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My summer in the Cohn Laboratory was spent trying to answer the question of whether the development of cartilage in *Limulus polyphemus* (horseshoe crab) involves a deeply conserved genetic pathway orthologous to vertebrate *Sox9-Sox5,6-Col2a1*. To test the hypothesis that invertebrates and vertebrates use a common genetic program to build cartilaginous tissues, we cloned and confirmed the expression of the orthologs of the vertebrate *Collagen2a1*, and *Sox9* in *Limulus polyphemus* (horseshoe crabs) prior to starting the internship.

My first goal during the Eden internship was to standardize the *SoxD* (ortholog of vertebrate *Sox5,6*) *in situ* hybridization protocol for *Limulus*. Picking up the expression of *SoxD* using traditional whole mount protocols based on the colorimetric reaction by alkaline phosphatases has been elusive up to date. To troubleshoot, we tried to perform *in situ* hybridizations on sections of *Limulus* embryos. We also tried another method of *in situ* hybridization using fluorescent detection by tyramide amplification (TSA). This technique is supposed to be more sensitive and than using colorimetric reactions. These *in situ* failed to detect *SoxD* expression and did not clearly support or discard expression in precartilaginous tissues.

Another goal of the summer was to explore functional experiments that would extend our understanding of the evolution of collagen based cartilage and suggest the existence of the underlying genetic mechanism in the common ancestor of protostomes and deuterostomes. One of these experiments is a gene specific knockdown experiment using RNA interference. The idea is that *Limulus* embryos would be injected with double stranded RNA molecules designed to silence *ColA*, *SoxE*, and *SoxD*. Knocking down expression of each of these genes individually will test the hypothesis that they play essential roles in horseshoe crab cartilage development. To begin these experiments, we attempted to clone the *Limulus* gene *Distal-less*, which is essential for appendage growth and development in arthropods. *Distal-less* will serve as our positive control for these RNAi experiments because the phenotype should be obvious to distinguish when expression is knocked down. However, our attempts to clone *Distal-less* was unsuccessful and more attempts need to be made.

The Eden internship allowed the project to expand into a broader molecular analysis of invertebrate cartilage. Vertebrate cartilage is defined as a semi-rigid connective tissue that is composed of large cells embedded within an extracellular matrix that has high amounts of fibrous protein (usually collagen or elastin) and glycosaminoglycans (usually chondroitin sulphate) (Cole and Hall 2003). The molecular components of invertebrate cartilage remain elusive. Histological staining of *Limulus* embryos with Masson's Trichrome indicated high collagen content in the developing endosternite. This summer, alcian blue staining was performed on sections of *Limulus* embryos and confirmed the presence of sulfated glycosaminoglycans in prechondrogenic tissues. Furthermore, the extracellular matrix of adult *Limulus* endosternite tissue also stained positive with alcian blue.

The next step in our molecular analyses was to determine the glycosamine content of the extracellular matrix in *Limulus* cartilage. I performed immunohistochemistry on *Limulus* adult cartilages for hylaronic acid, an abundant glycosaminoglycan in vertebrate cartilage extracellular matrix. This experiment has proved unsuccessful despite several troubleshooting techniques.

During the internship, not every experiment worked, which is part of the challenge when you start working with a non-model organism. The Eden internship was successful because it brought us closer to determining the mechanism of invertebrate chondrogenesis and allowed the project to expand into molecular studies of horseshoe crab cartilage.

Cole, A.G., and Hall, B.K. (2004). Cartilage is a metazoan tissue; integrating data from nonvertebrate sources. *Acta Zool. (Stockholm)* 69,70, 73-77.

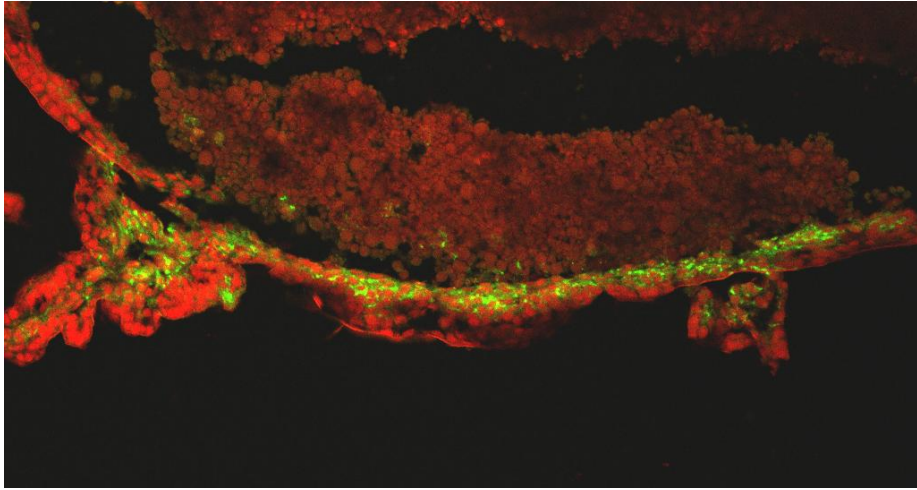


Figure 1: Transverse section of *Limulus ColA in situ* hybridization using fluorescent detection by tyramide amplification (TSA). Green staining shows expression in the developing endosternite. This our positive control to prove that our in situ protocol worked.

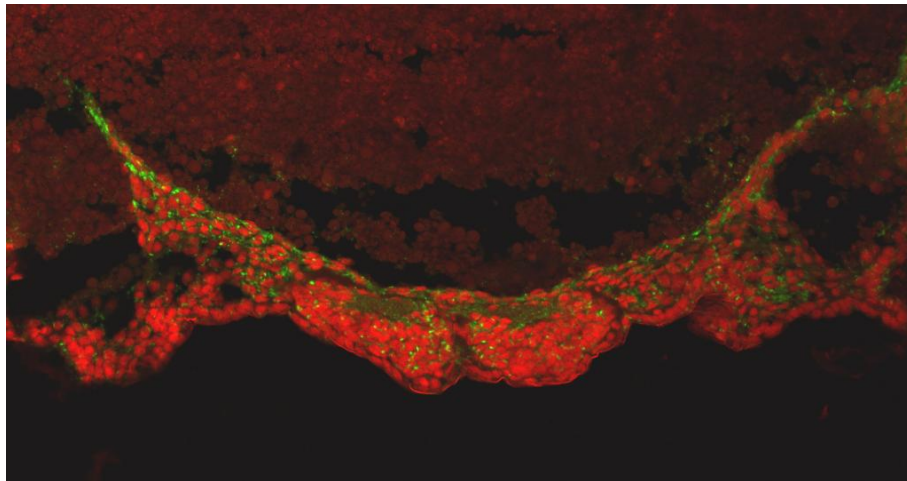


Figure 2: Transverse section of *Limulus SoxD in situ* hybridization using fluorescent detection by tyramide amplification (TSA). Green staining shows expression. These *SoxD in situs* are not very clean and more troubleshooting needs to be done with this method.

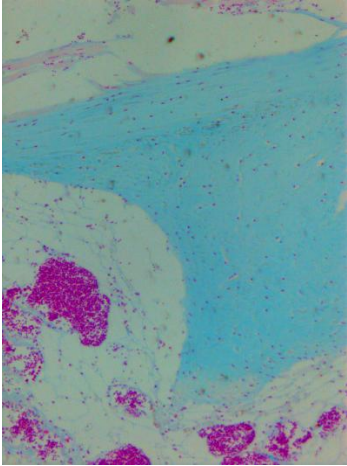


Figure 3: Alcian blue staining of adult endosternite tissue, showing the presence of sulfated glycosaminoglycans in *Limulus* cartilage.

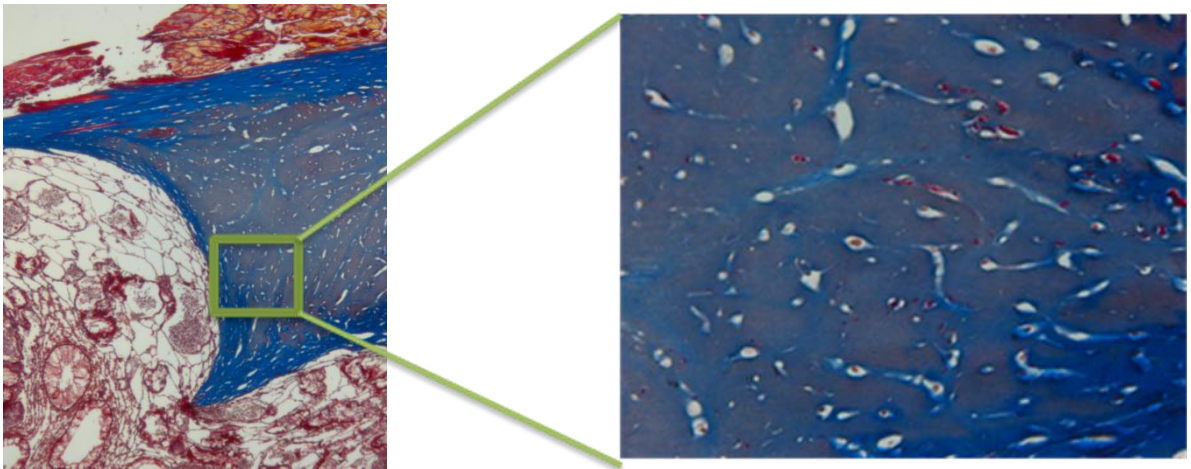


Figure 4: Masson's trichrome staining of adult endosternite tissue, showing the presence of collagen in *Limulus* cartilage.