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Research Article

A Speculative Role for Perlecan, an Instructive Multifunctional Proteoglycan with Matrix Stabilising and Cell Regulatory Properties in Anterior Cruciate Ligament Repair

James Melrose*

Raymond Purves Bone and Joint Research Laboratory, Kolling Institute Northern Sydney Local Health District, St. Leonards, NSW 2065, Australia

Sydney Medical School, Northern, The University of Sydney, Royal North Shore Hospital, Australia

Graduate School of Biomedical Engineering, University of New South Wales, Kensington, NSW 2052, Australia

***Corresponding author**

James Melrose, Raymond Purves Bone and Joint Research Laboratories, Institute of Bone and Joint Research, Kolling Institute of Medical Research, Level 10, B6, The Royal North Shore Hospital, St. Leonards, NSW 2065, Australia, Email: james.melrose@sydney.edu.au

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Abstract

Purpose/aim of the study: To immunolocalize perlecan in anterior cruciate ligament (ACL) and review its potential to promote ACL repair.

Materials and Methods: Perlecan immunolocalisations were undertaken with an antibody to perlecan domain IV (MAb A7L6), toluidine blue staining visualized ACL glycosaminoglycan. Primary ACL cells were cultured in monolayers, cellular morphologies were compared with meniscal, synovial fibroblast and articular chondrocytes from the same knee joints.

Results: The ACL contained toluidine blue stained proteoglycan associated with linear arrays of fusiform cells between collagen fibre bundles and plumper cells in vascular channels. Perlecan interconnected aligned fusiform cells in collagenous regions and rounded cells in vascular channels. Prominent actin cytoskeletons in monolayer culture demonstrating a contractile phenotype similar to myofibroblasts.

Discussion: This study showed perlecan interconnected strings of ACL cells in anteriomedial and posteriolateral collagenous bundles in the ACL connecting the tibia and femur providing rotational stability in the knee joint. Type I collagen (70-80%), elastin (5-6 %) and proteoglycans (2-5%) are major ACL components. A review of perlecan's roles in matrix stabilisation, mechanotransduction, growth factor mediated cellular proliferation and differentiation and its co-localisation with elastin in tensional and weight bearing connective tissues suggest it has potential contributions to make in functional ACL repair. Recently developed methods that have provided improved immobilization and a stable repair environment conducive to ACL repair indicate that the roles of perlecan in ACL repair processes deserve evaluation based on its known functional properties in other tensional and weight bearing connective tissues.

ABBREVIATIONS

ACL: Anterior Cruciate Ligament; AF: Annulus Fibrosus; CS: Chondroitin Sulfate; DMEM: Dulbecco's Modified Eagles Medium; ECM: Extracellular Matrix; EGF: Epidermal Growth Factor; FCS: Foetal Calf Serum; FGF: Fibroblast Growth Factor; HRP: Horse Radish Peroxidase; HS: Heparin Sulfate; IGF-I: Insulin-Like Growth Factor-I; KS: Keratin Sulfate; Mab: Monoclonal antibody; MMP: Matrix Metalloprotease; OA: Osteoarthritis; NP: Nucleus Pulposus; PCM: Pericellular Matrix; PDGF: Platelet Derived Growth Factor; PBS: Phosphate Buffered Saline; SLRP: Small Leucine Repeat Proteoglycan; TBS: Tris Buffered Saline; TGF-β: Transforming Growth Factor-beta; TIMP: Tissue Inhibitor of Matrix Metalloprotease; TNFα: Tumour Necrosis Factor-alpha; VEGF: Vascular Endothelial Cell Growth Factor

INTRODUCTION

The aim of this study was to demonstrate perlecan was a component of anterior ligament (ACL) and to assess its potential roles in ACL repair responses. Type I collagen is the dominant protein of tendon and ligament and it provides strength and stiffness. However, the stressed collagen fibres in these tissues are virtually inextensible which is an important requirement for load transmission in tendon. Ligaments have a greater elastin content than tendon and are thus more compliant and resilient. Elastin provides tendon and ligament with re-coil properties, returning stressed collagen fibres to their pre-stressed dimensions once they are un-loaded. In tendons the elastin content varies between 2-4% of the tissue dry weight while ligaments have higher elastin content (5-6%) [1,2]. Furthermore, perlecan co-localises with elastin in many connective tissues and has known roles in ECM

stabilisation through its ability to interact with a vast repertoire of ECM components.

The anterior cruciate ligament (ACL) has an important stabilising role in the knee-joint, a major multidirectional weight bearing and articulatory structure. The anteriomedial and posteriolateral bundles of the ACL resist anterior tibial translation and provide rotational stability however they are frequently injured during high impact or sporting activities [3]. Ruptured ACLs do not heal spontaneously like other ligaments in the knee joint and currently require surgical intervention to stabilise the knee-joint which allows functional recovery of its tissues. ACL tears are one of the most common knee injuries, with over 100,000 tears occurring annually in the United States [4]. It is important that normal knee-joint kinematics and stabilisation are rapidly re-established in the injured knee since an unstable knee predisposes knee joint tissues to premature onset of osteoarthritis (OA) [4].

The knee joint ligaments are composed of parallel tightly packed collagen fibre bundles arranged in hierarchical arrays, an elastin content of 5-6% provides visco-elastic compliancy and resilient properties to the ACL [5]. The ACL is composed of two spirally arranged anteromedial and posterolateral bundles that connect the tibia and femur providing mechanical stability [5]. Approximately 65-70% of a ligament is water. Type I collagen constitutes 70-80%, type III collagen 8% and type V collagen 12% of the ligament dry weight. Ligaments also have an appreciable elastin content which provides them with dynamic viscoelastic compliancy. A number of ligament proteoglycans have also been identified. Aggrecan and versican are present in the fibrocartilaginous insertions and ligament mid-substance. Two members of the small leucine rich proteoglycan (SLRP) family, decorin and biglycan are associated with collagen fibre bundles with decorin constituting ~90% of the total ligament proteoglycan while versican is the major large CS-proteoglycan of ligament. This study has shown that the large modular, HS-proteoglycan perlecan is also present in ligament. Perlecan has previously been identified in the infraspinatus tendon and in a rotator cuff tendinopathy model [6] and colocalizes with elastin in the ACL. Perlecan is a modular instructional multifunctional proteoglycan [7] with cell regulatory properties [8] and is an ECM component expected to allow ligaments to adapt to alterations in mechanical loading or in response to exercise in a similar manner to its biomechanical roles in articular cartilage and meniscus. Perlecan has also been immunolocalised in macroscopic views of the ACL and shown to co-localise with elastin, another important functional component of ligaments [9] but no studies have so far demonstrated high power localisations of perlecan in ligament. The aim of this study was to remedy this deficiency and to compare the synthesis of perlecan by ligament cells in culture compared to other cell types of the knee joint including chondrocytes, synovial cells and meniscal cells. A further aim of this study was to assess what potential roles perlecan might have in ligament repair processes based on perlecan's known properties in the stimulation of tissue repair through its ability to promote proliferation and differentiation of resident cell populations through growth factor sequestration and matrix stabilization through interactions with multiple ECM components. Platelets attach to perlecan through interactions with perlecan domain III and V [10]. Platelets express factors that promote tissue repair effecting angiogenesis, inflammation and the immune response. Platelets promote the formation of a fibrin clot at sites of vascular damage limiting blood loss. The release of platelet factors including growth factors, chemokines and cytokines from its α-granules creates chemotactic gradients stimulating cell migration and differentiation, recruitment of stem cells promotes repair of regions of tissue damage [11]. Platelets are essential for the innate immune response to combats viral, bacterial and fungal infection. Platelets also help maintain and modulate inflammation and are a major source of pro-inflammatory molecules thus while they initiate the inflammatory process which is beneficial in the early stages of wound repair they also down regulate inflammation in the latter stages of wound repair preventing any potential tissue damage which can result if inflammation spirals out of control [12]. Perlecan domain V also promotes tissue repair by influencing cell migration, adhesion and ECM stabilisation through its multifunctional interactions with a range of ECM components.

Recent studies have also shown that perlecan has biomechanical roles as a pericellular component of chondrocytes where it modulates the compressive loads transmitted to the chondrocytes by type VI collagen in the chondron structure [13]. FGF-2 has chondroprotective mechanotransducer roles in articular cartilage through interactions with perlecan domain-I HS which sequesters FGF-2 in this tissue [14]. These mechanotransductive events orchestrate cell-matrix communication critical for the maintenance of optimal tissue composition, structural organisation and functional properties of tensional tissues.

MATERIALS AND METHODS

Chondroitinase-ABC and clostridial collagenase were obtained from Sigma-Aldrich, Castle Hill, NSW, Australia. Menzel and Glaser SuperFrost ultraPlus, positively charged microscope sides were obtained from Fisher Scientific, Braunschweig, GmbH. NovaRED peroxidase substrate was obtained from Vector Laboratories (Burlingame, CA, USA). Rat monoclonal anti-perlecan domain-IV antibody (mAb A7L6) was obtained from abcam Sydney, Australia. This monoclonal antibody raised against bovine perlecan in rats is also cross-reactive with ovine perlecan, it works well in formalin fixed paraffin embedded tissue sections and has been used extensively in a range of sheep as well as human and murine tissues [15-20]. Monoclonal antibody A76 has also recently been employed in the 3D localisation of perlecan in the ovine annulus fibrosus and nucleus pulposus demonstrating perlecan is an intracellular and pericellular proteoglycan [21].

Biotinylated anti-rat IgG secondary antibody, avidin HRP conjugate and non-protein block were obtained from Dako, Botany, NSW, Australia. Foetal calf serum was obtained from Trace Biosciences Pty. Ltd., Castle Hill, NSW, Australia.

Tissues and cells

Ligaments were harvested from twelve 2 year old merino castrated male control wether sheep from a related project. Ovine synovial fibroblasts, meniscal cells and articular chondrocytes from ovine stifle joints were available in-house from our cryo-storage cell collection. All animal welfare and ethics for this work

were approved by the University of Sydney Animal Care and Ethics Committee under ethics approval A45/6-2011/3/5544.

Isolation and monolayer culture of ACL cells

Pooled ACL were separated into anteriomedial and posteriolateral bundles from left and right stifle joints of six 2 year old sheep, finely diced and the cells released by enzymatic digestion with pronase and clostridial collagenase and established in monolayer culture in Dulbeccos modified eagles medium (DMEM)-F-12 containing 10% foetal calf serum (FCS) and cultured for 6 days. The media was changed on days 2 and 4 and on day 6 the monolayers were fixed in 10% neutral buffered formalin for 60 min, rinsed in PBS and primary antibody to perlecan domain IV MAb A7L6 (2 µg/ml) diluted in Tris 0.15 M NaCl 2% BSA pH 7.2 (TBS-BSA) added at 4°C overnight. Following rinsing, HRP conjugated anti rat IgG (5 µg/ml) in TBS-BSA was added and after 1h binding at room temp the slides were washed again in TBS and NovaRED substrate added for colour development at room temp.

Preparation of ACL Histology specimens

ACL specimens were separated into anteriomedial and posteriolateral bundles and fixed in 10% neutral buffered formalin for 8h, dehydrated in sequential ethanol washes then embedded in paraffin. Four micron longitudinal sections were cur from the paraffin blocks and attached to SuperFrost ultraPlus positively charged microscope slides, de-paraffinised in xylene (2 changes x 5 min), and re-hydrated in graded ethanol (100-70% v/v) to water.

HISTOCHEMISTRY

Toluidine blue staining

ACL tissue sections were stained for 10 min with 0.04% w/v toluidine blue in 0.1 M sodium acetate buffer, pH 4.0, to visualize the anionic glycosaminoglycans and counterstained for 2 min in 0.1% w/v fast green FCF.

Haematoxylin and Eosin staining

Cellular morphology was routinely assessed by staining tissue sections with Mayers Haematoxylin (5 min), rinsed in tap water blued in Scotts Blueing solution (1 min) and counterstained in 0.0001% eosin (5 min), dehydrated in 95% ethanol, absolute ethanol, cleared in xylene and mounted.

Immunolocalisation of perlecan in ACL tissue sections.

Re-hydrated tissue sections were incubated with 0.3% H_2O_2 for 10 min to inactivate endogenous peroxidase then blocked in DAKO non-protein blocking agent and pre-digested with chondroitinase ABC (0.1 U/ml) in 50mM Tris HCl pH 7.2 2% BSA for 1h. MAb A7L6 (2 µg/ml) in Tris buffered saline (TBS, 50mM Tris-HCl pH 7.2 buffer containing 100 mM NaCl and 2% w/v BSA) was added to the tissue sections for 18h at 4°C overnight and following washing in TBS the slides were incubated with biotinylated anti-rat IgG secondary antibody for 1h followed by avidin horseradish peroxidase conjugate for 1h at 4°C. Colour development was undertaken with NovaRED peroxidase substrate for 20 min at room temp. Negative control sections were also run omitting primary Ab or by substituting an irrelevant primary antibody for the authentic one. Both yielded negative results.

RESULTS

Cells isolated from anteriomedial and posteriolateral ACL bundles, synovial fibroblasts, articular chondrocytes and outer and inner meniscal cells were grown in monolayer culture for 6 days (Figure 1a-h). The ACL cells synthesised perlecan in culture like the other knee-joint cells (Figure 1e,f). The perlecan pericellular arrangements assembled by ligament cells were subtly different from one another and with the synovial fibroblasts which assembled the most extensive perlecan networks (Figure 1c). Differences were also evident in the density of the perlecan layed down by the outer and inner meniscal cells with the former assembling denser networks (Figure 1a,b). In general cells isolated from fibrocartilaginous tissues synthesised higher levels of perlecan.

Ligament cells also assembled a well defined actin cytoskeleton in culture (Figure 1 g,h) indicating that they had a contractile phenotype like myofibroblasts and may have appropriate properties to participate in wound repair and contraction.

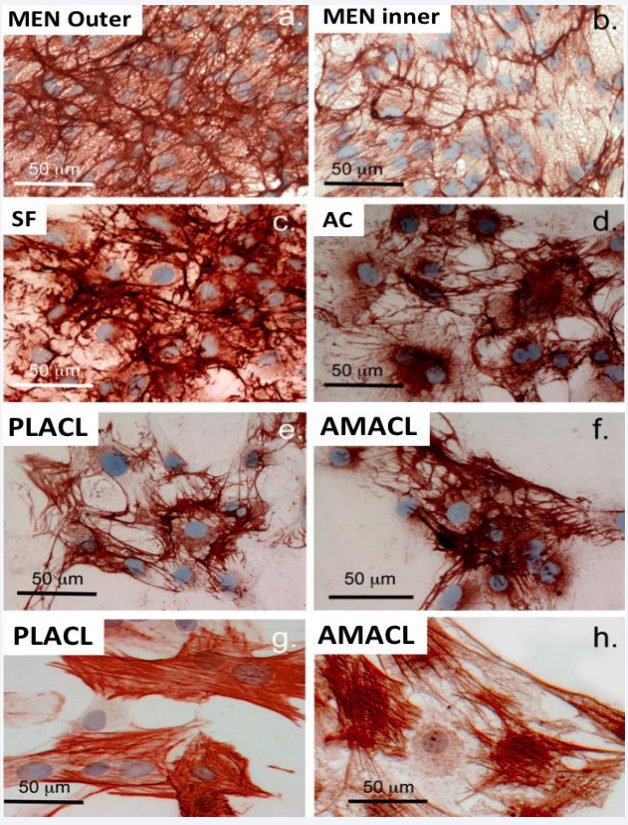

Figure 1 Immunolocalization of perlecan synthesised by connective tissue cells from a single stifle joint in monolayer culture for 6 days. (a) Meniscus outer zone, (b) Meniscus inner zone, (c) Synovial fibroblasts (SF), (d) Articular chondrocytes (AC), (e) Posteriolateral cruciate ligament (PLACL), (f) anteriomedial cruciate ligament (AMACL). Visualisation of the actin cytoskeleton synthesised by PLACL (g) and AMACL (h) cells in monolayer culture. Chromogen NovaRED, DAPI nuclear stain.

Perlecan was immunolocalised to interconnected fusiform ligament cells in the mid region of the anteriomedial and posteriolateral ACL bundles (Figure 2a,b) and to plumper cells in channels between the collagen fibre bundles (Figure 2e,f). Perlecan had a polarised distribution at the end of the fusiform ligament cells but had more of a pericellular distribution in the plumber ligament cells found in small vascular channels between the collagenous fibre bundles in ligament. This polarised distribution of perlecan may facilitate cell-cell communication in regions of the tendon subject to high tensile load where mechanotransductive processes may provide important regulatory feedback to the tendon cells that aids in the maintenance of a correct cell phenotype and tissue homeostasis.

DISCUSSION

In the present study, ligament cells were shown to synthesise slightly lower perlecan levels in monolayer culture than the other knee joint cells that were cultured at the same cell density including cells from the meniscus, synovium or articular cartilage (Figure 1). Ligament cells however clearly synthesised appreciable perlecan levels in a relatively short culture period (6 days) and confirmed the perlecan immunolocalised in ACL tissue sections (Figure 2). Perlecan displayed a pericellular distribution in monolayer culture but was also formed into fibrillar structures which extended across to adjacent cells in all of the cell types examined. These perlecan positive fibrillar structures are probably not due to perlecan per-se since perlecan has not been demonstrated to form fibrils in other studies on perlecan in a range of tissues but may be due to perlecan interacting with some other un-identified secreted proteins in the present study which form these structures. Perlecan certainly has interactive capabilities with a wide range of proteins found in connective tissues (Table I) which might facilitate this and also has self-aggregative properties through interactions with components in perlecan domain IV or V. In particular, perlecan has interactive properties with the fibril forming type VI and XI collagens which are also synthesized by chondrocytes, IVD cells, synoviocytes and meniscal cells and these have a pericellular localization pattern [15,19]. Perlecan also has self-aggregative properties which may also contribute to the formation of these structures and this may relate to the immunolocalisations of perlecan observed in the present study (Figure 2). Within tissues, perlecan

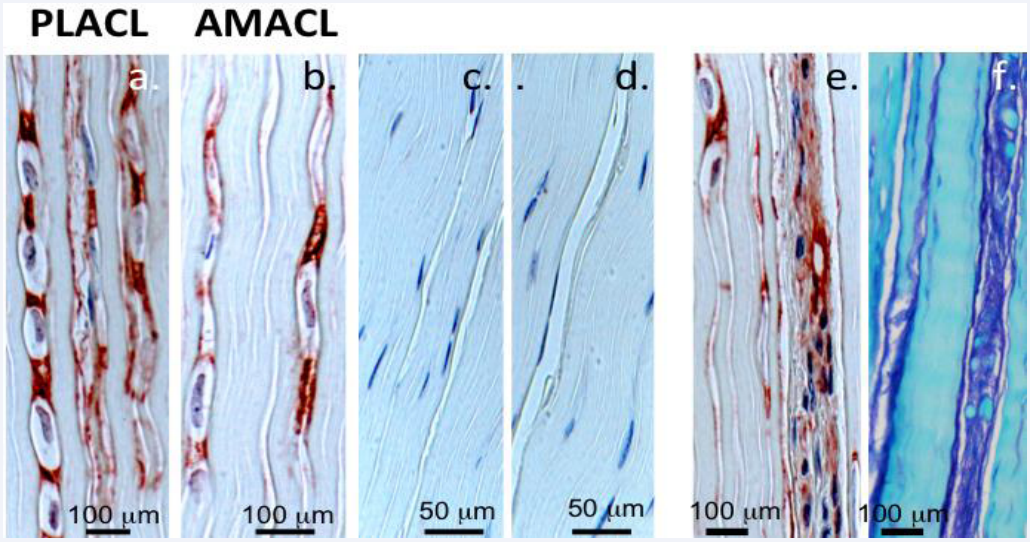

Figure 2 Immunolocalization of perlecan in the strings of fusiform cells (a, b) in the AMACL (a) and the PLACL (b). Negative control sections prepared with an irrelevant primary antibody are also shown (c, d). Perlecan was also immunolocalised in vascular channels adjacent to collagen fibre bundles (e). Toluidine blue-fast green stained longitudinal sections of ACL showing GAG localisation in vascular channels and fusiform cells in collagen fibre bundles (f). Negative control images are provided at lower magnification to provide greater tissue coverage.

Table 1: Perlecan Interactive ligands for domains I-V.

Domain-I	Domain-II	Domain-III	Domain-IV	Domain –V
Laminin-1, Collagens IV, V, VI, XI Fibronectin, PRELP, WARP, Fibrillin-1, Thrombospondin FGF-1, 2, 7, 9, 18, BMP-2, 4; PDGF, VEGF, IL-2, Ang-3, Activin A, Heparanase, Histone-H1, G6b-BR	VLDL LDL Wnt SHH	FGF-7, 18, FGFBP, PDGF, WARP, Collagen VI, Tropoelastin	Nidogen-1, 2, Fibronectin, Collagen IV, VI, PDGF, Fibulin-2, Tropoelastin, MMP-7	Nidogen-1, 2,b1-integrin, Collagen VI,a-dystroglycan, FGF-7, Endostatin, ECM-1, Progranulin, N-Acetylcholinesterase, Tropoelastin

Abbreviations: PRELP: Proline-arginine-Rich End Leucine-Rich Repeat Protein Prolargin; WARP: von Willebrand factor A Domain-Related Protein; FGF: Fibroblast Growth Factor; BMP: Bone Morphogenetic Protein; PDGF: Platelet Derived Growth Factor; VEGF: Vascular Endothelial Cell Growth Factor; Ang3: Angiogenin-3; G6b BR: Megakaryocyte and Platelet Inhibitory Receptor G6b**;** VLDL: Very Low Density Lipoprotein; LDL: Low Density Lipoprotein; Wnt: a condensed term created from the names Wingless and Int-1; SHH: Sonic Hedgehog; FGFBP: Fibroblast Growth Factor Binding Protein; ECM-1: Extracellular Matrix Protein-1.

also forms communicating structures between linear arrays of cells [22] and intralammellar cross-bridging structures within tensional tissues such as the collagenous lamellar tissues of the fibrocartilaginous annulus fibrosus and meniscus. These strings of cells have a similar morphology to the arrays of cells observed in the present study which were also interconnected by perlecan. Furthermore, the ligament cells also displayed a contractile phenotype in culture assembling prominent actin cytoskeletons and stress fibres (Figure 1 g,h). These are features that are also evident in cultured myofibroblasts and considered to facilitate the contractile roles that myofibroblasts play in tissue repair processes and wound contraction. In ruptured ACLs a significant decrease has been reported in the number of myofibroblast-like ligament cells expressing α-smooth muscle cell actin [23]. However, in dense connective tissues undergoing a reparative response, fibroblast proliferation, expression of alpha-smooth muscle actin, and revascularization are prominent features of the repair process. Perlecan promotes tissue repair, has angiogenic properties through the sequestration of growth factors like FGF-2, VEGF, and PDGF that promote re-vascularization and the proliferation of resident connective tissue cell populations that lay down ECM components to effect tissue repair [24,25]. Perlecan thus has potential beneficial properties that would be expected to play prominent roles in ACL repair processes.

Perlecan is a cell instructive, modular multifunctional proteoglycan

Previous studies have demonstrated a plethora of components that are interactive with perlecan [7,8,26]. Such interactions are critical in the regulation of many important physiological processes. Perlecan also has biomechanical properties in weight bearing tissues [22]. Of particular relevance to ACL repair is the ability of perlecan to promote tissue repair, perlecan's roles in ECM stabilisation, perlecan's biomechanical mechanotransductive roles in weight bearing and tensional tissues [13] and perlecan's ability to promote vascular repair of tissues [26]. Table 1 and Table 2 summarise the perlecan interactive ligands and the physiological processes that perlecan regulates through these ligands. Recombinant human perlecan domain V has recently been shown to promote growth factor signaling and enhanced angiogenesis and vascularization of implanted biomaterials. This biologically active perlecan fragment has potential in the treatment of native and bioengineered tissues and thus would be useful in bioaugmentation approaches in ACL repair.

The poor healing potential of ACL injuries

The ACL exists in a hostile weighted microenvironment where twisting knee movement and synovial fluid flow over the ACL prevents fibrin clot formation occurring as an initial step in wound repair. An inability to form a stabilizing clot at an ACL defect site is a major reason why these do not undergo spontaneous repair like defects in the MCL. After ACL injury MMP-1 and MMP-13 are up-regulated and secreted into the synovial fluid with MMP-13 up-regulated 100 fold within a few days of ACL transection (ACLT). The degenerate ACL is a major contributor to the degradative synovial enzyme pool that exists in the knee joint following trauma leading to degenerative changes in the articular cartilage predisposing the knee to the development of OA. Knee joint menisci also degenerate following ACLT and contribute MMP-1 and MMP-13 to this synovial degradative enzyme pool in response to the inflammatory cytokines, IL-1 and TNFα, that are also released into the synovial fluid following ACL transection [27]. Thus not only does the ACL fail to heal, but its injury leads to the development of post-traumatic OA and long-term degenerative effects on other knee joint tissues [28].

ACL proteoglycans

Ligament insertion sites into the femur and tibia contain aggrecan which is consistent with the cartilage like appearance of these compressed regions of ligament, versican however is a major large CS-proteoglycan in the main substance of ligament subjected to tension. Decorin, constitutes ~90% of the total proteoglycan in ligament. The present study has now shown perlecan is a pericellular component of fusiform

Table 2: Functional properties of Perlecan domains and the physiological processes they regulate.

Domain-I	Domain-II	Domain-III	Domain-IV	Domain –V
HS mediated PDGF, VEGF, HGF, BMP, GM-CSF, FGF,Ang-3 Activin-A, IL-2, IL-8 binding, delivery and receptor activation. ECM stabilization by interactions with WARP/ PRELP, laminin, Collagen IV, V, VI, XI; Nidogen-1, Fibronectin, TSP-1, Fibrillin-1 regulates platelet aggregation and fibrosis	LDLR-like domain binds and transports poorly soluble Wnt and SHH with roles in tissue morphogenesis Roles in LDL and VLDL clearance from circulation. Binds CTGF promotes tissue growth and repair, TGF-b mediates ECM production and fibrosis of tissues	Contains cryptic cell interactive peptide modules. Non-HS mediated binding of FGF-7, FGF-18, PDGF promotes cell signaling, cell proliferation	Multiple Ig repeats RGDS cell signaling Chondrogenic properties, roles in cell motility, cell adhesion Multiple perlecan mutations in prostate cancer and SJS. Highly susceptible to MMP-7. Mast cells produce perlecan with truncated domain IV	LG domains interact with DG stabilizing muscle, synaptic and NMJ BMs. LG1LG2LG3 perlecan domain V is angiogenic, promotes tissue repair, and is neuroprotective following stroke and TBI, SCI promoting neurogenesis, BBB and BM repair following ischaemic stroke. LG1LG2 and LG3 cryptic domains have anti-angiogenic, apoptotic properties, inhibiting VEGFA cell signaling and cell proliferation, and α2β1 integrin endothelial cell interactions that prevent tube formation and angiogenesis

Abbreviations (see abbreviations given in Table I also): TSP-1: Thrombospondin-1; TBI: Traumatic Brain Injury; SCI: Spinal Cord Injury; LDLR: Low Density Lipoprotein Receptor; CTGF: Connective Tissue Growth Factor; TGF-β: Transforming Growth Factor-β; RGDS: Arginine-Glycine-Aspartic acid-Serine cell attachment peptide; MMP: Matrix Metalloprotease; LG: Laminin G Domain; αDG: α-dystroglycan; AD: Alzheimers Disease; BBB: Blood Brain Barrier; BM: Basement Membrane; VEGFA: Vascular Endothelial Growth Factor-A; SJS: Schwartz-Jampel Syndrome.
Modified from {Melrose, 2020 #145} with permission © Melrose 2020.

ligament cells and plumper ligament cells in vascular channels in ligament. Perlecan may have roles in cell-cell and cell-ECM mechanotransductive communication in the ACL as has been proposed in other weight and tension bearing tissues [13]. Perlecan modulates mechanostransductive events in articular cartilage and is chondroprotective [14], promotes ECM assembly, repair and tissue homeostasis [26]. Perlecan is a highly interactive proteoglycan sequestering a number of growth factors and morphogens promoting cell proliferation, differentiation and matrix deposition in weight bearing and tensional tissues [24-26]. Expression of IGF-I, TGF-β, PDGF, VEGF, EGF and FGF family members are altered in healing ligaments [29]. Therapeutic use of growth factors either singly or in combination have been evaluated to improve ACL healing but with limited success. Repair of ruptured ACLs is undertaken by surgical reconstruction with harvested mid-patellar or hamstring tendon which do not replicate the complex tissue organization or biomechanical performance of native ACL and consequently have met with limited success. Significant advances have been made in the development engineered ligament constructs containing cells of an appropriate phenotype to effect bio-integration and matrix repair and in approaches using double bundle ACL reconstructions [30]. An experimental approach where ACL cells are pre-conditioned to an appropriate phenotype using micropatterned substrates subjected to mechanical stimuli, is also a promising approach. With a greater understanding of the functional organization and biomechanical properties of the ACL and regulation of the resident ligament cell populations we will be one step closer to achieving effective repair of this difficult connective tissue. Perlecan's ability to promote coacervation of tropoelastin during elastogenesis and elastic microfibril formation in-vitro and in-vivo is a further aspect of the pathobiology of perlecan that could be envisaged to aid in ligament repair [9]. Elastin provides ligaments with viscoelasticity, normal ligaments contain 5-6% elastin on a dry weight basis, a level comparable to that found in skin. These properties will need to be reproduced by engineered ACL constructs if they are to be successful ligament tissue replacements.

Potential roles for perlecan in ligament repair.

Perlecan promotes tissue repair by sequestering angiogenic growth factors like FGF-2, VEGF, and PDGF. These growth factors also promote re-vascularization of tissues and the proliferation of resident connective tissue cell populations that lay down ECM components for tissue repair [24]. ACL repair with the ligament advanced reinforcement system (LARS) have emphasised the importance of vascular processes in ACL repair. Perlecan also has roles in the regulation of elastin and microfibril assembly in tensional tissues. Perlecan, LTBP-1 and fibrillins are implicated in the incorporation of elastin into elastin microfibrils, and in the integration and stabilisation of elastin fibrils in tendon and ligament. The HS chains of perlecan promote coacervation of tropoelastin *in-vitro* and perlecan core protein interactions further enhance *de-novo* elastin deposition [9]. Moreover, elastin has stimulatory properties over ligament cells in culture that promote regeneration of the rabbit medial collateral ligament. Micro-fibrils have mechanosensory functions in tensional connective tissues such as ligament and tendon and also act as growth factor repositories providing feedback cues received by the resident cell populations that regulate tissue homeostasis and maintain optimal tissue function [31,32]. Bioaugmentation (administration of therapeutic cells, growth factors, scaffolds, and mechanical stimuli) of ACL surgical interventions is still in the exploratory stages and more evidence from preclinical and clinical studies are required before such procedures will enter clinical practice [33]. However, as shown in this study perlecan has considerable potential in this area and would be expected to improve on functional outcomes following surgical treatment of ACL injuries [34,35] . A major impediment to effective ACL repair processes has been how the knee should be stabilised to prevent disruption of the ACL repair site by excessive tibial translation. An evaluation of current ACL stabilisation procedures however has demonstrated unacceptably high complication rates and more effective procedures need to be developed [36]. A recent study in an ACL rupture model in rats showed that if knee kinematics are controlled appropriately to minimise abnormal tibial translation then spontaneous repair of the transected ACL can occur and recovery of 50% of the ACL biomechanical properties in an 8 week recovery period [37]. Use of perlecan-FGF-2 in similar therapeutic approaches in the future would be expected to improve on functional outcomes in ACL repair, ultimately perfection of such procedures may even eventually obviate the requirement for surgical intervention in the repair of ACL injuries.

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