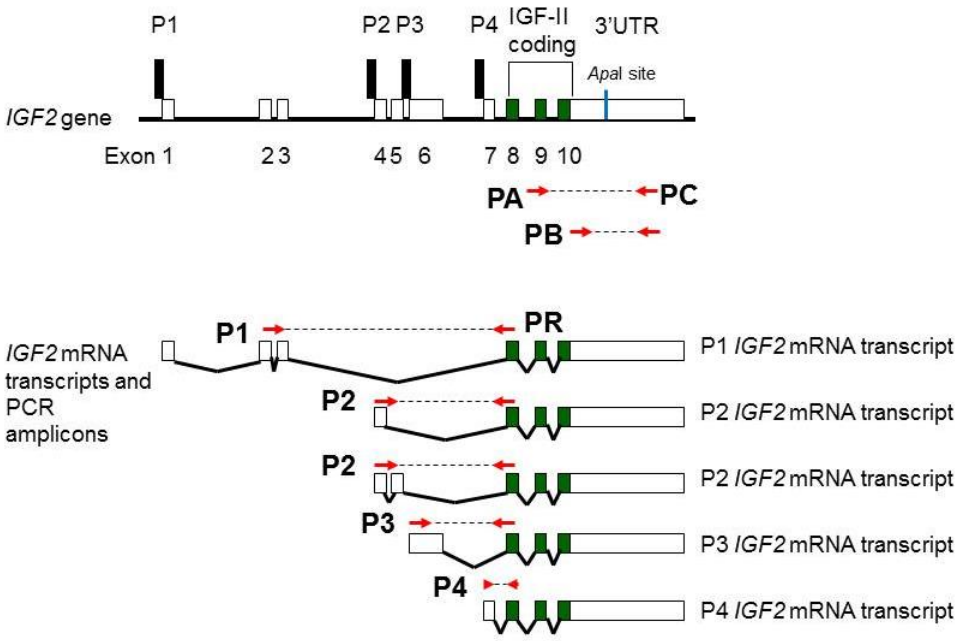

Figure 4-2: Primer map for *IGF2* gene

Primer map depicting position of primers used for promoter usage analysis (P1-P4, PR) and loss of allele-specific expression (PA, PB, PC). There are 4 distinct promoters that can initiate transcription of the IGF2 gene. The ApaI restriction fragment length polymorphism used for the loss of allele-specific expression analyses is located in the 3' untranslated region.

4.2.6 Epigenetic effects on *IGF2* expression

DD, PF and CT cells were cultured on type I rat tail collagen (~1.8 mg/ml stock concentration) to better replicate *in vivo* conditions. Neutralization solution (3 parts 10X Waymouth media (Sigma Aldrich, St. Louis, MO), 2 parts 0.34 M NaOH) was added to the collagen solution in a 1:4 ratio and 1 ml of this solution was added to coat the bottom of each well in a 6-well tray. The collagen was allowed to set in these trays at 37°C for 30 min. $1x10^5$ cells were seeded in each well of the collagen-coated tray and grown for 2 days in growth media. After 2 days, cells were rinsed twice in PBS and incubated with serum-free media overnight. Treatments were added to media containing 2% FBS, and incubated at 37°C with 5% CO_2. Each day, fresh media, containing new inhibitor was added until each treatment regime was completed. Treatments were as follows: decitabine, a DNA methyltransferase (DNMT) inhibitor (Cedarlane, Burlington, ON, Canada) was used at 10 µM for 72 hrs and trichostatin A (TSA), a histone deacetylase inhibitor (Cedarlane, Burlington, ON, Canada) was used at 300 nM for 48 hrs. Cells were then washed in PBS and incubated with 1 mL of 0.25 mg/ml collagenase XI (Sigma Aldrich, St. Louis, MO) in Hank's Balanced Salt Solution media (Sigma Aldrich, St. Louis, MO) at 37°C for 25 min with rocking. Once the cells were detached, the trays were placed on ice to inactivate the collagenase. The cells were collected and centrifuged (Hettich Zentrifugen Universal 32) at 2000 rpm for 5 minutes. The supernatant was removed, and the pellet was rinsed in 1X PBS and stored at -20ºC prior to RNA isolation and subsequent real-time PCR analysis of *IGF2* and *H19* expression. Experiments were performed on 4 patients (N = 4, n = 1). Paired t tests were used to determine significant changes in *IGF2* or *H19* expression when $p < 0.05$.

4.3 Results

4.3.1 Loss of imprinting in Dupuytren's Disease cells

IGF2 expression is increased in primary DD fibroblasts relative to controls, but the mechanisms involved in this induction are currently unknown. Loss of imprinting (LOI) results in biallelic expression and increased *IGF2* transcript levels in cancers; however there is no evidence of LOI expression of *IGF2* in benign fibroses to our knowledge. To

assess *IGF2* imprinting, DD, PF and CT cells were screened to determine whether they were "informative" for these analyses. The 3' untranslated region (3'UTR) of the *IGF2* gene contains a restriction fragment length polymorphism (RFLP) that introduces an *Apa*I restriction enzyme site. An *Apa*I restriction digest of genomic DNA amplified from the *IGF2* 3'UTR allowed for the identification of individuals with one or more of these RFLPs. In those individuals with an RFLP in only one allele (deemed "informative"), the two alleles could be distinguished by restriction digest analyses with *Apa*I and routine agarose gel electrophoresis. If normal *IGF2* imprinting is occurring, only one of these alleles (with or without the RFLP) will be identified in cDNA derived from reverse transcribed mRNA. If *IGF2* imprinting is lost and both alleles are transcribed, restriction mapping of cDNA derived from reverse transcribed mRNA is predicted to yield two DNA species, one with and one without an *Apa*I restriction site.

Eight PF/DD (N = 8, n = 1) and 7 CT patients (N = 7, n = 1) were screened for heterozygosity of the *IGF2* alleles. 3 PF/DD patients (#188, #265 and #274) were found to be informative based on the presence of two bands after the *Apa*I restriction digest of the *IGF2* 3'UTR PCR product derived from the genomic DNA by agarose gel electrophoresis (Figure 4-3A). Unfortunately, none of the 7 CT patient cell lines were informative (data not shown), with 4/7 containing- and 3/7 lacking the polymorphic *Apa*I site in both alleles. When mRNA samples derived from the informative DD cell lines were reverse transcribed into cDNA, 2 of the 3 informative patient samples yielded restriction patterns similar to their genomic DNA samples, indicating loss of *IGF2* imprinting. Restriction digest analysis of cDNA derived from patient #274 yielded a single band indicating the expression of a single allele that lacked an *Apa*I restriction site. Interestingly, evidence of LOI of *IGF2* was also evident in the PF cells derived from patients #188 and #265, whereas maintenance of *IGF2* imprinting was evident in PF cells derived from patient #274 (Figure 4-3B).

Biallelic expression of *IGF2* or LOI at the *IGF2* locus is usually associated with a reciprocal loss of *H19* expression [26]. To assess this in DD, *IGF2* and *H19* mRNA levels were assessed by real-time PCR in the 3 informative patients identified in the LOI studies. Consistent with previous analyses, all 3 DD cell lines exhibited higher *IGF2*

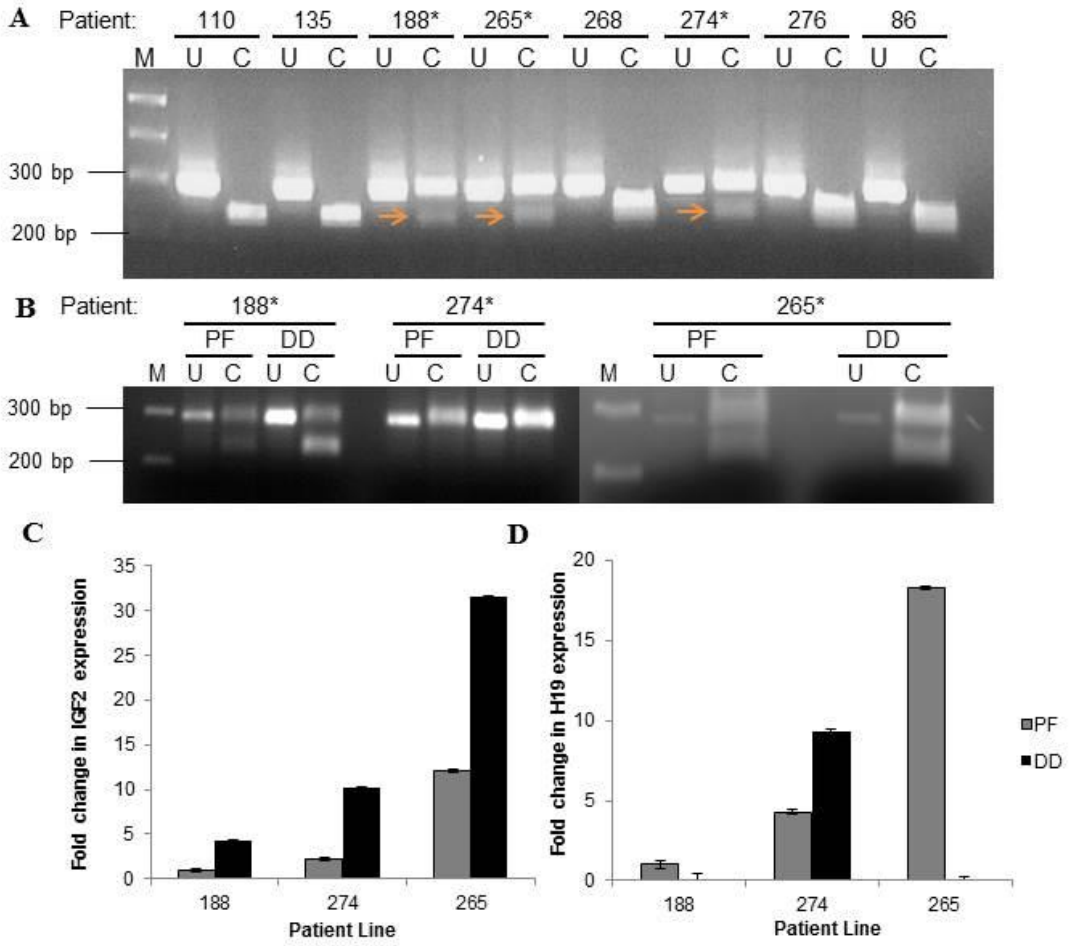

Figure 4-3: Loss of imprinting at the *IGF2* locus is detected in DD cells.

A) Genomic DNA samples from fibroblasts derived from patients with DD were screened for heterozygosity of an *Apa*I restriction site in the 3' UTR of the *IGF2* gene by PCR. U = uncut DNA, C = restriction digested with *Apa*I. Three patients (*, #188, #265 and #274) were found to be "informative" as indicated by the presence of two bands in the "C" lane (orange arrows) corresponding to one allele with and one without an *Apa*I restriction site. B) Reverse transcribed mRNA (cDNA) of these 3 patient-derived cells were analyzed by PCR to determine whether *Apa*I restriction digests of this region in *IGF2* gene resulted in 2 bands (confirming LOI) or a single band (maintenance of imprinting). C) QPCR analysis of *IGF2* and D) *H19* expression in PF (grey bars) and DD (black bars) cells derived from the 3 informative patients (N = 3, n = 1)

expression than their corresponding PF cells (Figure 4-3C). *H19* expression was undetectable after 45 PCR cycles in the DD cells derived from the two patients which displayed loss of imprinting (#188 and #265), indicating that the *H19* transcript levels were either absent or below detectable limits (Figure 4-3D). In contrast, *H19* mRNA was readily detectable in DD cells derived from patient #274, consistent with maintenance of normal *IGF2*/*H19* imprinting. In comparison to DD cells derived from the same patients, *H19* expression was readily detectable in all three sets of PF cells.

4.3.2 Histone deacetylase and DNA methyltransferase inhibitors increases *IGF2* and *H19* expression, respectively

Previous reports have shown that histone deacetylases (HDACs) regulate many imprinted genes, including *IGF2* in human fibroblasts [45]. To determine if HDACs and other epigenetic modifiers were having distinct effects in PF and DD cells, the effects of broad spectrum inhibitors of HDACs and DNA methyltransferases (DNMTs) on *IGF2* and *H19* expression were assessed. Trichostatin A, an established HDAC inhibitor [46,47], was shown to significantly induce *IGF2* expression (Figure 4-4A) and in PF ($p < 0.05$) and DD ($p < 0.01$) cells, and significantly attenuate *H19* expression (Figure 4-4B) in PF cells ($p < 0.01$), relative to vehicle treatment. Decitabine, an established DNMT inhibitor [48], induced *H19* expression (Figure 4-4B) in PF cells ($p < 0.05$) only.

4.3.3 Multiple promoters are active in Dupuytren's Disease

IGF2 transcripts can be derived from 4 different promoters (P1-P4) and their activities are reported to vary with tissue type, developmental stage and disease state [17,27,49]. To identify the *IGF2* promoters that were active in DD, PF and CT cells, promoter-specific primers were designed based on previous studies [29] and the expression levels of promoter-specific transcripts were assessed by qualitative reverse transcription PCR. RNA transcribed from HepG2 cells was used as a positive control for these analyses. Unexpectedly, despite P1 activity normally being highest in the liver [27], P1 transcripts were not detected in these liver carcinoma cells, which is in accordance with a previous study reporting that *IGF2*-P1 is silent in HepG2 cells [50]. As shown in Figure 4-5, *IGF2* transcripts from P3 and P4 were consistently expressed in all of the DD, PF and CT cell

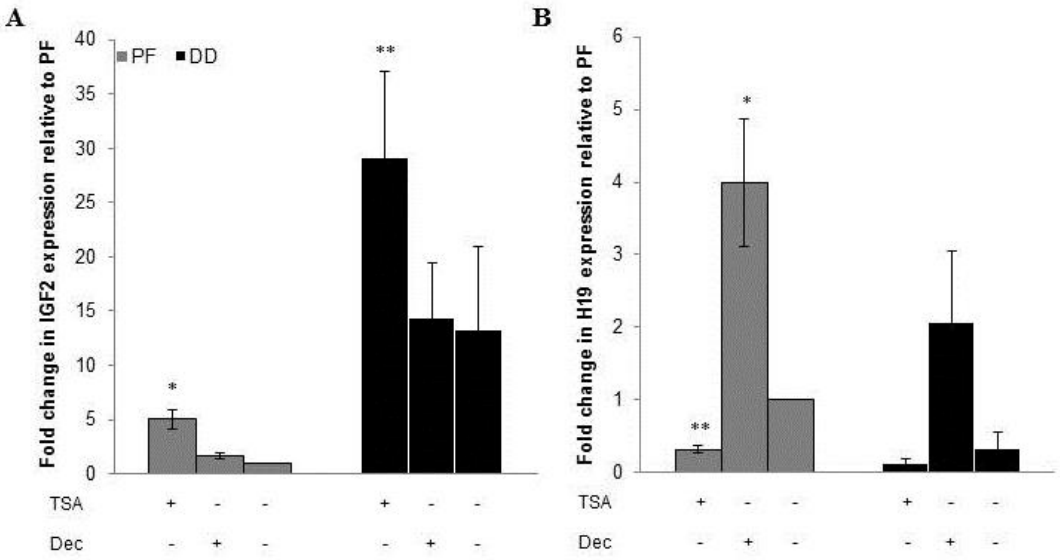

Figure 4-4: Inhibition of histone deacetylases induces *IGF2* expression in DD and PF cells and attenuates *H19* expression in PF cells.

The effects of trichostatin A (TSA, 300 nM for 48 hrs), a histone deacetylase inhibitor, and decitabine (Dec, 10 uM for 72 hrs), a DNA methyltransferase inhibitor were assessed on A) *IGF2* and B) *H19* expression in PF (grey bars) and DD cells (black bars) cultured on type I collagen (N = 4, n = 1). Treatment were refreshed each day in media containing 2% FBS. Significant treatment effects were determined by paired t test and are denoted as * $p < 0.05$ and ** $p < 0.01$. (Data is plotted as mean ± SE)

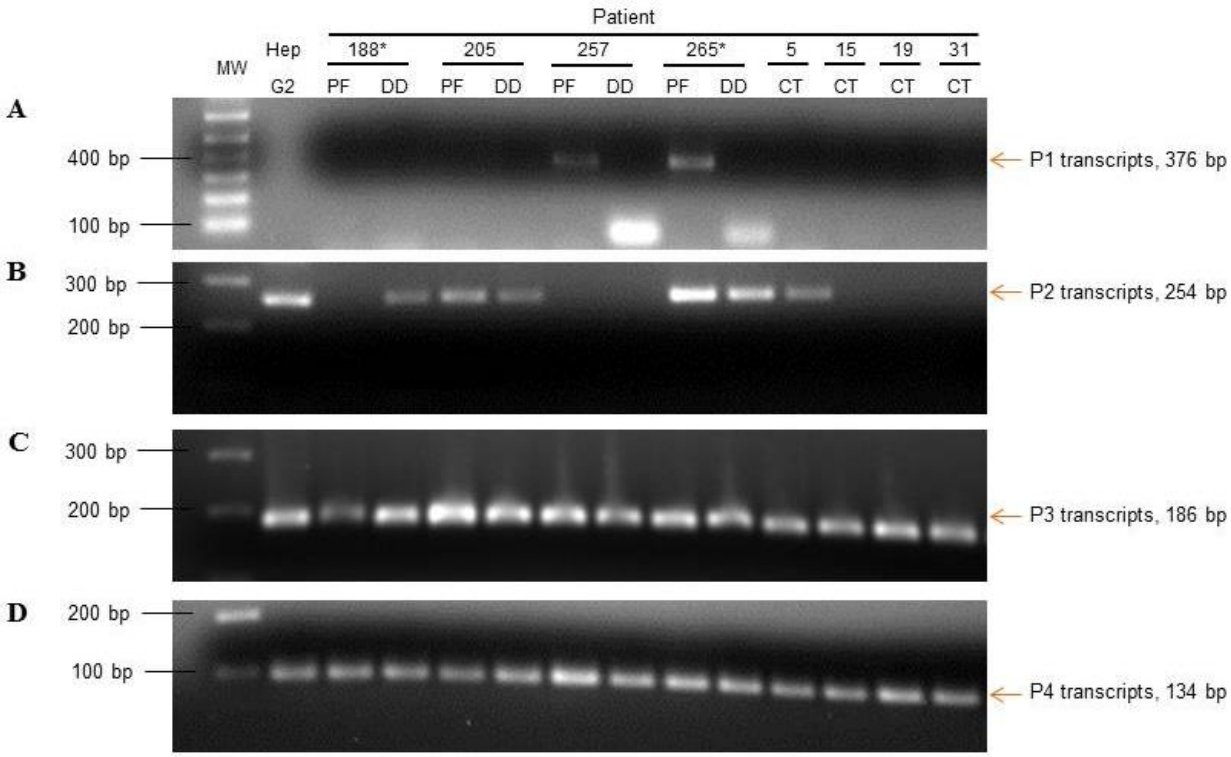

Figure 4-5: DD cells express *IGF2* transcripts derived from P2, P3 and P4.

PCR analysis of A) P1, B) P2, C) P3 and D) P4 promoter-derived *IGF2* transcripts in primary DD, PF and CT cells (N = 4 each, n =1) . Reverse transcribed RNA from HepG2 cells was used as a positive control in this analysis. As shown in A) *IGF2* transcripts originating at P1 were only evident in 2 PF cells isolates. B) *IGF2* transcripts originating at P2 were evident in a subset of DD, PF and CT cells. In contrast, *IGF2* transcripts originating at P3 (C) and P4 (D) were evident in all 3 cell types. (* denotes patient identified to display loss of imprinting).

lines assessed (N = 4/group, n = 1). In contrast, 3/4 of the DD cells, 2/4 of the PF cells and 1/4 of the CT cells utilized P2 to transcribe *IGF2*. Transcripts from P1 were identified in 2/4 PF patients (including patient #265, identified to have LOI of *IGF2*) whereas no evidence of P1 activity was evident in any of the DD or CT cell lines assessed.

4.4 Discussion

In this study, loss of imprinted *IGF2* expression and aberrant *IGF2* promoter usage were investigated as possible explanations for the increased *IGF2* expression in DD. Loss of imprinting was detected in a subset of DD patients and multiple *IGF2* promoters were activated in DD cells, both of which are likely to contribute to the increased *IGF2* expression in DD cells. Loss of imprinting of *IGF2* has been demonstrated in a variety of cancers [24] and the functional consequences of this are typically: increased *IGF2* expression and cell proliferation [51,52], presumably due to increased IGF-II signalling. LOI of *IGF2* was detected in two of the three "informative" patients with Dupuytren's Disease identified in this pilot study. Many more informative patients would need to be identified to gain further insight into how prevalent LOI of *IGF2* is in DD.

Interestingly, LOI of *IGF2* was also detected in PF cells derived from the non-fibrotic palmar fascia of these patients. While the palmar fascia from which PF cells are derived is phenotypically normal, it is immediately adjacent to fibrotic DD cord tissue and may be in an activated "pre-disease state". Genetic predisposition to DD development is also evident in many patient families, and thus PF cells may be predisposed to fibrosis relative to normal palmar fascia fibroblasts (CT cells). These PF cells exhibited LOI of *IGF2* but maintained detectable *H19* expression, unlike the DD cells derived from these patients. While preliminary, these data may be suggestive of progressive changes in the *IGF2*/*H19* axis during disease development. Additional studies will be necessary to determine if loss of *IGF2* imprinting precedes loss of *H19* expression during DD development.

The proportion of the population that are heterozygous for the *Apa*I polymorphism in the 3'UTR of *IGF2* was reported to be ~44% [53], which correlates well with our findings of 37.5% of DD patients being heterozygous for this RFLP. 62.5% of DD patients and 57%

of CT patients were homozygous for the *Apa*I polymorphism, which is higher than the 48.3% reported in larger population studies [53]. In contrast to CT patients, none of the DD patients that were screened in this analysis lacked the RFLP, a genotype which is reported to represent only ~8% of the population [53]. Taking all cell groups together, of the 15 patients screened for heterozygosity of the *Apa*I polymorphism, 20% were heterozygous, 60% were homozygous for the site, while 20% lacked the polymorphism. While the different frequencies of the genotypes observed in this study are likely to be skewed by the small sample size, it is also plausible that the frequencies differ between disease and normal populations. Larger scale studies would be required to establish more accurate genotype frequencies within Dupuytren's Disease populations relative to normal.

Since no heterozygous (informative) CT patients were identified in this study, no conclusions can be drawn in the context of a "baseline" for LOI of *IGF2* in the normal population. Previous studies have indicated that 20 to 25% of Caucasians with a mean age of 60 years demonstrate LOI of *IGF2* [54]. LOI of *IGF2* may be inherited or congenitally acquired and be stable over time in these patients [54]. It is unclear if the findings of this study, indicating LOI of *IGF2* in 2/3 informative patients, indicate chance findings or if LOI of *IGF2* is a risk factor for DD development. Additional, much larger scale studies will be required to address these possibilities.

The use of *IGF2* promoter P1was identified in 50% of the PF cells assessed in this study, but not in any of the DD or CT cells assessed. P1 transcripts are normally evident in the liver and in those parts of the brain where biallelic expression of *IGF2* is the norm. Two of the four patients from whom these PF cells were derived also demonstrated LOI of *IGF2*, however only one expressed P1 transcripts. These P1 transcripts were expressed in PF cells, but not in DD cells derived from the same patients with increased *IGF2* expression. This is not the first study in which the presence of *IGF2* transcripts from P1 has failed to correlate with LOI of *IGF2* [29] and further analyses will be required to determine the relevance of these findings.

Further studies will be required to gain a better understanding of LOI of *IGF2* in DD. These studies should include investigations of CCCTC binding factor (CTCF) interactions with the H19 imprinting control region (ICR), assessments of the methylation status of the H19 ICR and of the *IGF2* differentially methylated region (DMR). Methylation at the H19 ICR would prevent CTCF from binding and allow enhancers to activate *IGF2* expression while silencing *H19*. The current dogma would predict that the H19 ICR will be methylated in patients demonstrating LOI of *IGF2* while the IGF2 DMR may be unmethylated. Previous studies in other systems would suggest that the methylation status of these loci are integral to maintaining imprinting at the *IGF2*-H19 axis [21,26,55]. The addition of a DNMT inhibitor to PF cells significantly increased *H19* expression and a non-significant trend ($p = 0.123$) towards increased *H19* expression was evident in DD cells. These findings are consistent with hypomethylation of the H19 ICR, which allows CTCF to bind and activate *H19* transcription. Unexpectedly, treatment of DD and PF cells with the DNMT inhibitor had no discernible effects on *IGF2* expression. Based on previous studies [56,57], decitabine (5-aza-2-deoxy-cytidine) treatment was predicted to initiate expression of *IGF2* from the maternal allele and to potentially increase P2-P4 promoter usage. One possible explanation is that decitabine only inhibits DNA methylation in newly replicated DNA [48]. Thus, it may be necessary to treat cells over a multiple passages to induce the increases in *IGF2* expression reported in other systems [57]. As P2 was activated more frequently in DD cells, it is possible that the *IGF2*-P2 promoters are in a less methylated state in these cells relative to the same sites in CT cells. Future studies should focus on determining the relative methylation status of the P2 and P3 promoter regions in DD, PF and CT cells.

Histone acetylation is an epigenetic mark that promotes opening of the chromatin structure and the activation of gene transcription [58]. Trichostatin A (TSA) has been shown to inhibit histone deacetylases (HDACs), the enzymes that inhibit gene transcription by removing these acetylation marks [46,47]. HDACs can regulate imprinted genes in human fibroblasts and HDAC inhibition by TSA has been previously demonstrated to induce LOI of *IGF2* [45]. TSA treatment of PF and DD cells increased *IGF2* expression in both groups, suggesting that histone acetylation is likely to have a role in regulating *IGF2* gene expression in these cells. Future studies could confirm

whether HDAC inhibition increased *IGF2* expression in PF and DD cells by inducing biallelic *IGF2* expression.

Both loss of imprinting and activation of the *IGF2*-P2 promoter are likely to contribute to the increased *IGF2* expression evident in DD cells. This increase in *IGF2* expression is predicted to cause increased IGF-II signalling and promote the contractility of DD cells. IGF-II inhibitors or factors that regulate *IGF2* expression may have potential as therapeutic targets to attenuate DD progression and recurrence. Additionally, if loss of imprinting is confirmed to be a relatively common component of DD development, biallelic *IGF2* expression may have utility as one of several markers to differentiate between the general population and those individuals who are predisposed to develop Dupuytren's Disease.

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Chapter 5

5 General Discussion and Conclusion

5.1 Thesis Summary

Dupuytren's Disease (DD) is a heritable and benign fibromatosis that affects the palmar fascia. While the name specifically refers to contractile fibrosis of this particular tissue, this disease displays characteristics that are common to other connective tissue fibroses [1]. Two of these characteristics are increased β-catenin levels and the repressed expression of *IGFBP6*. In chapter 2 of this thesis, β-catenin was shown to associate with the promoter region of *IGFBP6* in PF and CT cells, which are derived from non-fibrotic tissues, but not fibrosis-derived DD cells. These findings correlated with increased *IGFBP6* expression levels in PF and CT cells relative to DD cells and were consistent with causative link between loss of β-catenin interactions with the *IGFBP6* promoter and *IGFBP6* expression. In addition to *IGFBP6*, more than 2 000 additional and novel genes were identified that have interactions with β-catenin that were specific to DD, PF or CT cells.

In chapter 3, the functional consequences of depleting *IGFBP6* expression was examined in the contexts of cellular proliferation, contractility and migration. Exogenous addition of IGFBP-6 was shown to inhibit the proliferation of DD, PF and CT cells. The major IGFBP-6 ligand, IGF-II, was found to be upregulated in DD, and distinct functional roles for this peptide were identified in CT, PF and DD cells. These novel roles included IGF-II induced increases in the cellular contractility and migration of DD cells, increased contractility of PF cells and increased proliferation of CT cells.

Lastly, the mechanisms that promote the overexpression of *IGF2* in DD cells were explored in chapter 4. Loss of *IGF2* imprinting was identified in a subset of DD patients, and cell-type specific aberrations in *IGF2* promoter usage were identified in DD, PF and CT cells. The findings reported in Chapters 2, 3 and 4 suggest that a complex array of transcriptional modifications and signalling events promote DD development. Some of these events can be envisaged as contributing to a potential feedback loop, where loss of

β-catenin interactions with the *IGFBP6* promoter region in DD cells leads to downregulation of *IGFBP6* expression and IGFBP-6 secretion, thereby enhancing IGF-II signalling, contraction of ECM by DD cells and, based on previous literature [2,3], IGF-II and mechano-transduction induced changes in the accumulation of β-catenin in palmar fascia fibroblasts.

5.2 Dupuytren's Disease and cancer development

The previous studies from our laboratory that identified β-catenin accumulation in DD cells [4] correlated with the increased levels of β-catenin interactions with the genome in DD cells relative to CT and PF cells reported in this thesis. In addition to the findings discussed in detail in Chapter 2, the Ingenuity analysis revealed that many of the genes specifically associated with β-catenin in DD cells have been previously reported to have roles in cancer development. These findings may have relevance to the increased incidence of cancer development in patients with a history of DD [5–7]. Correlations between types of abnormal wound repair including fibrosis and cancer development have been extensively reviewed in the literature [8], and there is evidence that some of the molecular mechanisms that promote fibrosis can lead to cancer development [9,10]. The stroma microenvironment around epithelial tumours has many similarities to the extracellular matrix in fibrosis, including the increased deposition of collagens and other ECM molecules, and these changes have been correlated with increased cancer risk [11–13] and enhanced myofibroblast differentiation [14].

The findings of loss of *IGF2* imprinting and aberrant *IGF2* promoter usage in DD and PF cells described in Chapter 4 may provide additional insights into the links between DD and tumour development. Loss of *IGF2* imprinting is a relatively common occurrence in cancers and has been implicated in tumour development [15–17]. While loss of *IGF2* imprinting is associated with overgrowth syndromes [18], a subset of the apparently normal population can also exhibit loss of *IGF2* imprinting in some tissues [19]. It is unclear whether these individuals are predisposed to cancer development, and/or from the findings in Chapter 4, DD development relative to the majority of the population exhibiting normal *IGF2* imprinting. Much larger scale studies will be required to

determine if loss of *IGF2* imprinting is over-represented in a subset of patients with DD, and if so, if those patients have an increased incidence of cancer development.

5.3 Origin of DD myofibroblasts

Cancer-associated fibroblasts (CAFs) have been shown to enhance epithelial-mesenchymal transition (EMT) and tumour progression by releasing growth factors and cytokines into the stroma [20,21]. β-catenin-mediated trans-regulation of gene transcription has a well-documented role in promoting EMT [2,22–24], and IGF-II is one of the growth factors shown to induce β-catenin nuclear translocation and EMT [2]. It is plausible that keratinocytes from the epidermis or endothelial cells from adjacent blood vessels may undergo epithelial or endothelial transition to fibroblasts or myofibroblasts (Figure 5-1) to promote DD progression. DD-associated fibroblasts have been shown to secrete factors, such as TGF-β, IGF-II and EGF, with established roles in promoting EMT through β-catenin-mediated processes [2,25–28]. As shown in Figure 5-2, TGF-β can downregulate *IGFBP6* expression and IGFBP-6 secretion, effects that are predicted to increase IGF-II signalling. Increased IGF-II signalling may promote nuclear translocation of β-catenin and facilitate EMT in DD. TGF-β signalling can also increase β-catenin levels in DD [3], however whether this increase in total cellular β-catenin induces nuclear localization of β-catenin in DD cells is yet to be explored. It is currently unclear if TGF-β can induce EMT independently of IGF-II or if synergistic interactions are required to activate this process. Future studies should address the possibility that TGF-β and/or IGF-II can induce EMT in a β-catenin-dependent manner and enhance DD progression. If this is shown to be the case, the palmar epidermis of individuals with DD may be compromised by the local fibrosis, and therapeutic interventions to EMT in the palmar epithelium may inhibit DD recurrence.

Interestingly, many of the genes shown to associate with β-catenin in DD and PF cells have established roles in cells of neuronal origin (See Appendices F and G). The neural crest has been hypothesized as a source of mesenchymal stem cells (MSCs) [29], and increased numbers of MSCs have been reported in DD relative to controls [30,31]. These cells were postulated to have been derived from both perinodular fat and disease cord tissues in DD patients and were not evident in palmar fascia samples derived from

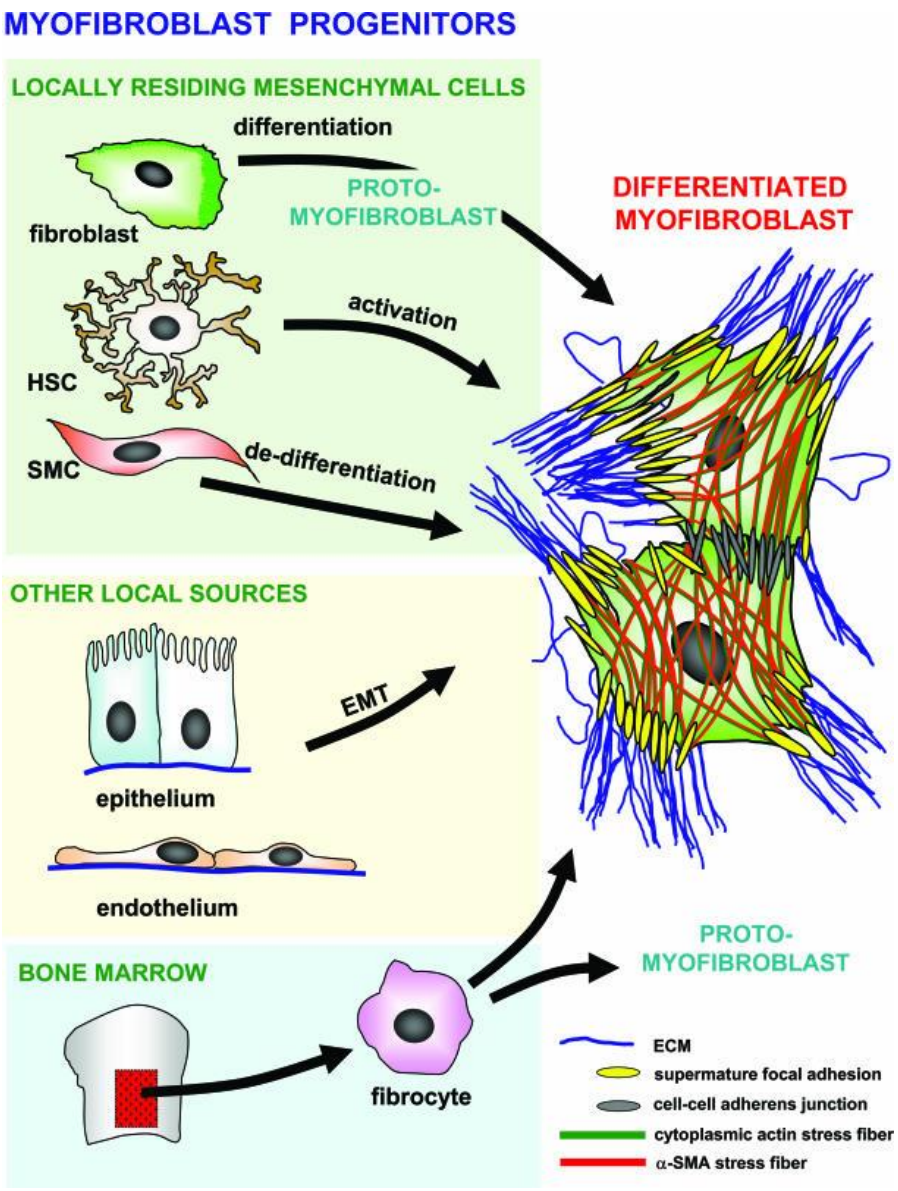

Figure 5-1: Origin of myofibroblasts.

Myofibroblasts can potentially be derived from many sources, including resident fibroblasts, hepatic stellate cells (HSCs), smooth muscle cells (SMCs), epithelium or endothelium (through epithelial- or endothelial- mesenchymal transition (EMT), or from the bone marrow, or from stem cells. (Reprinted with permission from Hinz *et al.* [32]). The source(s) of the myofibroblasts that induce finger contractures in DD are yet to be determined.

patients undergoing carpal tunnel surgeries [30]. Unfortunately, it is unclear if this increase in MSC numbers in DD tissues was truly associated with DD development or simply a consequence of a wound healing event in the palmar fascia that was not evident in the unwounded palmar fascia used as controls. Increased numbers of MSCs are a normal finding during the repair of many tissues [33–35]. Another possibility is that DD cells are derived from pericytes, a type of perivascular cell that is derived from the neural crest, exhibit neural epitopes including NG2, are α-SMA-positive and are highly contractile [36,37]. Future studies should include the analyses of DD cells for NG2 and other neural crest markers to confirm the origins of these cells.

5.4 Is *IGFBP6* a "fibrosis suppressor gene"?

The findings reported in Chapter 3 demonstrated that IGFBP-6 can inhibit basal proliferation and IGF-II induced contraction of DD cells. As *IGFBP6* expression and IGFBP-6 secretion are attenuated in DD, this molecule could represent a "fibrosis suppressor" that is turned off by the loss of β-catenin interactions during DD development. Thus, re-expression of *IGFBP6* in DD cells to the levels evident in CT cells may drive palmar fascia fibroblasts towards conditions approaching normal homeostasis where fibrosis is inhibited.

Bexarotene (trade name Targretin, LGD1069) is a retinoid X receptor agonist (rexinoid) that has been shown to increase IGFBP-6 levels in other systems [38]. This therapeutic intervention may potentially inhibit connective tissue fibroses including DD. Bexarotene is an FDA approved cutaneous T cell lymphoma treatment [39] that is currently being assessed for its efficacy against other cancers [40,41] and neurological diseases [42,43]. Previous studies in breast cancer indicate that the enhanced expression and secretion of IGFBP-6 is a primary mechanism by which bexarotene inhibits breast cancer cell growth through an AP-1/c-fos/c-jus-mediated mechanism [38,44]. Additionally, bexarotene has been shown to repress *CCND1* [45], encoding the proliferation-associated cell-cycle protein cyclin D1, and to inhibit cyclooxygenase-2 (COX-2) [44], which is reported to increase stromal fibrosis in colorectal cancer [46]. COX-2 inhibitors have been shown to reduce fibrosis in other systems [47]. It is plausible that bexarotene would increase IGFBP-6 secretion in fibroblasts and repress *CCND1* to limit fibroproliferation in DD.

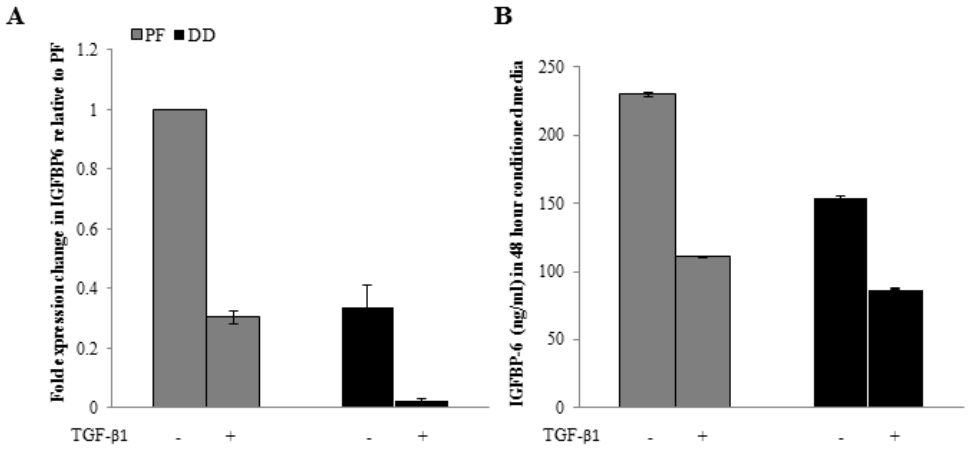

Figure 5-2: TGF-β1 treatment of PF and DD cells represses A) *IGFBP6* expression and B) IGFBP-6 secretion.

PF and DD cells were treated with TGF-β1 (12.5 ng/ml) for 48 hours. RNA was extracted and reverse transcribed for analysis by real-time PCR for *IGFBP6* expression. Media was collected and assessed by Luminex Multiplex assay for IGFBP-6 protein levels. TGF-β1 further repressed both mRNA and protein levels relative to vehicle treated cells.

Enhanced IGFBP-6 levels would also be predicted to inhibit IGF-II signalling, thereby attenuating IGF-II induced myofibroblast contractility. While outside of the main scope of this thesis, pilot studies were performed to assess the effects of bexarotene treatment of DD cells *in vitro*. The preliminary findings indicate that treatment with 1 μM bexarotene induces *IGFBP6* expression by approximately 2-fold and inhibits the proliferation of DD fibroblasts by approximately 30% over 7 days without affecting PF cell proliferation (data not shown). These preliminary findings support the hypothesis that bexarotene may have utility as an inducer of endogenous *IGFBP6* expression and inhibitor of fibroproliferation in DD, and these studies are ongoing in our laboratory.

5.5 Mechanisms of IGF-II induced contraction

A novel role for IGF-II as an inducer of DD myofibroblast contractility was identified in Chapter 3. Intriguingly, IGF-II induced contraction of DD and PF cells without inducing any significant effects on α-smooth muscle actin (α-SMA) expression or levels, suggesting that this increased contractility was independent of the differentiation state of these cells. In addition, IGF-II was shown to induce the ability of DD cells to contract unstressed collagen lattices. This effect was interpreted as likely to be due to an increase in three-dimensional migration within the collagen lattice based on the similarities between the effects of IGF-II and platelet derived growth factor (PDGF), shown to induce migration in these lattices in previous studies [48,49]. While the signalling pathways induced by IGF-II to elicit these effects are yet to be elucidated, an intriguing possibility is that they are mediated through the activation of focal adhesion kinase (FAK). FAK activation is an established component of integrin signalling and cell migration (reviewed in [50,51]). Links between ligand-induced activation of the IGFIR and FAK activity have been reported in cancer cell models [52]. These studies reported IGF-I induced migration of breast cancer cells through RACK1 (receptor for activated C kinases), a scaffold protein which links the IGF and integrin pathways [53,54]. IGF-I signalling through the IGFIR can recruit RACK1 to induce an association with the β1 integrin subunit through FAK to facilitate cell migration [53]. It is currently unclear if IGF-II signalling can induce similar effects through the IGFIR, and this should be a priority for future studies.

In addition to its effects on DD migration in three dimensional collagen matrices, pilot studies beyond the scope of this thesis have provided preliminary evidence that IGF-II can induce the expression of *CDH11*, encoding OB-cadherin, in stressed FPCLs. Increases in *CDH11* expression and OB-cadherin levels have been correlated with increased myofibroblast contractility in previous studies [55]. The inclusion of OB-cadherin in adherens junctions has been shown to enhance myofibroblast contractility by increasing the strength of cell-cell contacts [55], and this may suggest an additional potential mechanism by which IGF-II might be inducing these effects. Interestingly, β-catenin has been shown to interact with the 3' untranslated region (UTR) of *CDH11* to stabilize *CDH11* expression [56]. Future studies would be required to determine if this β-catenin associates with *CDH11* in DD and if IGF-II can induce *CDH11* expression in a β-catenin-dependent manner.

5.6 Toward novel therapeutic interventions in Dupuytren's Disease

In contrast to its well-established roles in fetal and tumour development, very little is known about IGF-II in the context of fibrosis. IGF-II signalling has been shown to induce collagen expression in systemic sclerosis-associated pulmonary fibrosis [57] and future studies should focus on determining if IGF-II plays a similar role during DD development. Excessive collagen induction is a hallmark of DD, as it is of most other fibroproliferative disorders [58–60]. While the findings reported in this thesis support distinct roles for IGF-II in promoting fibroblast migration and myofibroblast contraction in DD, it is important that future studies assess the combinatorial roles of IGF-II with other growth factors and cytokines that contribute to the DD microenvironment. The suppression of IGF-II signalling may help to ameliorate DD severity or recurrence, but it is unlikely that inhibition of IGF-II signalling in isolation will be sufficient. Therapeutic interventions that increase endogenous IGFBP-6 levels, such as bexarotene, may inhibit fibro-proliferation through IGF-II dependent and IGF-II independent mechanisms and be more effective than targeting IGF-II alone. However, the most effective therapeutic approach may be to inhibit fibroproliferation by attenuating β-catenin-mediated trans-regulation of gene transcription in DD.

Resveratrol (3,4',5-trihydroxystilbene) is a natural phytoalexin which has been demonstrated to decrease survival of colorectal cancer and Wnt-stimulated cells. Its mechanism of action appears to be dependent on inhibiting β-catenin interaction with TCF3, thereby suppressing the expression of c-Myc, Axin2 and cyclin D1 and potentially other targets of the Wnt signalling pathway [61]. Additionally, Nefopam (5-methyl-1-phenyl-1,3,4,6-tetrahydro-2,5-benzoxazocine) has recently been identified as having utility in reducing β-catenin levels in aggressive fibromatosis. This drug can inhibit proliferation of fibroblasts derived from aggressive fibromatosis and hypertrophic scars without discernible effects on control cells, and has been shown to reduce scar size *in vivo* [62]. It may be possible to combine multiple treatments to target both the IGF-II and β-catenin signalling pathways in Dupuytren's Disease to inhibit disease progression and/or recurrence. It is hoped that the findings presented in this thesis will provide an incremental advance in our knowledge of the pathophysiology of DD and facilitate the development of more effective therapeutic interventions for these patients.

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Appendices

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Appendix C: Western University Research Ethics Board for Health Sciences Research involving Human Subjects (HSREB protocol # 08222E)

Research Western

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. Bing Siang Gan
Review Number: 08222E
Review Level: Delegated
Approved Local Adult Participants: 1000
Approved Local Minor Participants: 0
Protocol Title: Molecular mechanisms of Dupuytren's Contracture, related fibroproliferative diseases and other human disorders.
Department & Institution: Surgery,
Sponsor:
Ethics Approval Date: November 08, 2011 **Expiry Date:** December 31, 2016
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Addition of Co-investigator	A new co-investigator has been added - Dr. Roel Ophoff from UCLA.	
Revised Study End Date	The researcher has requested a 5 year extension on this study - December 31, 2016.	
Revised UWO Protocol	With this new co-investigator samples will now be sent off site. This has been revised in the letter of information.	
Revised Letter of Information & Consent	September 2011 - Controls	
Revised Letter of Information & Consent	September 2011 - Study Subjects	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The UWO HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer to Contact for Further Information

Janice Sutherland (jsuther1@uwo.ca)	Grace Kelly (grace.kelly@uwo.ca)	Shantel Walcott (swalcot@uwo.ca)

The University *of* Western Ontario
Office of Research Ethics

Appendix D: PERL script used to process raw ChIP Seq data and align to genome

```
for d in $( ls ./ | grep _lib ); do
      echo $d
      mkdir md5
      mv $d/*.md5 md5/
      for f in $( ls $d/ | grep -v md5 | grep fastq ); do
            echo $d/$f
            gunzip $d/$f
      done
done

for f in $( ls ./ | grep fastq ); do
      echo $f
      cat $f >> $SAMPLE.all.fastq

      echo "removing $f"
      rm $f
done
```

Appendix E: PERL Script used to map common peaks back to human genome

```
#!/usr/bin/env perl -w
use strict;

#open the sorted bed file and create an array
#of positions for a chromosome (specified on the command line)
#containing accesssion numbers

#ARGV[0] is sorted bed file
#ARGV[1] is the chromosome
#ARGV[2] is the filtered file
print "to run:\n./merge_tabular_bed_efficient.pl
../Downloads/Galaxy47-\[Sorted_RefSeq_Upstream_50kb\].bed
../Downloads/Galaxy147-\[Filter_on_data_145\].tabular chr1 >
test_chr1.txt \n" if ! $ARGV[0];
exit if ! $ARGV[0];
#print out accession for start and stop positions in filtered
file
my @start = my @stop; my %id;
open (IN, "< $ARGV[0]") or die;
      while(my $l = <IN>){
            my @l = split/\t/, $l;
            chomp $l;
            if ($l[0] eq$ARGV[2]){
                  push @start, $l[1];
                  push @stop, $l[2];
                  my @nm = split/_/, $l[3];
                  $id{$l[1]} = $nm[0] . "_$nm[1]";
            }
      }
close IN;

my %seen;
open (IN, "< $ARGV[1]") or die;
      while(my $l = <IN>){
            chomp $l;
            my @l = split/\t/, $l;
            if ($l[0] eq $ARGV[2]){
                  for (my $i=0; $i < @start; $i++){
                        if ($l[1] >= $start[$i] && $l[2] <=
$stop[$i]){

                        print "$id{$start[$i]}\n" if !defined
$seen{$start[$i]};
                        $seen{$start[$i]} = "";

                        }
                  }
            }
      }
close IN;
```

Appendix F: Select β-catenin associations within intronic regions of a gene in PF cells, but not corresponding DD cells

RefSeq Accession Number	Gene Symbol	Gene Description	Chromosomal Location
NC_000005.10	ADAMTS 19	ADAM metallopeptidase with thrombospondin type 1 motif, 19	5q23.3
NC_000012.12	ADAMTS 20	ADAM metallopeptidase with thrombospondin type 1 motif, 20	12q12
NC_000007.14	ADCY1	adenylate cyclase 1 (brain)	7p13-p12
NC_000008.11	ADCY8	adenylate cyclase 8 (brain)	8q24
NC_000005.10	AFAP1L1	actin filament associated protein 1-like 1	5q32
NC_000015.10	APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2	15q11-q12
NC_000004.12	APBB2	amyloid beta (A4) precursor protein-binding, family B, member 2	4p13
NC_000004.12	ARHGEF3 8	Rho guanine nucleotide exchange factor (GEF) 38	4q24
NC_000010.11	ARMC3	armadillo repeat containing 3	10p12.31
NC_000002.12	BMP10	bone morphogenetic protein 10	2p13.3
NC_000020.11	BMP7	bone morphogenetic protein 7	20q13
NC_000009.12	CACNA1 B	calcium channel, voltage-dependent, N type, alpha 1B subunit	9q34
NC_000003.12	CACNA1 D	calcium channel, voltage-dependent, L type, alpha 1D subunit	3p14.3
NC_000003.12	CADM2	cell adhesion molecule 2	3p12.1
NC_000012.12	CD163L1	CD163 molecule-like 1	12p13.3
NC_000001.11	CD247	CD247 molecule	1q24.2
NC_000001.11	CD48	CD48 molecule	1q21.3-q22
NC_000005.10	CDH12	cadherin 12, type 2 (N-cadherin 2)	5p14.3
NC_000016.10	CDH13	cadherin 13	16q23.3
NC_000020.11	CDH4	cadherin 4, type 1, R-cadherin (retinal)	20q13.3

NC_000003.12	CNTN4	contactin 4	3p26.3
NC_000011.10	CNTN5	contactin 5	11q22.1
NC_000006.12	COL19A1	collagen, type XIX, alpha 1	6q12-q13
NC_000007.14	COL26A1	collagen, type XXVI, alpha 1	7q22.1
NC_000002.12	CTNNA2	catenin (cadherin-associated protein), alpha 2	2p12-p11.1
NC_000010.11	CTNNA3	catenin (cadherin-associated protein), alpha 3	10q22.2
NC_000007.14	EGFR	epidermal growth factor receptor	7p12
NC_000022.11	FAM19A5	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5	22q13.32
NC_000009.12	FAM219A	family with sequence similarity 219, member A	9p13.3
NC_000013.11	FGF14	fibroblast growth factor 14	13q34
NC_000007.14	FOXP2	forkhead box P2	7q31
NC_000002.12	GPR155	G protein-coupled receptor 155	2q31.1
NC_000007.14	GPR37	G protein-coupled receptor 37 (endothelin receptor type B-like)	7q31
NC_000011.10	GRIK4	glutamate receptor, ionotropic, kainate 4	11q22.3
NC_000012.12	GRIP1	glutamate receptor interacting protein 1	12q14.3
NC_000006.12	GRM1	glutamate receptor, metabotropic 1	6q24
NC_000001.11	HHAT	hedgehog acyltransferase	1q32
NC_000009.12	JAK2	Janus kinase 2	9p24
NC_000018.10	LAMA3	laminin, alpha 3	18q11.2
NC_000011.10	METTL15	methyltransferase like 15	11p14.1
NC_000016.10	MIR3680-2	microRNA 3680-2	
NC_000023.11	MIR548F5	microRNA 548f-5	Xp21.1
NC_000015.10	MYO1E	myosin IE	15q21-q22
NC_000002.12	MYO3B	myosin IIIB	2q31.1-q31.2
NC_000010.11	PCDH15	protocadherin-related 15	10q21.1
NC_000005.10	PCDHA1	protocadherin alpha 1	5q31

NC_000005.10	PCDHA10	protocadherin alpha 10	5q31
NC_000005.10	PCDHA11	protocadherin alpha 11	5q31
NC_000005.10	PCDHA12	protocadherin alpha 12	5q31
NC_000005.10	PCDHA13	protocadherin alpha 13	5q31
NC_000005.10	PCDHA2	protocadherin alpha 2	5q31
NC_000005.10	PCDHA3	protocadherin alpha 3	5q31
NC_000005.10	PCDHA4	protocadherin alpha 4	5q31
NC_000005.10	PCDHA5	protocadherin alpha 5	5q31
NC_000005.10	PCDHA6	protocadherin alpha 6	5q31
NC_000005.10	PCDHA7	protocadherin alpha 7	5q31
NC_000005.10	PCDHA8	protocadherin alpha 8	5q31
NC_000005.10	PCDHA9	protocadherin alpha 9	5q31
NC_000006.12	PKIB	protein kinase (cAMP-dependent, catalytic) inhibitor beta	6q22.31
NC_000006.12	PLG	plasminogen	6q26
NC_000007.14	RELN	reelin	7q22
NC_000009.12	SLC24A2	solute carrier family 24 (sodium/potassium/calcium exchanger), member 2	9p22.1
NC_000002.12	SLC25A12	solute carrier family 25 (aspartate/glutamate carrier), member 12	2q24
NC_000008.11	SLC30A8	solute carrier family 30 (zinc transporter), member 8	8q24.11
NC_000002.12	SP3	Sp3 transcription factor	2q31
NC_000019.10	ZNF431	zinc finger protein 431	19p12
NC_000007.14	ZNF716	zinc finger protein 716	7p11.2
NC_000007.14	ZNF804B	zinc finger protein 804B	7q21.13

Appendix G: Select β-catenin associations within intronic regions of a gene in DD cells, but not corresponding PF cells

RefSeq Accession Number	Gene Symbol	Gene Description	Chromosomal Location
NC_000008.11	ADAM18	ADAM metallopeptidase domain 18	8p11.22
NC_000008.11	ADAM2	ADAM metallopeptidase domain 2	8p11.2
NC_000007.14	ADAM22	ADAM metallopeptidase domain 22	7q21
NC_000002.12	ADAM23	ADAM metallopeptidase domain 23	2q33
NC_000008.11	ADAM28	ADAM metallopeptidase domain 28	8p21.2
NC_000004.12	ADAM29	ADAM metallopeptidase domain 29	4q34
NC_000008.11	ADAM32	ADAM metallopeptidase domain 32	8p11.22
NC_000008.11	ADAM5	ADAM metallopeptidase domain 5, pseudogene	8p11.22
NC_000005.10	ADAMTS 16	ADAM metallopeptidase with thrombospondin type 1 motif, 16	5p15
NC_000016.10	ADAMTS 18	ADAM metallopeptidase with thrombospondin type 1 motif, 18	16q23
NC_000005.10	ADAMTS 19	ADAM metallopeptidase with thrombospondin type 1 motif, 19	5q23.3
NC_000007.14	ADCY1	adenylate cyclase 1 (brain)	7p13-p12
NC_000005.10	ADCY2	adenylate cyclase 2 (brain)	5p15.3
NC_000002.12	ADD2	adducin 2 (beta)	2p13.3
NC_000008.11	ADRA1A	adrenoceptor alpha 1A	8p21.2
NC_000002.12	ALK	anaplastic lymphoma receptor tyrosine kinase	2p23
NC_000015.10	APBA2	amyloid beta (A4) precursor protein-binding, family A, member 2	15q11-q12
NC_000018.10	APCDD1	adenomatosis polyposis coli down-regulated 1	18p11.22
NC_000002.12	ARHGEF4	Rho guanine nucleotide exchange factor (GEF) 4	2q22
NC_000010.11	ARMC3	armadillo repeat containing 3	10p12.31
NC_000010.11	ARMC4	armadillo repeat containing 4	10p12.1-p11.23

NC_000006.12	BAI3	brain-specific angiogenesis inhibitor 3	6q12
NC_000020.11	BMP7	bone morphogenetic protein 7	20q13
NC_000001.11	BRINP3	bone morphogenetic protein/retinoic acid inducible neural-specific 3	1q31.1
NC_000003.12	CADM2	cell adhesion molecule 2	3p12.1
NC_000018.10	CCDC102B	coiled-coil domain containing 102B	18q22.1
NC_000018.10	CCDC11	coiled-coil domain containing 11	18q21.1
NC_000001.11	CD244	CD244 molecule, natural killer cell receptor 2B4	1q23.3
NC_000001.11	CD247	CD247 molecule	1q24.2
NC_000002.12	CD302	CD302 molecule	2q24.2
NC_000011.10	CD3E	CD3e molecule, epsilon (CD3-TCR complex)	11q23
NC_000003.12	CD86	CD86 molecule	3q21
NC_000003.12	CD96	CD96 molecule	3q13.13-q13.2
NC_000005.10	CDH12	cadherin 12, type 2 (N-cadherin 2)	5p14.3
NC_000016.10	CDH13	cadherin 13	16q23.3
NC_000005.10	CDH18	cadherin 18, type 2	5p14.3
NC_000018.10	CDH19	cadherin 19, type 2	18q22.1
NC_000020.11	CDH4	cadherin 4, type 1, R-cadherin (retinal)	20q13.3
NC_000018.10	CDH7	cadherin 7, type 2	18q22.1
NC_000016.10	CDH8	cadherin 8, type 2	16q22.1
NC_000013.11	CDK8	cyclin-dependent kinase 8	13q12
NC_000001.11	CHRM3	cholinergic receptor, muscarinic 3	1q43
NC_000015.10	CHRNA7	cholinergic receptor, nicotinic, alpha 7 (neuronal)	15q14
NC_000006.12	COL19A1	collagen, type XIX, alpha 1	6q12-q13
NC_000008.11	COL22A1	collagen, type XXII, alpha 1	8q24.3
NC_000005.10	COL23A1	collagen, type XXIII, alpha 1	5q35.3
NC_000007.14	COL28A1	collagen, type XXVIII, alpha 1	7p21.3

NC_000002.12	COL4A4	collagen, type IV, alpha 4	2q35-q37
NC_000003.12	COL6A5	collagen, type VI, alpha 5	3q22.1
NC_000006.12	COL9A1	collagen, type IX, alpha 1	6q13
NC_000002.12	CTNNA2	catenin (cadherin-associated protein), alpha 2	2p12-p11.1
NC_000010.11	CTNNA3	catenin (cadherin-associated protein), alpha 3	10q22.2
NC_000005.10	CTNND2	catenin (cadherin-associated protein), delta 2	5p15.2
NC_000011.10	DRD2	dopamine receptor D2	11q23
NC_000014.9	ESRRB	estrogen-related receptor beta	14q24.3
NC_000001.11	ESRRG	estrogen-related receptor gamma	1q41
NC_000015.10	FAM189A1	family with sequence similarity 189, member A1	15q13.1
NC_000003.12	FAM19A1	family with sequence similarity 19 (chemokine (C-C motif)-like), member A1	3p14.1
NC_000003.12	FGF12	fibroblast growth factor 12	3q28
NC_000013.11	FGF14	fibroblast growth factor 14	13q34
NC_000004.12	GABRA2	gamma-aminobutyric acid (GABA) A receptor, alpha 2	4p12
NC_000015.10	GABRA5	gamma-aminobutyric acid (GABA) A receptor, alpha 5	15q12
NC_000004.12	GABRB1	gamma-aminobutyric acid (GABA) A receptor, beta 1	4p12
NC_000001.11	GBP7	guanylate binding protein 7	1p22.2
NC_000005.10	GHR	growth hormone receptor	5p13-p12
NC_000010.11	GPR158	G protein-coupled receptor 158	10p12.1
NC_000005.10	GPR98	G protein-coupled receptor 98	5q13
NC_000011.10	GRIA4	glutamate receptor, ionotropic, AMPA 4	11q22
NC_000010.11	GRID1	glutamate receptor, ionotropic, delta 1	10q22
NC_000011.10	GRIK4	glutamate receptor, ionotropic, kainate 4	11q22.3
NC_000016.10	GRIN2A	glutamate receptor, ionotropic, N-methyl D-aspartate 2A	16p13.2
NC_000001.11	HHAT	hedgehog acyltransferase	1q32
NC_000004.12	HHIP	hedgehog interacting protein	4q28-q32

NC_000004.12	IL15	interleukin 15	4q31
NC_000001.11	IL23R	interleukin 23 receptor	1p31.3
NC_000002.12	IL36B	interleukin 36, beta	2q14
NC_000002.12	ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	2q31.3
NC_000016.10	ITGAD	integrin, alpha D	16p11.2
NC_000009.12	KGFLP2	keratinocyte growth factor-like protein 2	9p12
NC_000006.12	LPA	lipoprotein, Lp(a)	6q26
NC_000001.11	LRRC52	leucine rich repeat containing 52	1q24.1
NC_000014.9	LRRC74	leucine rich repeat containing 74	14q24.3
NC_000017.11	MAP2K4	mitogen-activated protein kinase kinase 4	17p12
NC_000015.10	MAP2K5	mitogen-activated protein kinase kinase 5	15q23
NC_000017.11	MAP2K6	mitogen-activated protein kinase kinase 6	17q24.3
NC_000019.10	MBD3L2	methyl-CpG binding domain protein 3-like 2	19p13.2
NC_000012.12	METTL25	methyltransferase like 25	12q21.31
NC_000010.11	MGMT	O-6-methylguanine-DNA methyltransferase	10q26
NC_000023.11	MIR548I4	microRNA 548i-4	Xq21.1
NC_000007.14	MIR548N	microRNA 548n	7p14.3
NC_000004.12	MIR548T	microRNA 548t	
NC_000016.10	MIR548W	microRNA 548w	
NC_000007.14	MIR5692 A1	microRNA 5692a-1	
NC_000014.9	MIR5694	microRNA 5694	
NC_000006.12	MYLK4	myosin light chain kinase family, member 4	6p25.2
NC_000013.11	MYO16	myosin XVI	13q33.3
NC_000022.11	MYO18B	myosin XVIIIB	22q12.1
NC_000010.11	MYO3A	myosin IIIA	10p11.1
NC_000011.10	NCAM1	neural cell adhesion molecule 1	11q23.1

NC_000021.9	NCAM2	neural cell adhesion molecule 2	21q21.1
NC_000001.11	NEGR1	neuronal growth regulator 1	1p31.1
NC_000008.11	NRG1	neuregulin 1	8p12
NC_000010.11	NRG3	neuregulin 3	10q22-q23
NC_000020.11	PAK7	p21 protein (Cdc42/Rac)-activated kinase 7	20p12
NC_000010.11	PCDH15	protocadherin-related 15	10q21.1
NC_000013.11	PCDH9	protocadherin 9	13q21.32
NC_000016.10	PRKCB	protein kinase C, beta	16p11.2
NC_000001.11	PTGER3	prostaglandin E receptor 3 (subtype EP3)	1p31.2
NC_000002.12	RAPGEF4	Rap guanine nucleotide exchange factor (GEF) 4	2q31-q32
NC_000007.14	RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5	7p15.3
NC_000007.14	RELN	reelin	7q22
NC_000020.11	SLC24A3	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	20p13
NC_000014.9	SLC25A21	solute carrier family 25 (mitochondrial oxoadipate carrier), member 21	14q11.2
NC_000003.12	SLC25A26	solute carrier family 25 (S-adenosylmethionine carrier), member 26	3p14.1
NC_000012.12	SLC2A13	solute carrier family 2 (facilitated glucose transporter), member 13	12q12
NC_000004.12	SLC2A9	solute carrier family 2 (facilitated glucose transporter), member 9	4p16.1
NC_000008.11	SLC30A8	solute carrier family 30 (zinc transporter), member 8	8q24.11
NC_000013.11	SMAD9	SMAD family member 9	13q12-q14
NC_000012.12	SOX5	SRY (sex determining region Y)-box 5	12p12.1
NC_000007.14	SP4	Sp4 transcription factor	7p15.3
NC_000011.10	SPON1	spondin 1, extracellular matrix protein	11p15.2
NC_000006.12	TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta)	6p12
NC_000002.12	TFCP2L1	transcription factor CP2-like 1	2q14

NC_000002.12	TGFA	transforming growth factor, alpha	2p13
NC_000001.11	TNFSF4	tumor necrosis factor (ligand) superfamily, member 4	1q25
NC_000001.11	WLS	wntless Wnt ligand secretion mediator	1p31.3
NC_000008.11	ZFAT	zinc finger and AT hook domain containing	8q24.22
NC_000008.11	ZMAT4	zinc finger, matrin-type 4	8p11.21
NC_000007.14	ZNF277	zinc finger protein 277	7q31.1
NC_000019.10	ZNF28	zinc finger protein 28	19q13.41

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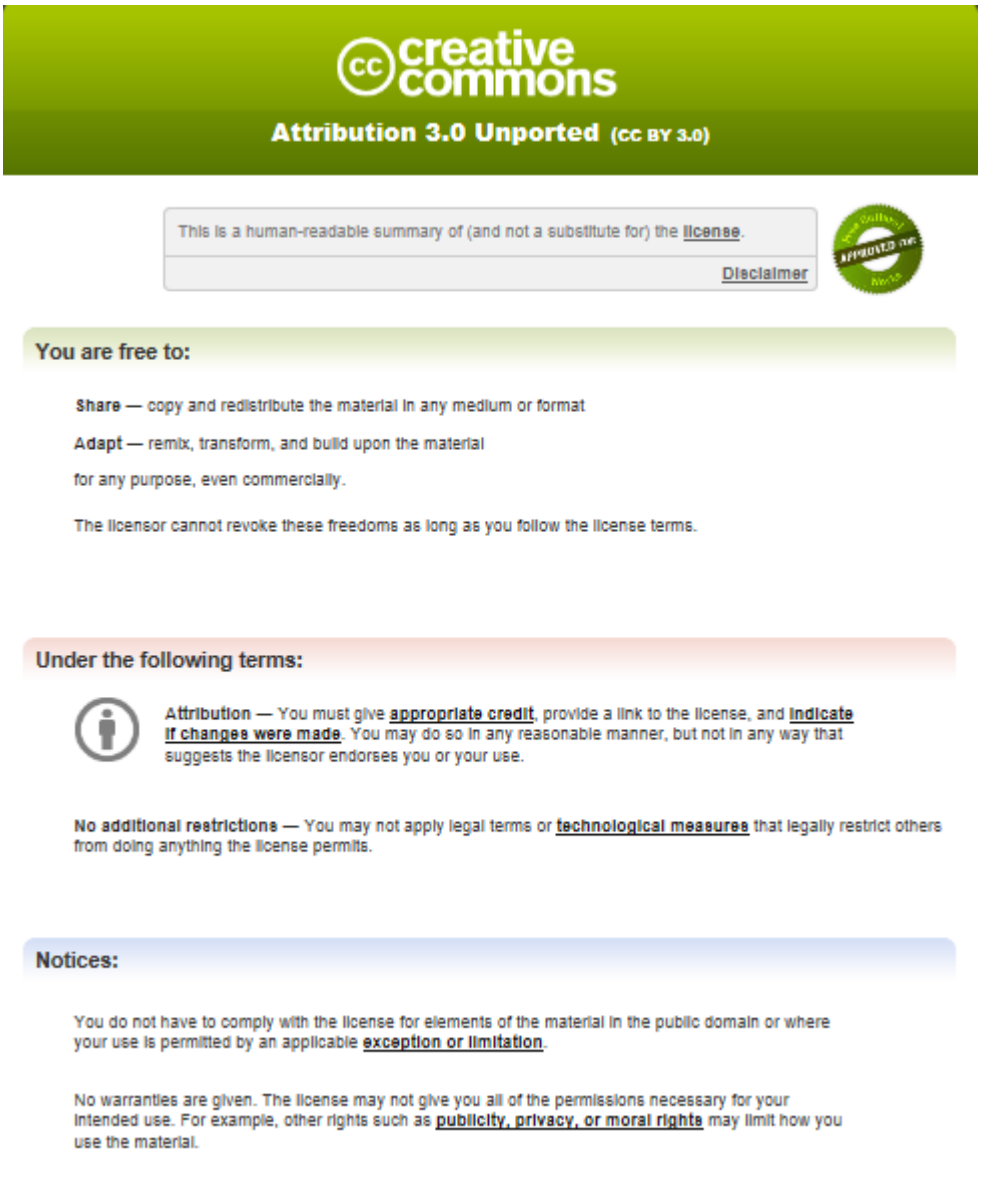

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Elsevier VAT number	GB 494 6272 12

Curriculum Vitae

Christina Raykha

Education

Western University, London, ON

Sept 2009 – Present	Doctor of Philosophy candidate in Biochemistry
Sept 2004 – June 2008	Honors Bachelor of Medical Sciences in Biochemistry of Infection and Immunity

Academic Awards & Accomplishments

Sept 2009 – Present	Western Graduate Research Scholarship
Mar 2013	IGF Society Travel Award
Oct 2012, Oct 2010	International Congress of the GRS and IGF Society Travel Award
June 2012	Canadian Student Health Research Forum – Canadian Institute of Health Research: Institute of Musculoskeletal Health and Arthritis Travel Funding Award
Sept 2010 – Apr 2011	Ontario Graduate Scholarship for Science and Technology
Sept 2008 – Dec 2008	Canadian Institute of Health Research Studentship Award, Institute of Musculoskeletal Health and Arthritis
Sept 2004 – Sept 2007	DaimlerChrysler Scholarship
Sept 2004	Western Scholarship of Excellence

Related Work Experience

Jan 2010 – April 2013	Graduate Teaching Assistant, Biochemistry 3380G Western University

Publications

Raykha C., Crawford J., Gan BS., Fu P., Bach LA., O'Gorman DB. IGF-II and IGFBP-6 regulate cellular contractility and proliferation in Dupuytren's Disease. Biochim Biophys Acta: 2013: 1832: p 1511-1519.

Satish L., O'Gorman DB., Johnson S., Raykha C., Gan BS., Wang JH., Kathju SH. Increased CCT-eta expression is a marker of latent and active disease and a modulator of fibroblast contractility in Dupuytren's contracture. Cell Stress Chaperones: 2013

Satish L., LaFramboise WA., Johnson S., Vi L., Njarlangattil A., Raykha C., Krill-Burger MJ., Gallo PH., O'Gorman DB., Gan BS., Baratz ME., Ehrlich GD., Kathju SH. Fibroblasts from phenotypically normal palmar fascia exhibit molecular profiles highly similar to fibroblasts from active disease in Dupuytren's Contracture. BMC Med Genomics: 2012: 5(1): p 15.

Raykha C, Crawford J, Gan BS, O'Gorman DB: Insulin-like Growth Factor Binding Protein (IGFBP)-6: A mediator of myofibroblast differentiation in Dupuytren's Disease. In: Dupuytren's Disease and Related Hyperproliferative Disorders. Springer Verlag. Dec 2011.

Oral Presentations

9 Jun 2014	Raykha C., Gan BS., O'Gorman D. "Identification of β-catenin gene targets by ChIP Sequencing in Dupuytren's Disease" Canadian Connective Tissue Conference, Delta Armouries, London, ON
18 Mar 2014	Raykha C., Gan BS., O'Gorman D. "IGF-II: A novel inducer of contraction in Dupuytren's Disease" London Health Research Day, London Convention Centre, London, ON
17 Jan 2014	Raykha C., Gan BS., O'Gorman D. "Bexarotene: a novel therapeutic approach to inhibiting connective tissue fibrosis" Bone and Joint Injury & Repair Conference, London Convention Centre, London, ON
20 Mar 2012	Raykha C., Crawford J., Gan BS., O'Gorman D. "Insulin-like Growth Factor Binding Protein-6 inhibits fibroblast proliferation and myofibroblast differentiation in Dupuytren's Disease" London Health Research Day, London Convention Centre, London, ON
6 Oct 2011	Raykha C., Crawford J., Gan BS., O'Gorman D.

	"Is Insulin-like Growth Factor Binding Protein-6 a 'fibrosis suppressor' in Dupuytren's Disease" 21st European Tissue Repair Society Annual Meeting, Felix Meritis, Amsterdam, Netherlands
6 Oct 2010	Raykha C., Crawford J., Gan BS., O'Gorman D. "IGFBP-6 and IGF-II: novel mediators of myofibroblast differentiation in Dupuytren's Disease?" Fifth International Congress of the GRS and IGF Society, Sheraton Hotel, New York, NY
17 Sept 2010	Raykha C., Crawford J., Gan BS., O'Gorman D. "Is Insulin-like Growth Factor Binding Protein-6 an endogenous inhibitor of myofibroblast differentiation in Dupuytren's Disease? " 20th European Tissue Repair Society Congress, Het Pand, Gent, Belgium
22 May 2010	Raykha C., Crawford J., Gan BS., O'Gorman D. "IGFBP-6: A novel mediator of myofibroblast differentiation in Dupuytren's Disease?" International Dupuytren Symposium, InterContinental Hotel, Miami, FL
27 Feb 2010	Raykha C., Gan BS., O'Gorman D. "Potential role of IGFBP-6 in the contractive hand disease: Dupuytren's Contracture." 23rd Western Research Forum, Western University, London, ON

Poster Presentations:

25 Mar 2014	Raykha C., Crawford J., Gan BS., O'Gorman D. "Identifying β-catenin gene targets during Dupuytren's Disease development by ChIP Sequencing" Fibrosis: From Bench to Bedside Keystone Conference, Keystone Resort, Keystone, CO
18-19 Mar 2013	Raykha C., Crawford J., Gan BS., O'Gorman D. "The effects of IGF-II and IGFBP-6 on fibroproliferation and contraction of Dupuytren's Disease cells" IGFs in Physiology and Disease Gordon Conference, Ventura Marriott, Ventura, CA
19 Oct 2012	Raykha C., Crawford J., Gan BS., O'Gorman D. "Regulation of Insulin-like Growth Factor Binding Protein-6: an inhibitor of fibroblast proliferation and myofibroblast

differentiation in Dupuytren's Disease"
Sixth International Congress of the GRS and IGF Society, Gasteig, Munich, Germany

13 Jun 2012 — Raykha C., Crawford J., Gan BS., O'Gorman D.
"Insulin-like Growth Factor Binding Protein-6, an inhibitor of fibroblast proliferation and myofibroblast differentiation in Dupuytren's Disease"
25th Annual Canadian Student Health Research Forum, Brodie Centre, Winnipeg, MB

28 Sept 2011 — Raykha C., Crawford J., Gan BS., O'Gorman D.
"The role of Insulin-like Growth Factor Binding Protein-6 as a potential 'fibrosis suppressor' in Dupuytren's Disease"
Musculoskeletal Research Symposium, Dr. Sandy Kirkley Centre for Joint and Musculoskeletal Health, London, ON

24 Mar 2010 — Raykha C., Crawford J., Gan BS., O'Gorman D.
"Potential role for Insulin-like Growth Factor Binding Protein (IGFBP)-6 in Dupuytren's Disease"
Lawson Research Day 2010, London Convention Centre, London, ON