Characterization of Stem Cells from Human Anterior Cruciate Ligament and Medial Collateral Ligament

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Introduction
ACL and MCL tears and ruptures are two of the most common orthopaedic injuries, occurring in both high level athletes and the weekend warrior. While the injured MCL tends to heal with conservative treatment, the injured ACL does not heal and almost invariably requires surgical intervention to minimize the risk of long-term instability and arthritic degeneration. The cellular basis for the difference in healing capacity between these two ligaments has not been completely understood. As it is now known that nearly all tissues contain adult stem cells, which are a natural reservoir for replenishing pools of specialized cells damaged in tissue injury, we hypothesized that both the ACL and MCL contain stem cells, but they exhibit distinctive characteristics. Using tissue culture techniques, we identified populations of ACL stem cells (ACL-SCs) and MCL stem cells (MCL-SCs), which exhibit the universal stem cell properties of clonogenicity, self-renewal capacity, and multipotency in culture; in addition, we showed that the two ligament stem cells differ from each other in their characteristics.

Materials and Methods
Human ACL and MCL samples were obtained from 6 donors (mean age 27.33 ± 5.20) without knee joint disease. Ligament tissues were minced, digested with collagenase, and centrifuged. The resulting cell pellet was then cultured. Stem cell colonies formed in culture were stained with methyl violet and counted using a hemocytometer. Population doubling time (PDT) was calculated to assess proliferative capacities of ACL-SCs and MCL-SCs. Immunochemistry was also performed to detect expression of stem cell markers, including Stro-1, Oct-4, and others.

In addition, the multi-differentiation potential of ACL-SCs and MCL-SCs was tested *in vitro* for adipogenesis, osteogenesis, and chondrogenesis. Both cell types were seeded at passage 1 in a 6-well plate at a density of 2.4 × 10⁵ cells/well and grown in differentiation-induction media. The gene expression of ACL-SCs and MCL-SCs was determined using quantitative real-time RT-PCR (qRT-PCR), and differentiation of the cells into adipocytes, osteocytes, and chondrocytes was detected using Oil Red O, Alizarin Red S, and Safranin O assays, respectively.

Results
After 3 days in culture, cell colonies were formed on culture plates. ACL-SCs were noted to form fewer and smaller colonies (Fig. 1) than MCL-SCs and also grew more slowly with a PDT nearly twice as long as that of MCL-SCs. Both populations underwent self-renewal, but MCL-SCs appeared to maintain self-renewal for a longer period of time (18 passages) compared to only 5 passages for ACL-SCs (Fig. 2). Both ACL-SCs and MCL-SCs expressed Stro-1 and Oct-4; however, their expression by ACL-SCs tended to be less extensive and weaker than that of MCL-SCs (Fig. 3). Additionally, both were similarly positive for Nanog, SSEA-4, CD44, and CD90 (data not shown).

Furthermore, when ACL-SCs and MCL-SCs were cultured in differentiation-induction media, lipid droplets, cartilage-like pellets, and calcium deposits formed, indicating that these cells were able to differentiate into adipocytes, chondrocytes, and osteocytes, respectively (data not shown). qRT-PCR analysis showed that both ACL-SCs and MCL-SCs in adipogenic, chondrogenic, and osteogenic media markedly increased expression of their respective markers, PPARγ/LPL, collagen II/Sox-9, and Runx2/ALP; however, higher levels of PPARγ, collagen II, and Sox-9 expression by ACL-SCs were noted compared to MCL-SCs (data not shown). Finally, ACL-SCs exhibited a much lower level of Oct-4 and Nanog expression than MCL-SCs (Fig. 4).

Discussion
We showed that human ACL and MCL contain stem cells, which exhibit clonogenicity, self-renewal, and multipotency, three universal criteria for stem cells. This finding is consistent with that of a previous study, which showed that multi-potent stem cells exist in ACL and PCL [1]. However, we further showed that these ligament stem cells, ACL-SCs and MCL-SCs, express multiple stem cell markers, including Oct-4, Nanog, SSEA-4, and nucleostemin, in addition to CD44 and CD90, two mesenchymal stem cell markers. We also noted that there are distinct differences between ACL-SCs and MCL-SCs: compared to MCL-SCs, ACL-SCs exhibited a slower proliferation rate, lower self-renewal capacity, higher expression of adipogenic and chondrogenic genes, and weaker expression of stem cell markers such as Stro-1 and Oct-4. The much lower expression of embryonic stem cell markers Oct-4 and Nanog by ACL-SCs suggests that ACL-SCs possess less "stemness" than MCL-SCs. These findings lend support for the first time to a stem cell-based mechanism by which ACLs display poorer healing capacity than MCLs after injury. Future studies should examine the possibility of using MCL stem cells to enhance repair or regeneration of the injured ACL.

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References