Isometric Contraction of Dupuytren's Myofibroblasts Is Inhibited by Blocking Intercellular Junctions

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Myofibroblasts (MFs) 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 MFs 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 downregulated the MF phenotype. Our data indicate that MFs 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.

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INTRODUCTION

Dupuytren's disease is a common fibroproliferative disorder with a prevalence of over 7% in the United States (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 (MF). MFs 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). MFs 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 MFs in Dupuytren's nodules coordinate their activity via intercellular junctions.

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Abbreviations: CFM, culture force monitor; Cx43, connexin 43; FBS, fetal bovine serum; HDF, human dermal fibroblast; MF, myofibroblast; mRNA, messenger RNA; siRNA, small interfering RNA; TGF-β1, transforming growth factor-β1; 3D, three-dimensional; α-SMA, α-smooth muscle actin

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Cells primarily communicate via three distinct means: adherens junctions, mechanosensitive junctions, 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 the epithelia (Hatta and Takeichi, 1986). Differentiation of primary rat lung or subcutaneous fibroblasts to MFs *in vitro* using transforming growth factor-β1 (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*, the development of MFs in granulating cutaneous wounds was associated with the 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 MF-populated collagen matrices (Hinz *et al.*, 2004), suggesting that OB-cadherin-containing intercellular contacts have an important role in coordinating the contraction of differentiated MFs.

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 1 KDa between cells via hydrophilic channels. Accumulating evidence demonstrates that gap junctions are also important in the generation of MFs 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 MFs (Ehrlich and Diez, 2003; Au and Ehrlich, 2007). TGF-β1, a potent inducer of MF phenotype (Hinz *et al.*, 2004; Follonier *et al.*, 2008), increased Cx43 and 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 MFs, and overall accelerated healing. This was accompanied by increased TGF-β1 and collagen-1α expression, and reduced numbers of neutrophils and macrophages and messenger RNA (mRNA) for TNF (Mori *et al.*, 2006).

We explored the hypothesis that myofibrobasts from patients with Dupuytren's disease act in a coordinated manner 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 used 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 (3D) collagen lattices under isometric conditions in a culture force monitor (CFM) (Verjee *et al.*, 2010). MFs 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, small interfering RNA (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 (Sukharev *et al.*, 1997; Ko *et al.*, 2001; Follonier *et al.*, 2008; Ermakov *et al.*, 2010). Here, we show that specific blockade of N- and OB-cadherins, mechosensitive channels, or intercellular gap junctions leads to reduced MF contractility *in vitro*, suggesting that the cells partly act in concert, effectively as a syncitium.

RESULTS

Dupuytren's MFs predominantly express OB-cadherin

We examined adherens and gap junctions in MFs and in non-palmar dermal fibroblasts (human dermal fibroblast (HDF)) from the same patients, and compared the relative expression of OB- and N-cadherins, and Cx43 in each cell type. As expected, MFs expressed higher levels of COL1 and α-SMA mRNA and higher levels of α-SMA protein than HDFs (Figure 1a). MFs also expressed more OB-cadherin at both message and protein levels compared with HDFs, but they expressed less N-cadherin (Figure 1b) and less Cx43 (Figure 1c) compared with HDFs. Within 3D collagen matrices, adherens junctions, visualized by immunofluorescent staining for β-catenin, were clearly observed between adjacent MFs (Figure 1d). HDFs in 3D collagen matrices did not demonstrate the distinctive intracellular organization of α-SMA in stress fibers that is characteristic of MFs, and intercellular adherens junctions between HDFs (Figure 1e) were less well defined than those between MFs. Gap junctions, visualized by immunostaining for Cx43, were more prominent in HDFs (Figure 1f). Although some of the labeling was localized to the perinuclear cytoplasm in both HDFs and MFs, distinctive labeling was visualized at sites of intercellular contact, particularly between HDFs (white arrows in Figure 1f).

Blocking OB- or N-cadherins downregulates the MF 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 the contraction of MFs, whereas control peptides had no effect (Figure 2a). Neither cadherin-blocking peptide had any effect on the low baseline contraction of HDFs (Figure 2b). Neutralizing antibodies for N-cadherin also resulted in reduced MF contractility in a dose-dependent manner, compared with isotype controls or antibodies to the epithelial-specific E-cadherin (Figure 2c). Blockade of OB-cadherin with peptides resulted in a reduction in α-SMA and COL1 message and α-SMA protein in MFs but not in HDFs (Figure 2d). Peptide blockade of N-cadherin also caused a significant reduction in α-SMA message and α-SMA protein in MFs but not in HDFs (Figure 2e). Cell viability was not impaired over 24 h after the addition of any of the peptides or antibodies. siRNA for OB-cadherin effectively reduced OB-cadherin mRNA and protein levels in MFs. Silencing of this cadherin also caused a reduction in α-SMA expression, and inhibited the isometric contraction of collagen matrices by MFs (Figure 2f). Similarly, siRNA for N-cadherin effectively reduced N-cadherin mRNA and protein levels, as well as α-SMA expression and MF contractility (Figure 2g). siRNA knockdown of OB-cadherin resulted in the reduction in OB-cadherin mRNA in HDFs, but had no effect on OB-cadherin protein or α-SMA expression (Supplementary Figure S1A online). siRNA knockdown of N-cadherin in HDFs resulted in a reduction in N-cadherin mRNA and protein, but had no effect on α-SMA expression (Supplementary Figure S1B online).

Blocking mechanosensitive junctions downregulates MF 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 a dose-dependent reduction in contractility

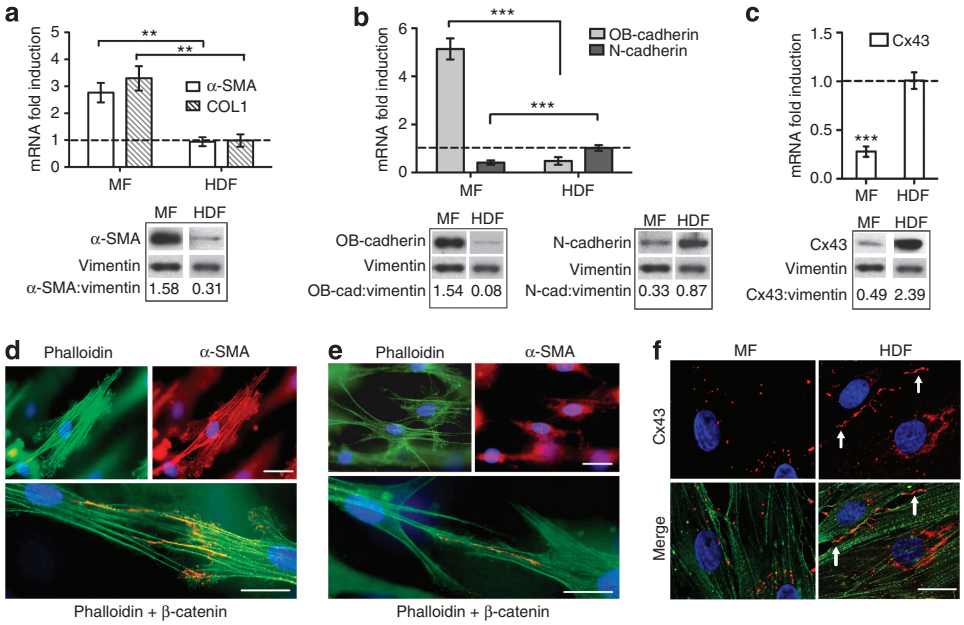

Figure 1. Dupuytren's myofibroblasts predominantly express OB-cadherin. Expression levels of (**a**) α-smooth muscle actin (α-SMA), and COL1, (**b**) OB- and N-cadherin, and (**c**) connexin 43 (Cx43) in myofibroblasts (MFs) and human dermal fibroblasts (HDFs). Messenger RNA (mRNA) expression levels are compared with baseline HDFs. $^{**}P<0.001$, $^{***}P<0.001$. $N\geqslant 3$. Immunofluoresence staining of (**d**) MFs and (**e**) HDFs cultured in three-dimensional collagen matrices for 12 hours. (Top panels) F-actin stained using phalloidin (green) and α-SMA (red), and nuclei stained with 4′6-diamidino-2-phenylindole (DAPI) (blue). (Lower panel) F-actin (green), β-catenin (red), and nuclei stained with DAPI (blue). (**f**) Immunofluoresence staining of MFs and HDFs 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). Bar = 10 μm.

of MFs (Figure 3a) but not of HDFs (Figure 3b). The reduced contractility of MFs was accompanied by a reduction of α-SMA and COL1 message expression, as well as α-SMA protein (Figure 3c) in MFs but not in HDFs. Cell viability was unaffected by gadolinium concentrations up to 600 μM, but reduced cell viability was observed at 1,200 μM.

Blocking gap junctions selectively inhibits MFs

Finally, we examined the effect of blockade of gap junctions in MFs and HDFs. Selective blockade of gap junctions by carbenoxolone resulted in a dose-dependent decrease in isometric contraction of MFs (Figure 4a) but not of HDFs (Figure 4b). Carbenoxolone also led to a reduction in the expression of COL1 mRNA and α-SMA mRNA and protein in MFs; a reduction in α-SMA mRNA only was observed in HDFs (Figure 4c). Impaired cell viability was not seen at 24 h after the addition of carbenoxolone up to a dose of 200 μM; higher concentrations (500–1,000 μM) resulted in cell death. siRNA knockdown of Cx43 in MFs reduced mRNA and protein expression of Cx43 and α-SMA, as well as a reduction in contractility of MFs in 3D collagen matrices (Figure 4d). Silencing of Cx43 in HDFs resulted in reduced expression of Cx43 and α-SMA mRNA and protein (Supplementary Figure S1C online). Combined treatment of MFs with neutralizing antibody to N-cadherin and carbenoxolone did not result in the reduction of contractility above that seen with carbenoxolone alone. However, contractility was completely abolished by the addition of cytochalasin D (Figure 4e).

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

Both MFs and fibroblasts contract 3D collagen lattices. However, although the latter reach tensional homeostasis at 6 hours, MFs continue to contract (Supplementary Figure S2 online) (Bisson *et al.*, 2004; Verjee *et al.*, 2010). In all the CFM experiments described above, the inhibitory compounds were added at the outset of the experiment. It is likely that the freshly disaggregated MFs 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 12 hours and cell–cell junctions established (Figure 1d–f). Comparison of the effect of addition of inhibitors of adherens, mechanosensitive, or gap junctions initially or 12 hours later showed no difference in the reduction of contractility compared with controls (Supplementary Figure S3A–C online).

DISCUSSION

We hypothesized that MFs *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 MFs derived after treatment of fibroblasts with TGF-β1 (Hinz *et al.*, 2004; Pittet *et al.*, 2008), we found that MFs from Dupuytren's tissue expressed more

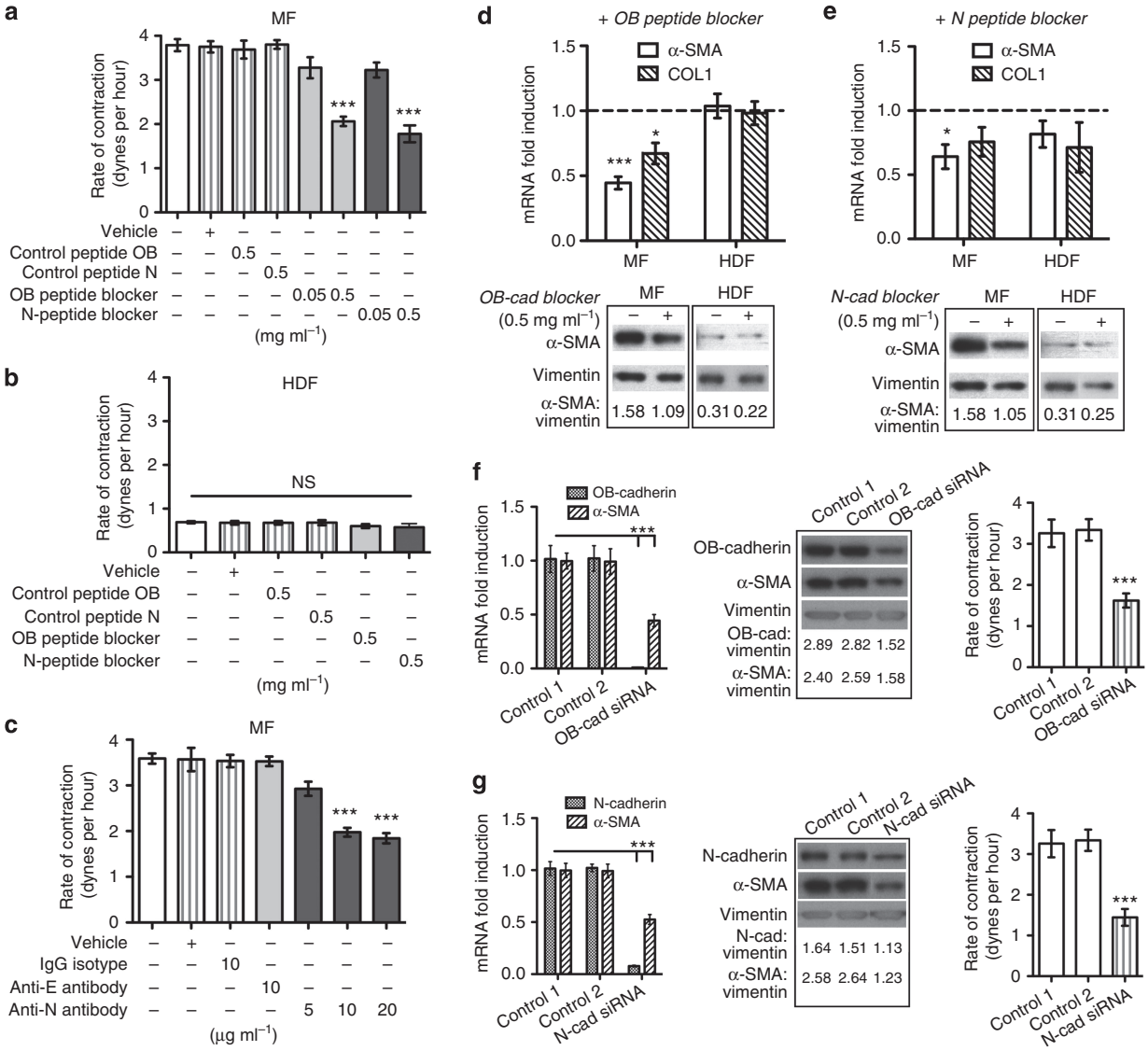

Figure 2. Blocking OB- or N-cadherins downregulates the myofibroblast phenotype. The effect of OB- or N-cadherin peptide blockers (0.05–0.5 mg ml^{-1}) on (**a**) myofibroblast (MF) and (**b**) human dermal fibroblast (HDF) contractility. (**c**) The effect of anti-N-cadherin and anti-E-cadherin antibodies (5–20 μg ml^{-1}) on MF contractility. The effect of (**d**) OB-cadherin peptide blocker (0.5 mg ml^{-1}) and (**e**) N-cadherin peptide blocker (0.5 mg ml^{-1}) on α-smooth muscle actin (α-SMA) and COL1 messenger RNA (mRNA) and α-SMA protein in MFs and HDFs. Effect of silencing of (**f**) OB-cadherin or (**g**) N-cadherin on cadherin and α-SMA expression and MF contractility. mRNA expression levels are compared with respective untreated cells. $N \geqslant 3$. *$P<0.05$, ***$P<0.001$. siRNA, small interfering RNA.

OB-cadherin at both message and protein levels than HDFs, but expressed lower levels of N-cadherin than HDFs. However, blocking either N- or OB-cadherin was effective in inhibiting isometric contraction of 3D collagen matrices by primary MFs from Dupuytren's nodules. This is in contrast to previous publications (Hinz *et al.*, 2004; Follonier *et al.*, 2008) that showed that only an OB-cadherin peptide blocker inhibited MF contraction, whereas 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 with MFs generated by exposure to TGF-β1 over 4 days and Hinz *et al* (2004) studied embryonic rat subcutaneous and lung fibroblasts and MFs generated by exposure to TGF-β1 over 5 days. An alternative explanation may be that intercellular adherence between MFs 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

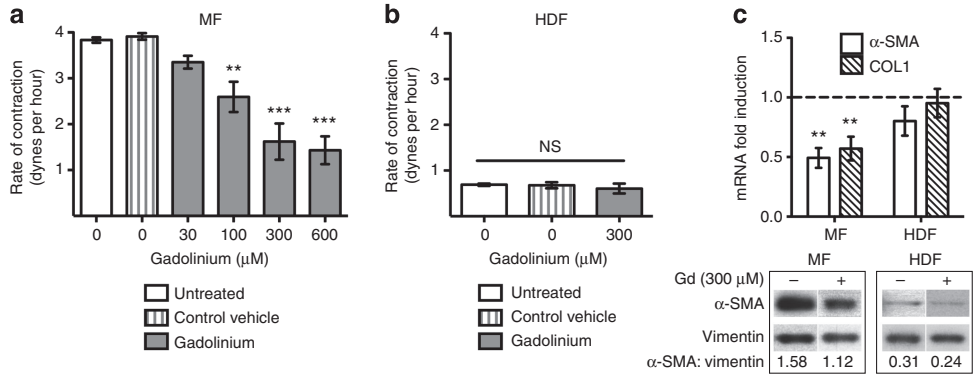

Figure 3. Blocking mechanosensitive junctions downregulates myofibroblast activity. The effect of gadolinium (300 μM) on (**a**) myofibroblast (MF) and (**b**) human dermal fibroblast (HDF) contractility. (**c**) The effect of gadolinium (300 μM) on α-smooth muscle actin (α-SMA) and COL1 messenger RNA (mRNA) and α-SMA protein expression in MFs and HDFs. mRNA expression levels are compared with baseline levels in respective untreated cells. $N \geqslant 3$. $^{**}P<0.01$, $^{***}P<0.001$. NS, not significant.

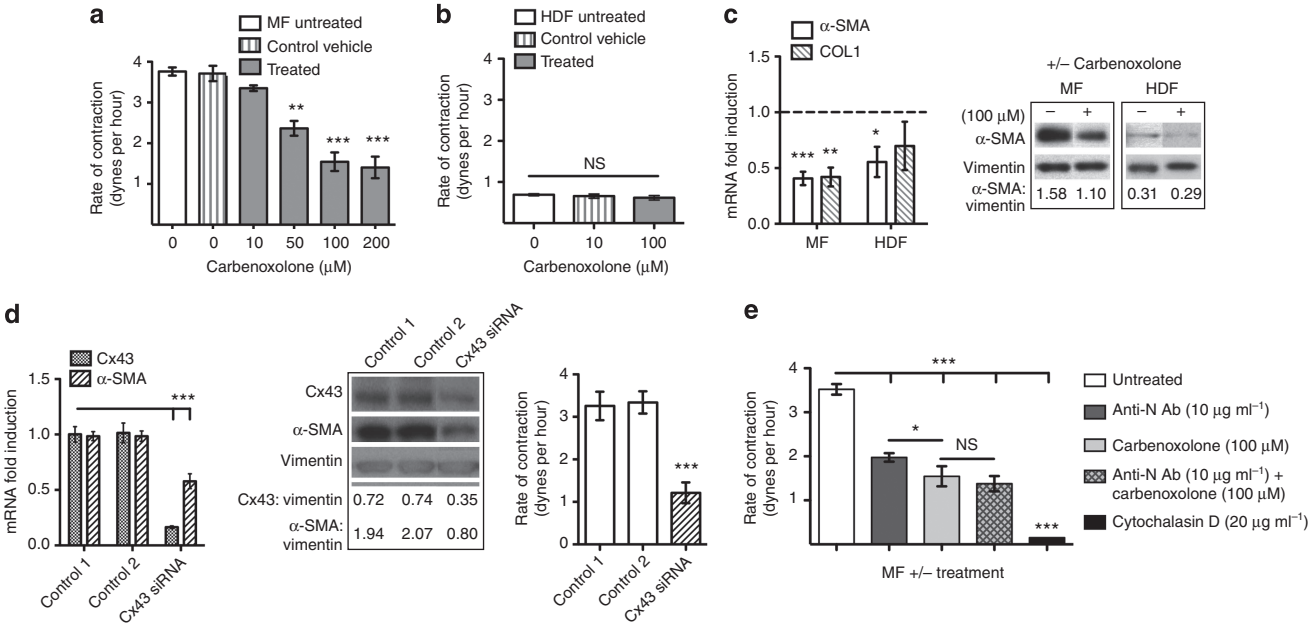

Figure 4. Blocking gap junctions selectively inhibits myofibroblasts. The effect of carbenoxolone (100 μM) on (**a**) myofibroblast (MF) and (**b**) human dermal fibroblast (HDF) contractility. (**c**) The effect of carbenoxolone (100 μM) on α-smooth muscle actin (α-SMA) and COL1 messenger RNA (mRNA) and α-SMA protein expression in MFs and HDFs. mRNA expression levels are compared with respective untreated cells. (**d**) The effect of silencing of connexin 43 (Cx43) on Cx43 and α-SMA gene, protein expression, and MF contractility. (**e**) The effect of addition of anti-N-cadherin antibody (10 μg ml^{-1}) in combination with carbenoxolone (100 μM) on MF contractility compared with carbenoxolone (100 μM) alone or to cytochalasin D (20 μg ml^{-1}). $N \geqslant 3$. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. NS, not significant.

resulted in almost complete abolition of their expression at the mRNA level and, over 24 hours, 30–50% reduction in protein expression. This was accompanied by a 40–50% reduction in α-SMA protein and 50% reduction in isometric contraction of collagen matrices by MFs. In contrast, we found no difference in contractility following OB- or N-cadherin blockade in HDFs. Although 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 α-SMA in HDFs. Similarly, silencing N-cadherin in HDFs had no significant effect on α-SMA expression despite relatively more N-cadherin. This may again be owing to there being far lower baseline α-SMA expression in HDFs compared with MFs.

HDFs expressed more Cx43 in the resting state and exhibited prominent immunofluorescent labeling compared with MFs. 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 with cells derived from matched nodules (Moyer *et al.*, 2002), which are especially rich in MFs (Verjee *et al.*, 2009). We have previously shown using immunohistochemistry that ~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 MFs. Furthermore, specific knockdown of gap junctions using siRNA directed against Cx43 resulted in a similar reduction in MF 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 MFs, whereas fibroblast Ca^{2+} oscillations were effectively desynchronized. Together, these data demonstrate that gap junctions have a key role in coordinating MF contraction but not in Ca^{2+} oscillations.

TGF-β1 is a potent inducer of the MF phenotype in quiescent fibroblasts (Hinz *et al.*, 2004; Follonier *et al.*, 2008), 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, whereas overexpression 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 antifibrotic 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 downregulation 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 MFs showed reduced Cx43 on the membrane after 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 MF contractility may have directly resulted in reduced α-SMA message, as a loss of MF tension has been shown to disassemble α-SMA stress fibers in MFs 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 MFs 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 the inhibition of contraction of the collagen gel populated by MFs 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 MF phenotype on the 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 ~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 coassembly of Cx43 and N-cadherin is required for gap and adherens junction formation, a process that probably 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 CFM. 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 MFs act in concert to contract 3D collagen matrices, and our *in vitro* findings indicate that this coordinated activity is responsible for ~50% of the contractile effect. The remainder is likely to be owing to the action of the individual MFs on the surrounding matrix. It has been suggested that the force generated by cell contraction is transmitted to the extracellular matrix, which is remodeled 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 MF activity, and hence contraction. This may offer a therapeutic approach to downregulate MF activity in cutaneous and musculoskeletal fibrotic disorders.

MATERIALS AND METHODS

Patient samples

Tissue samples were obtained after 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

HDFs were isolated from non-palmar skin, and Dupuytren's MFs were isolated from α-SMA-rich nodules (Verjee *et al.*, 2010). Tissue samples were dissected into small pieces and digested in DMEM (Lonza, Cambridge, UK) with 1% penicillin–streptomycin (PAA) and 5% fetal bovine serum (FBS) (Gibco, Paisley, UK) with type I collagenase (Worthington Biochemical Corporation, Reading, UK) + DNase I (Roche Diagnostics, West Sussex, UK) for up to 2 hours 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

Measurement of the isometric contractile forces generated by cells within 3D collagen matrices was performed as previously described (Verjee *et al.*, 2010). Briefly, 2×10^6 cells were seeded in 2.5 ml of type I collagen gel (FirstLink, Birmingham, UK), and the resulting 3D matrices were suspended between two flotation bars and held stationary at one end, whereas the other was attached to a force transducer. Fibroblast-populated collagen lattice–generated tensional forces were continuously measured for 24 hours and data logged every minute (dynes: 1×10^{-5} N). Cell-populated matrices were cultured in DMEM with 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified incubator for 24 hours with 5% CO_2 and treated with either N- or OB-cadherin peptide blockers (Adherex, Durham, NC) and respective control peptide blockers, anti-N-cadherin antibody (Sigma, Gillingham, UK), anti-OB-cadherin antibody (Santa Cruz, Mubio, Maastricht, The Netherlands), anti-E-cadherin antibody (Invitrogen, Paisley, UK), IgG isotype control, gadolinium, or carbenoxolone (Sigma). Compounds were added to CFM culture constructs at the start of the experiment, except as in Supplementary Figure S3 online, where compounds were added either at the start or 12 hours later. Cytochalasin D (Sigma) was added at a saturating dose of 20 μg ml^{-1} 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 ± SEM from $n \geqslant 3$ patients.

Quantitative reverse transcription PCR

Cells were cultured in monolayer and treated with either N- or OB-cadherin peptide blockers, gadolinium, or carbenoxolone for 24 hours, and total RNA was extracted from each sample using the QIAamp RNeasy Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. Isolated RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, NJ). 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, Paisley, UK) with Reverse Transcriptase qPCR Mastermix No ROX (Eurogentec, Seraing, Belgium). 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 with the level of gene expression in either baseline-respective cell types or with the level of gene expression in HDFs, which were assigned the value of 1 using delta CT analysis performed with the SDS software (Applied Biosystems). Data are shown as the mean ± SEM from $n \geqslant 3$ 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 24 hours before 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, West Sussex, UK) and then electrophoresed on 10% SDS polyacrylamide gels (Life Technologies, Paisley, UK), followed by electrotransfer of proteins onto PVDF transfer membranes (Perkin Elmer Life Sciences, Cambridge, UK). Membranes were blocked in 5% BSA/Tris-buffered saline + 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, Cambridge, UK). Horseradish peroxidase–conjugated anti-mouse IgG (Dako, Cambridge, UK) or anti-rabbit IgG (Amersham Biosciences, Amersham, UK) 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. Semiquantitative analysis of protein expression was performed using densitometry analysis software (Phoretics International, Manchester, UK). Gels shown are representative of three patients.

Small interfering RNA

Cells were seeded in six-well plates at a density of 200,000 cells per well and cultured with DMEM, 10% FBS, and 1% penicillin–streptomycin for 16 hours. Cells were then washed in phosphate-buffered saline and transfected with 90 pmol siRNA and Lipofectamine (both Invitrogen) according to the manufacturer's protocol. The assay was carried out with siRNA complexes diluted in optiMEM (Invitrogen) with 2% FBS. Inventoried silencer-select reagents and respective nontargeting 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 24 hours of incubation, the transfection medium was replaced by DMEM, 10% FBS, and 1% penicillin–streptomycin, and the cells were incubated for a further 24 hours. PCR and western blot analysis were 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 ± SEM from $n \geqslant 3$ 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 24 hours and then fixed for 10 minutes with 3% paraformaldehyde in phosphate-buffered saline 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 4′6-diamidino-2-phenylindole (Sigma). Secondary antibody alone was used as an immunolabeling control. Images were acquired using confocal microscopy oil-immersion objectives ($\times 60$), and the signal was analyzed by Ultraview confocal microscopy (Perkin Elmer, Waltham, MA). The compact configuration of MFs and HDFs 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 five patients.

Statistics

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

CONFLICT OF INTEREST

A patent has been filed on inhibiting fibrosis by blocking intercellular junctions (JN, KSM, and LSV).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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