Isometric contraction of Dupuytren's myofibroblasts is inhibited by blocking intercellular junctions.

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Short title: Myofibroblast intercellular junction blockade

Abbrevations used:

MF: myofibroblasts

HDF: human dermal fibroblasts

CFM: Culture Force monitor

α-SMA: α-smooth muscle actin

Cx43: connexin 43

Myofibroblasts are responsible for both physiological wound and scar contraction. However, it is not known whether these cells act individually to contract the surrounding matrix or whether they behave in a coordinated manner. Therefore, we studied intercellular junctions of primary human myofibroblasts derived from patients with Dupuytren's disease, a fibrotic disorder of the dermis and subdermal tissues of the palm. The cells were maintained in anchored three-dimensional collagen lattices to closely mimic conditions in vivo. We found that selective blockade of adherens, mechanosensitive or gap junctions effectively inhibited contraction of the collagen matrices and down-regulated the myofibroblast phenotype. Our data indicate that myofibroblasts in part function as a coordinated cellular syncytium and disruption of intercellular communication may provide a therapeutic target in diseases characterized by an overabundance of these contractile cells.

Introduction

Dupuytren's disease is a common fibroproliferative disorder with a prevalence of over 7% in the USA (Dibenedetti *et al.*, 2011). The most frequent manifestation is progressive flexion deformities of the digits of the hand, resulting in significantly compromised function. The cell responsible for the contraction of the palmar fascia and hence the digits in Dupuytren's disease is the myofibroblast. Myofibroblasts characteristically express α-smooth muscle actin (α-SMA), which is the isoform present in vascular smooth muscle cells, in addition to the β- and γ-cytoplasmic actins that are traditionally found in fibroblasts (Skalli *et al.*, 1986). Myofibroblasts in Dupuytren's tissue exist in aggregates both at the early phase of the disease (Rombouts *et al.*, 1989) and also in mature Dupuytren's cords, where they represent the vast majority of the cells in the histological nodules that are found overlying the finger joints (Verjee *et al.*, 2009). We hypothesized that the myofibroblasts in Dupuytren's nodules coordinate their activity via intercellular junctions.

Cells primarily communicate via three distinct means; adherens junctions, mechanosensitive and gap junctions. Adherens junctions are composed of cadherins that extend through the plasma membrane and mediate calcium-dependent cell-cell adhesion by homophilic association of their ectodomains (Yap *et al.*, 1997). The cadherin receptors associate intracellularly with several structural and signaling proteins, most notably the catenins, as

well as the actin cytoskeleton (Yap *et al.*, 1997). Cadherins comprise a large family, of which most members exhibit a tissue specific distribution. For example, E-cadherins are exclusively expressed in epithelia (Hatta and Takeichi, 1986). Differentiation of primary rat lung or subcutaneous fibroblasts to myofibroblasts in vitro using TGF-β1 is accompanied by an increase of OB-cadherin expression and a decrease of N-cadherin expression, with concomitant upregulation of α-SMA. In vivo, development of myofibroblasts in granulating cutaneous wounds was associated with upregulation of cadherin expression, and increased α-SMA expression correlated with markedly higher levels of OB-cadherin (Hinz *et al.*, 2004). OB-cadherin junctions exhibit higher adhesion strength than N-cadherin junctions (Pittet *et al.*, 2008). Inhibition of OB-cadherin, but not of N-cadherin, with specific peptides reduced the contraction of myofibroblast-populated collagen matrices (Hinz *et al.*, 2004), suggesting that OB-cadherin containing intercellular contacts play an important role in coordinating the contraction of differentiated myofibroblasts.

Mechanosensitive ion channels open when force is transmitted by an adjacent cell via adherens junctions, allowing an influx of cations such as calcium. They have been described in a variety of cells ranging from bacteria (Haswell *et al.*, 2011) to mammalian fibroblasts (Ko *et al.*, 2001). In fibroblasts, calcium ion influx through mechanosensitive channels opened by tension applied via N-cadherins at the adherens junctions has been shown to recruit intracellular actin filaments at the intercellular contacts (Ko *et al.*, 2001).

Adjacent cells can also communicate directly via gap junctions. Gap junctions are composed of six connexin 43 (Cx43) molecules. Hexamers in adjoining cells make direct contact to allow passage of molecules of up to 1kD between cells via hydrophilic channels. Accumulating evidence demonstrates that gap junctions are also important in the generation of myofibroblasts from precursor cells. Infusion of gap junction inhibitors into polyvinyl sponges implanted subcutaneously into rats led to reduced collagen deposition and numbers of α-SMA positive myofibroblasts (Au and Ehrlich, 2007; Ehrlich and Diez, 2003). TGF-β1, a potent inducer of myofibroblast phenotype (Follonier *et al.*, 2008; Hinz *et al.*, 2004), increased Cx43 as well as α-SMA expression in cardiac fibroblasts (Asazuma-Nakamura *et al.*, 2009). Antisense oligodeoxynucleotides to Cx43 applied to excisional skin wounds in mice led to earlier appearance followed by the disappearance of myofibroblasts, and overall

accelerated healing. This was accompanied by increased TGF-β1 and collagen-1α expression and reduced numbers of neutrophils and macrophages and mRNA for TNF-α (Mori *et al.*, 2006).

We explored the hypothesis that myofibrobasts from patients with Dupuytren's disease act in a coordinated fashion by communicating with each other through adherens, mechanosensitive or gap junctions. There is no animal model for Dupuytren's disease. In an effort to emulate the in vivo situation as closely as possible, we employed a validated system whereby we studied cells isolated from nodules excised from patients with Dupuytren's disease and cultured up to a maximum of passage 2 in three dimensional collagen lattices under isometric conditions in a culture force monitor (Verjee *et al.*, 2010). Myofibroblasts only maintain their phenotype under stress and loss of tension is associated with disassembly of α-SMA within minutes (Hinz *et al.*, 2001). To assess the relative contribution of each junction type respectively, we used specific blocking peptides to N- and OB-cadherins (Hinz *et al.*, 2004) as well as inhibitory cadherin antibodies, siRNA blockade of OB- or N-cadherin, carbenoxolone, which is a saponin that inhibits gap junction communication by disassembling Cx43 containing plaques (Guan *et al.*, 1996) and gadolinium, which is an inhibitor of cationic mechanosensitive channels (Ermakov *et al.*, 2010; Follonier *et al.*, 2008; Ko *et al.*, 2001; Sukharev *et al.*, 1997). Here we show that specific blockade of N- and OB-cadherins, mechosensitive channels or intercellular gap junctions leads to reduced myofibroblast contractility in vitro, suggesting that the cells partly act in concert, effectively as a syncitium.

Results

Dupuytren's myofibroblasts predominantly express OB-cadherin

We examined adherens and gap junctions in myofibroblasts (MF) and in non-palmar dermal fibroblasts (HDF) from the same patients, and compared the relative expression of OB- and N-cadherins, and Cx43 in each cell type. As expected, MF expressed higher levels of COL1 and α-SMA mRNA and higher levels of α-SMA protein than HDF (Fig 1A). MF also expressed more OB-cadherin at both message and protein levels compared to HDF, but expressed less N-cadherin (Fig 1B) and less Cx43 (Fig 1C) than HDF. Within 3D collagen matrices, adherens junctions, visualized by immunofluoresecent staining for β-catenin, were clearly observed between adjacent MF (Fig 1D). HDF in 3D collagen matrices did not demonstrate the distinctive intracellular organization of α-SMA in

stress fibers that is characteristic of MF, and intercellular adherens junctions between HDF (Fig 1E) were less well defined than those between MF. Gap junctions, visualized by immunostaining for Cx43, were more prominent in HDF (Fig 1F). Whilst some of the labeling was localized to the perinuclear cytoplasm in both HDF and MF, distinctive labeling was visualized at sites of intercellular contact, particularly between HDF (white arrows in Fig 1F).

Blocking OB- or N-cadherins downregulates the myofibroblast phenotype

We next examined the contribution of each type of cell junction to MF and HDF contraction using a number of different approaches. Inhibition of either OB- or N-cadherins with specific blocking peptides resulted in a dose dependent reduction in contraction of MF whilst control peptides had no effect (Fig 2A). Neither cadherin blocking peptide had any effect on the low baseline contraction of HDF (Fig 2B). Neutralizing antibodies for N-cadherin also resulted in reduced MF contractility in a dose dependent manner, compared to isotype controls or antibodies to the epithelial specific E-cadherin (Fig 2C). Blockade of OB-cadherin with peptides resulted in a reduction in α-SMA and COL1 message and α-SMA protein in MF but not HDF (Fig 2D). Peptide blockade of N cadherin also caused a significant reduction in α-SMA message and α-SMA protein in MF but not HDF (Fig 2E). Cell viability was not impaired over 24h following addition of any of the peptides or antibodies. siRNA for OB-cadherin effectively reduced OB-cadherin mRNA and protein levels in MF. Silencing of this cadherin also caused a reduction in α-SMA expression, and inhibited the isometric contraction of collagen matrices by MF (Fig 2F). Similarly, siRNA for N-cadherin effectively reduced N-cadherin mRNA and protein levels as well as α-SMA expression and MF contractility (Fig 2G). siRNA knockdown of OB-cadherin resulted in reduction in OB-cadherin mRNA in HDF but had no effect on OB-cadherin protein or α-SMA expression (Supplemental Fig 1A). siRNA knockdown of N-cadherin in HDF resulted in a reduction in N-cadherin mRNA and protein, but had no effect on α-SMA expression (Supplemental Fig 1B).

Blocking mechanosensitive junctions downregulates myofibroblast activity

Cells also communicate via mechanosensitive junctions that open as a result of force transmitted through

adherens junctions. Addition of the mechanosensitive channel blocker gadolinium led to dose-dependent reduction in contractility of MF (Fig 3A) but not of HDF (Fig 3B). The reduced contractility of MF was accompanied by a reduction of α-SMA and COL1 message expression as well as α-SMA protein (Fig 3C) in MF but not in HDF. Cell viability was unaffected by gadolinium concentrations up to 600μM, but reduced cell viability was observed at 1200μM.

Blocking gap junctions selectively inhibits myofibroblasts

Finally, we examined the effect of blockade of gap junctions in MF and HDF. Selective blockade of gap junctions by carbenoxolone resulted in a dose dependent decrease in isometric contraction of MF (Fig 4A) but not of HDF (Fig 4B). Carbenoxolone also led to a reduction in expression of COL1 mRNA and α-SMA mRNA and protein in MF; a reduction in α-SMA mRNA only was observed in HDF (Fig 4C). Impaired cell viability was not seen after 24h following addition of carbenoxolone up to a dose of 200μM; higher concentrations (500-1000μM) resulted in cell death. siRNA knockdown of Cx43 in MF reduced mRNA and protein expression of Cx43 and α-SMA, as well as a reduction in contractility of MF in 3D collagen matrices (Fig 4D). Silencing of Cx43 in HDF resulted in reduced expression of Cx43 and α-SMA mRNA and protein (Supplemental Fig 1C). Combined treatment of MF with neutralizing antibody to N-cadherin and carbenoxolone did not result in reduction of contractility above that seen with carbenoxolone alone. However, contractility was completely abolished by addition of cytochalasin D (Fig 4E).

Inhibitors of intercellular junctions are equally effective before or after establishment of junctions in vitro

Both myofibroblasts and fibroblasts contract 3D collagen lattices. However, whilst the latter reach tensional homeostasis at 6h, myofibroblasts continue to contract (Supplemental Fig 2) (Bisson *et al.*, 2004; Verjee *et al.*, 2010). In all the culture force monitor experiments described above the inhibitory compounds were added at the outset of the experiment. It is likely that the freshly disaggregated myofibroblasts need time to re-establish intercellular junctions and cell-matrix adhesion complexes. To discriminate between the possibility that blockade of intercellular communication merely prevents the formation of intercellular contacts, rather than inhibiting

established cell connections, inhibitors were also added after cells had been cultured in matrices for 12h and cell-cell junctions established (Fig 1D-F). Comparison of the effect of addition of inhibitors of adherens, mechanosensitive or gap junctions initially or 12h later, showed no difference in the reduction of contractility compared to controls (Supplemental Fig 3A-C).

Discussion

We hypothesized that myofibroblasts in vivo may contract in concert, with the activity of groups of cells being coordinated via adherens, mechanosensitive or gap junctions. Consistent with previous reports for myofibroblasts derived following treatment of fibroblasts with TGF-β1 (Hinz *et al.*, 2004; Pittet *et al.*, 2008), we found that myofibroblasts from Dupuytren's tissue expressed more OB-cadherin at both message and protein levels than HDF, but expressed lower levels of N-cadherin than HDF. However, blocking either N- or OB-cadherin was effective in inhibiting isometric contraction of 3D collagen matrices by primary myofibroblasts from Dupuytren's nodules. This is in contrast to previous publications (Follonier *et al.*, 2008; Hinz *et al.*, 2004) that showed only an OB-cadherin peptide blocker inhibited myofibroblast contraction whilst a N-cadherin peptide blocker only inhibited fibroblast contraction. A possible explanation of the variance of our findings is the source of our cells. We used early passage primary human cells from patients with Dupuytren's disease whereas Follonier et al (2008) studied subcutaneous rat fibroblasts up to passage 7 and compared them to myofibroblasts generated by exposure to TGF-β1 over 4 days and Hinz et al (2004) studied embryonic rat subcutaneous and lung fibroblasts and myofibroblasts generated by exposure to TGF-β1 over 5 days. An alternative explanation may be that intercellular adherence between myofibroblasts is crucial to their function such that blockade of even the relatively few N-cadherin-containing adherens junctions had a profound effect. We also found that blockade of N-cadherin was equally effective using either a peptide inhibitor or a neutralizing antibody but we are unable to locate an effective commercially available neutralizing antibody to OB-cadherin. These data were confirmed by gene silencing studies. siRNA inhibition of OB-cadherin or N-cadherin resulted in almost complete abolition of their expression at mRNA level and, over 24h, 30-50% reduction in protein expression. This was accompanied by 40-50% reduction in α-SMA protein and 50% reduction in isometric contraction of collagen matrices by MF. In

contrast, we found no difference in contractility following OB- or N-cadherin blockade in HDF. Whilst siRNA inhibition of OB-cadherin in HDF resulted in reduced OB-cadherin expression, no reduction in α-SMA expression was observed. This may be explained by low baseline expression of OB-cadherin in HDF. Similarly, silencing N-cadherin in HDF had no significant effect on α-SMA expression despite relatively more N-cadherin. This may again be due to there being far lower baseline α-SMA expression in HDF compared with MF.

HDF expressed more Cx43 in the resting state and exhibited prominent immunofluorescent labeling compared to myofibroblasts. The only previous study on gap junctions using cells derived from Dupuytren's tissue found that cord-derived cells showed increased intercellular passage of a dye through their gap junctions compared to cells derived from matched nodules (Moyer *et al.*, 2002), which are especially rich in myofibroblasts (Verjee *et al.*, 2009). We have previously shown using immunohistochemistry that approximately 83 - 92% of cells in Dupuytren's cords are fibroblasts and do not express α-SMA (Verjee *et al.*, 2009). We found that carbenoxolone, which disrupts gap junctions by dephosphorylation, effectively inhibited isometric contraction of collagen lattices populated by Dupuytren's myofibroblasts. Furthermore, specific knockdown of gap junctions using siRNA directed against Cx43 resulted in a similar reduction in myofibroblast contractility. Follonier et al (2008) found that the gap junction inhibitors palmitoleic acid or carbenoxolone did not inhibit the spontaneous Ca^{2+} oscillations exhibited by rat myofibroblasts, whereas fibroblast Ca^{2+} oscillations were effectively desynchronized. Together these data demonstrate that gap junctions play a key role in coordinating myofibroblast contraction but not in Ca^{2+} oscillations.

TGF-β1 is a potent inducer of the myofibroblast phenotype in quiescent fibroblasts (Follonier *et al.*, 2008; Hinz *et al.*, 2004) and there is evidence that TGF-β1 and Cx43 are interlinked. However, the precise relationship between the two is unclear and varies according to the system studied. TGF-β1 upregulated Cx43, α-SMA and matrix components in primary human aortic smooth muscle cells (Rama *et al.*, 2006) and it has been shown that Cx43 positively regulated TGF-β1 activity by competing with Smad 2/3 for binding to microtubules. In neonatal rat cardiac fibroblasts, knockdown of the endogenous Cx43 activity with antisense deoxyneucleotides inhibited α-SMA expression whilst over-expression of Cx43 increased α-SMA expression, and Cx43 acted cooperatively with TGF-β1 to induce α-SMA expression (Asazuma-Nakamura *et al.*, 2009). The authors speculated that anti-fibrotic

therapies based on modification of Cx43 activity may be more appropriate than blocking TGF-β1 signaling by antagonizing TGF-β1 receptors or Smads. Although there was a significant increase in TGF-β1 levels in the tissue post myocardial infarct, with no difference in levels between $Cx43^{-/-}$ and control animals, there was significant down regulation of phosphorylated Smads, which are downstream signaling mediators in the TGF-β1 pathway, in Cx43 deficient mice (Zhang *et al.*, 2010). Therefore, it is possible that in the heart Cx43 does not directly affect TGF-β1 but instead influences downstream Smad signaling. However, in other systems the relationship between TGF-β1 and Cx43 appears to be reversed. TGF-β1 inhibited Cx43 expression in human detrusor smooth muscle cells found in the bladder (Neuhaus *et al.*, 2009) and human suburothelial myofibroblasts showed reduced Cx43 on the membrane following exposure to exogenous TGF-β1 (Heinrich *et al.*, 2011). It is possible that in our system the carbenoxolone had an effect on the TGF-β1 pathway. Alternatively, carbenoxolone inhibition of myofibroblast contractility may have directly resulted in reduced α-SMA message as a loss of myofibroblast tension has been shown to disassemble α-SMA stress fibers in myofibroblasts within minutes (Hinz *et al.*, 2001). A direct link between gap junction inhibition and α-SMA protein expression and cell contractility is more likely as we observed similar effects with specific knockdown of Cx43 with siRNA.

It has been shown in rat myofibroblasts that coordination may be controlled through calcium ion influx via mechanosensitive channels, as detected by monitoring periodic Ca^{2+} oscillations between cells (Follonier *et al.*, 2008). Gadolinium is a specific mechanosensitive channel blocker that acts by altering the packing of membrane phospholipids and applying lateral pressure that squeezes the channels closed (Ermakov *et al.*, 2010). We found that inhibition of contraction of the collagen gel populated by myofibroblasts from Dupuytren's nodules occurred on exposure to gadolinium in a dose-dependent manner and was accompanied by reduced expression of COL1 and α-SMA message as well as α-SMA protein levels. Again, this may reflect the rapid reversal of the myofibroblast phenotype on release of external stress (Hinz *et al.*, 2001).

We have described the effects of inhibition of each individual form of intercellular junction in vitro and the maximal inhibition in contractility using blocking peptides, neutralizing antibodies or siRNA was approximately 50%. In vivo the various junctions are likely to function concurrently. For example, it has been shown that there is an intimate association between gap junctions and adherens junctions, although the latter are classically

considered to transmit mechanical force between cells. In 3T3 cells intracellular co-assembly of Cx43 and N-cadherin is required for gap and adherens junction formation, a process that likely underlies the close association between gap and adherens junction formation (Wei *et al.*, 2005). Furthermore, mechanosensitive channels are dependent on force transmitted by adherens junctions. Inhibition of each type of intercellular junction individually failed to abolish tension in the collagen matrices completely. Of all the blockers of intercellular junctions, carbenoxolone had the greatest inhibitory effect on cell contraction in the culture force monitor. Combined inhibition of gap junctions and adherens junctions did not result in any further reduction over that achieved by gap junction blockade alone. However, the addition of cytochalasin D, an actin depolymerizing agent (Cooper, 1987), resulted in complete abolition of contractility. These data confirm that MF contraction is wholly dependent on an intact cellular cytoskeleton and suggest that in the absence of effective intercellular communication residual tension exerted by individual cells on the matrix also contributes to MF contraction. Taken together our data suggest that myofibroblasts act in concert to contract 3D collagen matrices and our in vitro findings indicate that this coordinated activity is responsible for approximately 50% of the contractile effect. The remainder is likely to be due to the action of the individual myofibroblasts on the surrounding matrix. It has been suggested that the force generated by cell contraction is transmitted to the extracellular matrix, which is remodelled to a shortened position, a mechanism termed 'lock-step' (Follonier *et al.*, 2008). Our data show that inhibitors that disrupt adherens, mechanosensitive or gap junctions can each result in a significant decrease in myofibroblast activity, and hence contraction. This may offer a therapeutic approach to downregulate myofibroblast activity in cutaneous and musculoskeletal fibrotic disorders.

Methods

Patient samples

Tissue samples were obtained following institutional approval of experiments, informed written consent and adherence to Helsinki Guidelines (REC 07/H0706/81). Dupuytren's nodular tissue and matched full-thickness skin (harvested from the groin or medial aspect of arm) were obtained from patients with Dupuytren's disease undergoing dermofasciectomy.

Cell culture

Human dermal fibroblasts (HDF) were isolated from non-palmar skin and Dupuytren's myofibroblasts (MF) were isolated from α-SMA-rich nodules (Verjee *et al.*, 2010). Tissue samples were dissected into small pieces and digested in Dulbecco's modified Eagle's medium (DMEM) (Lonza) with 1% penicillin–streptomycin (PAA) and 5% fetal bovine serum (FBS) (Gibco) with type I collagenase (Worthington Biochemical Corporation) + DNase I (Roche Diagnostics) for up to 2h at 37°C. Cells were cultured in DMEM with 10% FBS and 1% penicillin–streptomycin at 37°C in a humidified incubator with 5% CO_2. Cells up to passage 2 were used for experiments.

Culture Force Monitor (CFM)

Measurement of the isometric contractile forces generated by cells within 3D collagen matrices was performed as previously described (Verjee *et al.*, 2010). Briefly, $2x10^6$ cells were seeded in 2.5 ml of type I collagen gel (FirstLink), and the resulting 3D matrices were suspended between two flotation bars and held stationary at one end whilst the other was attached to a force transducer. Fibroblast populated collagen lattice generated tensional forces were continuously measured for 24h and data logged every minute (dynes: $1x10^{-5}$ N). Cell populated matrices were cultured in DMEM with 10% FBS and 1% penicillin–streptomycin at 37°C in a humidified incubator for 24h with 5% CO_2 and treated with either N- or OB-cadherin peptide blockers (Adherex) and respective control peptide blockers, anti-N-cadherin antibody (Sigma), anti-OB-cadherin antibody (Santa Cruz, Mubio), anti-E-cadherin antibody (Invitrogen), IgG isotype control, gadolinium or carbenoxolone (Sigma). Compounds were added to CFM culture constructs at the start of the experiment except as in supplemental figure 3, where compounds where added either at the start or 12 h later. Cytochalasin D (Sigma) was added at a saturating dose of 20μg/ml cytochalasin D (Sigma) to fibroblast populated collagen matrices (Townley *et al.*, 2009). Experiments using each patient sample were performed in triplicate. Data are shown as the mean ± S.E.M from n≥3 patients.

Quantitative RT-PCR

Cells were cultured in monolayer and treated with either N- or OB-cadherin peptide blockers, gadolinium or carbenoxolone for 24h and total RNA was extracted from each sample using the QIAamp RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Isolated RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). For real-time quantitative reverse transcription PCR, Inventoried TaqMan® Gene expression Assays were used for α-SMA (Hs00426835-g1), COL1 (Hs00164004-m1), N-cadherin (Hs00362037-m1), OB-cadherin (Hs00901475-m1) and Cx43 (Hs00748445-s1) (Applied Biosystems) with Reverse Transcriptase qPCR™ Mastermix No ROX (Eurogentec). Samples were run on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Expression was normalized to GAPDH (Hs02758991-g1) (Applied Biosystems) and compared to the level of gene expression in either baseline respective cell types or to the level of gene expression in HDF, which were assigned the value of 1 using delta CT analysis performed with SDS software (Applied Biosystems). Data are shown as the mean ± S.E.M from n≥3 patients (each assay was performed in triplicate).

Western blots

Cells were cultured in monolayer and treated with either N- or OB-cadherin peptide blockers, gadolinium or carbenoxolone for 24h prior to protein extraction. Cell lysates were prepared in lysis buffer (25 mM HEPES (pH 7.0), 150 mM NaCl, and 1% Nonidet P-40), containing protease inhibitor cocktail (Roche Biochemicals) and then electrophoresed on 10% SDS polyacrylamide gels (Life Technologies), followed by electrotransfer of proteins onto PVDF transfer membranes (Perkin Elmer Life Sciences). Membranes were blocked in 5% BSA/TBS + 0.05% Tween and incubated overnight at 4°C with primary antibodies against α-SMA primary, anti-N-cadherin (Sigma), anti-OB-cadherin (Mubio), Cx43 or vimentin (Abcam). Horseradish peroxidase-conjugated anti-mouse IgG (Dako) or anti-rabbit IgG (Amersham Biosciences) were used as secondary antibodies. Bound antibody was detected using the enhanced chemiluminescence kit and visualized using Hyperfilm MP (Amersham Biosciences). Protein expression was assessed using vimentin as a loading control. Semi-quantitative analysis of protein expression was performed using densitometry analysis software (Phoretics International, UK). Gels shown are representative of 3 patients.

siRNA

Cells were seeded in 6 well plates at a density of 200,000 cells per well and cultured with DMEM, 10% FBS and 1% penicillin–streptomycin for 16hr. Cells were then washed in PBS and transfected with 90 pmol siRNA and Lipofectamine (both Invitrogen) following the manufacturers protocol. The assay was carried out with siRNA complexes diluted in optiMEM (Invitrogen) with 2% FBS. Inventoried silencer-select reagents and respective non-targeting negative controls were used for N-cadherin (Hs464829), OB-cadherin (Hs116471) and Cx43 (Hs.368353) (Applied Biosystems). siRNA for mechanosensitive junctions is not available. Negative control siRNAs 1 and 2 (Applied Biosystems) were used with sequences that do not target any gene product and provide a baseline to compare siRNA-treated samples. After 24hr of incubation, the transfection medium was replaced by DMEM, 10% FBS and 1% penicillin–streptomycin and the cells were incubated for a further 24hr. PCR and western blot analysis was used to quantify knockdown of gene and protein expression, and these cells were also used for experiments in the CFM. Data are shown as the mean ± S.E.M from n≥3 patients, with each assay was performed in triplicate).

Immunofluoresence

Cells were cultured in monolayer or 3D collagen matrices with DMEM, 10% FBS and 1% penicillin–streptomycin for 24h then fixed for 10 min with 3% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 (Sigma). Cells were stained with either a rabbit monoclonal anti-β catenin or a rabbit polyclonal anti-Cx43 (Abcam) followed by Alexa Fluor 568-conjugated goat anti-rabbit antibody (Invitrogen), Alexa Fluor 488 Phalloidin (Invitrogen) and DNA with DAPI (Sigma). Secondary antibody alone was used as an immunolabelling control. Images were acquired using confocal microscopy oil immersion objectives (60x) and the signal was analyzed by Ultraview confocal microscopy (PerkinElmer). The compact configuration of MF and HDF in 3D collagen lattices meant that we were unable to reliably assess Cx43 labeling under these conditions and therefore staining for Cx43 was performed in monolayer cultures. Cell viability was assessed using a Live/Dead

Viability/Cytotoxicity Kit (Invitrogen). Immunofluoresence images shown are representative of cells from 5 patients.

Statistics

The rate of MF or HDF populated collagen lattice contraction (dynes/h) was calculated by measuring the average gradient of the curve between 20 and 24 h. One-way analysis of variance (ANOVA) using Bonferroni's multiple comparison test was used for comparing all conditions. All statistical analyses were performed using software (GraphPad Software version 5.0c). Significance was achieved if $p<0.05$.

Conflict of Interest

Patent filed on inhibiting fibrosis by blocking intercellular junctions (JN, KM and LSV).

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Figure legends

Fig 1. Dupuytren's myofibroblasts predominantly express OB-cadherin

Expression levels of (A) α-SMA, and COL1, (B) OB- and N-cadherin and (C) Cx43 in MF and HDF. mRNA expression levels are compared to baseline HDF. *P < 0.05, ** P < 0.001, *** P < 0.001. N≥3. Immunofluoresence staining of (D) MF and (E) HDF cultured in a 3D collagen matrices for 12h. (Top panels) F-actin stained using phalloidin (green), α-SMA (red) and nuclei stained with DAPI (blue). (Lower panel) F-actin (green), β-catenin (red) and nuclei stained with DAPI (blue). **(F)** Immunofluoresence staining of MF and HDF cultured in monolayer showing Cx43 (red) and nuclei stained with DAPI (blue). Lower panels show merged images of the same cells also stained for F-actin using phalloidin (green). Scale bar: 10μm.

Fig 2. Blocking OB- or N-cadherins downregulates the myofibroblast phenotype

The effect of OB- or N-cadherin peptide blockers (0.05-0.5mg/ml) on MF (A) and HDF (B) contractility**. (C)** The effect of anti-N cadherin, and anti-E cadherin antibodies (5-20μg/ml) on MF contractility. The effect of OB-cadherin peptide blocker (0.5mg/ml) **(D)** and N-cadherin peptide blocker (0.5mg/ml**) (E)** on α-SMA and COL1 mRNA and α-SMA protein in MF and HDF. Effect of silencing of OB-cadherin (F) or N-cadherin (G) on cadherin and α-SMA expression and MF contractility. mRNA expression levels are compared to respective untreated cells. N≥3. *P < 0.05, *** P < 0.001**.**

Fig 3. Blocking mechanosensitive junctions downregulates myofibroblast activity

The effect of gadolinium (300μM) on MF (A) and HDF (B) contractility. (C) The effect of gadolinium (300μM) on α-SMA and COL1 mRNA and α-SMA protein expression in MF and HDF. mRNA expression levels are compared to baseline levels in respective untreated cells. N≥3 **P < 0.01, *** P < 0.001**.**

Fig 4. Blocking gap junctions selectively inhibits myofibroblasts

The effect of **c**arbenoxolone (100μM) on (A) MF and (B) HDF contractility. (C) The effect of **c**arbenoxolone

(100μM) on α-SMA and COL1 mRNA and α-SMA protein expression in MF and HDF. mRNA expression levels are compared to respective untreated cells (D) The effect of silencing of Cx43 on Cx43 and α-SMA gene, protein expression and MF contractility. **(E)** The effect of addition of anti-N cadherin antibody (10μg/ml) in combination with carbenoxolone (100μM) on MF contractility compared to carbenoxolone (100μM) alone or to cytochalasin D (20μg/ml). N≥3. *P < 0.05, *** P < 0.001