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Glycoproteomics of Lubricin-Implication of Important Biological Glyco- and Peptide-Epitopes in Synovial Fluid

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1. Introduction

The dynamic milieu of synovial fluid is of particular interest for biomarker discovery of joint related diseases as it is composed not only of ultra-filtrated proteins originating in serum, but also proteins exclusively expressed and secreted by cells localized within the synovial membrane, fluid or cartilage. Lubricin (proteoglycan 4, prg4) is an abundant mucinous and secretory glycoprotein (~227 to 345 kDa) in synovial fluid (SF) and one of the factors considered responsible for boundary lubrication of diarthrodial joints (Swann et al., 1981; Swann et al., 1985; Jay, 1992). Lubricin is encoded by gene *PRG4* and synthesized in synovial fibroblasts (synoviocytes) and superficial zone chondrocytes. Different transcripts of *PRG4* have been referred to as superficial zone protein (SZP), megakaryocyte stimulating factor (MSF) precursor, camptodactyly arthropathy coxa vara pericarditis (CACP) protein, and hemangiopoietin (HAPO), which has recently been reviewed by Bao et al (Bao et al., 2011). As a primarily lubricating glycoprotein, lubricin has been found in SF, superficial layer of articular cartilage, tendons, and menisci (Schumacher et al., 1994; Schumacher et al., 1999; Rees et al., 2002; Rhee et al., 2005b; Schumacher et al., 2005; Sun et al., 2006). This tissue-specific distribution makes lubricin a potential biomarker during the exacerbation of chronic articular inflammation.

Human synovial lubricin (1404 amino acids) has a large and central mucin-like domain characterized with 59 imperfect repeating units of EPAPTTPK which is subject to extensive *O*-linked glycosylation. The abundance of negatively charged sugars in this domain contributes to the protein's boundary lubrication of the cartilage surface due to strong repulsive hydration forces (Jay, 1992). The mucin domain is flanked by a *C*-terminal hemopexin (PEX)-like domain and two somatomedin B (SMB)-like domains at its *N*-terminus (Flannery et al., 1999; Schumacher et al., 1999; Ikegawa et al., 2000). The two *N*-terminal SMB-like domains have 60% similarity to that of vitronectin, while *C*-terminal PEX-like domain also shows similarity to domains in vitronectin (40-50%) as well as to the matrix metalloproteinase (MMPs) family. Purified serum hemopexin has been showed to interact with hyaluronan, suggesting that the PEX-like domain in lubricin may also medicate the binding of lubricin to hyaluronan at or near cartilage surface (Hrkal et al., 1996). In addition to boundary lubrication, lubricin protects cartilage surfaces from protein deposition and cell adhesion (Rhee et al., 2005b).

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During inflammation, glycosylation properties such as sialylation, sulfation, and fucosylation, are regulated to manipulate cell adhesion, differentiation, maturation, and activation in the case of immune cells. Bone and cartilage cells like osteoblasts, synovial fibroblasts and chondrocytes have been shown to possess the enzymes necessary for the synthesis of *N*- and *O*-glycans of glycoproteins, among which some activities are regulated by cytokines found in inflamed joints (Brockhausen & Anastassiades, 2008). Based on the established biosynthetic pathways, it was reported that human joint glycoproteins mainly had complex bi-antennary *N*-glycans and *O*-glycans with core 1 and the branched core 2 structures (Brockhausen & Anastassiades, 2008). In our previous study, the *O*-linked oligosaccharides of lubricin were characterized (Estrella et al., 2010). On lubricin, core 1 *O*-linked oligosaccharides are the predominant structures. Removal of sialic acid and core 1 oligosaccharides caused loss of boundary lubrication (Jay, 1992; Jay et al., 2001), showing that these structural elements are sufficient for providing lubricating property of lubricin. With aid of liquid-chromatography-mass spectrometry (LC-MS), small proportion of sialylated core 2 oligosaccharides were also found on lubricin both with and without sulfation. This indicates that lubricin glycosylation also have other task requiring complex *O*-glycosylation. In summary, both core 1 and core 2 glyco-epitopes on lubricin have the potential of excessive interactions with glyco-binding proteins, such as selectins and galectins, to facilitate inflammation.

Degenerative joint disease and joint injury are associated with increased turnover of articular cartilage proteins, inflammation, and alterations to other joint tissue proteins (Goldring & Goldring, 2007). So far, several synovial joint-specific biomarkers have been identified in adults, such as calgranulin A, B, and C (Sinz et al., 2002; Liao et al., 2004), fibrinogen β-chain, fructose bisphosphonate aldolase A, alpha-enolase (Tilleman et al., 2005), tenascin-C (Hasegawa et al., 2004), serum amyloid A (SAA), and broader inflammatory biomarkers, such as C-reactive protein (Kuhn et al., 2004) and haptoglobin (Sinz et al., 2002; Kantor et al., 2004). Lubricin as one important synovial component to monitor the state of a joint is less investigated, despite its highly relevant function as a biolubricant. Because of the size and posttranslational modifications of lubricin, it is not readily detectable by traditional two-dimensional electrophoresis (2-DE). However, a decreased expression of lubricin together with increased degradation of lubricin have been associated with more aggressive rheumatoid arthritis (RA) and osteoarthritis (OA). This strongly indicates that lubricin may be a good joint-specific biomarker. For example, *in vitro* boundary lubricating test indicated that SF from chronic inflammatory RA patients had decreased lubricating ability in comparison with SF from acute knee joint synovitis patients and cartilage transplant donors (Elsaid et al., 2005). According to the expression level of lubricin in synovium, RA patients could be classified into two groups, of where lower expression level of lubricin was associated with a more aggressive disease stage (Ungethuem et al., 2010). As for OA, animal models of OA also feature reduced levels of lubricin, particularly in the early stage of the disorder (Young et al., 2006; Elsaid et al., 2007). Also, when applied exogenous lubricin in an animal model of OA, it appears to be chondroprotective and to reduce structural damage (Flannery et al., 2009; Teeple et al., 2011). It has been demonstrated that lubricin expression is down-regulated by proinflammatory cytokines (e.g., interleukin (IL)-1β, tumor necrosis factor α (TNFα), and IL-6) (Flannery et al., 1999; Rhee et al., 2005b; Young et al., 2006; Schmidt et al., 2008). Decreased synovial lubricin level may be caused by degradation with neutrophil elastase,

cathepsin B, and MMPs (Jones et al., 2003; Elsaid et al., 2005). MMPs are an enzyme family of calcium-dependent zinc-containing endopeptidase which is known to play important roles in tissue remodeling during physiological as well as pathological processes. In cartilage, MMPs are the principal proteases capable of degrading a wide variety of the extracellular matrix components (Nagase & Woessner, 1999). The released fragment of lubricin together with other synovial residual proteins and cartilage matrices floating in synovial fluid may be detected by biochemical or immunochemical assay. The profile of proteins or fragments within SF may represent diagnostic or prognostic biomarker for degenerative joint diseases.

Defect of lubricin function leads to CACP syndrome in human, which is a rare and Mendelian genetic arthropathy causing juvenile-onset, inflammatory, precocious joint failure (Marcelino et al., 1999). Although *Prg4*$^{-/-}$ mice did not have noticeably reduced fertility or life span, with aging knockout mice underwent synovial hyperplasia, subintimal fibrosis, proteinaceous deposits on the cartilage surface, irregular cartilage surface and endochondral growth plates, and ultimate invasion of the cartilage surface by synoviocytes reminiscent of human CACP and the cartilage invasion of RA joints by the inflammatory pannus (Rhee et al., 2005b).

As all these studies indicate, it is reasonable to speculate that inflammation-induced alterations of both the level, degradation and glycosylation of lubricin that occur in the joints of patients with RA and OA may accelerate the destruction of joints and exacerbate the disease. Monitoring new glyco-epitopes and/or proteolytic fragments of lubricin may serve as a potential biomarker for advanced diagnosis of early stage. To perform this, it is necessary to fully characterize the lubricin molecule by glycoproteomics. In this study, we used various biotinylated lectins or anti-carbohydrate antibodies together with MS to characterize glyco-epitopes on lubricin. The results confirm that lubricin contains immunologically important *O*-linked oligosaccharide epitopes that are capable of binding selectins and galectins. Proteomic analysis indicated that not all repeat units are occupied with *O*-linked oligosaccharides and also revealed several fragments of lubricin in synovial fluid.

It is known that joint damage may progress despite decreased inflammatory activity and erosions may develop in patients with few signs of inflammation by conventional assessments (Flato et al., 2003). Therefore, predicting the progression and consequences of inflammatory pathology are essential for optimal clinical management. The ideal biomarker of persistent inflammation in arthritis should fulfill a number of criteria including: detectable levels in early disease, expression which coincides with each inflammatory episode and expression that is restricted to the inflamed joint. The identification of differentially expressed proteins contributes to understanding the molecular factors of the disease better and paves the way for new diagnostic and prognostic markers, and eventually to novel targets in the development of therapeutic strategies.

2. Glycoproteomic characterization of synovial lubricin

2.1 Materials and methods

2.1.1 Enrichments of lubricin from synovial fluid

Synovial fluid samples from RA patients were collected during therapeutic joint aspiration at the Rheumatology Clinic, Sahlgrenska University Hospital (Gothenburg, Sweden). All patients gave informed consent and the procedure was approved by the Ethics Committee

of Sahlgrenska University Hospital. All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA (Arnett et al., 1988). The samples were clarified by centrifugation at 10,000 g for 10 minutes and stored at -80°C before use. The acidic proteins were purified as previously described (Estrella et al., 2010). In brief, synovial fluid sample was diluted with washing buffer (250 mM NaCl, 20 mM Tris-HCl, 10 mM EDTA, pH 7.5) before applying to 1 mL DEAE FF Hi-Trap column (GE Healthcare, Uppsala, Sweden). Enriched glycoproteins were eluted with 1 M NaCl in washing buffer. Lubricin containing fractions were precipitated with 80% ethanol for 16 hours at -20°C. The precipitate was collected by centrifugation at 12,100 g for 20 minutes and re-suspended in phosphate buffered saline (PBS) at pH 7.4 after air-dry. Protein concentration was determined by BCA protein assay kit (Thermo Scientific, San Jose, CA, USA) using bovine serum albumin (BSA) as standard.

For sandwich ELISA, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-lubricin polyclonal antibody (Thermo Scientific) in 0.1 M carbonate buffer, pH 9.5, at a concentration of 2 ng/mL and 4°C overnight. The plates were then blocked with 1% BSA in TBS-T buffer (Tris-buffered saline with 0.01% Tween 20) at 37°C for 1 hour. Fractions were diluted with 1% BSA in TBS-T buffer, added to each well, and incubated at 37°C for 1 hour. After washing with TBS-T buffer, diluted anti-lubricin mouse monoclonal antibody (Pfizer Research, Cambridge, MA, USA) was added to each well and incubated at 37°C for 1 hour. After extensive wash, horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin antibody (DakoCytomation, Glostrup, Denmark) was added. Color was developed by using tetramethyl benzidine (TMB) buffer (Sigma-Aldrich, St. Louis, MO, USA) as substrate for 10 minutes at room temperature; and reaction was stopped by adding 1 M H_2SO_4. The optical density was measured at 450 nm wavelength.

2.1.2 Western blot and lectin immunoblot

Samples were reduced with 10 mM dithiothreitol (Sigma-Aldrich) and denatured by heating at 95°C for 20 minutes, and then alkylated with 25 mM iodoacetamide (Sigma-Aldrich) for 1 hour at room temperature in the dark. As for non-reduced samples, protein samples were mixed with SDS loading buffer and heated at 95°C for 20 minutes. The samples were then applied to a 3-8% Tris/acetate NuPAGE gel (Invitrogen AB, Stockholm, Sweden) or agarose-polyacrylamide gel (AgPAGE) which was made as described previously (Schulz et al., 2002). The samples were blotted onto PVDF membrane (Immobilon P, Millipore, Billerica, MA, USA) using a semi-dry blotter (Bio-Rad, Hercules, CA, USA).

PVDF membranes were blocked for 1-2 hour at room temperature in TBS-T buffer containing 1% BSA at room temperature on a shaker, and then incubated with primary antibodies or biotinylated lectins at the appropriate concentration diluted in TBS-T buffer with 1% BSA for 1 hour at room temperature on a shaker. After washing the blots three times with TBS-T, blots were incubated with secondary antibodies or streptavidin labeled with HRP for 1 hour at room temperature. After wash, bound antibodies and lectins were detected by using SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific).

Anti-carbohydrate antibodies used in study including anti-T antigen (mAb 3C9), anti-Tn antigen (mAb 5F4 and 1E3), and anti-sialyl Tn (mAb TKH2 and 3F1), which were kindly provided by Prof. Henrick Clausen and Prof. Ola Blixt (University of Copenhagen,

Denmark). Mouse anti-3'-sulfo-Le^a was kindly provided by Dr. Antoon J Ligtenberg (Department of Oral Biochemistry, University of Amsterdam, The Netherlands). The other anti-carbohydrate antibodies tested in this study were mouse anti- sialyl Lewis x (sLe^x , CD15s, or mAb CSLEX1, BD Biosciences, Franklin Lakes, NJ, USA), MECA-79 (CD62L, BD Biosciences), mouse anti-chondroitin sulfate (mAb CS56, Sigma-Aldrich), mouse anti-sLe^a (mAb CA19-9, Abcam, Cambridge, MA, USA), and mouse anti-Le^b (mAb 2-25LE, Abcam). Biotinylated lectins were also used in this study including ConA (concanavalin A), MAA-I (*Maackia amurensis* lectin I), WGA (succinylated wheat germ agglutinin), and AAL (*Aleuria aurantia* lectin), all from Vector (Vector Laboratories, Burlingame, CA, USA). Biotinylated PNA (*Arachis hypogaea* lectin) and HAA (*Helix aspersa* agglutinin) were from Sigma-Aldrich. Secondary antibodies used were HRP conjugated rabbit anti-mouse IgG, HRP conjugated rabbit anti-rat IgG+IgM (Jackson ImmunoResearch, Suffolk, UK). For biotin labeled lectin, HRP conjugated streptavidin (Vector Laboratories) was used. The immunoassay was validated and optimized with human salivary mucin as described previously (Issa et al., 2010) and bovine fetuin (Sigma-Aldrich).

2.1.3 Glycomic analysis of lubricin *O*-glycan structures

O-linked oligosaccharides were released by reductive β-elimination (Schulz et al., 2002). In brief, membrane strips were incubated with 50 µL of 1.0 M $NaBH_4$ in 100 mM NaOH for 16 hours at 50°C. Reactions were quenched with 1 µL of glacial acetic acid. Samples were then desalted and dried for capillary graphitized carbon LC-MS and LC-MS^2 in negative ion mode using an LTQ Ion Trap (Thermo Scientific). Oligosaccharides were identified from their MS^2 spectra using the UniCarb-DB (2011 version) (Hayes et al., 2011) and validated manually.

For deglycosylation, the reduced and alkylated samples (20 µg) were incubated with 5 mU of sialidase A (Prozyme Inc., Oxford, UK) to remove sialic acids at 37°C for 16 hours. An aliquot of sample was also treated with 2.5 mU *O*-glycanase (endo-α-*N*-acetylgalactosaminidase, Prozyme Inc.), which cleaves core 1 type *O*-linked glycan on glycoproteins and glycopeptides, at 37°C for 16 hours. The reaction was stopped by heating at 95°C for 10 minutes in SDS-loading buffer, and enzymes were removed by electrophoresis.

2.1.4 Proteomic characterization of lubricin

Coomassie blue-stained protein bands in Tris/acetate NuPAGE gels were excised and digested with trypsin as described (Kuster et al., 1997). The resultant peptides were subjected to nano-LC-MS^2 using LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). Peptide MS/MS spectra were searched against UniProt and NCBI human protein databases using GPM (Zhang et al., 2011) and Mascot software (v.2.2.04, Matrix Science Inc., MA, USA). Only peptides with a mass deviation lower than 10 ppm were accepted and two peptide sequences with manual inspection were used for positive protein identification.

Enriched synovial lubricin sample was also treated with *O*-sialoglycoprotein endopeptidase from *Pasteurella haemolytica* (Cedarlane Laboratories, Ontario, Canada). 5 µg of samples were incubated with endopeptidase in PBS (pH 7.4) at 37°C; and small aliquots were taken out at 0, 3, 6, and 16 hours. The reaction was stopped by adding SDS-loading buffer with boiling.

2.2 Result

2.2.1 Enrichment of synovial lubricin

Synovial lubricin is a heavily negatively charged glycoprotein that can be enriched by ion-exchange chromatography (Fig. 1A). Lubricin containing fractions which were determined by sandwich ELISA were pooled and precipitated by 80% ethanol. The amount of protein in these fractions corresponded to 1.38 mg/mL synovial fluid (mean, n=5). Considering that lubricin has been shown to be in the range of 0.2-0.5 mg/mL(Marcelino et al., 1999; Schmid et al., 2001; Elsaid et al., 2005), this indicates that additional proteins (*e.g.*, albumin) and glycoproteins (*e.g.*, fibronectin, aggrecan) are co-purified (See proteomic section).

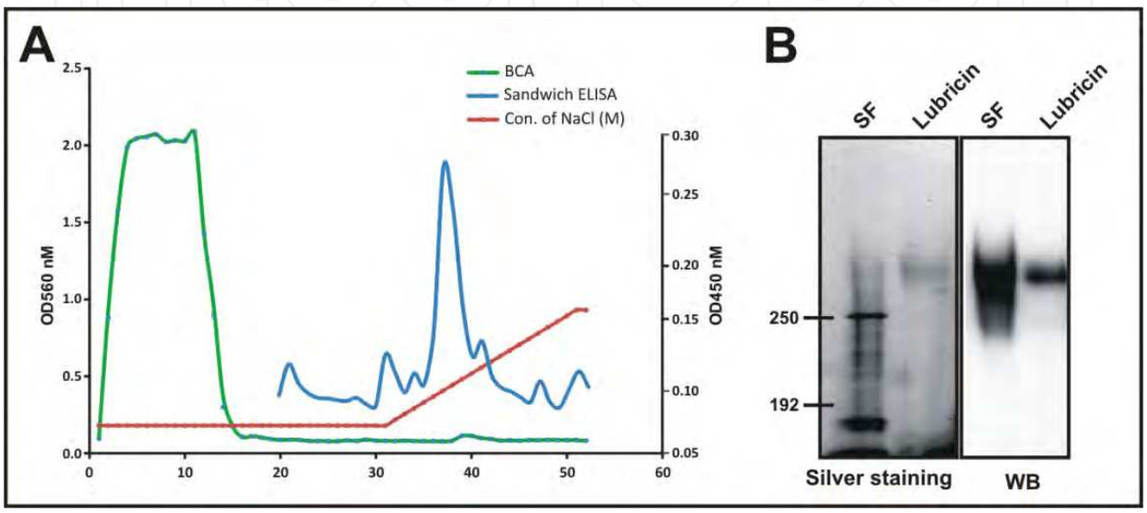

Fig. 1. Enrichment of human lubricin from synovial fluid. (A) Representative elution profile of DEAE ion-exchange chromatography. Protein levels in each fraction were determined by BCA method, while lubricin-containing fractions (Fr. 35-41) were pooled and precipitated with 80% ethanol. (B) Reduced and alkylated synovial fluid (SF, 1 μL) and enriched sample (Lubricin, 2 μg) were separated by Ag-PAGE. Protein bands were visualized by silver nitrate or detected by Western blot (WB).

As shown in Fig. 1B, silver staining of Ag-PAGE showed one major band around 300 kDa indicating that the majority of synovial proteins were removed during enrichment. When more samples were loaded, additional faint bands were also detected (Fig. 4B-2). Both mouse monoclonal (Fig. 1B) and rabbit polyclonal antibody (not shown) specifically react to prepared lubricin, respectively. Though there are few bands smaller than lubricin, none of them reacted with lubricin-specific antibodies.

2.2.2 Glyco-epitope on synovial lubricin verified by immunoassay

To examine the glycan profile on lubricin, purified samples were primarily analyzed by immunoassay with lectins or anti-carbohydrate antibodies (Fig. 2). Synovial lubricin was positive to the lectins specific to sialic acid and T antigen, such as WGA (sialic acid and terminal GlcNAcβ1,4), MAA-I (specific to α2,3-linked sialic acid), and PNA (T antigen, Galβ1,3GalNAcα1-*O*-Ser/Thr). Lubricin also reacted with HAA, a lectin specific to terminal GalNAcα1- including Tn antigen (GalNAcα1-*O*-Ser/Thr). Lectin immunoblot of synovial lubricin was also negative to ConA, which binds to branched Manα1- on high-mannose and

hybrid type *N*-glycans, suggesting *N*-glycans were absent on lubricin or in very low amounts. The same negative results were obtained with *Aleuria aurantia* lectin (AAL), which recognized both peripheral and core fucosylated glycans.

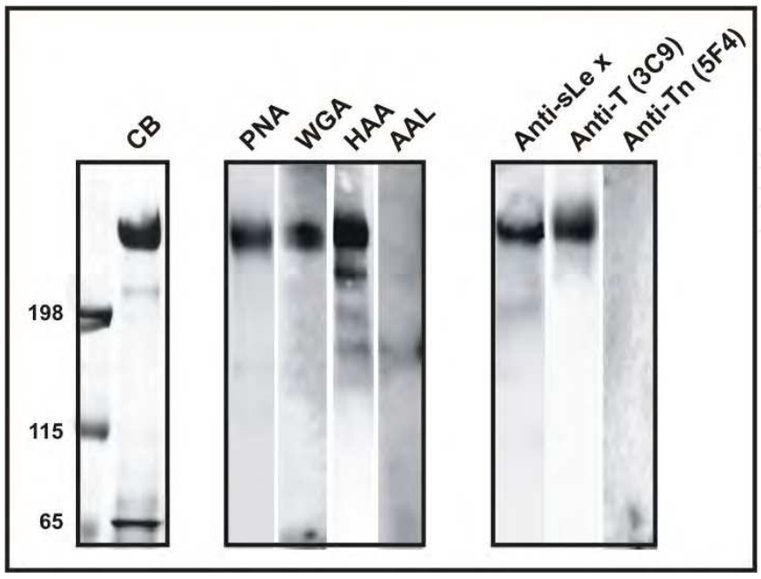

Fig. 2. Glyco-epitope on lubricin analyzed by immunoblot. Reduced and alkylated enriched lubricin sample (6 μg/lane) was separated by 3-8% Tris/acetate NuPAGE and blotted to PVDF membrane. Strips were incubated with various lectins or anti-carbohydrate antibodies after blocking with 1% BSA in TBS-T buffer. After incubating with HRP conjugated corresponding secondary antibodies and streptavidin, bands were developed by SuperSignal West Femto maximum sensitivity substrate. CB, Coomassie blue stained gel; PNA, peanut agglutinin; WGA, wheat germ agglutinin; AAL, *Aleuria aurantia* lectin; HAA, *Helix aspersa* agglutinin; Anti-sLe^x, sialyl Lewis x-specific antibody; T, T antigen, Galβ1,3GalNAc-*O*-Ser/Thr; Tn, Tn antigen, GalNAc-*O*-Ser/Thr.

When synovial lubricin was investigated by anti-carbohydrate antibodies (Fig. 2), lubricin was suggested to have T antigen and sialyl Lewis x (sLe^x, structure in Fig. 3C). Western blot showed (data not presented) that lubricin was negative for anti-carbohydrate antibodies specific to chondroitin sulfate (mAb CS56), sLe^a [NeuAcα2,3Galβ1,3(Fucα1,4)GlcNAcβ1-], (mAb CA19-9), 3′-sulfo-Le^a [NeuAcα2,3Gal(3S)β1,3(Fucα1,4)GlcNAc-], Le^b [Fucα1,2Galβ1,3(Fucα1,4)GlcNAcβ1-], (mAb 2-25LE], MECA-79 epitopes, Tn antigen (mAb 5F4 and 1E3), and sialyl Tn antigen [NeuAcα2,6GalNAc-*O*-Ser/Thr], (mAb TKH2 and 3F1). Results obtained from anti-carbohydrate antibodies agree with results from the lectin immunoblot except for lectin HAA. Together with the lectin immunoblot, these results demonstrated synovial lubricin had sialylated glycans, core 1 *O*-glycan and peripheral sLe^x epitope. In order to reveal the identity of the sLe^x containing *O*-glycans and identify other glycan epitopes not recognized by the antibodies and lectin used, additional experiments were carried out.

2.2.3 Glyco-epitope on synovial lubricin verified by LC-MS

Though immunoassay with lectins and anti-carbohydrate antibodies is convenient to detect glyco-epitopes, inner structural information is commonly scant. Furthermore, some glyco-

epitopes may not be detectable because of hindrance in space or detect limitations. In addition, certain glyco-epitopes are short of specific antibodies. For example, there is currently no antibody available that could distinguish 3-*O*-sulfation from 6-*O*- sulfation. Therefore, to scrutinize the result obtained by Western blot/lectin blot of *O*-linked oligosaccharides on lubricin, purified samples were also subjected to β-elimination with mild base. Released oligosaccharides were then analyzed by LC-MS equipped with online graphitized carbon column as previously described (Estrella et al., 2010).

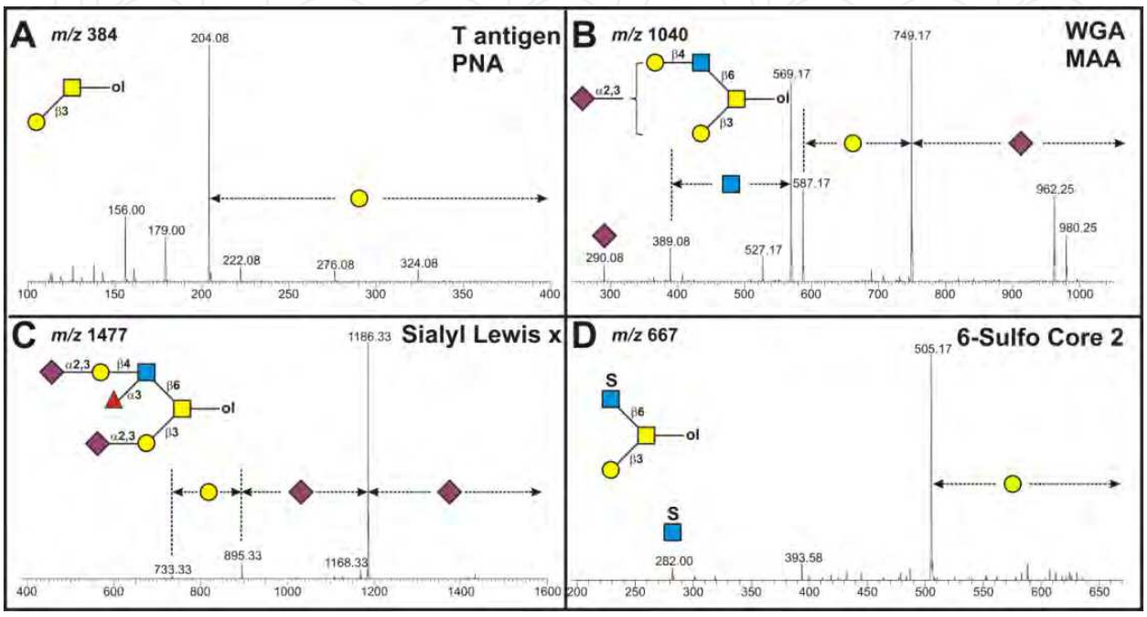

Fig. 3. Examples of core 1 and 2 *O*-linked oligosaccharides found on synovial lubricin determined by LC-MS using the [*M*-H]$^-$-ions as precursors. (A) MS^2 spectra of core 1 *O*-glycan (T antigen) at *m/z* 384; (B) MS^2 spectra of mono-sialylated core 2 *O*-glycan with one α2,3-linked NeuAc at *m/z* 1040; (C) MS^2 spectra of ion at *m/z* 1477 indicating a terminal sLe^x [NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc] epitope; (D) MS^2 spectra of ion at *m/z* 667, in which produced ion at *m/z* 282 indicate a sulfate group linked to GlcNAc. Purple diamond stands for sialic acid (NeuAc); yellow circle for galactose (Gal); blue square for *N*-acetylglucosamine (GlcNAc); yellow square for *N*-acetylgalactosamine (GalNAc); red triangle for fucose (Fuc); S for sulfate.

Consistent with findings from a previous study of lubricin (Estrella et al., 2010), core 1 *O*-linked oligosaccharides including T antigen (Galβ1,3GalNAc-*O*-Ser/Thr) and sialyl T antigen were the predominant *O*-linked oligosaccharides. As illustrated in Fig. 3A, MS^2 of ion at *m/z* 384 ([M-H]$^-$) indicates a composition of $Hex_1HexNAc_1$, corresponding to a T antigen. The presence of the Z ion fragment at *m/z* 204.1 is consistent with a composition of reduced HexNAc, while C ion fragment at *m/z* 179.0 indicates a terminal Hex. In comparison with MS^2 spectra in the database of UniCarb-DB (2011 version) (Hayes et al., 2011), the structure is consistent with Galβ1,3GalNAc, and its amount approximately accounts for 10% of total *O*-glycans on lubricin. Together with mono-sialylated [NeuAcα2,3Galβ1,3GalNAc] and [Galβ1,3(NeuAcα2,6)GalNAc] and di-sialylated [NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAc] structures, core 1-based structures accounted for up to 82% of total *O*-glycan, based on the total ion count. A small proportion of core 2 oligosaccharides, which account for the

remaining 18% of the total *O*-glycans detected, were found in this and a previous study (Estrella et al., 2010). Three representative MS^2 spectra of core 2 *O*-linked oligosaccharide is shown, with ions at *m/z* 1040, 1477 and 667 (Fig. 3B, C and D). The $[M-H]^-$-ion at *m/z* 1040 ($NeuAc_1Hex_2HexNAc_2$) demonstrates a mono-sialylated core 2 *O*-linked oligosaccharide, while ion at *m/z* 1477 ($[M-H]^-$, $NeuAc_2Hex_2deHex_1HexNAc_2$) is the same core with one additional sialic acid and one fucose. This structure has a sequence indicative of a sialyl Lewis-type terminal glyco-epitope. These types of sialylated structures together with sialylated core 1 *O*-glycan are consistent with the positive WGA and MAA lectin blots. The Western blot results showed that lubricin were only positive to sLe^x-specific antibody but negative to sLe^a. This suggests that synovial lubricin carries sLe^x [NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc] epitope (spectrum in Fig. 3C) on core 2 structures. Sulfated core 2 *O*-glycans were also found in this study (Fig. 3D) and previous study (Estrella et al., 2010). Due to lack of good antibodies and lectins, this epitope could only be identified by MS but not by lectin analysis. This argues for LC-MS and lectin as complementary techniques that need to be applied in glycomics studies.

2.2.4 Identification of synovial lubricin fragment by proteomic analysis

Though several proteomic analyses using synovial fluid samples have been carried out (Ruiz-Romero & Blanco, 2010), lubricin (or its fragments) appeared in only a few reports (Gobezie et al., 2007; Kamphorst et al., 2007; Estrella et al., 2010). To fully characterize synovial lubricin, the enriched samples were also subjected to proteomic analysis.

When the dominating band (area 2, Fig. 4B-2) was analyzed, 28.5% of the lubricin sequence could be identified and believed to represent the fully glycosylated full-length secreted lubricin. The unidentified portion was mostly located to the mucin-like domain of lubricin (Fig. 4C). Lubricin was also detected in all other pieces of the gel indicating that lubricin existed as fragments or splice variants. Sequences of all exons could be detected except exon 1, consisting of the *N*-terminal 24 amino acid-signal sequence. In addition to the area 2 (Fig. 4B-2) where full-length lubricin was detected, remarkably high sequence recovery of lubricin was also found in the low mass region below 65 kDa (Fig. 4B-5). Identified peptides were from both *N*- and *C*-terminal implying these fragments were generated by proteolytic cleavage close to or within mucin-like domain. Examples of LC-MS^2 spectra of tryptic peptides from *N*-and *C*-terminal region of lubricin is shown in Fig. 5. Both *N*- and *C*-terminal fragments of lubricin have been found in other studies (Flannery et al., 1999; Rhee et al., 2005b). These data together with our presented data suggest that lubricin is present in synovial fluid as both full-length and degraded proteins. Few peptides (7.7%) were recovered from the area higher than lubricin area (Fig. 4B-1). This is probably caused by inefficient reduction and trace amount of multimer of lubricin which has been found in synovial fluid recently (Schmidt et al., 2009). The dominating bands in area 3 and 5 are fibronectin and the *C*-terminal fragment of lubricin, respectively (Fig. 4B, Jin et al., unpublished results).

In addition to detection of lubricin, co-purified proteins were also identified by the proteomic approach. Table 1 listed top 3 proteins identified in each gel area, which consisted of 7 unique proteins and their fragments. Except serum albumin, other proteins are glycoproteins. The presence of the lower molecular weight fibronectin in high molecular area (area 1) confirmed inefficient reduction and suggest the presence of fibronectin dimers or oligomers. Alternatively, both serum albumin and fibronectin have both been reported to bind to lubricin *in vitro* (Schmid et al., 2002) and may have been attached to lubricin during the purification. The possible association of lubricin with these proteins or their fragments is under investigation by our group.

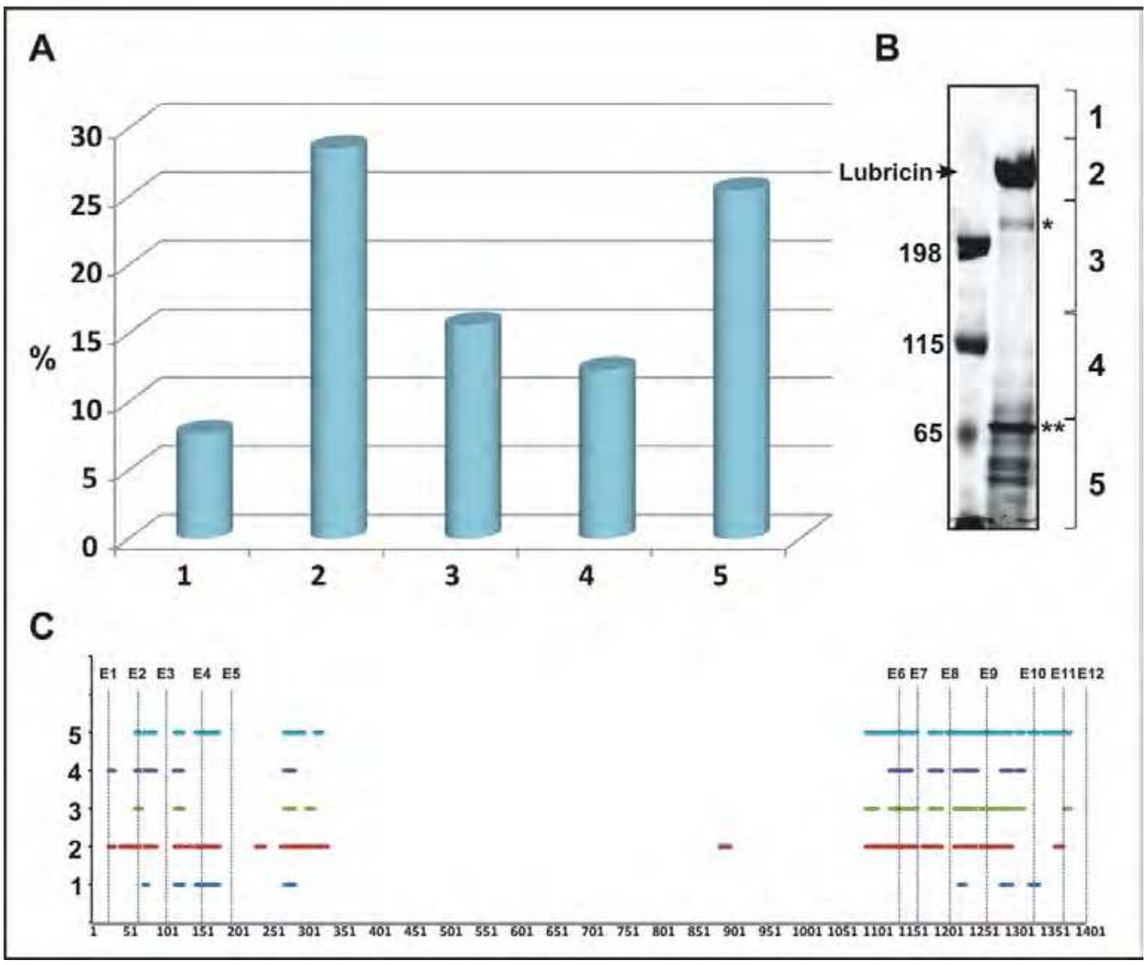

Fig. 4. Proteomic analysis of enriched synovial lubricin. (A and B) Reduced and alkylated lubricin sample was separated by 3-8% Tris/acetate NuPAGE. Protein bands were visualized by Coomassie blue. Gel slab was cut into five pieces (1 to 5) and subjected to LC-MS/MS analysis after trypsin digestion. The graph (A) shows the recoveries (%) of lubricin sequence from different cut areas. (C) Peptide map of lubricin recovered from different gel areas. The horizontal axis stands for the lubricin amino acid sequence (in total 1404 amino acids). E1 to E12 indicates the end of exon. *fibronectin; **C-terminus of lubricin

2.2.5 Characterization of lubricin mucin-like domain

Because the sequence in mucin-like domain is still largely unknown, several ways were tried to characterize this heavily *O*-glycosylated domain. As shown in Fig. 4C, resolved peptides from lubricin contain both *N*- and *C*-terminus (Fig. 5). Sequenced *N*-terminus spanned from residue 25 to 334, while *C*-terminus spanned from residue 1094 to 1383 (1404 amino acids in full-length). Only one peptide (A^{888}LENSPKEPGVPTTK^{902}) within mucin-like domain (348-855) containing 59 imperfect/perfect 8-amino acid repeats (KxPxPTTx) was found in area 2. It is believed that because of heavy *O*-glycosylation, the protein domain with this modification is normally not accessible to proteases and hence the low recovery obtained. In the case of synovial lubricin, however, it could be completely digested with trypsin in both reducing and non-reducing condition (Fig. 6A). The digestion was so complete that lubricin-

Gel area	Protein identified	MW (kDa)*	Peptide identified	Protein ID	Coverage (%)
1	Fibronectin	262.4	42	P02751	24.9
	Basement membrane-specific heparan sulfate proteoglycan core protein	468.5	14	P98160	4.0
	Apolipoprotein B-100	515.2	13	P04114	4.0
2	Lubricin	151.0	69	Q92954	28.5
	Alpha-2-macroglobulin	163.2	15	P01023	15.0
	Aggrecan core protein	250.0	15	P16112	6.9
3	Apolipoprotein B-100	515.2	111	P04114	30.9
	Fibronectin	262.4	85	P02751	44.9
	Alpha-2-macroglobulin	163.2	36	P01023	36.2
4	Fibronectin	262.4	51	P02751	30.9
	Serum albumin (HSA)	69.2	50	P02768	75.5
	Apolipoprotein B-100	515.2	52	P04114	16.9
5	Lubricin	151.0	39	Q92954	25.4
	Fibronectin	262.4	38	P02751	23.1
	Serum albumin	69.3	33	P02768	60.4

* Molecular weight of apoprotein obtained from protein database.

Table 1. Proteins identified in enriched synovial fluid sample. Reduced and alkylated lubricin sample was separated by 3-8% Tris/acetate NuPAGE. The entire gel line (Fig. 4B) was cut into five pieces (1 to 5). Gel pieces were subjected to in-gel digestion with trypsin. The resultant peptides were applied to nano-LC-MS^2. The proteins were identified from peptide MS/MS spectra, searched against Uniprot human protein database using GPM software. The 3 top ranked proteins from 1-5 cut areas with their molecular weight in kDa and with the number of unique peptides for each protein are listed in the table 1. The recoveries (%) of the 3 top ranked proteins sequence and their UniProt identification numbers are also listed.

specific antibodies showed negative in Western blot (data not shown). This data suggests that the mucin domain of lubricin is different from mucin domains of traditional mucous mucins which are not susceptible to trypsin. Difference in glycosylation between lubricin and traditional mucins was also suggested by the treatment with *O*-sialoglycoprotein endopeptidase from *Pasteurella haemolytica* which cleaves heavily sialylated mucin-domain, but only had minor effect on lubricin. As shown in Fig. 6B, after overnight incubation, the density of Coomassie blue stained band was significantly diminished. However, when the digested same samples were probed with mouse monoclonal antibody, Western blot showed that epitope of the antibody, recognizing part of the unglycosylated *N*-terminal region, was still attached to the large mucin domain. Only a small shift in the migration in SDS-PAGE was observed after the endopeptidase treatment.

In order to show that the reason for low recovery of the mucin domain was due to glycosylation, sialidase A and *O*-glycanase were used to remove the majority of *O*-linked oligosaccharides (Fig. 6C and 7). Desialylation with sialidase A decreased the size of lubricin on Ag-PAGE verified that synovial lubricin contained sialic acid (Fig. 6C). When further treated with *O*-glycanase, which cleaves core 1 type *O*-linked glycan (Galβ1,3GalNAcα1-*O*-Ser/Thr) on glycoproteins and glycopeptides, the size of lubricin decreased dramatically and was close to the calculated molecular weight of lubricin without posttranslational modification, i.e. 148 kDa. These results suggested that lubricin is heavily glycosylated and core 1 type *O*-linked oligosaccharides are the predominant *O*-glycans on lubricin. Bands after sialidase A treatment with or without subsequent *O*-glycanase treatment were subjected to LC-MS/MS analysis after trypsin digestion. Sialidase A alone recover 19.1% of lubricin sequence (Fig. 7), most of the peptides were located in the mucin-like domain including 18 random repeats of EPAPTTPK. In contrast, removal of glycosylation using both sialidase and *O*-glycanase gave up to 48% recovery of the lubricin sequence (Fig. 7). By removal of core 1 *O*-glycans, more protein core was revealed and made accessible for digestion providing peptides from the mucin domain repeated to be recovered and detected by LC-MS. Resolved sequence covered almost entire mucin-like domain of lubricin and repeat region without glycosylation could be identified (Fig. 8).

2.3 Discussion

Though several biomarkers in SF and serum have been associated with RA and OA, no single biomarker has sufficient discriminating power to clearly indicate prognosis. Hence, the quest to find new, more efficient single biomarker for cartilage degrading diseases remains. On the other hand, measurement of multiple biomarkers at the time of diagnosis would improve diagnosis accuracy and even early diagnosis. As a candidate biomarker, SF lubricin has been found to be an important lubricant in SF, but expression level is also associated with inflammation. Lubricin has not been characterized fully because of its size and heavily *O*-glycosylation. In this study, SF lubricin was characterized by both glycomic and proteomic means, indicating that in addition to the level of lubricin in SF, both the glycosylation and its degradation are potential marker for disease progression and inflammation.

In combination with our previous study (Estrella et al., 2010) and this study (Fig. 2 and 3), synovial lubricin was shown to possess predominantly core 1 *O*-linked oligosaccharides. Even in a low amounts, with the aid of liquid chromatography-mass spectrometry (LC-MS), small proportions of core 2 oligosaccharides were found to carry sulfate group. In addition,

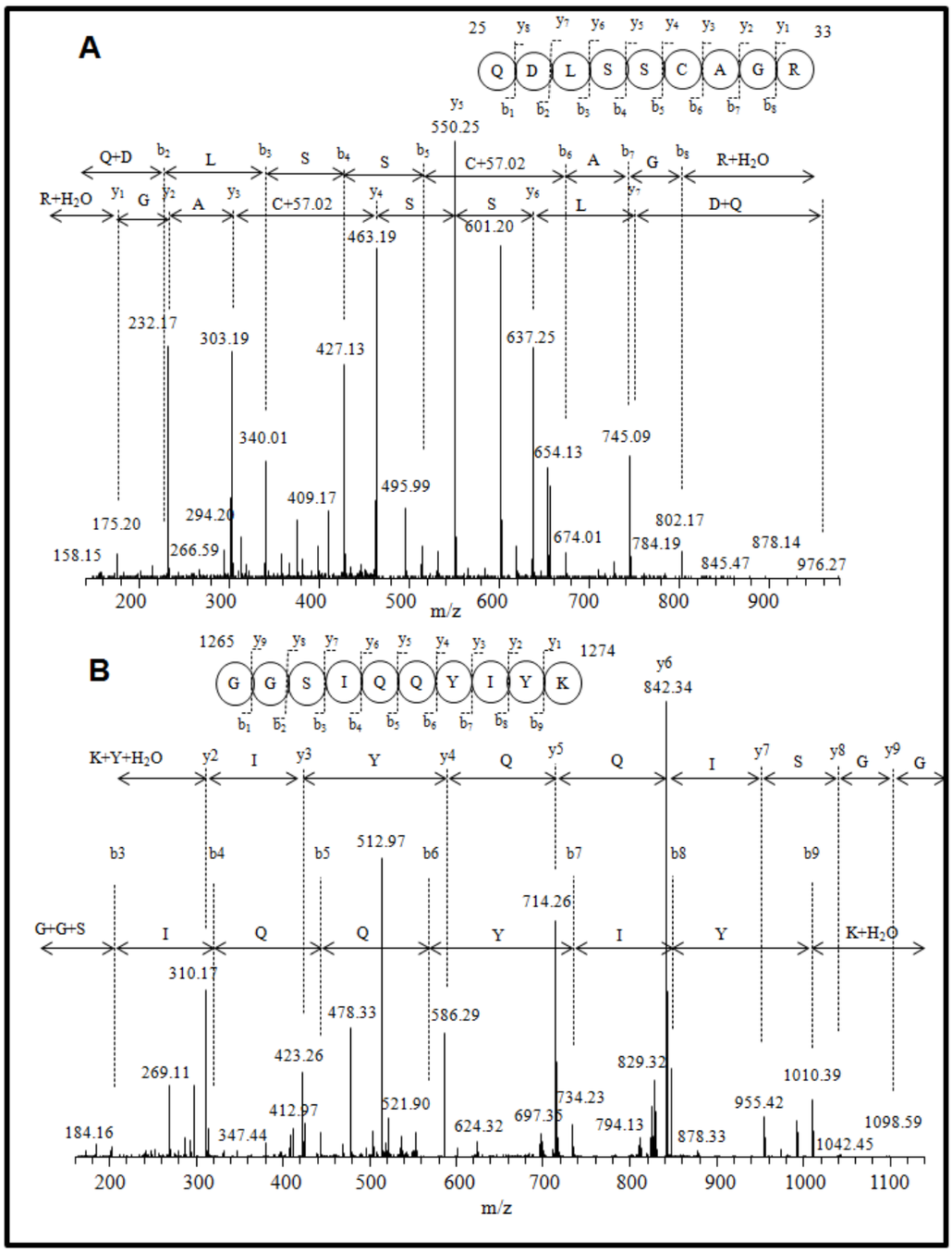

Fig. 5. MS² spectra of one *N*-terminal (A) and one *C*-terminal (B) peptide derived from reduced and alkylated lubricin that was searched against UniProt and NCBI human protein database using GPM software. The position of the *N*-terminal peptide in the protein sequence starts from amino acid 25 and ends at 33. The *m/z* 976.41 is the $[M+H]^+$ precursor ion and *m/z* 488.71 is the $[M+H]^{2+}$. The assigned ID number for this peptide in the GPM database is 1193. The position of the *C*-terminal peptide starts from amino acid 1265 and ends at 1274. The *m/z* 1156.59 is the $[M+H]^+$ precursor ion and *m/z* 578.80 is the $[M+H]^{2+}$.The assigned ID number for this peptide in the GPM is 2538.

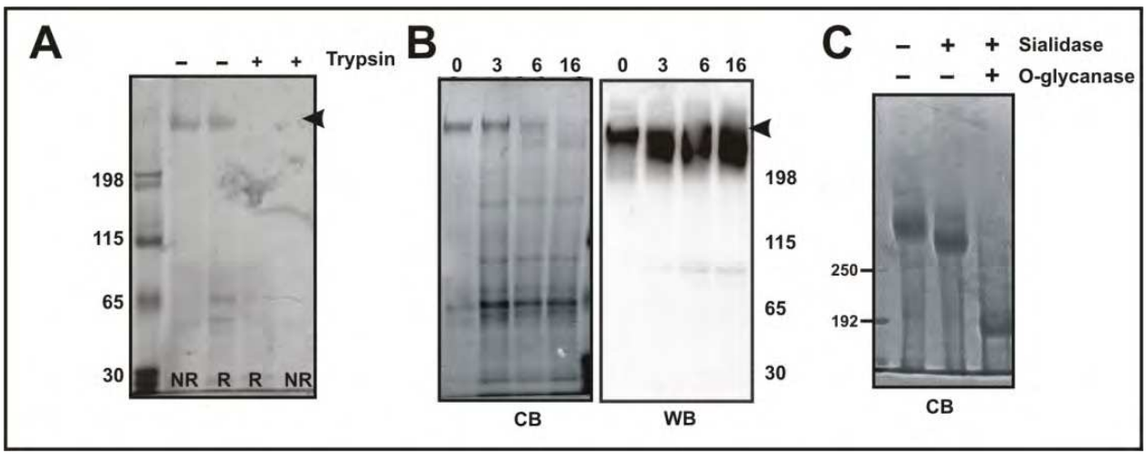

Fig. 6. Proteomic analysis of lubricin under various conditions. All purified samples were reduced and alkylated before separation by 3-8% Tris/acetate NuPAGE gel. (A) Samples (5 µg) were incubated with trypsin. Aliquots were taken out at different time (0 to 16 hours). SDS-PAGE gel was stained with Coomassie blue. (B) Samples (5 µg) were treated with *O*-sialoglycoprotein endopeptidase from *Pasteurella haemolytica.* A duplicated gel was blotted to PVDF membrane and probed with moue monoclonal anti-lubricin antibody. (C) Enriched lubricin sample (8 µg/lane) was treated with sialidase in absence or presence of *O*-glycanase at 37°C overnight. The resultant products were separated by Ag-PAGE under reducing condition.

a sLe^x epitope was also found present on a small proportion of the core 2 oligosaccharides. However, unlike sulfation, the level of fucosylation on lubricin was very low. Though in comparison with LC-MS, immunoassay seems less efficient but very specific to certain glyco-epitopes. For example, MS^2 spectra of ion at *m/z* 1477 suggested a Lewis type epitope. Without further fragmentation and known retention time on LC, it is not easy to define this structure of sLe^a or sLe^x. With sLe^a- and sLe^x-specific antibody, immunoblot demonstrated lubricin was modified with sLe^x. HAA is a lectin specific to terminal GalNAcα1- on *N*- or *O*-glycans. The lack of antibody recognition to Tn-antigen despite HAA reactivity indicated that exposed GalNAcα1- to protein backbone was only sparingly found. Additionally, synovial lubricin was shown to contain PNA binding epitopes (Fig. 2B). This is consistent with that PNA can been used as an affinity ligand to enrich synovial lubricin (Jay et al., 2001; Teeple et al., 2011). The result from the glycomic study using both $LC\text{-}MS^n$ and antibody/lectins showed the presence of a trace amount of Tn antigen, high abundant sialylated and unsialylated core 1 and several sialylated, fucosylated and sulfated core 2 oligosaccharides to be present on lubricin.

Suggestions of lubricin involvement in disease and inflammation can be identified from its glycosylation. Glycomic analysis showed that approximately 50% of the lubricin *O*-glycans contain terminal galactose, such as the T antigen. It makes lubricin a potential ligand for galectins, which are a mammalian lectin family recognizing terminal galactose. Increased expression of galectin-3 has been reported in synovial fluid from RA patients (Ohshima et al., 2003). Galectin-3 is believed to play a pro-inflammation role in joint diseases in which galectin-3 together with soluble fibrinogen was found to regulate neutrophil activation, degranulation and survival (Fernandez et al., 2005). Another attractive glyco-epitope on lubricin, sLe^x, is reminiscent of selectin ligands which are involved in leukocyte trafficking. For instance, although it is in a low amount, L-selectin on the surface of synovial neutrophils as well as soluble L-selectin are reported in synovial fluid (Humbria et al., 1994; De Clerck et al., 1995).

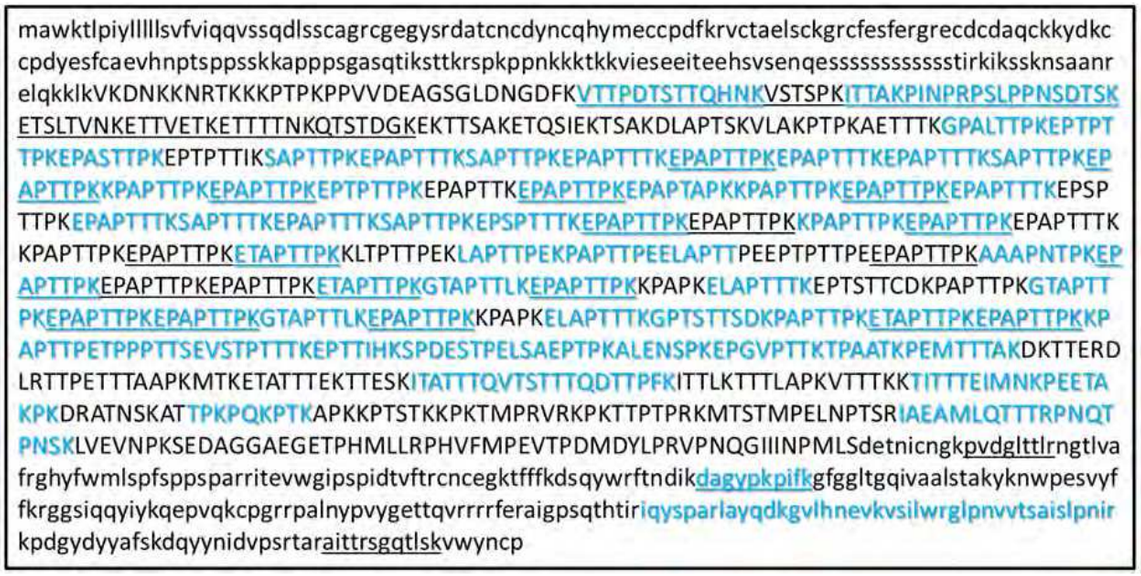

Fig. 7. Peptides recovery of bands excised from sialidase A and *O*-glycanase (Fig. 6C). The mucin-like domain is in capital (encoded by exon 6). Sequences recovered after sialidase A treatment were underlined, while sequences recovered after sialidase A and *O*-glycanase treatment were in blue.

With increased mechanical stress and protease activity associated with OA and RA, the fragmentation of lubricin shown here opens up a new possibility for disease-specific biomarkers. A few fragments of lubricin were detected in synovial fluid, which were enriched together with intact protein. The *O*-glycosylation domain is supposed to protect against proteolytic cleavage. In the case of lubricin, however, it was extensively degraded by trypsin but resistant to *O*-sialoglycoprotein endopeptidase (Fig . 5A). Similarly, lubricin has been found to be extensively degraded by papain, trypsin and pronase and to a lesser extent by pepsin (Flannery et al., 1999). Other proteases, such as neutrophil elastase (a serine protease) and cathepsin B (a cysteine protease), are also able to degrade lubricin *in vitro* (Jones et al., 2003; Elsaid et al., 2005). Interestingly, lubricin tryptic peptides were detected as low as the 30-65 kDa region (Table 1 and Fig. 4). These fragments are unlikely to contain the full mucin-like domain, but more likely an *N*- or *C*-terminal domain with a portion of mucin-like domain (non-glycosylated *N*-terminus has a mass of 33.8 kDa and the *C*-terminus 35.4 kDa). So far, it is not clear whether they were from unique cleavages along lubricin sequence or just randomly excised *in vivo*. Evidence has indicated *N*-terminus of lubricin is more sensitive to neutrophil elastase (Elsaid et al., 2005). Purified neutrophil elastase has been shown to damage cartilage explants *in vitro* (Burkhardt et al., 1988). Also neutrophil elastase, and not MMPs, can destroy the superficial layer of cartilage where lubricin locates. Consequently, MMPs have better access to cartilage molecules in less superficial layers of cartilage (Jasin & Taurog, 1991).

Are lubricin fragments associated with inflammation or pathophysiology of degenerative joint disease? Or does lubricin fragmentation patterns in OA or RA differ from those in healthy individuals? Recent studies reported the fragment of lubricin in SF (Gobezie et al., 2007; Kamphorst et al., 2007). In the work of Kamphorst et al., they found two lubricin *C*-terminal fragments (R^{1285}PALNYPVYGETTQV^{1299} and D^{1373}QYYNIDVPSRTA^{1385}) in OA SF but not in healthy SF (Kamphorst et al., 2007). In the current study with enriched RA synovial lubricin,

the first sequence (1285-1300) was found distributed throughout the gel; while the second sequence (1373-1385) was only detected in area 3 and 5, areas lower than the lubricin area (Fig. 4B and C). Additionally, several new peptides derived from both *N*-terminus and *C*-termini were found in this study. These fragments could be solely by-products of degenerative joint. Alternatively, these fragments might play a regulatory role. For example, fragments from fibronectin and aggrecan have been reported to correlate with joint diseases (Homandberg et al., 1997a; Homandberg et al., 1997b; Struglics et al., 2009). Interestingly, these two proteins were also found in this study (Table I). It is not clear whether they form a protein complex together with lubricin or just happened to be co-purified with lubricin.

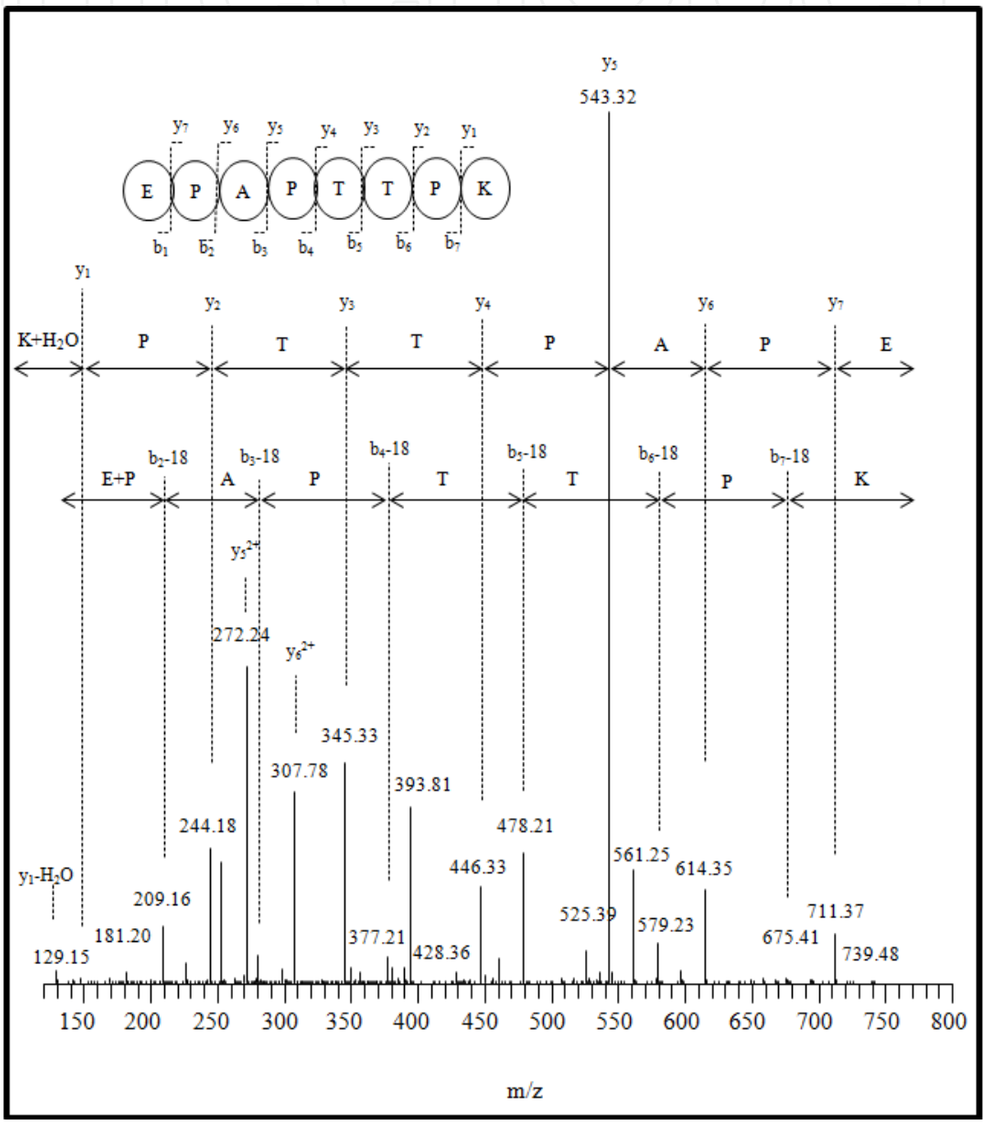

Fig. 8. MS² spectrum of the peptide derived after desialylated and *O*-glycanase treated lubricin sample searched against UniProt and NCBI human protein database using GPM software. The *m/z* 420.73 is the $[M+H]^{2+}$ precursor ion. The assigned ID number for the peptide in the GPM database is 3740. This peptide sequence is in the mucin domain of lubricin and is repeated 18 times in lubricin.

Besides two flanking protein domains, in the middle of lubricin there is a mucin-like domain. To our knowledge, this is the first report of a protein sequence within this domain (Fig. 7 and 8). It should be noted that among 59 imperfect repeat units, 18 have a perfect repeating unit of EPAPTTPK (Fig. 7 and 8). *O*-glycanase treatment greatly increased the recovery. As discussed above, proteolytic cleavage sites on lubricin are probably located within the mucin-like domain, and here we show that the mucin domain of lubricin is indeed accessible to proteolytic enzymes. This is probably due to a dispersed *O*-glycosylation in contrast to continuous *O*-glycosylation as the heavily bottle-brush-like *O*-glycosylation on traditional mucins, which will hinder the access to the cleavage site. Being able to sequence mucin-like domain in lubricin will facilitate the mapping of authentic proteolytic cleavage sites on lubricin *in situ* and to investigate the effect of cytokine regulated proteases on synovial lubricin during joint diseases.

3. Conclusion

In summary, using glycoproteomics we fully characterized the major glycoprotein in SF as lubricin. With knowledge of *O*-glycosylation and proteomic properties of lubricin, it allowed us to identify RA or OA-specific glyco-epitopes and fragments, enabling us to better understand how the glycosylation of lubricin is influenced by inflammation of the joint.

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5. References

Arnett, F. C., Edworthy, S. M., Bloch, D. A., McShane, D. J., Fries, J. F., Cooper, N. S., Healey, L. A., Kaplan, S. R., Liang, M. H., Luthra, H. S., Medsger, T. A., Mitchell, D. M., Neustadt, D. H., Pinals, R. S., Schaller, J. G., Sharp, J. T., Wilder, R. L. & Hunder, G. G. (1988). The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum*, Vol.31, No.3, (Mar 1988), pp. 315-24, ISSN 0004-3591

Bao, J. P., Chen, W. P. & Wu, L. D. (2011). Lubricin: a novel potential biotherapeutic approaches for the treatment of osteoarthritis. *Mol Biol Rep*, Vol.38, No.5, (Jan 2011), pp. 2879-85, ISSN 0301-4851

Bayless, K. J., Meininger, G. A., Scholtz, J. M. & Davis, G. E. (1998). Osteopontin is a ligand for the alpha4beta1 integrin. *J Cell Sci*, Vol.111, No.9, (May 1998), pp. 1165-74, ISSN 0021-9533

Brockhausen, I. & Anastassiades, T. P. (2008). Inflammation and arthritis: perspectives of the glycobiologist. *Expert Rev Clin Immunol*, Vol.4, No.2, (Mar 2008), pp. 173-91, ISSN 1744-8409

Burkhardt, H., Rehkopf, E., Kasten, M., Rauls, S. & Heimann, P. (1988). Interaction of polymorphonuclear leukocytes with cartilage in vitro. Catabolic effects of serine

proteases and oxygen radicals. *Scand J Rheumatol*, Vol.17, No.3, (Jan 1988), pp. 183-95, ISSN 0300-9742

De Clerck, L. S., De Gendt, C. M., Bridts, C. H., Van Osselaer, N. & Stevens, W. J. (1995). Expression of neutrophil activation markers and neutrophil adhesion to chondrocytes in rheumatoid arthritis patients: relationship with disease activity. *Res Immunol*, Vol.146, No.2, (Feb 1995), pp. 81-7, ISSN 0923-2494

Elsaid, K. A., Jay, G. D. & Chichester, C. O. (2007). Reduced expression and proteolytic susceptibility of lubricin/superficial zone protein may explain early elevation in the coefficient of friction in the joints of rats with antigen-induced arthritis. *Arthritis Rheum*, Vol.56, No.1, (Jan 2007), pp. 108-16, ISSN 0004-3591

Elsaid, K. A., Jay, G. D., Warman, M. L., Rhee, D. K. & Chichester, C. O. (2005). Association of articular cartilage degradation and loss of boundary-lubricating ability of synovial fluid following injury and inflammatory arthritis. *Arthritis Rheum*, Vol.52, No.6, (Jun 2005), pp. 1746-55, ISSN 0004-3591

Estrella, R. P., Whitelock, J. M., Packer, N. H. & Karlsson, N. G. (2010). The glycosylation of human synovial lubricin: implications for its role in inflammation. *Biochem J*, Vol.429, No.2, (Jul 2010), pp. 359-67, ISSN 1470-8728

Fernandez, G. C., Ilarregui, J. M., Rubel, C. J., Toscano, M. A., Gomez, S. A., Beigier Bompadre, M., Isturiz, M. A., Rabinovich, G. A. & Palermo, M. S. (2005). Galectin-3 and soluble fibrinogen act in concert to modulate neutrophil activation and survival: involvement of alternative MAPK pathways. *Glycobiology*, Vol.15, No.5, (May 2005), pp. 519-27, ISSN 0959-6658

Flannery, C. R., Hughes, C. E., Schumacher, B. L., Tudor, D., Aydelotte, M. B., Kuettner, K. E. & Caterson, B. (1999). Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and Is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. *Biochem Biophys Res Commun*, Vol.254, No.3, (Jan 1999), pp. 535-41, ISSN 0006-291X

Flannery, C. R., Zollner, R., Corcoran, C., Jones, A. R., Root, A., Rivera-Bermudez, M. A., Blanchet, T., Gleghorn, J. P., Bonassar, L. J., Bendele, A. M., Morris, E. A. & Glasson, S. S. (2009). Prevention of cartilage degeneration in a rat model of osteoarthritis by intraarticular treatment with recombinant lubricin. *Arthritis Rheum*, Vol.60, No.3, (Mar 2009), pp. 840-7, ISSN 0004-3591

Flato, B., Lien, G., Smerdel, A., Vinje, O., Dale, K., Johnston, V., Sorskaar, D., Moum, T., Ploski, R. & Forre, O. (2003). Prognostic factors in juvenile rheumatoid arthritis: a case-control study revealing early predictors and outcome after 14.9 years. *J Rheumatol*, Vol.30, No.2, (Feb 2003), pp. 386-93, ISSN 0315-162X

Gobezie, R., Kho, A., Krastins, B., Sarracino, D. A., Thornhill, T. S., Chase, M., Millett, P. J. & Lee, D. M. (2007). High abundance synovial fluid proteome: distinct profiles in health and osteoarthritis. *Arthritis Res Ther*, Vol.9, No.2, (Apr 2007), pp. R36, ISSN 1478-6362

Goldring, M. B. & Goldring, S. R. (2007). Osteoarthritis. *J Cell Physiol*, Vol.213, No.3, (Dec 2007), pp. 626-34, ISSN 1097-4652

Hasegawa, M., Hirata, H., Sudo, A., Kato, K., Kawase, D., Kinoshita, N., Yoshida, T. & Uchida, A. (2004). Tenascin-C concentration in synovial fluid correlates with

radiographic progression of knee osteoarthritis. *J Rheumatol*, Vol.31, No.10, (Oct 2004), pp. 2021-6, ISSN 0315-162X

Hasegawa, M., Nakoshi, Y., Iino, T., Sudo, A., Segawa, T., Maeda, M., Yoshida, T. & Uchida, A. (2009). Thrombin-cleaved osteopontin in synovial fluid of subjects with rheumatoid arthritis. *J Rheumatol*, Vol.36, No.2, (Feb 2009), pp. 240-5, ISSN 0315-162X

Hayes, C. A., Karlsson, N. G., Struwe, W. B., Lisacek, F., Rudd, P. M., Packer, N. H. & Campbell, M. P. (2011). UniCarb-DB: a database resource for glycomic discovery. *Bioinformatics*, Vol.27, No.9, (May 2011), pp. 1343-4, ISSN 1367-4811

Homandberg, G. A., Davis, G., Maniglia, C. & Shrikhande, A. (1997a). Cartilage chondrolysis by fibronectin fragments causes cleavage of aggrecan at the same site as found in osteoarthritic cartilage. *Osteoarthritis Cartilage*, Vol.5, No.6, (Nov 1997a), pp. 450-3, ISSN 1063-4584

Homandberg, G. A., Hui, F., Wen, C., Purple, C., Bewsey, K., Koepp, H., Huch, K. & Harris, A. (1997b). Fibronectin-fragment-induced cartilage chondrolysis is associated with release of catabolic cytokines. *Biochem J*, Vol.321, No.3, (Feb 1997b), pp. 751-7, ISSN 0264-6021

Hrkal, Z., Kuzelova, K., Muller-Eberhard, U. & Stern, R. (1996). Hyaluronan-binding properties of human serum hemopexin. *FEBS Lett*, Vol.383, No.1-2, (Mar 1996), pp. 72-4, ISSN 0014-5793

Humbria, A., Diaz-Gonzalez, F., Campanero, M. R., Arroyo, A. G., Laffon, A., Gonzalez-Amaro, R. & Sanchez-Madrid, F. (1994). Expression of L-selectin, CD43, and CD44 in synovial fluid neutrophils from patients with inflammatory joint diseases. Evidence for a soluble form of L-selectin in synovial fluid. *Arthritis Rheum*, Vol.37, No.3, (Mar 1994), pp. 342-8, ISSN 0004-3591

Ikegawa, S., Sano, M., Koshizuka, Y. & Nakamura, Y. (2000). Isolation, characterization and mapping of the mouse and human PRG4 (proteoglycan 4) genes. *Cytogenet Cell Genet*, Vol.90, No.3-4, (Aug 2000), pp. 291-7, ISSN 0301-0171

Issa, S., Moran, A. P., Ustinov, S. N., Lin, J. H., Ligtenberg, A. J. & Karlsson, N. G. (2010). O-linked oligosaccharides from salivary agglutinin: Helicobacter pylori binding sialyl-Lewis x and Lewis b are terminating moieties on hyperfucosylated oligo-N-acetyllactosamine. *Glycobiology*, Vol.20, No.8, (Aug 2010), pp. 1046-57, ISSN 1460-2423

Jasin, H. E. & Taurog, J. D. (1991). Mechanisms of disruption of the articular cartilage surface in inflammation. Neutrophil elastase increases availability of collagen type II epitopes for binding with antibody on the surface of articular cartilage. *J Clin Invest*, Vol.87, No.5, (May 1991), pp. 1531-6, ISSN 0021-9738

Jay, G. D. (1992). Characterization of a bovine synovial fluid lubricating factor. I. Chemical, surface activity and lubricating properties. *Connect Tissue Res*, Vol.28, No.1-2, (Jan 1992), pp. 71-88, ISSN 0300-8207

Jay, G. D., Harris, D. A. & Cha, C. J. (2001). Boundary lubrication by lubricin is mediated by O-linked beta(1-3)Gal-GalNAc oligosaccharides. *Glycoconj J*, Vol.18, No.10, (Oct 2001), pp. 807-15, ISSN 0282-0080

Jones, A. R. C., Hughes, C. E., Wainwright, S. D., Flannery, C. R., Little, C. B. & Caterson, B. (2003). Degradation of PRG4/SZP by matrix proteases [abstract]. *Trans Orthop Res Soc*, Vol.28, (Feb 2003), pp. 133, ISSN 0065-9533

Kamphorst, J. J., van der Heijden, R., DeGroot, J., Lafeber, F. P., Reijmers, T. H., van El, B., Tjaden, U. R., van der Greef, J. & Hankemeier, T. (2007). Profiling of endogenous peptides in human synovial fluid by NanoLC-MS: method validation and peptide identification. *J Proteome Res*, Vol.6, No.11, (Nov 2007), pp. 4388-96, ISSN 1535-3893

Kantor, A. B., Wang, W., Lin, H., Govindarajan, H., Anderle, M., Perrone, A. & Becker, C. (2004). Biomarker discovery by comprehensive phenotyping for autoimmune diseases. *Clin Immunol*, Vol.111, No.2, (May 2004), pp. 186-95, ISSN 1521-6616

Kuhn, E., Wu, J., Karl, J., Liao, H., Zolg, W. & Guild, B. (2004). Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and 13C-labeled peptide standards. *Proteomics*, Vol.4, No.4, (Apr 2004), pp. 1175-86, ISSN 1615-9853

Kuster, B., Wheeler, S. F., Hunter, A. P., Dwek, R. A. & Harvey, D. J. (1997). Sequencing of *N*-linked oligosaccharides directly from protein gels: in-gel deglycosylation followed by matrix-assisted laser desorption/ionization mass spectrometry and normal-phase high-performance liquid chromatography. *Anal Biochem*, Vol.250, No.1, (Jul 1997), pp. 82-101, ISSN 0003-2697

Liao, H., Wu, J., Kuhn, E., Chin, W., Chang, B., Jones, M. D., O'Neil, S., Clauser, K. R., Karl, J., Hasler, F., Roubenoff, R., Zolg, W. & Guild, B. C. (2004). Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. *Arthritis Rheum*, Vol.50, No.12, (Dec 2004), pp. 3792-803, ISSN 0004-3591

Marcelino, J., Carpten, J. D., Suwairi, W. M., Gutierrez, O. M., Schwartz, S., Robbins, C., Sood, R., Makalowska, I., Baxevanis, A., Johnstone, B., Laxer, R. M., Zemel, L., Kim, C. A., Herd, J. K., Ihle, J., Williams, C., Johnson, M., Raman, V., Alonso, L. G., Brunoni, D., Gerstein, A., Papadopoulos, N., Bahabri, S. A., Trent, J. M. & Warman, M. L. (1999). CACP, encoding a secreted proteoglycan, is mutated in camptodactyly-arthropathy-coxa vara-pericarditis syndrome. *Nat Genet*, Vol.23, No.3, (Nov 1999), pp. 319-22, ISSN 1061-4036

Nagase, H. & Woessner, J. F., Jr. (1999). Matrix metalloproteinases. *J Biol Chem*, Vol.274, No.31, (Jul 1999), pp. 21491-4, ISSN 0021-9258

O'Regan, A. & Berman, J. S. (2000). Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. *Int J Exp Pathol*, Vol.81, No.6, (Dec 2000), pp. 373-90, ISSN 0959-9673

Ohshima, S., Kuchen, S., Seemayer, C. A., Kyburz, D., Hirt, A., Klinzing, S., Michel, B. A., Gay, R. E., Liu, F. T., Gay, S. & Neidhart, M. (2003). Galectin 3 and its binding protein in rheumatoid arthritis. *Arthritis Rheum*, Vol.48, No.10, (Oct 2003), pp. 2788-95, ISSN 0004-3591

Oldberg, A., Franzen, A. & Heinegard, D. (1986). Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. *Proc Natl Acad Sci U S A*, Vol.83, No.23, (Dec 1986), pp. 8819-23, ISSN 0027-8424

Rees, S. G., Davies, J. R., Tudor, D., Flannery, C. R., Hughes, C. E., Dent, C. M. & Caterson, B. (2002). Immunolocalisation and expression of proteoglycan 4 (cartilage superficial zone proteoglycan) in tendon. *Matrix Biol*, Vol.21, No.7, (Nov 2002), pp. 593-602, ISSN 0945-053X

Rhee, D. K., Marcelino, J., Al-Mayouf, S., Schelling, D. K., Bartels, C. F., Cui, Y., Laxer, R., Goldbach-Mansky, R. & Warman, M. L. (2005a). Consequences of disease-causing

mutations on lubricin protein synthesis, secretion, and post-translational processing. *J Biol Chem*, Vol.280, No.35, (Sep 2005a), pp. 31325-32, ISSN 0021-9258

Rhee, D. K., Marcelino, J., Baker, M., Gong, Y., Smits, P., Lefebvre, V., Jay, G. D., Stewart, M., Wang, H., Warman, M. L. & Carpten, J. D. (2005b). The secreted glycoprotein lubricin protects cartilage surfaces and inhibits synovial cell overgrowth. *J Clin Invest*, Vol.115, No.3, (Mar 2005b), pp. 622-31, ISSN 0021-9738

Ruiz-Romero, C. & Blanco, F. J. (2010). Proteomics role in the search for improved diagnosis, prognosis and treatment of osteoarthritis. *Osteoarthritis Cartilage*, Vol.18, No.4, (Apr 2010), pp. 500-9, ISSN 1522-9653

Schmid, T., Homandberg, G., Madsen, L., Su, J. & Kuettner, K. (2002). Superficial zone protein (SZP) binds to macromolecules in the lamina splendens of arthicular cartilage [abstract]. *Trans Orthop Res Soc*, Vol.27, (Feb 2002), pp. 359, ISSN 0065-9533

Schmid, T., Lindley, K., Su, J., Soloveychik, V., Block, J., Kuettner, K. & Schumacher, B. (2001). Superficial zone protein (SZP) is an abundant glycoprotein in human synovial fluid and serum. *Trans Orthop Res Soc*, Vol.26, 2001), pp. 82,

Schmidt, T. A., Gastelum, N. S., Han, E. H., Nugent-Derfus, G. E., Schumacher, B. L. & Sah, R. L. (2008). Differential regulation of proteoglycan 4 metabolism in cartilage by IL-1alpha, IGF-I, and TGF-beta1. *Osteoarthritis Cartilage*, Vol.16, No.1, (Jan 2008), pp. 90-7, ISSN 1063-4584

Schmidt, T. A., Plaas, A. H. & Sandy, J. D. (2009). Disulfide-bonded multimers of proteoglycan 4 PRG4 are present in normal synovial fluids. *Biochim Biophys Acta*, Vol.1790, No.5, (May 2009), pp. 375-84, ISSN 0006-3002

Schulz, B. L., Packer, N. H. & Karlsson, N. G. (2002). Small-scale analysis of O-linked oligosaccharides from glycoproteins and mucins separated by gel electrophoresis. *Anal Chem*, Vol.74, No.23, (Dec 2002), pp. 6088-97, ISSN 0003-2700

Schumacher, B. L., Block, J. A., Schmid, T. M., Aydelotte, M. B. & Kuettner, K. E. (1994). A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch Biochem Biophys*, Vol.311, No.1, (May 1994), pp. 144-52, ISSN 0003-9861

Schumacher, B. L., Hughes, C. E., Kuettner, K. E., Caterson, B. & Aydelotte, M. B. (1999). Immunodetection and partial cDNA sequence of the proteoglycan, superficial zone protein, synthesized by cells lining synovial joints. *J Orthop Res*, Vol.17, No.1, (Jan 1999), pp. 110-20, ISSN 0736-0266

Schumacher, B. L., Schmidt, T. A., Voegtline, M. S., Chen, A. C. & Sah, R. L. (2005). Proteoglycan 4 (PRG4) synthesis and immunolocalization in bovine meniscus. *J Orthop Res*, Vol.23, No.3, (May 2005), pp. 562-8, ISSN 0736-0266

Sinz, A., Bantscheff, M., Mikkat, S., Ringel, B., Drynda, S., Kekow, J., Thiesen, H. J. & Glocker, M. O. (2002). Mass spectrometric proteome analyses of synovial fluids and plasmas from patients suffering from rheumatoid arthritis and comparison to reactive arthritis or osteoarthritis. *Electrophoresis*, Vol.23, No.19, (Sep 2002), pp. 3445-56, ISSN 0173-0835

Struglics, A., Larsson, S., Hansson, M. & Lohmander, L. S. (2009). Western blot quantification of aggrecan fragments in human synovial fluid indicates differences in fragment patterns between joint diseases. *Osteoarthritis Cartilage*, Vol.17, No.4, (Apr 2009), pp. 497-506, ISSN 1522-9653

Sun, Y., Berger, E. J., Zhao, C., Jay, G. D., An, K. N. & Amadio, P. C. (2006). Expression and mapping of lubricin in canine flexor tendon. *J Orthop Res*, Vol.24, No.9, (Sep 2006), pp. 1861-8, ISSN 0736-0266

Swann, D. A., Silver, F. H., Slayter, H. S., Stafford, W. & Shore, E. (1985). The molecular structure and lubricating activity of lubricin isolated from bovine and human synovial fluids. *Biochem J*, Vol.225, No.1, (Jan 1985), pp. 195-201, ISSN 0264-6021

Swann, D. A., Slayter, H. S. & Silver, F. H. (1981). The molecular structure of lubricating glycoprotein-I, the boundary lubricant for articular cartilage. *J Biol Chem*, Vol.256, No.11, (Jun 1981), pp. 5921-5, ISSN 0021-9258

Teeple, E., Elsaid, K. A., Jay, G. D., Zhang, L., Badger, G. J., Akelman, M., Bliss, T. F. & Fleming, B. C. (2011). Effects of supplemental intra-articular lubricin and hyaluronic acid on the progression of posttraumatic arthritis in the anterior cruciate ligament-deficient rat knee. *Am J Sports Med*, Vol.39, No.1, (Jan 2011), pp. 164-72, ISSN 1552-3365

Tilleman, K., Van Beneden, K., Dhondt, A., Hoffman, I., De Keyser, F., Veys, E., Elewaut, D. & Deforce, D. (2005). Chronically inflamed synovium from spondyloarthropathy and rheumatoid arthritis investigated by protein expression profiling followed by tandem mass spectrometry. *Proteomics*, Vol.5, No.8, (May 2005), pp. 2247-57, ISSN 1615-9853

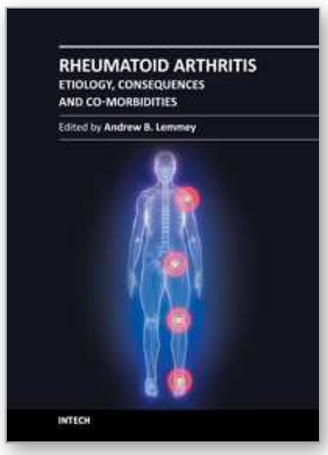
Ungethuem, U., Haeupl, T., Witt, H., Koczan, D., Krenn, V., Huber, H., von Helversen, T. M., Drungowski, M., Seyfert, C., Zacher, J., Pruss, A., Neidel, J., Lehrach, H., Thiesen, H. J., Ruiz, P. & Blass, S. (2010). Molecular signatures and new candidates to target the pathogenesis of rheumatoid arthritis. *Physiol Genomics*, Vol.42A, No.4, (Nov 2010), pp. 267-82, ISSN 1531-2267

Yamamoto, N., Nakashima, T., Torikai, M., Naruse, T., Morimoto, J., Kon, S., Sakai, F. & Uede, T. (2007). Successful treatment of collagen-induced arthritis in non-human primates by chimeric anti-osteopontin antibody. *Int Immunopharmacol*, Vol.7, No.11, (Nov 2007), pp. 1460-70, ISSN 1567-5769

Yokosaki, Y., Matsuura, N., Sasaki, T., Murakami, I., Schneider, H., Higashiyama, S., Saitoh, Y., Yamakido, M., Taooka, Y. & Sheppard, D. (1999). The integrin alpha(9)beta(1) binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. *J Biol Chem*, Vol.274, No.51, (Dec 1999), pp. 36328-34, ISSN 0021-9258

Young, A. A., McLennan, S., Smith, M. M., Smith, S. M., Cake, M. A., Read, R. A., Melrose, J., Sonnabend, D. H., Flannery, C. R. & Little, C. B. (2006). Proteoglycan 4 downregulation in a sheep meniscectomy model of early osteoarthritis. *Arthritis Res Ther*, Vol.8, No.2, (Jan 2006), pp. R41, ISSN 1478-6362

Zhang, C. C., Rogalski, J. C., Evans, D. M., Klockenbusch, C., Beavis, R. C. & Kast, J. (2011). In silico protein interaction analysis using the global proteome machine database. *J Proteome Res*, Vol.10, No.2, (Feb 2011), pp. 656-68, ISSN 1535-3907



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The purpose of this book is to provide up-to-date, interesting, and thought-provoking perspectives on various aspects of research into current and potential treatments for rheumatoid arthritis (RA). This book features 16 chapters, with contributions from numerous countries (e.g. UK, USA, Japan, Sweden, Spain, Ireland, Poland, Norway), including chapters from internationally recognized leaders in rheumatology research. It is anticipated that Rheumatoid Arthritis - Etiology, Consequences and Co-Morbidities will provide both a useful reference and source of potential areas of investigation for research scientists working in the field of RA and other inflammatory arthropathies.

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