Ageing does not result in a decline in cell synthetic activity in an injury prone tendon

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Abstract

Advancing age is a well-known risk factor for tendon disease. Energy storing tendons, (e.g. human Achilles, equine superficial digital flexor tendon (SDFT)) are particularly vulnerable and it is thought that injury occurs following an accumulation of micro-damage in the extracellular matrix (ECM). Several authors suggest that age-related micro-damage accumulates due to a failure of the aging cell population to maintain the ECM or an imbalance between anabolic and catabolic pathways. We hypothesised that ageing results in a decreased ability of tendon cells to synthesise matrix components and matrix degrading enzymes, resulting in a reduced turnover of the ECM and a decreased ability to repair micro-damage. The SDFT was collected from horses aged 3-30 years with no signs of tendon injury. Cell synthetic and degradative ability was assessed at the mRNA and protein levels. Telomere length was measured as an additional marker of cell ageing. There was no decrease in cellularity or relative telomere length with increasing age, and no decline in mRNA or protein levels for matrix proteins or degradative enzymes. The results suggest that the mechanism for age-related tendon deterioration is not due to reduced cellularity or a loss of synthetic functionality and that alternative mechanisms should be considered.

Keywords:

- **Tendon**
- **Ageing**
- **Degeneration**
- **Tenocyte**
- **Metabolism**
- **Telomere**

Introduction

Ageing results in a gradual and inevitable decline in the performance of physiological systems in the body. Changes to the musculoskeletal system are easily observed as individuals lose their strength and flexibility and athletic performance declines. Furthermore, susceptibility to skeletal tissue injury increases with advancing chronological age. Several epidemiological studies have shown a marked increase in the incidence of tendon and ligament injuries in older age groups, beyond the age of peak athletic ability (Clayton and Court-Brown, 2008; Hess, 2010; Raikin et al., 2013). For example, in the patellar tendon the peak in incidence of injury occurs in the seventh decade of life and the incidence of Achilles tendon injury peaks in middle age (40-60 years) (de Jonge et al., 2011), with a smaller peak in women in their seventh decade (Clayton and Court-Brown, 2008). These studies suggest there is an age related decline in tendon health rather than simple mechanical overload.

Tendon injuries are also prevalent in horses; most injuries occur to the superficial digital flexor tendon (SDFT) (Ely et al., 2009). Injuries to the SDFT are remarkably similar to those seen in the human Achilles tendon, both in terms of risk factors and the pathology. It is well documented that older horses are at a higher risk of SDFT injury than younger horses (Avella et al., 2009; Ely et al., 2009; Williams et al., 2001). The equine SDFT and human Achilles tendon have a spring-like function, storing and releasing elastic strain energy (Lichtwark and Wilson, 2005; Wilson et al., 2001). Tendons with this specialised function appear to be particularly prone to age-related pathologies.

The equine SDFT and human Achilles tendon, in their energy storing roles, are subjected to high strains. These tendons therefore require mechanical properties that allow them to withstand repeated cycles of loading and unloading at strains of up to 16% and 11% respectively (Lichtwark and Wilson, 2005; Stephens et al., 1989). These properties are

provided by a complex hierarchical arrangement of collagen molecules interspersed with non-collagenous components throughout the matrix such as proteoglycans and glycoproteins (see Thorpe et al., 2013 for review). The fibril forming type I collagen forms about 75% of the dry weight of tendon (Birch et al., 2008a). A relatively sparse population of tendon cells or tenocytes maintains this large amount of load-bearing collagenous matrix and the supporting non-collagenous extracellular matrix.

While tendon ruptures can occur due to an acute overloading event, or laceration, tendon injuries are often accompanied by evidence of chronic degeneration (Thomopoulos et al., 2015), suggesting that damage has been accumulating within the tendon. This accumulation of microdamage is thought to result in a gradual weakening of the hierarchical structure, but the nature of this micro-damage and the pathways by which it forms are not clear. Several authors however suggest a failure of the cell population to maintain the extracellular matrix or an imbalance between anabolic and catabolic pathways favouring matrix degradation (Riley, 2008). The degradation of the matrix occurs through the combined action of members of the matrix metalloproteinase (MMP) and A Disintegrin And Metalloproteinase with ThromboSpondin motif (ADAMTS) family. Only the secreted collagenases (MMP-1 and MMP-13) are able to cleave the intact collagen triple helix away from the cell surface, however once cleaved, the denatured collagen molecule can then be degraded by other proteases.

Early observations in ageing research established that both protein synthesis and degradation decline during ageing (Tavernarakis, 2008) resulting in a limited capacity of the cellular maintenance, repair and turnover pathways and possible accumulation of age related damage. In keeping with this, our research has shown that, although cell density does not decline (Birch et al., 1999), collagen half-life, calculated by measuring D and L forms of aspartate in an injury prone tendon, increases with increasing age (Thorpe et al., 2010), suggesting a

decline in the ability of the tendon cells (tenocytes) to synthesise new matrix components. In a more recent study, we have used proteomic analysis to show that levels of some matrix components, including several proteoglycans, decrease with ageing (Peffers et al., 2014). The results of our previous work also suggest a reduced ability of the tenocytes to degrade the matrix components, as the levels of type I collagen crosslinked telopeptide (ICTP), a marker of mature collagen degradation, decline with increasing age (Thorpe et al., 2010). Our previous work was not able to determine whether reduced collagen turnover results from a failure of the tendon cell population to synthesise high levels of MMPs or whether changes in the matrix render it less digestible. The aim of the current study was therefore to assess changes in cell metabolism and the balance between anabolic and catabolic potential with advancing age.

We hypothesised that ageing results in a decreased ability of tendon cells to synthesise matrix components and matrix degrading enzymes, resulting in a reduced turnover of the extracellular matrix. We have used the equine SDFT for our studies as this tendon is injury prone and shows a clear age related increase in susceptibility to damage. We have collected tendons from a wide age range of horses and investigated in situ cell synthetic ability by assessing expression of matrix proteins at mRNA and protein level and the ability to degrade the matrix by measuring expression of matrix degrading enzymes and enzyme activity. In addition, relative telomere length was measured as a marker of ageing based on cellular growth and proliferation rather then extracellular matrix synthesis.

Materials and Methods

Sample Collection

The right forelimbs of skeletally mature horses with a wide age range (n = 32, age range 3 to 30 years) euthanased for reasons other than tendon injury were collected from a commercial

abattoir. Horses above the age of 20 years are considered to be geriatric (Brosnahan and Paradis, 2003). The SDFT was dissected free from the limbs at the level of the metacarpophalangeal joint and examined for macroscopic signs of injury. Only tendons with no signs of injury were included in the study. Tissue was harvested from the mid-metacarpal region of each SDFT. For DNA and protein analysis, samples were snap frozen in hexane cooled on dry ice and stored at -80 ºC prior to processing. When required, snap-frozen tendon samples were thawed and the periphery of the tendon removed. Samples were lyophilised at -40 ºC until a constant weight was reached, then reduced to a fine powder using a micro-dismembrator (Sartorius, Germany) at 3000 rpm for 2 minutes, and stored at -80 ºC prior to analysis. Samples for RT-qPCR were stored in RNAlater at 4 °C for 24 hours, before being stored at -20 °C until required for analysis.

Tendon DNA Content

Lyophilised samples were accurately weighed and papain digested according to Birch *et al*. (1998). Immediately after papain digestion the DNA content of the tendons was measured using the bisbenzimidazole dye Hoechst 33258, according to Kim *et al. (1988)* and expressed as μg per mg dry weight tendon tissue.

RT-qPCR

Tissue samples stored in RNAlater (Ambion) were chopped and pulverised for 2 min. at 2000 oscillations/minute in a liquid nitrogen cooled dismembranator (Braun Mikro-Dismembrator Vessel, Braun Biotech International, Melsungen, Germany). RNA was extracted using a phenol-chloroform extraction (TriReagent™, Sigma) followed by purification using a commercially available kit (RNeasy Mini Kit, Qiagen), including an on-column DNA digestion step (RNase-Free DNase Set, Qiagen). RNA was stored at -80°C prior to reverse transcription. RNA concentration was measured (ND-1000 spectrophotometer Nanodrop Technologies) and 1 μg RNA was used to prepare 20μl cDNA using M-MLV reverse

transcriptase and random primers according to the manufacturer's instructions (Promega). Samples were stored at -20°C prior to relative quantification of gene expression. Primers were designed using Primer Express (Applied Biosystems) software and selected to span predicted exon boundaries where possible. BLAST searches were performed for all sequences to confirm gene specificity. Target and reference gene primers were synthesized by Eurogentec. Specificity and efficiency between 95 - 105 % was confirmed for all primers (Taylor et al., 2009). GeNorm (Vandesompele et al., 2002) identified HIRA-interacting protein 5 (HIRP-5) and mitochondrial ribosomal protein S7 (MRPS-7) as the two most stably expressed genes from a panel of 8 candidate reference genes. All data were therefore normalised to the average expression of HIRP-5 and MRPS-7 using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). RT-qPCR assays were performed in triplicate using the 7900 HT Fast Real-Time PCR System (Applied Biosystems; Warrington, UK) in 384 well plates. Reaction volume in each well was 10 µl (4.6 µl cDNA, 5 µl Power SYBR mastermix (Applied Biosystems), 0.1 µl DEPC water, 0.15 µl 3 µM forward primer and 0.15 µl 3 µM reverse primer). The cycling conditions comprised 10 min polymerase activation at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Data were then analysed using Sequence Detection Systems Software v2.2.1 (Applied Biosystems; Warrington, UK).

Western Blotting for PINP

Collagen synthesis was assessed at the protein level in the SDFT from 14 horses selected from the sample group based on age range (6 – 30 years). Pro-collagen was detected using an antibody for the N-terminal propeptide of type-I collagen (PINP) using Western blotting; this antibody (SP1.D8, Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa, UK) has been previously shown to cross react with the horse (Young et al., 2009).

PINP was extracted from the tendon matrix using Guanidine-HCl (GndHCl) extraction followed by precipitation according to Birch *et al.* (2008b). The remaining pellet was reconstituted in 100 µl 2x reducing buffer (10% mecaptoethanol in 125 mM Tris, 2% SDS, 10% glycerol, pH 6.8) and heated at 60 ºC for 5 min. before loading 20 µl of each sample onto the gels. Proteins were separated by SDS-PAGE on a 5 % acrylamide gel; a molecular weight standard (10 µl; Precision Plus Standards, Biorad Laboratories Ltd., Hemel Hempstead, UK) was run alongside the samples on each gel. Proteins were separated by applying a constant current of 20 mA per gel to the gels for 55 min.

After electrophoresis, proteins were transferred to PVDF membranes (Amersham Hybond-P, GE Healthcare, Amersham, UK) by blotting for 75 min at 100 V. After washing, membranes were probed with the PINP primary antibody (SP1.D8, diluted 1 in 1000 in TBS-Tween) for 2 hours at room temperature, followed by incubation with an enhanced chemi-luminescent (ECL) peroxidase labelled anti-mouse secondary antibody (GE Healthcare, diluted 1 in 10 000 in TBS-T). Blots were developed with Amersham ECL Plus™ Western blotting detection reagents (GE Healthcare) according to the manufacturer's instructions, with an x-ray exposure time of 10 min. Resulting x-ray films were photographed on a lightbox (Nikon Coolpix, 5700) and band areas corresponding to α2 chain with PINP and PICP (Fig. S1) were quantified using Scion Image (Version 4.0.3.2, Scion Corporation, Maryland, USA).

Fluorescent Substrate Assay (MMP-13)

A fluorogenic assay was used to assess protein levels of latent and active forms of MMP-13 in the SDFT according to Birch et al. (2008b). Briefly, lyophilised tendon tissue was suspended in extraction buffer (50 mM HEPES, 200 mM NaCl, 1 mM CaCl2, 0.01% BRIJ-35, pH 7.3) and incubated at 22 °C for 60 min. Samples were centrifuged (16 000 g, 5 min.) and the supernatant was removed and stored at -80 °C. Aliquots of supernatant were pipetted

into a 96 well fluorometer plate in duplicate. Fluorescence was measured in the presence and absence of amino-phenyl mercuric acetate to determine the amount of latent active MMP-13 in the samples. A quenched fluorescence substrate for MMP-13 (Calbiochem) was added to each well to give a final concentration of 0.1 mM. Plates were incubated at 37 °C and fluorescence measured after 2 h on a fluorescence reader (Bio-tek Instruments Inc. FLX800 microplate reader) with excitation and emission wavelengths of 325 nm and 393 nm respectively. Activity was expressed as relative fluorescence units per mg tissue.

Gelatin and Casein Zymography

Lyophilised tendon tissue was incubated in 2x non-reducing sample buffer (approximately 15 mg tissue in 600 µl; 125 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue at pH 6.8) at 22 ˚C for 30 min. The samples were centrifuged (16 000 g, 2 min.) and the supernatant was removed and stored at -80 °C. Samples were separated on 8 % acrylamide gels containing bovine gelatin type B (1 mg/ml), and 10 % gels containing β-casein (1 mg/ml). A molecular weight marker and an MMP-9 standard prepared from equine neutrophil cells were included on each gel (see supplementary information).

After electrophoresis, gels were washed in 2.5% Triton X-100 solution for 1 hour, rinsed in incubation buffer (50 mM Tris, 0.2% Sodium Azide, 5 mM Calcium chloride, pH 7.6) and then incubated in incubation buffer (as above) at 37 °C for 40 hours. Gels were then rinsed and stained for 30 min in Coomassie Blue (0.5% Coomassie Blue R250, 30% methanol, 10% acetic acid). After staining, the gels were de-stained (10% methanol, 5% acetic acid) for 20 hours. Gels were photographed (Nikon Coolpix, 5700) and analysed using Scions β 4.0.2 image analysis software (Scion Corporation, Maryland, USA). For gelatin zymography, digestion bands were expressed per mg of tissue and relative to the MMP-9 standard. For casein zymography, an SDFT sample with a relatively high concentration of MMP-3 was

identified and run on every gel, and the activity of MMP-3 in all other samples were expressed relative to this sample.

Telomere Length Measurements

Genomic DNA was extracted from the SDFT of 32 horses using the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions for the purification of total DNA from animal tissues. Relative telomere lengths were then determined using the singleplex qPCR method described in detail by Cawthon (2002). One SDFT DNA sample served as a reference sample and was serially diluted by three-fold per dilution to give five DNA concentrations ranging from 0.185 to 15 ng/µl, which were then added to the standard curve wells of a 96-well PCR plate in 10 µl aliquots. For all other samples, 16.7 ng DNA (middle of the standard curve range) was added to each sample well in triplicate. Separate master mixes were prepared for the telomere (T) and single copy gene (S) primer pairs (3 µM) using the GoTaq qPCR SYBR green master mix (Promega) and each sample was assayed for each primer pair separately. The vertebrate telomere primer sequences were (5' to 3'): Tel1, GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT; Tel2, TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA; and the single copy gene (equine GAPDH) primer sequences were: forward, GCATCGTGGAGGGACTCA; reverse, GCCACATCTTCCCAGAGG. All PCR was performed on an Applied Biosystems 7300 instrument.

For the standard curves, the average Ct values for each DNA standard were plotted for both the T and S primer pairs. The linear regression equations produced for each primer pair was then used to generate the T and S values for each of the SDFT samples using the average SDFT Ct values. The T/S ratio, for each SDFT sample was then determined to give a relative measure of telomere length.

Statistical Analysis

Data were tested for normality using Kolmongorov-Smirnov analysis (Minitab, version 15). Correlation was assessed using Pearson Product Moment Correlation for parametric data and Spearman's rank correlation for non-parametric data (SPSS, version 14). Statistical significance was set at $p<0.05$. All data are displayed as mean ± SEM.

Results

Cell number does not decline with increasing age

To give an indication of tendon tissue cellularity and any change in cell concentration with increasing horse age, the amount of DNA was assayed in papain-digested samples of tendon tissue. Our results show DNA levels of 0.54 ± 0.02 μg/mg (mean ± SEM) and no significant change in DNA levels with ageing in the equine SDFT (Fig. 1).

Expression of matrix proteins in relation to donor age

To assess the potential for matrix synthesis, we quantified levels of mRNA for some of the main protein components of the extracellular matrix in tendon tissue. These included the α2 chain of type I collagen, α1 chain of type III collagen, α1 chain of type V collagen, α1 chain of type XII collagen, decorin, biglycan, fibromodulin, lumican, aggrecan and collagen oligomeric matrix protein (COMP). In addition, we measured expression of scleraxis and tenascin, two genes coding for proteins considered to be markers of a tenogenic phenotype (Jelinsky et al., 2010). Expression of all genes was detected (Table 1) however none of the mRNA transcript levels correlated with horse age (Table 1 & Fig. 2).

Evidence for the synthesis of new collagen at the protein level was assessed by measuring levels of the N-terminal propeptide of type I collagen (PINP) in tissue extracts using Western blotting. Four bands were visible on the blot ranging from approximately 200 kDa to 155 kDa

molecular weight representing the α1 chain with PINP and C-terminal propeptide (PICP) attached, α2 chain with PINP and PICP, α1 chain with PINP and α2 chain with PINP (see supplementary information Figure S1). The α2 chain with PINP and PICP gave the clearest band in the majority of samples and so the area of this band was used for relative quantification of PINP levels between samples. The level of pro-collagen varied widely between individual horses and was not detectable in some samples, however levels did not relate to horse age (Fig. 3a). There was no relationship between the level of pro-collagen extracted from the tissue and the expression of Col1A2 at the mRNA level (Fig. 3b).

Expression of matrix degrading enzymes in relation to donor age

Expression of matrix degrading enzymes was measured at the mRNA level for the following enzymes; collagenases (MMP-1, MMP-13), stromelysins (MMP-3, MMP-10), gelatinases (MMP-2, MMP-9), MMP-23, ADAM-12, ADAM-17, and ADAMTS-2. In addition, the expression of tissue inhibitor of metalloproteinases (TIMP-3 and TIMP-4) was measured. Expression of all proteolytic enzymes and TIMPs was detected (Table 2) however none of the mRNA transcript levels correlated with horse age (Table 2 & Fig. 4) except for MMP-10 expression, which increased significantly with increasing horse age ($p=0.005$). While not significant, there was a trend for increased MMP-3 expression with increasing age ($p=0.054$).

Latent and active forms of matrix degrading enzymes at the protein level were quantified using a fluorogenic assay (MMP-13), gelatin zymography (MMP-2 and MMP-9) and casein zymography (MMP-3). MMP-13 was detected only in the active form (45.46 ± 5.66 RFU/mg tissue) and levels did not correlate with horse age. The gelatinases, MMP-2 and MMP-9, were detected in all tendon samples. MMP-9 was present entirely in the active form whereas MMP-2 was detected mainly in the latent form although small amounts of the active form were also present in most tendon samples (see supplementary information Figure S2). Neither

MMP-2 nor MMP-9 correlated with horse age and there was no correlation between MMP-9 mRNA and protein levels. Casein zymography showed that MMP-3 was present mainly in the latent form (Pro MMP-3) with small amounts of active enzyme present in some tendon samples. Both pro MMP-3 and active MMP-3 levels increased significantly with increasing horse age (Fig. 5a). Total MMP-3 levels showed a weak but significant positive correlation with MMP-3 mRNA levels (Fig. 5b).

Relative telomere length

Relative telomere length was determined in DNA extracted from tendon tissue samples using a quantitative PCR technique (Cawthon, 2002). The results showed that relative telomere length, expressed as T/S ratios, did not decrease significantly with increasing horse age (Fig. 6).

Discussion

The results of our study do not support our hypothesis and unexpectedly show that synthesis of matrix proteins and matrix proteases in the SDFT does not decline with increasing horse age. In a wide variety of other tissues and organisms, a decrease in protein synthesis is one of the most common age associated changes (Rattan, 1996) and has been linked to age-related neurodegenerative (Douglas and Dillin, 2010), cardiovascular, muscle and bone disease. Furthermore decline in the synthesis of matrix components has been shown in other skeletal tissues such as cartilage where it has been linked to osteoarthritis (Peffers et al., 2013). Our finding is therefore contrary to the widely accepted notion that decreased protein synthesis represents a universal phenomenon (Ryazanov and Nefsky, 2002).

Tendon is characterised by a large amount of extracellular matrix, which is predominately protein and a relatively sparse population of cells. The tendon cells have the task of

maintaining proteostasis throughout life and in the face of mechanical challenges to the integrity of the matrix. One way this may be comprised is by declining cell numbers with increasing age. A number of previous studies have reported a decrease in cell number with increasing age in rat Achilles tendon (Yu et al., 2013), rat tail tendon (Lavagnino et al., 2013) and mice patellar tendons (Dunkman et al., 2013). However it is difficult to separate maturation and ageing effects in short lived species such as rats where growth plates remain open throughout life (Kilborn et al., 2002). Furthermore small mammals do not have tendons that are specialised for an energy storing role (Alexander, 2002), making extrapolations to age related deterioration to injury prone tendons in larger mammals difficult. The results of our study showed no decrease in DNA content with increasing horse age indicating that in an energy-storing tendon from a long-lived species decrease in cell number does not occur.

In our study, we examined protein turnover by measuring synthesis at the transcriptional and translational levels and assessed the potential for degradation by measuring expression of matrix degrading enzymes. Proteostasis however involves additional regulatory steps of protein folding, processing, assembly, disassembly and localisation. Taking a more global view, we have previously shown, using aspartic acid racemisation, that the half-life of the collagenous component of the equine SDFT increases with increasing horse age suggesting a decrease in the rate of collagen synthesis (Thorpe et al., 2010). The results of the present study however demonstrate that the synthesis of collagen does not decline and suggest an alternative mechanism; that there is reduced ability to dissemble and reassemble the collagenous matrix. The first step in collagen degradation relies on the action of collagenases (MMP-1 and MMP-13) and we did not show reduced expression or activity of these enzymes. Interestingly, our previous work on the equine SDFT demonstrated an accumulation of the type I collagen neoepitope generated by collagenase activity in old tissue and reduced ability to extract partially degraded collagen from the tendon tissue (Thorpe et

al., 2010). Further, we have recently shown that overall protein extractability is reduced with ageing in the SDFT (Peffers et al., 2014). Taken together with these previous findings, our results suggest that the ageing process results in modifications to matrix proteins rendering them resistant to turnover rather than reduced cellular capacity for matrix metabolism.

Our previous work showed a distinction between collagenous and non-collagenous protein turnover and age related changes (Thorpe et al., 2010). The non-collagenous matrix had a lower half-life showing turnover is more rapid and in keeping with this, we find in this study that the levels of expression of non-collagenous proteins such as decorin, biglycan, lumican and COMP are higher than expression of collagens. Furthermore, this previous work found no age related decline in non-collagenous protein turnover and the data presented here show the levels of stromelysins responsible for proteoglycan degradation (MMP-3 and MMP-10) actually increase with increasing horse age. The differences observed between collagen and non-collagenous components may relate to the specialised role of the equine SDFT as an energy store and the mechanism by which specialised mechanical properties are attained. Recent work has demonstrated that high strains, which are essential for function in energy storing tendons, are achieved by sliding between fascicles and an inter-fascicular matrix with low stiffness (Thorpe et al., 2012). The majority of non-collagenous proteins reside in the inter-fascicular matrix and their important role in the mechanical function may necessitate a higher rate of turnover.

It is of interest to note that although degeneration is associated with ageing, the results of this study highlight differences between the two processes. Gene expression studies on degenerate human Achilles tendon tissue found decreased expression of MMP-3 and MMP-10 in painful tendons and decreased expression of MMP-3 in the ruptured group (Jones et al., 2006). In another study, dysfunctional posterior tibialis tendons showed decreased expression of MMP-3 (Corps et al., 2012), while our study showed that expression of both MMP-3 and MMP-10

increases with advancing age. Indeed previous analysis of degenerated equine SDFT (Birch et al., 1998), ruptured human supraspinatus tendon (Riley et al., 2002) and dysfunctional human posterior tibialis tendon (Corps et al., 2012) all demonstrated changes indicative of matrix remodelling, changes which this study suggests are not characteristic of ageing. Therefore while ageing may predispose to degeneration these appear to be two distinct processes.

Although we interpret our data as demonstrating that ageing in tendon is a matrix phenomenon and not cell mediated, it may be that older cells are not able to respond to mechanical stimuli as efficiently as younger cells. It was not possible to obtain the exercise history of the horses used in this study, and as it is unlikely that any horse underwent high intensity exercise prior to euthanasia, the resident cells would have experienced low levels of stimuli. Therefore it is possible that aged cells may show a decreased response to loading. In addition, older cells may also show a reduced response to anabolic stimuli after injury. Following micro-trauma or gross injury an immediate inflammatory response is initiated (Dakin et al., 2012) and the release of a cascade of growth factors including IGF-1, TGFβ, PDGF, VEGF and bFGF follows, which stimulates the repair process (Molloy et al., 2003). Evidence from in vitro studies of chondrocytes (Loeser et al., 2000; Martin et al., 1997) and in vivo study of osteoblasts (Cao et al., 2007) suggests that cells in older individuals have a reduced capacity to respond to IGF-1. Recent work however has shown that local injection of IGF-1 into the patellar tendon of young and old men is able to stimulate protein synthesis to an equal degree demonstrating that the ability to respond to anabolic stimuli is maintained in older age (Nielsen et al., 2014).

As we chose to focus on matrix proteins and matrix degrading proteins, our study would not detect other important age-related changes to tendon cell gene expression that may occur. A

previous study of ageing in cartilage, using Next Generation Sequencing to assess the full transcriptomic phenotype of chondrocytes, reported that 396 transcribed elements were differentially expressed with ageing, the majority of which were down-regulated in old samples (Peffers *et al*., 2013). Further, our recent work characterising the proteome of the SDFT from young and old individuals has shown that abundances of 34 proteins were altered with ageing, with decreased amounts of several proteoglycans, and increased cytoskeletal proteins in aged tendon (Peffers et al., 2014). While the abundance of major tendon matrix proteins were not altered with ageing, the change in amounts of some minor proteins may have important consequences for tendon health (Peffers et al., 2014). Future work should therefore attempt to characterise full transcriptomic signatures in tendon to further investigate tendon cell ageing, and relate this to changes seen at the protein level.

Tendon is known to contain distinct populations of cells including a sub-population of progenitor cells (Bi et al., 2007). It is not known at present how these different cell populations contribute to tendon maintenance and anabolic response to injury. Recent work has shown that progenitor cells from aged/degenerate human Achilles tendon have reduced self-renewal and clonal capacity, leading to the proposal that reduced functional fitness of the stem cell pool is responsible for tendon degeneration (Kohler et al., 2013). While these findings may appear to contradict the results of our study, it is more likely that the studies are looking at different aspects. We assessed relative telomere length of the total cell population, whereas Kohler *et al*. (2013) studied ageing in a specific sub-population of tendon cells. The proportion of these progenitor cells relative to total tendon cell population is unclear, but it is likely that the majority of cells in tendon are a stable resident population of cells which are terminally differentiated, rather like osteocytes in bone tissue, hence telomere length does not shorten. Additional work is required to elucidate cell specialisation within the tendon structure.

Further, we choose to focus on healthy tendon over a range of ages with the intention of identifying age-related changes that may predispose to degeneration (micro-damage). Kohler *et al*. (2013) did not differentiate between cells from aged or degenerated tendons and it may be that the differences observed are due to degeneration rather than healthy ageing.

In conclusion, the results of this study suggest that an important mechanism for age related tendon deterioration lies around protein post-translational modifications thereby disrupting the assembly and disassembly process of the functionally important collagenous scaffold and that future work should focus around understanding these processes. It is likely that similar processes occur in other collagen rich tissues with a relatively slow turnover. These matrix changes have implications for cell-based therapies, which aim to enhance synthetic capability, and suggest additional strategies must be adopted to tackle age related decline of skeletal tissues. As collagen is a ubiquitous and abundant protein, the results of this study are far reaching and findings in tendon are likely to translate to other skeletal tissues and connective tissue components of other organs where half-life is long, in addition to fibrotic tissue diseases.

Perspectives

While it is well established that the risk of tendon injury increases with ageing, the aetiology of this is unclear. In the current study, we have shown that there is no overall decline in tendon cell synthetic or degradative capacity with ageing, suggesting that other mechanisms result in age-related tendon deterioration. The results of this and previous studies (Thorpe et al., 2010) alternatively suggest that age-related alterations to the collagenous matrix may render the tendon matrix more resistant to degradation and remodelling, such that the cells are less able to repair areas of damage. This information will help guide the development of effective treatment options for age-related tendon injury.

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Supporting information

Table S1. Forward and reverse primer sequences

Figure S1. Representative blot showing the 4 bands detected by the PINP antibody

Preparation of MMP-9 Standard

Figure S2. Levels of pro- and active forms of MMP-2 and -9 in the SDFT assessed by gelatin zymography

Tables

	Relative mRNA levels		Correlation with age
	Per mg tissue	**Corrected for DNA content**	**p value**
Col1a2	34.93±7.23	19.42±4.30	0.85
Col3a1	18.56±3.93	10.72±2.75	0.25
Col5a1	1.49±0.40	0.84±0.22	0.36
Col12a1	9.51±1.38	5.20±0.79	0.27
Aggrecan	5.55±1.81	3.07±0.96	0.64
Biglycan	63.27±13.76	35.84±8.44	0.63
Decorin	1343.42±302.85	780.99±198.98	0.63
Fibromodulin	32.80±6.68	18.53±3.90	0.51
Lumican	48.50±7.45	27.62±5.30	0.98
Tenascin	1.86±0.49	1.07±0.29	0.80
Scleraxis	3.79±0.99	2.35±0.64	0.62
COMP	2674.33±570.18	1516.06±320.36	0.61

Table 1. Average gene expression of matrix proteins in the SDFT per tissue weight and corrected for DNA content. Data are displayed as Mean±SEM. Correlations are shown between horse age and mRNA levels corrected for DNA content.

	Relative mRNA levels		Correlation with age
	Per mg tissue	**Corrected for DNA content**	**p value**
MMP-1	0.03±0.01	0.01±0.01	0.46
MMP-3	3.52±0.83	1.89±0.45	0.054
MMP-9	0.43±0.20	0.23±0.11	0.36
MMP-10	17.46±4.48**	8.61±2.09**	0.0045
MMP-13	0.16±0.12	0.07±0.05	0.76
MMP-23	0.03±0.01	0.02±0.01	0.46
TIMP-3	55.08±9.29	31.01±6.19	0.98
TIMP-4	0.53±0.15	0.27±0.07	0.52
ADAM-12	0.05±0.01	0.03±0.01	0.86
ADAM-17	1.21±0.22	0.65±0.13	0.47
ADAMTS-2	1.22±0.27	0.64±0.12	0.90

Table 2. Average gene expression of matrix degrading enzymes in the SDFT per tissue weight and corrected for DNA content. Data are displayed as Mean±SEM. Correlations are shown between horse age and mRNA levels per cell. * indicates significant correlation with age. ** p>0.005.

Figure Legends

Fig. 1 Tendon DNA content plotted as a function of horse age.

Fig. 2 Gene expression levels of tendon extracellular matrix molecules as a function of horse age. There was no correlation between horse age and mRNA levels of Col1A2 (a), Col3A1 (b), decorin (c), or biglycan (d).

Fig. 3 Levels of the N-terminal propeptide of type I collagen (PINP) in SDFT tissue. PINP levels showed no relationship with horse age (a), and did not correlate with mRNA levels of Col1A2 (b).

Fig. 4 Expression of matrix degrading enzymes as a function of horse age. MMP-3 mRNA levels did not show a significant relationship with horse age (a), but MMP-10 expression increased significantly with age ($p=0.005$).

Fig. 5 Pro and active MMP-3 levels as a function of horse age measured using casein zymography. Both pro (•; ———) and active (■; --------) forms of MMP-3 increased significantly with horse age (a, $p<0.02$). There was a weak, but significant, correlation between total MMP-3 protein levels and MMP-3 mRNA levels (b, $p=0.02$).

Fig. 6 Estimated relative telomere length plotted as a function of horse age. There was no correlation between horse age and relative telomere length.