University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student

   Surname: Nelis Forename: Briana

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2. Supervisor:

   Surname: Millar Forename: Neal

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3. Research Project Report

   3.1 Project Title (maximum 20 words): Danger signals in Human Tendon Disease

   Project Lay Summary (copied from application):

   Tendon injuries are a common cause of morbidity and significant health burden on society. One in three GP consultations are the result of soft tissue tendon disease with an annual estimated cost to the NHS of £250 million. The current treatment armentarium available to clinicians remains weak and a large proportion of patients remain under treated.

   We plan to investigate a key damage molecule (HMGB1) in damaged tendon removed from patients during routine surgery. Ultimately we wish to understand how this molecule is

involved in tendon damage/pain at a cellular level and translate this to improving tendon disease clinically.

3.2 Start Date: 6th June 2016 Finish Date: 8th July 2016

3.3 Original project aims and objectives (100 words max): Based on the key role of HMGB1 that is emerging in inflammatory/fibrotic disease, and our preliminary data, we hypothesize that HMGB1 plays a crucial role in matrix regulation and inflammatory responses in tendinopathy.

This project aims to:

1: evaluate the expression of HMGB1 and its cognate receptors (such as TLRs) in tendinopathy and assess the contribution of exogenous HMGB1 to tenocyte activity in vitro.

2: evaluate the functional contribution of HMGB1 +ve/-ve tenocyte populations on the resolution of tendon damage.

3.4 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

The study comprised of a cell culture phase in which key HMGB1-mediated biologic activities of tendon-derived fibroblasts were performed. Human tendon tissue was obtained at rotator cuff surgery and tissue was snap frozen/formalin fixed/placed in RNAlater for *in situ* histological analysis or mRNA.

Aim 1: To evaluate the contribution of exogenous HMGB1 on human tenocyte activity in vitro.

Explant cultures from tendon tissues obtained as above from 3 patients were treated with exogenous HMGB1 at concentrations of 1, 2 and 4ug/ml. RNA was extracted from tenocytes and cDNA was made and submitted for gene expression analysis of key inflammatory cytokines, chemokines and matrix proteins via qPCR. Molecular presence

of proteins IL-6, CCL2, s100a8 and s100a9 were analysed using ELISA tests on cell supernatant. The student was trained in both techniques to obtain an inflammatory profile of HMGB1 in tenocytes, evaluating fibroblast behaviours such as: proliferation, collagen synthesis, matrix regulation, cytokine and chemokine release. Experiments established the effector profile of 'tendinopathy-derived' compared to 'control derived' explant cultures upon exposure to HMGB1.

Aim 2: evaluate the functional contribution of HMGB1 +ve/-ve tenocyte populations on the resolution of tendon damage.

Student was trained in sterile techniques for plating and counting tenocytes using haemocytometer, diluting to optimal density and staining intracellularly with GFP-labelled anti-HMGB1 antibody. DAPI stain (blue) was used to identify tenocyte nuclei, and Green Fluorescing Protein (GFP) indicated HMGB1 positive and negative cells.

3.5 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

Aim 1: To evaluate the contribution of exogenous HMGB1 on human tenocyte activity in vitro.

In comparison to untreated cells, IL-6 protein expression increased with increasing HMGB1 concentration from 0 to 4μg/ml (Figure 1). IL-6 gene expression increased markedly in cells (Figure 2). IL-33 RNA expression had a 35-fold increase from untreated tenocytes at 4μg/ml HMGB1 (Figure 3).

CCL2 gene expression was found to increase with increasing HMGB1 concentration (Figure 4). Expression at protein level further increased with treatment of 1μg/ml to 2μg/ml HMGB1, however, CCL2 protein levels were decreased in tenocytes treated with 4μg/ml, although remaining higher than untreated (Figure 5).

Collagen I gene expression increased 2-fold with tenocytes in 2μg/ml HMGB1, however, a decreased change was found in tenocytes at 4μg/ml cf. untreated (Figure 6). A similar trend was found with Collagen III, except with a greater fold-change at HMGB1 concentrations (Figure 7).

Tenascin C gene expression increased 2-fold at 2μg/ml (Figure 8), however HMGB1 mRNA synthesis did not show marked changes from untreated samples (Figure 9).

Decorin expression had an increase in fold-change with increasing HMGB1 concentration (Figure 10). Stem Cell Factor (SCF) expression had 3-fold change at 2μg/ml HMGB1, however this was not sustained at 4μg/ml (Figure 11). Periostin had a similar trend at lower fold-change (Figure 12), and CXCL12 demonstrated this pattern with increased fold-change (Figure 13).

IL-1β expression had 25-fold change at 4μg/ml (Figure 14). With S100A8 and A9, no greater than 2-fold was achieved with increasing HMGB1 concentrations (Figure 14 & 15). S100A8 and A9 were not detected at protein level.

Aim 2: evaluate the functional contribution of HMGB1 +ve/-ve tenocyte populations on the resolution of tendon damage.

Due to poor resolution of GFP in cell nuclei under fluorescent microscopy, positive/negative HMGB1 expression in tenocyte nuclei could not be identified.

3.6 Discussion (500 words max):

HMGB1 is a transcription factor implicated in tendinopathy however the exact molecular action is unknown. (1). From artificial application of HMGB1 cells *in vitro*, it is evident that IL-6 and IL-33 are amplified in a dose-dependent relationship with HMGB1. Although these results were consistent in the majority of donor cells, repetition of this experiment would be beneficial to support this cause. Notably, cells from one donor

gave a consistently lower level of gene expression than the other donors at 4μg/ml, suggesting that cells were too necrotic at high dose HMGB1. Due to amplification of inflammatory interleukins, HMGB1 may have clinical potential as an early danger signal in tendonitis.

Expression of CCL2 increased as HMGB1 concentration applied to cells increased, highlighting HMGB1 as a direct or indirect transcription promoter of chemokine expression, thus promoting inflammatory cell migration (2). Although protein levels did not support, this may due to the rapid turnover of chemokine *in vitro* at high HMGB1 concentrations. CXCL12 had increased expression in tenocytes in the presence of HMGB1, further supporting that HMGB1 is induces immune cell recruitment and extracellular matrix proliferation in tendonitis immunopathogenesis (3). However, notably CXCL12 is self-propagating, unlike HMGB1 (as highlighted in our results), hence it is difficult to provide a comparison of gene expression induced by HMGB1 alone. Although IL-1β expression also appears to be of direct cause of HMGB1 application, it must be noted that this experiment is not fully representative of tendonitis, whereby mechanical load and inflammatory cytokines cause necrosis and extracellular matrix damage (1,4). Investigating this synergism further with HMGB1 application to stretched tendon may provide a model for human tendonitis. Our results also highlight increased SCF expression in tenocytes treated with HMGB1 cf. untreated which, *in vivo,* would promote migration and maturation of inflammatory cells, namely mast cells (5).

Collagen I and III appeared to have marked increases in expression following tenocyte exposure to HMGB1, Collagen III doing so to a greater extent. This suggests the role of HMGB1 in altering tendon physiology during damage, whereby a higher proportion of weaker, dysregulated Collagen III to stronger, organised fibrils of Collagen I is made for healing/remodelling (6). mRNA synthesis of Tenascin C increased 2-fold, supporting that

Tenascin C is implicated in tendon remodelling and induced by HMGB1 (7). Decorin, also involved in collagen fibrillogenesis, demonstrated a positive relationship with HMGB1, suggesting that HMGB1 encourages tenocyte release of cell matrix regulators in an attempt to repair damage. In contrast, periostin, involved in alignment of tenocytes, had inconsistencies in expression at 2μg/ml HMGB1. No clear graphical trend of marked increase was found. Repeating this experiment to a larger scale may generate a more reliable representation of the impact of HMGB1 on tenocytes.

Calcium regulators S100A8/A9 are assumed as alarmins in rheumatoid arthritis and diseases whereby tendonitis often manifests (1). However, from this experiment, it cannot be presumed that such molecules are directly influenced by HMGB1.

4. Reflection by the student on the experience and value of the studentship (300 words max):

Ultimately, I aspire, to follow a career as a medical doctor, however, since, partaking in the studentship, I have developed a fascination in current medical research. I was naïve to the manifestations of tendonitis prior to this project, where I witnessed the few medical treatments available to offer to suffering patients at my supervisor's clinic. In the lab, working alongside post-doctoral researchers, PhD students and undergraduate students, I learned skills which will be invaluable to me during my Honours degree project. Such skills included setting up human tissue cultures and maintaining sterility of samples, cell-counts, fluorescent staining and using a fluorescent microscope, measuring RNA purity, and performing multiple qPCR and ELISA analyses. After a couple of weeks, I became confident that I could carry out the above tasks independently. Being embedded in an active research environment and working alongside active researchers fuelled my excitement to generate results of my own. My results have importantly highlighted mechanisms of a key danger molecule in tendonitis, and with further research, may be targeted in diagnosis and future treatments of tendinopathy.

5. Dissemination: (note any presentation publications submitted/planned from the work):

Planned presentation at Orthopaedic Research Society Annual Meeting, San Diego March 2017

6. Signatures:

Supervisor  Date 02/08/16

Student  Date 02/08/16

References

1. Millar N, Murrell G, McInnes I. Alarmins in tendinopathy: unravelling new mechanisms in a common disease. Rheumatology. 2013;52(5):769-779. Stålman A, Bring D, Ackermann P. Chemokine expression of CCL2, CCL3, CCL5 and CXCL10 during early inflammatory tendon healing precedes nerve regeneration: an immunohistochemical study in the rat. Knee Surgery, Sports Traumatology, Arthroscopy [Internet]. 2014 [cited 1 August 2016];23(9):2682-2689. Available from: http://link.springer.com/article/10.1007%2Fs00167-014-3010-9
2. Shen W, Chen X, Chen J, Yin Z, Heng B, Chen W et al. The effect of incorporation of exogenous stromal cell-derived factor-1 alpha within a knitted silk-collagen sponge scaffold on tendon regeneration. Biomaterials [Internet]. 2010 [cited 1 August 2016];31(28):7239-7249. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20615544
3. Archambault J, Tsuzaki M, Herzog W, Banes A. Stretch and interleukin-1β induce matrix metalloproteinases in rabbit tendon cells in vitro. Journal of Orthopaedic Research [Internet]. 2002 [cited 1 August 2016];20(1):36-39. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11853088
4. Hashimoto T, Nobuhara K, Hamada T. 2003.Pathologic evidence of degeneration as a primary cause of rotator cuff tear. Clin Ortop Relat Res. pp111-120.
5. Behzad H, Scott A. EFFECTS OF MAST CELLS ON THE FUNCTION OF ISOLATED HUMAN TENOCYTES. British Journal of Sports Medicine. 2013;47(9):e2.40-e2.
6. Riley GP, Harrall RL, Cawston TE, Hazleman BL, Mackie EJ. Tenascin-C and human tendon degeneration. *The American Journal of Pathology*. 1996;149(3):933-943.
7. Chiquet-Ehrismann RTucker R. Connective tissues: signalling by tenascins. The International Journal of Biochemistry & Cell Biology [Internet]. 2004 [cited 1 August 2016];36(6):1085-1089. Available from: http://www.sciencedirect.com/science/article/pii/S1357272504000172

Appendix

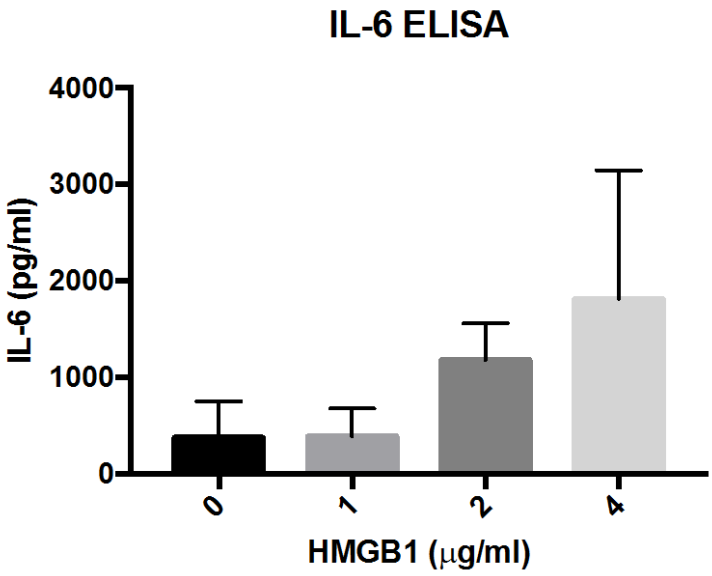

Figure 1: average concentration of IL-6 detected via ELISA from three donor tenocyte samples treated with increasing concentrations of HMGB1. Error bars indicate standard deviation for tenocytes treated with HMGB1.

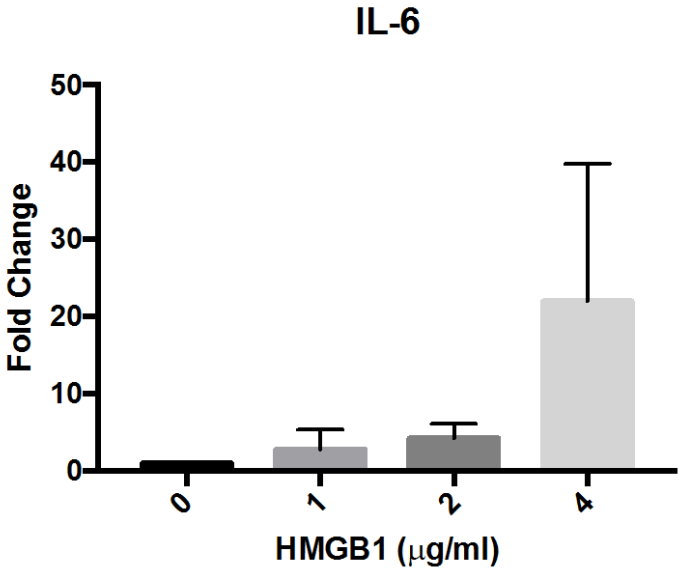

Figure 2: Fold change of IL-6 mRNA expression detected by qPCR of cDNA extracted from tenocytes from 3 patients treated with increasing HMGB1 concentration. Error bars indicate standard deviation.

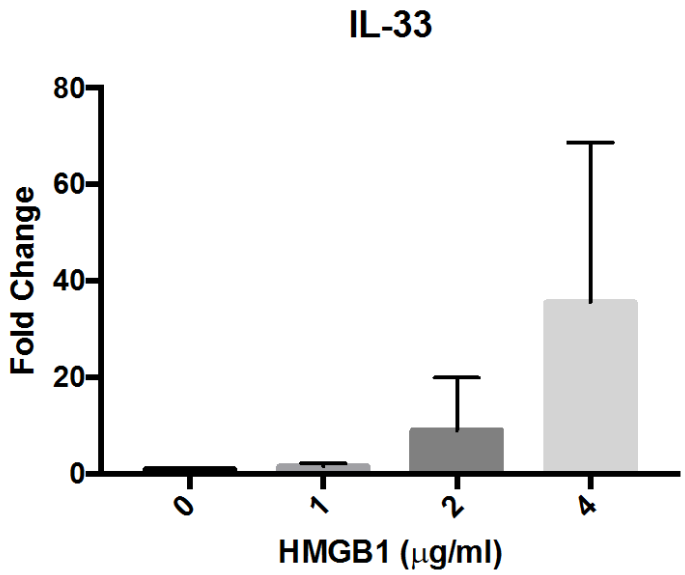

Figure 3: Fold change in intracellular RNA expression of IL-33 in tenocytes from 3 patients, treated with HMGB1 concentrations of 1, 2 and 4ug/ml. Error bars indicate standard deviation.

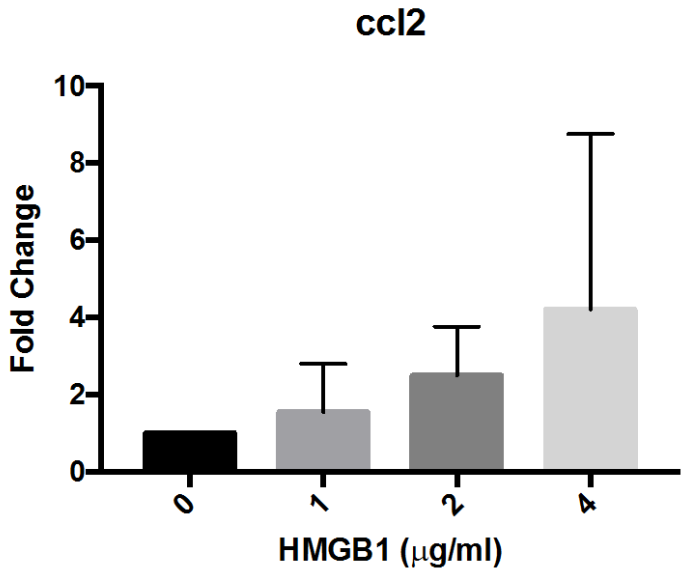

Figure 4: Fold change in CCL2 RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

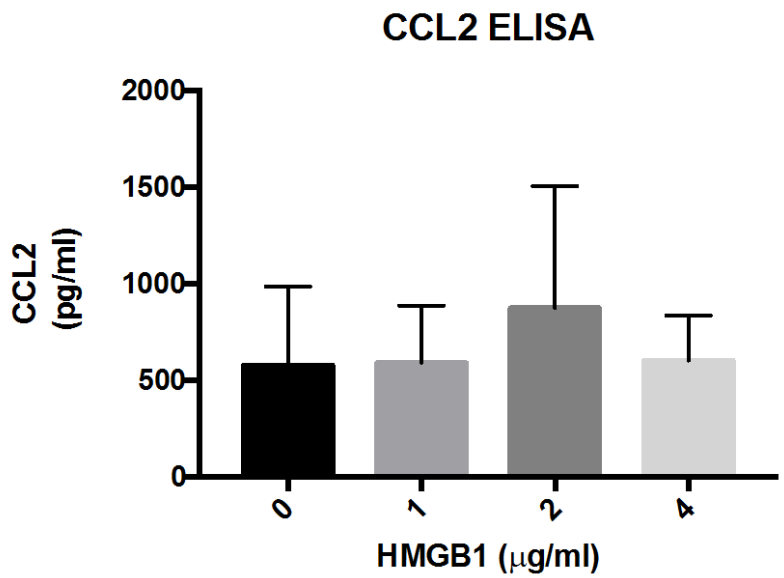

Figure 5: average concentration of CCL2 detected via ELISA from three donor tenocyte samples treated with increasing concentrations of HMGB1. Error bars indicate standard deviation for tenocytes treated with HMGB1

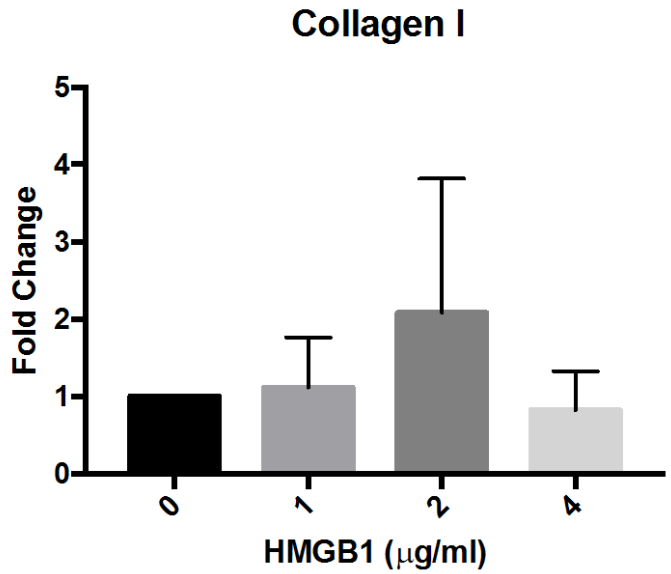

Figure 6: Fold change in Collagen I RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

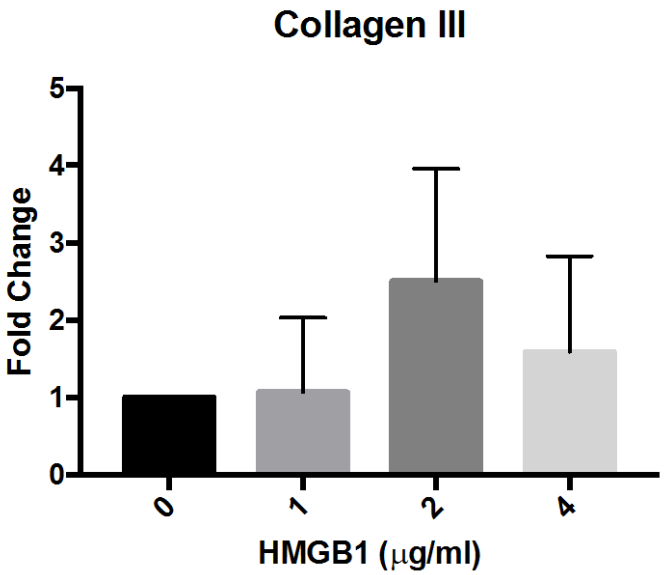

Figure 7: Fold change in Collagen III RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

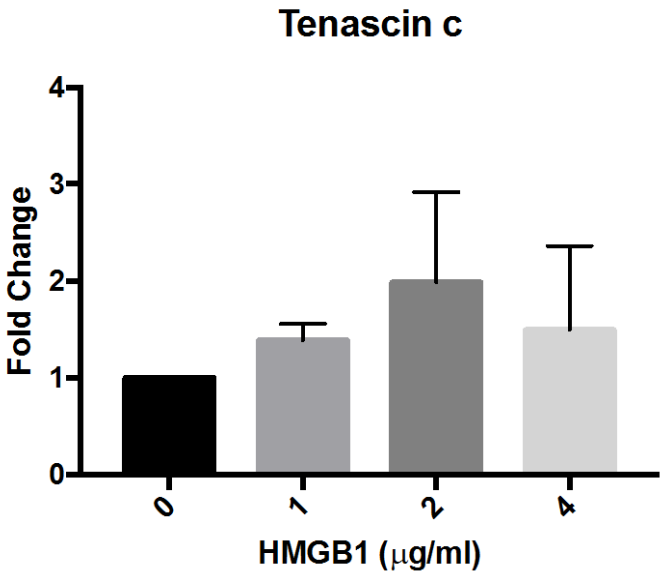

Figure 8: Fold change in Tenascin C RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

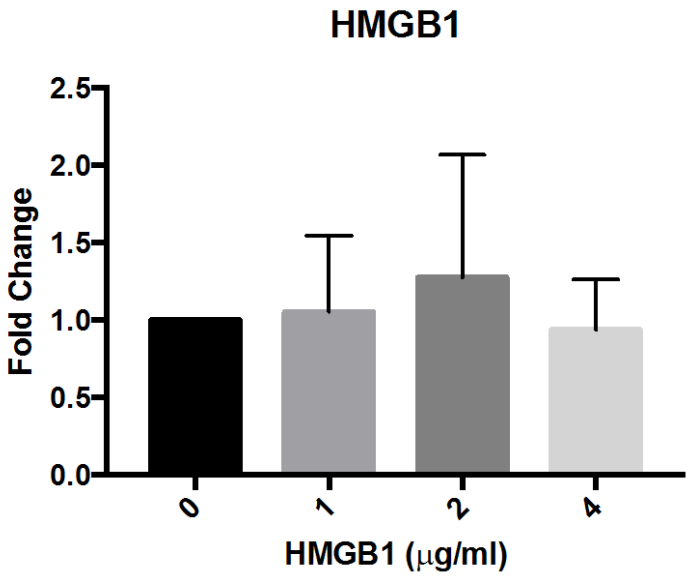

Figure 9: Fold change in HMGB1 RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

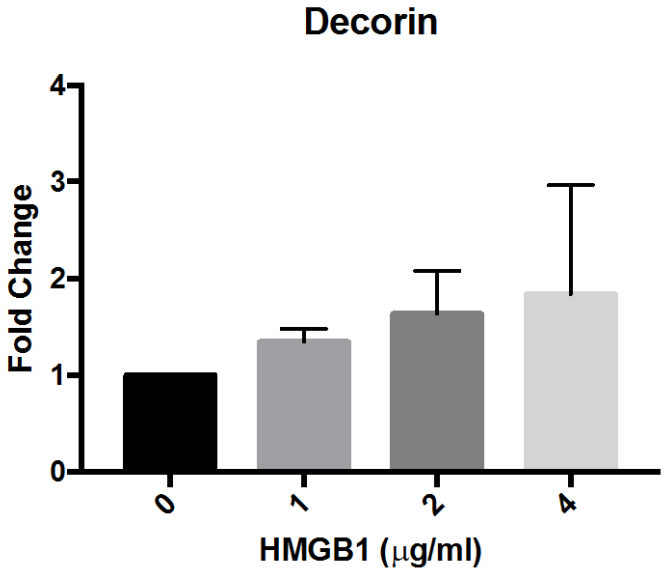

Figure 1o: Fold change in decorin RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

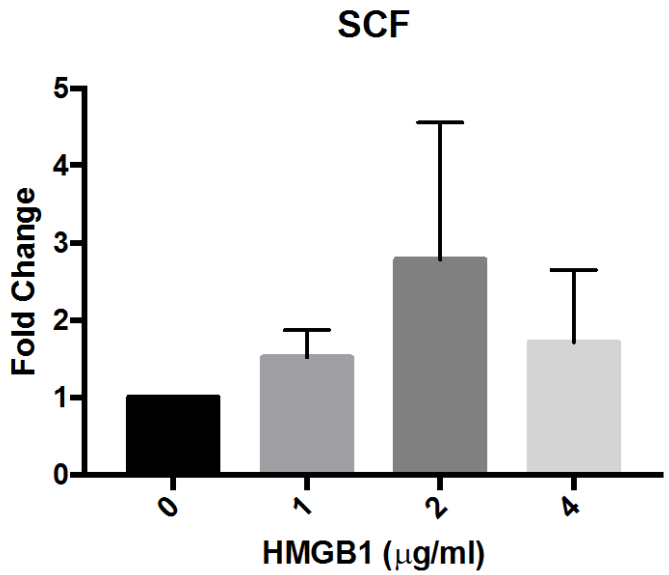

Figure 11: Fold change in SCF RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

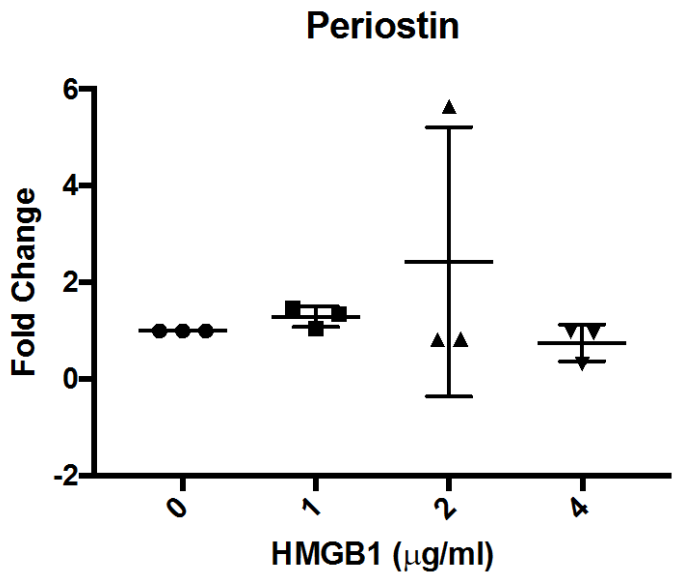

Figure 12: Fold change in periostin RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

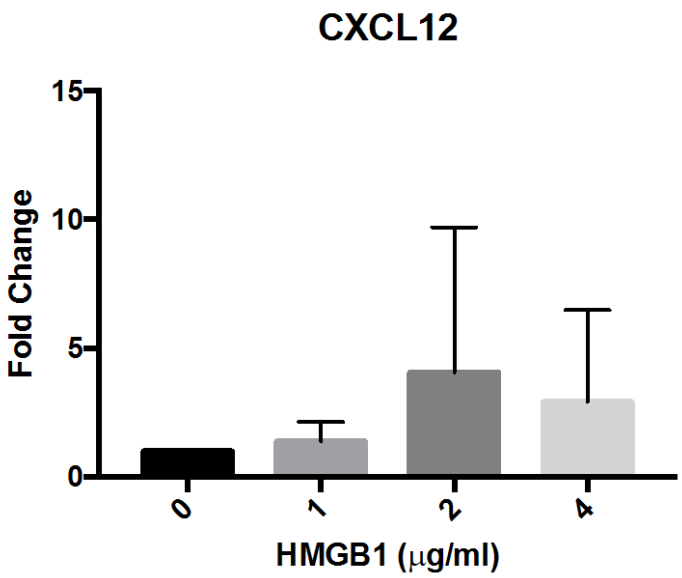

Figure 13: Fold change in Collagen III RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

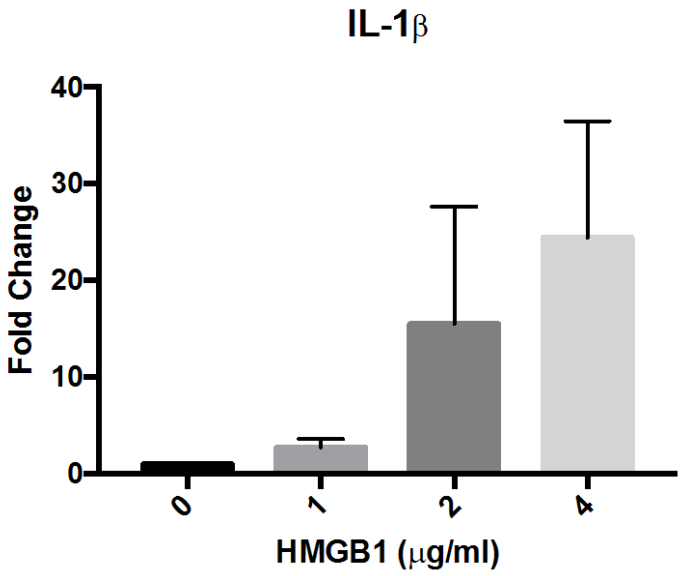

Figure 14: Fold change in IL-1β RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

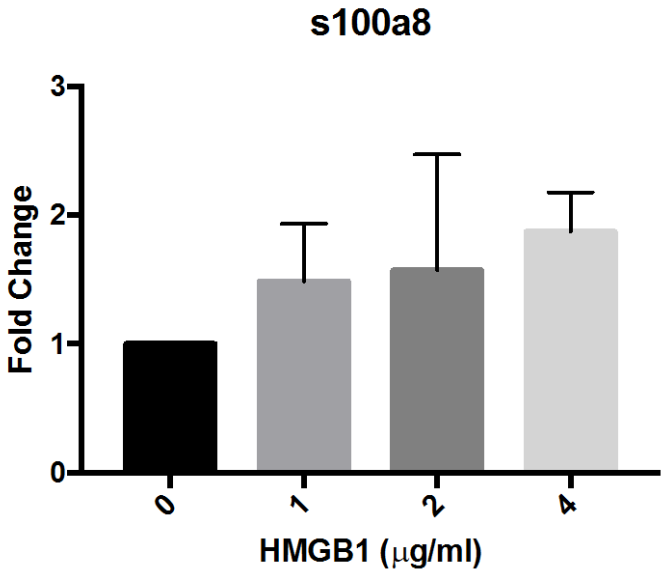

Figure 15: Fold change in S100A8 RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.

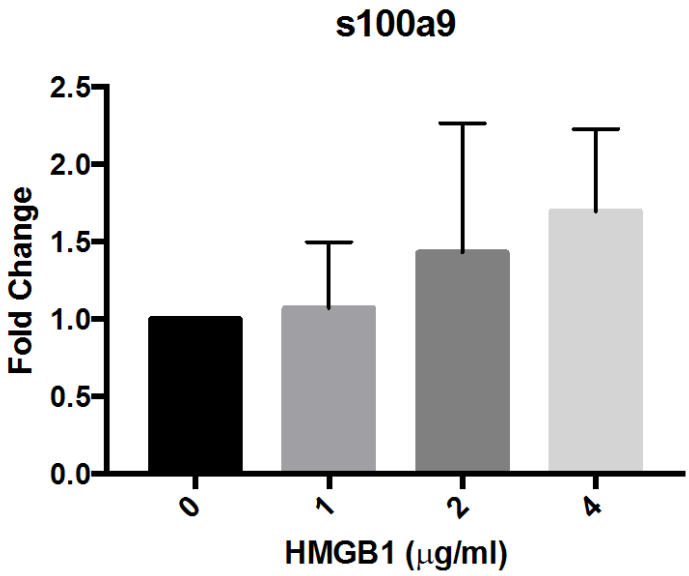

Figure 16: Fold change in S100A9 RNA expression In tenocytes from 3 patients, treated with HMGB1 at concentrations of 1, 2 and 4μg/ml. Error bars indicate levels of standard deviation.