View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by University of Liverpool Repository

Accepted Article

Proteomic differences between native and tissue engineered tendon and ligament

Yalda A. Kharaz[1], Simon R. Tew[1], Mandy Peffers[1, 2], Elizabeth G. Canty-Laird[1, 2] and Eithne Comerford[1*]

[1]Department of Musculoskeletal Biology, Institute of Ageing and Chronic Disease, University of Liverpool, Leahurst Campus, Neston CH64 7TE

[2]The MRC-Arthritis Research UK Centre for Integrated Research into Musculoskeletal Ageing (CIMA)

[*]Correspondence to: Eithne Comerford

Tel: 0151 7956100 Email: eithne.comerford@liv.ac.uk

Total word count = 5829

Key Words: 3D tissue engineered tendon constructs, 3D tissue engineered ligament construct, tendon, ligament, proteomics

Abbreviations: 3D: Three dimensional, AB-PAS: Alcian blue and periodic acid Schiff stain, ACL: Anterior cruciate ligament, ALC: Average local confidence score, ECM: Extracellular matrix: HCD: High energy collisional dissociation, LC-MS/MS: Liquid chromatography tandem mass spectrometry, LDET: Long digital extensor tendon: LF: Label-free, TE: Tissue engineered, TL: Tendon and ligament, sGAG: Sulphated glycosaminoglycan, TEM: Transmission electron microscopy

Received: 11/17/2015; Revised: 03/02/2016; Accepted: 04/08,2016

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/pmic.201500459.

This article is protected by copyright. All rights reserved.

Accepted Article

Abstract

Tendons and ligaments (T/Ls) play key roles in the musculoskeletal system, but they are susceptible to traumatic or age-related rupture, leading to severe morbidity as well as increased susceptibility to degenerative joint diseases such as osteoarthritis. Tissue engineering represents an attractive therapeutic approach to treating T/L injury but it is hampered by our poor understanding of the defining characteristics of the two tissues. The present study aimed to determine differences in the proteomic profile between native T/Ls and tissue engineered (TE) T/L constructs. The canine long digital extensor tendon (LDET) and anterior cruciate ligament (ACL) were analysed along with 3D TE fibrin-based constructs created from their cells. Native tendon and ligament differed in their content of key structural proteins, with the ligament being more abundant in fibrocartilaginous proteins. 3D T/L TE constructs contained less ECM proteins and had a greater proportion of cellular-associated proteins than native tissue, corresponding to their low collagen and high DNA content. Constructs were able to recapitulate native T/L tissue characteristics particularly with regard to extracellular matrix (ECM) proteins. However 3D T/L TE constructs had similar ECM and cellular protein compositions indicating that cell source may not be an important factor for T/L tissue engineering.

This article is protected by copyright. All rights reserved.

Accepted Article

Statement of significance

Tissue engineering approaches have the potential to provide materials for treatment of tendon and ligament injuries. To date, no studies have characterised the proteome of engineered tendon or ligament using the increasingly popular 3D fibrin-based culture system. In this paper, we report the first proteome profile of 3D tendon and ligament TE constructs and have performed a comprehensive proteomic analysis to reveal differences between constructs and native tissues.

1 Introduction

Tendons and ligaments (T/Ls) are dense connective tissues that play key roles in musculoskeletal system. Both tissues have specialised functions required for efficient locomotion [1]. T/L injuries are increasingly common in humans, in comparative species such as the dog and the horse [2-5] and are caused as result of degeneration or trauma/acute tears. There are currently more than 30 million tendon injures per year worldwide [6], with 30-50% of these being sports-related [7]. With regards to tendon injuries, rotator cuff tears in humans [8] and superficial digital flexor tendinopathy in the horse [9] are the most common. The aethiopathogenesis of this tendinopathy is thought to be caused by repetitive micro trauma resulting in degenerative changes subsequently leading to injury [1]. The anterior cruciate ligament (ACL) is one of the most frequently injured ligaments in humans with 30% caused by trauma and 70% due to degenerative and non-contact injuries [10]. Both ACL and medial collateral ligament (MCL) injuries account for 95% of all multi-ligament injuries in the knee joint [11], resulting in significant joint instability and

This article is protected by copyright. All rights reserved.

Accepted Article

morbidity [12]. Ligament injury can also lead to significant functional impairment in athletes resulting in degenerative joint diseases such as osteoarthritis (OA) [13, 14]. Severe T/L injuries are presently treated with autografts or allografts, but these are associated with complications such as infection [15], disease transmission and graft rejection [15, 16], chronic pain [17], decreased muscle strength [18] and donor site morbidity [19].

Tissue engineering offers great potential for the treatment of T/L injury by aiming to provide a biological replacement that mimics the structure, function and longevity of native tissue [20]. The tissue engineering approach involves the acquisition and cultivation of an adequate source of cells, addition of growth inducing stimuli and provision of an artificial ECM (scaffold) in which cells can proliferate and differentiate enabling new tissue generation [21]. Fibrin is a natural biomaterial which has been used for the creation of engineered T/L constructs using variety of cell sources including rat and chick tendon fibroblasts [22-25], human bone marrow stem cells [26], human adult ACL [27] and tendon cells [28]. To date an understanding of the proteins that comprise these tendon and ligament-like structures is unknown. The hypotheses of this study were that 1) native tendons and ligaments have different structural protein content and that 2) three dimensional (3D) tissue TE constructs formed from T/L cells possess the proteome characteristics of the original tissues. Therefore, this study aimed to identify the differences between the proteomes of native T/L as well as those from engineered T/L 3D constructs. In this study a proteomics workflow using a gel-free separation technique with label-free (LF) quantification was used to identify differences in protein abundance. A detailed proteomic comparison between native and TE tendons and ligaments has not previously been reported.

This article is protected by copyright. All rights reserved.

Accepted Article

2 Material and Methods

All chemicals were supplied by Sigma–Aldrich, Dorset, UK unless otherwise stated.

2.1 Tissue collection and preparation

ACLs and long digital extensor tendons (LDETs) were harvested immediately after euthanasia from five paired cadaveric canine knee joints. The knee joints were from skeletally mature Staffordshire bull terrier dogs (2-5 years old) with a healthy body score (4-5/9). The joints were assessed as disease-free by gross inspection. The dogs were euthanased for purposes not related to this study and ethical approval for use of the cadaveric material was granted by Veterinary Research Ethics Committee, School of Veterinary Science (VREC64). Tissues from the right knee joint were used for protein isolation and proteomic analysis of the native tissues. Tissues from the left knee joint were used for cell isolation and creation of engineered tissues, which were subsequently used for protein extraction and proteomic analysis. All samples were snap frozen in liquid nitrogen and stored at -80°C until required.

2.2 Tendon and ligament 3D TE construct formation

The 3D TE constructs were created using a 3D fibrin-based culture system with isolated ACL and LDET cells as described previously [22] with minor modifications as detailed in the supplementary method information.

2.3 Protein extraction

Native tendon, ligament and harvested T/L constructs samples were freeze dried overnight and the dry weight then measured. Approximately 3mg of each lyophilised sample was disrupted using a micro-dismembrator (B.Braun Biotech. International, Germany). Each sample was digested with 1U/ml chondroitinase ABC in 100mM tris

This article is protected by copyright. All rights reserved.

Accepted Article

acetate pH 8, containing mini protease inhibitor cocktail with EDTA (Roche, UK) for 6 hours at 37°C with end-over-end mixing. The supernatant was removed following centrifugation at 15000rpm at 4°C for 15 minutes. Tissue was extracted in 500μl 4M guanidine hydrochloride (GnHCl), 65mM dithiothreitol, 50mM sodium acetate, pH 5.8 for 48 hours at 4°C with shaking. The samples were then centrifuged at 15000rpm at 4°C for 15 minutes and the soluble fraction was removed. The protein concentration of each soluble fraction was estimated using a Pierce^{TM} 660 nm protein assay (Thermo scientific, Hertfordshire, UK) and aliquots analysed by 1D SDS-PAGE gel electrophoresis to grossly assess the protein expression profile between samples.

2.4 In-solution trypsin digestion

Prior to trypsin digestion the GnHCL soluble fraction was diluted 8-fold with 100 mM ammonium bicarbonate and further normalised to the sample that had the lowest protein concentration. In-solution tryptic digestion was carried out as described previously [29].

2.5 Liquid chromatography and tandem mass spectrometry

LC-MS/MS was performed using an Ultimate 3000 nano system (Dionex/Thermo Fisher Scientific) coupled online to a Q-Exactive Quadrupole-Orbitrap instrument (Thermo-Fischer Scientific) using 10μl aliquots of tryptic peptides equivalent to 93 ng protein per sample. Samples were randomised and run on a 1 hour gradient with 30 min blanks in between runs as detailed in the supplementary methods.

2.6 Proteomic data analysis

Mass spectrometry data was analysed for identification of protein composition and LF quantification using PEAKS® (Version 6, Bioinformatics Solutions, Waterloo, Canada) and Progenesis^{QI} LC-MS (Waters, Elstree Hertfordshire, UK) software.

This article is protected by copyright. All rights reserved.

Accepted Article

Mass spectrometry data is available in PRIDE database (http://www.ebi.ac.uk/pride/) at the European Bioinformatics Institute under accession number PXD003094.

To identify the protein composition in each group (native ligament, native tendon, 3D ligament and 3D tendon) raw MS/MS data were imported into PEAKS® and searches then performed against the Ensembl canine taxonomy ((http://www.ensembl.org/info/data/ftp/index.html).

Instrument configuration was set as Orbitrap (Orbi-Orbi) and HCD fragmentation. The following parameters were used for the PEAKS® search; parent mass error tolerance, 10 ppm; fragment mass error tolerance, 0.1 Da, precursor mass search type, monoisotypic; enzyme, trypsin; max missed cleavage, 1; non-specific cleavage, 1; fixed modification; carbamidomethylation, variable modification; oxidation, methionine, hydroxylation, and variable PTMs per peptide, 3. The results were filtered on the basis of the following parameters; *de-novo* average local confidence score (ALC) % threshold, 50; protein -10lgP> 20; FDR at peptide spectrum matches, 1%; and unique peptides $\geq$ 2. The ensemble protein accessions were input into Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA, USA) and gene symbol with protein description and protein subcellular locations were then mapped. Proteins were classified into ECM categories according to the Matrisome Project [30, 31]. The remaining proteins were categorised according to UNIPROT function description [32]. Gene ontology (GO) and protein network analysis was carried out using the String bioinformatics tool, version 10 [33]. LF quantitative analysis was performed using Progenesis^{QI} LC-MS software. Search results in PEAKs® were adjusted to 1% FDR, unique peptides $\geq$ 2 and ALC > 50% and search hits were imported into Progenesis^{QI} [34].

This article is protected by copyright. All rights reserved.

Accepted Article

2.7 Biochemical analysis

The biochemical composition of native T/L tissues or 3D TE T/L constructs (n=5) was determined by measuring double stranded DNA, collagen and sulphated glycosaminoglycan (sGAG) content using previously described protocols [35-37].

2.8 Histology and Immunohistochemistry

Native T/L samples and TE constructs created from isolated cell samples (n=3) were fixed in 4% paraformaldehyde for 48 hours, embedded in paraffin wax and 4 µm longitudinal sections mounted on polylysine-coated slides. Sections were stained with H&E and Alcian blue-periodic acid Schiff stain (AB-PAS) (TCS, Biosciences Ltd, Buckingham, UK) [38].

Immunohistological staining was performed on native T/L tissue for asporin, aggrecan, versican and collagen type III on deparaffinised sections. The immunohistochemistry procedure and antibodies details are provided in the supplementary methods.

2.9 Transmission electron microscopy

Transmission electron microscopy (TEM) of T/L 3D TE constructs (n=3) was performed following fixation in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (Agar Scientific, Essex, UK) for 8 hours, followed by a second fixation and contrast stain with 0.1% osmium tetroxide for 90 minutes. Samples were stained with 8% uranyl acetate in 0.69% maleic acid for 90 minutes, dehydrated in ascending ethanol concentrations and embedded in epoxy resin (all from TAAB Laboratories Equipment Ltd, Berks, UK). Ultrathin cross-sections (60-90 nm) were cut with a Reichert- Jung Ultracut ultramicrotome (Leica Microsystems Ltd, Milton Keynes, UK) using a diamond knife. Sections were then mounted on 200 mesh copper grids and stained

This article is protected by copyright. All rights reserved.

Accepted Article

with 'Reynold's Lead citrate' stain (VWR, Leicestershire, UK) for 4 minutes. Images were obtained using a Philips EM208S Transmission Electron Microscope at 80kv.

2.10 Statistical analysis

Statistical analysis for proteomic LF data sets was performed by Progenesis^{QI} on all detected features using transformed normalised abundances for one-way ANOVA. Identification of proteins with two or more peptides, greater than 2 fold abundance and with a q value (p-value adjusted to FDR) <0.05 were considered significant. Quantitative analysis was initially performed by comparing the four groups of tissue samples together. After that pair-wise comparisons were performed between native ligament and tendon, native ligament and 3D TE ligament construct, native tendon and 3D TE tendon construct, and 3D TE ligament and 3D TE tendon constructs. Data sets for biochemical analysis were first assessed for normality using a Kolmogorov-Smirnov test (Graphpad Software, Version 6, La Jolla, California). All data sets were normal distributed and were analysed using one-way ANOVA with a Bonferroni post-hoc test using Graphpad Prism. The significance level was set at $p<0.05$.

3 Results

3.1 Engineered 3D tendon and ligament constructs display a loose architecture with a high degree of cellularity

Histological observation of native tendon demonstrated a dense, parallel aligned architecture and long elongated cellular morphology, however native ligament had less compact collagen fibre alignment and a more rounded cell morphology (Figure 1A and B). H&E staining of 3D constructs indicated that both tendon and ligament constructs had a loose architecture and a high degree of cellularity with a fibroblastic

This article is protected by copyright. All rights reserved.

Accepted Article

cellular morphology (Figure 1C and D). The presence of collagen fibrils was confirmed using TEM, where close-packed narrow diameter collagen fibrils were visible in the extracellular space (Figure 1E and F). Collagen fibrils were also found to be located in collagen fibripositors (Figure 1E and F), which are actin rich plasma membrane protrusions that mediate collagen fibril organisation in embryonic tendon [39].

3.2 Matrisomal proteins and gene ontology terms associated with ECM organisation were more strongly represented in native tissue than in engineered 3D constructs

An average protein content of (µg/mg dry weight) of 181 was measured for native T/L and 284 for 3D T/L constructs. A total of 3569, 3743, 4481 and 5790 peptides assigned to 167, 215, 442 and 561 proteins each were identified in native ligament, native tendon, 3D ligament and 3D tendon, respectively. Between both native tissues and 3D tissues 93 proteins were common, which included several ECM proteins such as collagen type I ,III, V,VI, decorin, biglycan, lumican, tenascin C, fibrillin 1, fibulin 1, thrombospondin 1 and cellular proteins such vimentin, ATP synthase and actin (Supplementary table 1).

The native T/L proteome contained 40% and 50% matrisomal proteins respectively, with 45% and 53% of proteins locations annotated to extracellular space, (Figure 2B, 2C). The remainder of the native T/L proteome was associated with cytoplasmic, nucleus and plasma membrane locations. In both 3D TE tendon and ligament constructs 66.3% of proteins were associated with cytoplasmic location whereas 22% of proteins were associated with translation and signalling and 13.1% were matrisomal proteins (Figure 2D and 2E).

This article is protected by copyright. All rights reserved.

Accepted Article

STRING protein-protein interaction network analysis and GO in both native T/L tissues resulted in connected clusters around ECM proteins and matrisomal associated proteins. The principal GO processes for both tendon and ligament tissues were identified as ECM organisation (FDR adjusted p-values 1.53^{-26} and $3.75e^{-27}$ respectively), wound healing (FDR adjusted p-value $1.94e^{-14}$ and 1.85^{-14}) and collagen fibril organisation (FDR adjusted p-values $2.01e^{-21}$and$1.19e^{-13}$) (Supplementary Figure 1A and 1B). Principle ontology for 3D ligament and 3D TE tendon constructs involved translational elongation (FDR adjusted p-values $3.71e^{-65}$ and $2.3e^{-63}$) and protein targeting to ER (FDR adjusted p-values $9.98e^{-64}$, and 1.75e-^{65}). The strongest predicted protein-protein interaction was between the ribosomal proteins (Supplementary Figure 1C and 1D).

3.3 Quantitative differences in protein composition were observed between native tendon and ligament but not between constructs formed from either tendon or ligament cells.

Quantitative LF analysis demonstrated a set of 387 proteins within the four tissue types with a fold change $\geq$ 2 and unique peptides > 2. PCA revealed that native ligament and tendon samples were distinctly grouped, whereas 3D tendon and tendon did not cluster into discrete groups (Figure 3A).

Pairwise quantitative comparison between native TL demonstrated that native ligament was more abundant in fibrocartilaginous proteins such as collagen type II, alpha 1, agreccan and chondroadherin, whilst tendon had more thrombospondin 4, asporin and collagen type XII (Figure 4A). No differentially expressed proteins were found between 3D TL constructs (Figure 4B). Quantitative differences between native tendon and 3D tendon resulted in 321 and 62 proteins being more abundant in 3D tendon and native tendon respectively. When native ligament and 3D ligament

This article is protected by copyright. All rights reserved.

Accepted Article

were compared 301 proteins were more abundant in 3D ligament and 62 proteins were more abundant in native ligament (Figure 4C and 4D).

Observation of abundant proteins in both native tendon and ligament when compared to 3D tendon and ligament engineered constructs demonstrated not only the presence of significantly more ECM proteins and enzyme enhancers but also more blood/plasma proteins in both native tissues. In contrast both 3D TE constructs had significantly more cellular proteins (Supplementary Tables 2 and 3).

3.4 Biochemical analysis confirmed differences in cellular and collagen content between native tissues and engineered

Both 3D T/L constructs demonstrated significantly higher DNA content (10.2 ± 1.5 mg/ dry weight) indicating a high cellularity of both constructs in comparison to the native tissues (3.43 ± 0.7) (Figure 3B). In contrast, the collagen content was significantly lower in the constructs (average of 3.2%) compared to the native tissues (average of 67.8%) (Figure 3C). Native ligament (15.1 ± 0.7) (Figure 5A) contained significantly more sGAG in comparison to the native tendon (8.3± 1.03) where higher GAG staining in native ligament (Figure 5B and 5C) was located between collagen fascicles, fibre bundles and surrounding cells. Each 3D TE tendon or ligament construct contained a comparable sGAG content to the native tissue (Figure 5A). Native ligament (15.1± 0.7) was significantly higher sGAG compared to native tendon. Only 3D ligament constructs (11.1 ± 0.7) had significantly more sGAG than native tendon. No significant differences were found between both 3D TL constructs.

This article is protected by copyright. All rights reserved.

Accepted Article

3.5 Immunohistochemistry supported differences in the abundance of versican between tendon and ligament and further demonstrated an altered tissue distribution of type III collagen, aggrecan and asporin.

Proteomic comparison identified native ligament to be more abundant in collagen type III, aggrecan and versican, whilst tendon contained more asporin. These findings were supported with immunohistochemical staining. In comparison to tendon, collagen type III was differentially distributed in ligament being more widespread in ligament substance whilst in tendon it was mainly present between collagen fascicles (Figure 5D and 5E). A marked presence of aggrecan (Figure 5F and 5G) and versican (Figure 5H and 5I) was noted in ligament between collagen fascicles in comparison to tendon. Aggrecan was also localised pericellularly in ligament and this could be a key characteristic of ligament cells. Asporin (Figure 5J and 5K) was found to be distributed between collagen fascicles and surrounding tenocytes in tendon, whilst in the ligament asporin was only localised around ligament cells.

4 DISCUSSION

In this study we have performed a comprehensive analysis of the proteomic composition of native T/L tissue and 3D TE fibrin-based constructs. The results support the hypothesis that there are key structural protein differences between native T/L and that 3D TE constructs share similar characteristics with native tissues particularly with regard to prominent ECM proteins.

The abundance of more fibrocartilaginous proteins such as collagen type II, aggrecan, versican, chondroadherin and hyaluronic acid link protein in native ligament (ACL) is most likely to be due to the physical adaptation of the tissue

This article is protected by copyright. All rights reserved.

Accepted Article

against compressive or shear forces generated during twisting of the ACL as the knee joint moves through normal range of motion [40]. The formation of fibrocartilage in TLs has been shown to occur in response to compression, primarily in regions where they approach or traverse bone [41]. Regional variations in cell morphology and glycosaminoglycan content in tendons has been reported [42] as well as in the canine ACL suggesting that the ligament is subjected to multiaxial stresses [43]. In the present study we did not discriminate between different regions of native ligament so the higher levels of fibrocartilaginous proteins may arise from the origin and insertion regions of the ACL. Nevertheless sGAG analysis, histological and immunohistochemical staining of aggrecan and versican in the ACL mid-substance indicates that these proteins are also up-regulated throughout the entire ligament. The higher proportion of collagen type III, aggrecan and versican observed in ligament agrees with a previous comparison between human ACL and patellar tendon [44].

In the current study 3D TE constructs were created from mature canine LDET and ACL fibroblasts using *in-vitro* 3D cell fibrin cultures [22]. Constructs from both tissues displayed a high degree of cellularity and collagen fibril content. Collagen fibrils were mainly located in extracellular space, but were also occasionally found in plasma membrane protrusions also known as fibripositors found previously in embryonic tendon [39, 45]. The proteomic comparison between native TL tissue and 3D TE constructs demonstrated significantly more ECM proteins in native tissues, whilst both 3D tissues had more cellular associated proteins. The higher levels of cellular associated proteins in 3D TE constructs is likely to be due to the greater cell content in these tissues compared to the native tissues. Their high DNA and low collagen

This article is protected by copyright. All rights reserved.

Accepted Article

content is indicative of a high cell-to-matrix ratio. In contrast 3D TE constructs were also found to contain high sGAG content, suggesting that the proteoglycans are rapidly acquired and mature much faster or may require less maturation than the collagen matrix. Proteoglycans have been shown to play role in regulation of tendon collagen fibrillogenesis [46] and prevent later fusion of collagen fibrils [47]. This higher proteoglycan content (based on sGAG measurement) might play an important role in organisation of the collagen fibrils and development of 3D TE constructs. Therefore our findings reflect the immature state of the constructs in this study and are consistent with previous observations in adult human tendon constructs [28, 48]. To date, the extent of maturation of 3D tissue engineered T/L constructs is not fully known. However, Herchenhan et al. [48] demonstrated a 5-fold and 50% increase of mechanical strength and collagen fibril diameter when constructs were subjected to static tension for 5 weeks. Other studies have identified that factors such as uniaxial cyclic stretch [49] and addition of growth factors such as transforming growth factor 1 or insulin growth factor 1 [23, 27] can increase collagen gene expression, content and fibril diameter in 3D fibrin constructs.

PCA analysis of our proteomic data suggested that there were no statistically significant differences between 3D T/L constructs. This was in contrast to native T/L, which separated into distinct groups, based upon their protein content. These findings suggest that fibroblasts of the T/L do not result in distinct 3D constructs during the 14-day culture period we have used. This might indicate that cell source is not an important factor for tissue engineering although longer term *in vitro* studies with more mature constructs would be required to test this. Proteomic composition between 3D T/L constructs that were derived from T/L cell source indicate that

This article is protected by copyright. All rights reserved.

Accepted Article

tendon and ligament fibroblasts are not phenotypically distinct when cultured *in-vitro*. This data indicates that fine-tuning ECM composition may be more significant challenge for tendon and ligament tissue engineering. It is yet to be determined whether T/L cells become a tendon/ligament or whether different cell sources such mesenchymal stem cells or skin dermal fibroblasts differentiate into ligament or tendon when situated in their native *in-vivo* environment.

A possible limitation to this study is the chondroitinase ABC treatment step which was included to produce a better protein separation of proteoglycan peptides and to facilitate trypsin digestion. The extreme charge density of the long GAG chains could have interfered with protein separation during ion exchange chromatography and could have reduced the efficiency of trypsin digestion, or introduced unwanted variability at this crucial step. However the chondroitinase treatment could have also extracted other chondroitinase sulphate binding proteins that may have been overlooked. It should also be considered that whilst most proteins were solubilised in all samples, a fraction of proteins were insoluble and retained in pellet form. This is most likely due to cross-linked collagen chains (Peffers et al. 2014). Future studies may involve optimisation of protein extraction using a combination of other chaotropic agents and LC-MS/MS analysis on the insoluble fraction.

In conclusion we have shown for the first time the differences between tendon, ligament and 3D TE tendon and ligament constructs. Our findings make a vital contribution to future tendon and ligament tissue engineering and regeneration strategies.

This article is protected by copyright. All rights reserved.

Accepted Article

5 ACKNOWLEDGMENTS

The authors wish to thank the Medical Research Council, Institute of Ageing and Chronic Disease and University of Liverpool Technology Directorate for proteomic funding, Dr Deborah Simpson for expert assistance with mass spectrometry and proteomic analysis and Marion Pope for expert technical assistance with transmission electron microscopy. We also thank Professor Bruce Caterson and Dr. Clare Hughes, University of Cardiff, for providing the aggrecan 7D1 antibody.

The authors have declared no conflict of interest.

This article is protected by copyright. All rights reserved.

Accepted Article

6 REFERENCES

[1] Birch, H. L., Thorpe, C. T., Rumian, A. P., Specialisation of extracellular matrix for function in tendons and ligaments. *Muscles Ligaments Tendons J* 2013, *3*, 12-22.

[2] Adirim, T. A., Cheng, T. L., Overview of injuries in the young athlete. *Sports Medicine* 2003, *33*, 75-81.

[3] Burt, C. W., Overpeck, M. D., Emergency visits for sports-related injuries. *Annals of emergency medicine* 2001, *37*, 301-308.

[4] Clegg, P., Musculoskeletal disease and injury, now and in the future. Part 2: Tendon and ligament injuries. *Equine veterinary journal* 2012, *44*, 371-375.

[5] Comerford, E. J., Smith, K., Hayashi, K., Update on the aetiopathogenesis of canine cranial cruciate ligament disease. *Vet Comp Orthop Traumatol* 2011, *24*, 91-98.

[6] Maffulli, N., Wong, J., Almekinders, L. C., Types and epidemiology of tendinopathy. *Clinics in sports medicine* 2003, *22*, 675-692.

[7] Rees, S., Dent, C., Caterson, B., Metabolism of proteoglycans in tendon. *Scandinavian journal of medicine & science in sports* 2009, *19*, 470-478.

[8] Butler, D. L., Juncosa-Melvin, N., Boivin, G. P., Galloway, M. T., *et al.*, Functional tissue engineering for tendon repair: A multidisciplinary strategy using mesenchymal stem cells, bioscaffolds, and mechanical stimulation. *Journal of Orthopaedic Research* 2008, *26*, 1-9.

[9] Thorpe, C., Clegg, P., Birch, H., A review of tendon injury: why is the equine superficial digital flexor tendon most at risk? *Equine veterinary journal* 2010, *42*, 174-180.

This article is protected by copyright. All rights reserved.

Accepted Article

[10] Cimino, F., Volk, B. S., Setter, D., Anterior cruciate ligament injury: diagnosis, management, and prevention. *American family physician* 2010, *82*, 917-922.

[11] Funakoshi, Y., Hariu, M., Tapper, J. E., Marchuk, L. L.*, et al.*, Periarticular ligament changes following ACL/MCL transection in an ovine stifle joint model of osteoarthritis. *Journal of orthopaedic research* 2007, *25*, 997-1006.

[12] Woo, S. L., Hildebrand, K., Watanabe, N., Fenwick, J. A.*, et al.*, Tissue engineering of ligament and tendon healing. *Clinical orthopaedics and related research* 1999, S312-323.

[13] Maffulli, N., Binfield, P. M., King, J. B., Articular cartilage lesions in the symptomatic anterior cruciate ligament-deficient knee. *Arthroscopy: The Journal of Arthroscopic & Related Surgery* 2003, *19*, 685-690.

[14] Daniel, D. M., Stone, M. L., Dobson, B. E., Fithian, D. C.*, et al.*, Fate of the ACL-injured patient a prospective outcome study. *The American journal of sports medicine* 1994, *22*, 632-644.

[15] Robertson, A., Nutton, R., Keating, J., Current trends in the use of tendon allografts in orthopaedic surgery. *Journal of Bone & Joint Surgery, British Volume* 2006, *88*, 988-992.

[16] Strickland, S. M., MacGillivray, J. D., Warren, R. F., Anterior cruciate ligament reconstruction with allograft tendons. *Orthopedic Clinics of North America* 2003, *34*, 41-47.

[17] Spindler, K. P., Kuhn, J. E., Freedman, K. B., Matthews, C. E.*, et al.*, Anterior cruciate ligament reconstruction autograft choice: Bone-tendon-bone versus hamstring does it really matter? a systematic review. *The American Journal of Sports Medicine* 2004, *32*, 1986-1995.

This article is protected by copyright. All rights reserved.

Accepted Article

[18] Beynnon, B. D., Johnson, R. J., Fleming, B. C., Kannus, P., *et al.*, Anterior cruciate ligament replacement: Comparison of bone-patellar tendon-bone grafts with two-strand hamstring grafts a prospective, randomized study. *The Journal of Bone & Joint Surgery* 2002, *84*, 1503-1513.

[19] Mastrokalos, D. S., Springer, J., Siebold, R., Paessler, H. H., Donor Site Morbidity and Return to the Preinjury Activity Level After Anterior Cruciate Ligament Reconstruction Using Ipsilateral and Contralateral Patellar Tendon Autograft A Retrospective, Nonrandomized Study. *The American journal of sports medicine* 2005, *33*, 85-93.

[20] Rodrigues, M. T., Reis, R. L., Gomes, M. E., Engineering tendon and ligament tissues: present developments towards successful clinical products. *Journal of tissue engineering and regenerative medicine* 2013, *7*, 673-686.

[21] Ahmed, T. A., Dare, E. V., Hincke, M., Fibrin: a versatile scaffold for tissue engineering applications. *Tissue Engineering Part B: Reviews* 2008, *14*, 199-215.

[22] Kapacee, Z., Richardson, S. H., Lu, Y., Starborg, T., *et al.*, Tension is required for fibripositor formation. *Matrix Biology* 2008, *27*, 371-375.

[23] Paxton, J. Z., Grover, L. M., Baar, K., Engineering an in vitro model of a functional ligament from bone to bone. *Tissue engineering. Part A* 2010, *16*, 3515-3525.

[24] Herchenhan, A., Kalson, N. S., Holmes, D. F., Hill, P., *et al.*, Tenocyte contraction induces crimp formation in tendon-like tissue. *Biomechanics and modeling in mechanobiology* 2012, *11*, 449-459.

[25] Calve, S., Dennis, R. G., Kosnik, P. E., Baar, K., *et al.*, Engineering of functional tendon. *Tissue engineering* 2004, *10*, 755-761.

This article is protected by copyright. All rights reserved.

Accepted Article

[26] Kapacee, Z., Yeung, C.-Y. C., Lu, Y., Crabtree, D., *et al.*, Synthesis of embryonic tendon-like tissue by human marrow stromal/mesenchymal stem cells requires a three-dimensional environment and transforming growth factor β3. *Matrix Biology* 2010, *29*, 668-677.

[27] Hagerty, P., Lee, A., Calve, S., Lee, C. A., *et al.*, The effect of growth factors on both collagen synthesis and tensile strength of engineered human ligaments. *Biomaterials* 2012, *33*, 6355-6361.

[28] Bayer, M. L., Yeung, C. Y., Kadler, K. E., Qvortrup, K., *et al.*, The initiation of embryonic-like collagen fibrillogenesis by adult human tendon fibroblasts when cultured under tension. *Biomaterials* 2010, *31*, 4889-4897.

[29] Peffers, M. J., Thorpe, C. T., Collins, J. A., Eong, R., *et al.*, Proteomic analysis reveals age-related changes in tendon matrix composition, with age-and 1 injury-specific matrix fragmentation. *Journal of Biological Chemistry* 2014, jbc. M114. 566554.

[30] Hynes, R. O., Naba, A., Overview of the matrisome—an inventory of extracellular matrix constituents and functions. *Cold Spring Harbor perspectives in biology* 2012, *4*, a004903.

[31] Naba, A., Hoersch, S., Hynes, R. O., Towards definition of an ECM parts list: An advance on GO categories. *Matrix Biology* 2012, *31*, 371-372.

[32] Reichenberg, E., Redlich, M., Cancemi, P., Zaks, B., *et al.*, Proteomic analysis of protein components in periodontal ligament fibroblasts. *Journal of periodontology* 2005, *76*, 1645-1653.

[33] Franceschini, A., Szklarczyk, D., Frankild, S., Kuhn, M., *et al.*, STRING v9. 1: protein-protein interaction networks, with increased coverage and integration. *Nucleic acids research* 2013, *41*, D808-D815.

This article is protected by copyright. All rights reserved.

Accepted Article

[34] Peffers, M. J., Beynon, R. J., Clegg, P. D., Absolute quantification of selected proteins in the human osteoarthritic secretome. *International journal of molecular sciences* 2013, *14*, 20658-20681.

[35] Singer, V. L., Jones, L. J., Yue, S. T., Haugland, R. P., Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. *Analytical biochemistry* 1997, *249*, 228-238.

[36] Bergman, I., Loxley, R., Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Analytical Chemistry* 1963, *35*, 1961-1965.

[37] Farndale, R. W., Buttle, D. J., Barrett, A. J., Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochimica et biophysica acta* 1986, *883*, 173-177.

[38] Bancroft, J. D., Gamble, M., *Theory and practice of histological techniques*, Elsevier Health Sciences 2008.

[39] Canty, E. G., Lu, Y., Meadows, R. S., Shaw, M. K., *et al.*, Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon. *J Cell Biol* 2004, *165*, 553-563.

[40] Young, R., Vaughan-Thomas, A., Wardale, R., Duance, V. C., Type II collagen deposition in cruciate ligament precedes osteoarthritis in the guinea pig knee. *Osteoarthritis and cartilage* 2002, *10*, 420-428.

[41] Benjamin, M., Ralphs, J. R., Fibrocartilage in tendons and ligaments—an adaptation to compressive load. *J Anat* 1998, *193*, 481-494.

[42] Vogel, K. G., Ordog, A., Pogany, G., Olah, J., Proteoglycans in the compressed region of human tibialis posterior tendon and in ligaments. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 1993, *11*, 68-77.

This article is protected by copyright. All rights reserved.

Accepted Article

[43] Smith, K. D., Vaughan-Thomas, A., Spiller, D. G., Clegg, P. D., *et al.*, Variations in cell morphology in the canine cruciate ligament complex. *Veterinary journal* 2012, *193*, 561-566.

[44] Little, D., Thompson, J. W., Dubois, L. G., Ruch, D. S., *et al.*, Proteomic Differences between Male and Female Anterior Cruciate Ligament and Patellar Tendon. *PloS one* 2014, *9*, e96526.

[45] Canty, E. G., Starborg, T., Lu, Y., Humphries, S. M., *et al.*, Actin filaments are required for fibripositor-mediated collagen fibril alignment in tendon. *Journal of Biological Chemistry* 2006, *281*, 38592-38598.

[46] Zhang, G., Young, B., Ezura, Y., Favata, M., *et al.*, Development of tendon structure and function: regulation of collagen fibrillogenesis. *J Musculoskelet Neuronal Interact* 2005, *5*, 5-21.

[47] Raspanti, M., Viola, M., Sonaggere, M., Tira, M. E., Tenni, R., Collagen fibril structure is affected by collagen concentration and decorin. *Biomacromolecules* 2007, *8*, 2087-2091.

[48] Herchenhan, A., Bayer, M. L., Svensson, R. B., Magnusson, S. P., Kjær, M., In vitro tendon tissue development from human fibroblasts demonstrates collagen fibril diameter growth associated with a rise in mechanical strength. *Developmental Dynamics* 2013, *242*, 2-8.

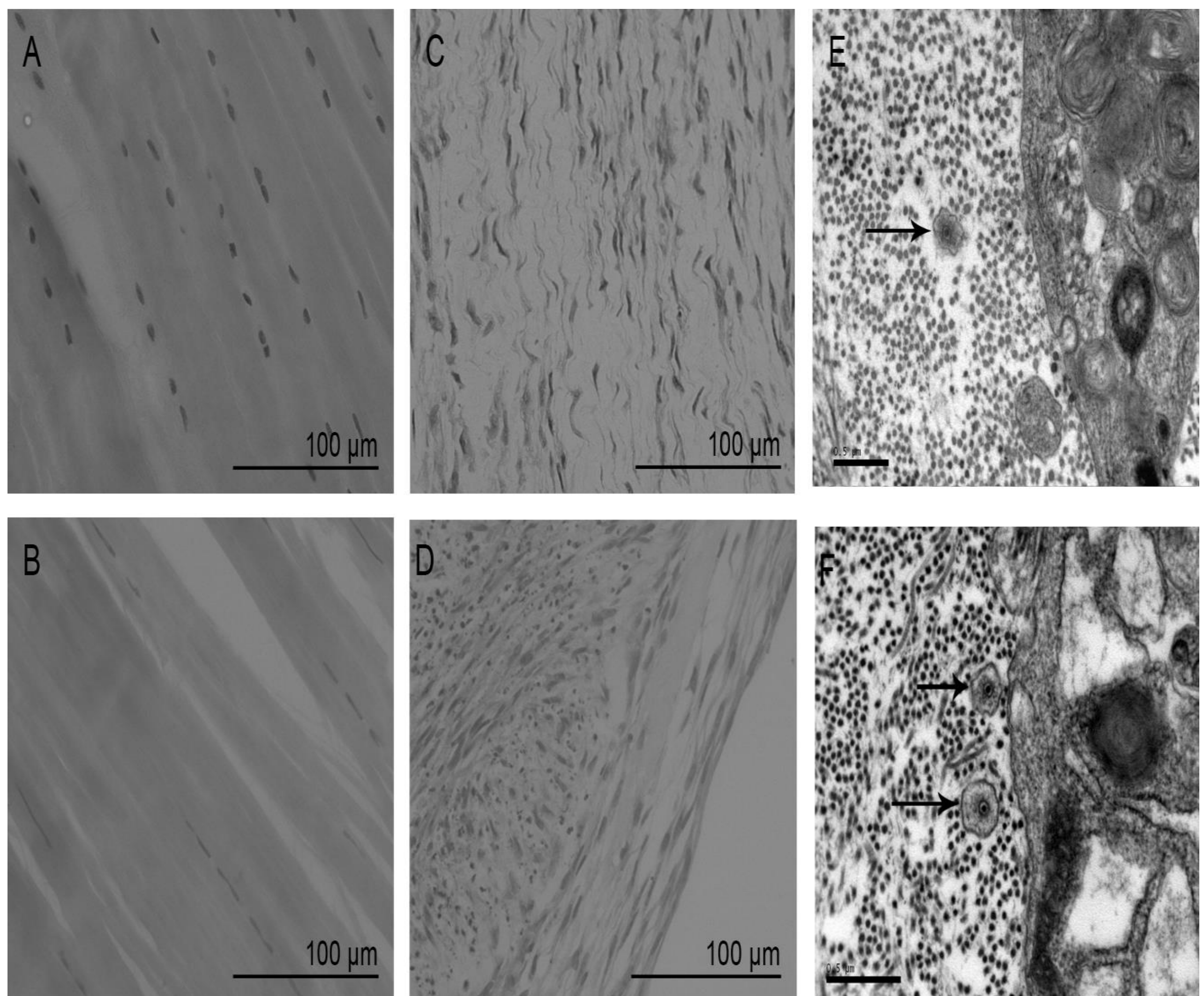
[49] Paxton, J. Z., Wudebwe, U. N., Wang, A., Woods, D., Grover, L. M., Monitoring sinew contraction during formation of tissue-engineered fibrin-based ligament constructs. *Tissue Engineering Part A* 2012, *18*, 1596-1607.

This article is protected by copyright. All rights reserved.

Accepted Article

Figure Legends

Figure 1. Ultrastructural images of native T/L and 3D TE constructs. H&E staining of native ligament (A), native tendon (B), 3D ligament constructs (C) and 3D tendon construct (D) (Bar 100μm). TEM of 3D TE tendon (E) and ligament (F) constructs indicate the presence of aligned extracellular collagen fibrils and fibripositors (black arrows) demonstrating that constructs have formed correctly.



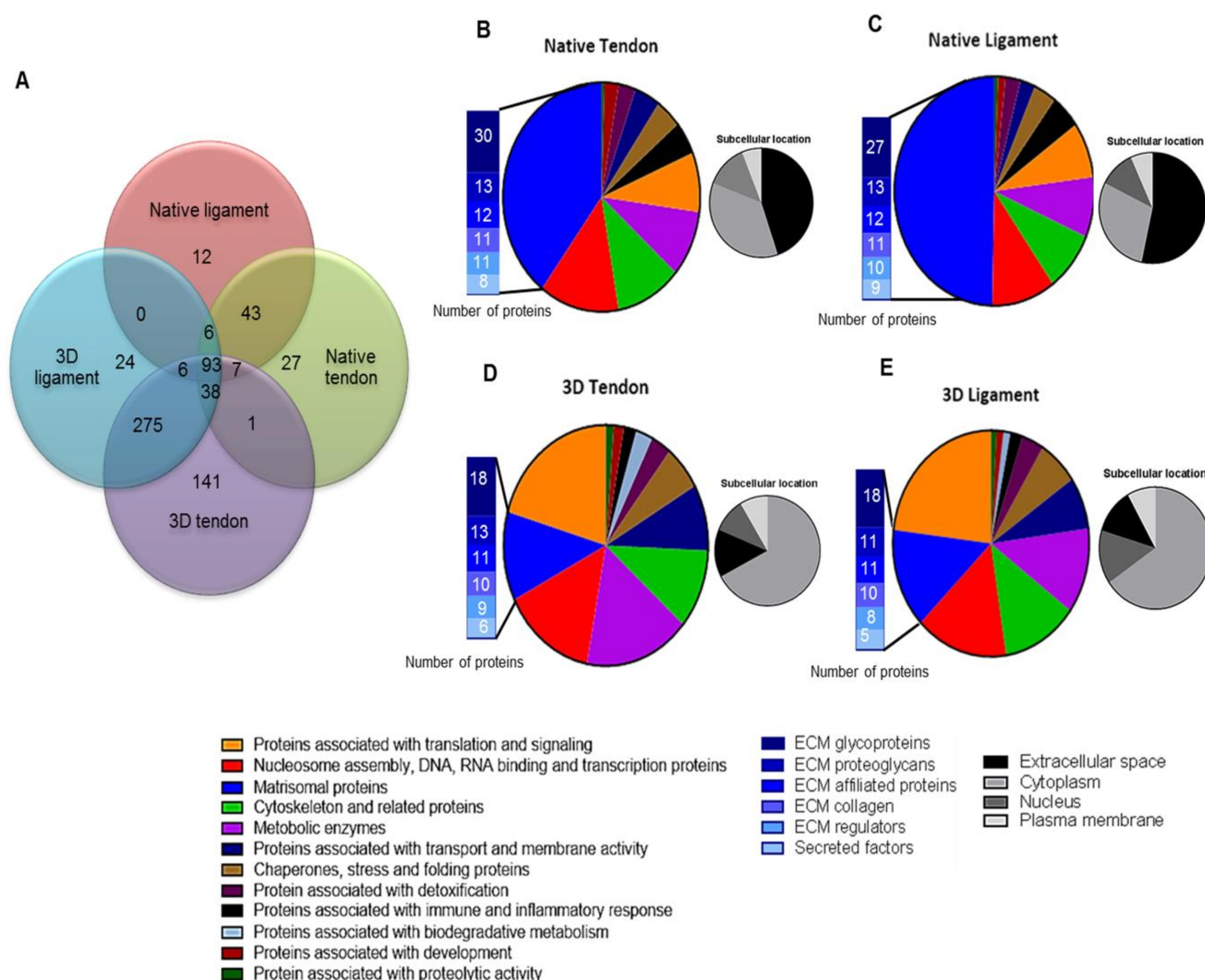
Received: 11/17/2015; Revised: 03/02/2016; Accepted: 04/08,2016

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/pmic.201500459.

This article is protected by copyright. All rights reserved.

Accepted Article

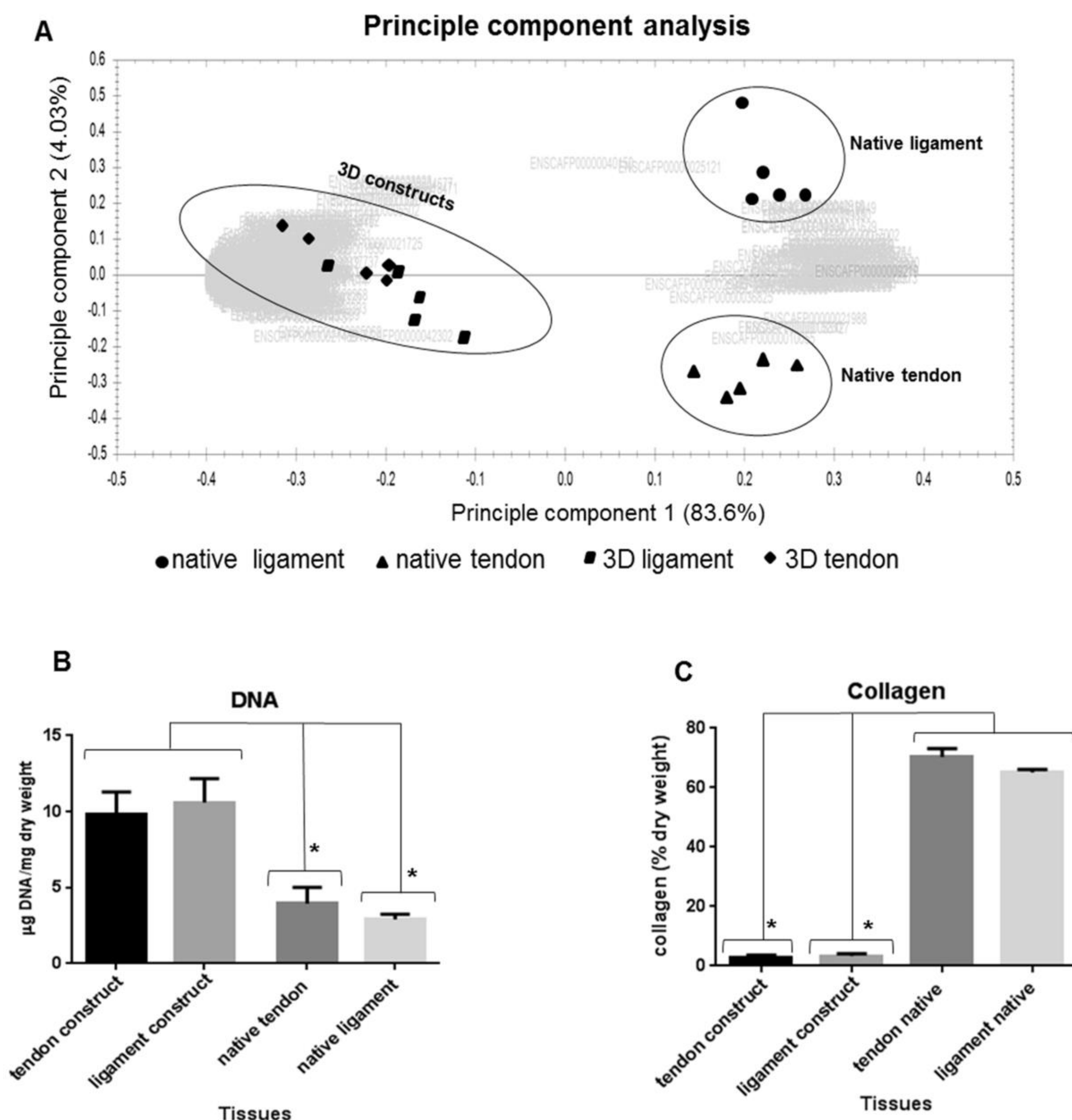
Figure 2. Protein composition of native T/Ls and 3D TE construct identified with PEAKS®. The total number of proteins identified following mass spectrometry in each tissue type as well as common proteins between the tissue types is presented (A). The proteomes of native tendon (B), ligament (C), 3D tendon (D) and 3D ligament (E) constructs were subdivided based on Uniprot function and matrisomal classification (Matrisome Project). The associated subcellular locations of the proteins are also shown (B-E).



This article is protected by copyright. All rights reserved.

Accepted Article

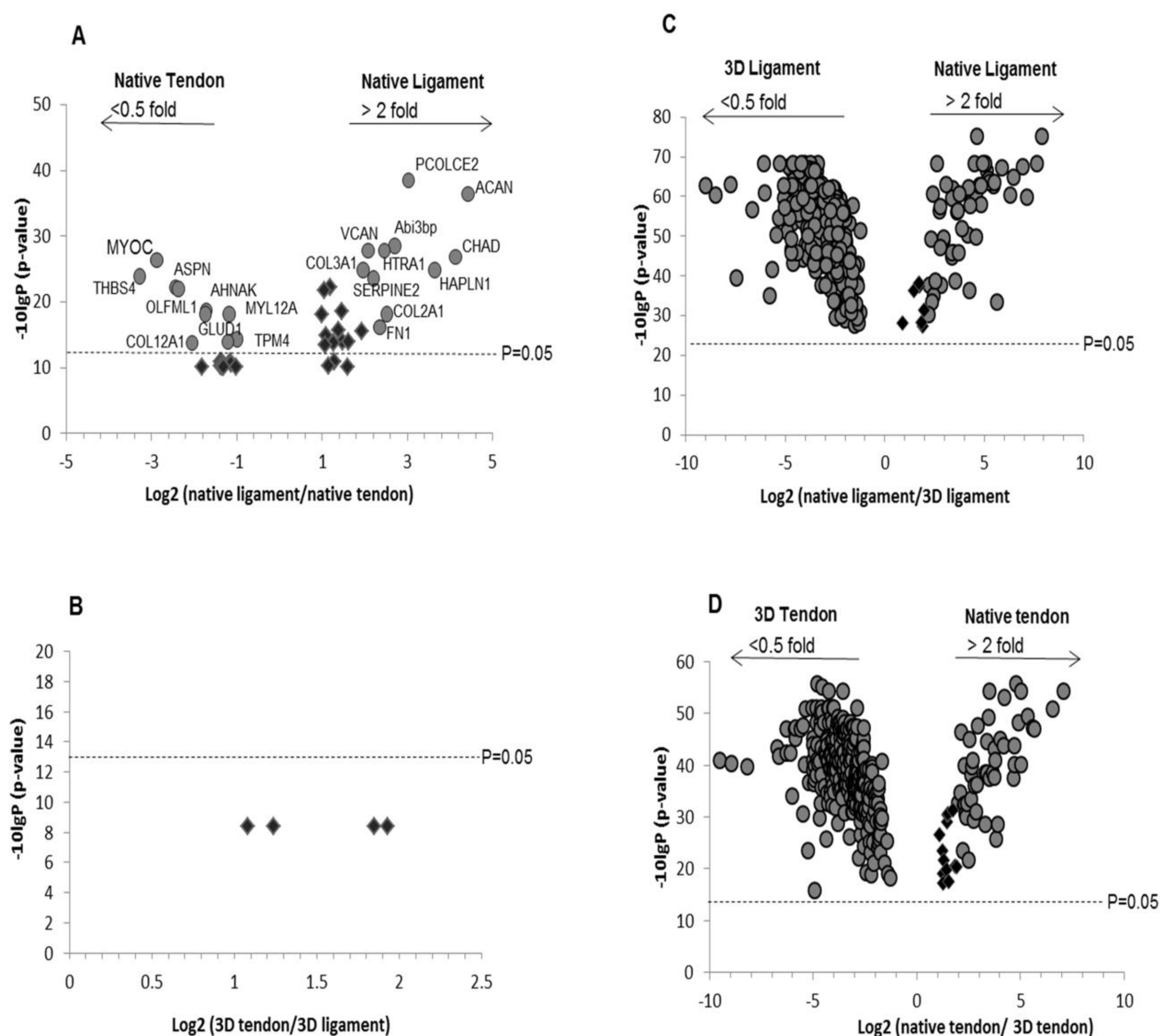
Figure 3. PCA and biochemical composition comparison between native tissues and 3D TE constructs. PCA (A) between native tendon, ligament and 3D TE constructs and a comparison of DNA (B) and collagen content (C) (% of dry weight) between native tissues and 3D constructs is shown. Values are mean and error bars represent standard error of mean (SEM). * indicates $p<0.05$.



This article is protected by copyright. All rights reserved.

Accepted Article

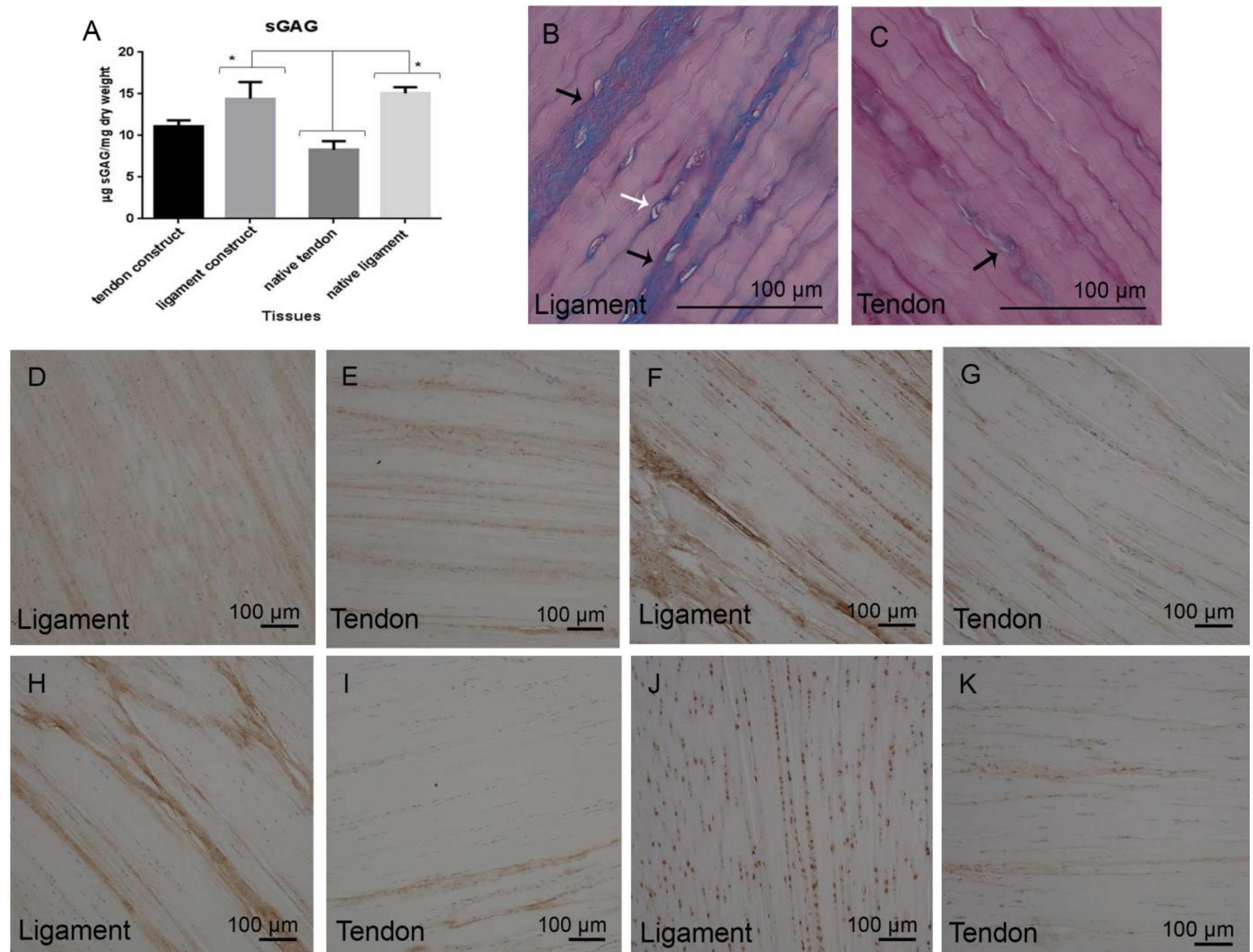
Figure 4. Label-free proteomic analysis of group comparisons between native tissues and 3D TE constructs using Progenesis^{QI} software. Volcano plots demonstrate proteins that were more abundant and significant ($p<0.05$) between native ligament versus native tendon (A), native ligament vs 3D ligament (C) and native tendon vs 3D tendon (D). No significant proteins were identified between 3D tendon and 3D ligament construct (B) as all protein p-values were greater than 0.05. Abundant proteins in panel C and D are highlighted in supplementary table 2 and 3.



This article is protected by copyright. All rights reserved.

Accepted Article

Figure 5. sGAG content in native tendon, native ligament and 3D TE constructs and validation of proteomic results. sGAG content measurement (A) (µg/mg dry weight). and Alcian blue-PAS histology staining of native ligament (B) and native tendon (C) (Bar 100µm) is demonstrated. Immunohistochemistry staining of native ligament and tendon for collagen type III (D, E), aggrecan (F, G) versican (H, I) and asporin (J, K) (Bar 100µm). Statistical values represent the mean, error bars represent SEM and * indicates $p<0.05$.



This article is protected by copyright. All rights reserved.