Doxycycline prevents matrix remodeling and contraction by trichiasis-derived conjunctival fibroblasts

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Word count: 3095

Grant information: Supported by Fight for Sight and the Special Trustees of Moorfields Eye Hospital (Ref 1086), and the Moorfields Eye Hospital and UCL Institute of Ophthalmology Biomedical Research Center for Ophthalmology (BMRC084), the Wellcome Trust (080741/Z/06/Z) and the British Council for the Prevention of Blindness (MJB). Laboratories and imaging facilities supported by the Wellcome Trust and the Medical Research Council.

Abstract

Purpose. Trachoma is a conjunctival scarring disease, which is the leading infectious cause of blindness worldwide. Elimination of blinding trachoma is being held back by the high rate of trichiasis recurrence following surgery. There is currently no treatment available to suppress the pro-fibrotic state and reduce recurrence. Although the mechanisms underlying trichiasis development are unknown, the pro-fibrotic phenotype has been linked to matrix metalloproteinase (MMP) expression. Doxycycline, a well-known tetracycline antibiotic, can act as a broad MMP inhibitor and has showed some success in preventing fibrosis in various clinical contexts. The purpose of this work was to assess the anti-scarring properties of doxycycline in an *in vitro* model of trichiasis fibroblast-mediated tissue contraction.

Methods. Primary cultures of fibroblasts were established from conjunctival samples obtained from normal donors or during surgery for trachomatous trichiasis. The effect of doxycycline on matrix contraction was investigated in our standard collagen gel contraction model. Cell morphology and matrix integrity were assessed using confocal reflection microscopy. Quantitative real time polymerase chain reaction (QRT-PCR) and a FRET-based assay were used to measure MMP expression and activity respectively.

Results. Doxycycline treatment successfully suppressed the contractile phenotype of trichiasis fibroblasts, matrix degradation, and significantly altered the expression of MMP1, 9 and 12 associated with the pro-fibrotic phenotype.

Conclusions. In view of the results presented here and the wider use of doxycycline in clinical settings, we propose that doxycycline might be useful as a treatment to prevent recurrence following trichiasis surgery.

Introduction

Trachoma is the leading infectious cause of blindness worldwide [1]. The disease begins with recurrent infection by the bacterium *Chlamydia trachomatis* in early childhood, promoting chronic inflammation of the upper tarsal conjunctiva, which leads to progressive scaring and distortion of the eyelid. The edge of the eyelid turns in (entropion), so that the lashes scratch the surface of the eye (trichiasis). This can result in corneal opacity and irreversible sight loss [2]. Trachoma is a public health problem in over 50 countries, predominantly in Sub-Saharan Africa, Middle East, the Indian Subcontinent, South-east Asia and South America [3]. The most recent global estimation from the World Health Organization (WHO) suggests that 40 million people currently have active trachoma, a further 8.2 million have trichiasis, and 1.3 million are estimated to be blind as a result [1]. The WHO is leading a Global Alliance to eliminate blinding trachoma by 2020. This focuses on the implementation of the SAFE Strategy: Surgery for trichiasis, Antibiotics for infection, Facial cleanliness, and Environmental improvements to reduce transmission of infection. However, there is growing evidence that the scarring complications can progress even in the absence of detectable chlamydial infection [4], and following trichiasis surgery, the anatomical abnormality can re-develop (from 10% at one year to 60% at three years), in part through an ongoing immuno-fibrogenic process [5, 6]. There is currently no adjuvant treatment available to suppress the pro-fibrotic state and reduce recurrence.

The mechanisms underlying post-surgical recurrence of trichiasis are not fully understood. However, the dysregulated extra cellular matrix (ECM) proteolysis observed following infection and inflammation is suggested to play a key role in the

development of fibrotic sequelae [6]. MMPs are a tightly regulated family of zinc-dependent enzymes responsible for degrading structural proteins of the ECM, and are produced by a variety of cell types after injury [7]. A number of MMPs have been found to associate with conjunctival scarring in *in vitro* models [8], as well as *in vivo* [9]. MMP-9 expression increases when conjunctival inflammation is associated with non-chlamydial bacterial infection in recurrent trichiasis [10], and an increased level of MMP7 gene expression was also identified in trichiasis conjunctival samples [11]. Moreover, microarray analysis has confirmed the increased expression of MM7, MMP9 and MMP12 in conjunctival samples from trichiasis subjects [12]. Overall this suggests that the accumulation of fibrotic tissue in trichiasis might be due at least in part to altered MMP expression.

Doxycycline, a well-known tetracycline antibiotic, is widely used to prevent and treat bacterial and parasite infection, including *Chlamydia trachomatis*. More recently, its role as MMP inhibitor and in apoptosis has gathered more attention in the context of vascular disease [13, 14], pulmonary fibrosis [15], periodontitis [16] as well as ocular pathology [17-19]. Doxycycline inhibits MMPs, and particularly MMP9, at sub-antimicrobial doses in patients [13, 14, 19, 20]. In addition, recent work suggests that doxycycline treatment can dampen local [21], as well as systemic [22], inflammation, thus making it a good candidate to prevent tissue remodeling and fibrosis in trachoma. Using for the first time conjunctival cells directly isolated from trachomatous trichiasis-affected individuals, we demonstrate that doxycycline significantly reduced collagen matrix remodeling and contraction, and specifically inhibited the mRNA expression of MMP1, 7, 9 and 12 during contraction, suggesting that it could be a potential adjuvant treatment following trichiasis surgery.

Material and methods

Ethics Statement:

This study adhered to the tenets of the Declaration of Helsinki. It was approved by the Tanzanian National Institute of Medical Research, the Kilimanjaro Christian Medical Centre, and the London School of Hygiene and Tropical Medicine Ethics Committees. The study was explained to potential study participants and written informed consent was obtained before enrolment.

Clinical Samples:

Conjunctival biopsies were obtained from the upper tarsal conjunctiva from Tanzanian patients undergoing trichiasis surgery. All cases had tarsal conjunctival scarring with entropion trichiasis. The eyelid was anaesthetized with an injection of 2% lignocaine and the eye cleaned with 5% povidone iodine. A biopsy sample was taken using a 3mm trephine from the tarsal conjunctiva, 2mm from the lid margin, at the junction of the medial ⅔ and lateral ⅓ of the everted lid. The biopsies were wrapped in sterile gauze, moistened with normal saline, and transported to the laboratory at +8°C.

Cell culture and reagents

The biopsies were mechanically dispersed and the tissue fragments were placed in tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) with 4.5g/L l-Glutamine (PAA), supplemented with 10% fetal bovine serum (FBS, Sigma), 100 IU/ml penicillin, 100 ug/ml streptomycin (Invitrogen) at 37 °C with 5% CO_2. Following growth from the explant, the fibroblast populations (F07, F09, F10 and F11) were trypsinized and maintained routinely in the above medium. All four cell lines were

126 tested for *C. trachomatis* infection using the Amplicor CT/NG Kit (Roche Molecular
127 Systems, Branchburg, NJ) and were found to be negative. The cells were used between
128 passage 4 and 9 for all experiments. For doxycyline treatment, a stock solution of 48.7
129 mM Doxycycline hyclate (Sigma) was made in sterile ultrapure water (Millipore
130 Biocel) and added to the cell culture medium at final concentrations of 104 and 416 uM.
131
132 **Collagen contraction assay**
133 The collagen contraction assays were performed as previously described [8]. Trachoma
134 cells were seeded in a 1.5 mg/ml collagen type-I matrix (First Link Ltd) at a
135 concentration of $7x10^4$ cells/ml. The gels were detached from the edge of the well, and
136 2 mL of DMEM with/without Doxycycline was added. Gel contraction was monitored
137 daily for 7 days by digital photography. Gel areas were measured using ImageJ software
138 (http://rsb.info.nih.gov/ij/), and the contraction was plotted as a percentage of gel area
139 normalized to original area (day 0 measurement).
140
141 **Cytotoxicity assay**
142 Cytotoxicity was determined using a Cytotoxicity Detection Kit (LDH) (Roche), on
143 media collected at the termination of the gel contraction experiment (in phenol red-free
144 DMEM) to measure the percentage of lactate dehydrogenase activity present in the
145 samples. The gels were lysed in 2% Triton X-100 (Sigma) in phenol red free, serum
146 free DMEM for 10min to achieve the maximum LDH release. Absorbances were
147 measured at 490 nm (Fluostar Optima) and the percentage of cytotoxicity was
148 calculated according to the manufacturer's protocol.
149
150

Cell and matrix imaging

Following contraction for 7 days with/without 416uM doxycycline, gels were fixed in 3.7% paraformaldehyde (Sigma) at room temperature for 30 min, followed by permeabilization with 0.5% Triton X-100 (Sigma) for 30 min, and staining with rhodamine-phalloidin (Invitrogen) for 1hr [8, 23]. Imaging was carried out on a Zeiss Axiovert S100/Biorad Radiance 2000 confocal laser scanning microscope using simultaneous reflection microscopy and fluorescence imaging [23]. Representative images were acquired as z-stacks using a long working distance objective (Zeiss 63X/0.75 plan neo fluar with correction collar). The resulting volumes were imported into Image J where the fluorescence channel (F-actin staining) was compressed to a single projection and merged with a representative section of the matrix.

Quantitative Real-Time PCR

Collagen gel contraction assays were ended at days 0, 3, and 7 by placing the gels straight into TRIzol Reagent (Invitrogen) at 4 °C for 1hr. Control mRNA at day 0 were obtained after 1hr of initial gel polymerization. Homogenization and phase separation were carried out according to the TRIzol manufacturer's instructions. The aqueous phase was harvested and used for RNA isolation using the RNeasy Mini Kit according to the standard protocol (Qiagen). Reverse transcription was carried out using the QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instructions. MMP gene expression was measured by QRT-PCR using validated primers and probes (Assay-on-Demand; Applied Biosystems). Assay identification numbers are MMP1 (Hs00899658_m1), MMP2 (Hs01548727_m1), MMP7 (Hs01042796_m1), MMP9 (Hs00234579_m1), and MMP12 (Hs00899662_m1). The HPRT1 gene was used as an endogenous control to normalize sample concentration. RT-PCR reactions were

performed on an HT7900 Fast Real-Time PCR system (Applied Biosystems), and the 2(-Delta Delta C(T)) Method (Livak and Schmittgen, 2001) was used for quantification of mRNA levels.

MMP activity assay

Total MMP activity was determined using a FRET-based MMP activity assay kit according to the manufacturer's protocol (Abcam, ab112147). In brief, 25ul of medium from control and doxycycline-treated collagen gel contraction cultures at day 0, 3 and 7 were added to 25ul of 2mM APMA solution and incubated at 37°C for 3hrs. 50ul of the MMP Red Substrate was then added and the mix was incubated at room temperature for 1hr. Fluorescence was measured at Ex/Em=540/590nM (Fluostar Optima).

Statistical analysis

All graphs display mean and standard error. Statistical analysis was performed using the Students t test to establish significant differences and individual P values displayed.

Results

Doxycycline prevents collagen matrix remodeling and contraction by trichiasis fibroblasts

We used our well-characterized *in vitro* model of cell-mediated matrix contraction [8, 23-26] to assess the contractile potential of primary fibroblasts isolated from the conjunctiva of patients with trachomatous trichiasis and evaluate the potential of doxycycline as a modulator of contraction. As expected from their conjunctival and fibrotic origin [23, 25], trichiasis fibroblasts (F07, F09, F10 and F11) contracted collagen matrices strongly, down to 20-30% of their original size over 7 days in the presence of 10% serum. The application of 104uM of doxycycline for 7 days was sufficient to reduce matrix contraction by 25% and more significantly, a 7-day treatment with 416uM of doxycycline prevented the contraction by up to 75% (Fig. 1 A). Figures 1B and 1C show 2 representative contraction kinetics from F10 and F11 fibroblast lines, illustrating that doxycycline treatment reduced gel contraction as early as at day 1, with the effect of the drug increasing with incubation time for the higher concentration. To confirm that this effect was not due to drug toxicity, a lactate dehydrogenase (LDH) assay was performed on the cells within collagen gels following 7-day doxycycline treatment at 104 or 416uM. We found no detectable toxicity effect for the drug at either concentration (Fig. 1D).

We have shown previously that fibroblast-mediated gel contraction is dependent on the ability of the cells to affect the organization of pericellular collagen fibers through both direct mechanical pulling on the fibers to align and compact them, as well as by matrix degradation through the release of MMPs [23, 24]. To determine how doxycycline prevented gel contraction, we used confocal microscopy to assess cell

morphology and pericellular matrix organization in the gels following doxycycline treatment. As all 4 cell lines behaved identically in terms of matrix contraction and response to doxycycline (data not shown), we selected 2 representative cell lines, F10 and F11, to perform these studies and further work. Trichiasis-derived fibroblasts had a stellate appearance in the gels, with long F-actin rich protrusions, as illustrated by the full projection of the cell volume[23] (Fig.2, red staining). In agreement with the toxicity data, the overall morphology of the cells appeared unaltered by the doxycycline treatment. Consistent with our previous work on other types of fibroblasts, the high contractile profile of the trichiasis fibroblasts was linked to extensive remodeling and degradation of the collagen matrix by day 7, as visualized by a lack of distinct collagen fibers following confocal reflection imaging[8, 23]. Areas of dense compacted poorly resolved collagen clumps could be seen as a bright white aura around the cells (Fig. 2, arrows), whilst the rest of the matrix shows a fuzzy appearance, characteristic of MMP-mediated degradation [23]. By contrast, in presence of 416uM doxycycline, the matrix fibers remained clearly defined and evidence of fiber alignment consecutive to active cell pulling on the matrix can be found surrounding most of the cells (Fig. 2, arrowhead).

Doxycycline reduces MMP expression during collagen matrix contraction

Our morphological analysis of the cells and matrix during contraction strongly suggested that doxycycline could act through a modulation of matrix degradation and thus likely MMP release. MMPs have long been connected to scarring processes. We have previously shown that matrix remodeling by MMPs plays an important role in tissue contraction, both in *in vitro* [8, 23], *ex vivo* [8], as well as in an ocular scarring model in the rabbit model of glaucoma filtration surgery [27]. In addition, our recent studies have

242 suggested a role for MMPs in the development of the fibrotic phenotype in trachoma [10,
243 11]. We thus used real-time PCR to evaluate MMP expression during matrix contraction.
244 We chose to investigate levels of MMP1 as a well known collagenase previously
245 implicated in our standard collagen contraction assay [26], MMP2 as a standard gelatinase,
246 and MMP7, MMP9 and MMP12 as these particular MMPs have been found enriched in
247 trichiasis samples [12]. The C_T values from the RT-PCR study demonstrated that all of
248 above MMPs were present in both F10 and F11 at Day 0. MMP1 and 2 were expressed
249 at significant levels, whilst MMP7 and 9 were naturally low (Table 1). All MMPs
250 showed an increased expression during contraction in the control group, although to
251 different extent and kinetics. While MMP1, MMP2 and MMP12 showed a sustained
252 increase throughout the contraction kinetics, MMP9 expression peaked at day 3 (Fig. 3).
253 Continuous treatment with 416uM doxycycline did not significantly affect MMP2
254 expression (Fig. 3C, D). However, MMP1 (Fig. 3 A, B), MMP9 (Fig. 3 E, F) and
255 MMP12 (Fig. 3 G, H) all show a strong reduction in expression in the presence of the
256 drug. We also observed a similar trend for MMP7 in F10 (Table 1, normalized
257 expression data not shown), but could not confirm this effect in F11 due to its lower
258 expression of MMP7 and the technical limitation of RT-PCR. To confirm that the effect
259 of doxycycline on MMP gene expression led to a reduction in protein expression and
260 activity, we measured the total MMP activity released in the medium during contraction.
261 As expected, the total MMP activity releasable from the medium increased significantly
262 during contraction, particularly in F10, matching the gene expression profile (Fig. 4).
263 Treatment with doxycycline completely abrogated MMP activity, even in medium at
264 day 0, suggesting that doxycycline affected both the MMP protein levels and the
265 activity of the MMPs present in the medium.
266
267

Discussion

Using our *in vitro* model of cell-mediated matrix contraction [8, 23-26], we found that doxycycline significantly reduced the contractile potential of primary fibroblasts isolated from the conjunctiva of patients with trachomatous trichiasis, whilst presenting only minimal toxicity. This low toxicity and strong effect on contraction compares favorably with previously studied inhibitors of matrix contraction targeting cell division [28], matrix metalloproteinase activity [8, 23, 29] or small Rho GTPases [26], which have been found to prevent tissue contraction *ex-vivo* [26, 29], and scarring *in vivo*, both in animal models [27] and in the clinic [30, 31]. Our results suggest that doxycycline's effect on contraction is at least partly mediated by its ability to inhibit MMP expression and activity, which we have shown is a major component of the contraction process [8, 23, 26]. In addition, doxycycline appears to selectively target the expression of MMP1, 7, 9 and 12, which have been linked to the fibrotic phenotype in trachoma. Doxycycline has previously been shown to both reduce MMPs expression levels [32, 33], and affect MMP activity [13, 19, 33]. In particular, it reduced MMP2 and MMP9 activity during gel contraction *in vitro* [34] and fibrosis *in vivo* [35], suggesting that doxycycline's effects on MMP underlies at least part of its strong effect on matrix remodeling in trachoma. Whilst the doxycycline inhibition of MMP activity is known to involve zinc chelation, the mechanism by which doxycycline affects MMP gene expression is still unclear. However, as doxycycline appears to broadly affect the pro-inflammatory response, it could affect MMP expression through a downregulation of MMP-inducing pro-inflammatory cytokines[21, 22, 32].

MMP1, one of the main collagenases, has been linked to pathological processes such as fibrotic diseases and cancer [36]. Although it has not been reported in association

293 with trichiasis, our previous work with human Tenons' capsule fibroblasts has shown
294 that it is heavily expressed during matrix contraction *in vitro* and its reduction is linked
295 to a decrease in contraction [26], suggesting that it may also functionally facilitate the
296 matrix remodeling process during trichiasis. MMP7 is expressed in epithelia and injured
297 tissue. It plays an important role in inflammation [37, 38]. MMP7 upregulation not only
298 participates in ECM regulation, but also correlates with many fibrotic diseases [39],
299 including trachoma [11] and tumor metastasis [40]. MMP9 is a major component of ECM
300 turnover during homeostasis and conjunctival scarring [41], and its expression is closely
301 linked to the degree of inflammation in the human conjunctival epithelium of children
302 with active trachoma [42, 43]. We found here that trichiasis-derived fibroblasts express low
303 levels of MMP7 and 9. However, both MMP levels are increased transiently during the
304 contraction process, suggesting that these MMPs may be functional and activated
305 mostly at the initial stage. The extremely low expression of MMP7 in F11 might be the
306 result of the natural biological variation of F11, together with the technical limit of
307 semi-quantitative RT-PCR. MMP12 on the other hand is mainly produced by
308 macrophages, its main function including degrading elastin and taking part in pro-
309 inflammatory processes [44]. Increased MMP12 expression has been reported in the
310 scarred conjunctiva of people with trichiasis either with or without inflammation [12]. Our
311 results showed MMP12 has a modest but consistent increasing during the matrix
312 contraction both in F10 and F11, suggesting that it could directly contribute to matrix
313 remodeling in trichiasis. Interestingly, though doxycycline treatment was shown to
314 significantly inhibit MMP12 expression in both cell lines studied, it was significantly
315 more efficient in preventing the contraction of F11, which did not express significant
316 levels of MMP7 and MMP9. This suggests that in the absence of MMP7 and MMP9,
317 MMP12 might be a significant factor driving trichiasis fibroblast-mediated contraction.

Doxycycline's potential as a MMP inhibitor has been extensively documented and it has proved useful in clinical settings [14, 20], with many reporting its strong effect on MMP9 [13, 14, 19]. Recent work suggests that it can also modulate inflammation [21, 22], thus making it a good candidate to prevent the immunofibrogenic process that underlies recurrent trachomatous trichiasis. We present here evidence that doxycycline prevents matrix remodeling and contraction by trichiasis-derived fibroblasts and leads to a significant down-regulation in MMP expression in these cells. The *in vitro* model of tissue contraction used here has already proved essential to the development of treatments for the prevention of scarring following glaucoma filtration surgery and a reasonable predictor of the clinical potential of anti-scarring treatments [30]. In the absence of any animal model for trichiasis development and recurrence, this *in vitro* model may facilitate the translational pathway to modeling the pathogenesis of trachoma and evaluating the effectiveness of new treatments in advance of clinical trials. In view of our results and the wider use of doxycycline in clinical settings, we propose that doxycycline might be useful as a treatment to prevent recurrence following trichiasis surgery.

Acknowledgments

The authors wish to thank Victor Hu for his help with the collection and testing of the samples for Chlamydial infection.

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Gene	MMP1		MMP2		MMP7		MMP9		MMP12		HPRT1	
Dox	-	+	-	+	-	+	-	+	-	+	-	+
F10												
Day 0	31.1 ± 0.4	29.1 ± 2.1	25.1 ± 0.5	24.5 ± 0.6	39.6 ± 0.3	38.2 ± 0.9	39.0 ± 0.3	37.5 ± 1.0	34.8 ± 0.8	34.1 ± 0.8	29.8 ± 0.8	29.8 ± 0.8
Day 3	23.3 ± 0.7	24.0 ± 0.5	23.4 ± 0.4	22.8 ± 0.2	35.9 ± 0.4	36.7 ± 0.7	33.9 ± 0.5	34.0 ± 0.7	31.7 ± 0.8	32.9 ± 0.7	30.9 ± 0.8	30.4 ± 0.5
Day 7	23.0 ± 0.4	25.9 ± 0.7	22.1 ± 0.2	22.2 ± 1.2	36.3 ± 0.2	37.2 ± 1.0	34.6 ± 0.5	35.5 ± 0.9	30.3 ± 0.8	32.7 ± 0.9	30.0 ± 0.7	30.1 ± 1.0
F11												
Day 0	30.4 ± 0.4	29.4 ± 0.1	26.5 ± 2.3	27.7 ± 3.4	37.0 ± 0.1	n/a	40.4 ± 0.9	37.0 ± 2.6	34.4 ± 1.3	34.4 ± 1.0	31.9 ± 0.9	32.1 ± 0.8
Day 3	26.3 ± 0.2	27.0 ± 0.5	23.5 ± 0.2	21.9 ± 0.9	41.3 ± 1.4	39.2 ± 0.6	39.0 ± 0.2	39.7 ± 0.5	34.1 ± 1.7	37.0 ± 0.4	32.0 ± 0.4	30.3 ± 1.2
Day 7	25.6 ± 0.5	27.6 ± 0.7	23.2 ± 0.2	22.4 ± 1.3	40.8 ± 0.9	40.5 ± 0.7	39.9 ± 0.6	41.1 ± 1.3	33.6 ± 1.4	38.5 ± 0.6	32.0 ± 0.6	30.6 ± 1.6

Table1: Quantitative RT-PCR C_T values for MMP mRNA expression levels during gel contraction. C_T values are averaged from n≥3 experiments.

Figure legends:

Figure 1: Doxycycline treatment prevents collagen matrix contraction by trichiasis fibroblasts. (A) Effect of Doxycycline on trichiasis fibroblasts (pooled data for F07, F09, F10 and F11) gel contraction at day7. Each data point was averaged from triplicate gels, n=3. *p<0.05, **p<0.01, ***p<0.001. (B, C) Representative collagen gel contraction profile for F10 and F11 (mean ± SEM, 3 gels each). (D) Cytotoxicity as measured by LDH activity release into the medium during contraction after 7-day. The data is shown as percentage cell survival (mean ± SEM, for n=3 gels each).

Figure 2: Doxycycline treatment prevents matrix degradation and remodeling. Trichiasis fibroblasts F10 and F11 were embedded in collagen gels in medium with/without 416 uM doxycycline. The gels were fixed and stained with Rhodamine phalloidin after 7 days. Shown are representative images of cells embedded in the matrix: red, 2D projection of the full cell F-actin volume; white, collagen matrix fibers viewed using confocal reflection microscopy. Arrows show pericellular collagen fibers compaction, arrowhead radial alignment consecutive to cell dynamic activity. Scale bar, 10 um.

Figure 3: Doxycycline inhibits MMP expression during contraction. Quantitative RT-RCR for MMP1 (A, B), MMP2 (C, D), MMP9 (E, F) and MMP12 (G, H) mRNA expression in trichiasis fibroblasts F10 and F11 during contraction with/without 416 uM doxycycline. Significant differences in expression during contraction with reference to the value at day 0 are expressed as *p<0.05, **p<0.01, ***p<0.001; significant

differences between control and treated samples on the same day are expressed as $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ (mean ± SEM, n=3 repeats).

Figure 4: Doxycycline inhibits MMP activity during contraction. The total MMP activity released in the medium by F10 and F11 cells with/without doxycycline treatment was measured at day 0, 3 and 7 during contraction using a FRET based assay. MMP activity is expressed as fluorescence levels (mean ± SEM; F10, n=3; F11 n=2).

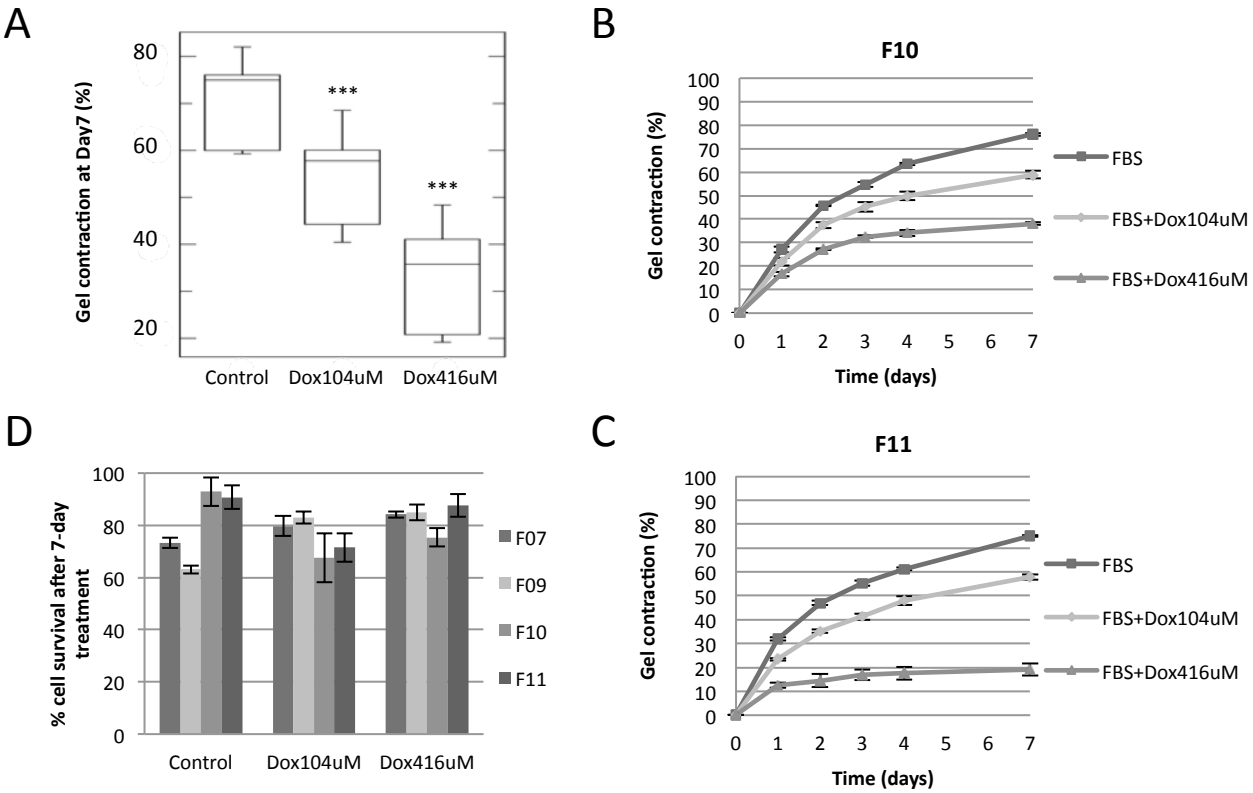

Figure 1, He *et al. revised*

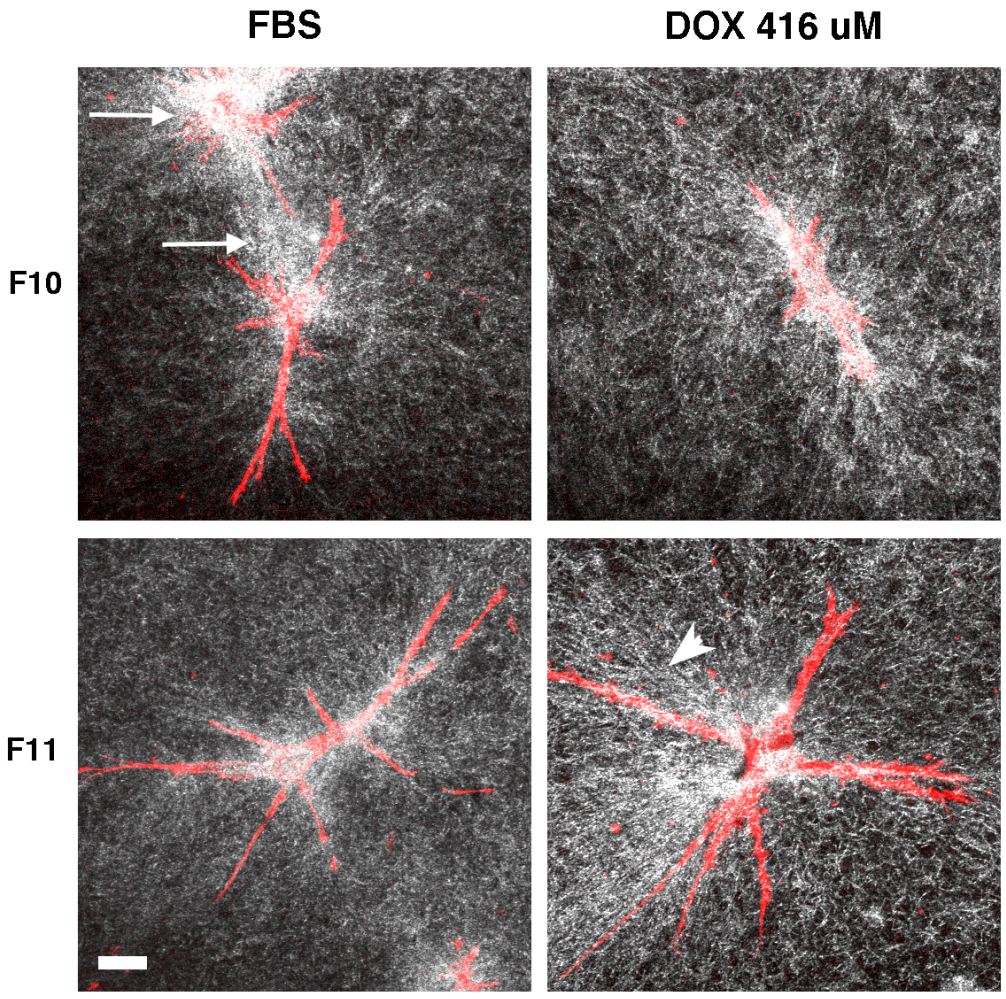

Figure 2, He *et al. revised*

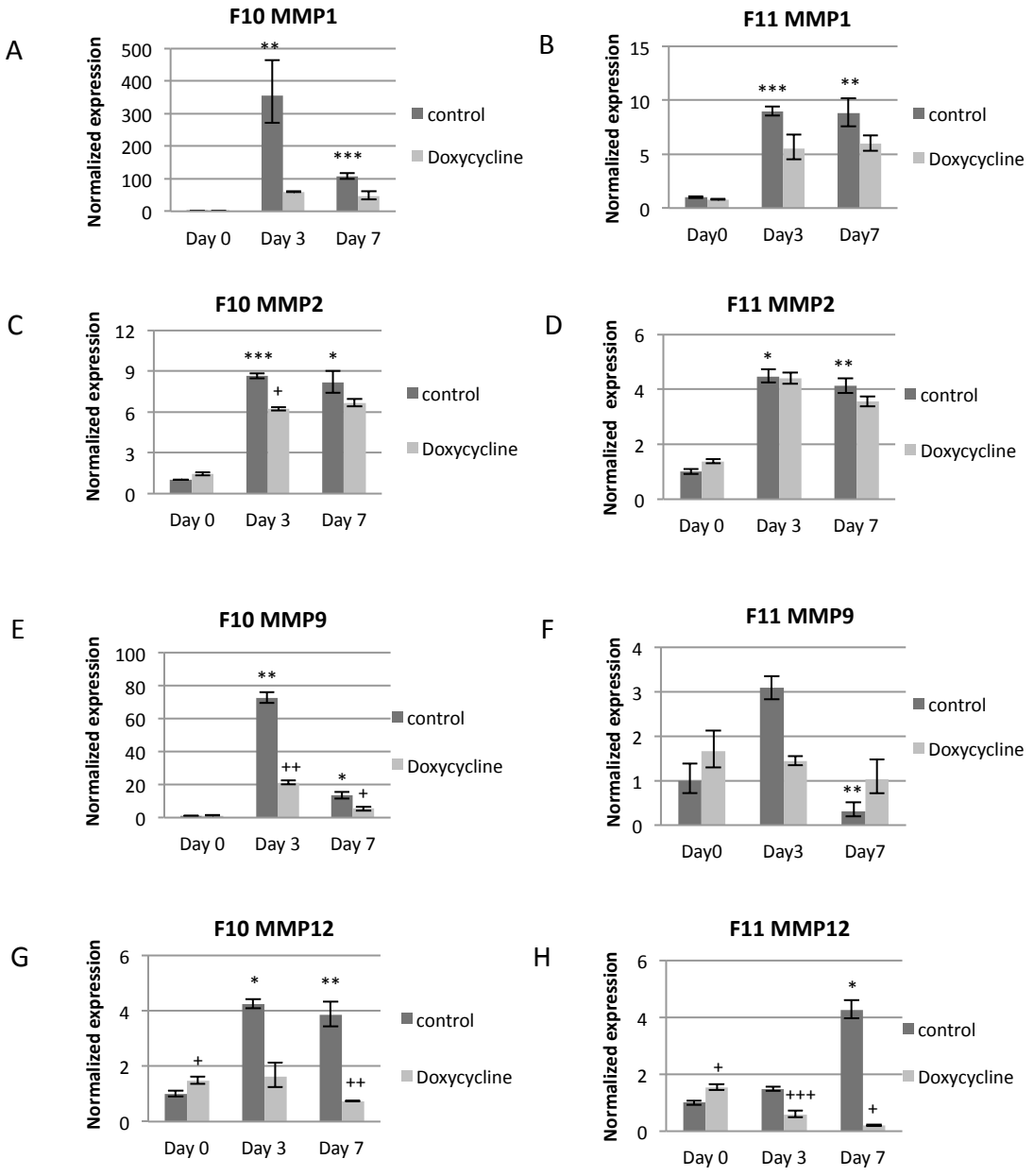

Figure 3, He *et al. revised*

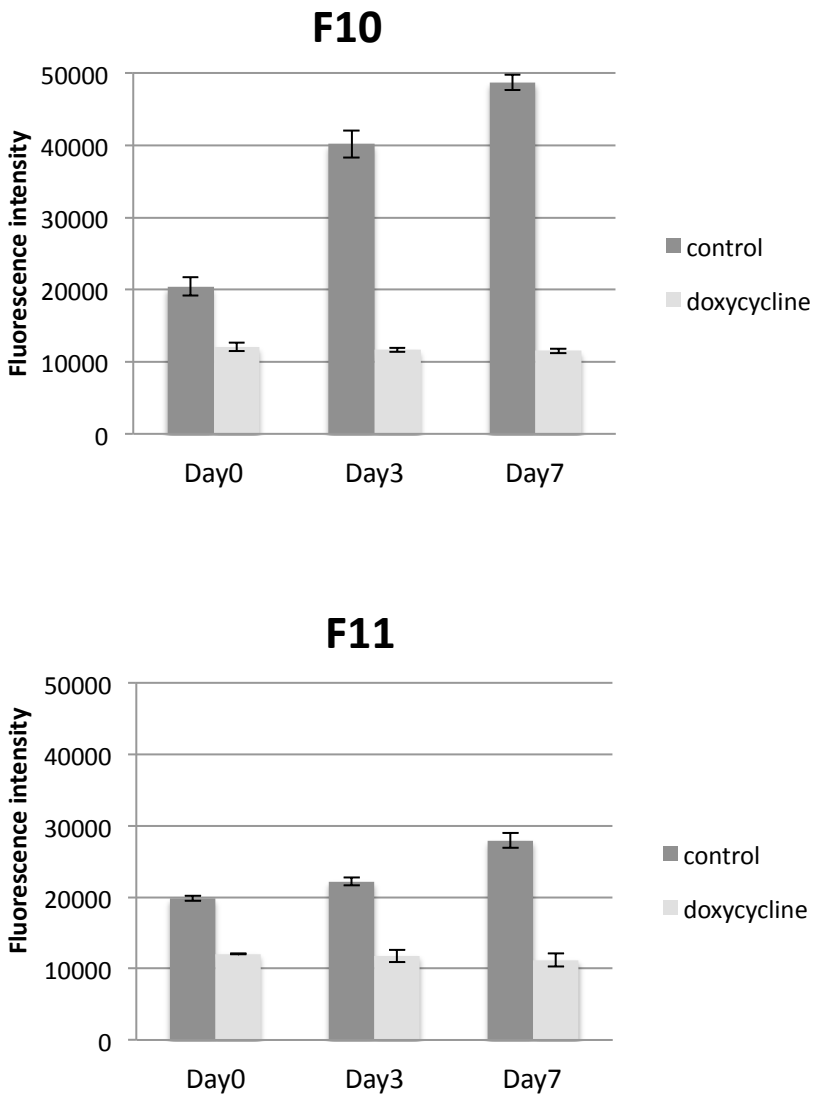

Figure 4, He *et al. revised*