A peer-reviewed version of this preprint was published in PeerJ on 29 June 2018.

View the peer-reviewed version (peerj.com/articles/5120), which is the preferred citable publication unless you specifically need to cite this preprint.

Shu CC, Smith MM, Appleyard RC, Little CB, Melrose J. 2018. Achilles and tail tendons of perlecan exon 3 null heparan sulphate deficient mice display surprising improvement in tendon tensile properties and altered collagen fibril organisation compared to C57BL/6 wild type mice. PeerJ 6:e5120
https://doi.org/10.7717/peerj.5120
Diverse effects of Perlecan-HS on cell and matrix regulation: Tendon destabilization in *Hspg2* Exon 3 null HS deficient mice reveals essential homeostatic roles for HS in cellular regulation and tendon functionality

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The aim of this study was to determine the regulatory role of the perlecan HS side chains on cell and matrix homeostasis in tail and Achilles tendons in 3 and 12 week old *Hspg2* exon 3 null HS deficient (*Hspg2*Δ3/-Δ3) and C57 BL/6 Wild Type (WT) mice. Tendons were biomechanically tested (ultimate tensile stress [UTS], tensile modulus [TM]) and glycosaminoglycan (GAG) and collagen (hydroxyproline) compositional analyses undertaken. Monolayer cultures of *Hspg2*Δ3/-Δ3 tenocytes stimulated with FGF-2 showed elevated *Adamts4*, *Mmp2*, 3, 13 gene expression compared to WT mice. *Col1A1*, *Vcan*, *Bgn*, *Dcn*, *Lum*, *Hspg2*, *Ltbp1*, *Ltbp2*, *Eln* and *Fbn1* showed no major differences between genotypes. Type VI collagen and perlecan were immunolocalised in tail tendon and collagen fibrils imaged using transmission electron microscopy (TEM). The amplified catabolic phenotype of *Hspg2*Δ3/-Δ3 mice may account for the age-dependent decline in GAG observed in tail tendon and changes in UTS/TM biomechanics. Collagen fibril diameter increased in WT but decreased in *Hspg2*Δ3/-Δ3 tail tendons over 3 to 12 weeks. Achilles tenotomy resulted in changes in tendon material properties in both genotypes but, *Hspg2*Δ3/-Δ3 mice had a slower recovery of UTS after tenotomy. HS deficiency in *Hspg2*Δ3/-Δ3 tendon impaired tenocyte repair responses to FGF-2 and led to deleterious changes in tendon organization which was reflected in changes in their material properties.
Diverse Effects of Perlecan-HS on Cell and Matrix Regulation: Tendon Destabilization in Hspg2 Exon 3 Null HS deficient Mice Reveals Essential Homeostatic Roles for HS in Cellular Regulation and Tendon Functionality.

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Running Title: Perlecan HS and tendon homeostasis

Keywords: Tendon; collagen; fibroblast growth factor; extracellular matrix; proteoglycan; heparan sulphate; tenocyte; biomechanics.
ABSTRACT.

The aim of this study was to determine the regulatory role of the perlecan HS side chains on cell and matrix homeostasis in tail and Achilles tendons in 3 and 12 week old Hspg2 exon 3 null HS deficient (Hspg2\(^{\Delta3/\Delta3}\)) and C57 BL/6 Wild Type (WT) mice. Tendons were biomechanically tested (ultimate tensile stress [UTS], tensile modulus [TM]) and glycosaminoglycan (GAG) and collagen (hydroxyproline) compositional analyses undertaken. Monolayer cultures of Hspg2\(^{\Delta3/\Delta3}\) tenocytes stimulated with FGF-2 showed elevated Adamts4, Mmp2, 3, 13 gene expression compared to WT mice. Col1A1, Vcan, Bgn, Dcn, Lum, Hspg2, Ltbp1, Ltbp2, Eln and Fbn1 showed no major differences between genotypes. Type VI collagen and perlecan were immunolocalised in tail tendon and collagen fibrils imaged using transmission electron microscopy (TEM). The amplified catabolic phenotype of Hspg2\(^{\Delta3/\Delta3}\) mice may account for the age-dependent decline in GAG observed in tail tendon and changes in UTS/TM biomechanics. Collagen fibril diameter increased in WT but decreased in Hspg2\(^{\Delta3/\Delta3}\) tail tendons over 3 to 12 weeks. Achilles tenotomy resulted in changes in tendon material properties in both genotypes but, Hspg2\(^{\Delta3/\Delta3}\) mice had a slower recovery of UTS after tenotomy. HS deficiency in Hspg2\(^{\Delta3/\Delta3}\) tendon impaired tenocyte repair responses to FGF-2 and led to deleterious changes in tendon organization which was reflected in changes in their material properties.
Introduction

Heparan sulphate (HS) is an ancient glycosaminoglycan (GAG) which has evolved over hundreds of millions of years of vertebrate and invertebrate evolution (Yamada, S., Sugahara, K., et al. 2011). HS has developed important cell regulatory and interactive properties with matrix components which stabilize the extracellular matrix (ECM) and maintain tissue homeostasis (Whitelock, J.M. and Iozzo, R.V. 2005). HS is attached to a number of matrix and cell associated proteoglycans (PGs) including, perlecan, agrin, type XVIII collagen, syndecan and glypican (Gallagher, J. 2015, Iozzo, R.V. and Schaefer, L. 2015). Perlecan is an important matrix organizational, stabilizing and cell-signaling hub in tissues. Besides its biodiverse range of interactive ECM components perlecan-HS also binds and delivers a number of growth factors (FGF-2, 7, 9, 18; VEGF; PDGF; Wnt; SHH; VEGF, BMPs) to their cognate receptors (Whitelock, J.M., Melrose, J., et al. 2008). The aim of the present study was to ablate these HS chains by deletion of exon 3 of perlecan core protein and determine what effect this had on the homeostasis and function of tendon. A level of redundancy is normally evident in physiological systems thus we envisaged that we should also see what other molecules assisted the HS chains of perlecan in such processes which maintain tissue functionality and homeostasis. As already noted, HS also occurs on a number of proteoglycans other than perlecan however it is not known to what extent these can fill-in for a deficit in the perlecan-HS chains. Our experimental design also allowed us to ascertain what accessory roles these may have in the maintenance of tissue function and homeostasis.
AFM studies have identified biomechanical roles for these pericellular components with perlecan providing a level of compliancy which may be cytoprotective (Li, Q., Doyran, B., et al. 2015, McLeod, M.A., Wilusz, R.E., et al. 2013, McNulty, A.L. and Guilak, F. 2015, Plodinec, M., Loparic, M., et al. 2010, Sanchez-Adams, J., Wilusz, R.E., et al. 2013, Taffetani, M., Raiteri, R., et al. 2015, Wang, M., Peng, Z., et al. 2012). Type VI collagen and perlecan interconnections between the cell and ECM, have mechanosensory roles. Perlecan is a minor proteoglycan in normal tendon but when tendon is damaged such as in a rotator cuff tendinosis model (Melrose, J., Smith, M.M., et al. 2013) the tenocytes dramatically increase their production of perlecan suggesting that it participates in a repair response. In the present study we were interested in ascertaining how ablation of the HS chains in perlecan of Hspg2 exon 3 null mice affected tendon organization and functional properties. We hypothesized that HS deficient tendons should be less capable of undergoing effective repair when challenged by a traumatic insult (tenotomy) or stress deprivation due to an inability of FGF-2 to promote repairative cell proliferation and matrix synthesis with mutant perlecan devoid of its interactive HS chains(Zhou, Z., Wang, J., et al. 2004). Hspg2 exon 3 null mice also lay down significantly lower tissue levels of TGF-β thus this important anabolic growth factor is unavailable to participate in such tissue repair processes(Shu, C., Smith, S.M., et al. 2016).
Materials and Methods

Ethics approval to conduct this study was obtained from The Animal Care and Ethics Review Board of The Royal North Shore Hospital, St. Leonards, Sydney, Australia. (RNS/UTS 0709-035A J Melrose, C Little, R Appleyard. Evaluation of Δ3-/Δ3- HSPG2 HS deficient mice).

Tissues

Hspg2Δ3-/Δ3- homozygous mouse breeding pairs backcrossed into a C57BL/6 background for 12 generations were kindly supplied by Dr R Soinninen, University of Oulu BioCentre, (Oulu, Finland). WT C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). All mice were caged in groups (n=2-5 mice per 500cm² cage floor space) and received acidified water and complete pelleted food ad libitum. All cages were individually ventilated with filter lids, sterilised Aspen chip bedding, environmental enrichment (tissues, house) and maintained at 20-22°C, 50-60% humidity, with a 12 hour light-dark cycle regimen. Only male mice were used for these studies.

Genotyping of Hspg2Δ3-/Δ3- mice

Genomic DNA was extracted from WT and Hspg2Δ3-/Δ3- mouse tail tips using commercial kits (Qiagen). Specific regions of the mouse perlecan gene were amplified by PCR using genotyping primers recognising intron 2 of mouse Hspg2 (GTA GGG ACA CTT GTC ATC CT), exon 3 (CTG CCA AGG CCA TCT GCA AG) and Hspg2Δ3-/Δ3- (AGG AGT AGA AGG TGG CGC GAA GG). The PCR products were identified by electrophoretic separation on 2% w/v agarose gels.
Isolation and identification of perlecan from skeletal muscle.

Muscle from the hind limbs of two WT and two Hspg2 exon 3 null mice were finely minced and extracted with 6M urea 50mM Tris-HCl pH 7.2 (15ml/g tissue) for 48h at 4°C. Perlecan was isolated using Resource Q anion exchange FPLC and electrophoresed on pre-poured 3-8% PAG Tris-acetate gradient gels, blotted to nitrocellulose and perlecan identified using MAb H300 (Santa-Cruz). The GAG side chains of these samples were also analysed by ELISA using MAb 10E4 and 3G10. Selected samples were pre-digested with Heparitinase III to generate Δ-HS stub epitopes to generate 3G10 reactivity.

Biomechanical assessment of murine tail and Achilles tendons

Tail tendons from 3, 6 and 12 wk mice were dissected from underlying bone and connective tissue within 1 h of death. Achilles tendons attached to the calcaneous and gastrocnemius muscles proximally were also collected. Biomechanical testing was undertaken in an Instron 8874 servo-hydraulic material testing apparatus. Tail tendons were marked with two reference points using Alcian blue and anchored in custom-built brass clamps. With Achilles tendons the calcaneous was anchored in the lower clamp, and gastrocnemius muscle attached to the upper clamp. Tendons were loaded to failure at a rate of 1mm/second (10% strain), real-time videos of each test were recorded at 100 frames per second using a high-speed camera mounted perpendicular to the tendon (Marlin F/145B camera, Allied Vision Technology, Massachusetts, USA). The videos of tendon deformation were analysed using LabView software (National Instruments, Austin, Texas, USA) and the data normalised to tendon cross-sectional area to calculate stress. Elastic modulus was calculated from the gradient of the linear region on the stress-strain curve.

Tendon compositional analyses
Finely-diced tail tendons were papain digested and sulfated GAG determined using 1,9-dimethylmethylene blue and bovine tracheal CS as standard (Farndale, R.W., Buttle, D.J., et al. 1986). Aliquots of the tissues were also hydrolysed in 6M HCl for hydroxyproline determinations by the dimethylaminobenzaldehyde procedure (Stegemann, H. and Stalder, K. 1967).

**Tendon injury models**

Isolated tail tendons from 12-week-old mice were cultured in DMEM/10% FBS/2mM L-glutamine for 5 days, then stored at –20°C wrapped in saline-gauze. This simulates acute stress deprivation.

**Achilles tendon tenotomy**

Under general anaesthesia (2% isofluorane) one Achilles tendon of 12wk mice in each genotype was sharply transected mid-way between the calcaneal attachment and muscle leaving the plantaris tendon intact. The skin incision was closed with a subcutaneous Vicryl 8/0 suture and sealed with cyanoacrylate tissue glue. Mice were returned to their pre-operative social groups post-tenotomy. Groups of mice (n=10) were sacrificed 2, 4 or 8 wk PO and Achilles material properties measured.

**Immunolocalisation of type XI collagen in annulus fibrosus monolayer cultures.**

Sheep AF cell monolayers were established as indicated earlier (Melrose, J., Smith, S., et al. 2003). After 3 days culture the cells were washed with TBS, fixed in ice-cold methanol, blocked 20 min in 0.01% H$_2$O$_2$ then 30 min in 5% BSA in TBS and primary antibody (5 µg/ml) was added (pAb HPA058335, Atlas Antibodies, Affinity purified polyclonal antibody to the type XI collagen α1-chain amino acid sequence (single letter code)
DCDSSAPKAQAQPQIDYEYDYEYHEEAESVTEGPTVTEETIAQTEANI
VDDFQEYNYG) overnight at 4°C. After washing, horse-radish peroxidase conjugated anti-rabbit IgG secondary antibody 1/1000 diln was used to visualize the bound primary antibody using diaminobenzidine as substrate.

Immunolocalisation of type VI Collagen and perlecan in tail tendon samples.

Longitudinal tail sections (4 μm) of 12 wk mice were attached to microscope slides, de-paraffinised in xylene and graded ethanol washes. Type VI collagen and perlecan were immunolocalised using rabbit anti type VI polyclonal and rat anti perlecan domain IV antibody MAb A7L6 (both obtained from abcam) (2μg/ml) overnight at 4° C. Alkaline phosphatase conjugated anti-mouse and anti-rat secondary antibodies were used for visualization using NovaRED chromogen.

Gene expression in tail tendons

Mouse tail tendons from 3, 6, and 12wk WT and Hspg2Δ3/Δ3- mice were pooled to provide ~50mg wet weight of tissue for each RNA isolation. Tendons were snap frozen in liquid nitrogen, freeze-shattered using a Mikro dismembranator (B. Braun Biotech International, Melsungen, Germany) and total RNA extracted using Trizol (Invitrogen, Mulgrave, VIC, Australia), purified using Qiagen RNeasy columns (Qiagen, Chadstone Centre, VIC, Australia) and quantified by NanoDrop (ThermoFisher Scientific, Scoresby, VIC, Australia). RNA (1μg) from each sample was reverse transcribed using Omniscript Reverse Transcription Kit (Qiagen) with random pentadecamers (50ng/ml, Sigma-Genosys, Castle Hill, NSW, Australia) and RNase inhibitor (10U per reaction, Bioline, Sydney, NSW, Australia). The cDNA was subjected to q RT-PCR in a Rotorgene 6000 (Qiagen) using Immomix (Bioline,
Sydney, NSW, Australia), SYBR Green I (Cambrex Bioscience, Rockland, ME, USA), and 0.3µM validated murine-specific primers. Relative copy numbers for genes of interest were determined using a standard curve generated from pooled cDNA normalised to Gapdh. PCR primer specificity was confirmed by sequencing (SUPAMAC, Sydney University). Genes, primers, and annealing temperatures are listed in Table 1.

Tendon outgrowth tenocyte culture

3 wk tail tendons were macerated and cultured in 2ml DMEM/10% FBS/2mM L-glutamine under an atmosphere of 5% CO₂, with media changes every 3-4 days. After 2 weeks the tissue was removed and the attached cells detached with trypsin-EDTA and sub-cultured in fresh media. Passage 3 cells were cryopreserved (10⁷ cells/ml, 0.5ml aliquots) in 10% v/v DMSO, 20% v/v FBS in DMEM. Tenocytes were re-seeded in 6-well plates at 2 x 10⁵ cells per well for 48 h. The cultures were washed three times in serum-free DMEM, and incubated in DMEM/1% v/v FBS containing 0, 1, 10 or 100ng/ml FGF-2 (PeproTech Inc, Rocky Hill, NJ, USA) for 24 h. Total RNA was extracted using Trizol, 1µg RNA was reverse-transcribed then qRT-PCR undertaken for Mmp2, Mmp3, Mmp13, Timp1, Timp3 and Adamts4.

Transmission electron microscopy of tail and Achilles tendon

Three tendons from 3- and 12-wk WT and Hspg²α³⁻/⁻ mice were washed in 0.1M sodium cacodylate buffer containing 3mM CaCl₂, 100mM sucrose at pH 7.4 and fixed in 2.5% v/v glutaraldehyde/0.5% v/v paraformaldehyde for 30 min at room temperature, 4°C for 24h, followed by storage in 70% v/v ethanol. The fixed tissues were trimmed and post-fixed in 2% w/v OsO₄ in 0.1M cacodylate buffer for 1-2h at 4°C followed by dehydration in graded ethanol washes (25%, 50%, 75%, 95%, 100%, 100%, all v/v). The tissues were infiltrated with
Spurrs resin/ethanol (1:1) overnight then with two overnight infiltrations of 100% resin then polymerised at 60°C for 48h. Ultra-thin transverse tissue sections (70 nm) were cut using an Ultracut T microtome and transferred to copper grids (200 mesh). The specimens were stained/contrasted for 10 min with 2 % w/v uranyl acetate and Reynold’s lead citrate (1.33g lead nitrate, 1.76g sodium citrate dihydrate, 5ml 1M NaOH, in 50ml H₂O final total volume). The specimens were examined in a JEOL1400 transmission electron microscope at 120 kV at 25,000× magnification. The images were analysed using ImageJ (public domain Java-based image processing software developed by NIH) to determine fibril diameters. Three separate regions of each specimen were photographed and all fibrils were measured in each image. When the fibril had a non-circular configuration the diameter across the minimum axis was measured. The frequency distribution of the collagen fibril diameters was calculated as a percentage of the total fibril numbers measured.

Statistical analyses

Parametric data (mechanical properties, hydroxyproline, sGAG, fibril diameters) were analysed by unpaired Students-t test for differences between age and genotypes. Non-Gaussian data (qRT-PCR) were analysed by Mann-Whitney U ranked tests. The alpha level was set at 0.05.
Results

Genotyping of mouse strains

The schema in Fig 1a depicts the replacement of perlecans exon-3 with a pGK-neo cassette in the Hspg2Δ3-/Δ3- mice. Hspg2Δ3-/Δ3- mice were fertile and litters were of expected size. There were no gross abnormalities or difference in appearance between WT and Hspg2Δ3-/Δ3- animals at birth. By 3 weeks of age, the previously reported microphthalmia in Hspg2Δ3-/Δ3- animals was evident. Age-matched Hspg2Δ3-/Δ3- mouse body weights were less than corresponding WT mice 10 to 18 weeks of age (Fig 1b). Although smaller, Hspg2Δ3-/Δ3- mice had similar skeletal proportions to WT mice and no apparent musculoskeletal abnormalities. Mutant mice were more docile when handled but no other behavioral abnormalities were noted.

Perlecans isolated from C57BL/6 Wild Type and Hspg2 exon 3 null mice.

Perlecans isolated from skeletal muscle demonstrated a core protein of ~420 kDa for WT perlecans while mutant perlecans had a molecular weight of ~300-390 kDa. ELISA analysis demonstrated that perlecans from C57BL/6 Wild Type mice contained HS chains while Hspg2 exon 3 null perlecans did not (Fig 1c).

Tendon material properties and biochemical composition

Ultimate tensile stress (UTS) (Fig 1d, e) and tensile modulus (TM) (Fig 1f, g) measurements of tail (Fig 1d, f) and Achilles (Fig 1e, g) 3-12 wk tendons demonstrated there were no difference in 3-wk tendons. UTS and TM of tail and Achilles tendons
increased with maturation (Fig 1 d-g). Tail tendon sGAG levels underwent a maturation-dependent decline in \(Hspg^{2\Delta3/-\Delta3}\) mice (Fig 1h), with 50% reduction in GAG content at 12 weeks compared to 3 weeks \((3 > 6 > 12 \text{ week})\), and lower GAG content in \(Hspg^{2\Delta3/-\Delta3}\) mice whereas hydroxyproline contents did not change significantly with age or genotype (Fig 1i).

Effect of stress-deprivation and Achilles tenotomy on tendon repair response

Increased UTS \((2.0\pm0.4 \text{ fold}; P = 0.026)\) and TM \((2.9\pm0.8 \text{ fold}; P = 0.026)\) was evident in cultured 12wk WT tail tendon compared to \textit{ex vivo} but no significant change in UTS \((1.5\pm0.2 \text{ fold})\) or TM \((1.5\pm0.3 \text{ fold})\) in \(Hspg^{2\Delta3/-\Delta3}\) tail tendons following 5 days of culture (data not shown). This differential response to stress-deprivation meant that after 5 days culture there was no longer a difference in material properties between WT and \(Hspg^{2\Delta3/-\Delta3}\) tail tendons. There was a significant reduction in UTS and TM at 2 weeks after Achilles tenotomy in both genotypes compared to the contralateral tendon (Fig 1j-m), and a progressive increase in material properties with time after injury. The injured WT Achilles tendon recovered to the contralateral value by 8 weeks however \(Hspg^{2\Delta3/-\Delta3}\) Achilles tendons did not recover to the same extent (Fig 1j-m).

Pericellular localization of perlecan and type XI collagen in monolayer cultures of annulus fibrosus disc cells and articular chondrocytes.

Fine microfibrillar perlecan positive material was observed in the pericellular matrix of monolayer cultures of annulus fibrosus disc cells (Fig 2a), type XI collagen had a similar localization pattern (Fig 2c). Studies with articular chondrocytes have also demonstrated a
pericellular localization of type XI collagen using antibodies to its HS binding regions (Fig 2d, e). This localization was HS dependent, pretreatment of chondrocyte cultures with heparitinase-III greatly diminished this type XI collagen localization pattern (Fig 2f, g).

Hierarchical organization of tendon collagen fibres/fibrils and tenocytes

Tendons are assembled from collagen fibre bundles, and each fibre is composed of a number of collagen fibrils. These are depicted diagrammatically (Fig 2h). Tenocytes are embedded within the collagen fibrillar arrangements and are an elongated cell type (Fig 2i). Type I collagen is the major tendon component, type VI collagen and elastin are also pericellular components around tenocytes. Minor amounts of type V and type XI collagen are distributed throughout the type I/XI/V heterofibril (Fig 2j). The \( \alpha_1 \) chain of collagen XI has a prominent N-terminal domain which protrudes through to the fibre surface and has two HS interactive sites which may attach the heterofibril pericellularly to HS chains of perlecan.

Immunolocalisation of type VI collagen and perlecan in tail tendon

Type VI collagen was immunolocalised in longitudinal sections of mouse tail tendons from C57BL/6 Wild Type and \( Hspg2 \) exon 3 null mice (Fig 2k, l). Higher power views showed a significant reduction in collagen VI deposition in the \( Hspg2 \) exon 3 null tendons. Perlecan was also localized in ovine ACL positive control, WT and \( Hspg2 \) exon 3 null tendons (Fig 2). Mouse tendon perlecan was evident at each pole of the tenocytes (Fig 2).

Tail tendon gene expression
qRT-PCR of selected ECM genes in mouse tail tendons, (\textit{Col1a1}, \textit{Vcn}, \textit{Bgn}, \textit{Dcn}), demonstrated a maturation-dependent decline in gene expression over 3 to 12 weeks in both genotypes (Figure 3a). Relative gene expression levels for perlecan core protein (\textit{Hspg2}) and the elastin micro-fibril associated proteins \textit{Ltbp1}, \textit{Fbn1}, and \textit{Eln} were significantly lower in \textit{Hspg2}^{\Delta3/\Delta3} mice compared to age-matched WT mice. \textit{Ltbp2} gene expression displayed an increase with ageing in both genotypes.

\textit{Tendon outgrowth cell responses to FGF-2}

\textit{Mmp2}, \textit{Mmp3}, \textit{Mmp13} and \textit{Adams4} expression were significantly higher in basal \textit{Hspg2}^{\Delta3/\Delta3} mice compared to WT tenocyte cultures (0ng/ml FGF-2). \textit{Mmp2} expression in \textit{Hspg2}^{\Delta3/\Delta3} cultures remained significantly higher than WT at all doses of FGF-2 examined. \textit{Mmp3} and \textit{Mmp13} expression increased dose-dependently in both genotypes, and this response was greater in \textit{Hspg2}^{\Delta3/\Delta3} tenocytes at high doses of FGF-2 (\textit{Mmp3} 33-36 fold versus 50 fold and \textit{Mmp13} 134-192 fold versus 226-248 fold at 10 and 100ng/ml in WT and \textit{Hspg2}^{\Delta3/\Delta3}) (Fig 3b). \textit{Adams4} gene expression was significantly decreased by FGF-2 treatment in \textit{Hspg2}^{\Delta3/\Delta3} tenocyte cultures but still remained significantly greater than in WT cultures at all doses. FGF-2 up-regulated \textit{Timp1} gene expression, less so in \textit{Hspg2}^{\Delta3/\Delta3} compared to WT cultures, favoring a pro-catabolic phenotype in the mutant mice. Expression of \textit{Timp3}, the naturally occurring inhibitor of the ADAMTS (\textbf{A}\textbf{D}isintigrin \textbf{A}\textbf{N}d \textbf{M}etalloprotease with Thrombospondin motifs) enzymes, while equivalent in basal culture, was decreased to a greater extent by FGF-2 in \textit{Hspg2}^{\Delta3/\Delta3} tenocyte cultures at the highest dose.

\textit{Tail tendon collagen fibril diameter by transmission electron microscopy}
Figure 4 shows the changes in collagen fibril diameter of tail and Achilles tendon measured from TEM images of 3 and 12 wk mouse tendons. In 3 wk mice there were small differences between genotypes in mean fibril diameter (±SD) in tail (WT = 202±60nm, \(Hspg^{2\Delta3/\Delta3}\) = 193±46nm; not significant) and Achilles (WT = 160±44nm, \(Hspg^{2\Delta3/\Delta3}\) = 139±37nm; \(P < 0.001\)) tendons (Fig 4a, b). This was reflected in minor differences in frequency distributions of collagen fibril diameters in the two genotypes in these immature mice (Fig 4c, d). An increase in average collagen fibril diameter was evident from 3 to 12 wk in the WT tail (202±60nm to 243±81nm; \(P < 0.0001\)) and Achilles (160±44nm to 210±63nm; \(P < 0.001\)) tendons, accompanied by an increase in collagen fibril diameter distribution in both tendons (Fig 4c, d). Mean Achilles collagen fibril diameter also increased from 3-12 wk in \(Hspg^{2\Delta3/\Delta3}\) mice (139±37nm to 150±34nm \(P < 0.001\)) to a lesser extent than in WT mice (8 versus 31%), and significantly decreased in \(Hspg^{2\Delta3/\Delta3}\) tail tendons (193±46nm to 84±28nm; \(P < 0.001\)). Thus differences between genotypes in mean collagen fibril diameter and distribution in tail and Achilles tendons were more marked by 12 weeks of age.
Discussion

In the present study we describe for the first time how ablation of HS chains from perlecan domain-I detrimentally affected tenocyte behavior, tendon material properties and collagen fibril structure in Achilles and tail tendons. HS interacts with soluble effectors (e.g. growth factors, morphogens, chemokines), membrane receptors and cell adhesion proteins such as fibronectin, fibrillin and several collagens including type IV, V, VI, XI collagen (Gallagher, J. 2015). The extensive interactive properties of HS, and its pericellular distribution provides a direct link between the cell and its extracellular microenvironment, both of these are critical in the maintenance of tissue homeostasis and functional properties.

The principal extracellular HS-PGs are perlecan, agrin and collagen XVIII which possess large modular core proteins that interact with a diverse repertoire of other ECM components and contribute significantly to matrix organization and stabilization (Whitelock, J. and Melrose, J. 2011). Two families of cell surface PGs are also substituted with HS the transmembrane syndecans and the GPI-anchored glypicans however neuropilin, betaglycan and CD44 also occasionally contain HS (Gallagher, J. 2015).

HS interacts with a number of growth factors including the FGF family, VEGF, PDGF, Wnt, SHH and BMPs. When attached to perlecan domain-1, HS equips perlecan with low affinity co-receptor properties sequestering these growth factors and presenting them to their cognate receptors(Whitelock, J.M., Melrose, J., et al. 2008).
In the present study we have proposed that HS-collagen V and XI interactions in the type I collagen heterofibril were critical to tendon stability. Thus in the HS deficient \textit{Hspg2} exon 3 null mouse we observed a destabilization of tail and Achilles tendons over time which was not observed in WT tendons and TEM demonstrated the appearance of collagen fibrils of small diameter over time in the HS deficient mice. This was accompanied by deleterious changes in tendon material properties in the HS deficient tendons. We also observed a maturational decline in the sulphated GAG content of murine tendons and this was more marked in the HS deficient tendons. The \textit{Hspg2} exon 3 null mouse also had a poor repair response following Achilles tenotomy. This is consistent with the tenocyte FGF-2 culturing experiments which demonstrated a disturbance in the ability of the HS deficient tenocytes to signal through FGF-2. FGF-2 failed to elicit an anabolic response in \textit{Hspg2} exon 3 null tenocytes but upregulated production of MMP-2, 3, 9, 13 and ADAMTS-4 and lowered expression of TIMP-1 and TIMP-3. MMP-2 not only cleaves collagens and other matrix components (Bauvois, B. 2012, Wells, J.M., Gaggar, A., et al. 2015) (reviewed in (Burrage, P.S., Mix, K.S., et al. 2006)) but it also releases the active FGFR1 ecto-domain (Levi, E., Fridman, R., et al. 1996). FGF-2 signalling through FGFR1 produces catabolic effects in cartilage leading to excessive ECM turnover and may also antagonise productive associations between FGF-2 with FGFR3, which promote anabolic responses (Levi, E., Fridman, R., et al. 1996, Yan, D., Chen, D., et al. 2011). FGF-2 is a mechanotransducer in articular cartilage acting via perlecan domain-I HS to sequester FGF-2 in the tissue (Vincent, T.L., McLean, C.J., et al. 2007). Unloading/injury causes release of FGF-2 in tendon and cartilage (Ellman, M.B., Yan, D., et al. 2013). By analogy with FGF-2 mediated mechanotransduction in cartilage (Vincent, T., Hermansson, M., et al. 2002, Vincent, T. and Saklatvala, J. 2006,
Vincent, T.L., Hermansson, M.A., et al. 2004) Hspg2^∆3-/∆3^- tendon displayed compromised FGF-2 dependent mechano-transductive cell signaling (Solchaga, L.A., Penick, K., et al. 2010). Findings of the present study showed that the presence of HS substitution on other matrix and cell associated proteoglycans does not appear to be able to compensate for the HS-deficiency in perlecan exon 3 mutant mice demonstrating the key role the HS chains of perlecan plays in tissue homeostasis and the maintenance of tendon functional properties.

Conclusions

Ablation of the HS side chains of perlecan in Hspg2 exon 3 null mice resulted in maturational changes in tendon organization and material properties and a reduction in the GAG content of tail and Achilles tendon. Collagen fibril organization was also disrupted with HS deficiency with the appearance of collagen fibrils of significantly smaller cross-sectional area. The ability of Achilles tendon to undergo repair was also diminished following tenotomy due to an inability of tenocytes of Hspg2 exon 3 null tendons to participate in anabolic repair processes and was also reflected in tendon material properties.

Monolayer cultures of tenocytes isolated from Hspg2 exon 3 null tendons were less responsive to FGF-2 stimulation in terms of synthesis of matrix components (collagen, GAG). Tenocytes from Hspg2 exon 3 null tendons however expressed elevated levels of active MMPs and reduced production of TIMPs correlating with reduced GAG levels at 12 weeks. The disruption in tendon organization in Hspg2 exon 3 null tendons apparently was due to a lack of HS mediated interactions with tendon Type VI and XI collagen which normally stabilize tendons and exacerbated by the catabolic phenotype of tenocytes in these tendons. Perlecan HS thus has important roles in the maintenance of tendon homeostasis and normal tenocyte function. The HS chains of other HS-proteoglycans such as type
XVIII collagen, agrin and the cell associated syndecan and glypican families were incapable of rescuing these degenerative changes in Hspg2 exon 3 null tendon.

Disclosure of potential conflict statement

The authors state they have no conflicts or financial interests to disclose.

Author Contributions.

CBL and JM conceived and designed the experiments, interpreted the results and made intellectual contributions to manuscript writing and editing. CS performed all of the experiments and data preparation and analysis and aided in manuscript preparation and editing. MMS undertook statistical analyses, critical editing of the manuscript and had intellectual input into experimental design. RCA designed the biomechanical apparatus and biomechanical protocols, supervised experiments, had input into data analysis and had input into manuscript preparation and editing. All authors approved the final version of the manuscript.

Acknowledgements

This study was funded by NHMRC Project Grant 1004032. Dr Joanna Peterson developed the tendon materials testing procedures used in this study as part of her PhD studies at The Murray-Maxwell Biomechanics Laboratory within the Institute of Bone and Joint Institute of The Kolling Institute of Medical Research. The expert assistance of Ms Susan Smith for the tendon immunolocalisations reported in this study is acknowledged.
Figure 1. (a) Genomic organisation of exons 2-5 of the WT and Hspg2\textsuperscript{2\Delta3/\Delta3} mice (a). Body weights of male WT (○-○) and Hspg2\textsuperscript{2\Delta3/\Delta3} mice (□-□) from 10-20 weeks of age (b). ELISA analysis of perlecan GAG side chains using MAAb 10E4 to native HS and MAAb 3G10 to the ΔHS stub epitope generated by heparitinase III pre-digestion demonstrating an absence of HS in the mutant perlecan. (c)

Tendon material properties: ultimate tensile stress (d, e) and tensile modulus (f, g). GAG (h) and HyPro (i) content of 3 to 12 week-old WT and Hspg2\textsuperscript{2\Delta3/\Delta3} tail tendons. Box plots show mean (line in box) and data range (box 25-75%, whiskers maximum-minimum). White bars: WT; Gray bars: Hspg2\textsuperscript{2\Delta3/\Delta3}. Bar graph shows mean ± standard error of mean. Brackets - $P < 0.05$ between samples, * $P < 0.05$ between genotypes. N = 6-8 for each sample.

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Immunolocalisation of type VI collagen and perlecan in mouse tail tendon. Vertical sections of mouse tails with collagen VI localization shown in C57 BL/6 Wild Type (k) and Hspg2 exon 3 null (l) mouse tails. Negative control slides are also shown for each phenotype (m, n). The boxed areas in (k) and (l) are also presented at higher magnification. Notice the reduced size of the Hspg2 exon 3 null tendons compared to WT. Immunolocalisation of perlecan in ovine ACL positive control tissue (o) and corresponding negative control (p) and in WT mouse tail tendon and Hspg2 exon 3 null tendon.

Figure 3. Comparative gene expression of selected extracellular matrix genes and elastin-associated protein genes in mouse ex vivo tail tendons at 3, 6 and 12 weeks old (a). * $P < 0.05$ between genotype. Data were normalised to Gapdh expression. White bars: WT; Gray bars: Hspg2\textsuperscript{2\Delta3/\Delta3}. Box plot shows mean (line in box), interquartile range (box) and data range (whiskers, maximum – minimum). N = 6 per sample.

Gene expression in 3 week-old mouse tail tendon outgrowth tenocytes cultured with FGF-2 (0, 1, 10 or 100ng/ml) (b). Data expressed as a fold change relative to the expression in the WT control (0 ng/ml FGF-2). White bars: WT; Gray bars: Hspg2\textsuperscript{2\Delta3/\Delta3}. Box plot shows mean (line in box), interquartile range (box) and maximum – minimum (whiskers). N = 6 for each.
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BIBLIOGRAPHY


**Figure 1** (on next page)

Genomic organisation, mouse weights, ELISA of perlecan GAGs and tendon material properties.

Flexible Roles for Proteoglycan Sulfation and Receptor Signaling **Figure 1.** (a) Genomic organisation of exons 2-5 of the WT and *Hspg2*Δ3-Δ3- mice (a). Body weights of male WT (■ -■ ) and *Hspg2*Δ3-Δ3- mice (□-□) from 10-20 weeks of age (b). ELISA analysis of perlecan GAG side chains using MAb 10E4 to native HS and MAb 3G10 to the ΔHS stub epitope generated by heparitinase III pre-digestion demonstrating an absence of HS in the mutant perlecan. (c) Tendon material properties: ultimate tensile stress (d, e) and tensile modulus (f, g). GAG (h) and HyPro (i) content of 3 to 12 week-old WT and *Hspg2*Δ3-Δ3- tail tendons. Box plots show mean (line in box) and data range (box 25-75%, whiskers maximum-minimum). White bars: WT; Gray bars: *Hspg2*Δ3-Δ3-. Bar graph shows mean ± standard error of mean. Brackets - *P* < 0.05 between samples, *P* < 0.05 between genotypes. N = 6-8 for each sample. Achilles tendon material properties at 2, 4 and 8 weeks after surgical tenotomy: normal contralateral (j, k) and surgical tenotomy (l, m), equivalent to 14, 16 and 20 weeks of age. White bars: WT; Gray bars: *Hspg2*Δ3-Δ3-. Box plot show median (line in box), inter-quartile range (box) and data range (whiskers, maximum – minimum). Bracket – *P* < 0.05 between samples. *P* < 0.05 between genotypes. # *P* < 0.05 to contralateral. N = 6-8.
Achilles tenotomy

Hspg2

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.26644v1 | CC BY 4.0 Open Access | rec: 8 Mar 2018, publ: 8 Mar 2018
**Figure 2** (on next page)

Immunolocalisation of type VI and XI collagen and perlecan in WT and HSPG2 exon 3 null tendons and schematic of tendon organisation.

**Figure 2.** Demonstration of pericellular perlecan (a) and type XI collagen (c) produced by AF cells in monolayer culture and pericellular type XI collagen produced by chondrocytes and identified with antibodies to its HS binding sites (d, e). Heparitinase-III treatment significantly reduced the pericellular localization of type XI collagen (f, g). Segments d-g reproduced from (Warner, L.R., Brown, R.J., et al. 2006). Diagram showing the hierarchical organization of collagen fibres and fibrils (h) in heterotopic type I/XI/V collagen fibres in tendon and the pericellular microenvironment of tenocytes. Pericellular perlecan/type VI collagen distribution pericellularly and strings of tenocytes (i). Perlecan C-terminal interaction with $\alpha_2\beta_1$ integrin and the $\alpha_1$ chain of type XI collagen with the HS chains of perlecan domain-I (j). Type XI and V collagen regulate fibrillogenesis and stabilize the collagen fibril. Immunolocalisation of type VI collagen and perlecan in mouse tail tendon. Vertical sections of mouse tails with collagen VI localization shown in C57 BL/6 Wild Type (k) and *Hspg2* exon 3 null (l) mouse tails. Negative control slides are also shown for each phenotype (m, n). The boxed areas in (k) and (l) are also presented at higher magnification. Notice the reduced size of the *Hspg2* exon 3 null tendons compared to WT. Immunolocalisation of perlecan in ovine ACL positive control tissue (o) and corresponding negative control (p) and in WT mouse tail tendon and Hspg2 exon 3 null tendon.
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