OATech Network+ end of project report

Hedgehog signalling in ligamentocytes: the role of mechanical loading

Background

Osteoarthritis (OA) is a whole joint condition with a well-established role for ligaments in its aetiology. Altered biomechanics arising from ligament derangements is a significant contributing factor in the initiation and progression of OA highlighting the importance of ligaments and ligament mechanobiology in joint health and OA[1]. Ligament failure can occur following a single incident but also following progressive degeneration associated with overload[2,3]. In OA, investigation of cell-level microscale mechanical failure has drawn attention to mechanotransducers such as primary cilia and the role of their associated Hedgehog pathway[1,4].

Hedgehog (Hh) signalling has been shown to be regulated by mechanical stimulation[5] and although it is context-dependent[6], in OA chondrocytes it has been associated with a catabolic response[7]. Hh signalling in tenocytes participates in matrix organisation during development[8,9] but there is no report of its role in the mechanobiology of ligamentocytes and the association with the initiation and progression of OA.

Here, we investigated the presence of Hedgehog signalling in healthy adult ligamentocytes and its regulation following mechanical loading and overload. This was undertaken in healthy equine ligamentocytes due to access to a plentiful supply of healthy ligaments. We assessed whether mechanical overload was followed by a catabolic response in healthy adult ligamentocytes and whether the Hedgehog pathway was involved in the regulation of the catabolic response.

Results

Healthy adult ligamentocytes were stimulated with 200 ng/mL recombinant mouse Sonic Hedgehog (r-Shh) to determine the presence of Hedgehog signalling. Following one hour of r-Shh stimulation, the mRNA expression of Smoothened (*SMO*) and zinc finger protein Gli (*GLI1*) was significantly upregulated compared to the unstimulated control ($p<0.05$) (Figure 1a). These results demonstrate that r-Shh activated the Hedgehog pathway and the presence of Hedgehog signalling in healthy adult ligamentocytes.

Healthy adult ligamentocytes were subsequently cyclically loaded within a physiological loading range using a Flexcell FX3000 system. Following 15 minutes of cyclic loading with 3% strain, the mRNA expression of *SMO*, *GLI1*, and Patched 1 (*PTCH1*) was significantly upregulated compared to

the unstimulated control, 67.2%, 81.5%, and 41.5% respectively ($p<0.05$) and Sonic Hedgehog (*SHH*) mRNA expression showed a strong tendency for upregulation, 57.5% ($p=0.0509$). Further cyclic loading for 30 minutes and one hour resulted in expression levels for *SMO*, *GLI1*, *PTCH1*, and *SHH* comparable to unstimulated control levels (Figure 1b). The Hedgehog pathway is therefore activated in response to physiological loading shortly after the initiation of loading. The ligamentocytes were then subjected to overload using 8% cyclic strain for six hours which resulted in a statistically significant downregulation in the expression of *GLI1* compared to the unstimulated control, 18% ($p<0.05$) and a tendency for downregulation in the expression of *SMO*, 7.5% ($p=0.0544$) (Figure 1c). These results would suggest a tendency for downregulation of Hedgehog signalling following mechanical overload of healthy adult ligamentocytes. Taken together, we have determined the presence of the Hedgehog pathway in healthy adult ligamentocytes and we have demonstrated regulation of the Hedgehog pathway following mechanical loading within physiologic range and mechanical overload with 8% strain for 6 hours.

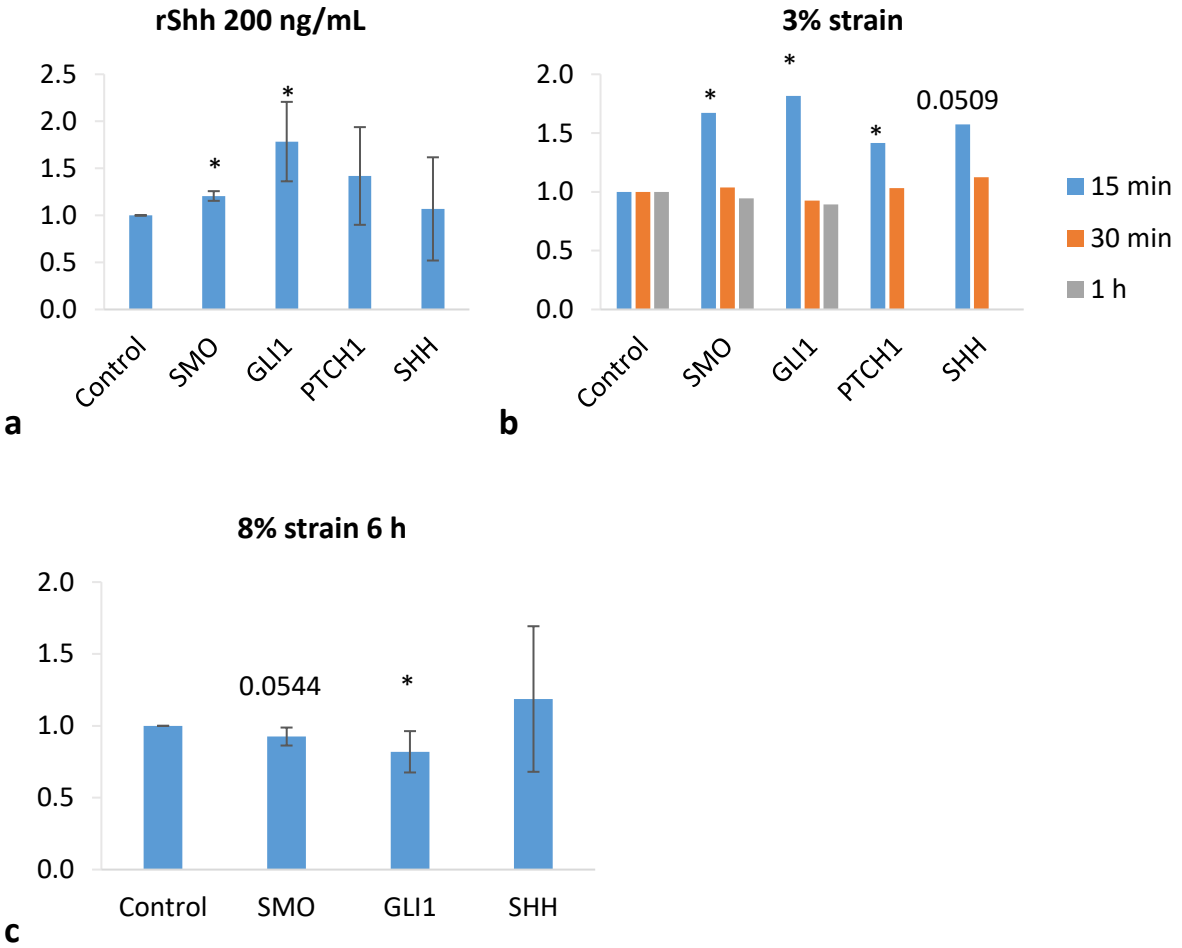

Figure 1. mRNA fold change of *SMO*, *GLI1*, *PTCH1*, and *SHH* in healthy adult ligamentocytes following stimulation with 200 ng/mL r-Shh (a), 3% cyclic strain for 15 mimutes, 30 minutes, and one hour (b)

and 8% cyclic strain for six hours (c) compared to unstimulated control. * indicates statistical significance with p<0.05.

Next, we investigated whether exposure to mechanical overload was followed by a catabolic response in healthy adult ligamentocytes. Following six hours of 8% cyclic strain, healthy adult ligamentocytes showed an upregulation in the expression of the pro-inflammatory enzyme *COX2* and the matrix metalloproteinase *MMP13*, 374.9% and 21.9% (p<0.05) (Figure 2a). To investigate the participation of the Hedgehog pathway in the observed upregulation in the expression of *COX2* and *MMP13* we treated the ligamentocytes with cyclopamine, a SMO antagonist that inhibits Hedgehog signalling. Using 20 µM, 25 µM, and 30 µM we were not able to confirm Hedgehog signalling inhibition in healthy adult ligamentocytes (Figure 2b) and therefore we were not able to investigate the participation of the Hedgehog pathway in the observed upregulation in the expression of *COX2* and *MMP13*.

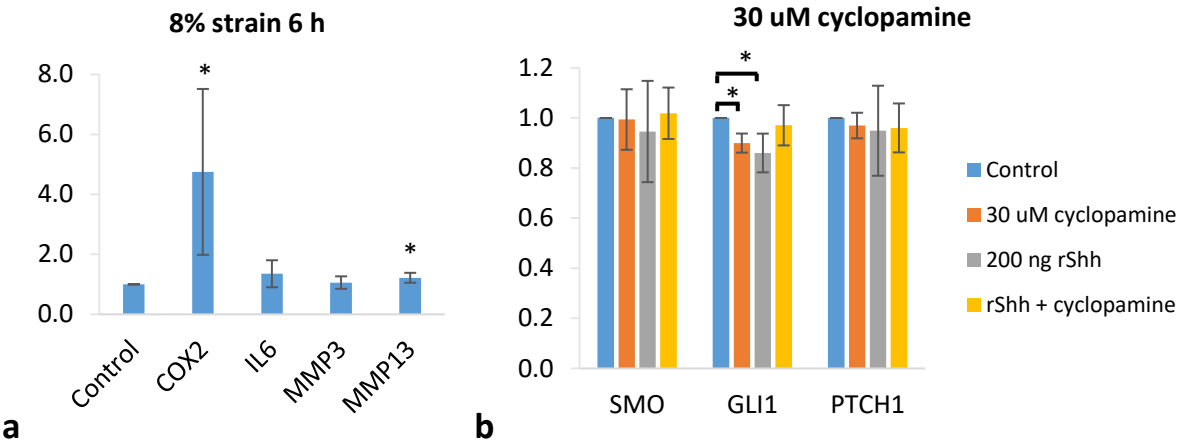

Figure 2. a. mRNA fold change of *COX2*, *IL6*, *MMP3*, and *MMP13* in healthy adult ligamentocytes following 8% cyclic strain for six hours compared to unstimulated control. b. mRNA fold change of *SMO*, *GLI1*, and *PTCH1* in healthy adult ligamentocytes following stimulation with 30 µM cyclopamine, 200 ng/mL r-Shh, and 200 ng/mL r-Shh and 30 µM cyclopamine compared to unstimulated control. * indicates statistical significance with p<0.05.

In conclusion, we successfully demonstrated the presence of a Hedgehog pathway in healthy adult ligamentocytes and that this pathway is activated rapidly following mechanical stimulation within physiologic range and shows a tendency for downregulation following mechanical overload. In addition, an upregulation of *COX2* and *MMP13* was noted following mechanical overload of healthy

adult ligamentocytes but the role of the Hedgehog pathway in the observed response could not be verified on this occasion.

Future plans: The results generated in this project are insufficient to generate a publication or to provide preliminary data for an application for further funding. However, they have certainly unveiled a new factor in the joint ligamentocytes mechanobiology by establishing the presence of a mechanoresponsive Hedgehog pathway and we are hoping that continuation of the work started with this pump priming project will lead to a grant application and a new collaboration.

Academic Impact: The results from this project present valuable information to both mechanobiologists and biologists studying OA and the communication of tissues in OA alike. They have introduced a new factor for consideration in the pathogenesis and the progression of OA in the joint adding to its complexity with the interplay between ligaments and articular cartilage potentially now also extending signalling through the Hedgehog pathway. In addition, it has allowed the PDRA working on this project to gain experience in the field of mechanobiology and in project management which will allow her to advance further her career in academia. Finally, this project has allowed for a new collaboration between molecular biologists from the University of Liverpool and mechanobiologists from the Queen Mary University of London.

Commercial exploitation: No commercial exploitation of intellectual property rights and results was generated in this project.