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Comparative transcriptional analysis of three human ligaments with distinct biomechanical properties

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Abstract

One major aim of regenerative medicine targeting the musculoskeletal system is to provide complementary and/or alternative therapeutic approaches to current surgical therapies, often involving the removal and prosthetic substitution of damaged tissues such as ligaments. For these approaches to be successful, detailed information regarding the cellular and molecular composition of different musculoskeletal tissues is required. Ligaments have often been considered homogeneous tissues with common biomechanical properties. However, advances in tissue engineering research have highlighted the functional relevance of the organisational and compositional differences between ligament types, especially in those with higher risks of injury. The aim of this study was to provide information concerning the relative expression levels of a subset of key genes (including extracellular matrix components, transcription factors and growth factors) that confer functional identity to ligaments. We compared the transcriptomes of three representative human ligaments subjected to different biomechanical demands: the anterior cruciate ligament (ACL); the ligamentum teres of the hip (LT); and the iliofemoral ligament (IL). We revealed significant differences in the expression of type I collagen, elastin, fibromodulin, biglycan, transforming growth factor β1, transforming growth interacting factor 1, hypoxia-inducible factor 1-alpha and transforming growth factor β-induced gene between the IL and the other two ligaments. Thus, considerable molecular heterogeneity can exist between anatomically distinct ligaments with differing biomechanical demands. However, the LT and ACL were found to show remarkable molecular homology, suggesting common functional properties. This finding provides experimental support for the proposed role of the LT as a hip joint stabiliser in humans.

Key words: biglycan; collagen; decorin; Sox9; transforming-growth-factor-beta.

Introduction

Ligaments are key structures for joint stability and dynamics. They block certain displacements and/or guide and restrict joint movements within their physiological ranges. Additionally, ligaments provide important functional substrates for the transmission of proprioceptive information. The gross structures of ligaments are based on fibroblasts immersed in a collagen-based extracellular matrix (ECM), with collagen organised within cross-linked fibrils that are aligned according to tensile stress (Amiel et al. 1984). Type I collagen is the predominant molecule in these tissues; however, different components, such as other collagens, elastins/fibrillins or proteoglycans (PGs), are also present in tendons and ligaments, in proportion to their biomechanical demands (Hurle et al. 1990; Ros et al. 1995; Vogel & Peters, 2001; Vogel, 2004; Yoon & Halper, 2005; Franchi et al. 2010; Young et al. 2011).

Ligaments are hypocellular tissues, although their fibroblasts are connected to one another through long cellular processes. The cellular net is interconnected via GAP junctions, permitting communication throughout the entire ligament. Such an organisation may enable the orchestration of coordinated responses to maintain and adapt the composition and organisation of the ECM to the mechanical stress (McNeilly et al. 1996; Benjamin & Ralphs, 1997, 2000; Lo et al. 2002).

Collagen fibrils constitute approximately 80% of the dry weight of ligaments (Amiel et al. 1984). Type I collagen constitutes more than 95% of the collagen contained in

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ligaments, with the remaining portion primarily consisting of type III collagen, as well as small amounts of types V, VI, XI and XIV collagens (Amiel et al. 1984; Liu et al. 1995; Frank, 2004). The collagen molecules are cross-linked by bonds that stabilise the fibres and improve the tensile strength of the ligaments (Fujii et al. 1994; Eleswarapu et al. 2011). Additionally, the collagen fibrils confer the capacity for elongation to ligaments under increased mechanical stress, based on the crimping disposition of the collagen molecule (Boorman et al. 2006).

Elastic fibres are important components of dense connective tissues and have two major constituents, namely the fibrillin-based microfibrillar scaffold and the elastin deposits (Hurle et al. 1990; Neurath & Stofft, 1992; Strocchi et al. 1992; Ros et al. 1995; Hurle & Colombatti, 1996; Reinboth et al. 2000; Frank, 2004). The ratio of elastic fibres in the ligaments has a major impact on the biomechanical properties of the tissue, and their abundance is usually proportional to the tensile load on the ligaments (Neurath & Stofft, 1992; Sherratt et al. 2003; Frank, 2004; Glab & Wess, 2008).

The other ECM constituents of dense connective tissues include PGs, which are molecules based on a protein core that binds to the anionic glycosaminoglycan (aGAG) sidechains (Scott et al. 1995). The PG protein core binds at specific sites on the collagen fibrils, whereas the aGAGs form filaments that bridge between and across the collagen fibrils (Scott, 1992, 1996). These interfibrillar aGAG bridges are important in the maintenance of tissue shape, as they organise the collagen fibrils by linking them together. The aGAGs confer a hydrophilic character to the PGs, allowing them to aggregate with hyaluronic acid and thus determine the tissue's water content, which accounts for 60–70% of the total ligament weight (Amiel et al. 1984; Woo & Buckwalter, 1988; Hannafin & Arnoczky, 1994). Importantly, the PGs also modulate fibrillogenesis to create collagen fibres that accommodate the tensile forces (Pogany et al. 1994; Svensson et al. 1995; Pins et al. 1997; Graham et al. 2000; Corsi et al. 2002). It has also been suggested that PGs regulate cell growth and differentiation by controlling the local bioavailability of growth factors and/or through interactions with tyrosine kinase receptors (Ruoslahti, 1989; Hausser et al. 1994; Iozzo, 1998, 1999).

Ligaments have often been considered homogeneous tissues with common biomechanical properties. However, the development of regenerative medicine and advances in tissue engineering research have highlighted the functional relevance of the organisational and compositional differences between ligament types, especially in those with higher risks of injury. In this study, we used quantitative polymerase chain reaction (PCR) and Western blotting to analyse compositional and transcriptional differences in three ligaments of clinical relevance: the anterior cruciate ligament (ACL) of the knee; the iliofemoral ligament (IL); and the ligamentum teres (LT) of the hip. These ligaments are subjected to different biomechanical demands, and the knee and hip joints are potential targets of regenerative medicine, due to the high prevalence of traumatic and degenerative diseases in these joints. In addition, the functional role of the LT is controversial. It has been proposed that the LT plays a mechanical role in hip stabilisation analogous to that of the ACL of the knee (Wenger et al. 2007; Bardakos & Villar, 2009; Cerezal et al. 2010). Hence, a comparative analysis of molecular differences between the LT and other ligaments with better functional characterisation may provide new insights into their biomechanical properties. For this study, we selected a panel of genes that included ECM components, transcription factors and growth factors.

Materials and methods

Human ligaments

In this study, we employed 10 round ligaments (LTs) of the hip, eight hip capsular ILs and eight ACLs from 20 different donors (Table 1). These ligaments were taken from donors lacking severe aging-related diseases (except hypertension), diagnosed with hip or knee arthrosis during the joint removal surgery for prosthesis substitution. Only ligaments without appreciable anatomical or gross structural anomalies were collected. After their collection, the samples were immediately frozen using liquid nitrogen immersion in the operating room before further processing. The donor ages ranged from 45 to 84 years. This project has been approved by the competent authority 'COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA DE CANTABRIA' (Certificate no. 2513).

Real-time quantitative PCR (Q-PCR) for gene expression analysis

Ligaments were homogenised using a hand-held rotor-stator homogeniser. RNA extraction was performed by the acid guanidinium thiocyanate–phenol–chloroform-based method (Chomczynski & Sacchi, 2006). Treatment with RNAse-free DNAse I (Ambion) and subsequent phenol extraction for RNA purification were performed. Purified RNA samples were quantified using a spectrophotometer (Nanodrop Technologies ND-1000). First-strand cDNA was synthesised by reverse transcriptase-PCR using random hexamers and M-MulV reverse transcriptase (Fermentas). The cDNA concentrations were measured in a spectrophotometer (Nanodrop Technologies ND-1000) and adjusted to 0.5 $\mu g\ \mu L^{-1}$. Q-PCR was performed using the Mx3005P system (Stratagene) with automation attachment. In this work, we used SYBRGreen (Takara)-based Q-PCR. *Gapdh* expression did not vary significantly across the sample set and therefore was chosen as the normaliser in our experiments. Mean gene expression levels were calculated for each gene to determine differences between different tissues. Expression levels were evaluated relative to a calibrator according to the $2^{-\Delta\Delta C_t}$ equation (Livak & Schmittgen, 2001). We randomly selected IL values as the calibrator for comparison purposes.

Each value in this work represents the mean ± SEM of all the obtained samples. Data were analysed using one-way analysis of variance followed by Bonferroni tests for *post hoc* comparisons of gene expression levels. Statistical significance was set at $P < 0.05$. All

Table 1 Data concerning the donors of the ligaments collected in this study.

Donor	Age (years)	Sex	Pathology	Ligament	Ligament gross anatomy
1	84	Male	Primary gonarthrosis	ACL	Normal
2	77	Female	Primary coxarthrosis	LT	Normal
3	80	Female	Primary gonarthrosis	ACL	Normal
4	54	Male	Primary gonarthrosis	ACL	Normal
5	75	Male	Primary coxarthrosis	LT	Normal
6	64	Male	Primary coxarthrosis	LT & IL	Normal
7	55	Male	Primary coxarthrosis	LT	Normal
8	76	Male	Primary coxarthrosis	LT & IL	Normal
9	75	Female	Primary gonarthrosis	ACL	Normal
10	54	Male	Primary coxarthrosis	IL	Normal
11	61	Female	Primary gonarthrosis	ACL	Normal
12	72	Female	Primary gonarthrosis	ACL	Normal
13	73	Female	Primary coxarthrosis	LT & IL	Normal
14	69	Male	Primary coxarthrosis	LT & IL	Normal
15	63	Male	Primary coxarthrosis	LT & IL	Normal
16	78	Male	Primary coxarthrosis	LT & IL	Normal
17	63	Female	Primary gonarthrosis	ACL	Normal
19	45	Female	Healthy body donor	ACL	Normal
20	68	Female	Primary coxarthrosis	LT & IL	Normal

ACL, anterior cruciate ligament; IL, Iliofemoral ligament; LT, ligamentum teres.

Table 2 Primers employed in this study.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Scleraxis (*SCX*)	gcaccaacagcgtgaaca	ggtgcgagatgtagctggag
Collagen 1a2 (*Col1a2*)	tctggagaggctggtactgc	tagaccacgttcacctctcg
Collagen 3a1 (*Col3a1*)	tagctggacctcgtggtagc	ccaggttcaccattctgtcc
Collagen 5a1 (*Col5a1*)	ccaccagaacgtcacctacc	gacatctcctcgtcgttgg
Collagen 9a1 (*Col9a1*)	gcagattcaggattcctctgg	tggagacttccatccagtcc
Collagen 2a1 (*Col2a1*)	tccagatgaccttcctacgc	agctgcttcgtccagatagg
Elastin (*Eln*)	gctaaggcagccaagtatgg	gacaccaacacctggaacg
Emilin 1 (*Emn*)	tcactgaatgagctccagacc	atgatacggtccttggttgc
Decorin (*Dcn*)	atcatcctccttctgcttgc	cggtcatcaggaacttctgg
Biglycan (*Bgn*)	cctccaggtggtctatctgc	ctgatgccgttgtagtaggc
Fibromodulin (*Fmod*)	cctccaacaccttcaattcc	ggtagaggttctccaggttgg
SRY (sex-determining region Y)-box 9 (*Sox9*)	tctgaacgagagcgagaagc	gcggctggtacttgtaatcc
Aggrecan (*Acan*)	caagtggttcctggtgtgg	gctcggtggtgaactctagg
Hypoxia inducible factor 1 alpha (*Hif1a*)	gaaggtattgcactgcacagg	agcaccaagcaggtcatagg
Bone morphogenetic protein 12 (*Bmp12*)	actacgaggcgtaccactgc	agcagcgtctgaatgatgg
Transforming growth factor β inducible gene (*Bigh3*)	caccatcaccaacaacatcc	cttcaagcatcgtgttgagc
Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)	tgcaccaccaactgcttagc	ggcatggactgtggtcatgag
Mohawk (*Mhk*)	cgtattggaaggagatcaacg	ggacgacttctggatgatgc
Tenomodulin (*Tnmd*)	tcctctggcatctgttagcc	ttgccatggtctctcagc
Transforming growth factor β 1 (*Tgfβ1*)	gatgtcaccggagttgtgc	gtccacttgcagtgtgttatcc
Transforming growth factor β 2 (*Tgfβ2*)	ctcagcaatggagaagaatgc	cgttgttcaggcactctgg
Transforming growth factor β 3 (*Tgfβ3*)	caacgaactggctgtctgc	ttctgctcggaataggttgg
Transforming growth inducible factor 1 (TGiF1)	gaaaggatggcaaagatcca	aggaatgaaatggggtctcc

the analyses were performed using SPSS for Windows version 18.0. Specific Q-PCR primers for human genes (Table 2) were designed using the PRIMER3 program (Sequence Analysis, Informagen). Furthermore, dissociation curves were evaluated in the PCR reaction to ensure specificity (Fig. S1).

Patients may exhibit inherent differences that could mask the results. One limitation of this study, which is common to reports of this type, is that sourcing ligaments from age-matched truly normal joints proved unfeasible. To discard distorted interpretations due to structural differences in the ligaments based on potential patient-

dependent variations, we analysed neutral adjacent tissues from affected joints (i.e. dermis; see Fig. S2). We used Q-PCR to analyse the gene expression levels of all the factors and proteins employed in this work in the control tissues. No statistically significant differences were found in these analyses, suggesting that the observed differences in the ligaments are not due to the characteristics of each patient.

Western blotting

Total protein extracts were obtained from the LT, IL and ACL samples. Cell lysis was performed with RIPA buffer [in mM: NaCl, 150; $MgCl_2$, 1.5; NaF, 10; glycerol, 10%; EDTA, 4; Triton X-100, 1%; sodium dodecyl sulphate (SDS), 0.1%; deoxycholate, 1%; HEPES, 50; pH 7.4] supplemented with the protease inhibitors phenylmethylsulphonyl fluoride (1 mM), leupeptin (10 μg mL^{-1}) and aprotinin (10 μg mL^{-1}) for 15 min on ice. The cell lysates were clarified of cellular debris by centrifugation (13 200 *g*) for 10 min at 4 °C. Proteins were separated by 10% polyacrylamide gel electrophoresis containing 0.1% SDS and were transferred to a polyvinylidene fluoride membrane (Bio-Rad). The membranes were incubated for 1 h at room temperature in bovine serum albumin and incubated overnight with the following antibodies: rabbit polyclonal antibody against SOX9 (Chemicon); rabbit polyclonal antibody against DECORIN (Cell Signalling); and rabbit polyclonal antibody against transforming growth factor TGFβ1 (Santa Cruz Sc-146). Protein bands were detected with an ODYSSEY infrared-imaging system (Li-Cor Bioscience) according to the ODYSSEY Western blot protocol. Immunoblots were developed with anti-mouse IRDye 800DX or anti-rabbit IRDye 680DX (Rockland Immunochemicals, USA). Three different samples of each ligament type were tested by Western blotting.

Results

Expression of ECM components

One of the main purposes of this study was to gain insight into the tissue identities of the different ligaments under study. Therefore, we first evaluated by Q-PCR the relative levels of gene expression of a set of ECM components that are characteristic of most connective tissues. The ECM is the main component of ligaments, and comparisons of gene expression by this technique would thus be very informative in terms of determining tissue identity.

We began by analysing the fibrillar components of the ECM. The LT and ACL showed equivalent levels of expression of *collagen Ia1* and *collagen Ia2* (data not shown; Fig. 1, respectively), and these were significantly higher than levels found in the IL. Similar findings were obtained for *type III collagen* and *type V collagen* (Fig. 1). Regarding specific differences, the *collagen IIa1* relative gene expression level was higher in the IL than in the LT and ACL (Fig. 1). However, differences in *collagen IIa1* relative gene expression level between the ACL and IL were not statistically significant. In addition, the LT and ACL exhibited equivalent relative levels of gene expression of *collagen IXa1* that were significantly lower than levels in the IL (Fig. 1). We found that *elastin* expression was equivalent in the ACL and LT, while these were higher levels than those observed in the IL (Fig. 1). Interestingly, other components of the elastic fibres, such as *emilin 1* and *emilin 3* (Hurle

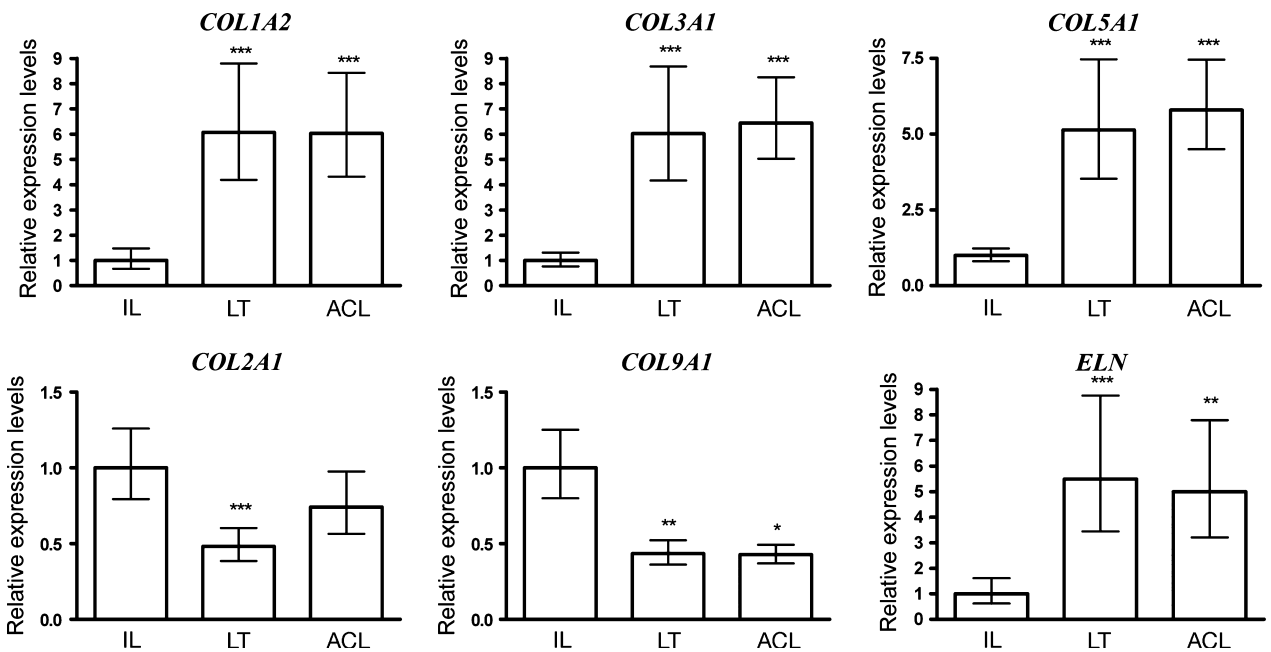

Fig. 1 Comparative analysis of relative gene expression levels of fibrillar components of the ECM in the iliofemoral ligament (IL), the ligamentum teres of the hip (LT) and the anterior cruciate ligament (ACL), as measured by Q-PCR. Note that the expression level profiles for fibrillar components of the ECM are comparable between the LT and ACL but different in the IL. The expression level in the IL was considered as the baseline for each gene (calibrator). n = 6 LT, 4 ACL and 4 IL. *P < 0.05, **P < 0.01 or ***P < 0.001 in ACL or LT vs. LT. *COL*, collagen; *ELN*, elastin. Each bar represents the mean value ± SEM.

et al. 1994), displayed no among-group differences (Table 3; data not shown, respectively).

We also studied non-fibrillar components of the ECM, namely PGs. *Decorin*, the most abundant PG in ligaments, was expressed in significantly higher levels in the ACL than in the LT and IL, with the latter ligament types showing equivalent levels of relative gene expression (Fig. 2). These differences were confirmed at the protein level by Western blotting (Fig. 4). The relative *Biglycan* gene expression levels were significantly higher in the LT than in the other tissues (Fig. 2). Furthermore, relative *Biglycan* gene expression levels were higher in the ACL than in the IL (Fig. 2). The relative *Fibromodulin* gene expression levels were considerably higher in the ACL and LT than in the IL. In addition, the relative *Fibromodulin* gene expression levels were significantly more elevated in the ACL than in the LT (Fig. 2). Finally, *Aggrecan* expression was equivalent in all three tissues (Table 1).

Finally, among other types of proteins found in the ECM of ligaments, we did not observe any differences in the relative gene expression levels of *Tenomodulin* (Table 3). However, we detected an elevated expression of *Transforming growth factor β induced gene* (*βigh3*) in the ACL and LT compared with the IL (Fig. 2).

Table 3 Average ± SEM expression values of genes studied in this work where no significant differences were found between LT, ACL and IL.

Ligament	Average	SE+	SE−
Emilin 1			
LT	1.043650666	0.321719658	0.245913456
ACL	0.943874313	0.256445056	0.201656248
IL	1	0.292435084	0.226266749
Aggrecan			
LT	0.726146896	0.242859486	0.18199226
ACL	0.686183655	0.227703431	0.170969012
IL	1	0.274261154	0.215231511
Tenomodulin			
LT	1.218058424	0.449715422	0.328449604
ACL	1.04367076	0.303202426	0.234946771
IL	1	0.271589072	0.213582421
Transforming growth factor beta 2			
LT	0.661025475	0.14738526	0.120514743
ACL	0.959541206	0.355001233	0.259130707
IL	1	0.18368741	0.155182364
Transforming growth factor beta 3			
LT	0.747712499	0.237316865	0.180141621
ACL	0.772219737	0.24932197	0.188471352
IL	1	0.156154722	0.135063862
Scleraxis			
LT	0.707174858	0.109757794	0.095011446
ACL	1.25375026	0.373929861	0.288026286
IL	1	0.23421779	0.189770227
Mohawk			
LT	1.19770929	0.857215424	0.499626517
ACL	1.030789681	0.322691998	0.245756989
IL	1	0.278379476	0.217759657

Note that the expression for the IL was taken as calibrator for each gene in the Q-PCR values.
ACL, anterior cruciate ligament; IL, Iliofemoral ligament; LT, ligamentum teres.

Expression of growth factors

As discussed above, the aim of this study was to obtain some insight into the tissue identity of the different ligaments under study, and growth factors are key players in the maintenance of ligament tissue integrity. Therefore, we chose to compare by Q-PCR the relative levels of gene expression of different widely recognised fibrogenic growth factors in the three ligaments. Members of the TGFβ superfamily are significantly involved in the development and maintenance of most connective tissues (Montero & Hurle, 2007; Montero et al. 2012). *TGFβ* genes have been recently shown to be absolutely required for the formation of dense connective tissue on the basis of *in vitro* evidence and in knock-out mice (Lorda-Diez et al. 2009; Pryce et al. 2009). In our study, differences in relative gene expression levels were only detected for *TGFβ1*, which was expressed at higher levels in the ACL and LT than in the IL (Fig. 3). *TGFβ2* and *TGFβ3* expression showed no differences between these tissues (Table 3). Analysis at the protein level confirmed the lower expression of TGFβ1 in IL (Fig. 4).

Bone morphogenetic protein 12 (BMP12) is a member of the TGFβ superfamily and is known to stimulate fibrogenesis (Haddad-Weber et al. 2010; Berasi et al. 2011). We observed higher *BMP12* relative gene expression levels in the IL than in the other ligaments; however, these differences did not reach statistical significance (Fig. 3).

Expression of transcription factors

Transcription factors are relevant molecular players that confer identities to different tissues. In fact it has been proposed that the balance between the expression levels of key transcription factors may specify different types of connective tissues as ligaments and tendons (Lorda-Diez et al. 2009; Furumatsu et al. 2010). In our comparative analysis of the gene expression levels in three ligaments, we chose three specific markers of ligament and tendon tissue identity, including *Scleraxis* (Lorda-Diez et al. 2009), *Mohawk* (Ito et al. 2010) and *transforming growth interacting factor 1* (*TGiF1*) (Lorda-Diez et al. 2009). We found no significant differences in the relative gene expression levels of *Scleraxis* and *Mohawk* among the ACL, LT and IL (Table 3). However, we found an almost twofold increase in *TGiF1* expression levels in the IL compared with the other two ligaments (Fig. 3).

In addition, chondrogenic markers are often expressed in ligaments, especially at the level of the enthesis (Smith et al. 2012). Thus, we analysed two major chondrogenic markers, *Sox9 SRY (sex-determining region Y)-box 9* (*Sox9*) (Chimal-

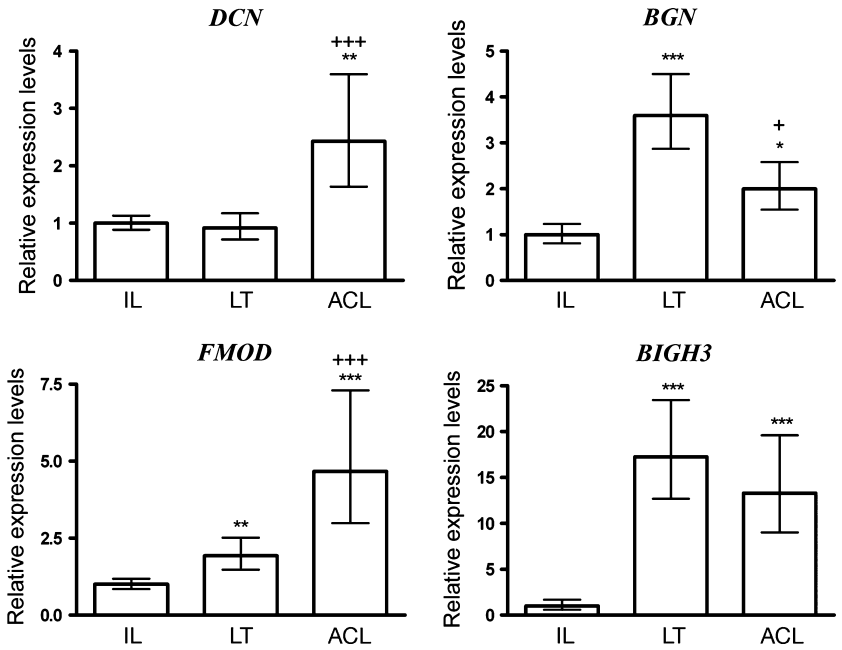

Fig. 2 Comparative analysis of relative gene expression levels of characteristic components of the ECM of ligaments in the iliofemoral ligament (IL), the ligamentum teres of the hip (LT) and the anterior cruciate ligament (ACL), as measured by Q-PCR. Note that the expression levels of the canonical ligament PGs *DCN*, *BGN* and *FMOD* are always higher in the ACL and/or LT than in the IL. The same pattern is observed for *BIGH3*, an important component of the ECM. The expression level in the IL was considered the baseline level for each gene (calibrator). *BGN*, *Biglycan*; *BIGH3*, *Transforming growth factor β induced gene*; *DCN*, *Decorin*; *FMOD*; *Fibromodulin*. $n = 6$ LT, 4 ACL and 4 IL. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ in ACL or LT vs. LT. $^{+}P < 0.05$ or $^{+++}P < 0.001$ in ACL vs. LT. Each bar represents the mean value ± SEM.

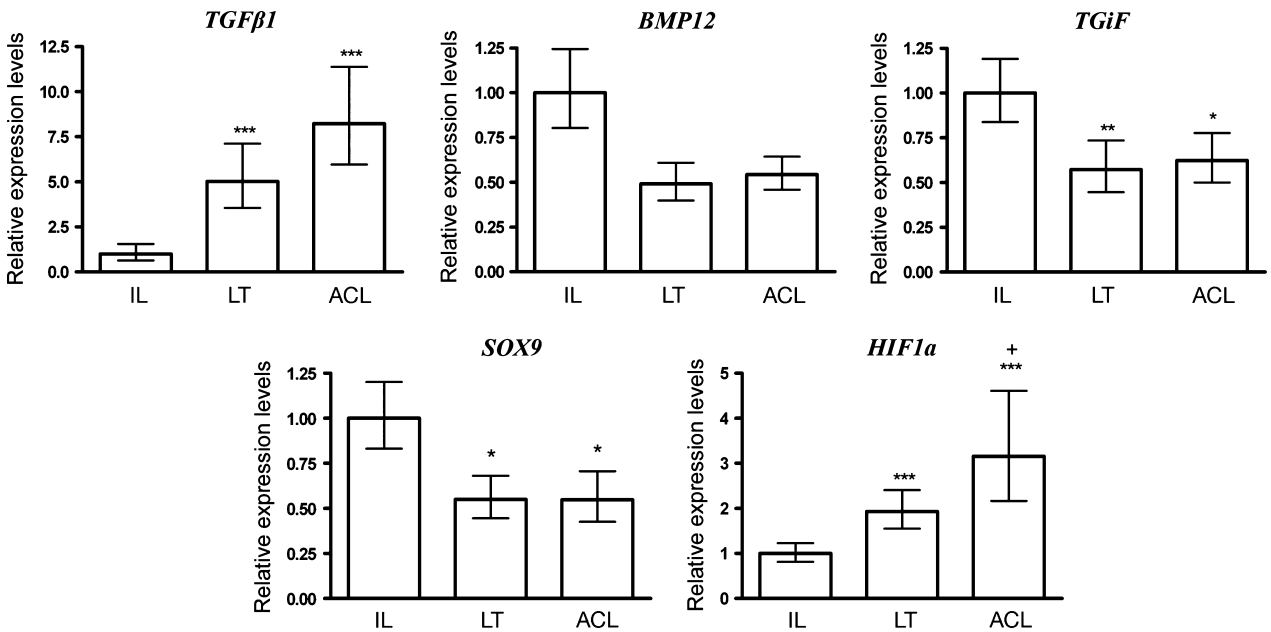

Fig. 3 Comparative analysis of relative gene expression levels of growth and transcription factor genes in the iliofemoral ligament (IL), the ligamentum teres of the hip (LT) and the anterior cruciate ligament (ACL) as evaluated by Q-PCR. Note that the expression levels of the growth and transcription factors illustrated in the figure are similar in the ACL and LT but distinct in the IL. The expression level in the IL was considered the baseline for each gene (calibrator). *BMP12*, *Bone morphogenetic protein 12*; *HIF1A*, *Hypoxia inducible factor 1α*; *SOX9*, *SRY (sex-determining region Y)-box 9 gen*; *TGFβ1*, *Transforming growth factor β1*; *TGiF1*, *Transforming growth interacting factor 1*. $n = 6$ LT, 4 ACL and 4 IL. $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ in ACL or LT vs. LT. $^{+}P < 0.05$ in ACL vs. LT. Each bar value represents the mean ± SEM.

Monroy et al. 2003) and *Hypoxia-inducible factor 1-alpha* (*Hif1a*) (Kanichai et al. 2008). Interestingly, the relative gene expression levels of *Sox9* were similar in the ACL and LT, but were significantly higher in the IL (Fig. 3). This finding was further demonstrated at the protein level by Western blotting (Fig. 4). Additionally, *Hif1a* relative gene expression levels were considerably higher in the ACL and LT than in the IL (Fig. 3). Finally, the ACL showed significantly higher expression levels of this gene than did the LT (Fig. 3).

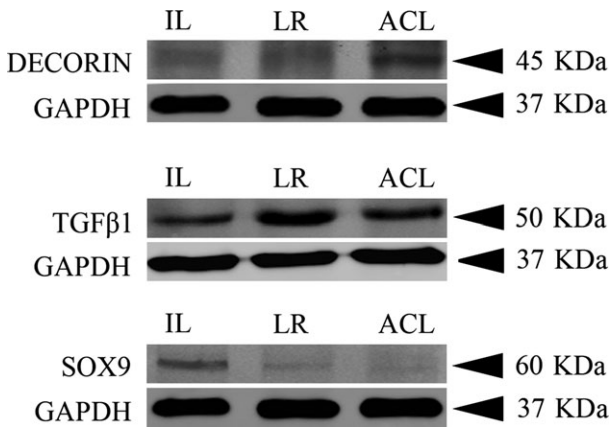

Fig. 4 Representative Western blots illustrating DECORIN, TGFβ1 precursor and SOX9 expression in the iliofemoral ligament (IL), the ligamentum teres (LT) and the anterior cruciate ligament (ACL). Note that the findings at the protein level reproduce to some extent those for gene expression. GAPDH was used as the loading control in all the experiments. n = 4 LT, 4 ACL and 4 IL.

Discussion

The precise knowledge of different ligaments is of considerable clinical importance, as this information might be relevant to the improved design of modern healing strategies based on tissue engineering. Rupture of the ACL is one of the most prevalent ligament injuries, especially among people practicing contact sports or hiking (Farshad et al. 2011). ACL disruption causes changes in the kinematics of the knee, and this injury often results in knee instability with accompanying degeneration of the menisci and articular cartilage, as well as subsequent functional disability and pain. The LT of the hip has traditionally been considered an embryological remnant, acting as a vessel-bearing structure that allows blood supply to the femoral head. However, increasing evidence supports its role as a hip joint stabiliser, especially during flexion, external rotation and abduction, similar to the functional role of the ACL in the knee (Cerezal et al. 2010; Kivlan et al. 2013; Martin et al. 2013). Alterations in the LT have been associated with hip pain in athletes (Byrd & Jones, 2004). Compared with the distinct characteristics of the LT and the ACL, the IL is a less specialised ligament. However, the IL is a significant joint stabiliser for the standing position, and it limits the external rotation and extension of the femur (Wagner et al. 2012).

The tensile and elastic biomechanical properties of ligaments rely largely on the collagen and elastic fibres. We found that both the ACL and LT exhibit similar expression levels of collagen and elastic fibre genes. In fact, for those collagens that are more characteristic of ligaments, including collagen types I, III and V, expression levels were higher in the ACL and LT compared with the IL. As mechanical loading is an important factor modulating gene expression in connective tissues (Murchison et al. 2007; Scott et al. 2011), these findings could suggest that the LT is subjected to specialised biomechanical demands and is not simply an embryonic vestige that functions as a passive blood vessel bearer. Our interpretation is consistent with previous clinical and *in vitro* biomechanical studies (Wenger et al. 2007; Bardakos & Villar, 2009; Cerezal et al. 2010).

We analysed a panel of small leucine-rich PGs (SLRPs), including *Decorin*, *Biglycan* and *Fibromodulin*, which are important ECM components with key functions in the formation and homeostasis of ligaments. These PGs include collagen- and growth factor-binding molecules that are involved in the modulation of collagen fibrillogenesis, cell shape, cell growth and cell signalling (Corsi et al. 2002; Ferdous et al. 2007, 2010; Kilts et al. 2009). Furthermore, it is well recognised that PGs favour tissue hydration, acting as a lubricant between collagen fibres. They are also critical for the viscoelastic properties that allow ligaments under tension to return to their original shapes once the tension is removed (Scott, 1988; Weiss et al. 2002). Our findings showed that the ACL has the highest levels of *Decorin* (the predominant PG in ligaments) and *Fibromodulin*, which may account for the stiffness of the ligament. Consistent with this interpretation, the ACL is stiffer than the LT. Accordingly, animal models lacking these PGs show a disorganisation of the collagen fibres accompanied by reduced ligament stiffness. In these models, the ACL appears hypertrophied and torn, and it may exhibit ectopic ossification (Gill et al. 2002; Zhang et al. 2006; Kilts et al. 2009). The LT showed substantially higher levels of *Biglycan* expression than the IL or ACL. Similar to *Decorin*, *Biglycan* is a proteodermatan sulphate SLRP that mediates ligament stiffness (Kilts et al. 2009), and it may compensate for a deficiency of *Decorin* (Corsi et al. 2002; Zhang et al. 2006). Hence, despite these compositional differences in SLRPs, the mechanofunctional properties of the ACL and LT may be similar to each other and therefore different from those of the IL.

Proteoglycans modulate the bioavailability of growth factors. Hence, the high expression levels of PGs in the LT and ACL correlate with the elevated expression of *TGFβ1* found in these ligaments. *Decorin*, *Biglycan* and *Fibromodulin* all bind TGFβ1, and they modulate its function in association with enzymatic processing (Hausser et al. 1994; Hildebrand et al. 1994). TGFβ1 has been involved in ligament development, homeostasis and healing, in turn regulating fibroblast differentiation, proliferation, adhesion and migration; furthermore, it promotes ECM synthesis and inhibits enzymatic degradation (Peltonen et al. 1991; Ghahary et al. 1993; Mauviel, 1993; Scherping et al. 1997; Uria et al. 1998; Evans, 1999; Lorda-Diez et al. 2009; Ferdous et al. 2010; Achari et al. 2011; Wang et al. 2011a). TGFβ1 also promotes collagen cross-linking, thereby contributing to ligament stiffness (Eleswarapu et al. 2011). These functions of TGFβ1 are regulated by mechanical stress, which can stimulate its production.

Given the findings mentioned above, the higher levels of expression for *TGFβ1* may reflect the greater demands of

the ACL and LT for self-renewal and strengthening, given their exposure to upper loading and compressive supported stress, in comparison with the IL. In this regard, the presence of high *βiGH3* expression levels in the LT and ACL is also suggestive of elevated TGFβ signalling activity in these ligaments. *βiGH3* is a gene that is directly inducible by TGFβ proteins, and it is known to modulate cell adhesion, cell migration and cell differentiation (Thapa et al. 2007). Importantly, it has been recently shown that it potentiates profibrogenic effects on connective tissue precursors under the control of TGFβ signalling (Lorda-Diez et al. 2013).

We found higher expression of *hypoxia inducible factor 1α* (*Hif1a*) in the LT and especially in the ACL, compared with the IL. This high expression is suggestive of a hypoxic environment. The presence of vessels might well be the cause of the lower expression of this factor in the LT compared with the ACL. However, the levels were still higher in the LT than in the IL. In other models, the *Hif1a* expression in cartilage has been associated with the inhibition of cell proliferation and tissue hypocellularity (Schipani, 2005); therefore, *Hif1a* could well be acting in a similar fashion in these ligaments. Furthermore, *Hif1a* expression has been linked to high matrix-metalloproteinase 2 activity in ligaments (Wang et al. 2011b). This could be associated with the weak healing capability of some ligaments, such as the ACL, as it would interrupt the necessary balance in the ECM remodelling (Zhou et al. 2005).

We did not find substantial differences in the expression levels of transcription factors related to fibrogenic induction, such as *Scleraxis* or *Mohawk*. However, we did indeed find higher expression of chondrogenic factors, such as *Sox9*, in the IL compared with the ACL or LT. Accordingly, we identified higher expression levels in the IL of *type II collagen* or *type IX collagen*, which are collagens that are more abundant and characteristic in cartilage and fibrocartilage (Eyre et al. 2004; Chen et al. 2012). Consistent with this expression pattern, the IL presents a prominent fibrocartilage interphase at the enthesis (Wagner et al. 2012), which may explain our findings of higher IL expression levels of *collagen II* or *collagen IX* than those in the LT. The ACL shows an intermediate profile for these genes, which is again consistent with the presence of fibrocartilaginous structures (Petersen & Tillmann, 1999). Finally, *TGiF* is a profibrogenic factor that exhibits higher expression in the IL compared with the ACL or LT, with an intermediated profile found for the ACL. Importantly, this transcription factor is involved in inhibiting the expression of the prochondrogenic *Sox9* gene (Lorda-Diez et al. 2009), and thus this transcription factor might be important in maintaining the identity of these capsular and knee ligaments.

In summary, our data complement traditional histological and functional studies of three representative human ligaments, and provide a transcriptomal characterisation of potential usefulness for modern regenerative medicine.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Graph showing the dissociation curves in a characteristic Q-PCR experiment for different genes employed in this study. Primer specificity was verified by the presence of only one amplicon at a specific temperature in each experiment. X_{axis}= fluorescence; Y_{axis}= temperature.

Fig. S2. Chart illustrating the comparison by Q-PCR of relative gene expression levels of several markers, in the dermis of the knee vs. the hip. Most of the relevant genes from this study are shown. The samples were taken from the knee and hip regions during the joint removal surgery procedures, at the same time that ligaments were collected. Each pair of bars represents the gene expression mean ± SEM values for dermis from the knee (left bar in each pair) and dermis from the hip (right bar in each pair). Note that the relative gene expression level in the knee was chosen as the calibrator for comparisons in each pair of bars. $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$. Note the absence of significant differences in any of the genes. The absence of significant differences precludes individual variations masking the differences observed between knee and hip ligaments. $n_{(knee\ dermis)} = 3$ and $n_{(hip\ dermis)} = 3$.