INTRODUCTION: Fibromodulin (FMOD) is an abundant small leucine-rich proteoglycan (SLRP) of the cartilage extracellular matrix where it regulates collagen fibrillogenesis and transforming growth factor (TGF)-β bioavailability. Proteolysis of FMOD may be important in degradation of cartilages, but the enzymes responsible in vivo have yet to be defined. MMP-13 has been shown to cleave in-vitro between residues 63 and 64 of the .PAYAG sequence in bovine FMOD. This cleavage only occurred when FMOD was in situ in cartilage but not in solution-phase digests with MMP13. ADAMTS-4 and -5 have been shown to cleave at the same site when bovine FMOD was digested in solution, but no data is available on cleavage of FMOD in cartilage. Whether similar FMOD cleavage occurs in pathological human cartilage in vivo has not been determined. In the present study we have compared the naturally-occurring FMOD core protein fragments in pathological human knee and hip articular cartilage and meniscus with those generated by in-vitro digestion of macroscopically normal knee articular cartilage with MMP-13, ADAMTS-4 or ADAMTS-5, three key enzymes involved in arthritis pathophysiology. Ovine articular cartilage (AC) explant cultures were also stimulated with interleukin (IL-1) and oncostatin-M (OSM) to a broad range MMP inhibitor to determine what FMOD and aggrecan species were attributable to MMP or ADAMTS cleavages. The FMOD core protein fragmentation patterns were identified by Western blotting using an antibody (TsYG11) to a linear N terminal FM sequence (TYGSPSPDP) which is immediately C-terminal to the putative MMP-13/ADAMTS cleavage site in human FMOD. An antibody directed to the C-terminal nonapeptide sequence (PR-184) of FMOD (LRLASLIEI) was also used to map FMOD core protein fragments to specific regions of the core protein.

METHODS: Surgically discarded human tissues from knee and hip replacements were obtained with informed consent of our institutional HREC. Normal, cadaveric, age-matched, knee femoral and tibial AC and lateral and medial menisci were obtained from The National Institute of Advancement in Medicine, Jessup, PA, USA.

FMOD fragmentation in pathological human joint tissues: Finely diced human knee and hip AC and knee joint menisci were extracted with GSHCl + proteinase inhibitors, and the extracts digested with chondroitinase ABC (0.2 U/ml), keratanase-I (0.1U/ml) and selected samples with N-glycanase (PNGase F). Samples were electrophoresed on 10% PAG Bis-Tris gels, blotted to nitro cellulose and probed with TsYG11 and PR-184 FMOD antibodies, with the standard 0.8 mg wet weight of tissue was loaded/lane.

Generation of FMOD fragments in ovine cartilage explant cultures: Full-depth 6-12 month ovine trochlear groove AC was cultured serum-free ± 10ng/ml IL-1α + 50ng/ml oncostatin M (IL-1/OSM) ± 300nM MMP-inhibitor which at this concentration inhibits MMP-1, -2, -3, -7, -8, -9, -13 and -14 but not the ADAMTSs. At day 5 or 12 the explants and corresponding media samples were harvested, digested with papain and the percent loss of proteoglycan and collagen quantified. The remaining explants were digested with 4M GSHCl and examined by Western blotting using PR-184.

Generation of FMOD fragments in vitro. Finely diced normal human AC samples (100mg wet weight) were either (i) digested directly with 4M GSHCl, or were dispersed in (ii) a solution (0.2ml) of 1mM APMA and MMP-13 (50µg/ml) in MMP digestion buffer (50mM Tris HCl 150mM NaCl 5mM CaCl2 1mM ZnCl2 0.01% Brij 35 pH 7.5); (iii) 1mM APMA in MMP digestion buffer; (iv) ADAMTS-4 or (v) ADAMTS-5 (50 µg/ml) in MMP digestion buffer. After 24h digestion at 37°C, digestion buffers were harvested and tissue residues extracted with 4M GSHCl and examined by Western blotting with TsYG11, PR-184 FMOD, and antibodies BC-3 and BC-14 to ADAMTS and MMP-generated aggrecan neoepitopes, respectively.

RESULTS and DISCUSSION: In pathological human tissues, few fragments of FMOD bearing the C-terminus were detected using Ab PR-184, while multiple bands with an intact N-terminus were observed (Fig 1A), suggesting that unlike other SLRPs, FMOD was extensively C-terminally processed. Western blots of explant AC stimulated with IL-1/OSM showed aggrecan loss was associated with increased cleavage by ADAMTS on day 5 but not MMPs. In contrast, collagenolysis did not occur until day 12 (10-20% loss), with 80% collagen loss by day 21. The MMP-inhibitor did not modulate aggrecan loss but completely abrogated collagen release. No evidence of FMOD fragmentation was detected on day 5 using Ab PR-184 despite active aggregcanolysis. Most of the FMOD was cleaved to a single 47kDa fragment by day 12 after the majority of the aggrecan had been released but before significant collagenolysis had occurred (Fig 1B). This was due to MMPs and could be blocked completely with the MMP-inhibitor which does not inhibit ADAMTS-driven aggregcanolysis. In this explant model of cartilage degradation induced by IL-1/OSM, FMOD breakdown appears to be driven by MMP rather than ADAMTS activity. Digestion of normal human knee AC with MMP-13 and ADAMTS-4 generated 35, 45 and 47 kDa FMOD fragments similar to those present in pathological AC (Fig 1C). Despite its superior aggrecanase activity (Fig 1D), ADAMTS-5 showed little activity against FMOD in cartilage. These studies have demonstrated that despite the potential for ADAMTS enzymes, and ADAMTS-4 in particular, to cleave FMOD in cartilage, MMP-13 plays a more significant role in this process. Nevertheless, FMOD catabolites that could not be attributed to any of the enzymes tested in vitro, were also identified in pathological human cartilage and meniscus.

Figure 1. FMOD fragmentation in Western blots A. Pathological and normal human cartilages, PR-184 and TsYG11 blots, B. II-1/OSM stimulated explant cultures, PR-184 blot. C. Ovine cartilage digests digested with MMP-13 and ADAMTS-4 and -5 TsYG11 blots D. MMP and ADAMTS aggrecan neoepitopes in cartilage digests.

Acknowledgements. Supported by NHMRC Project Grant 352562.

Poster No. 1030 • 55th Annual Meeting of the Orthopaedic Research Society