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INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN (*IGFBP6*) IS A CROSS-SPECIES TENDON MARKER

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Abstract

The main challenge in tendon injury management is suboptimal tissue healing that fails to re-establish original tendon function. Tissue bioengineering is a promising approach for tendon therapy, with potential to improve its functional outcomes. However, evaluation criteria for tissue-engineered tendon are unclear due to the lack of specific markers of differentiated tendon. The study aim was to identify a panel of genes that characterised tendons in comparison to cartilage or muscles and validate those genes, both in human and key species used as models for tendon diseases. Gene expression profiling of rat tendon and cartilage in whole-tissue samples and primary tenocytes and chondrocytes was undertaken using two independent microarray platforms. Genes that demonstrated high expression correlation across two assays were validated by qRT-PCR in rat tendon relative to cartilage and muscle. Five genes demonstrating the highest tendon-related expression in the validation experiment (*ASPN*, *ECM1*, *IGFBP6*, *TNMD*, *THBS4*) were further evaluated by qRT-PCR in ovine, equine and human tissue. The group of tendon markers, identified by unbiased transcriptomic analysis of rat musculoskeletal tissues, demonstrated species-dependent profiles of expression. Insulin-like growth factor binding protein 6 (*IGFBP6*) was identified as the only universal tendon marker. Further investigation in equine tendon showed that *IGFBP6* expression was not affected by ageing or tendon function but decreased in anatomical regions subjected to elevated compressive force. *IGFBP6* is a robust cross-species marker of tendon phenotype and may find application in evaluation of tendon physiology and guided differentiation of permissive cells towards functional tenocytes.

Keywords: Bioengineering, tissue engineering, tendonitis, biomarker, translational biology.

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Introduction

The tendon is a highly specialised connective tissue that transmits the mechanical force of muscle contraction to the bone. Tendon histological structure, dominated by dense extracellular matrix (ECM) with sparsely distributed resident cells, reflects adaptation to high physical demands of tensile strength resistance (Thorpe and Screen, 2016). Despite their small contribution to the overall tissue mass, tendon cells (tenocytes) are primarily responsible for its mechano-physical properties, through synthesising the bulk of ECM mass during development and modifying its composition according to the prevailing biomechanical stimuli (Kjær *et al.*, 2009; Testa *et al.*, 2017; Zhang *et al.*, 2010). Aberrant cell response to injury is thought to be one of the causes underlying the development of chronic tendinopathies and is characterised by changes in ECM organisation which are inconsistent with optimal tendon function (Titan and Andarawis-Puri, 2016). Alterations in resident cell phenotype, primarily chondrogenic differentiation, are widely identified in tendinopathies in association with degenerate tendon ECM (Asai *et al.*, 2014; Burssens *et al.*, 2013; Clegg *et al.*, 2007; Titan and Andarawis-Puri, 2016). Therefore, promoting functional tendon tissue repair at the injury site and directing appropriate cell response subsequent to injury are key goals for tendinopathy treatment.

Tendon bioengineering involves the application of tenocytes as well as mesenchymal stem cells (MSCs) to develop engineered tendon that may be subsequently used as grafts for tendon injury repair (Müller *et al.*, 2013). This promising therapeutic approach is challenged by the tendency of tendon cells to undergo phenotypic transitions when maintained under cell-culture conditions (Mueller *et al.*, 2016; Taylor *et al.*, 2009; Yao *et al.*, 2006). Tenocyte- and stem-cell-

derived constructs developed *in vitro* are at risk of cell dedifferentiation and aberrant differentiation towards chondrogenic and osteogenic lineages, possibly due to the common mesenchymal origin of musculoskeletal tissues (Harris *et al.*, 2004; Tan *et al.*, 2012). In order to evaluate outcomes of tendon tissue-engineering and further optimise culture conditions promoting teno-lineage differentiation, molecular markers of a functional adult tendon need to be defined. Genes currently investigated in tenocyte phenotype characterisation, such as scleraxis (*SCX*), tenomodulin (*TNMD*), tenascin C (*TNC*) and type 1 collagen α1 chain (*COL1A1*), are often primarily derived from developmental studies and their validity as markers of mature tenocytes can vary depending on tendon type, anatomical region, species and age (Howell *et al.*, 2017; Jelinsky *et al.*, 2010; Kuemmerle *et al.*, 2016; Mehr *et al.*, 2000; Murchison *et al.*, 2007; Peffers *et al.*, 2015; Taylor *et al.*, 2009). Moreover, their expression can also be maintained in tendons undergoing pathological transformation such as fibrosis, cartilage formation or endochondral ossification (Agarwal *et al.*, 2017; Howell *et al.*, 2017; Mehr *et al.*, 2000) further questioning their utility as markers of normal tendon phenotype. Several studies utilising unbiased approaches to identify novel tendon-selective markers by comparative expression profiling of various musculoskeletal tissues have been reported (Jelinsky *et al.*, 2010; Kuemmerle *et al.*, 2016). However, resilience of these new targets to the above described variables has yet to be defined, limiting their application to the species, tendon type and region they were derived from.

Rat, sheep and horse are frequently used animal models for tendon research (Lui *et al.*, 2011). Rat Achilles tendon shows anatomical complexity similar to that of humans (Szaro *et al.*, 2012) and are suitable for manipulation methods which are impractical in larger species (Andarawis-Puri and Flatow, 2011; Brunton *et al.*, 2018). The size of sheep and equine flexor tendons makes them more suitable for investigating the efficacy of clinical interventions in experimentally induced tendon lesions (Dahlgren *et al.*, 2002; Hausmann *et al.*, 2009; Song *et al.*, 2010). Additionally, horse and sheep tendons exhibit a hierarchical microstructure analogous to humans, with two distinct anatomical compartments: collagen-rich fascicles and looser interfascicular matrix (IFM). The fascicle-IFM interaction is considered to play a major role in tendon mechanical behaviour (Thorpe *et al.*, 2015); therefore, conservation of that microstructure is a highly desirable feature in a relevant animal model. The horse is of particular interest for tendon research due to the natural occurrence of overuse tendinopathy and age-related degeneration (Patterson-Kane *et al.*, 2012; Peffers *et al.*, 2014), which are also the main disorders affecting tendon health in humans (Scott *et al.*, 2015).

The present study aimed at identifying novel gene markers that exhibited clearly higher expression in adult tendon relative to cartilage and muscle and validated them across the key model species, rat, sheep and horse, as well as human tendons.

Materials and Methods

Tissue collection

Tissue collection from different species was individually approved by the University of Liverpool Institute of Veterinary Sciences Research Ethics Committee. Details of sample origin and donor demographics are presented in Table 1. Tissues from rat were collected under the Schedule I of the UK Animal Scientific Procedures Act (1986). Equine samples were collected as a by-product of the agricultural industry. Sheep tissues were collected as part of routine *post-mortem* examinations for *Fasciola hepatica* at the School of Veterinary Science, University of Liverpool. Human cadaveric Achilles tendon samples were obtained following ethical approval by North East Tyne and Wear South Research Ethics Committee (14/NE/0154). Human cadaveric femorotibial joint cartilage was obtained from a commercial biorepository (ProteoGenex, Inglewood, CA, USA) and provided with proof of donor consent approval by the local ethical authority at the sample collection site. Human muscle samples were represented by primary myoblasts isolated from muscle biopsy material collected intraoperatively from patients undergoing surgical treatment for *hallux valgus*, with patient consent and approval from the University of Liverpool, University Hospital Aintree Hospital and South West Wales Research Ethics Committee (13/WA/0374). Rat, sheep and equine whole tissue specimens were dissected and stored immediately upon collection in RNAlater™ Stabilisation Solution (Invitrogen) according to the manufacturer's protocol (24 h at 4 °C followed by long-term storage at − 80 °C). According to the information provided by the biobank, human cadaveric tendon and cartilage samples were preserved by flash-freezing within 30 to 60 min of excision in liquid nitrogen and stored at − 80 °C prior to RNA isolation.

Histology

To evaluate phenotype of different regions of equine SDFT, samples of SDFT midportion and sesamoid region were fixed in 10 % buffered formalin for 48-72 h, dehydrated and embedded in paraffin-wax on their longitudinal axis. Then, the tissue blocks were cut into 5-µm-thick sections, collected on polylysine slides and stained with haematoxylin and eosin (H&E).

Cell isolation

Human

Human primary myoblast isolation was undertaken according to a previously published method (Soriano-Arroquia *et al.*, 2017). Briefly, samples were washed in Dulbecco's phosphate-buffered saline

Table 1. Detailed description of samples used for respective experiments. Information on donor sex in equine samples was not available.

Microarray			
Rat, F344	12 weeks; male	*n* = 5	Tendon (AT, DDFT, tail tendon)
			Cartilage (coxo-femoral and femorotibial joint)
Rat, Lewis	12 weeks; male	*n* = 4	Primary tenocytes (AT, DDFT)
			Primary chondrocytes (coxo-femoral and femorotibial joint)
RT-qPCR cross-species validation			
Rat, Lewis	12 weeks; male	*n* = 6	Tendon (AT, DDFT)
			Cartilage (coxo-femoral and femorotibial joint)
			Muscle (*quadriceps femoris*)
Sheep	Adult female	*n* = 6	Tendon (DDFT)
			Cartilage (metatarsophalangeal joint)
			Muscle (*quadriceps femoris*)
Horse	9-14 years (11.9 ± 2.2 years)	*n* = 8	Tendon (SDFT midportion)
	4-15 years (8.4 ± 6 years)	*n* = 5	Cartilage (metatarsophalangeal joint)
		n = 4	Muscle (*trapezius*)
Human	56-93 years (82.4 ± 15.4 years); 3 female, 2 male	*n* = 5	Tendon (AT midportion)
	32-75 years (56 ± 20.8 years); male	*n* = 6	Cartilage (femorotibial joint)
	31-38 years (32.2 ± 2.7 years); female	*n* = 6	Primary myoblasts (*extensor digitorum brevis*, *tibialis anterior*, *abductor halluces*)
RT-qPCR *IGFBP6* in tendon ageing and function			
Horse	0 d	*n* = 4	SDFT midportion
	0-1 months	*n* = 3	
	3-6 months	*n* = 4	
	12-24 months	*n* = 4	
	3-5 years	*n* = 5	
	9-11 years	*n* = 5	
	18-22 years	*n* = 4	
	3-5 years	*n* = 5	SDFT midportion
			CDET
	5-7 years	*n* = 5	SDFT midportion
			SDFT sesamoid region

and dissected in a sterile Petri dish in warm (37 °C) collagenase-dispase-$CaCl_2$ solution [1.5 U/mL of collagenase D, 2.4 U/mL of Dispase II and 2.5 mM $CaCl_2$ in serum-free Dulbecco's modified eagle medium (DMEM)] followed by a 40 min incubation in 50 mL Falcon tube. Digestion was stopped by adding sterile growth medium (4 mL of DMEM containing 20 % foetal bovine serum, 1 % L-glutamine and 1 % penicillin-streptomycin) and the sample was filtered through a 70 μm cell strainer. Cells were pelleted by centrifugation at 443 ×*g* for 5 min at room temperature and stored in TriReagent (Sigma-Aldrich) at − 80 °C for RNA isolation.

Rat

In addition to whole tissue samples, expression profiling of rat primary tenocytes and chondrocytes was undertaken. Primary tenocytes were isolated from Achilles tendon (AT) and deep digital flexor tendon (DDFT), chondrocytes from femorotibial and coxo-femoral joint articular cartilage; both were pooled for each individual. Tissue samples were minced and washed twice in serum-free DMEM, containing penicillin (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (2 μg/mL). Cells were isolated by collagenase digestion (0.4 %, type 2, Worthington) for over 20 h at 37 °C. Next, samples were centrifuged: cartilage for 8 min at 500 ×*g* and tendon for 15 min at 1,000 ×*g*, to ensure that partially digested fascicular material would be spinned-down (Ritty *et al.*, 2003). Supernatant was removed and samples incubated for 4 h at 37 °C in 0.25 % bovine pancreas trypsin (Sigma-Aldrich) in serum-free DMEM. Samples were centrifuged as

before, washed with complete medium, re-suspended and filtered through a 70 μm sterile cell strainer. Cells were pelleted by centrifugation and cell pellets stored in TriReagent.

RNA extraction

Whole tissue samples were pulverised under liquid nitrogen. Ground-up tissue and cell pellets were incubated for 10 min at room temperature in TriReagent. RNA was extracted using an acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 2006). Following RNA resuspension in 75 % ethanol, DNAse digestion and purification were performed using RNeasy Mini Kit (QIAGEN). RNA was quantified using an ND1000 spectrophotometer (Thermo Fischer Scientific). For the microarray experiment, RNA integrity was measured using a Bioanalyser (Agilent), with all samples obtaining RNA integrity number (RIN) scores > 8. In samples used for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), RNA purity was assessed based on the A_{260}/A_{280} absorbance ratio and samples with ratio < 1.8 were not accepted for cDNA preparation. RNA quantity used for the reverse transcription was normalised across different tissue types within each species for cross-tissue comparison of gene expression.

Rat microarray

Samples

Gene expression profiling of rat tendon was undertaken using two independent microarray platforms: i) Illumina RatRef v12 platform (ArrayExpress accession code: E-MTAB-4800) as previously described (Mueller *et al.*, 2016) and ii) Affymetrix GeneST Rat platform. Sample preparation for the two platforms differed as follows: i) RNA was extracted from whole tendon and cartilage from 12-week-old male F344 rats (n = 5) for profiling by Illumina microarray; ii) RNA extraction for Affymetrix profiling was derived from primary tenocytes and chondrocytes isolated as described above from tissues obtained from 12-week-old male Lewis rats (n = 4). Sample preparation for each platform followed the standard protocol recommended by each manufacturer. Illumina microarray profiling was performed by The Genome Centre, Queen Mary, University of London, UK. Affymetrix microarray profiling was performed by Tepnel Pharma Services, Hologic Ltd., Manchester, UK.

Data analysis

Raw gene expression data from both platforms was pre-processed and interrogated using software packages implemented in *R* (Web ref. 1). Expression data were $\log_2$-transformed and normalised (loess) across all samples. Differential expression analysis was performed using methods previously described (Smyth, 2005). Pearson's correlation coefficient was calculated for genes differentially expressed in tendon in both platforms.

Quantitative real-time PCR

Samples

Candidate marker genes identified in the rat microarray were further validated in different musculoskeletal tissues of human and key model species by qRT-PCR. A summary of sample information is presented in Table 1. Briefly, tissue samples included: i) whole tendon, cartilage and muscle from rat, sheep and horse; ii) human whole tendon, cartilage and isolated primary myoblasts.

Insulin-like growth factor binding protein 6 (*IGFBP6*) expression was further analysed in equine superficial digital flexor tendon (SDFT) in relation to age and anatomical location. RNA extracted from the SDFT midportion was analysed across different age groups, from full formed foetuses to old horses (Table 1). To investigate the impact of anatomical differences on *IGFBP6* expression in tendons, the SDFT midportion was compared to the SDFT sesamoid region (compressed by proximal sesamoid bones) in young mature horses. Additionally, expression in energy-storing SDFT and positional common digital extensor tendon (CDET) was compared to determine the relation between tendon function and *IGFBP6* expression.

Primers

Primers were designed using NCBI PrimerBlast (Ye *et al.*, 2012) against the most recent published record (04/2016). All primers were species-specific and amplification efficiency was calculated using tendon cDNA in six 10-fold serial dilutions. Primer efficiencies were used to correct normalised cycle threshold (C_t) values (Pfaffl, 2006). All primers were sourced from Eurogentec S.A. (Liege, Belgium) except for equine *TNMD*, which was custom designed by PrimerDesign (UK), and human *IGFBP6* and *TNMD*, which were provided by Bio-Rad. The sequences of custom-designed primers are provided in Table 2.

cDNA production and qRT-PCR

Reverse transcription was performed using M-MLV reverse transcriptase and random-hexamer oligonucleotides (all Promega). Quantitative PCR (qPCR) was performed on a LightCycler 480 II (Roche) using 96-well plates (STARLAB). Negative controls (cDNA) were included for each primer test. A proprietary qPCR SYBR™ master mix (Takyon™, no ROX passive reference dye, deoxythymidine triphosphate (dTTP); Eurogentec S.A.) was used for all analyses apart from human *IGFBP6* and *TNMD* where Sso Advanced™ master mix by Bio-Rad was used. The manufacturer's recommended protocol for PCR was used: briefly, enzyme activation (3 min, 95 °C) followed by 40 cycles consisting of denaturation (3 s, 95 °C) and annealing-extension (20 s, 60 °C). Products of qPCR amplification were

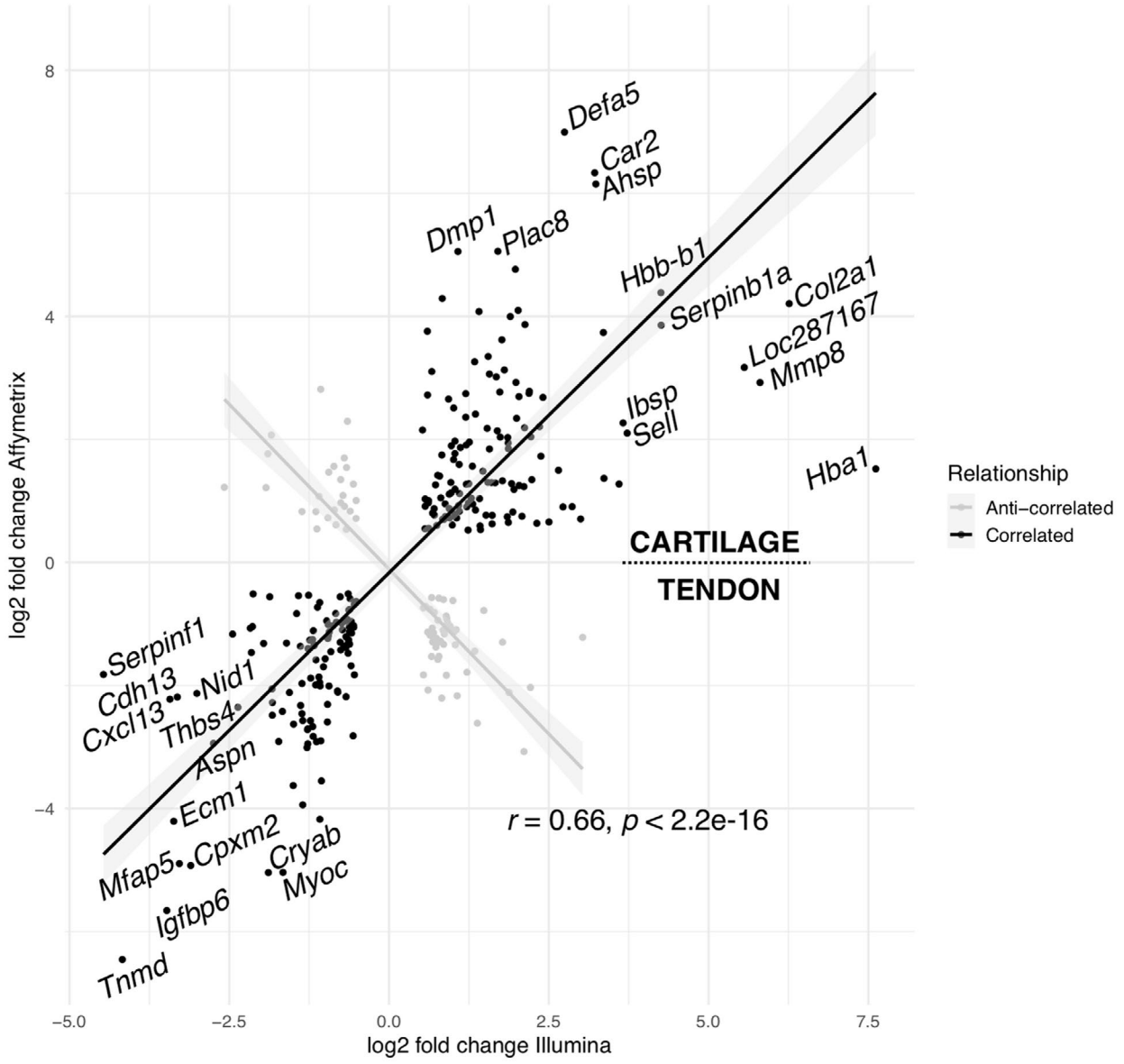

Fig. 1. Correlation plot of log_2 fold changes of independent gene expression profiling studies of rat tendons. Genes found to be differentially expressed between native cartilage (top right quadrant) and tendons (bottom left quadrant) in both Illumina (x-axis) and Affymetrix (y-axis) datasets plotted by log_2 fold-change. For clarity only, some data points are annotated. Data points are defined as correlated or anti-correlated in the relationship key. Where genes have the same directional change, the data point is defined as a dark dot, whereas genes with conflicting (anti-correlated) expression changes are defined as grey points (see relationship legend). For tendons, *Tnmd*, *Igfbp6*, *Serpin1*, *Mfap5* and *Ecm1* are all highly expressed in two independent datasets.

assessed for specificity by melting curve analysis. Gene expression levels were normalised to reference genes, using the efficiency corrected comparative C_t method (Pfaffl, 2006), with all qPCR reactions performed in triplicate. Reference genes ribosomal protein S20 (*Rps20*) and ribosomal protein L13a (*Rpl13a*) were shown to have the most stable expression across sample types in rat using the geNorm algorithm (Vandesompele *et al.*, 2002) and geometric mean of their expression used for relative expression quantification in that species. Therefore, *RPS20* was used as reference gene in sheep and horse. In human samples, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was identified as the most stable gene in all tissue types and used for relative expression quantification. Normalised C_t data were converted to the linear form ($E^{-\Delta Ct}$) for statistical analysis.

Data analysis

Following Shapiro-Wilks test for normality and Levene's tests for equality of variances, differences between conditions were analysed by one-way ANOVA with Tukey *post-hoc* test (equal variance between groups), Welch ANOVA with Games-Howell *post-hoc* test (unequal variance between groups) or Kruskal-Wallis test with Dunns pairwise comparison (non-parametric data).

Results

Microarray analysis of rat tendon expression markers

Between rat cartilage and tendon gene expression profiles, 311 genes were commonly differentially expressed across two studies. Of these, 71 did not match in the direction of the fold change (decrease or increase in expression) between microarray platforms (Fig. 1). A moderately high correlation was observed between the fold changes of the 311 common genes (cor = 0.66, $p < 2.2e\text{-}16$). Genes that demonstrated the highest expression in tendon relative to cartilage in both studies included: *Tmnd*, *Serpinf1*, *Igfbp6*, *Cpxm2*, *Cryab*, *Ecm1*, *Mfap5*, *Myoc*, *Aspn*, *Thbs4* ($\log_2$ fold change > 1.5) (Table 2). Other tendon-related genes demonstrated more variable expression. A list of the top tendon-related genes in rat with their respective $\log_2$ fold-change values is presented in Table 3. When genes showing higher expression in tendon were functionally annotated using gene ontology terms, the following biological process and cell compartment terms were significant after adjustment for multiple testing ($p < 0.05$): blood vessel development, developmental process, extracellular matrix. Interestingly, annotations specifically referencing tendon were not identified.

qPCR validation of putative markers

Validation of relevant tendon markers from a group of genes defined in the correlation study was undertaken relative to both cartilage and skeletal muscle in rats. This represented a third independent cohort. Genes selected for validation were inferred from correlation plot (Fig. 1) and highlighted in Table 3. Expression of developmental tendon markers *Scx* and *Mustn1* was additionally investigated alongside target genes derived from the microarray experiment. Significantly higher ($p < 0.05$) expression in tendon as compared to cartilage and muscle was detected for *Aspn*, *Cpxm2*, *Ecm1*, *Igfbp6*, *Mfap5*, *Myoc*, *Serpinf1*, *Tnmd* and *Thbs4*. Expression of the tendon development gene *SCX* was increased relative to cartilage, but not to muscle. *Mustn1* and *Cryab* expression was similar in tendon as compared to other musculoskeletal tissues.

Table 2. Primer sequences for custom-designed oligonucleotides.

Gene	Forward	Reverse
Rat		
Aspn	CCGAAAGGACTACCACCAACT	TTGTTTCCAAGACCCAGCCT
Cpxm2	GGCCTATGAAGGAGGTTCCG	CTCCCAGAGCAGCGTGTTTA
Cryab	AAGAGCGCCAGGACGAAC	ACTCCATCCGATGACAGGGA
Ecm1	CCGTGACCAGTTCTTACCCC	CTGAAACCTTGAAGGCTCCCT
Igfbp6	CCGTCGGAAGAGACTACCAAG	CTTGAACAGGACTGGGCCTT
Mfap5	GCCAACGAGGAGATGACGTA	GTCATCCGTGGAAGGTGTGA
Mustn1	TTGCCTGTGGCTACTGCCTGC	GAGTGCCAGCCTCGGACATGG
Myoc	GAGGGAGACAAAGGATGTGGAG	CAGTGATTGTCTCAGCTGTCCT
Scx	CCAGAGACGGCGGCGAGAAC	TTGGCTGCTGTGGACCCTCCT
Serpinf1	TGCCTTACTTCAAGGGGCAG	GTCCTGTCCTCGTCCAGGTG
Thbs4	AGTACCGCTGTAACGACACC	GGTTTGGGCGTTTGAGAAGG
Tnmd	CCGCCGCACCAGACAAGCAA	GGCAGTAGCGGTTGCCTCGAC
Rps20	CCGCTGTTCGCTCCTGCTGA	TGCGGCTGGTGAGCGTGATT
Sheep		
ASPN	GATCTGCAAAGGCTGGGTCT	TCACACGTGGTATGTTAGCAAGA
ECM1	GCTGTGCTGAGGAGGAGAAAT	GCAGAGTCTCGCCAGAAGTT
IGFBP6	AGGAACTCGGGGACCTCTAC	CAGCACGGAGTCCAGATGTT
THBS4	GACACCAGAGACGGCTTTCA	GGGGTGGTACTTGCACTCAT
TNMD	ATCCCACTCTAATAGCAGTTTCAGA	CACCCACTGCTCGTTTTGTT
RPS20	GAAGGTGTGTGCTGACCTGA	CTCAGAGTCTTGGTAGGCATCC
Horse		
ASPN	ACGCTTTACACGTTCTGGAGAT	CTCCTTCAAATGCCCCTGGT
ECM1	CCAGGGTGAGACCCTCAATTT	GCATCCTCCCACACGAGTT
IGFBP6	GAACCGCAGAGACCAACAGA	ACGGGCCCATCTCCGT
THBS4	AATCCTGACAGACCCCACCC	GGTAGCGGAGGATGGCTTTGTT
RPS20	TTTGGAGAAGGTGTGTGCTGA	GTCTTGGTGGGCATCCGAA
Human		
THBS4	GTTGCAGAACCTGGCATTCAG	CCCTGGACCTGTCTTAGACTTCA
GAPDH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGATGACC

Table 3. Top 40 differentially expressed genes in rat tendon as compared to cartilage resulting from two independent microarray studies. Only the genes with matching direction of log_2 fold change between platforms were included. Genes highlighted in bold were selected for validation with RT-qPCR in rat tendon, cartilage and muscle.

Gene symbol	Gene name	Log_2 fold change	
		Ilumina	Affymetrix
Serpinf1	**Serpin family F member 1**	4.4682858	1.8204873
Tnmd	**Tenomodulin**	4.1729279	6.4517042
Igfbp6	Insulin-like growth factor binding protein 6	3.4772336	5.6566756
Cxcl13	Chemokine (C-X-C motif) ligand 13	3.4282285	2.222346
Ecm1	**Extracellular matrix protein 1**	3.3686932	4.2056954
Cdh13	Cadherin 13	3.3125358	2.1872266
Mfap5	**Microfibril associated protein 5**	3.2807773	4.8933617
Cpxm2	**Carboxypeptidase X, M14 family member 2**	3.103086	4.9246271
Nid1	Nidogen 1	3.0072092	2.128184
Aspn	**Asporin**	2.7472203	2.9400469
Lamc1	Laminin subunit gamma 1	2.4477262	1.164511
Thbs4	**Thrombospondin 4**	2.36365	2.3545135
Clic5	Chloride intracellular channel protein 5	2.1707479	1.0681173
Lama4	Laminin subunit alpha 4	2.1538213	1.463447
Has1	Hyaluronan synthase 1	2.1369792	1.0421126
Nexn	Nexilin	2.1266246	0.5113326
Cd151	Cluster of differentiation 151	1.9624259	1.316076
Cryab	**Crystallin alpha b**	1.8879595	5.0390885
Meox2	Mesenchyme homeobox 2	1.8666559	0.5582083
Ccdc3	Coiled-coil domain containing 3	1.8304224	2.0541286
Prss23	Serine protease 23	1.8265794	2.4828796
S100a4	S100 calcium binding protein A4	1.8254947	2.2765567
Itgbl1	Integrin subunit beta like 1	1.7264565	2.909726
Crip2	Cysteine rich protein 2	1.6683555	2.4204807
Myoc	**Myocilin**	1.6593264	5.0361836
Agtr1a	Angiotensin II receptor type 1a	1.6040215	1.3105763
Lmcd1	Lim and cysteine rich domains 1	1.5583528	2.1135088
Nbl1	NBL1, DAN family BMP antagonist	1.4990361	3.6254119
Fbln2	Fibulin-2	1.4933224	2.6278954
Rgs4	Regulator of G protein signaling 4	1.4458768	0.8312598
Cygb	Cytoglobin	1.4163564	0.5405094
Sncg	Synuclein gamma	1.3867141	1.3627389
Procr	Protein C receptor	1.3833375	2.3241227
Thbd	Thrombomodulin	1.3626751	2.4577927
Chrnb1	Cholinergic receptor nicotinic beta 1 subunit	1.3621048	1.9674221
Ltbp4	Latent transforming growth factor β binding protein 4	1.3502595	3.938885
Lamb2	Laminin subunit beta 2	1.3499291	2.5700232
Cfb	Complement factor b	1.2835458	3.008987
Lama2	Laminin subunit alpha 2	1.2763259	2.717257
Mustn1	**Musculoskeletal, embryonic nuclear protein 1**	1.2715101	2.9484326

Tendon marker analysis in human and model species

Genes showing the highest expression in tendon (*ASPN, ECM1, IGFBP6, TNMC* and *THBS4*), with $p < 0.001$, were selected for further evaluation in sheep and horse (Fig. 2**a**). Unlike in rat, *ECM1* expression was higher in cartilage for both sheep and horse; *ASPN* had high expression in horse tendon, but not in sheep. Therefore, only *IGFBP6*, *TNMD* and *THBS4* were additionally analysed in human musculoskeletal tissues (Fig. 2**a**). Expression of *THBS4* was highly variable but showed a trend towards higher expression in all species' tendons. Higher expression of *TNMD* was confirmed in rat, sheep and human but not equine tendon. Only *IGFBP6* demonstrated significantly higher expression in tendon relative to cartilage and muscle in all species (Fig. 2**a**). Therefore, nucleotide sequence

identity of *IGFBP6* across the species was evaluated using NCBI BLAST (Altschul *et al.*, 1997) (Fig. 2**b**).

IGFBP6 in equine tendon with relation to function and age

Expression of *IGFBP6* in a region of equine SDFT subjected to compressive force and, thus, displaying more fibrocartilage-like morphology was lower than in the SDFT tensional midportion (Fig. 3**b**). No significant difference in expression was observed between energy-storing (SDFT) and positional tendon (CDET), with SDFT expression results showing bigger individual variation than CDET (Fig. 3**c**). *IGFBP6* expression increased between 0 d and 12-24 months of age, followed by stabilisation at lower level in the older age groups (3-5, 8-11 and 18-22 years); however, none of those age-related differences achieved statistical significance (Fig. 3**d**).

Discussion

Evaluation of bioengineered tendon constructs and native tendon tissue is based predominantly on histological examination and a limited pool of molecular markers often not tested for potential species-, age- and function-related differences among tendons. In the present study, a group of genes, identified by unbiased microarray analysis in rat native tendon and primary tenocytes, was validated across several species relevant for tendon studies and confirmed in human. Among those targets were genes previously recognised as tendon-specific (*TNMD, THBS4*) (Docheva *et al.*, 2005; Hauser *et al.*, 1995; Jelinsky *et al.*, 2010) or known to be expressed in tendon but not subjected to direct comparisons with other adult musculoskeletal tissues (*IGFBP6*, *ASPN*, *ECM1*) (Dahlgren and Nixon, 2005; Henry *et al.*, 2001; Jelinsky *et al.*, 2010; Kuntz *et al.*, 2018). It is worth noting that potential markers identified were limited to genes included in the microarray platforms and other tendon-specific genes could be identified should a similar approach be employed using RNA sequencing.

Primarily, candidate tendon markers should be able to differentiate clearly between tendon and cartilage as chondrogenic transformation is a common feature of degenerated tendon (Asai *et al.*, 2014; Burssens *et al.*, 2013; Clegg *et al.*, 2007; Titan and Andarawis-Puri, 2016). *ECM1* and *ASPN*, although highly expressed in rat tendons, showed similar or higher expression level in sheep and horse cartilage, thus reducing their usability as tendon markers. That ambiguity in gene expression is in concordance with previous studies. *ECM1* is more expressed in porcine Achilles tendon enthesis (tendon-bone

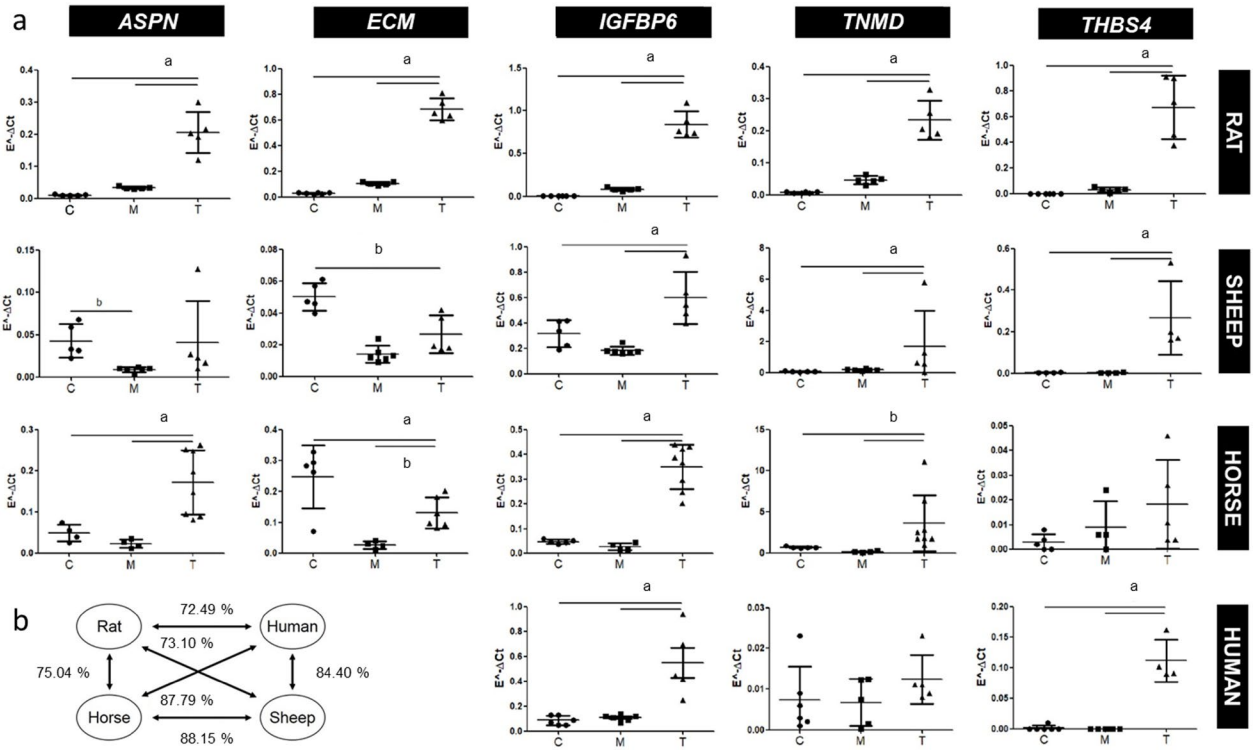

Fig. 2. RT-qPCR validation of candidate marker genes identified using microarray analysis across musculoskeletal tissues in rat, sheep, horse and human. (**a**) Data were normalised to *Rps20* and *Rpl13* (rat), *RPS20* (horse and sheep) and *GAPDH* (human) and presented as efficiency corrected $2^{-\Delta Ct}$. Bars represent the average expression and standard deviation for each group. A significant difference between average expression in cartilage (C), muscles (M) and tendons (T) was marked as: [a] $p < 0.001$, [b] $p < 0.05$ as determined using one-way (equal variances) or Welch (unequal variances) ANOVA. (**b**) Nucleotide sequence identity of *IGFBP6* across the species investigated determined as statistically significant by NCBI BLAST (E-value < 1e-92).

insertion) than tendon body (Kuntz *et al.*, 2018) and *ASPN* in human adult cartilage, although with no reference to expression levels in other tissues (Henry *et al.*, 2001). Interestingly, *SCX* used in the rat RT-qPCR experiment as a benchmark tendon marker (not derived from the current microarray analysis) showed substantial variability between individual tendon samples, not observed in muscle or cartilage and contributing to loss of significance when comparing average expression among tissues (Fig. 2**a**). Similarly, Mohawk (*MKX*) gene, known to be critical in tendon development, despite being present in both microarray platforms, was not significantly higher in tendons than in cartilage. *SCX* is known for its key role in tendon development and differentiation (Schweitzer *et al.*, 2001); however, its expression in tendons declines with their maturation (Chen *et al.*, 2017) and can be affected by tendon mechanical environment (Murchison *et al.*, 2007; Scott *et al.*, 2011). Therefore, it is possible that *SCX* expression in adult tendons is more susceptible to differences in local biomechanical factors or individual activity levels. *SCX* regulates expression of another tendon marker investigated, *TNMD* (Shukunami *et al.*, 2006). Results confirmed tendon-specific expression of *TNMD* in all species apart from horse, where high variability among biological replicates and no clear difference in expression among tissues were found (Fig. 2**a**). This finding is supported by previous equine tendon marker studies (Kuemmerle *et al.*, 2016; Taylor *et al.*, 2009) and may challenge the frequent application of *TNMD* as a marker of tendon healing and tenogenic differentiation of stem cells in horse (Barsby and Guest, 2013; Durgam *et al.*, 2016; Smith *et al.*, 2015). Regulation of *TNMD* transcription is complex and involves *SCX*-independent mechanisms, such as Wnt/β-catenin signalling (Katsuhiko *et al.*, 2014), which is responsive to mechanical stimulation in other musculoskeletal tissues (Lara-Castillo *et al.*, 2015; Niu *et al.*, 2016), potentially enhancing individual differences in *TNMD* mRNA levels. The reason for variable expression of *THBS4* in human musculoskeletal tissues in the present study was not clear. *THBS4* may be abundantly expressed in muscle (Frolova *et al.*, 2014); however, it is expected to show good separation between tendon and cartilage (Jelinsky *et al.*, 2010). Recent work [Simpson *et al.*, (2019) Isotopic labelling reveals diverse rate of protein turnover within tendon. In: 2019 Annu Meet Orthop Res Soc Austin, TX, USA] on use of isotopic labelling for evaluation of protein turnover rate within tendons has demonstrated that *THBS4* is the most quickly metabolised ECM protein, which perhaps is also reflected by fluctuations at the gene

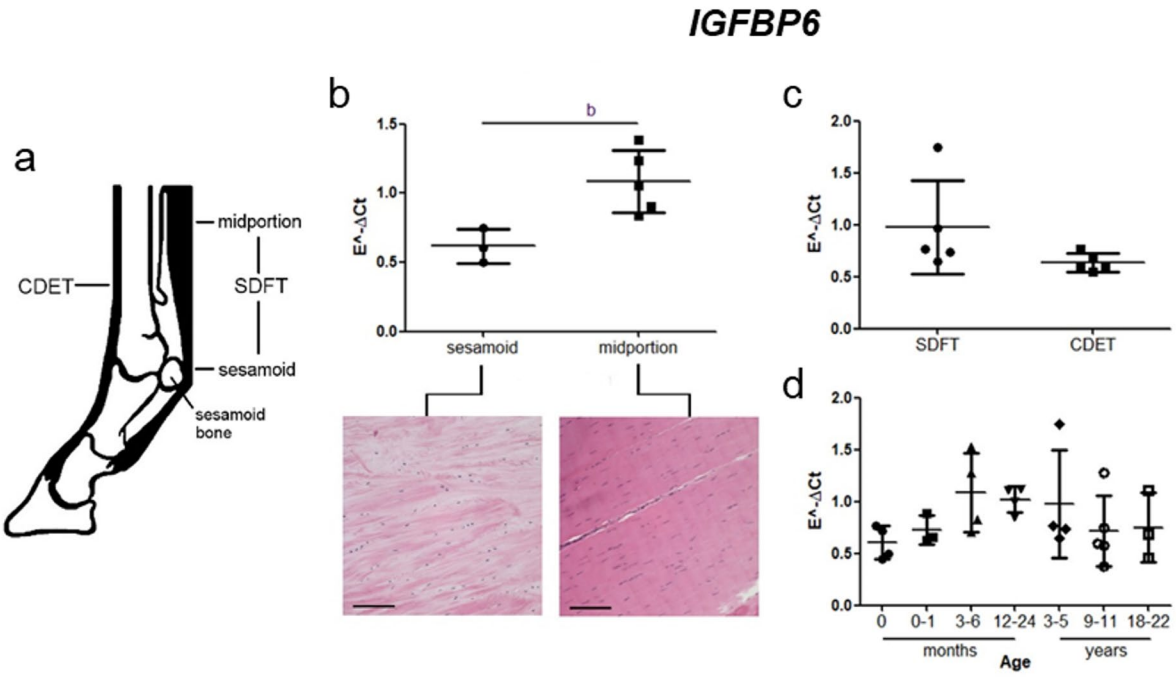

Fig. 3. The effect of age and anatomical location on *IGFBP6* expression in equine tendons determined by RT-qPCR. (**a**) Schematic view of investigated structures in equine distal limb (tendons marked in black). CDET function is to stabilise the limb joints (positional tendon) while SDFT plays a role in locomotion (energy-storing tendon). SDFT sesamoid region is subjected to compression by adjacent proximal sesamoid bones affecting tendon morphology. Expression of *IGFBP6* was compared in (**b**) two regions of SFDT – sesamoid and midportion. Images below the graph present differences in histological structure of the two regions (haematoxylin and eosin stain, scale bar = 100 μm); (**c**) functionally distinct tendons, SDFT and CDET; (**d**) SDFT during postnatal development and ageing. Data were normalised to *RPS20* and presented as efficiency corrected $2^{-\Delta Ct}$. Bars represent the average expression and standard deviation for each group. Significant difference between average expression in each group was marked as: [a] $p < 0.001$, [b] $p < 0.05$ as determined using (**a**,**b**) *t*-test or (**c**) Kruskal-Wallis test with Dunns pairwise comparisons.

expression level. Results discussed above add to the general conclusion that species can have a significant effect on tendon gene expression and that an optimal tendon marker should facilitate translation between animal experiments and human clinical studies.

Among the initial pool of candidate tendon markers, *IGFBP6* was the only gene consistently more expressed in tendons as compared to cartilages and muscles across different species. This finding, combined with high nucleotide-sequence identity (Fig. 2**b**), may suggest *IGFBP6* homology and conservation of its evolutionary function across species. Expression of *IGFBP6* in tendon has previously been shown in a human microarray study comparing a wider group of tissue types including bone, bone marrow, cartilage, fat and muscle (Jelinsky *et al.*, 2010). *IGFBP6* is the main regulator of insulin growth factor II (IGF-II) availability and it also displays several IGF-II-independent actions affecting cell proliferation, survival, differentiation and migration (Bach, 2016). IGFs stimulate proteoglycan, collagen and non-collagen synthesis in flexor-tendon-derived cells (Abrahamsson, 1997) and causes cell proliferation in both the epitenon and endotenon (Murphy and Nixon, 1997). Thus, proteins regulating their activity may have a significant role in ECM remodelling relevant for tendon physiology. Decreased expression of *IGFBP6* was described in an equine model of tendon injury and human fibroblasts from Dupuytren's disease tissue (Dahlgren *et al.*, 2006; Raykha *et al.*, 2013) and altered methylation of *IGFBP6* promoter was detected in a chemical model of murine tendinopathy (Trella *et al.*, 2017). In the present study, fibrocartilaginous transformation of the equine SDFT region compressed by sesamoid bones (similar to patellar compression of quadriceps femoris tendon in humans) was associated with lower *IGFBP6* expression (Fig. 3), indicating that not only can it discriminate between different mesenchymal tissue lineages but also react to phenotype change within fully differentiated tendon tissue. A trend for increasing levels of *IGFBP6* in SDFT postnatal development could reflect gradual restriction of growth factor accessibility corresponding with reaching musculoskeletal maturity (two years of age in the horse), when *IGFBP6* level stabilised (Fig. 3**d**). The lack of a significant effect of ageing or function (positional *versus* energy-storing tendon) on *IGFBP6* expression suggests its robustness as a biomarker of tendon phenotype, which may be desirable in the context of a practical application. In the light of the current evidence, *IGFBP6* expression appears to be useful in recognition of a functional tendon phenotype and may potentially become employed in evaluating tendon degeneration in intra-operative and biopsy tendon samples. However, that would require further investigation of *IGFBP6* expression in fibrous scar tissue resulting from tendon injuries, for instance by analysing overload lesions frequently diagnosed in human and equine athletes. Fibrous tissue produced during tendon healing has altered microstructure and ECM composition (*e.g.* increased proportion of type III to type I collagen) which results in compromised mechanical properties and high risk of re-injury (Yang *et al.*, 2014). A gene marker differentiating intact from repair tissue could inform decision on the extent of tendon dissection during surgical management of shoulder tendon injuries. Another important direction in tendon marker application is quality assessment of engineered tissues for use in tendon lesion repair. Previously published datasets comparing transcriptomic profiles of rat tenocytes and chondrocytes maintained in different culture systems and in native tissue (Mueller *et al.*, 2016) showed that *IGFBP6* expression in tendon-derived cells is consistently higher than in those derived from cartilage, independently of the culture method [monolayer or three-dimensional (3D) tissue construct]. Particularly interesting is that *IGFBP6* maintained higher expression in late passage monolayer tenocytes despite the general loss of a tissue-specific transcriptomic signature. Dedicated studies investigating *IGFBP6* at different stages of tendon construct creation and relating the expression level to structural and functional outcomes are needed to validate its use in directed tendon construct differentiation.

Limitations associated with the study, mainly pertained to collection of a sample range from different tissue types and species. Unlike rat, sheep and horse, human tendon, cartilage and muscle samples were collected from different donor groups, with muscle donors being considerably younger than cartilage and tendon donors. That difference is related to the fact that healthy cartilage and tendons for research are mainly derived from cadaveric sources, while muscles can be collected intraoperatively or as biopsy samples. Age disparity could have potentially affected comparability of expression results from different musculoskeletal tissues. Nevertheless, two studies analysing age-related changes in human tendon and cartilage transcriptome by RNA sequencing did not identify any of the marker genes investigated in the present study to be differentially expressed between young old subjects (Peffers *et al.*, 2013; 2015). Results of *IGFBP6* analysis in horse further demonstrated that growth and development are more likely to affect expression of tendon marker genes than ageing. Another potential source of variation in gene expression levels was the circadian rhythm, as it was not possible to control for sample collection time in most species, except for rats. The available studies investigating circadian changes in mouse tendon indicated that only *THBS4* expression is rhythmic (Yeung *et al.*, 2014). It is also unclear if species considered in the present study have the same tendon circadian clock. Additionally, analysis of bone alongside tendon, cartilage and muscle would be desirable as ectopic ossification is one of the manifestations of tendon degeneration (Agarwal

et al., 2017). However, it is suspected that appearance of osteoblasts, responsible for tissue mineralisation in tendon, is preceded by fibrocartilaginous metaplasia of tenocytes. According to the present study, that change is associated with a marked decrease in *IGFBP6* expression level. *IGFBP6* has been also shown to be involved in mechanisms inhibiting human osteoblast phenotype development (Yan *et al.*, 2001). Whilst *IGFBP6* expression appears to be a reliable marker of tendon tissue, similar relations may not necessarily occur at the protein level. Validation of the results described in the present study would be required to introduce the use of *IGFBP6* in protein-based assays, opening additional avenues for its clinical application. In order to establish *IGFBP6* as clinical marker of healthy tendon phenotype, present results would need to be validated by an independent cohort study comparing the effect of age, sex and pathologies on *IGFBP6* expression.

Conclusion

IGFBP6 was identified as a universal transcriptomic marker in rat, sheep, equine and human tendons and may constitute a potential reference biomarker for evaluation of tendon physiological phenotype and directed development of engineered tendons.

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Discussion with Reviewers

Linda Dahlgren: Do you think that *IGFBP6* would be differentially expressed between tendon and a general fibrous connective tissue? Or between tendon fibroblasts passaged in culture and primary tendon fibroblasts?

Authors: Gene expression profiles of rat primary tenocytes, tenocytes cultured in monolayer (passage 3) and 3D fibrin constructs (unpublished data, presented at British Society for Matrix Biology Spring Meeting, 2019) were compared. *IGFBP6* was significantly more expressed [$\log_2$ fold change > ± 0.5, false disovery rate (FDR) < 0.01, log-odds ratio of expression > 0] in primary cells and monolayer

culture than in 3D constructs. Also, higher expression of *IGFBP6* was observed in equine tendon cultured in monolayer (passage 3) than in 3D fibrin construct, as measured by RT-qPCR. Tenocytes cultured in monolayer lose their specific phenotype (Muller *et al.*, 2016; Yao *et al.*, 2006). Therefore, higher expression of *IGFBP6* in primary than in cultured tenocytes could further support its importance as a tendon marker. However, it is unclear why monolayer cells would show higher *IGFBP6* transcript level than tenocytes cultured in a 3D system showing more similarity to the native tissue in global expression profile (Yao *et al.*, 2006). This observation might question *IGFBP6* usability for tendon engineering purposes or the value of the applied 3D culture technique for tendon phenotype maintenance. It is likely that *IGFBP6* declines with culture time as generating 3D bioengineered tendon requires significantly more time than reaching confluence in cell monolayer. Evaluating *IGFBP6* expression in a range of tendon *in vitro* models, including novel multifactorial culture systems, is needed to validate its application as tendon marker for bioengineering application.

Brianne Connizzo: Is *IGFBP6* expressed highly in other non-musculoskeletal tissues that might be present *in vivo*, such as vascular or adipose tissues?
Authors: *IGFBP6* is expressed across multiple tissues. The Human Protein Atlas (additional Web ref. 1) utilising mRNA expression data derived from RNA deep-sequencing studies of healthy tissues reports high expression levels of *IGFBP6* in adipose tissue, endocrine and gastrointestinal systems, further confirmed by the report of The Genotype-Tissue Expression (GTEx) portal (additional Web ref. 2). All tendon samples analysed in the present study were dissected from the tendon midportion, limiting the possibility of including adipose or loose vascular connective tissue located in the paratenon. *IGFBP6* cannot be considered as a tendon-specific gene, however, the expression difference between tendon and other musculoskeletal tissues and between different tendon phenotypes might be relevant in the context of tendon tissue engineering and detecting fibrocartilaginous degeneration.

Brianne Connizzo: How much of the conclusions do you think could be driven by variability in the data? Or what does the higher variability in tendon as compared to other tissues indicate about expression of these genes?
Authors: Target genes were chosen based on expression across two microarray platforms that were highly correlated for tendon (that was the selection criteria). Relative expression of many of these genes in cartilage and muscle is very low and should not be confused with 'very consistent'. Expression of certain genes in tendon may vary with site, age, injury and what seen may reflect the variation in the population. However, answering such a question was beyond the scope of the study due to the limited sample size and demographic characterisation of the investigated tissues.

Brianne Connizzo: How does expression of *IGFBP6* change in injured or diseased tendon? Can this be used to identify varying levels of injury?
Authors: Previous studies report decreased expression of *IGFBP6* in animal models of tendon injury and human fibrotic disease of the palmar fascia (Dupuytren's disease) (see Discussion). Time-series study utilising collagenase-induced lesions of equine SDFT showed that *IGFBP6* expression declines in response to the insult, however, it returns to pre-injury level within three months of injury (Dahlgren *et al.*, 2006). That may suggest that tracking *IGFBP6* expression change could indicate healing progress in acute tendon injuries if those finding would have been validated in naturally occurring disease. Preliminary experiment (data not published) demonstrated a significant decrease in *IGFBP6* expression in early but not advanced rotator cuff tendinopathy as compared to healthy hamstring tendon. Those promising results warrant further investigation of *IGFBP6* expression in tendon health and disease. However, discussing *IGFBP6* role in tendon pathology was beyond the scope of the study as results described gene expression only in healthy tissue samples.

Additional Web References

1. https://www.proteinatlas.org [28.06.2019]
2. https://www.gtexportal.org/home/gene/IGFBP6 [19.07.2019]

Editor's note: The Scientific Editor responsible for this paper was Juerg Gasser.