A High Content Cryohistological Evaluation Of The Destabilized Mouse Knee

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Disclosures:

Introduction: Joint destabilization secondary to transection of a ligament or meniscectomy is a frequently utilized model to study the development of osteoarthritis in a variety of rodent models. While most of the attention is focused on the articular cartilage, it is important to assess the response of the surviving ligaments and entheses to better appreciate the global impact of this model on joint biology.

Methods: Using transgenic mice harboring a variety of GFP reporter constructs, a study of the knee joint was performed after 4 weeks of destabilization induced by transection of the anterior cruciate ligament (ACL). The reporters included Dkk3eGFP (ligament cells and tendon insertion sites), Col3.6GFPCyan (strong in osteoblasts and weaker in fibrochondrocytes) and ColXRFPChry (hypertrophic chondrocytes). One day prior to sacrifice, the animals received a single dose of alizarin complexone (AC) which produces a red line at sites of active mineralization. After sacrifice, the dissected lower limb was fixed for 3 days in 10% buffered formalin, equilibrated to 30% sucrose and embedded in cryomedium. The knees were sectioned either frontally in the plane of the patellar tendon or sagittal at varying depths to visualize specific features of the complex knee anatomy. The sections were captured using cryotape and bonded to glass slides with the section side up. Four rounds of imaging, staining and re-imaging were performed on the same section: endogenous signals: dark field for mineral, red for AC and RFPChry, blue for GFPcyan, and green for eGFP. Demineralization and TRAP: The acid conditions required for the tartrate-resistant acid phosphatase decalcifies the section and removes the mineralization dyes. The enzyme is detected with the yellow fluorescent substrate, ELF97; Alkaline phosphatase (AP) and cell nuclei: This section is adjusted to neutral pH and the AP is detected by fast red substrate, while cell nuclei detected with the DAPI stain are color coded white; Chromogenic: The final step is a toluidine blue stain performed in water without dehydration to avoid sample shrinkage. The 8 individual image files are aligned and stacked in Photoshop to inter-relate the fluorescent and light signals.

Results: Normal joint: This histology clearly distinguishes the growth plate activity of endochondral cartilage from articular cartilage and the fibrocartilage that forms the enthesis. In the growth plate, there is weak mineralization line at the site of forming cartilage mineralization within the band of the ColXRFPChry and AP positive hypertrophic chondrocytes. It is followed by a strong mineralization line that is associated with strong Col3.6GFPCyan and AP+ osteoblasts and is in close proximity of mineralizing trabecular bone and large TRAP+ osteoclasts. Additionally some of the stacked AP+ hypertrophic chondrocytes in the vicinity of the cartilage mineralization line also exhibit punctate TRAP activity. In contrast, the articular cartilage has a well defined layer of AP+ cells that are ColXRFPChry+ at the site of cartilage mineralization that can also show a faint AC+ mineralization line. However beneath the AP+ zone is a layer of mineralized cartilage, which distinctly separates the AP layer from the underlying bone. Few if any of the hypertrophic cells are TRAP+. The fibrocartilage of the enthesis (figure) is characterized by columns of strong Dkk3eGFP+-elongated ligament cells that merge with columns of the AP+ and TB+ hypertrophic chondrocytes, which in turn merge with the mineralized cartilage. Like the articular cartilage, there is a faint line of AC mineralization at the tidemark and it is in this region that a small number of ColXRFPChry cells can be found. However unlike the articular cartilage, the hypertrophic cells can have a relatively strong punctate TRAP signal. In addition, a few cartilage cells reside at the interface between the AP+ and Dkk3+ cells that have a faint Col3.6GFPCyan signal.

Destabilized joint: X-ray examination of the knees bearing a 10 gm weight showed a 10 fold increase in anterior displacement of the tibial plateau relative to the femur confirming the functional destabilization that resulted from the ACL transection. The X-rays and μCT scans revealed significant regions of new mineralization in the horns of the meniscus and in protuberances arising from the lateral tuberosities of both the femur and tibia. The major features of the structures of the knee with the resected ACL were:

1. The PCL and lateral collateral ligaments show major increase in the number of Dkk3+ cells that represent both a higher density and increase ligament volume. Dkk3+ remains strong in the stump of the transected ACL.

2. At the enthesis of the intact ligaments there is a) increased AC mineralization line intensity; b) increased AP activity of the bone embedded cartilage cells; c) marked increase in the punctate TRAP activity that is localized to the embedded cartilage cells; d) increased activity of Col3.6GFPCyan expressing fibrochondrocytes; e) increased toluidine blue staining of the proteoglycans deposited by chondrocytes; f) increased number of ColX-RFPChry cells. This overall cellular activity is exaggerated in the enthesis of the resected ACL.

3. The meniscus showed a lateral expansion of the Dkk3GFP cells and a more central differentiation of the DKK3+ and Col3.6+ cells toward AP+ and ColX hypertrophic chondrocytes that are undergoing mineralization and generating a strong mineralization line. TRAP positive chondrocytes are present at these sites. Overall the meniscus is mineralizing in an outward direction.
4. Mineralizing fibrocartilage nodules develop either within the patellar tendon or at ligament insertion sites in a process similar to the activated enthesis but progressing to the development of a central core of bone and bone marrow.

5. Disorganization and expansion of the AP+ and ColXRFPChry+ hypertrophic chondrocytes a few of which have become TRAP+, but otherwise a surprising lack of cellular activity. In fact in certain regions of the superficial cartilage zone there is a complete loss of DAPI stained nuclei while there is thinning of the mineralized cartilage region.

**Discussion:** Upon joint destabilization there is a major response of the ligamentous and fibrocartilaginous structures with an increase in the remodeling activity at the enthesis. Most striking is the increase in mineralized cartilage forming in the region of the hypertrophic chondrocytes. In addition, TRAP activity develops in a punctate pattern presumably within lysosomes as previously seen in other non-osteoclastic cells (1,4). Past studies have demonstrated TRAP activity in columnar chondrocytes (2,3) but it has not been described as a feature of the enthesis. The lack of a cellular response of the articular chondrocyte relative to the neighboring fibrocartilage is particularly striking.

**Significance:** Enhanced remodeling of the fibrocartilaginous enthesis, as seen by a strong mineralization line, increased AP and punctate TRAP activity in the hypertrophic chondrocytes and expansion of the Col3.6GFP chondrocytes, is a clear signal of ligamentous stress associated with joint destabilization. This activity may contribute to the degenerative process of the joint including to development of osteophytes. The disorganized cellular response within the articular cartilage underlies the limited regenerative capacity of this tissue. Thus therapeutic options need to consider all the cellular components that constitute the joint.

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**References:**
Figure: Large panel: Inter-condylar groove of the normal knee joint. 1, 3 are the enthesis of the ACL and PCL while 2 is a ligament attachment of the meniscus. The individual images that form the composite are shown in the smaller panels. A. Dkk3eGFP (green) and DAPI (blue). B. Panel A with TRAP (yellow) added. C. Panel B with AP added. D. Composite of A, B and C. E. Toluidine blue showing the deep red staining of cartilage proteoglycans. F. Composite of E and D.

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