The Mechanical Environment in Dupuytren's Contracture Determines Cell Contractility and Associated MMP-Mediated Matrix Remodeling

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ABSTRACT: Matrix metalloproteinases (MMPs) are expressed in Dupuytren's contracture and play a role in matrix remodeling. We tested the role of tension on contractility and MMP expression in Dupuytren's nodule and cord cells. Cells were subjected to predetermined loading patterns of known repeatable magnitudes (static load, unloading, and overloading) and tested for MMP gene expression (MMP-1, -2, -9, -13, and TIMP-1, -2) and force generation using a tension-culture force monitor. Matrix remodeling was assessed by addition of cytochalasin D and residual matrix tension was quantified. Nodule compared to cord and control cells demonstrate greater force generation and remodeling ($p < 0.05$). Nodule cells subjected to a reduced load and overloading led to threefold increase of MMP-1, -2, and -9 compared to static load, whilst cord and control cells only showed a twofold increase of MMP-9. Nodule cells subjected to overloading showed a twofold increase in TIMP-2 expression, whilst cord and control cells showed a twofold increase in TIMP-1 expression. Nodule cells differ from cord cells by increased force generation in response to changes in the mechanical environment and related MMP/TIMP-mediated matrix remodeling. In turn this may lead to permanent matrix shortening and digital contracture. Interventional therapies should be aimed at nodule cells to prevent contraction and subsequent permanent matrix remodeling. © 2012 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 31:328–334, 2013

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Dupuytren's contracture is a fibroproliferative condition of the palmar fascia, typically described in terms of contractile cellular nodules that progress to form fibrotic cords and ultimately lead to digital contracture.[1,2] The mainstay for treatment is surgery although there has been recent interest in the role of enzymatic treatment with injecting bacterial collagenase clostridium histolyticum,[3] leading to disruption of the contracted Dupuytren's cord. It has been proposed that the mechanism of contracture is a result of two separate but related processes: cell mediated contraction of the matrix, whereby cells contract to cause a physical deformation[4] and secondly, continuous matrix remodeling, leading to the permanence of contracture.[5]

Tension is known to be a contributing factor in Dupuytren's contracture[6] and alterations in the mechanical environment in which Dupuytren's cells reside can lead to changes in the production of matrix metalloproteinases (MMPs).[7] MMPs are a major group of zinc-dependent endopeptidases that function in the degradation of the extracellular matrix (ECM) as a part of controlled matrix remodeling.[8] Under physiological conditions MMPs are usually present at low levels and the activity of these enzymes is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs).[8] An imbalance of these enzymes has been reported to be associated with Dupuytren's contracture.[9–11] Therefore, we tested the effect of externally applied mechanical tension on contractility and MMP expression in fibroblast populated collagen lattices (FPCLs) seeded with Dupuytren's nodule and cord cells compared to control flexor retinaculum (FR) cells.

METHODS

Cell Culture

Following local ethical committee approval and informed written consent, Dupuytren's samples were obtained from patients undergoing primary fasciectomy. Dupuytren's nodule and cord ($n = 5$) tissue were distinguished clinically based on gross morphology and by histological evaluation.[12,13] In line with previous studies, age- and sex-matched FR tissue ($n = 5$) was used as control and excised from patients with no evidence of coexisting Dupuytren's contracture at routine carpal tunnel decompression. The mean age of the patients was 65 years (range: 56–71). Dupuytren's patients were classified into Tubiana stages, the mean Tubiana's grade was 1.8 (range: 1–3). An explant method was used to establish cell cultures, which were maintained in culture with normal growth medium (NGM), consisting of Dulbecco's Modified Eagle's medium, supplemented with 2 mM Glutamax, penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% foetal calf serum (all Gibco, Paisley, UK), at 37°C and 5% CO_2. All cultures were studied at or below passage 5.

Tension Culture Force Monitor

The tension culture force monitor (t-CFM; Fig. 1A) is an instrument which measures isometric contractile forces generated by cells within a 3D FPCL and applies load to the FPCL via a microprocessor-controlled stepping motor (Michromech, Braintree, UK), as previously described.[14] It comprises of a rectangular, three-dimensional FPCL, which is cast and

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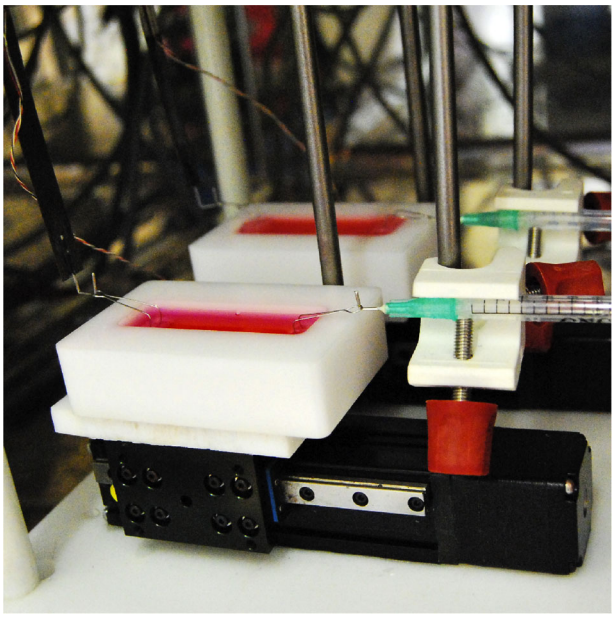

Figure 1. The tension culture force monitor (t-CFM). The collagen gels were cast and floated in medium in a mould positioned on a platform on a microprocessor-controlled stepping motor. The collagen gel was tethered between two floatation bars one is attached to a fixed point, whilst the other is attached to a force transducer.

floated in medium, tethered to two flotation bars on either of the short edges, and attached to a fixed point at one end and a force transducer at the other. Briefly, 5×10^6 cells from either Dupuytren's nodule, Dupuytren's cord or FR cells were dispersed uniformly through 5 ml of neutralized type I collagen gel (2.3 mg/ml; First link, West Midlands, UK), the gel was set for 30 min prior to being floated in 20 ml NGM.

For the MMP force generation, data were collected every second and a contractility profile was generated over 22 h. After this period, the FPCLs were allowed to contract for a further 2 h (static load), subjected to a series of uniaxial reductions of load forces (unload), or subjected to a series of rapid uniaxial loading forces (overload). A reduction in load and an overload was applied to the FPCLs via a microprocessor-controlled stepping motor. The motion of the culture platform was calibrated against the force transducer. Since the force transducer has a linear response the relationship between force and movement was simple to derive.[14] This ensured us reproducibly and precise loading and unloading rates.

The loading cycle comprised a decrease or an increase in tension of 30 dynes[15] over a 30-s period, followed by a 30-min resting phase. This process was repeated for four cycles.

Quantification of Residual Matrix Tension (RMT)

For the permanent matrix remodeling experiments, cell contractile profiles within the t-CFM were generated for 12-, 24-, and 48-h periods. Following these different time periods in culture, cell-mediated force generation was abolished by addition of a saturating dose of 20 μg/ml cytochalasin D as previously described.[16,17] Eliminating cell-generated force revealed force components, force due to active cellular contraction and force due to fixed tension within the matrix due to collagen remodeling by the cells (RMT; Fig. 1B). RMT was measured 2-h post-delivery of cytochalasin-D as changes in cellular contractility after this period had reached a plateau.

Gene Expression

At the conclusion of the t-CFM experiments, RNA was extracted from FPCLs using an RNeasy MiniKit (Qiagen, Crawley, UK) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA which was used in PCR reactions with primers specific for MMP-1, -2, -9, -13, TIMP-1, -2, and GAPDH. The sequences of the primers are shown in Table 1. PCR products were run on a 2% agarose gel containing ethidium bromide. The expression levels of the genes of interest were normalized in relation to control GAPDH.

Immunofluorescence Staining

Following contraction in the t-CFM, FPCLs were fixed under tension[18] with 4% paraformaldehyde for 30 min prior to being processed for immunofluorescence labeling. To visualize cellular morphology F-actin was stained using Phalloidin-Alexa Fluor 594 and DAPI for nuclear staining (Invitrogen, Paisley, UK).[19]

Statistical Analysis

Results are presented as means of three independent experiments and standard error of the mean (SEM) was calculated

Table 1. Sequences of the Primers Used in Assessment of Gene Expression

Gene	Forward Primers	Reverse Primers
MMP-1	5′-CGA CTC TAG AAA CAC AAG AGC AAG A-3′	5′-AAG GTT AGC TTA CTG TCA CAC GCT T-3′
MMP-2	5′-GTG CTG AAG GAC ACA CTA AAG AAG A-3′	5′-TTG CCATCC TTC TCA AAG TTG TAG G-3′
MMP-9	5′-CAC TGT CCA CCC CTC AGA GC-3′	5′-GCC ACT TGT CGG CGA TAA GG-3′
MMP-13	5′-TGC TGG CTC ATG CTT TTC CTC-3′	5′-GGT TGG GGT CTT CAT CTC CTG-3′
TIMP-1	5′-ACC ACC TTATAC CAG CGT TAT GAG-3′	5′-GAG GAG CTG GTC CGT CCA CAA GCA-3′
TIMP-2	5′-CGC TGG ACG TTG GAG GAAAGAAGG-3′	5′-GGG TCC TCG ATG TCG AGAAAC TCC-3′
GAPDH	5′-AAG AAG ATG CGG CTG ACT GTC GAG CCA CAT-3′	5′-TCT CAT GGT TCA CAC CCA TGA CGA ACA TG-3′

for each experiment analyzed for Dupuytren's nodule, cord and control FR cells. Comparisons between groups were made using a Mann–Whitney's non-parametric test to compare intragroup data, or a one-way ANOVA for intergroup data, using Prism software (GraphPad Software). Significance was achieved if $p < 0.05$.

RESULTS

Effect of a Single Static Load on FPCLs Contractility and MMP/TIMP Expression

Force generation in nodule cells was significantly greater ($p < 0.001$), compared to cord and FR cells. Nodule cells continued to contract to a maximum of 137 dynes (mean: 3.3 ± 1.1 dynes/h) at 24 h and did not reach tensional homeostasis, for cord cells this was a maximum of 72.8 dynes (mean: 2.0 ± 0.9 dynes/h; Fig. 2A and D). In contrast, FR cells contracted to a maximum force of 48 dynes at 24 h and reached tensional homeostasis on average after 15 h. Under static load there was no significant difference in expression of MMP-1, -2, -9, -13, TIMP-1, and -2 in all three cell types ($p = 0.3$). There was a higher TIMP/MMP mRNA expression ratio amongst all cell types, with significantly more TIMPs expressed compared to MMPs ($p = 0.02$; Fig. 3).

Effect of Unloading on FPCL Contractility and MMP/TIMP Expression

Next, we tested whether mechanically unloading the FPCL led to changes in contractile profiles and gene expression. All three cell types responded to this reduction in load by significantly increasing cellular contraction (Fig. 2B and E). Nodule cells subjected to a reduced load significantly upregulated expression of MMP-1 (threefold), -2 (fivefold), and -9 (threefold) compared to a static load ($p = 0.01$; Fig. 3). In contrast, cord and FR cells subjected to a reduced load only upregulated expression of MMP-9 (twofold) compared to a static load ($p = 0.02$). Nodule cells showed no changes in the expression of TIMPs after a reduction in load although cord cells significantly upregulated TIMP-1 expression (twofold) after a reduction in load ($p = 0.04$).

Effect of Overload on Contractility and MMP/TIMP Expression

Next we assessed whether externally applied overloads led to changes in contractile profiles and gene expression. Nodule subjected to overload responded by significantly increased cellular contraction ($p < 0.01$; Fig. 2C and F), a greater increase compared to cord cells. In subsequent three post-overload periods the nodule and cord cells responded by decreasing cell generated force to a negative gradient, which corresponded to gradients of FR cells. Nodule cells subjected to an overload upregulated expression of MMP-1 (threefold), -2 (fivefold), -9 (fourfold), and -13 (twofold) compared to a static load ($p = 0.02$). Conversely, cord and FR cells only significantly upregulated MMP-9 (threefold) compared to static load ($p = 0.03$; Fig. 3). TIMP-2 expression was significantly increased (twofold) in nodule cells ($p = 0.03$), but not for cord and FR cells ($p = 0.7$). In contrast, TIMP-1 expression was significantly increased in cord (twofold) ($p = 0.02$) and FR cells (fourfold) ($p < 0.001$), but not for nodule cells ($p = 0.3$).

Permanent Matrix Remodeling Measured by Residual Matrix Tension (RMT)

We quantified permanent matrix remodeling by calculating the RMT following the addition of a saturating dose of cytochalasin D (Fig. 4). After 12 h the RMT

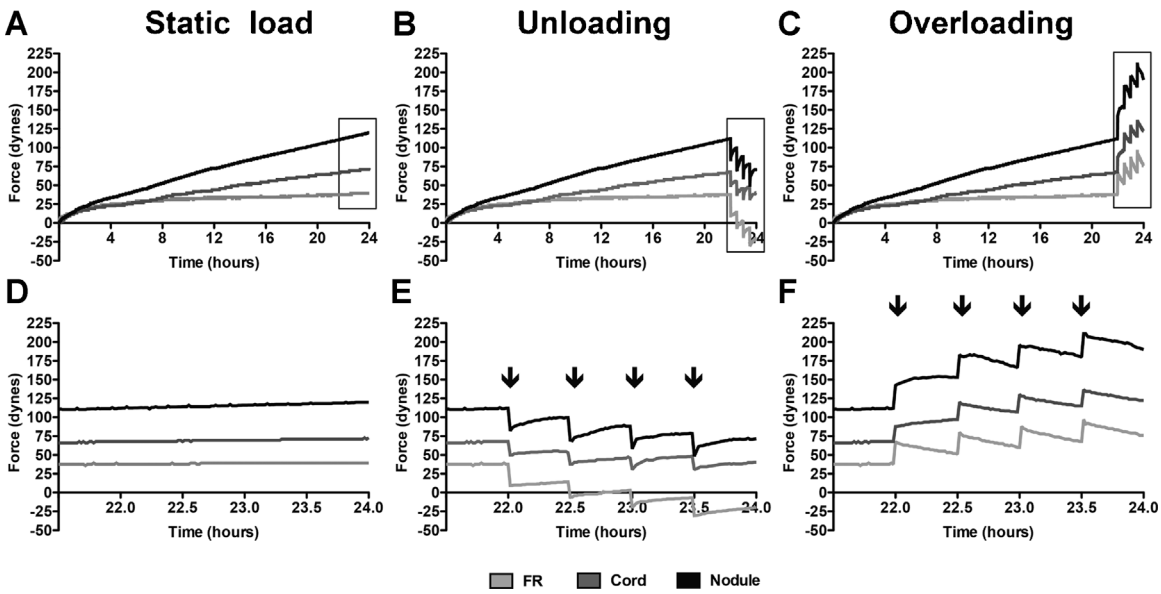

Figure 2. Effect of mechanical loading on FPCL contraction. Isometric contraction of collagen gels seeded with 5 million cells from either FR, cord or nodule ($n = 5$). (A–F) FPCLs were cultured for 24 h in the t-CFM and subjected to a static load (A, magnified in D), to 4 sequential uniaxial reductions in load (B, magnified in E) and overloadings (C, magnified in F) of 30 dynes, each separated by 30 min. Real-time isometric force contraction was quantified. Data shown represent the mean of triplicate experiments.

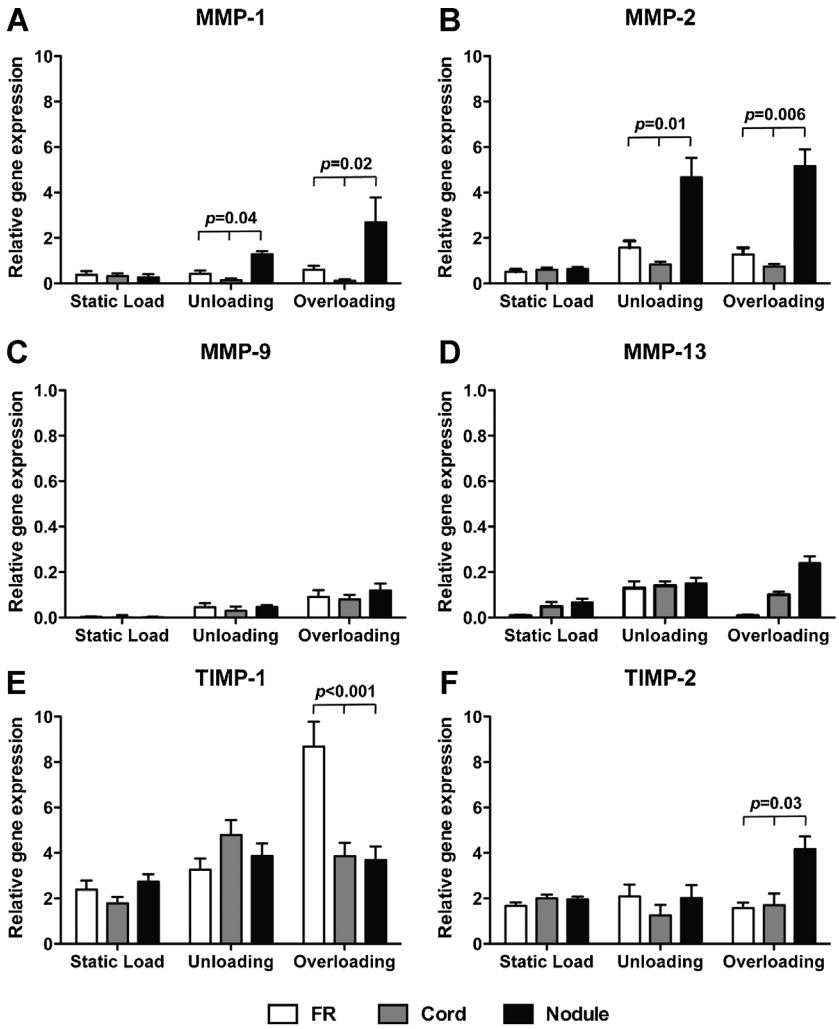

Figure 3. MMP/TIMP expression with mechanical loading. Relative gene expression of MMPs and their TIMPs in collagen gels seeded with 5 million cells from either FR, cord or nodule ($n = 5$). (A–F) FPCLs were cultured for 24 h in the t-CFM and subjected to a static load, unloading and overloading. After 24 h cells from the FPCLs were isolated and levels of MMP-1, -2, -9, -13, TIMP-1, and -2 mRNA were compared to GAPDH by quantitative PCR. Data are shown as the mean of triplicate experiments (±SEM).

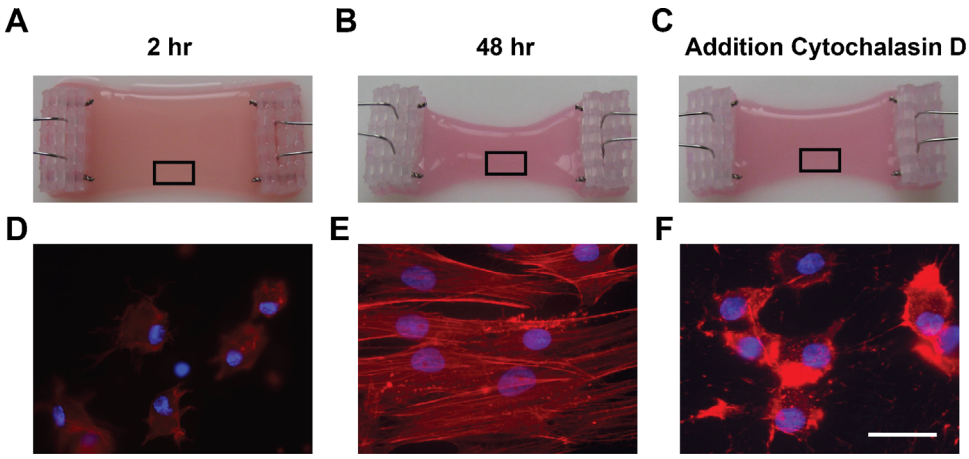

Figure 4. Immunofluorescence labeling and matrix remodeling over 48 h. Fibroblast seeded collagen gels with embedded flotation bars cultured in the t-CFM, tethered between a fixed point and a force transducer. (A–C) Cells contract and remodel the collagen gel in a time-dependent manner. (D–F) To visualize the cellular morphology collagen gels seeded with nodule cells were fixed after 2 h (A,D), 48 h (B,E) and after the addition of cytochalasin D (C,F) to abolish cell-mediated force generation. Representative sections of FPCL highlighted in section (A–C) were processed for immunofluorescence using phalloidin (red) and DAPI (blue). Scale bar: 30 μm.

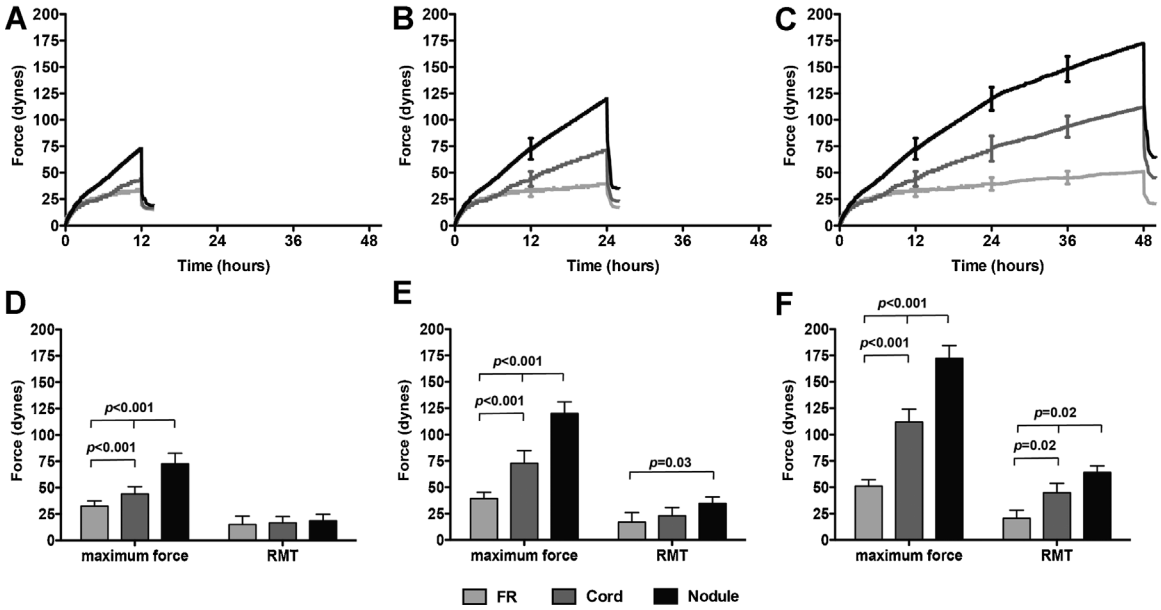

Figure 5. Force generation and matrix remodeling over 48 h in the t-CFM. FPCLs were seeded with 5 million cells from either FR, cord or nodule cells ($n = 5$). (A–C) FPCLs were cultured for 12 h (A), 24 h (B), and 48 h (C) in the t-CFM and real-time isometric force contraction was quantified. Following these time points, cell-mediated force generation was abolished by addition of a saturating dose of cytochalasin D. Data shown represent the mean of triplicate experiments. (D–F) The total force generated by the FPCLs and the residual matrix tension, after cell-mediated force generation was abolished, over 12 h (D), 24 h (E), and 48 h (F) is shown. Data are shown as the mean of triplicate experiments (±SEM).

was minimal, between 15 and 19 dynes for all three cell types, showing no evidence of permanent remodeling (Fig. 5A and D). RMT at 24 h was significantly higher for nodule 35 (±6) dynes ($p = 0.03$) compared to FR 17 (±9) dynes, for cord cells this was 22.9 (±8) dynes. The RMT increased further at 48 h: nodule was significantly higher, 64 (±6) dynes ($p = 0.02$), compared to cord, 44.8 (±9) dynes, and FR cells, 21 (±8) dynes.

DISCUSSION

Our findings suggest significant differences between Dupuytren's nodule and cord fibroblast in their response to mechanical stimulation, with respect to contractility, MMP/TIMP gene expression and permanent matrix remodeling. Nodule-derived cells are more contractile than cord or FR cells. Nodule cells respond to externally applied forces by further increasing contractility and this difference was mirrored by changes in MMP/TIMP expression, this increase in contractility was greater compared with cord and FR cells. We also found that nodule cells exerted greater RMT compared to cord and FR cells, suggesting that these cells not only have a highly contractile phenotype but also play a crucial role in matrix turn over and remodeling. These findings would be consistent with the in vivo observation, whereby nodules in active disease are comprised mainly of contractile myofibroblasts and fibrous, less cellular cord represents residual or burnt out disease.[12]

We measured isometric cell contractility in constrained FPCLs that generate a static load.[20] Nodule cells significantly increased contraction of FPCLs, compared to cord and control cells ($p < 0.001$). Previous studies have shown these differences between Dupuytren's nodule compared to cord and FR cells[2,13,21] or with matched dermal cells.[22] In line with these studies, we found that control cells reached tensional homeostasis, whereas Dupuytren's derived cells continued to contract over a 24-h test period.

However, the ECM is not static but is constantly being remodeled in response to the local, mechanical environment. Therefore, we also tested nodule, cord, and FR cells contractility in response to altered mechanical load. Overloading FPCLs seeded with Dupuytren's derived cells led to increased cellular contraction in the first overload, whereas FR cells responded to this externally applied load, by decreasing cell generated force to a negative gradient. All cell types decreased cell generated force in subsequent overloads. We also tested the effect of a reduced load on FPCLs and, interestingly, all cell types responded to this reduction in load by significantly increasing cellular contraction. This suggests that Dupuytren's derived cells have an innate ability to continue to contract in response to externally applied overload, as compared to control cells, whereas all the cell types respond to underloads by increasing contractility equally. An explanation for the latter is tensional homeostasis, whereby cells will establish a level of tension within the collagen matrix, and in the case of a reduced load act to maintain the level of tension against the opposing influence of mechanical unloading.[17,23–25] This mirrors the situation in tendons. In

lax tendons, native tendon cells have the ability to contract and re-establish tensional homeostasis. Tendon cells have also been shown to develop a cytoskeletal tensional homeostasis that, in turn, calibrates their mechanoresponsiveness to externally applied loads and, ultimately, their gene expression.[24]

Cellular responses to tension include changes not only in contractility, but also in synthesis of matrix components and regulatory enzymes such as MMPs.[26–28] In Dupuytren's tissue MMP-1, -2, -9, and -13 are significantly increased compared to control palmar fascia.[11,29,30] Therefore, we chose to test these MMP expressions in FPCLs subjected to different loads and looked at expression of their TIMPs which are specific inhibitors that act to control the local activities of MMPs.[8] Under static loads we found very low expression levels of MMPs and TIMPs, implying low matrix turnover. Very low-measured basal levels of MMPs and TIMPs are found in non-wounded skin, which is not under altered mechanical tension.[31] However, in Dupuytren's contracture the mechanical environment of the ECM is likely to be more dynamic as it is subjected to frequent loads applied to the hand. Indeed, we found that with both a reduction in load and overloading, nodule cells significantly increased ($p < 0.02$) expression of MMP-1, -2, -9, -13 and no significant change in TIMP 1 expression. In contrast, we observed downregulation of MMP-1 after mechanical stimulation in cord cells and in parallel an upregulation of TIMP-1 in cord and FR cells suggesting differential ECM remodeling in nodule compared to cord and FR cells. The upregulation of MMP-1, -2, and -9 has also been demonstrated in Dupuytren's tissue subjected to defined creep loading in a custom built tensiometer, which simulated the loading regime, found in the continuous elongation technique.[32] However, our findings suggest these changes in MMP expression in Dupuytren's contracture also occur with mechanically unloading. This may be of importance in the clinical setting, when mechanically unloading of the palmar fascia has been observed after fasciotomy[33] and residual Dupuytren's tissue appears to regress only temporarily with high rates of recurrence of 85% within 5 years.[34] These changes in MMP expression and increase in force generation may also explain why when external splints or fixation devices have been used to stretch and straighten contracted digits forcibly, Dupuytren's contractures recurred when the device was removed without surgical excision.[35]

The contractile nodule derived cells hold the matrix in a newly shortened position (RMT), while concurrently cells act to permanently remodel the surrounding matrix as suggested by an increase in MMP/TIMP expression. Taken together these findings show that nodule cells have a significantly increased remodeling capacity compared to cord and control cells. Although blocking MMP activity using a broad-spectrum MMP inhibitor, ilomastat, has been shown to decrease RMT and matrix remodeling by Dupuytren's cells using the same model system,[36,37] the fundamental problem still appears to be determined by an abnormality of nodule derived cell contraction. Cell contraction over time will lead to subsequent matrix remodeling and in turn digital contracture. Therefore interventional therapies should target nodule cells to prevent permanent matrix remodeling and subsequent contracture.

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