The Spatio-Temporal Dynamics of Ligament Healing
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Introduction:
The onset of soft tissue injury characterizes three distinct, but highly inter-dependent stages including the inflammatory, proliferative, and remodeling phases which result in a functional scar. The inflammatory phase typifies the onset of neutrophils and macrophages, which phagocytose bacteria and debris within the wounded region and stimulates the release of pro-inflammatory cytokines and growth factors. The proliferative phase involves the upregulation of fibroblasts, angiogenesis, and granulation tissue. Finally, the remodeling phase denotes the formation of scar tissue via collagen remodeling and loss of blood vasculature. Although soft tissue injury is characterized in several tissues, a complete spatio-temporal description of ligament healing has yet to be elucidated. Identifying the healing mechanisms that guide scar formation could ultimately lead to therapeutic interventions for the regeneration of damaged tissue. Therefore, the objective of this study was to spatially and temporally characterize the cellular components involved during ligament healing in an MCL model.

Methods:
The study was performed according to a protocol approved by the University of Wisconsin Institutional Animal Use and Care Committee. Skeletally mature Wistar rats were randomly placed in 1 of 10 groups (n=3). MCLs were subjected to complete bilateral transection. Another group of 3 animals did not undergo transection and served as intact controls. At 1, 3, 5, 7, 9, 11, 14, 21 or 28 days post-injury, MCL measurements were collected, animals were sacrificed and the left MCLs were obtained and snap-frozen in liquid nitrogen. Tissue was cryosectioned at 5 μm, mounted on superfrost-plus microscope slides. Sample were subjected to immunohistochemistry/immunofluorescence to detect polymorphonuclear (PMN), circulating monocytes/macrophages (ED1), resident macrophages (ED2), t lymphocytes (CD3), hematopoietic cells (CD43), proliferating cells (proliferating cell nuclear antigen; PCNA), endothelial cells (thrombomodulin), vascular endothelial growth factor (VEGF), myofibroblasts (α-smooth muscle actin), total cells (propidium iodide), apoptotic cells (TUNEL), type I procollagen, type III collagen, fibromodulin, and decorin. Tissue was also subjected to H&E staining to determine general morphology of the healing MCL. Six images from each MCL were collected for each IHC/IF experiment. Cell numbers were then quantified via Image J (NIH). To test for differences in cell counts across time, one way analysis of variance (ANOVA) was used for each of the responses. The actual response was the square root of the cell counts, since this yielded more homogeneous variances.

Results
Immunohistochemistry and immunofluorescence results demonstrate overlapping temporal localization of healing factors (Figure 1). The inflammatory, angiogenic, and mitotic markers including the neutrophils, monocytes/macrophages, t lymphocytes, VEGF, thrombomodulin, and PCNA positive cells appear within the first 7 days of healing. Apoptosis and the ECM components including myofibroblasts, decorin, fibromodulin, type III collagen, and type I collagen respond to healing beyond day 7. Spatially, the majority of markers localizes to the epiligament of the healing ligament but progressively infiltrate the ligament body with healing (Table 1).

Ligament healing also exhibits expansion of granulation tissue into the uninjured portion of the ligament, otherwise known as creeping substitution (Figure 2). Immunohistochemistry results demonstrate the involvement of specific healing factors in creeping substitution (Table 1). During this process the cells concentrate to the region. The cells then localize to the healing region edges and eventually the distal and proximal ends of the ligament.

Discussion:
The current results indicate a dynamic healing response of the injured ligament. Interestingly, most cells analyzed concentrate within the epiligament. The cells progressively infiltrate the MCL body and exhibit creeping substitution. Creeping substitution ultimately results in a larger hypercellular, hypervascular and disorganized wound region. These results suggest an inefficient wound healing process of the ligament. Control of creeping substitution may limit the extent of injury and the time necessary for healing.

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| Table 1: Primary localization of IHC staining and those exhibiting creeping substitution. |
|------------------------------------|----------------------------------|
| Epiligament                         | Ligament Body                    |
| Neutrophils                        | Circulating macrophage (creep)   |
| Resident Macrophages               | Fibromodulin                     |
| T lymphocytes                      | Type III collagen (creep)        |
| Mitotic Cells                      | Type I procollagen (creep)       |
| Apoptotic Cells                    | Myofibroblasts                   |
| Total Cells (creep)                | VEGF (creep)                     |
| Myofibroblasts                     | Endothelial Cells (creep)        |
| APC (creep)                        | Decorin (creep)                  |

Figure 1: Overview of healing.
Figure 2: Example of creeping substitution.