



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



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ABSTRACT

Dupuytren's disease (DD) is a common and heritable fibrosis of the palmar fascia that typically manifests as permanent finger contractures. The molecular interactions that induce the development of hyper-contractile fibroblasts, or myofibroblasts, in DD are poorly understood. We have identified *IGF2* and *IGFBP6*, encoding insulin-like growth factor (IGF)-II and IGF binding protein (IGFBP)-6 respectively, as reciprocally dys-regulated genes and proteins in primary cells derived from contracture tissues (DD cells). Recombinant IGFBP-6 inhibited the proliferation of DD cells, patient-matched control (PF) cells and normal palmar fascia (CT) cells. Co-treatments with IGF-II, a high affinity IGFBP-6 ligand, were unable to rescue these effects. A non-IGF-II binding analog of IGFBP-6 also inhibited cellular proliferation, implicating IGF-II-independent roles for IGFBP-6 in this process. IGF-II enhanced the proliferation of CT cells, but not DD or PF cells, and significantly enhanced DD and PF cell contractility in stressed collagen lattices. While IGFBP-6 treatment did not affect cellular contractility, it abrogated the IGF-II-induced contractility of DD and PF cells in stressed collagen lattices. IGF-II also significantly increased the contraction of DD cells in relaxed lattices, however this effect was not evident in relaxed collagen lattices containing PF cells. The disparate effects of IGF-II on DD and PF cells in relaxed and stressed contraction models suggest that IGF-II can enhance lattice contractility through more than one mechanism. This is the first report to implicate IGFBP-6 as a suppressor of cellular proliferation and IGF-II as an inducer of cellular contractility in this connective tissue disease.

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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

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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 *TGFB1*, encoding transforming growth factor- β 1 (TGF- β 1) 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 bio-availability [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 inhibit the proliferation of DD, PF and CT cells, and co-treatment with IGF-II was mostly 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 fibro-contractile diseases.

2. Methods

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).

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 μ m), 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).

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.

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 μ g 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 $\Delta\Delta$ Ct 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.

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. 15% PAGE 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 (albumin) levels were assessed by Ponceau S staining (Sigma Aldrich, Oakville, ON, Canada).

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 read-out 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).

2.7. WST-1 cell proliferation assay

The WST-1 assay (Millipore/Chemicon, Billerica, MA) 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 $60 \mu\text{l}$ 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 performed at 450 nm and 650 nm (reference wavelength). This assay measures total cellular mitochondrial activity and we have demonstrated a linear correlation between manual cell counts and WST-1 absorbance values over a range of 0.1 to 0.4 absorbance units at 450 nm for cells grown on type-1 collagen substrates (data not shown). We interpret these 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.

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 $400 \mu\text{l}$ collagen (final collagen concentration of 1.8 mg/ml), $100 \mu\text{l}$ 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.

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. Results

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 [19,21], corresponding changes in IGFBP-6 levels have not been reported. As shown in Fig. 1A, a corresponding decrease in 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 patients (Fig. 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 Fig. 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 Fig. 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 them 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 relative to PF cells and CT cells over 48 h when cultured under identical serum free conditions (Fig. 1D).

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 (Fig. 2A). In contrast to *IGF2*, the expression of *IGF1*, encoding insulin-like growth factor-I, was unchanged between DD and PF cells (Fig. 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 Fig. 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, on the net proliferation and contractility of DD and PF cells.

3.3. IGFBP-6 inhibits DD cell proliferation in an IGF-II independent manner

Based on our analyses of IGFBP-6 secretion by CT cells (Fig. 1D) and previous studies in tumor cells [30], we chose to assess the effects

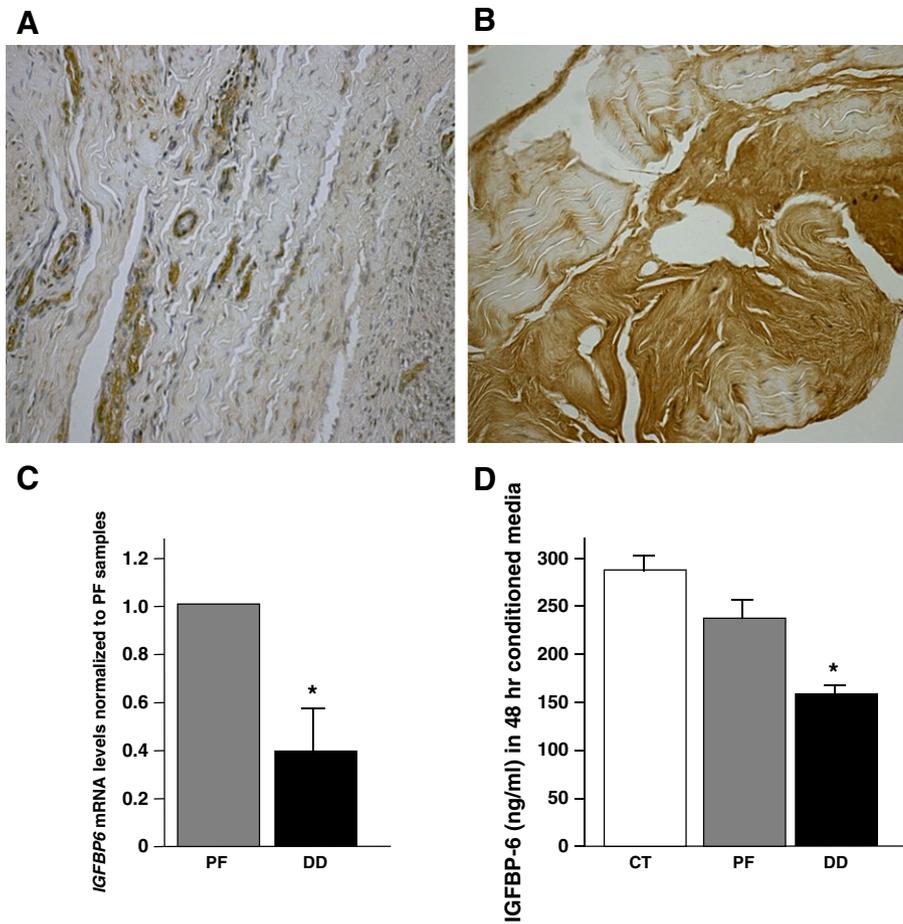


Fig. 1. *IGFBP6* expression and IGFBP-6 secretion are downregulated in Dupuytren's disease (DD). Sections of paraffin embedded DD "cord" tissue (A) and adjacent, phenotypically unaffected palmar fascia tissue (B) were assessed for IGFBP-6 immunoreactivity, evident as brown (diaminobenzidine, DAB) staining. 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, $N = 3$). 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, $N = 3$).

of 400 ng/ml of recombinant IGFBP-6 on net cellular proliferation. As shown in Fig. 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 over the subsequent 7 days relative to vehicle treated cells. While a single 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 (Fig. 3B).

As shown in Fig. 3C, 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) [31] at 400 ng/ml. IGFBP-6 and mIGFBP-6 had similar inhibitory effects on the proliferation of PF cells ($p < 0.05$, Fig. 3D) 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 ($p < 0.01$).

3.4. 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) [28,32–34] and stressed FPCLs (sFPCLs) [21,35,36]. 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 [37] from the immediate effects of treatment that are measured in rFPCLs [33].

DD cells were more contractile than PF cells in rFPCL assays in the absence of treatment (Fig. 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 or CT cell contractility (Fig. 4A). Platelet derived growth factor (PDGF), an established inducer of fibroblast contractility in rFPCLs [33], 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 (Fig. 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- β 1 treatment (12.5 ng/ml) significantly enhanced DD cell ($p < 0.01$) contractility in these assays, whereas a significant TGF- β 1 treatment/time interaction was evident in PF cells ($p < 0.01$) 6 h after lattice release ($p < 0.01$) (Fig. 4B). IGF-II did not significantly affect sFPCL contraction by CT cells (data not shown). Co-treatments with equimolar

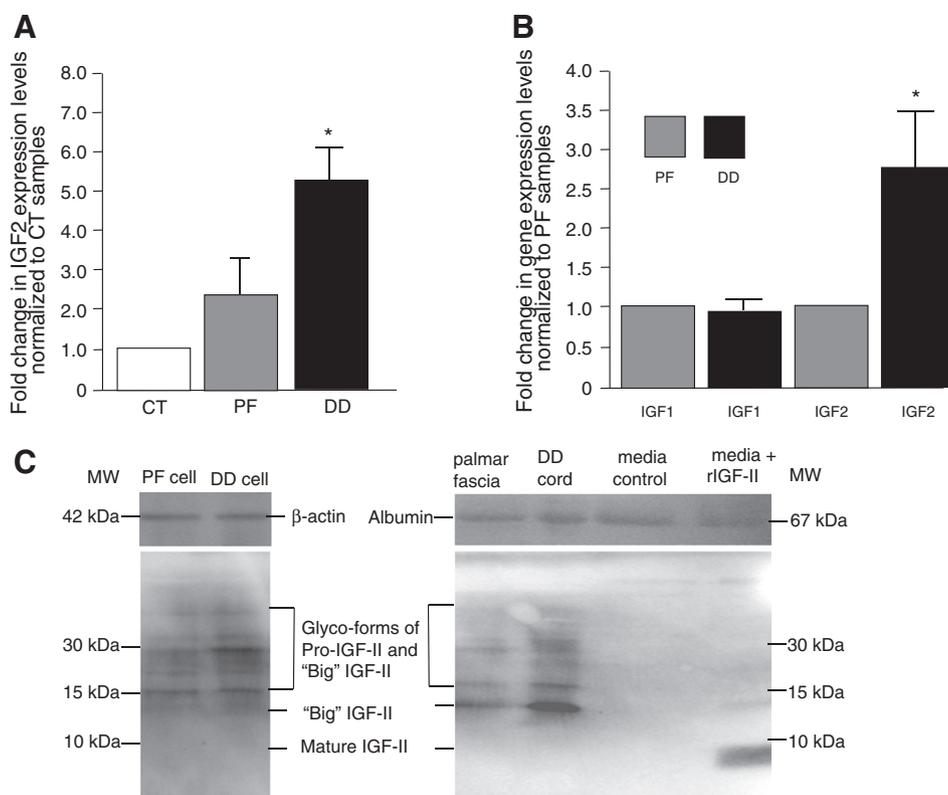


Fig. 2. *IGF2* expression and IGF-II levels are upregulated in DD. A) QPCR analysis of *IGF2* mRNA levels in CT, PF and DD cells (* = $p < 0.05$ for DD vs CT samples, $n = 6$ patients). B) QPCR analyses of *IGF1* and *IGF2* mRNA levels in primary PF cells (grey bars) and DD cells (black bars) (* = $p < 0.05$ for DD vs PF samples, $n = 6$ patients). C) Representative Western immunoblotting analyses of DD and PF cell lysates and palmar fascia tissue 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.

IGFBP-6 and IGF-II abrogated the IGF-II induced contractility of both DD and PF cells (Fig. 5A).

As TGF- β 1 signaling intermediates and IGF-II have been reported to elicit combinatorial effects on myofibroblast differentiation in murine fibroblasts [38], we assessed the effects of treating PF and DD cells with both growth factors in combination (Fig. 5B). While TGF- β 1 and IGF-II treatments significantly enhanced the contractility of PF and DD cells relative to vehicle treated controls, no combinatorial treatment effects were detected over treatment with either factor in isolation.

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 the increased TGF- β 1 expression and sensitivity, enhanced contractility, α smooth muscle actin (α SMA) expression and myofibroblast differentiation in DD cells [39–41], features shared with primary myofibroblast-like cells derived from other fibroses [42–44]. Other than TGF- β 1, 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 *IGFBP6* expression and upregulated *IGF2* expression jointly contribute to DD progression by promoting fibroblast proliferation, contractility and palmar fascia contraction in DD (Fig. 6).

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 [45–48] and inhibit apoptosis [49,50], and IGFBP-6 can antagonize these effects by sequestering

and inhibiting IGF-II signaling [30,51–53]. 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 relative to native IGFBP-6. These data suggest that IGFBP-6 inhibits 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 [31,54,55]. In contrast to PF and CT cells, mIGFBP-6 was significantly less effective at inhibiting the 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 (Fig. 3B). 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 (Fig. 5A). Overall, our data imply two distinct consequences of IGFBP-6 depletion in DD that are likely to enhance disease progression; the attenuation 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.

The increased TGF- β 1 expression and sensitivity and enhanced basal contractility of DD cells [39–41] suggest that DD cells have differentiated further toward a myofibroblast phenotype than PF cells.

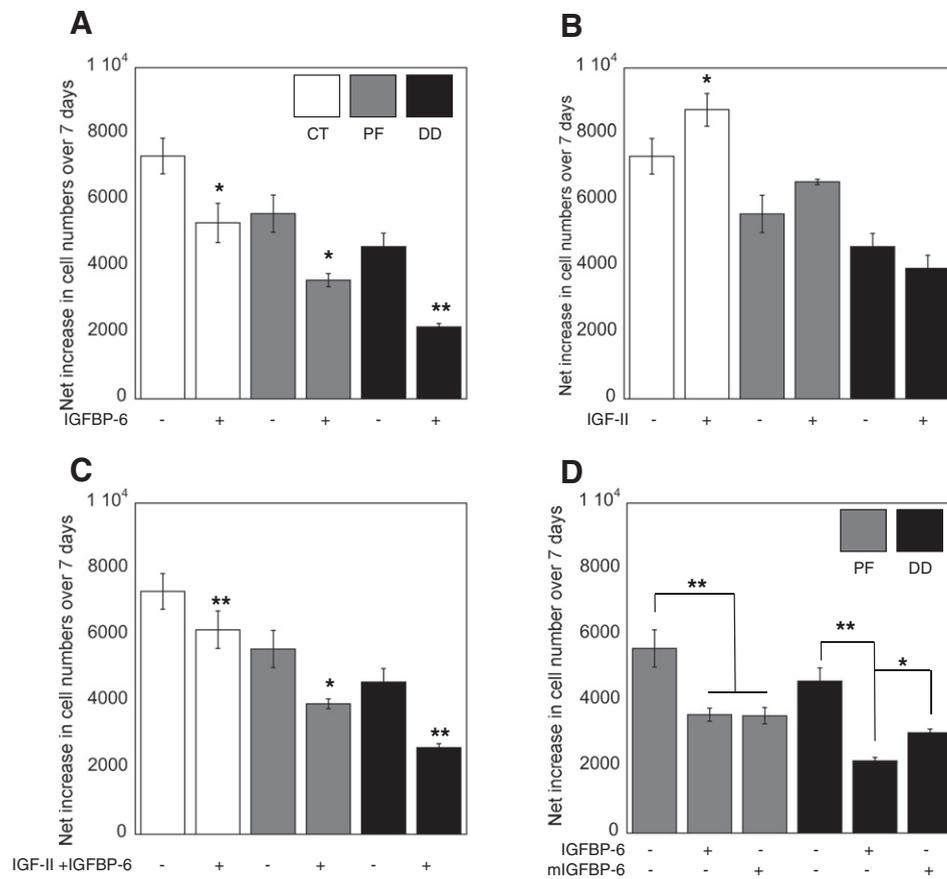


Fig. 3. IGFBP-6 inhibits the proliferation of DD, PF and CT cells. Net proliferation (growth and/or apoptosis) of CT (white bars, N = 3 patients), PF (grey bars, N = 4 patients) and DD (black bars, N = 4 patients) cells cultured on type-I collagen substrates was assessed using the WST-1 assay. Data are shown as change scores (cell numbers on day 7 minus cell numbers on day 1). Significant effects of treatment vs vehicle are indicated as * $p < 0.05$ and ** $p < 0.01$. Treatments were the following: A) IGFBP-6 treatment (400 ng/ml) vs vehicle, B) IGF-II treatment (100 ng/ml) vs vehicle, C) IGF-II + IGFBP-6 treatment (100 ng/ml and 400 ng/ml respectively) vs vehicle, and D) mIGFBP-6 (400 ng/ml) or IGFBP-6 (400 ng/ml) vs vehicle.

Our analyses of DD and PF cells treated with TGF- β 1 or IGF-II revealed some interesting, divergent behaviors in these genetically matched cells at differing stages of differentiation. TGF- β 1 treatment significantly enhanced DD cell contractility ($p < 0.01$) but no significant treatment/time interactions were evident, suggesting that the pro-contractile effects of TGF- β 1 treatment were induced prior to, and not after, lattice release. In contrast, no significant TGF- β 1 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- β 1 in PF cells occurred during the lattice contraction phase after release. These differences are visually evident in the parallel versus divergent contraction curves in Fig. 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- β 1 may activate distinct pro-contractile signaling pathways in DD and PF cells. Despite the potential for IGF-II and TGF- β 1 to induce cellular contraction through different signaling pathways, we were unable to detect significant additive effects of TGF- β 1 and IGF-II treatment in our analyses. 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 (Fig. 4A), whereas TGF- β 1, which significantly enhanced DD cell contraction in sFPCLs (Fig. 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 [56–58] 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.

5. Conclusion

These novel findings support our hypothesis that the attenuation of *IGFBP6* expression and enhancement of *IGF2* expression promote

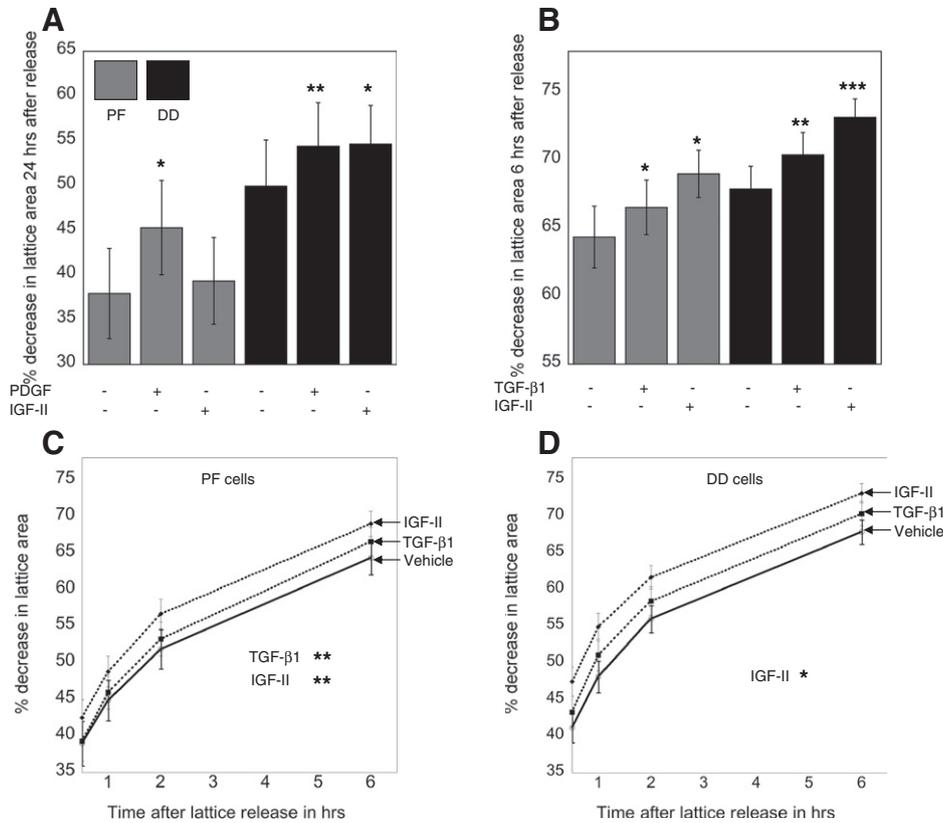


Fig. 4. IGF-II enhances DD cell contraction of collagen lattices. The effects of IGF-II on PF (grey bars) and DD (black bars) cell contractility were assessed in relaxed fibroblast populated collagen lattice assays (rFPCLs) over 24 h (A) and stressed FPCLs (sFPCLs) over 6 h (B, C and D) as described 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). 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). 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- β 1 (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- β 1 or IGF-II treatment. D) Contraction curves for DD cells from 0.5 h to 6 h after lattice release with vehicle, TGF- β 1 or IGF-II treatment. Significant treatment/time interactions are denoted as * $p < 0.05$ and ** $p < 0.01$.

DD development. IGFBP-6 inhibited DD proliferation, while exogenous IGF-II potentially 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 *IGFBP6* and *IGF2* as novel regulators of DD cell proliferation and contractility that may have utility as targets to inhibit DD progression and recurrence.

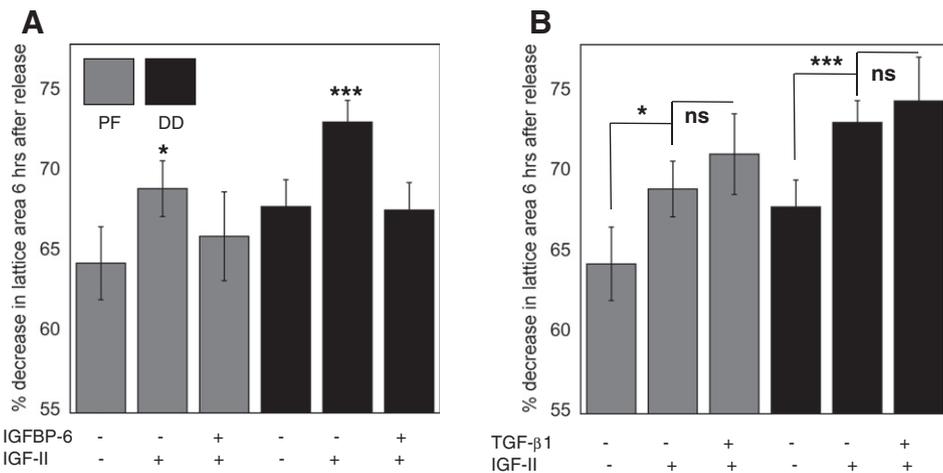


Fig. 5. IGF-II-induced PF and DD cell contractility is attenuated by IGFBP-6, but not enhanced by TGF- β 1. The effects of co-treatment with IGF-II and IGFBP-6 (N = 3 patients), and IGF-II and TGF- β 1 (N = 6 patients) were assessed on PF (grey bars) and DD (black bars) cell contractility in stressed 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) IGF-II (100 ng/ml) and IGFBP-6 (400 ng/ml) and B) IGF-II (100 ng/ml) and TGF- β 1 (12.5 ng/ml).

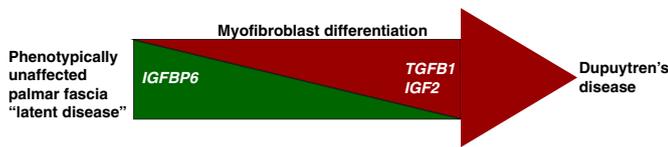


Fig. 6. Downregulated IGFBP-6 and upregulated IGF-II levels contribute to DD progression. We hypothesize that the differing IGFBP-6 and IGF-II levels evident between cells derived from “latent disease” tissues (PF cells) and diseased tissues (DD cells) reflect the roles played by these factors in promoting DD development. Downregulation of IGFBP-6 promotes cellular proliferation through IGF-II dependent and IGF-II independent mechanisms. In parallel, the upregulation of IGF-II signaling enhances cellular contractility through mechanisms that may be distinct from those induced by TGF- β 1.

Author contributions

CR and JC conducted the in vitro studies. BSG provided the primary surgical isolates from which primary cells were derived by CR and JC and contributed conceptually to the project. PF and LAB supplied the mutant IGFBP-6 and contributed conceptually to the project. DBO originally conceived of the project and coordinated all experimental approaches. CR, JC, BSG, LAB and DBO contributed to the drafting of the final manuscript.

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