MMP-14 and MMP-2 are key metalloproteases in Dupuytren's disease fibroblast-mediated contraction

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ABSTRACT

Dupuytren's disease (DD) is a common fibrotic condition of the palmar fascia, leading to deposition of collagen-rich cords and progressive flexion of the fingers. The molecular mechanisms underlying the disease are poorly understood. We have previously shown altered expression of extracellular matrix-degrading proteases (matrix metalloproteases, MMPs, and 'a disintegrin and metalloprotease domain with thrombospondin motifs', ADAMTS, proteases) in palmar fascia from DD patients compared to control and shown that the expression of a sub-set of these genes correlates with post-operative outcome. In the current study we used an *in vitro* model of collagen contraction to identify the specific proteases which mediate this effect. We measured the expression of all MMPs, ADAMTSs and their inhibitors in fibroblasts derived from the palmar fascia of DD patients, both in mono-layer culture and in the fibroblast-populated collagen lattice (FPCL) model of cell-mediated contraction. Key proteases, previously identified in our tissue studies, were expressed *in vitro* and regulated by tension in the FPCL, including *MMP1, 2, 3, 13 and 14*. Knockdown of *MMP2* and *MMP14* (but not *MMP1, 3 and 13*) inhibited cell-mediated contraction, and knockdown of *MMP14* inhibited proMMP-2 activation. Interestingly, whilst collagen is degraded during the FPCL assay, this is not altered upon knockdown of any of the proteases examined. We conclude that MMP-14 (via its ability to activate proMMP-2) and MMP-2 are key proteases in collagen contraction mediated by fibroblasts in DD patients. These proteases may be drug targets or act as biomarkers for disease progression.

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1. Introduction

Dupuytren's disease (DD) is a common condition causing fixed flexion deformity of the digits with reported prevalence between 0.2% and 56% depending on age, population and methodology of the study [1]. Clinically, it is characterised by fibrosis of the palmar fascia with contraction of the digits potentially mediated by the formation and proliferation of myofibroblasts. Three distinct histological phases have been described [2] with: a proliferative phase leading to the development of a nodular lesion, an involutional phase where cells align themselves to lines of stress with concomitant contraction of the tissue, and a residual phase leaving a scar-like cord tissue. Risk factors include age, sex, alcohol and tobacco intake, trauma, diabetes, epilepsy and hyperlipidaemia [3]. The disease is often familial but the mode of

inheritance is currently unknown [4]. A recent genome wide association study highlighted loci relevant to the Wnt signaling pathway as involved in the genetic susceptibility to Dupuytren's disease [5].

Mechanical disruption of the fibrotic material through surgical intervention to allow finger extension remains the most common treatment. Techniques range from minimally invasive needle fasciotomy, excision of the fibrous cords (fasciectomy) or most aggressively, excision of skin and DD material with full thickness skin grafts (dermofasciectomy). None of these operations, which can be associated with significant complications, fundamentally affect underlying disease activity and this is confirmed by high recurrence rates [3]. Enzymatic fasciotomy using clostridium-derived collagenases has recently been approved as a non-surgical alternative [6] but mirrors needle fasciotomy with only partial improvement and high recurrence rates of proximal interphalangeal joint contractures. Disease progression and post-surgical prognosis is difficult to predict and there are no useful biomarkers.

The matrix metalloproteases (MMPs) are a family of 23 enzymes in man, which include the only mammalian enzymes able to degrade the collagen triple helix in a specific manner under physiological

conditions [7]. The 'classical' collagenases are MMP-1, -8 and -13 of the human enzymes. More recently, MMP-2 (gelatinase A) and MMP-14 (MT1-MMP) have also been shown to make this specific cleavage though with less catalytic efficiency [8–10]. Other MMPs are also implicated in collagen turnover, e.g. MMP-3, by virtue of its ability to activate the pro-enzyme form of MMP-1 [11]. A related family of metalloproteases, the ADAMTSs (a disintegrin and metalloprotease domain with thrombospondin motif), number 19 enzymes in man and are also implicated in extracellular matrix (ECM) metabolism. These include enzymes capable of degrading the proteoglycan, aggrecan (at least ADAMTS-1, -4, -5, -8, -9 and -15), and three procollagen N-propeptidases (ADAMTS-2, -3 and -14). Many other members of this family are of unknown function [12]. A family of four specific inhibitors, the TIMPs, have been described [13]. Whilst the ability of the four TIMPs to inhibit MMPs is largely promiscuous, a number of functional differences have been noted, e.g. TIMP-2, -3 and -4, but not TIMP-1, are effective inhibitors of the membrane-type metalloprotease (MT-MMP) subclass. Specificity amongst the TIMPs for inhibition of the ADAMTS family of metalloproteases has also been described with TIMP-3 being a potent inhibitor of e.g. ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) [14]. In many fibrotic diseases such as those affecting liver, lung, and skin, MMPs (and related metalloproteases) and TIMPs play an important role. Normal ECM turnover can be viewed as a balance between protease and inhibitor activities (presuming synthesis remains unaltered), with fibrosis coming from an imbalance away from proteolysis [7].

In the 1980s and 1990s, several small molecule inhibitors of MMPs underwent clinical trials in a variety of cancers [15]. The major side-effect of these drugs was a so-called 'musculoskeletal syndrome', often referred to as musculoskeletal pain accompanied by tendonitis [16,17]. This was dose- and time-dependent and reversible on stopping treatment, but did not respond well to NSAIDs or low dose steroid treatment. The clinical presentation, where reported in detail, is described as frozen shoulder or a condition resembling Dupuytren's disease [18]. Both of these conditions involve similar fibrotic mechanisms (of the shoulder joint capsule in the case of frozen shoulder), the laying down of a collagen-rich ECM and the involvement of myofibroblast-mediated contraction [19,20]. Whilst Dupuytren's disease and frozen shoulder have very different natural histories (the former a progressive disease, the latter usually self-limiting and resolving in time), they may well share common pathways leading to contracture [19]. The MMP inhibitors that cause the 'musculoskeletal syndrome' are 'pan-MMP' inhibitors, showing an approximately nanomolar (or lower) inhibition constant against many of the MMPs tested. Moreover, there is good evidence that they may also inhibit related metalloproteases e.g. ADAMTSs. Indeed, the musculoskeletal syndrome is usually ascribed to the inhibition of non-target metalloproteases.

Previously, measurement of small sub-sets of MMPs and TIMPs had been undertaken in DD tissues or in patient sera, but none of these was comprehensive [21–24]. We recently assayed the expression of the entire *MMP*, *TIMP* and *ADAMTS* gene families in DD tissue (nodule and cord) compared to normal palmar fascia using qRT-PCR [25]. The expression of a number of enzymes was raised in DD nodule including four collagenolytic proteases, *MMP1*, *MMP2*, *MMP13* and *MMP14*, as well as *TIMP1*. We also followed DD patients for 2 years and assessed their hand function. We discovered that the expression of key proteases (e.g. all four collagenases mentioned) correlated with poor progression post-fasciectomy [26]. This reinforces their role as key mediators of the disease process.

Whilst there is no animal model of Dupuytren's disease, a number of *in vitro* models have been used to mimic aspects of the disease. These include the fibroblast-populated collagen lattice assay which measures cell-mediated contraction [27]. Fibroblasts from Dupuytren's disease patients have been shown to generate enhanced contraction compared to controls in the fixed, or attached-delayed release (ADR) format of the FPCL assay e.g. [28]. Broad spectrum small molecule inhibitors of

metalloproteases have been shown to inhibit contraction in this and other similar models [29,30]. This blockade is difficult to dovetail with the reported side-effects of pan-MMP inhibitors in clinical trials; however, this reflects our increasing understanding that MMPs can mediate both positive and negative effects and the need is to identify specific MMPs as therapeutic targets [31].

In this study we measured metalloprotease gene expression in the FPCL model, in lattices populated with fibroblasts derived from Dupuytren's disease palmar fascia. We then used siRNA knockdown of individual MMPs to probe function. We also measured collagen breakdown across the assay to determine if inhibition of collagenolysis was responsible for inhibition of cell-mediated contraction. We found that, of the collagenolytic MMPs, only knockdown of either *MMP14* or *MMP2* inhibited contraction, with no concomitant change in collagen breakdown. This, and our earlier research in man, establishes these enzymes as key players in Dupuytren's disease, highlighting them as potential drug targets and/or biomarkers of disease progression.

2. Material and methods

2.1. Cell culture

Primary fibroblasts were derived by explant outgrowth from the nodular palmar fascia of Dupuytren's disease patients undergoing fasciectomy (as previously described [32]) and used up to passage 5. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C, 5% CO_2.

2.2. Fibroblast-populated collagen lattice (FPCL)

Collagen contraction was assessed using a fixed FPCL format (n = 4). Rat tail type 1 collagen (First Link, UK) was combined 9:1 with 10× serum-free DMEM to give a final concentration of 1.85 mg/ml collagen and neutralized with 10 M NaOH. Collagen was then combined at a ratio of 1:1 with cells suspended at 4×10^5 cells/ml in serum-free DMEM. Final concentration of collagen and cells were 0.925 mg/ml and 2×10^5 cells/ml respectively. The collagen/cell suspension was added 0.5 ml/well in a 24-well plate and allowed to form a gel at 37 °C for 1 h. Serum-free medium (0.5 ml/well) was added and collagen lattices, attached to the wells, were allowed to develop tension over 24, 48 and 72 h prior to their release or harvest. One hour before the release of gel (using a pipette tip to detach the gel from the plastic of the well), the serum-free medium was replaced with complete medium containing 10% FCS. All conditioned media were harvested and stored at −80 °C for downstream analysis.

Collagen lattices were harvested directly in Trizol reagent (Invitrogen) after 24, 48 and 72 h of tension and also at 3 h and 24 h post release. Contraction was monitored using a flatbed scanner (HP Scanjet 3800) on transient removal of plates from the incubator and quantified using the image processing software Image J (http://rsbweb.nih.gov/ij/). Images were taken at release and across the subsequent 24 h.

2.3. RNA extraction and synthesis of complementary DNA

Gels (and the equivalent cells from monolayer culture) were harvested directly into Trizol (Invitrogen, UK). After complete suspension, chloroform was added (200 μl/ml Trizol), vortexed and centrifuged at 12,000 g for 15 min at 4 °C. The clear aqueous layer was placed into a separate tube and a total of 0.5× volume of 100% ethanol was added and mixed. Samples were then applied onto spin columns (RNeasy Mini Kit, Qiagen, UK) and RNA purification performed according to the manufacturer's instructions. RNA samples were quantified using a NanoDrop spectrophotometer (NanoDrop technologies, Wilmington, DE) and stored at −80 °C. RNA (250 ng) was converted

to cDNA using Superscript II reverse transcriptase (Invitrogen) in a final volume of 20 μl according to the manufacturer's instructions and stored at −20 °C.

2.4. Quantitative real time-PCR

Quantitation of single gene expression was carried out on the ABI Prism 7500 or 7900 sequence detection system (Applied Biosystems) following the manufacturer's protocol. The sample (5 ng cDNA or 1 ng for 18S rRNA analyses) was added to the PCR reaction mixture containing 50% 2× mastermix, 100 nmol/l of forward and reverse primer, 200 nmol/l probe in a final volume of 25 μl. The PCR protocol involved 2 min at 50 °C, 10 min at 95 °C, then 40 cycles each consisting of 15 s at 95 °C and 1 min at 60 °C. The expression of MMPs, ADAMTSs and TIMPs, 18S rRNA and β-actin housekeeping genes was measured using a custom Taqman® Low Density Array (TLDA) (Applied Biosystems) on the ABI Prism 7900 system. For the amplification reactions 100 ng/μl of cDNA was combined with master mix to a final volume of 100 μl and applied to each port of the TLDA. In order to compare expression of all genes, expression was normalised to either β-actin or 18S rRNA using a transformation proportional to normalised copy number ($2^{-\Delta C_T}$), where ΔC_T is C_T of the gene of interest − C_T of housekeeping gene (C_T = threshold cycle).

2.5. Hydroxyproline assay

Hydroxyproline (OH-Pro) was measured in conditioned medium using a microtitre modification of the method described by Bergman and Loxley (1963) [33] to quantify collagen release.

2.6. RNA interference (siRNA)

Efficacy of knockdown for all siRNAs was initially assayed in cells in monolayer culture using qRT-PCR to measure expression of the target gene. In order to knock down gene expression in the FPCL assay, two rounds of siRNA transfection were used. Cells were plated in a 6 well plate (2×10^5 cells/well) and at ~80–90% confluency, transfected (using DharmaFECT1 4 μl/well) with 5 nM siRNA (Qiagen) individually targeting *MMP1, MMP2, MMP3, MMP13 or MMP14* or a non-targetting siRNA control (AllStars, Qiagen). A mock transfection containing no siRNA and a negative control with no manipulation of the cells were also included. Cells were incubated for 48 h before a second round of transfection of the same siRNAs for 24 h. Cells were then trypsinised and seeded into the FPCL assay as before.

2.7. Gelatin zymography

Samples were electrophoresed under nonreducing conditions by SDS–PAGE in 10% polyacrylamide gels copolymerised with 1% gelatin. Gels were washed vigorously twice for 15 min in 2.5% Triton X-100 to remove SDS, then incubated overnight in 50 mM Tris/HCl, pH7.5, 5 mM CaCl2 at 37 °C. Gels were then stained with Coomassie Brilliant Blue and quantified using the Odyssey imaging system (Li-COR, USA). Parallel gels were incubated in buffers containing 5 mM EDTA to show that lysis of gelatin was due to metalloprotease activity.

2.8. Statistical analysis

Data are presented as mean value +/− SEM. Statistical analysis utilised either pairwise comparison by two-tailed Student's *t*-test or for groups of three of more, ANOVA with Tukey's post test in either Microsoft Excel or GraphPad Prism 4 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Fibroblast-populated collagen lattice model

Initially we verified that fibroblasts derived from the palmar fascia of Dupuytren's disease patients would reproducibly contract a collagen lattice. Fig. 1 shows contraction in the fixed FPCL format using cells derived from four independent patients. The kinetics of contraction was similar across the patient cell lines with initial rapid contraction upon release of the lattice, followed by a slower phase to 24 h. This is in broad agreement with published data for this assay [28,29,34]. Expression of α-smooth muscle actin was detected in tissue, cells in monolayer culture and in the FPCL by immunochemistry (data not shown).

3.2. Protease gene expression

In order to ascertain if the pattern of metalloprotease gene expression in the isolated cells was similar to that seen in the tissue, the
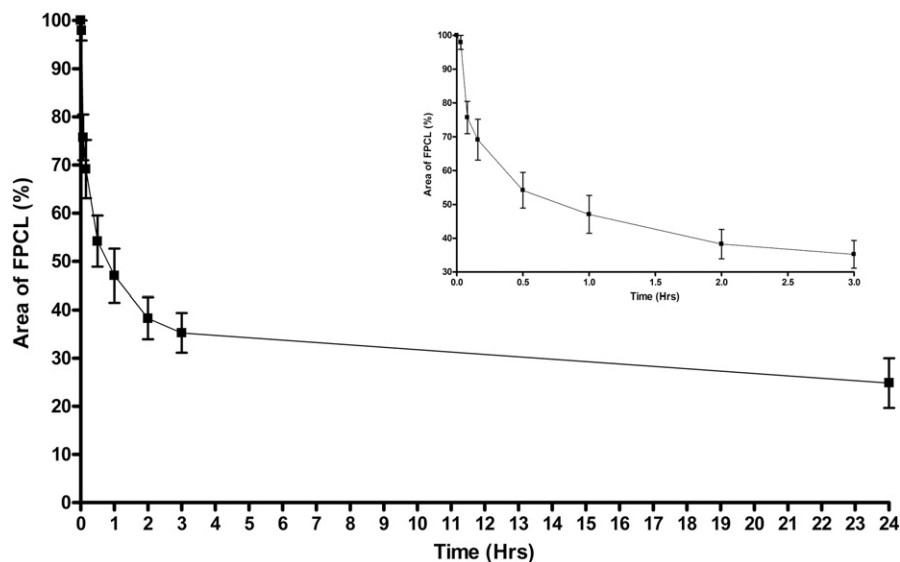

Fig. 1. Fixed fibroblast-populated collagen lattice model. Fibroblasts derived from the nodules of four independent Dupuytren's disease patients were used in the FPCL model (see Material and methods). Contraction is shown as percentage of the lattice compared to $t = 0$. The insert shows the first 3 h of the time course on different scale axes for clarity. Error bars are s.e.m.

expression of metalloproteases was measured in cells derived from two independent Dupuytren's disease patients and cultured in monolayer. Fig. 2 shows e.g. high expression of *MMP2, MMP14, ADAMTS1, ADAMTS2, TIMP1, TIMP2, TIMP3* with lower expression of *MMP1*, *MMP3* and *MMP13* in a similar pattern to that seen in our previous tissue study [25].

Cells were then seeded into the FPCL assay and allowed to develop tension for 24, 48 and 72 h prior to RNA isolation. Lattices were released at 48 h and RNA was also isolated at 3 h or 24 h after release. The expression of *MMP1, MMP2, MMP3, MMP13, MMP14* and *TIMP1*, as genes encoding proteins involved in collagen turnover, were then measured by qRT-PCR. Fig. 3 shows that for all genes measured, expression was higher in the three-dimensional collagen lattice at 24 h, compared with monolayer culture. The expression of *MMP1*,

MMP3, MMP14 and *TIMP1* then decreased as tension was allowed to develop over 72 h, whereas the expression of *MMP2* was unchanged and that of *MMP13* increased. Upon release, the expression of all genes except *MMP2* increased, with *MMP14* and *TIMP1* just below significance. All of these apart from *MMP1* increased in expression between the 3-h release and the 24-h release time point giving a greater difference between tension compared to tension and release at the 24-h compared to the 3-h release time point.

3.3. The function of specific metalloproteases in cell-mediated contraction

For each of *MMP1, MMP2, MMP3, MMP13* and *MMP14*, four individual siRNAs per gene were tested for their ability to knock down expression of the target gene, but not the other four *MMP*s. A concentration range of
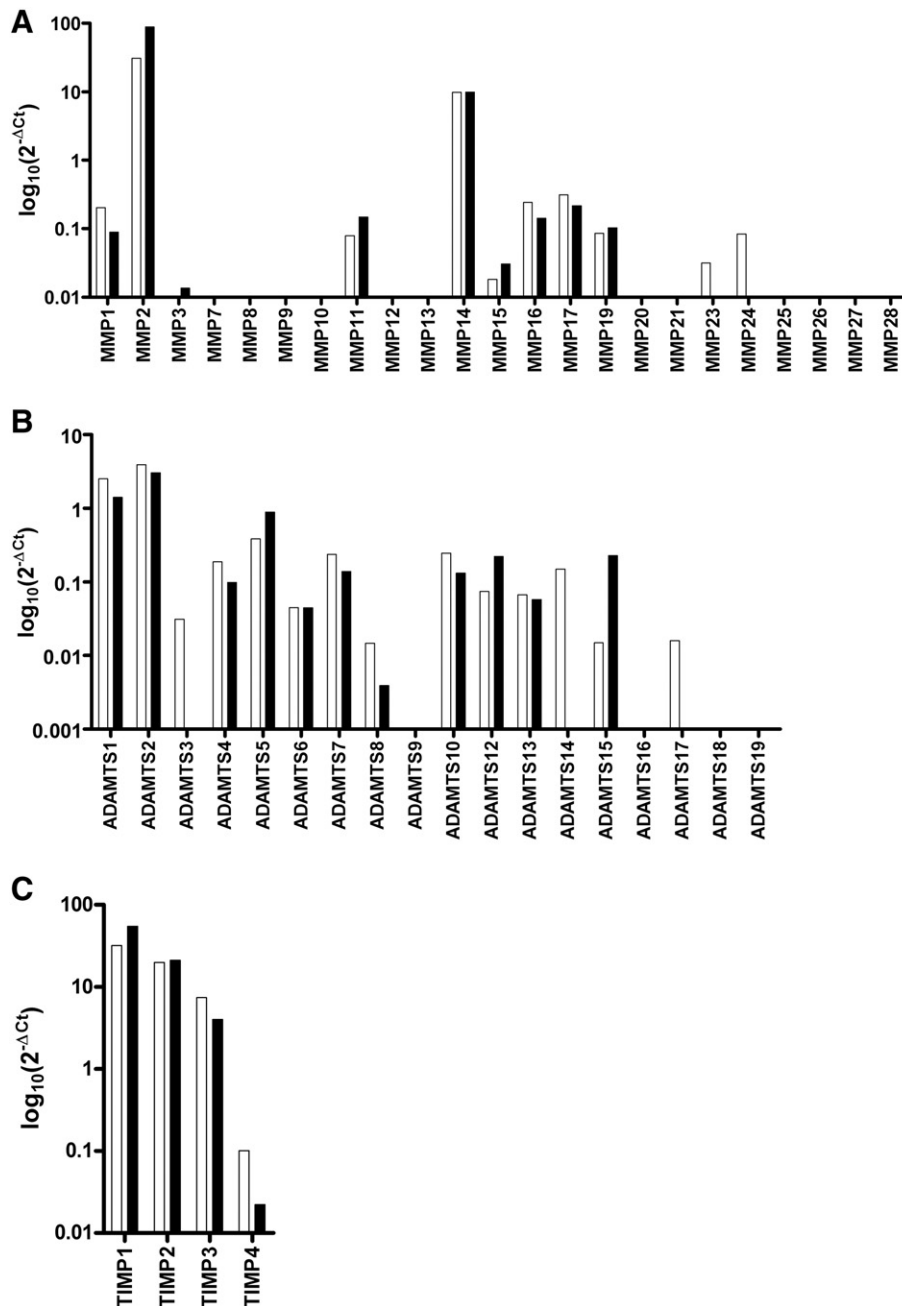

Fig. 2. Metalloprotease gene expression in Dupuytren's disease fibroblasts. Fibroblasts were isolated from the nodules of two independent Dupuytren's disease patients (empty and filled bars) and expanded in monolayer culture to passage 5. A., MMP, B., ADAMTS and C., TIMP expression was analysed using Taqman® Low Density Array and normalised to the β-actin housekeeping gene.
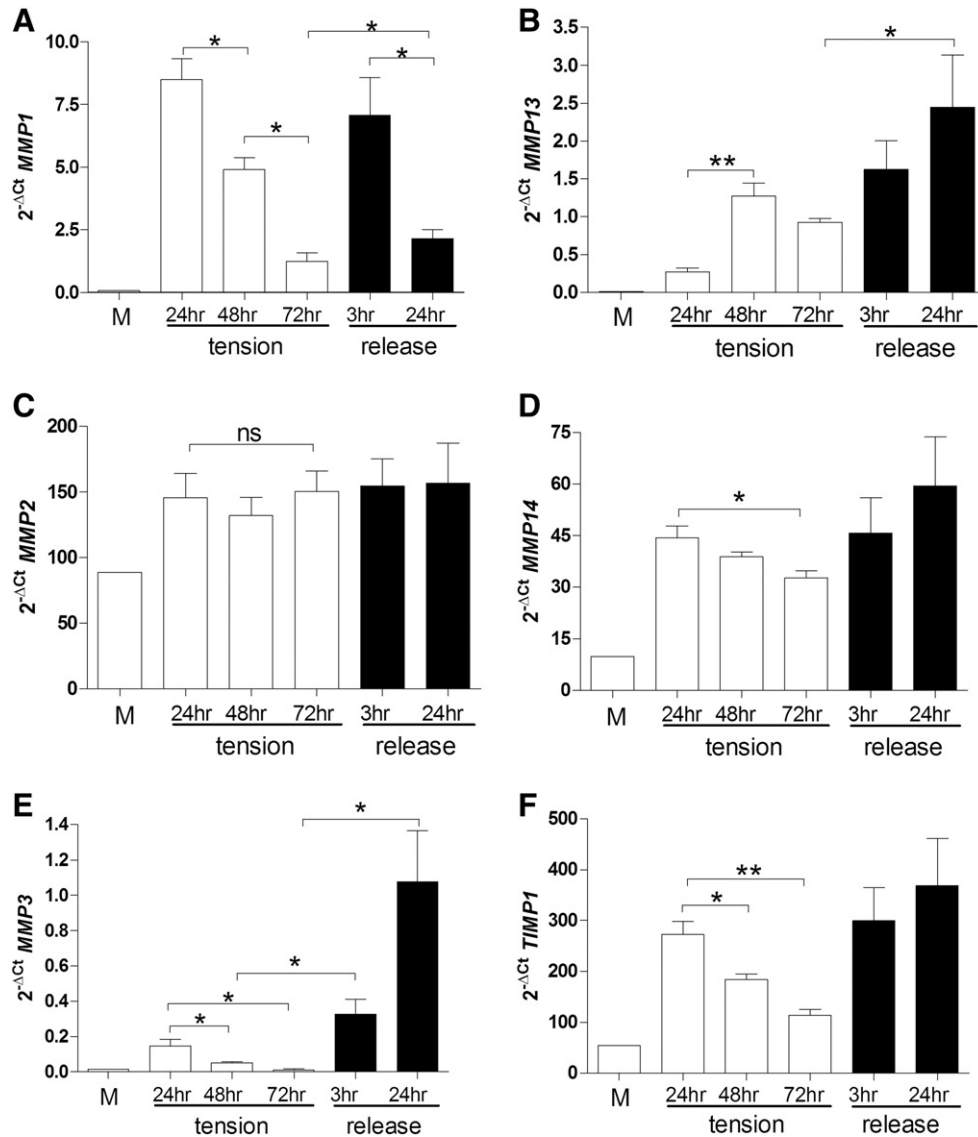
Fig. 3. Metalloprotease and inhibitor gene expression during the FPCL model. Dupuytren's disease fibroblasts were expanded in monolayer culture (M) and seeded into the FPCL model. Lattices were harvested directly into Trizol at 24, 48 and 72 h and also at 3 or 24 h after release (following 48 h of tension). Gene expression (A., MMP-1; B., MMP-13; C., MMP-2; D., MMP-14; E., MMP-3; F., TIMP-1) was measured using Taqman® Low Density Array and normalised to the β-actin housekeeping gene. Data are shown as mean $+/-$ s.e.m., $n = 3$ and compared using ANOVA with Tukey's post test for samples across tension, or t-test for pairwise comparison of tension vs. release: *, $p < 0.05$; **, $p < 0.01$.

1–50 nM siRNA was tested, measuring gene expression at 24, 48 and 72 h (data not shown). Two siRNAs were then selected for trial in the FPCL assay, where two rounds of transfection with the siRNA allowed robust knock-down at 5 nM concentration. Fig. 4A and B shows knock-down of *MMP2* and *MMP14* in both monolayer culture (72 h with transfection of siRNA at $t = 0$ and 48 h) and after a further 48 h in the FPCL assay using this format (see Material and methods).

Examining the combined data from cells isolated from three independent Dupuytren's patients in the FPCL assay, transfection of the non-targeting siRNA did not alter the amount or kinetics of contraction compared to the mock transfection (data not shown). Fig. 5A shows that knockdown of *MMP3* or *MMP13* did not alter contraction, whilst knockdown of *MMP1* increases contraction significantly in the first 30 min after release, though this then equalises at 1 h and beyond. Knockdown of *MMP2* and particularly *MMP14* slows contraction across the time course, with overall contraction at 24 h remaining significantly diminished (Fig. 5B and C).

3.4. Gelatin zymography

Since MMP-14 can activate proMMP-2, the activity of MMP-2 was measured in the conditioned medium from the FPCL assay by gelatin zymography. Fig. 4C shows that knockdown of *MMP2* decreases activity due to proMMP-2 though there remains some active MMP-2 whilst knockdown of MMP-14 has no effect on proMMP-2, but completely abrogates active MMP-2 (note that residual proMMP-2 activity comes from the FCS in the culture medium as shown).

3.5. Collagen turnover

Initial data demonstrate that the concentration of hydroxyproline in the conditioned medium increases across 72 h of tension in the FPCL format but is further increased upon lattice release and contraction and this is replicated in the later knockdown experiments (Fig. 6A and B). Specific knockdown of *MMP1*, *MMP2*, *MMP3*,
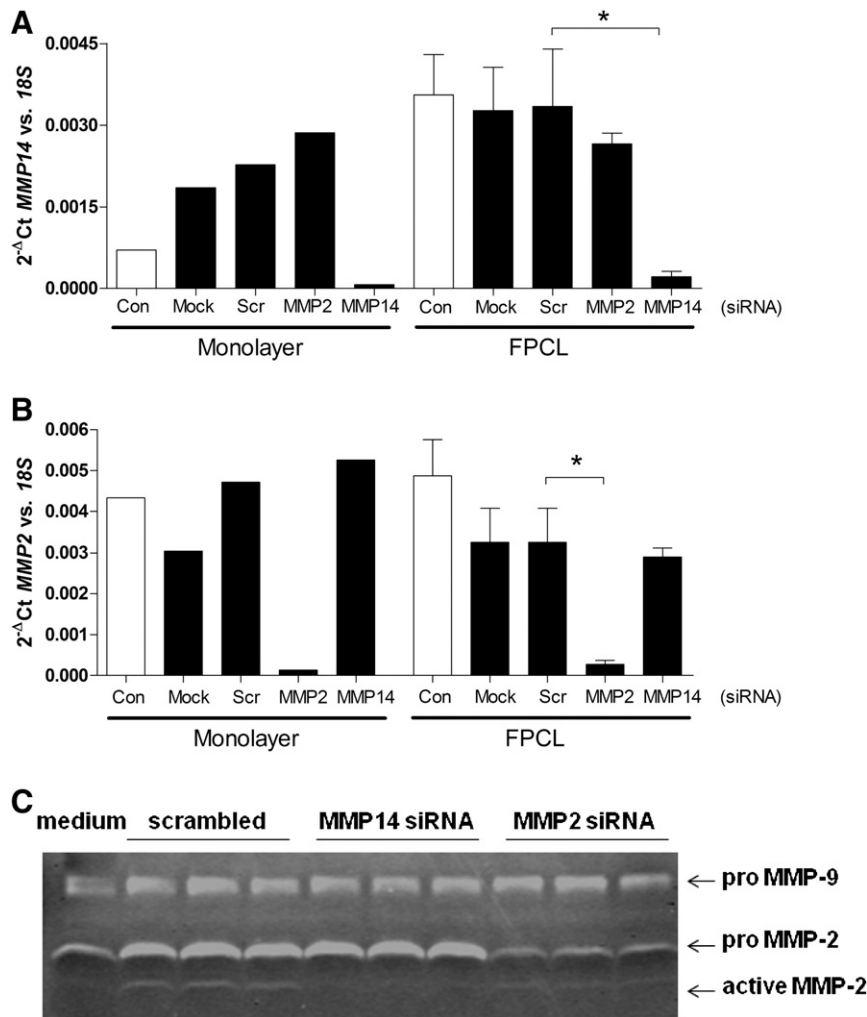
Fig. 4. Knockdown of MMP gene expression using siRNA. Dupuytren's disease fibroblasts were expanded in monolayer culture and either untreated (Con), mock transfected (Mock) or transfected with 5 nM siRNA, non-targetting (Scr) or targeting MMP-2 or MMP-14. Cells were re-transfected at 48 h, incubated for a further 24 h and seeded into the FPCL model. Gene expression (A., MMP-14 and B., MMP-2) was monitored by qRT-PCR prior to seeding into the FPCL (monolayer) and at the end of the contraction phase (FPCL). Data are n = 1 for monolayer and mean +/− s.e.m., n = 3 for FPCL and compared using t-test: *, p<0.05; **. C. Conditioned medium from the end of the contraction phase was subjected to gelatin zymography.

MMP13 or MMP14 has no significant impact on collagen degradation by this measure, either during tension or release phase of the FPCL assay (Fig. 6B).

4. Discussion

The molecular mechanisms underlying Dupuytren's disease are complex. The accumulation of a collagen-rich extracellular matrix suggests decreased proteolytic activity, whereas the irreversible contraction of the cords suggests matrix remodelling must still occur. Since several broad spectrum MMP inhibitors, trialled in cancer, displayed a musculoskeletal toxicity which included a Dupuytren's-like contracture, it is important to understand the function of specific MMPs in the pathophysiology of the disease. There are no animal models of Dupuytren's disease therefore we sought to do this *in vitro*.

Cells were isolated from the nodules of Dupuytren's disease palmar fascia by explant outgrowth onto tissue culture plastic. The expression of the entire *MMP*, *ADAMTS* and *TIMP* families was measured in cells cultured from two independent patients using a quantitative Taqman® low density array method. A broad correlation in the relative levels of many of these genes (e.g. *MMP1*, *MMP2*, *MMP3*, *MMP13*, *MMP14*, *ADAMTS1*, *ADAMTS2*, *TIMP1*, *TIMP2*, *TIMP3*) was observed between the cells in culture and that measured directly in patient tissue in our earlier study [25]. Since the cells were at passage

five in culture, this may suggest a stable genetic or epigenetic component to their regulation, either directly on individual genes, or more likely via an autocrine mechanism of cell signalling (e.g. Wnt or TGFβ [5,35]). Measurement of steady state mRNA as a surrogate for protein or activity levels ignores the potential for these post-transcriptional or post-translational levels of regulation, but allows for a greater range of proteases and inhibitors to be measured and increased sensitivity.

Several studies have compared the contractile properties of Dupuytren's cord- or nodule-derived fibroblasts with control cultures in the fibroblast-populated collagen lattice model [28,36–38]. Results varied depending on the model of contraction used, but the consensus appears to be that Dupuytren's fibroblasts can generate significantly increased contractile force compared with control cells, with nodule-derived cells showing greatest contraction. We therefore chose to dissect MMP function in the fixed FPCL. In such models, broad-spectrum synthetic MMP inhibitors have been shown to inhibit contraction [29,39,40]. This blockade of contraction is difficult to dovetail with the reported side effects of some of these MMP inhibitors in clinical trials; however, this may reflect the fact that culture models of collagen lattice contraction only represent a single facet of a more complex disease process. Ilomastat, an inhibitor with nanomolar Ki against many MMPs [41], only inhibits contraction by DD fibroblasts in the FPCL at 100 μM [29], though this may reflect the
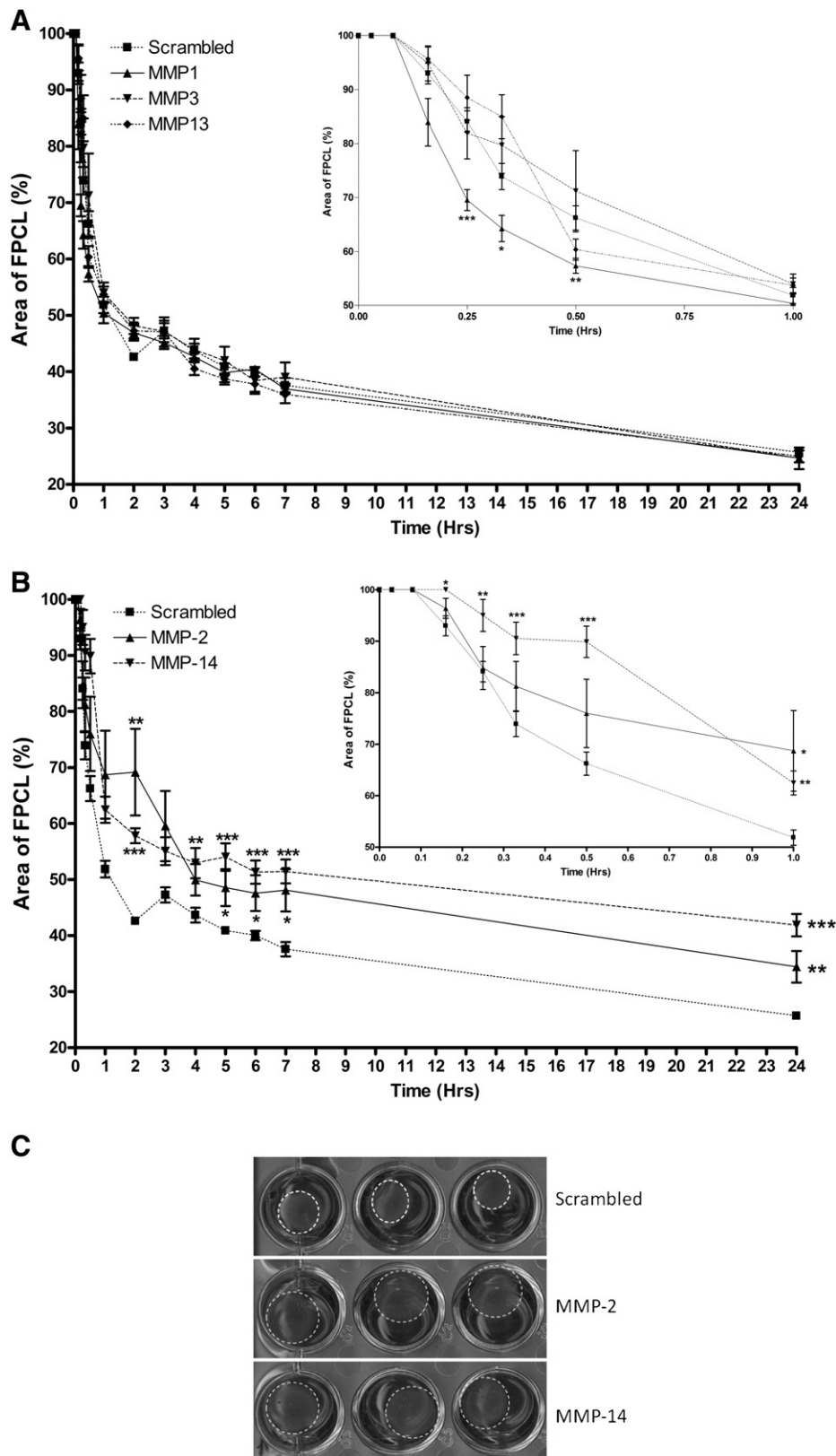
Fig. 5. The impact of specific MMP knockdown of collagen gel contraction. Dupuytren's disease fibroblasts transfected with 5 nM siRNA targeting specific MMPs were seeded into the FPCL model (see Material and methods). A. Non-targetting siRNA (scrambled), compared to siRNA targeting MMP-1, MMP-3 or MMP-13. B. Non-targetting siRNA (scrambled) compared to siRNA targeting MMP-2 or MMP-14. Data shown are mean of three experiments (n = 3 each, therefore n = 9) +/−s.e.m. using independent Dupuytren's disease patients. The insert shows the first hour of the time course on different scale axes for clarity. Targeting siRNA is compared to scrambled using t-test: *, p<0.05; **, p<0.01; ***, p<0.001. C., An example of collagen lattices (as before) at the end of the contraction phase.
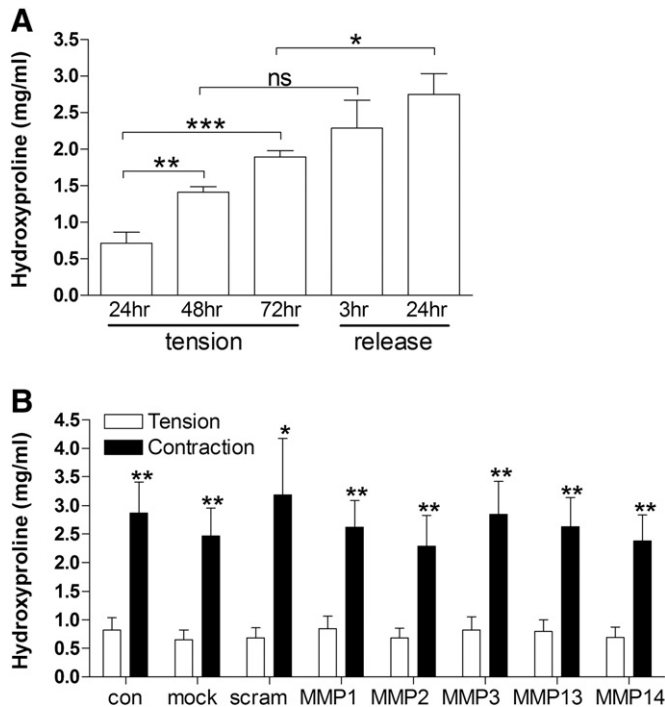
Fig. 6. Collagen degradation in the FPCL model. Hydroxyproline release into the conditioned medium was assayed as a measure of collagen breakdown. A., at 24, 48 and 72 h of tension and also at 3 or 24 h after release (following 48 h of tension); B., at the end of the tension (empty bars) and release (filled bars) phase of the FPCL model. Data are shown as mean $+/-$ s.e.m., $n=3$ and compared using ANOVA with Tukey's post test for samples across tension, or t-test for pairwise comparison of tension vs. release: *, $p<0.05$; **, $p<0.01$; ***, $p,0.001$.

high effective concentration of collagen in the assay. In our hands, Ilomastat inhibits MMP activity in conditioned medium effectively at 25 μM (though this still provides no information about inhibition in the collagen lattice itself), but, at this concentration, has no effect on contraction (data not shown).

We probed function of individual proteases in cell-mediated contraction by using siRNA to knockdown MMPs involved in collagen metabolism, i.e. MMP1, MMP2, MMP3 (as an activator of procollagenases), MMP13 and MMP14. Using a double transfection protocol, we minimised the concentration of siRNA needed to achieve efficient knockdown, thus abrogating problems of toxicity that might confound the contraction assay at higher concentrations. Knockdown of MMP3 or MMP13 had no impact on contraction, whereas knockdown of MMP1 gave more rapid contraction in the period immediately post release. The action of MMP-1 might therefore be to decrease tension in the fixed phase of the FPCL via reorganisation of the collagen lattice. The inability of MMP-8 or MMP-13, the other two classical collagenases, to compensate, may reflect their comparatively low expression compared to MMP-1 (see Fig. 2A). Knockdown of either MMP14 or MMP2 led to a decreased contraction of the collagen lattice with slower kinetics, particularly for MMP14. MMP-14 can activate proMMP-2 [42] at the cell surface in a process also involving TIMP-2 and we show that knockdown of MMP14 decreases the level of active MMP-2 in the conditioned medium of the contracted collagen lattices without altering the level of proMMP-2. It is uncertain if the action of MMP-14, in promoting contraction, is solely via proMMP-2 activation, since MMP-14 is a collagenase in its own right. Both enzymes also have activities outside of their matrix-degrading capabilities, e.g. MMP-14 mediates the ectodomain shedding of the CD44 hyaluronan receptor [43] and of syndecan 1 [44] with effects on cell migration and can also enhance latent TGFβ activation [45]; MMP-2 can cleave MCP-3/CCL7 [46] which itself displays cross-talk with TGFβ in fibrosis [47]. Interestingly, in tumour cells, constitutive overexpression of MMP-14, accompanied

by activation of endogenous proMMP-2 leads to enhanced contraction of collagen gels [48]. MMP-14 is also insensitive to inhibition by TIMP-1, the gene for which is highly expressed in DD tissue and in DD-derived fibroblasts [49].

Release of hydroxyproline, a marker for collagen breakdown, was seen across the FPCL model (Fig. 6), however, no alteration in the level of the marker was seen upon knockdown of each MMP. Interestingly, Ilomastat, as a pan-MMP inhibitor, did abrogate hydroxyproline release, despite having no significant effect on contraction (data not shown). If pericellular collagen turnover is the essential feature of contraction, it is likely that this is simply overwhelmed by overall collagenase activity in the bulk of the gel and is therefore obscured in the conditioned medium. In a study utilising fibroblasts derived from MMP null mice deficient in single collagenases, only MMP-14 was essential for cell-associated collagen degradation [50]. In the presence of plasminogen (providing a pathway for activation of proMMPs apart from proMMP-14), soluble collagenase activity was shown to mediate collagenolysis distal from the cell, but the absence of any single collagenase (MMP2, MMP8, MMP9, MMP13, MMP14 or MMP16) was unable to abrogate this [50]. MMP-14 has also been shown to be essential for the invasion of tumour cells into native (i.e. cross-linked) collagen gels [51] and is a key player in cell migration [49], whilst MMP14 null mice display a significant musculoskeletal phenotype, with decreased collagen turnover [52].

5. Conclusion

In conclusion, our data highlight the potential for MMP-14 and/or MMP-2 to be therapeutic targets in Dupuytren's disease, whilst inhibition of MMP-1 may potentiate cell-mediated contraction. Despite the phenotype of the MMP-14 homozygous null mouse [52], the heterozygotes do not display similar defects of collagen turnover. A specific peptide inhibitor of MMP-14 has been used in short term cancer models in mice with no reported toxicity [53] and a function-blocking anti-MMP-14 antibody was tested in rats for 28 days without adverse clinical or histological events [54]. Potent inhibitors of MMP-2 and MMP-14 inhibitors have also been synthesised [55]. Particularly if topical application of such agents were developed, we suggest that these would be useful in slowing Dupuytren's disease progression or preventing post-operative recurrence of contraction. Data from the Dupuytren's disease-derived fibroblasts described here and from our previous tissue studies [25,26], also suggest that the level of circulating MMP-2 or MMP-14, or their ratio with TIMP-1, may provide a biomarker for disease progression or be predictive of post-operative recurrence.

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