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Post-Transcriptional Regulation of α-Smooth Muscle Actin Determines the Contractile Phenotype of Dupuytren's Nodular Cells

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The objective was to study Dupuytren's myofibroblast cells in constrained collagen matrices in order to more closely emulate their in vivo environment and, to correlate their contractility with α-smooth muscle actin (α-SMA) expression and determine if dermal fibroblasts regulate Dupuytren's myofibroblast phenotype. Isotonic and isometric force contraction by cells isolated from Dupuytren's nodules, palmar and non-palmar skin fibroblasts was measured in collagen matrices. The effect of co-culturing nodule cells with dermal fibroblasts on isometric contraction was examined. Isometric contraction was correlated with levels of α-SMA mRNA by pcr and protein by Western blotting, and α-SMA distribution assessed by immunofluorescence. Dupuytren's nodule cells exhibited similar levels of isotonic contraction to both palmar and non-palmar dermal fibroblasts. However, nodule cells generated high levels of isometric force (mean: 3.5 dynes/h), which continued to increase over 24 h to a maximum of 173 dynes. In contrast, dermal fibroblasts initially exhibited low levels of contraction (mean: 0.5 dynes/h) and reached tensional homeostasis on average after 15 h (range: 4–20 h), with a maximum force of 52 dynes. Although all three cell types had similar α-SMA mRNA levels, increased levels of α-SMA protein were observed in nodule cells compared to dermal fibroblasts. α-SMA localised to stress fibres in 35% (range: 26–50%) of nodule cells compared to only 3% (range:0–6%) of dermal fibroblasts. Co-cultures of Dupuytren's cells and dermal fibroblasts showed no contractile differences. The contractile phenotype of Dupuytren's myofibroblasts is determined by increased α-SMA protein distributed in stress fibres, not by cellular mRNA levels. Dupuytren's cell contractility is not influenced by dermal fibroblasts.

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Dupuytren's disease is a fibrotic disorder characterised by thickening and contraction of the fascia of the palm and digits, resulting in progressive deformities and impairment of hand function. Severe contractures may require amputation of the finger. Dupuytren's is a very common condition, affecting 4–6% of all Caucasians and more than 25% of males and 17% of females over the age of 65 years (Ling, 1963).

During the fibroproliferation that is the hallmark of Dupuytren's disease, new tissue is deposited along lines of tension and in relation to normal fascial bands within the hand. Ultrastructural and histopathological studies have shown the diseased tissue is heterogenous composed of both hypercellular myofibroblast-rich nodules and collagen rich fibrotic cords, which are relatively cellular throughout. Cells in cord tissue are aligned longitudinally with collagen fibres and a small proportion of these cells are also myofibroblasts (Chiu and McFarlane, 1978; Verjee et al., 2009).

Myofibroblasts are characterised by the presence of α-smooth muscle actin (α-SMA) (Tomasek et al., 2002; Hinz, 2007). Dupuytren's nodule-derived cells have been shown to express high levels of α-SMA protein compared to normal fibroblasts (Benzonana et al., 1988), cells from cords and a human foreskin fibroblast cell line (Dave et al., 2001). α-SMA is a mechanosensitive protein recruited to stress fibres under high tension in rat lung myofibroblasts (Goffin et al., 2006) and, in three-dimensional collagen matrices, transforming growth factor β-1 (TGFβ-1) differentiated human gingival myofibroblasts exhibit increasing levels of α-SMA protein expression with mechanical load (Arora et al., 1999). Using isotonic collagen gel contraction models, increased contractility is observed with rat lung fibroblasts expressing high levels of α-SMA, compared to rat subcutaneous fibroblasts expressing low levels of α-SMA (Hinz et al., 2001a). However, studies using isotonic contraction to compare Dupuytren's nodule-derived cells, cord and carpal ligament-derived cells have yielded inconsistent findings. Some reported no differences in contraction (Rayan and Tomasek, 1994; Tarpila et al., 1996), whilst others have reported increased contraction only with early passage nodule-derived cells (Moyer et al., 2002) or in cells expressing higher levels of α-SMA (Tomasek and Rayan, 1995). More recently, isometric contractile forces within constrained fibroblast populated collagen lattices

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(FPCLs) have been studied using a culture force monitor (CFM). This allows real-time quantification of uniaxial isometric forces generated by FPLCs (Eastwood et al., 1994, 1996). Using this model, dermal fibroblasts reached tensional homeostasis (Brown et al., 1998). Dupuytren's nodules and cord-derived cells did not reach tension homeostasis and generated significantly greater isometric contractile force than control carpal ligament fibroblasts over a 20-h test period (Bisson et al., 2004). However, comparative differences between cells derived from genetically matched human samples have not been previously analysed in both isotonic and isometric model systems and correlation of α-SMA expression with isometric contractility has also not been undertaken in any cell type.

Currently, the commonest treatment for patients with significant Dupuytren's contracture is surgery, although recurrence rates following surgical excision of the affected fascia (fasciectomy) are approximately 40–50% (Hueston, 1961; Tonkin et al., 1984; Foucher et al., 1992). Recurrence following dermofasciectomy, where the overlying palmar skin is replaced by non-palmar skin grafts is 1–5% (Tonkin et al., 1984; Brotherston et al., 1994; Hall et al., 1997). However, the mechanism of the reduced recurrence rate following dermofasciectomy remains unexplained.

Here we compare the isotonic and isometric contractile profiles of genetically matched primary human cells isolated from Dupuytren's nodules, non-palmar and palmar skin samples following Dupuytren's dermofasciectomy in three-dimensional collagen lattices. We assess α-SMA mRNA levels, α-SMA protein expression and α-SMA distribution in these lattices. Finally, based on the observation that recurrence rates are significantly reduced following dermofasciectomy, we co-cultured Dupuytren's nodule cells with dermal fibroblasts to investigate whether the contractility of Dupuytren's cells is down regulated by non-palmar dermal fibroblasts.

Materials and Methods

Patient samples

Tissue samples were obtained from 10 patients undergoing dermofasciectomy for Dupuytren's disease between September 2006 and 2008 with informed consent (COREC no. 06/Q0403/95). The mean age of patients was 58 years (range: 38–72 years) and the male-to-female ratio was 7:3. Three types of tissue were obtained from each patient: Dupuytren's tissue, palmar skin (uninvolved skin overlying Dupuytren's tissue) and non-palmar skin (full-thickness skin harvested from the groin).

Cell culture

Dermal fibroblasts (non-palmar and palmar) were isolated from whole skin samples. Dupuytren's nodule cells were specifically isolated from α-SMA-rich nodules within Dupuytren's tissue samples (Verjee et al., 2009). Tissue samples were dissected into small pieces and digested in 2 mg/ml of type I collagenase (Worthington Biochemical Corporation, USA), and 0.15 mg/ml of DNase I (Roche Diagnostics, Germany) for up to 3 h at 37°C. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (PAA) supplemented with 1× penicillin–streptomycin (PAA) and 10% foetal bovine serum (FBS) (Gibco, UK) at 37°C in a humidified incubator with 5% CO_2. Cells between passages 1 and 2 were used for the experiments except where stated otherwise. Cell viability was assessed using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) based assay (Sigma-Aldrich, UK).

Isotonic contraction

Cells were cultured within stabilised collagen lattices and FPCL contraction assessed using a stress-release model (Tomasek et al., 1992; Grinnell, 1994). Following trypsinisation, 1×10^5 cells were seeded in 250 μl of neutralised type I rat tail collagen (FirstLink Ltd., UK). FPCLs were allowed to polymerise at 37°C for 30 min in 24-well tissue culture plates and then cultured with 1 ml DMEM (with 10% FBS) at 37°C in 5% CO_2 for 24 h. At 24 h, the constructs were mechanically released from the plate using a sterile needle and lattice contraction was digitally recorded at regular time intervals over 48 h. Gel contraction was quantified by measuring changes in lattice surface area using Openlab software.

Isometric contraction

Measurement of the contractile forces generated by FPCLs was performed as previously described (Eastwood et al., 1996; Tomasek et al., 2002) (Fig. 1). Briefly, 1.5×10^6 cells from either nodule-derived cells, palmar or non-palmar fibroblasts were seeded in 2.5 ml of type I collagen gel (FirstLink), and FPCLs tethered between two flotation bars and held stationary at one end whilst the other attached to a force transducer. FPCL generated tensional forces were continuously measured and data logged every 15 sec (dynes: 1×10^{-5} N) (Eastwood et al., 1994). To ensure nodule-derived cells were within the linear range of contraction, FPCLs were also seeded with reducing cell densities (1.125, 0.75, 0.375 and 0.15×10^6 million cells) in the same final volume of collagen gel. FPCLs were also seeded with co-cultures of 1.5 million nodule-derived cells and dermal fibroblasts, of different cell proportions (75% nodule-derived cells + 25% non-palmar or palmar fibroblasts; 25% nodule-derived cells + 75% non-palmar or palmar fibroblasts). As high aspect-ratio FPCLs (Fig. 1D) have been shown to generate high strain patterns and better reflect cellular activity of Dupuytren's tissue (Eastwood et al., 1998), we also compared identical co-culture FPCLs of nodule-derived cells and dermal fibroblasts contraction using low-aspect-ratio FPCLs (Fig. 1E).

Immunofluorescence

For monolayer studies, 20,000 nodule-derived cells, non-palmar fibroblasts and palmar fibroblasts were cultured on glass coverslips in DMEM and 10% FBS for 24 h. Cells were then fixed for 10 min with 3% paraformaldehyde in PBS and permeabilised with 0.2% Triton X-100 (Sigma) for 5 min. Cells were stained with a mouse monoclonal α-SMA (Cat no: A2547, Sigma) followed by Alexa Fluor 568-conjugated rabbit anti-mouse antibody (Invitrogen, UK). F-actin was probed with Phalloidin-Alexa Fluor 488 (Invitrogen) and DNA with DAPI (Sigma). Secondary antibody alone was used as an immunolabelling control. Following 24-h contraction in the CFM, FPCLs were fixed whilst under tension for 30 min in 3% paraformaldehyde, then embedded in paraffin wax and 10 μm sections cut and stained as described above. Images were acquired using oil immersion objectives (40× and 60×) and a Nikon TE2000 microscope fitted with a camera.

RNA isolation

After 24 h contraction in the CFM, RNA was extracted from gels using an RNeasy kit (Qiagen, UK) according to the manufacturer's instructions. RNA (0.5 μg) was reverse transcribed into cDNA which was used in PCR reactions with primers specific for α-SMA and GAPDH.

The following primers were used to detect α-SMA: 5′-GTC CAC CGC AAA TGC TTC TAA-3′ (forward) and 5′-AAA ACA CAT TAA CGA GTC AG-3′ (reverse), and primers used to amplify GAPDH were: 5′-GGT GAA GGT CGG AGT CAA CGG A-3′ (forward) and 5′-GAG GGA TCT CGC TCC TGG AAG A-3′ (reverse). PuReTaq Ready-To-Go PCR beads (GEHealthcare Amersham, UK) were used following the manufacturer's instructions and PCR products run on a 1% agarose gel containing ethidium bromide. Levels of α-SMA were also analysed by quantitative RT-pcr using ABI TaqMan probes specific for α-SMA (Hs00426835-g1) (Applied Biosystems, USA) in a Corbett Rotor-gene 6000 machine (Corbett Research, UK). The expression levels were normalised to the levels of ribosomal

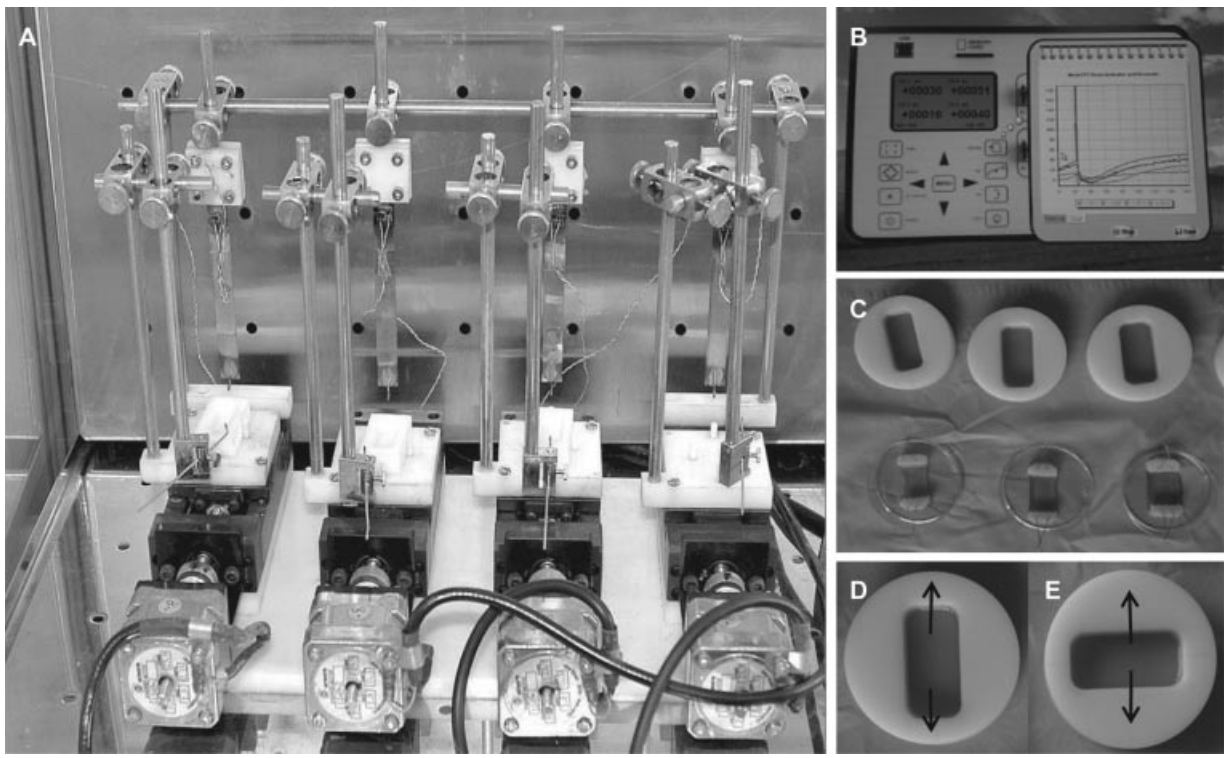

Fig. 1. The culture force monitor (CFM). (A) Rectangular seeded collagen gels were cast and floated in medium, tethered between two flotation bars one of which is held stationary whilst the other is attached to a force transducer. (B) Cell-generated tensional forces in the collagen gel are detected by the force transducer, and live data are logged every 15 sec providing a continuous output of force (dynes, 1×10^5 N) generated. (C) After 24-h contraction, gels are harvested and processed for α-SMA mRNA, protein and Immunofluorescence. (D) Cells were routinely seeded in gels with a high aspect-ratio collagen lattice, although low aspect-ratio lattices (E) were also used in experiments to compare effects of less strain on cell contractility.

protein control RPLPO (Taq man probe set 4310879E) (Applied Biosystems) and to the level of gene expression in unrelated non-palmar skin fibroblasts which were assigned the value of 1 (ΔΔCT method).

Western blot analysis

Fibroblasts within 3-D collagen matrices following 24 h FPCL contraction were harvested, sheared using a 21 gauge needle, and proteins extracted using 2% sodium dodecyl sulphate (SDS). Proteins were separated by SDS–polyacrylamide gel electrophoresis using 7.5% or 10% polyacrylamide gels. Proteins were then transferred to nitrocellulose, and detected using anti α-SMA primary antibody (Sigma) and vimentin (Abcam, UK), appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO, Denmark) and an enhanced chemiluminescence kit (Amersham). Semi-quantitative analysis of protein expression was performed using densitometry analysis software (Phoretics International, UK).

Statistics

Statistical analyses were determined using Prism software (GraphPad Software). The rate of FPCL contraction (dynes/h) was calculated by measuring the average gradient of the curve between 6 and 24 h. Contraction gradients and different aspect-ratio FPCLs were compared using unpaired *t*-tests. Analysis of single variance was used for comparisons of matched set contraction profiles and percentage stress fibres, and also for comparisons of co-cultures. Significance was achieved if $P < 0.05$.

Results

Isotonic versus isometric force contraction of FPCLs

To examine the differences in contractility of Dupuytren's nodule-derived cells, non-palmar and palmar fibroblasts from matched patient samples we used two methods. To assess isotonic contraction, cells were seeded in constrained collagen gels for 24 h. Following 24 h, FPCLs were released from the plate edges, and FPCL surface area measured using digital images recorded at regular intervals over 48 h. Polymerised collagen alone without cells did not contract. Cells isolated from Dupuytren's nodule, non-palmar and palmar skin contracted rapidly over time to the same extent (Fig. 2A), and no significant difference in contraction was observed between these cells at 48 h (Fig. 2B).

Next we examined isometric force contraction. The CFM is capable of measuring minute forces exerted by cells within a collagen lattice over 24 h (Eastwood et al., 1994). Dupuytren's nodule-derived cells, non-palmar and palmar fibroblasts from matched patient samples were seeded in high aspect-ratio FPCLs, attached to a CFM force transducer and isometric force generated was quantified in real time. FPCLs seeded with 1.5 million non-palmar fibroblasts (Fig. 3A) or palmar fibroblasts (Fig. 3B) initially contracted (mean: 0.5 ± 0.2 dynes/h) and reached tensional homeostasis on average after 15 h (range: 4–20 h), with a maximum force of 52 dynes. The same number of nodule-derived cells continued to contract to a maximum of 173 dynes (mean: 3.5 ± 1.1 dynes/h) and did not reach tensional homeostasis (Fig. 3C). Although the absolute rate of force contraction varied between patients, nodule-derived cells consistently generated significantly higher force

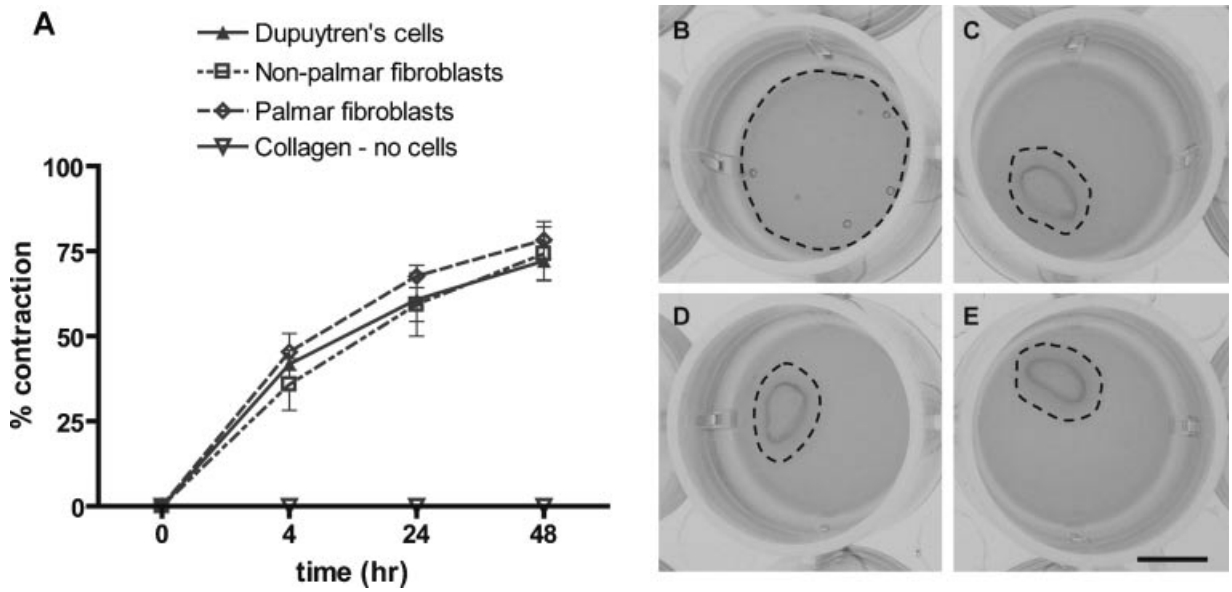

Fig. 2. Isotonic contraction of collagen gels by dermal fibroblasts and Dupuytren's nodule-derived cells. Collagen gels were seeded with non-palmar fibroblasts, palmar fibroblasts or Dupuytren's nodule cells and cultured for 24 h. Control gels were cultured for 24 h in the absence of any cell type. (A) After 24 h collagen gels were released from the edge of the culture dish and gel contraction was assessed by quantifying change in gel surface area over time up to 48 h. Data are shown as the mean % contraction (±SEM) from triplicate experiments using cells derived from one patient and are representative of values from a total of five different patients. B–D: Representative images of collagen gels without cells (B) or seeded with non-palmar fibroblasts (C), palmar fibroblasts (D) or Dupuytren's nodule cells (E) after 48 h of contraction. The dashed black line indicates the outer edge of each collagen gel. Scale bar = 5 mm.

than dermal fibroblasts (Fig. 3D) and never reached tensional homeostasis. Dose–response experiments were carried out to determine if nodule-derived cell contraction was dependent on cell numbers and at what cell density they reached tension homeostasis. The rate of nodule-derived cell contraction was proportional to cell number and, tension homeostasis was reached with 150,000 nodule-derived cells (Fig. 3E).

It is recognised that cells change phenotype when cultured in vitro (Bell et al., 1979; Moyer et al., 2002) therefore, we analysed the effect of passage on cell contractility in both nodule cells and dermal fibroblasts. Collagen gels seeded with cells at passage 1 and 5 were attached to the CFM for 24 h. Using nodule-derived cells, isometric force generation significantly decreased (Fig. 3E) between passages 1 and 5, whereas mean isometric force generation increased with non-palmar skin fibroblasts.

These data show that differences in contraction between nodule-derived cells and dermal fibroblasts are only seen under isometric and not isotonic conditions. Compared with dermal fibroblasts, nodule-derived cells continued to contract in a dose-dependent manner and did not reach tension homeostasis. These contractile differences were seen consistently between different patients. Furthermore, with increasing cell passage, nodule-derived cells lost their contractility whereas dermal fibroblasts became more contractile.

Post-transcriptional regulation of α-SMA drives myofibroblast contractility

To examine α-SMA expression in nodule-derived cells and dermal fibroblasts from matched patient samples, collagen gels seeded with each cell type underwent isometric contraction for 24 h. Cells were then harvested and α-SMA mRNA levels, α-SMA protein levels and α-SMA localisation analysed. Nodule-derived cells contracted at a significantly greater rate over the 24 h test period than dermal fibroblasts (mean rate of contraction, nodule-derived cells = 4.3 dynes/h; non-palmar fibroblasts = 0.5 dynes/h; palmar fibroblast = 0.6 dynes/h) (Fig. 4A). However, between the cell types no significant difference ($P = 0.9$) was seen in total α-SMA mRNA levels when compared to RPLPO (Fig. 4B) and GAPDH (Fig. 4C), although nodule-derived cells expressed more α-SMA protein than dermal fibroblasts compared to vimentin loading controls (Fig. 4D). The ratio of α-SMA-to-vimentin was 1.74:0.66:0.42 (nodule-derived cells/non-palmar fibroblasts/palmar fibroblasts). Furthermore, whilst non-palmar and palmar fibroblasts frequently demonstrated α-SMA staining in a cytosolic peri-nuclear arrangement, in nodule cells α-SMA was typically distributed throughout the cell processes in stress fibres up to the sites of cell–matrix attachment (Fig. 4E). These data suggest change that post-transcriptional changes in α-SMA regulate contractility of Dupuytren's nodule-derived cells.

To quantify the differences in α-SMA distribution between the matched cell types, immunofluorescence was also carried out on cells in monolayer. In nodule-derived cells, α-SMA protein frequently localised to cytoskeletal stress fibres, whereas in dermal fibroblasts α-SMA protein was typically seen in the peri-nuclear cytoplasm (Fig. 5A). The number of cells with positive α-SMA stress fibres was counted and expressed as a percentage of total cells. Nodule-derived cells had significantly more cells with α-SMA stress fibres than dermal fibroblasts (mean α-SMA stress fibres: nodule-derived cells = 35 ± 13%; dermal fibroblasts = 3 ± 0.5%) (Fig. 5B). These data further suggest that α-SMA is more frequently seen to localise to stress fibres in nodule-derived cells.

Dermal fibroblasts co-cultured with nodule-derived cells show no difference in isometric force contraction

Co-culture experiments were performed to test the hypothesis that the contractility of Dupuytren's nodule-derived

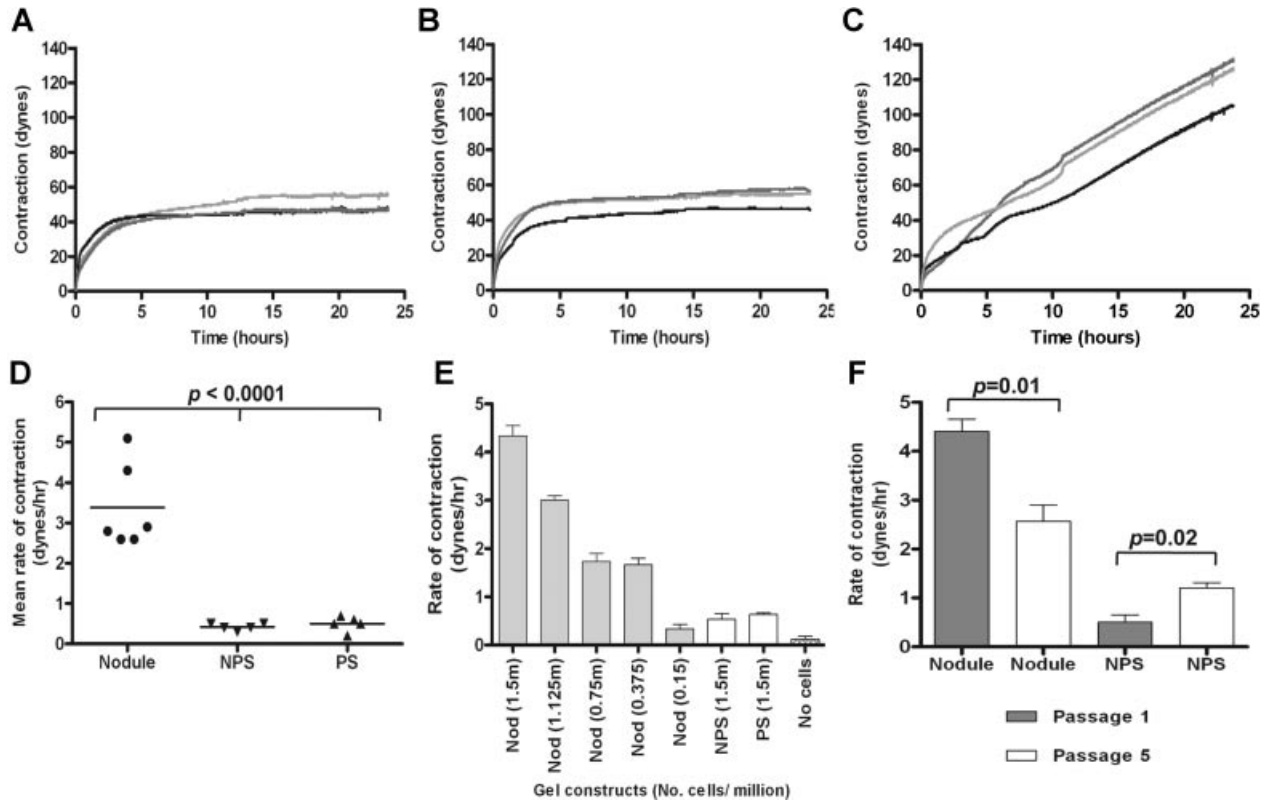

Fig. 3. Isometric contraction of collagen gels by dermal fibroblasts and Dupuytren's nodule-derived cells. (A–C) Collagen gels were seeded with 1.5 million non-palmar fibroblasts (A), palmar fibroblasts (B) or Dupuytren's nodule-derived cells (C), cultured for 24 h in the CFM and real-time isometric force contraction was quantified. Data shown represent triplicate experiments using cells derived from one patient. (D) The average rate of contraction between 6 and 24 h for nodule-derived cells (nodule), non-palmar (NPS) and palmar fibroblasts (PS) is shown for five different matched patient samples. Horizontal bars represent mean rates of contraction (dynes/h). (E) Collagen gels were seeded with decreasing numbers of nodule-derived cells (1.5, 1.125, 0.75, 0.375, 0.15 m) and either non-palmar or palmar fibroblasts (1.5 m each). Collagen alone was included as a control. The average rate of contraction (dynes/h) between 6 and 24 h was quantified. (F) 1.5 million nodule-derived cells and dermal fibroblasts from cell passages 1 and 5 were seeded in collagen gels and analysed for isometric force contraction. (E,F) Data are shown as the mean of triplicate experiments (±SEM) using cells derived from one patient and representative of results from three different patient samples.

cells is negatively regulated by non-palmar dermal fibroblasts. A total of 1.5 million cells at different ratios of nodule-derived cells to dermal fibroblasts (either non-palmar or palmar fibroblasts) were seeded in FPCLs and attached to the CFM for 24 h. The co-culture cell ratios were: 75% nodule-derived cells and 25% dermal fibroblasts (either palmar or non-palmar fibroblasts), or vice versa. As nodule cells were the contractile cell type, isometric forces generated from co-culture FPCLs were compared with control FPCLs seeded with equivalent densities of nodule cells (i.e. 25% nodule cells co-culture was compared with 375,000 PIPJ nodule cells and 75% with 1.125 m cells). No significant difference was observed in isometric force contraction with different ratios of the matched cells over 24 h (Fig. 6A).

To further investigate the potential effects of longer term co-cultures, cells were co-cultured together for 5 days at the same cell ratios as described above. After 5 days, isometric forces generated were measured over 24 h (Fig. 6B). No significant difference was observed with these co-cultures (75% nodule-derived cells + 25% dermal fibroblasts; 25% nodule-derived cells + 75% dermal fibroblasts). There was no difference in both proliferation rates of the individual cell types and the relative proportion of nodule cells to dermal fibroblasts in each co-culture construct ($P = 0.2$, $P = 0.7$) and, there was no change in cell viability over time ($P = 0.3$) (data not shown).

We also compared co-cultures in both low and high aspect-ratio FPCLs to examine the dependence of strain on cell contractility. Identical co-cultures of 50% dermal fibroblasts and 50% nodule cells were seeded in collagen gels and compared with high and low aspect-ratio lattices. No significant difference in the rate of isometric force generation ($P = 0.1$) was observed between FPCLs with different aspect-ratios (high aspect-ratio = 2 ± 0.2 dynes/h; low aspect-ratio = 1.6 ± 0.1 dynes/h). However, overall force generation was significantly greater ($P = 0.005$) with high aspect-ratio FPCLs after 24 h contraction (mean high aspect-ratio = 91 ± 1 dynes/24 h, and mean low aspect-ratio mean = 77 ± 4 dynes/24 h) (data not shown).

Discussion

Myofibroblasts are best studied in 3D collagen lattices under isometric conditions. Several authors have studied myofibroblasts derived from rat subcutaneous and lung tissue (Hinz et al., 2001a; Goffin et al., 2006; Hinz, 2007; Follonier et al., 2008), and with or without treatment with TGFβ-1 (Arora et al., 1999; Dugina et al., 2001). We studied early passage (1–2) myofibroblasts from patients with a common musculoskeletal disorder and compared them with matched fibroblasts obtained from the same patients. Whereas previous studies on Dupuytren's disease have compared Dupuytren's

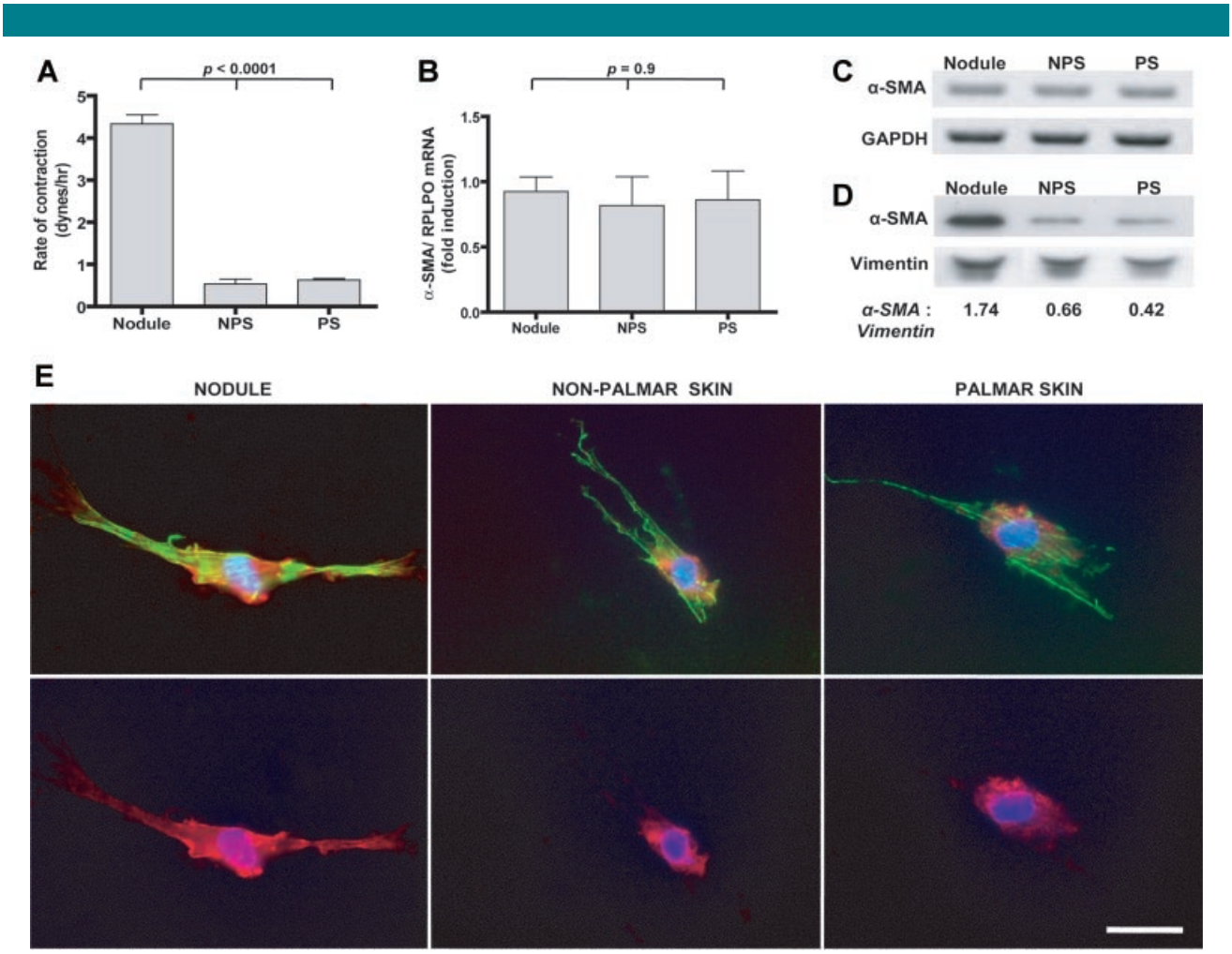

Fig. 4. Contractility of Dupuytren's nodule-derived cells is regulated by post-transcriptional changes in α-SMA. (A) Isometric force contraction in collagen gels seeded with nodule-derived cells (nodule), non-palmar (NPS) and palmar fibroblasts (PS) over 24 h. Experiments were performed in triplicate and values represent the mean ± SEM. After 24 h cells were isolated from gels and levels of (B) α-SMA mRNA compared to RPLPO by quantitative RT-pcr (C) α-SMA mRNA compared to GAPDH (D) levels of α-SMA protein compared with vimentin and (E) α-SMA protein localisation analysed by immunofluorescence. (E) Representative immunofluorescence images of nodule-derived cells, palmar and non-palmar fibroblasts are taken from 7 μm thick sections fixed and stained with α-SMA antibodies (red), phalloidin (green) and DAPI (blue) are shown in the higher parts. The lower parts are images of the same cells showing only α-SMA staining (red) and DAPI (blue). Scale bar: 30 μm. Data are shown from experiments using cells derived from one matched patient sample and are representative of a total of three different patients.

nodule and cord cells with non-matched carpal ligament derived cells (Brickley-Parsons et al., 1981; Murrell et al., 1991; Rayan and Tomasek, 1994; Tomasek and Rayan, 1995; Bisson et al., 2003, 2004), we report for the first time comparisons between genetically matched cells from Dupuytren's nodules, non-palmar and palmar skin with regards to their contractile phenotypes in 3D collagen lattices.

Initially, we measured isotonic contraction in stress-released circular collagen lattices and found no differences in contraction between Dupuytren's cells and dermal fibroblasts. Several authors have used this model to examine Dupuytren's cells and similar findings to ours have been reported (Rayan and Tomasek, 1994). Although others have demonstrated increased contraction with nodule cells compared to cord cells or control carpal ligament fibroblasts (Tomasek and Rayan, 1995; Moyer et al., 2002), these differences were only maintained in earlier cell passage (Moyer et al., 2002) and when nodule derived cells were subdivided in to high α-SMA expression groups (Tomasek and Rayan, 1995). Interestingly, one study demonstrated increased contraction by dermal fibroblasts compared to nodule-derived cells (Tarpila et al., 1996) and attributed this to late stage Dupuytren's disease samples that had lost their contractile phenotype.

It is well recognised that in 3D collagen lattices, migrating fibroblasts develop tractional forces that increase matrix stiffness by reorganising the loose collagen matrix (Grinnell, 2003). The presence of TGFβ-1 (Desmouliere et al., 1993), ED-A fibronectin (Serini et al., 1998) and continued high levels of extracellular stress (Arora et al., 1999; Hinz et al., 2001b) in constrained FPCLs, leads to the incorporation of α-SMA in to stress fibres, development of prominent matrix focal adhesions and myofibroblast differentiation (Hinz, 2007). However, upon release of FPCLs, cell-generated tension within the lattice is immediately lost, with subsequent change in fibroblast morphology as rapid isotonic contraction occurs (Tomasek et al., 1992; Grinnell, 2003). This has been likened to wound healing and granulation tissue contraction (Grinnell, 1994) and more importantly, the loss of tension has been shown to result in rapid disassembly of stress fibres and degradation of α-SMA (Hinz et al., 2001b). Such changes in stress fibres following release have been observed within 2 min and after 10 min almost all stress fibres have disappeared (Tomasek et al., 1992).

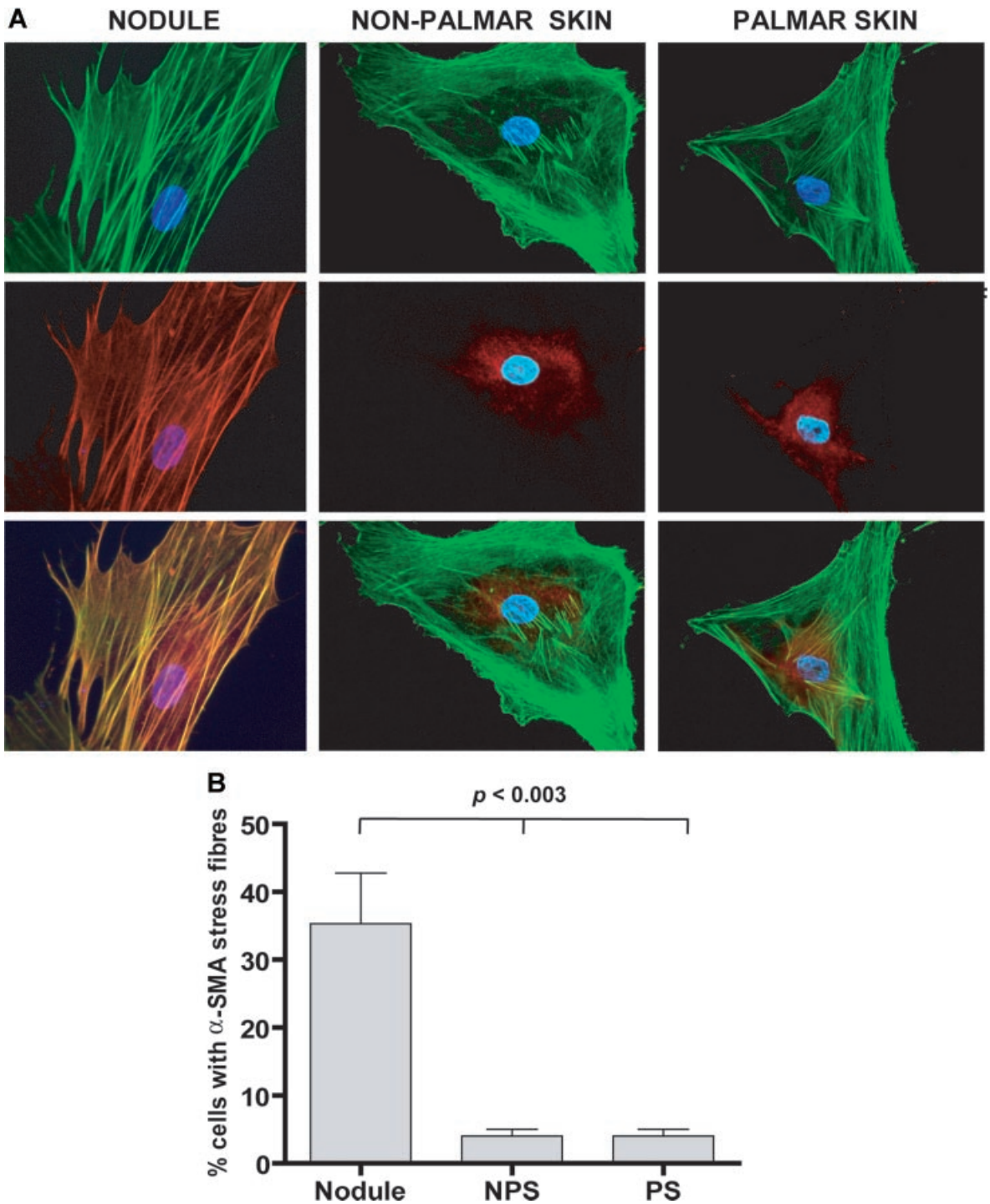

Fig. 5. Quantification of α-SMA localisation to stress fibres in dermal fibroblasts and Dupuytren's nodule-derived cells. Cells were cultured on glass coverslips for 24 h, fixed and then immunofluorescently labelled using α-SMA antibodies (red), phalloidin (green) and DAPI (blue). (A) Representative images are shown for Dupuytren's nodule-derived cell, non-palmar fibroblast and palmar fibroblast. The higher parts show staining for phalloidin (green) and DAPI (blue). The middle parts show α-SMA (red) and DAPI (blue), and the lower parts represent merged images with α-SMA (red), phalloidin (green) and DAPI (blue) staining. Scale bar: 20 μm. (B) The numbers of cells with α-SMA positive stress fibres in nodule-derived cells, palmar and non-palmar fibroblasts were counted on digital images (over 300 cells counted per cell type) and expressed as a percentage of total cells (original magnification 60×). Experiments were performed in triplicate and data are shown as the mean (±SEM) from a total of three different patients.

The CFM permits real-time quantification of uniaxial isometric forces generated in constrained high aspect-ratio rectangular FPCLs (Eastwood et al., 1996; Bisson et al., 2004). Using this more representative model which would preserve the phenotype of myofibroblasts and hence more closely emulate the in vivo situation, we compared Dupuytren's nodule-derived cells with matched dermal fibroblasts. In line with previous studies, we found that dermal fibroblasts reached

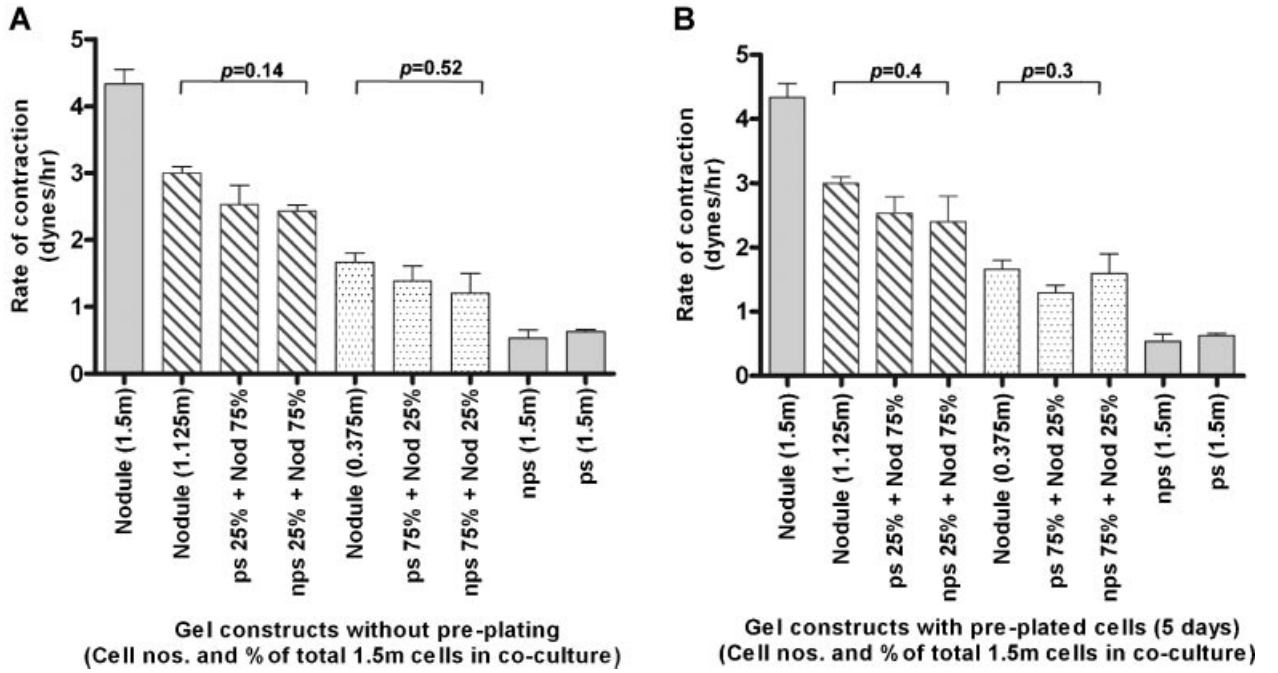

Fig. 6. Isometric contraction of collagen gels seeded with co-cultures of dermal fibroblasts and Dupuytren's nodule-derived cells. (A) Co-cultures of nodule-derived cells and dermal fibroblasts (either palmar or non-palmar) were seeded in collagen gels at different cell ratios of nodule to dermal fibroblasts. A total of 1.5 m cells were seeded in gels with co-cultures or dermal fibroblasts alone. Nodule-derived cells were seeded at equivalent cell densities to nodule-derived cells in co-culture (1.125 m nodule cells = 75% nodule cells in co-culture; 0.375 m nodule cells = 25% nodule cells in co-culture). Contractile profiles of co-cultures were then compared to nodule-derived cells of equivalent cell densities. (B) Co-cultures were also pre-plated for 5 days using the same cell ratios, then seeded in collagen gels and isometric force contraction measured over 24 h. Data are shown as the mean (±SEM) from triplicate experiments using cells derived from one patient, and are representative of values from a total of three different patients.

tension homeostasis, whereas nodule-derived cells continued to contract in a dose-dependent manner over a 24-h test period (Brown et al., 1998; Bisson et al., 2004). In addition, by harvesting FPCLs following 24 h contraction in the CFM, we compared α-SMA mRNA levels, α-SMA protein expression and α-SMA protein localisation by immunofluorescence in the matched cell types. Interestingly, no differences in α-SMA mRNA levels were seen between nodule-derived cells and dermal fibroblasts, although greater α-SMA protein levels were seen in nodule-derived cells compared with matched dermal fibroblasts. Furthermore, by immunofluorescence we demonstrated that in dermal fibroblasts, α-SMA was typically distributed in a 'halo' within the peri-nuclear cytoplasm, whereas in nodule-derived cells, α-SMA was frequently localised in stress fibres throughout the cell processes up to cell–matrix attachment sites. These differences seen with nodule-derived, non-palmar and palmar skin cells from matched samples have not been previously reported. Previous studies have reported only differences in α-SMA protein expression and localisation between nodule- or cord-derived Dupuytren's cells when compared to non-matched control carpal ligament cells. The comparative levels of α-SMA mRNA in these cell types have not been analysed (Benzonana et al., 1988; Dave et al., 2001). Here we simultaneously examined α-SMA protein levels, protein localisation and mRNA levels in cells isolated from the same patient. Our findings suggest post-transcriptional changes in α-SMA occur in genetically matched cells to mediate the Dupuytren's myofibroblast cell phenotype. Recent studies on cellular mechanisms of local protein synthesis have now also suggested that, not only is mRNA subject to translation, but that cells can instruct the cytoskeleton and translation apparatus where to transport and when to translate the intended protein (Rodriguez et al., 2008). Therefore, post-transcriptional changes in α-SMA expression may include mRNA stability, mRNA localisation, translation, cytoskeletal localisation and stress fibre formation. Further studies using Dupuytren's matched cells are required to investigate these possibilities.

Previous studies have reported that nodule-derived cells maintain significant contractile differences up to cell passage 4–5 when compared to control fibroblasts (Moyer et al., 2002; Bisson et al., 2004). However, our data suggest that contractility of nodule cells significantly decreases by cell passage 5. We found that increasing cell passage was correlated with a consistent decrease in α-SMA stress fibres (not shown). Interestingly, we observed the opposite in dermal fibroblasts, with a significant increase in contractility with cell passage although no change was seen in α-SMA stress fibres. This suggests that culturing dermal fibroblasts on rigid plastic substrates may over time lead to increased cell-generated tension and promote the development of a more contractile protomyofibroblast phenotype (Tomasek et al., 2002; Hinz, 2007). The tendency towards a similar contractile phenotype between nodule-derived cells and dermal fibroblasts may reflect contractile adjustments by these cells as a consequence of the identical in vitro culture conditions. All our experiments were performed with cells from cell passage 1 or 2.

We have previously shown that excised Dupuytren's tissue often has multiple α-SMA-rich nodules which may also vary significantly in size and that a proportion of myofibroblasts are found distributed outside nodules and throughout cord tissue (Verjee et al., 2009). Whilst this may explain the variation in contractility we observed between samples with different proportions of α-SMA-rich cells, it also represents a significant

methodological issue in Dupuytren's cell culture-based studies as no reliable myofibroblast cell lines are available. Therefore, in this study to ensure that we isolated proportionally more α-SMA-rich nodule cells, digital cord samples were bisected and half processed for cell culture, whilst the cut surface of the mirror half was processed to identify samples with α-SMA-rich nodules by immunohistochemistry. Subsequent quantification by immunofluorescence demonstrated on average 35% of cells express α-SMA stress fibres in histology confirmed nodular samples, as compared to 10% α-SMA stress fibres in non-nodular samples. Although this still does not constitute a homogenous population of myofibroblasts, our method of sampling α-SMA-rich cells represents a significant improvement on previous studies which have reported on average between 9.7% and 15% α-SMA-positive cells isolated from clinical and not histology defined nodules (Tomasek and Rayan, 1995; Bisson et al., 2003).

Finally, we also tested the hypothesis that non-palmar dermal fibroblasts may downregulate Dupuytren's myofibroblasts based on the surgical observation that recurrence following dermofasciectomy is much lower than after fasciectomy (Tonkin et al., 1984). We found no differences in isometric force contraction with co-cultures when compared to controls of equivalent numbers of nodule cells alone, either over 24 h or with longer-term cultures. No differences in contractility were observed with different ratios of nodule-derived cells to dermal fibroblasts and controlling for cell proliferation rates in co-culture. Our co-cultures were only performed over a period of 5 days and we recognise the limitation of CFM co-culture system in that it does not reflect the processes involved in vivo for dermofasciectomy and, recurrent disease may evolve over several months or years.

It has been demonstrated previously that isometric contraction of a 3D collagen lattice by resident fibroblasts causes strains to develop within the lattice and these strains can be increased or decreased by altering the FPCL aspect-ratio (ratio of length/width/thickness) (Eastwood et al., 1998) to modulate cellular activity. Dupuytren's cord tissue in vivo has a high aspect-ratio and is subjected to considerable tensile force that causes high strain gradients, and these may in turn dictate cellular alignment along the major axis of the cord. Consequently, iso-strains run parallel with the long axis, which in Dupuytren's cord is reflected by fibroblasts aligned longitudinally with collagen fibres (Luck, 1959; Verjee et al., 2009). Interestingly, it has been observed clinically that after simple division of Dupuytren's tissue (fasciotomy), cords which are no longer under tension, soften and temporarily disappear (Hueston, 1992). Furthermore, it has also been suggested that reducing skin tension following fasciotomy by a single Z-plasty significantly reduced recurrence at 2 years (Citron and Hearnden, 2003). We therefore compared identical co-cultures in both low and high aspect-ratio FPCLs to examine the dependence of tension and strain on cell contractility. Although we found no significant difference in the rate of contraction (dynes/ hour) between the different aspect-ratios, a significant decrease in overall force generation was observed with low aspect-ratio FPCLs. This implies that Dupuytren's cells remain significantly more contractile than dermal fibroblasts even in the absence of high strain gradients, although nodule-derived cells may prefer an environment with higher baseline levels of tensile force. Indeed, it may be that dermofasciectomy, with resurfacing of the cutaneous defect in the digit with a full-thickness skin graft, may alter myofibroblast activity through a combination of reduced tension and more complete excision, as it can be difficult to accurately differentiate the surgical plane between Dupuytren's cord and palmar skin at the time of fasciectomy.

Our histological findings based on 103 digital cord samples showed that α-SMA-rich nodules occurred with equal frequency in primary and recurrent disease samples and were also not significantly different in size (Verjee et al., 2009). Recurrences were seen following previous fasciotomy, fasciectomy and firebreak dermofasciectomy but not radical dermofasciectomy. It is possible that residual unexcised Dupuytren's tissue following less radical surgery may serve as a trigger for recurrence and, indeed, the increased motion following initial surgery may facilitate myofibroblast differentiation and persistence. This may also explain why radical dermofasciectomy has a lower rate of recurrence (1–5%) (Tonkin et al., 1984; Brotherston et al., 1994; Hall et al., 1997) than 'firebreak' dermofasciectomy (12%) (Ullah et al., 2009) or fasciectomy (40–50%) (Hueston, 1961; Tonkin et al., 1984; Foucher et al., 1992).

In conclusion, isometric force contraction measured by the CFM represents the best model available for the study of cell contractility in Dupuytren's disease. Using matched patient samples we demonstrated that post-transcriptional changes of α-SMA expression mediate the contractile phenotype of Dupuytren's cells. The different possible mechanisms for post-transcriptional modification warrant further investigation. Our co-cultures data suggests that contractility of Dupuytren's cells is not influenced by dermal fibroblasts. Reduced recurrence rate following dermofasciectomy may be due to other factors, such as more complete excision of affected tissue combined with reduced tension following application of full thickness skin grafts.

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