Early Changes in Synovial Protease Gene Expression after Surgical Induction of Post-Traumatic Osteoarthritis in a Porcine Large Animal Model

Jakob T. Sieker, MD¹, Ugur M. Ayturk, PhD¹, Benedikt L. Proffen, MD¹, Braden C. Fleming, PhD²,³, Martha M. Murray, MD¹.

¹Department of Orthopaedic Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA, ²Warren Alpert Medical School, Brown University and Rhode Island Hospital, Providence, RI, USA, ³Center for Biomedical Engineering, Brown University, Providence, RI, USA.


Introduction: Protease mediated breakdown of cartilage extracellular matrix molecules (i.e. type II collagen and aggrecan) is an established molecular mechanism leading to OA and is mediated by a variety of proteases¹. However, early mechanisms of OA are still not fully understood². Joint trauma has a clearly established link to subsequent post-traumatic osteoarthritis (PTOA) development³,⁴ and thus offers a unique opportunity to study mechanisms of PTOA development, including those in non-cartilaginous tissues such as the synovial membrane. The transection of the anterior cruciate ligament (ACL) is an established method to induce PTOA in small⁵,⁶ and large animal models⁵,⁷. Previous studies have suggested an increased gene expression of selected proteases, including Matrix metalloproteinase-1 (MMP-1, encoded by \textit{MMP1}), MMP-3 (encoded by \textit{MMP3}), MMP-13 (encoded by \textit{MMP13}) and ADAMTS4 with magnitudes up to 350-fold in the synovial membrane⁸,⁹. However, fold-changes do not inform whether a gene is expressed in a dominant or a marginal quantity within the tissue of interest. Rather, low expression levels could lead to over-estimations of the fold-change after PTOA induction. To date it is not well known which proteases are expressed at dominant levels in the synovial membrane, neither in the healthy-state nor after joint injury. Whole Transcriptome Sequencing (RNA-Seq) is capable of quantifying the number of specific transcripts in relation to the total number of transcripts present within the tissue of interest¹⁰. In this study, we used RNA-Seq to address the following objectives: (1) to describe the relative abundance of mRNAs encoding proteases, their activators and inhibitors in the synovial membrane prior and after surgical PTOA induction, (2) to describe the changes in expression of those genes in the early timecourse (1, 5, 9 and 14 days) after PTOA induction. (3) To identify protease-encoding genes, in which a change in transcript levels results in the change of the corresponding synovial fluid protein concentration.

Methods: Unilateral ACL transection was performed in 24 Yucatan mini-pigs. Synovial tissue was collected at 1, 5, 9, and 14 days after injury (n=6 for all time points), as well as from 6 healthy animals. Total RNA was extracted from the synovial membranes, followed by polyA+ mRNA enrichment and cDNA library creation using the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA). Libraries were pooled and sequenced on 4 lanes of an IlluminaHiSeq 2000. Raw reads were aligned to the August 2011 assembly of the sus scrofa genome (susScr3, derived from the UCSC genome browser). Uniquely mapped reads were used to detect differential gene expression between the healthy animals and each time point after ACL transection using R in combination with the edgeR package. The \( p \) values
were adjusted to account for the transcriptome-wide analysis and differential expression was considered as statistically significant when \( p < 0.05 \). *Reads per kilobase of exon model per million mapped reads* (RPKM) values were calculated to quantify the relative abundance of mRNA species. Further, synovial fluid was drawn from a subset of animals at each time point and the relative quantities of synovial fluid proteins were determined using Liquid chromatography-mass spectrometry based proteomics. When changes in mean mRNA and protein abundances corresponding to a respective gene changed in the same direction in the comparison of two time-points, a score of 1 was assigned (mRNA-protein-correlation score). The sum of these scores (max=10 indicating concordant changes in all possible comparisons of time points) was used in ranking mRNA and protein pairs with respect to the agreement between them. A comprehensive set of 47 genes encoding for proteases and modulators of their activity was derived from a literature review and used to address the objectives of this analysis of the Transcriptome-wide data.

**Results:** mRNA abundance: Transcripts of *TIMP2* and *TIMP3* (encoding for Metalloproteinase inhibitor-2 and-3) were the most abundant in the synovial membrane of healthy joints, while *MMP1* and *MMP2* were most abundant after ACL transection (Figure 1A). Expression after ACL transection: The expressions of 26 out of 47 assessed genes were found to change significantly in the synovium after ACL transection (Figure 1B). Most genes encoding for proteases with aggrecanase function (except *ADAMTS5*) and collagenase function (including *MMP1*, *MMP2* and *MMP13*) were upregulated post-ACL transection. Further, *PCSK5* and *PCSK7*, encoding for aggrecanase activators, as well as *PLAU*, encoding the potential collagenase activating Urokinase-type plasminogen activator, were also significantly upregulated. *TIMP2* and *TIMP3* were significantly downregulated after ACL transection (all adjusted \( p \) values < 0.001). Concordance of synovial membrane expression with synovial fluid protein levels: A similar behavior of the changes in synovial membrane gene expression and synovial fluid protein levels were observed for *MMP2* and *PROC*, as indicated by high mRNA-protein-correlation scores (Figure 2C).

**Discussion:** After surgical PTOA induction via ACL transection, we observed a net increase in the expression of genes encoding proteases and their activators in the synovial membrane of the knee joint. These changes were concordant with an increased presence of MMP-2 protein in the synovial fluid. MMP-2 is a gelatinase which is also expressed in osteoarthritic synovial membranes and synovial fluid\(^{11,12}\). This current study suggests this enzyme may be secreted in the early stage of PTOA development by the synovial membrane as well, suggesting a potential role of the synovial membrane in the onset of PTOA. While additional studies are needed, this data suggests the response of the synovial membrane to joint injury should be considered in studying the mechanisms of onset and progression of PTOA.

**Significance:** The increased *MMP2* expression and reduced *TIMP2* expression in the synovial membrane after surgical ACL injury should be considered as potential mechanisms of early PTOA and thus might resemble a target for early structure-modifying therapies in ACL injured patients. The response of the synovial membrane to joint injury should be considered in future studies of PTOA.
Figure 1. (A) Relative abundance of selected transcripts encoding proteases, their activators and inhibitors in the healthy-state synovial membrane (Intact=12, dark red) and after PTOA induction by ACL transection (ACLT, n=24, light green). Bars depict the means and 95% Confidence Intervals of RPKM values. Genes are ranked by the healthy-state transcript abundance. (B) Significantly up- and downregulated genes at day 1, 5, 9 or 14 after ACL transection. The heatmap depicts fold changes comparing the respective day (n=6 each) with Intacts (n=12). Hierarchical clustering reveals one cluster of upregulated (red) and one of downregulated (green) genes. Multiple transcripts were detected for two genes (*, **). (C) The concordance between the abundance of detected proteins in the synovial fluid and mRNA abundance in the synovial membrane is depicted as bar graph of the mRNA-Protein-Correlation score. Four genes including MMP2, PROC, SERPINA1 and ITIH3 demonstrated a concordant change of mean protein and transcript abundance levels within more than half of all possible comparisons of time points.

ORS 2015 Annual Meeting
Poster No: 1233