**Tissue Engineering Anterior Cruciate Ligament**

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**Introduction.** Tissue engineering utilizes both scientific and engineering disciplines to address clinical needs. To support the in vitro development of a tissue engineered ACL, the following system components must be appropriately chosen: (i) cell type, (ii) matrix material, geometry, design and mechanical properties to support and direct tissue ingrowth, (iii) biochemical milieu, (iv) material surface chemistry to enhance cell-matrix and cell-cell interactions, (v) cell seeding strategies for uniform cell-matrix distribution, (vi) bioreactor systems with enhanced fluidic and environmental controls to address mass-transfer limitations, and (vii) mechanical growth regimes including rates and percents of physiologically relevant mechanical strains that induce optimal ligament development in vitro.

An autologous tissue engineered ACL generated from a patient’s own bone marrow stromal cells (BMSCs) would eliminate the side effects associated with today’s most popular autologous and allogenic reconstruction options including donor site morbidity and the potential of disease transmission. The future advancement of ACL reconstruction lies with tissue engineered ligaments incorporating autologous adult stem cells, a mechanically robust biodegradable matrix and the appropriate developmental stimuli applied in vivo. We have shown that physiologically relevant, multidimensional mechanical stimuli applied in vitro to a 3-D collagen gel system induced BMSC ligament specific differentiation and development. Recent focus has been placed on the development of a novel silk protein matrix that is biocompatible, mechanically robust and biodegradable over the long-term in vivo. Utilizing a wire rope geometry, the matrix can be designed to desired ACL mechanical specifications including yield point and stiffness, while supporting BMSC ligament specific differentiation in vitro.

An advanced bench-top bioreactor system with enhanced multidimensional strain, fluidic and biochemical (e.g., oxygen tension) controls was developed to mimic some aspects of the in vivo knee joint environment thus increasing the number of system parameters that could be manipulated to induce ligament development in vitro.

We describe the optimization of an in vitro ligament development strategy; human BMSCs, an RGD tripeptide modified silk matrix with ACL relevant mechanical properties and a newly developed automated cell seeding apparatus (Fig. 1) in combination with the advanced bioreactor system were employed.

**Materials and Methods.** Human BMSCs were isolated and culture expanded to P3, prior to seeding and silk matrices, 3 cm in length were prepared as previously described. Multiple silk matrix variations were explored to optimize cell and ECM ingrowth. RGD peptide concentrations were 0.01-0.2 mg/ml were used for covalent coupling to the silk matrix through EDC/NHS activation as described.

An automated motor controlled seeding apparatus was designed and implemented in combination with the recently developed advanced bench-top bioreactor system. The apparatus provides a pseudo-static environment for the uniform circumferential seeding of the silk matrices. Cell seeding concentrations between 0.1 to 4.0 million cells/ml were explored. Standard tissue culture conditions were employed. Utilizing the advanced bench-top system, translational strains from –10% to 10% and rotational strains 0 to 25% in combination with strain rates between 1.6x10⁻³ Hz and 6.9x10⁻¹ Hz (as well as a true static environment) were explored within the experimental design.

Seeded matrices cultured over a 14-day growth period (N=3 per time point and assay) were prepared for SEM, histology and immunohistochemistry (against collagen type I) as described. MTT staining as a measure of cell density, total RNA isolation and real-time RT-PCR (against collagen types I & III, tenascin-C as markers of ligament differentiation and collagen type II and bone sialoprotein as markers of cartilage and bone differentiation, respectively, normalized to GAPDH) were employed as previously described.

**Results.** Covalent modification of the silk matrix with RGD at a coupling concentration of 0.1 mg RGD/ml optimally enhanced rates of human BMSC attachment and spreading resulting in a 2.5 fold increase in cell density 1 day post seeding (Fig. 2A) compared to non-modified control silk matrices.

A seeding regime incorporating 4 inoculations each with 2.4x10⁵ cells/ml (800 µl total volume) applied at 0°, 180°, 90° and 270° with a 0.5 hr static period following each inoculation supported optimal cell attachment. Cell density 24 hrs post seeding was 2-fold greater when compared to manual seeding methods previously reported. SEM confirmed the presence of uniform cell and ECM coverage of the matrix by 5 days post seeding when cultured in a static environment; cell density, as determined by MTT analysis, increased by 250% when compared to the highest values reported over 14 days of culture for manually seeded matrices.

The application of mechanical stimuli, regardless of type, frequency or percent strain, prior to uniform matrix coverage (e.g., 5 days) was detrimental to cell growth and ECM development. Concurrent cyclic translational strains of –3.3% to 3.3% and rotational strains of 0 to 12.5% applied after 5 days of static culture at a frequency of 6.9x10⁻¹ Hz induced the greatest cell density 10 days post seeding. Cells and ECM (including collagen type I as determined by immunohistochemistry) were organized along the longitudinal axis of the silk matrix 10 days post seeding (Fig. 2B). Ligament specific marker transcripts increased with time over the culture period up to 14 days to levels comparable with human ACL marker transcription as determined by real-time RT-PCR; non-specific cartilage or bone differentiation marker transcripts were not detected.

A matrix geometry of 48 cords each consisting of 3 bundles of 12 individual fibers supported optimal ingrowth. Cell and collagenous ingrowth was observed throughout the transverse section of the matrix 14 days post seeding as determined by H&E. Maximum cell density was achieved 10 days post seeding while maximum ligament specific matrix transcription was observed after 14 days of culture indicating the transition from a proliferative state to one of a differentiation state.

**Discussion.** The combination of the optimized growth regime incorporating physiologically relevant mechanical stimulation, the novel mechanically robust long-term biodegradable silk matrix and autologous BMSCs suggests the engineered ligament may serve in vivo as functional equivalents to the native ACL. The contribution of the engineered tissue to the mechanical integrity of the ACL construct as well as the optimization of additional manipulated parameters including extended growth periods and biochemical milieu such as serum supplements and growth factors is currently under investigation.


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