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Ligament Tissue Engineering

Jingsong Chen[1], Jodie Moreau[1], Rebecca Horan[1], Adam Collette[1], Diah Bramano[1], Vladimir Volloch[1], John Richmond[2], Gordana Vunjak-Novakovic[3], David L. Kaplan[1], and Gregory H. Altman[1]

Department of Biomedical Engineering[1], Bioengineering Center, Tufts University, Medford, Massachusetts 02155; Department of Orthopaedics[2], New England Baptist Hospital, Boston, Massachusetts; Department of Biomedical Engineering, Columbia University, 363G Engineering Terrace, Mail Code 8904, 1210 Amsterdam Avenue, New York, NY 10027

Corresponding author: Gregory.Altman@tufts.edu

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1. BACKGROUND

1.1. Context of Tissue Engineering

In any tissue engineering effort, the preparation of cells is a critical system component along with the biomaterial matrix and bioreactor environment. Cell preparation typically involves the appropriate choice of cell type (e.g., differentiated or progenitor, human or animal), expansion, seeding, and genotypic and phenotypic characterization of cells within the context of the specific tissue engineering goal (tissue-specific outcomes).

1.2. ACL Injury and Limitations of Prevalent Treatments

The need to explore tissue engineering options for the anterior cruciate ligament (ACL) has arisen from the inability of currently available clinical options to fully restore knee function. Over the last twenty years, orthopedic sports medicine has been faced with continued repair needs associated with tears and ruptures of the ACL, a major cause of athletic disability. Presently, more than 200,000 ACL ruptures occur in the United States each year. Injury to the ACL and the high frequency of subsequent knee instability often result in further damage to the joint, motivating improvements in ACL reconstructive techniques. Currently, autologous tendon grafts harvested at the time of reconstruction are the most widely utilized ACL replacement tissues, but this practice typically results in tendon donor site morbidity. Allograft tendons have gained acceptance because they alleviate donor site morbidity problems associated with autograft harvest, but cost, risk of infection, and disease transmission remain problematic. Synthetic polymers have also been widely used as ligament replacements, including polytetrafluorethylene (Gore-tex®), polyester (Dacron®), carbon fiber, and polypropylene ligament augmentation devices (LAD). Complications due to stress shielding, particulate debris, and early mechanical failures are commonly associated with these prostheses.

1.3. Tissue Engineering May Be the Solution for ACL Replacement

Tissue engineering may potentially provide improved clinical options in orthopedic medicine through the generation of biologically based functional tissues in vitro for transplantation at the time of injury or disease. A tissue-engineered

ACL with the appropriate biological and mechanical properties would eliminate many of the deleterious effects associated with current clinical options for restoring knee function. In addition, model systems available in vitro which better represent tissues in vivo may provide new opportunities for the study of disease onset, disease prevention, pharmaceuticals, and fundamentals of tissue structure and function.

1.4. Criteria for the Tissue-Engineered ACL

The failure of the ruptured ACL to heal is related to the lack of vascular supply, deficits in intrinsic cell migration, impaired growth factor availability, and environmental effects of the synovial fluid on cell morphology. These problems have led to research on the biology of the ACL, particularly its response to injury and wound healing, providing the backdrop of issues important to consider in any tissue engineering strategy, such as marker profiling [Bramono et al., 2004; Frank et al., 1999; Lo et al., 1998, 2003]. Accumulated data from clinical studies combined with fundamental insight into the biological response to ACL rupture provide criteria to consider for any tissue engineering strategy to achieve success for ACL replacement. These criteria include (1) minimal patient morbidity, (2) surgically simple insertion with reliable methods of fixation that will withstand aggressive rehabilitation, (3) generation and maintenance of immediate knee stability without tissue-fixation device creep to allow rapid return to preinjury function, (4) minimal risk to the patient for infection or disease transmission, (5) biocompatibility and minimal host immune response, (6) support of host tissue ingrowth without causing stress shielding (i.e., adequate communication of environmental signals including mechanical, biochemical, and transport) to the developing host tissue such that ingrowth is properly directed and organized, and (7) biodegradation at a rate that provides adequate mechanical stability during replacement by new extracellular matrix (ECM).

2. PRINCIPLES OF METHODOLOGY

Through the incorporation of an appropriate biomaterial matrix, judiciously selected cells, and appropriate differentiation signaling, tissue engineering can offer options for generating an unlimited amount of autologous ligament tissue in vitro.

2.1. Background of ACL Tissue Engineering

A tissue engineering approach to ACL replacements has been under investigation since the early 1990s. A ligament prosthesis combining the advantages of synthetic materials (high strength, simple fabrication, and storage) and biological materials (biocompatibility and ingrowth promotion) was initially reported using a collagenous composite consisting of reconstituted type I collagen fibers in a

collagen I matrix with polymethylmethacrylate bone fixation [Dunn et al., 1992, 1994]. However, inconsistent neoligament formation and significant weakening of the prosthesis in a rabbit model were observed. Collagen fiber-poly(L-lactic acid) (PLA) composites were explored to improve mechanical integrity and allow for neoligament tissue ingrowth; however, mechanical integrity could not be maintained for the rigorous ACL rehabilitation protocols.

2.2. Silk As a Candidate for ACL Tissue Engineering

Native silkworm silk is being explored as a scaffold for ACL tissue engineering because of silk's superior mechanical and biological properties. Historically, two common misconceptions have limited silk's broader use as a biomaterial in tissue engineering: first, that silk is immunogenic and second, that it is nondegradable. We have recently shown that silk fibroin, when contaminating sericin proteins secreted by the silkworm have been properly extracted, is nonantigenic, biocompatible, and capable of supporting BMSC attachment, spreading, growth, and differentiation (Fig. 8.1) [Altman et al., 2002a]. In vivo studies indicate that sericin-extracted silk induces a foreign body response comparable to most common degradable synthetic and natural polymers such as poly(glycolic acid) (PGA)-PLA copolymers and collagen [Altman et al., 2003]. With regard to biodegradability, silk maintains its mechanical integrity in tissue culture conditions, but in vivo is susceptible to proteolytic degradation resulting from a foreign body response. The slow rate of degradation in comparison to other degradable natural and synthetic polymers (e.g., collagen, polyesters) is viewed as a benefit, meeting criteria established for ACL tissue engineering [Weitzel et al., 2002]. The slow rate of silk degradation allows for the gradual transfer of stabilizing properties from the matrix to the new tissue without exposing the patient to periods of joint destabilization.

2.3. Choice of Cells for ACL Tissue Engineering

The cells incorporated into tissue-engineered constructs can provide signals needed for tissue regeneration. Two types of autologous cells were explored for reaching the goal of tissue engineering ACL: ACL fibroblasts and bone marrow stromal cells (BMSC). The first approach required a surgical procedure (e.g., arthroscopy) to harvest ACL tissue from the patient's knee and a relatively long period for cell culture expansion because of the limited proliferation capability of adult ACL fibroblasts. BMSCs attained via a small bone marrow aspirate (5–10 ml harvested from the patient's iliac crest in the physician's office) proved a better cell source because of their potential for differentiation into ligament lineage and superior growth ability compared to ACL fibroblasts [Chen et al., 2003]. In addition, this process does not involve the knee, thus eliminating the risk of local infection.

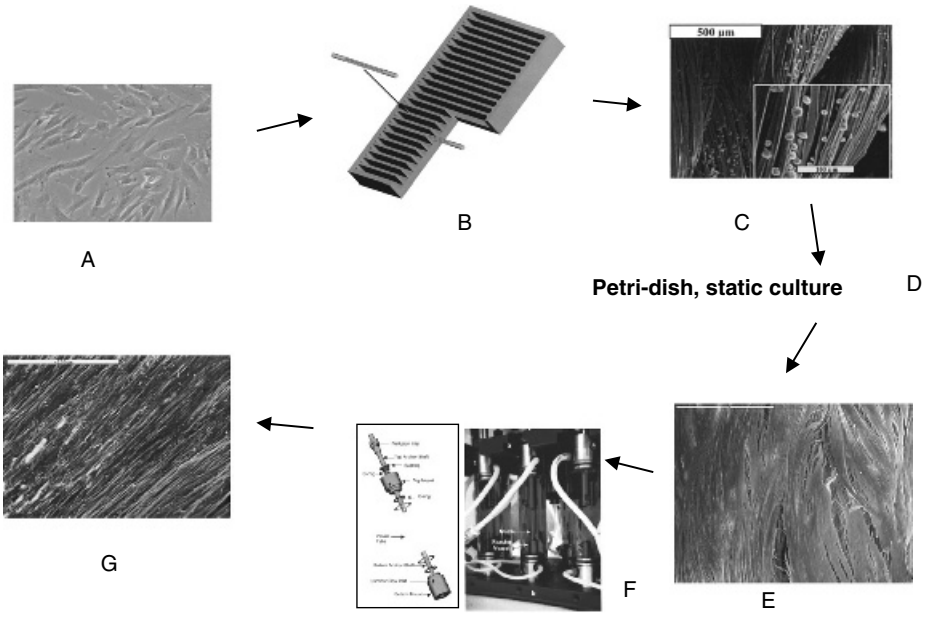

Figure 8.1. Cell culture for ligament tissue engineering. A. Step 1—Cell expansion: Human BMSCs were plated on tissue culture plastic at a density of 5000/cm^2 in DMEM-10% FCS containing 1 ng/ml bFGF (functions as the mitogen to enhance BMSC proliferation and as differentiation inhibitor) and culture-expanded for 6 days (Chen et al, 2003). B. Step 2—Cell seeding: Before confluence, BMSCs were trypsinized, centrifuged, and resuspended at a concentration of 2 million/ml in DMEM-10% FCS. BMSCs were seeded onto RGD-modified silk matrices in a custom-designed seeding vessel (Chen et al, 2003) to increase cell-matrix contact. C. As early as 5 min after seeding, BMSCs attached to RGD-modified silk matrices and started to spread (Chen et al, 2003). D. Step 3—Cell cultivation on silk matrices: Two hours after seeding, matrices were transferred from seeding vessel into tissue culture flasks, followed by a 2-week cultivation in DMEM-10% FCS at 37 °C/5% CO_2. E. After the 2-week static culture, BMSCs reached confluence and fully covered the silk matrix. Cell/ECM was not aligned (Chen et al, 2003). F. Step 4—Bioreactor and mechanical stimulation: Cell-seeded silk matrices were then transferred into bioreactor and cultured under dynamic conditions with direct cyclic mechanical stimulation for 6 days (Altman et al, J Biomech Eng. 2002 Dec;124(6):742–9). Culture medium was reflushed for 1 h/day. G. The mechanically stimulated matrices showed highly organized cell/ECM alignment (Chen et al, 2003).

2.4. Rationale for Using Mechanical Stimulation as ACL Differentiation Inducer

BMSCs have the potential to differentiate into multiple lineages such as bone, cartilage, muscle, ligament, and tendon. Therefore, ACL-specific differentiation cues must be developed in order to direct BMSC differentiation toward ligament tissue. Recent research has focused on the impact of mechanical forces on progenitor cell differentiation toward ligament-like tissues. The motivation for this approach was based on two major factors: the finding that most fibroblastic cells, including those of the ACL, are load responsive and the absence of defined biochemical/chemical signaling factors to direct progenitor cells to differentiate into ligament cells. The focus on mechanical forces imparted through the bioreactor environment and transmitted by the matrix to the cells is an important principle to consider in tissue engineering ligaments.

3. PREPARATION OF REAGENTS

3.1. Bone Marrow Stromal Cell Culture Medium (BMSC Medium)

BMSC medium is prepared from Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 40 U/ml penicillin and 40 μg/l streptomycin (P/S), and 1 ng/ml basic fibroblast growth factor (bFGF) (Table 1A).

3.2. Engineered Ligament Culture Medium (Ligament Medium)

Ligament medium is prepared from DMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, P/S, and 100 μM L-ascorbic acid 2-phosphate (Table 1B).

3.3. Freezing Medium

Freezing medium is prepared from DMEM supplemented with 10% FBS and 8% DMSO. Prepare fresh solution before use.

3.4. Trypsin-EDTA

Trypsin-EDTA is prepared from 0.15 M NaCl supplemented with 0.25% trypsin and 1mM EDTA.

3.5. 3-[4,5-dimethylthiazol-2-y]-2,5-dipheynyl tetrazolium bromide (MTT) Solution

MTT solution is prepared from ligament medium supplemented with 5 mg/ml MTT to make a 10× stock solution. Sterilize by filtration. Dilute into 1× with ligament medium for use. Avoid light and store at 4 °C.

Table 1. Preparation of cell culture media.

Reagent	Stock solution concentration	Volume of reagent needed (ml)	Final concentration
A. Media Preparation for Bone Marrow Stromal Cell Culture			
DMEM	1×	438	1×
FBS	—	50	10%
Nonessential amino acids	10 mM	5	0.1 mM
P/S antibiotics	P: 10,000 U/ml, S: 10 mg/ml	2	40 U/ml
BFGF	250 μg/ml	5	1 ng/ml
Total volume of reagents		500	
B. Media Preparation for Engineered Ligament Culture			
DMEM	1×	442	1×
FBS	—	50	10%
Nonessential amino acids	10 mM	5	0.1 mM
P/S antibiotics	P: 10,000 U/ml, S: 10 mg/ml	2	40 U/ml
L-Ascorbic acid 2-phosphate	50 mM (0.128 g in 10 ml H_2O)	1	100 μM
Total volume of reagents		500	

3.6. Buffers for Scanning Electron Microscopy (SEM)

Sodium Cacodylate Buffer (0.2 M)

Sodium cacodylate	4.28 g
Calcium chloride	25 g
Hydrochloric acid, 0.2N	2.5 ml
Dilute with distilled water, pH 7.4, to	200 ml

Karnovsky Fixative Stock Solution

Paraformaldehyde	2.0 g
Sodium hydroxide, 1 M	2–4 drops
Glutaraldehyde, 50%	5.0 ml
Dilute with 0.2 M cacodylate buffer, pH 7.4, to	25 ml

Mix the paraformaldehyde with 25 ml of distilled water. Heat to 60 °C on a stir plate. When moisture condenses on the sides of flask, add sodium hydroxide drop-wise and stir the solution until it clears. Cool solution in cold water bath. Filter and then add glutaraldehyde and 0.2 M buffer, pH range 7.2–7.4.

△ ***Safety note.*** Use caution when preparing the solutions. Work in a well-ventilated area; wear gloves and a lab coat.

3.7. Collagen ELISA Buffers

Acetic acid, 0.05 M, Containing 0.5 M NaCl, pH 2.9

Acetic acid (17.4 M stock solution)	1.436 ml
NaCl (MW: 58.44)	14.61 g
dH_2O to	500 ml
Adjust to pH 2.9 with formic acid	

Tris-Base, 1 M

Tris-Base (MW: 121.14)	6.057 g
dH_2O to	50 ml

Tris-HCl, 0.1 M, Containing 0.2 M NaCl and 0.05 M $CaCl_2$, pH 7.8

Tris-HCl (1 M stock solution)	5 ml
NaCl (MW: 58.44)	0.584 g
$CaCl_2$ (MW: 111)	0.276 g
dH_2O to	50 ml

3.8. Pepsin

Pepsin solution is made by dissolving 10 mg/ml in 0.05 M acetic acid.

3.9. Elastase

Elastase solution is made by dissolving 1 mg/ml pancreatic elastase in Tris-HCl buffer containing 0.2 M NaCl and 0.05 M $CaCl_2$ at a pH of 7.8.

4. TISSUE HARVEST AND CELL ISOLATION

A variety of different cell types have been considered and compared for developing tissue-engineered ligaments. Human BMSC (hBMSC) and differentiated human ACL fibroblasts were culture-expanded and seeded onto silk matrices. Rat bone marrow stem cells were isolated, culture-expanded, and assayed by flow cytometry to clarify the mesenchymal stem cell phenotype.

4.1. Human Bone Marrow Stromal Cells (hBMSCs)

Stromal cells were isolated from unprocessed whole bone marrow aspirates obtained from donors <25 years of age (See Sources of Materials).

Protocol 8.1. Primary Culture of Human Bone Marrow Stromal Cells

Reagents and Materials

Sterile or aseptically collected

- ❑ Human bone marrow (See Sources of Materials)
- ❑ BMSC medium (See Section 3.1)
- ❑ Culture flasks or Petri dishes
- ❑ Trypsin-EDTA (See Section 3.4)
- ❑ Freezing medium (See Section 3.3)

Protocol

(a) Suspend the marrow in BMSC medium (25 ml whole bone marrow aspirate in 100 ml medium).
(b) Plate at 8 μl aspirate/cm^2 in flasks or Petri dishes.
(c) Change medium after 4 days, removing nonadherent hematopoietic cells with the culture medium.
(d) Change the medium twice per week thereafter.
(e) Detach primary hBMSCs just before confluence, using trypsin-EDTA.
(f) Replate at 5×10^3 cells/cm^2.
(g) Trypsinize first-passage (P_1) BMSCs just before they reach confluence; resuspend in freezing medium, and store in liquid nitrogen for future use.

4.2. Human ACL Fibroblasts (ACLFs)

Fibroblasts were obtained by an explant culture method. ACL tissue was harvested from patients undergoing total ACL reconstruction and transferred to the laboratory in saline.

Protocol 8.2. Primary Explant Culture of Human Ligament

Reagents and Materials

- ❑ ACL tissue
- ❑ Culture flasks, 25 cm^2
- ❑ Scalpels, #11 blade
- ❑ Ligament medium (See Section 3.2)

Protocol

(a) Clean ligament of all synovial tissue and cut into ~1-mm^3 pieces.
(b) Transfer explants into a culture flask at ~1 piece per cm^2.
(c) Add 1 ml ligament medium and culture at 37 °C under 5% CO_2.
(d) Add additional ligament medium for a total of 5 ml once explants have attached.
(e) Change medium twice per week until the fibroblastic outgrowth reaches near-confluence, usually within 1–2 weeks.
(f) Detach primary ACLFs with trypsin-EDTA and replate at 5×10^3 cells/cm^2.
(g) Trypsinize first-passage (P_1) ACLFs; resuspend in freezing medium (See Section 3.3), and store in liquid nitrogen for future use.

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5. SILK MATRICES AND RGD SURFACE MODIFICATION

5.1. Silk Matrices

Matrices were prepared from raw *Bombyx mori* silkworm silk as previously described [Altman et al., 2002a]. Bundles of 12 twisted fibers were generated and complete sericin removal was achieved through batch extraction in an aqueous solution of 0.02 M Na_2CO_3 and 0.3% (w/v) detergent at 90 °C for 1 h. Polyolefin heat shrink tubing was used to cuff the first and fifth centimeters (creating a 3-cm exposed sample length in 5-cm total matrix length) of 24 parallel bundles anchored under equal tension.

5.2. Surface Modification

We recently demonstrated that arginine-glycine-aspartic acid (RGD) surface modification of silk fibers enhanced BMSC adhesion and collagen matrix production [Chen et al., 2003]. RGD peptides were covalently coupled to the matrices as previously described [Chen et al., 2003; Sofia et al., 2001].

Protocol 8.3. Surface Modification of Silk Fibers with RGD for Bone Marrow Stromal Cell Adhesion

Reagents and Materials

- ❑ PBSA: Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}, pH 6.5
- ❑ EDC and NHS solution: 0.5 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 0.7 mg/ml N-hydroxysuccinimide in PBSA
- ❑ GRGDS peptide: glycine-arginine-glycine-aspartate-serine, 0.4 mg/ml in PBSA
- ❑ Silk matrices (See Section 5.1)

Protocol

(a) Hydrate in PBSA for 1 h.
(b) Activate exposed carboxyl groups of aspartate and glutamate on silk with 20 ml EDC and NHS solution for 15 min at room temperature.
(c) Rinse matrices extensively with PBSA to remove excess EDC/NHS and react with 1 ml GRGDS peptide in PBSA for 2 h at room temperature with gentle shaking.
(d) Rinse matrices thoroughly in PBSA and then in UPW, and dry in air.

6. CELL SEEDING AND CULTIVATION

6.1. For Study of Silk Matrix Design (See Section 9.1.1)

A custom seeding chamber was designed with 24 wells, each 3.2 mm wide by 8 mm deep by 40 mm long (1 ml total volume) machined into a Teflon block designed to fit within a 15-cm Petri dish. Frozen first passage (P_1) BMSCs were used for matrix seeding within the Teflon wells. Sterilized silk cords and bundles were seeded with cells in the customized seeding chambers to minimize the cell to medium volume and increase cell-matrix contact. Cords were seeded with 3.3×10^6 cells in 1 ml of cell culture medium without bFGF as described in Protocol 8.4.

Protocol 8.4. Seeding Silk Matrix Cords with BMSCs

Reagents and Materials

Sterile

- ❑ Frozen P_1 BMSC cells (See Protocol 8.1)
- ❑ BMSC medium without bFGF (See Section 3.1)
- ❑ Silk cords sterilized in ethylene oxide
- ❑ Teflon seeding block, steam sterilized (See Section 6.1)

Protocol

(a) Thaw frozen P_1 BMSCs.
(b) Plate at 5×10^3 cells/cm^2 (P_2).

(c) Trypsinize when near ~85% confluence and resuspend at 1×10^6 cells/ml in BMSC medium (See Section 3.1)
(d) Place sterilized cords within the wells of the steam-sterilized Teflon block.
(e) Add 250 μl cell suspension (1×10^6cells/ml) to the wells containing cords.
(f) Incubate at 37 °C under 5% CO_2 for 30 min.
(g) Rotate the cords through 90° and add a second 250 μl of the cell suspension.
(h) Incubate for a further 30 min.
(i) Repeat the procedure twice more for a total of 270° of rotation to uniformly seed the cords.

After seeding, the cords were cultured independently in an appropriate amount of cell culture medium for 0 (immediately postseeding), 1, 7, and 14 days ($n = 2$ per time point for SEM and 2 for DNA), at which times cell morphology, growth, and marker expression ($n = 3$ at day 14) were assessed.

6.2. Modification for Study of Silk Surface Modification (See Section 9.1.2)

Frozen P_1 BMSCs or ACLFs were defrosted and replated at 5×10^3 cells/cm^2 (P_2), trypsinized when near confluence, and used for matrix seeding. The seeding procedure was dependent on matrix morphology. For single-side seeding, fibrous matrices were inoculated with 1 ml of cell suspension at a concentration of 2×10^6 cells/ml by direct pipetting, incubated for 2 h at 37 °C/5%CO_2, and transferred to tissue culture flasks for experiments in an appropriate amount of cell culture medium without bFGF.

6.3. Modification for Study of Mechanical Stimulation as the Ligament Differentiation Inducer (See Section 9.2)

For multiple (4-time) seeding, matrices were loaded into a custom Teflon seeding vessel designed to allow circumferential cell seeding while minimizing the volume of cell suspension (0.8 ml total) required for full coverage. Each RGD-coupled silk matrix was suspended horizontally (~4mm above the well surface) and anchored between two stainless steel shafts. Once matrices were loaded, the complete vessel was sterilized with ethylene oxide. After aeration, the matrices were wet with PBSA in their independent wells. P_3 BMSCs were delivered via four inoculations of 0.75 ml of cell suspension (3×10^6 cells/ml) at 0°, 180°, 90°, and 270° of rotation. Each inoculation was followed by an incubation period of 30 min at 37 °C under 5%CO_2. After seeding, matrices were incubated in the seeding vessel with culture medium overnight and subsequently transferred to Petri dishes for 2D culture.

7. BIOREACTOR

We have developed a specialized bioreactor to establish conditions in vitro that mimic the knee environment in vivo in terms of mechanical and biochemical conditions. This computer-controlled bench-top bioreactor system can accommodate

up to 12 reactor vessels (2.5 cm in diameter × 8 cm long). Each vessel provides an environment for the growth of one vertically oriented BMSC-seeded silk matrix attached longitudinally between two anchors positioned 3 cm apart. Tube dimensions were determined by culture volumes previously used to grow ligaments (20 ml per ligament). Anchor placement was selected based on a human ACL length of 28 mm. Silk matrices were seeded four times with BMSCs in the Teflon chamber as described above (See Protocol 8.4), followed by 6 days of incubation in a static environment, then loaded into bioreactors, and cultured in DMEM with 10% FBS at 37 °C for 6 days. Gas exchange was controlled by recirculating growth medium through a custom-designed environmental chamber (pO_2 21%, pCO_2 10%, pN_2 69%, and pH 7.4). Mechanical stimulation (linear displacement: 0.5 mm; rotational displacement: 45° from the neutral position) was applied with a frequency of 1.39e-4 Hz.

8. ANALYTICAL ASSAYS

8.1. Mechanical Analysis

Mechanical testing was performed with a servohydraulic Instron 8511 tension/compression system with Fast-Track software. Fatigue analysis was performed on single ACL matrix cords. The data for a single cord were extrapolated to represent the six-cord ACL matrix because the cords were designed to be loaded in parallel. Single pull-to-failure testing was performed at a strain rate of 100%/s, and data were analyzed with Instron Series IX software. Fatigue testing to determine cycles to failure at ultimate tensile strength (UTS), 1680 N, and 1200 N ($n = 5$ for each load) was conducted with an H-sine wave function at 1 Hz generated by Wavemaker32 version 6.6 (Instron). Fatigue testing was performed in neutral phosphate-buffered saline (PBSA) at 23 °C. Samples were stored after preparation for no more than 7 days at room temperature before testing.

8.2. Cell Viability

Cell viability was determined by MTT staining. Seeded matrices were incubated in MTT solution (0.5 mg/ml MTT in cell culture medium) at 37 °C under 5%CO_2 for 2 h. The intense purple-colored formazan derivative formed via cell metabolism was eluted and dissolved in 0.04 M HCl in 95% isopropanol, and the absorbance was measured at 570 nm with a reference wavelength of 690 nm. Cell number was correlated to optical density (OD).

8.3. Morphology

Phase-contrast light microscopy was used to observe the morphology of hBMSCs with a Zeiss Axiovert S100 light microscope. Digital image analysis incorporated a CCD color video camera (DXC-390, Sony), a frame grabber card (CG-7 RGB, Scion), and Scion Image software version 1.9.1.

SEM was used to determine cell morphology on matrices. After harvest, seeded matrices were immediately rinsed in 0.2 M sodium cacodylate buffer and fixed in Karnovsky fixative overnight at 4 °C. Samples were then dehydrated through exposure to a gradient of alcohol followed by 1,1,2-trichlorotrifluoroethane and allowed to air dry in a fume hood. Specimens were sputter coated with gold, using a Polaron SC502 Sputter Coater, and examined with a JEOL JSM-840A scanning electron microscope at 15 kV.

8.4. Transcript Levels

RNA Isolation and Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cells were detached with 0.25% trypsin solution and collected after brief centrifugation (1000 *g*, 10 min). RNA was isolated from the collected cells, using the QuiAmp DNA mini kit according to the manufacturer's protocol (Quiagen). The RNA samples were reverse transcribed with oligo(dT) selection (Superscript Preamplification System). Transcripts were assessed with suitable primer sets to target specific markers, using an ABI Prism 7000 real-time system. PCR reaction conditions were 2 min at 50 °C, 10 min at 95 °C, and then 50 cycles at 95 °C for 15 s, and 1 min at 60 °C. The expression data were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences for the human GAPDH gene were (5′-3′) : forward primer ATG GGG AAG GTG AAG GTC G, reverse primer TAA AAG CCC TGG TGA CC, probe CGC CCA ATA CGA CCA AAT CCG TTG AC. Primers and probes for specific markers of ligament formation can be purchased from Applied Biosciences (Assay on Demand).

8.5. Protein Formation—Extracellular Matrix

Collagen type I protein content was assessed with a commercially available ELISA kit against human collagen type I according to the manufacturer's protocol (Chondrex) and measured at 405 nm with a Spectra Max 250 microplate spectrophotometer; collagen type I concentration was determined by generating a standard curve of known human collagen type I (from ELISA kit) concentrations correlated to optical density.

Protocol 8.5. Isolation of Collagen from Cell-Seeded Silk Matrices

Reagents and Materials

- ❑ PBSA
- ❑ Acetic acid, 0.05 M in 0.5 M NaCl, pH 2.9–3.0 (adjust the pH with formic acid)
- ❑ Pepsin (10 mg/ml) dissolved in 0.05 M acetic acid (See Section 3.8)

- ❑ Pancreatic elastase (1 mg/ml) (See Section 3.9)
- ❑ 1 M Tris-base

Protocol

(a) Rinse the silk matrices with PBSA.
(b) Transfer the matrices to tubes that contain 1.0 ml 0.05 M acetic acid containing 0.5 M NaCl, pH 2.9–3.0.
(c) Add 0.1 ml of pepsin (10 mg/ml) and shake at 4 °C for 48 h

(Pepsin digests telopeptides located on both N- and C-terminals of the collagen molecule and solubilizes the collagen from collagen fibrils; however, pepsin cannot digest the intra- and intermolecular cross-linkages)

(d) Add 0.1 ml 1 M Tris-base to neutralize the pH to 7.5–7.8.
(e) Add 0.1 ml pancreatic elastase (1 mg/ml) and shake at 4 °C overnight.

(Elastase digests the collagen molecule at the N-terminal region, which contains the intra- and intermolecular cross-linkages. As a result of N-terminal cleavage by elastase, the dimer and trimer of collagen are converted into monomeric collagen. ***Note:*** **Elastase digests the denatured collagen into small fragments, so avoid increasing the sample temperature**.)

(f) Centrifuge at 10,000 rpm in a microfuge for 5 min and collect supernate.

(***Note***: Tissue should be completely solubilized; however, a trace of insoluble materials might remain.)

(g) Dilute the supernate at desired times with the Sample Dilution Buffer provided in Collagen Capture ELISA Kits.

8.6. Statistical Analysis

Data for cell attachment, viability, and collagen matrix production were analyzed by one-way ANOVA to evaluate differences between groups. Post hoc comparison of means is accomplished with the Student-Newman-Keuls test to determine significance between groups in which a p value <0.05 is typically defined as significant. All data are reported as means $\pm$ the standard error of the mean.

9. REPRESENTATIVE STUDIES

9.1. Silk Fibroin as the Scaffold for ACL Tissue Engineering

9.1.1. Silk Matrix Design

The geometry of the knee and kinematics related to ACL structure must be incorporated into any design strategy if a tissue-engineered ACL generated in vitro is to stabilize the knee successfully and function correctly in vivo. A mismatch in the ACL structure-function relationship would result in graft failure. Our hypothesis is that mechanical signals applied in vitro to a growing ACL will induce the formation of the structural and functional features required to meet performance requirements in vivo. The objective of the study was to engineer a mechanically

and biologically functional matrix for ACL tissue engineering. Proper matrix design is essential to a successful tissue-engineered ACL and provides a foundation upon which to explore mechanics and biological function.

A silk fiber-based matrix was used in combination with a wire rope design to mirror the ACL in ultimate tensile strength (UTS) and linear stiffness. Having already been used clinically as suture material for decades [Inouye et al., 1998; Minoura et al., 1990; Santin et al., 1999; Sofia et al., 2001], silk was selected from the array of alternative synthetic and natural fibers for this application because it was the only protein-based fiber that: (1) could match the required mechanical properties of an ACL, (2) was biocompatible when properly prepared [Inouye et al., 1998], (3) avoided bioburdens associated with mammalian-derived materials, (4) maintained mechanical tensile integrity in vitro in tissue culture conditions, and (5) exhibited slow degradation in vivo [Greenwald et al., 1994], allowing adequate time for host tissue infiltration and eventual stabilization.

The number of fibers and geometry of the silk matrix were designed to mirror human ACL in UTS, linear stiffness, yield point, and percent elongation at break, as well as to support cell seeding and tissue ingrowth. Sericin removal, UTS measured in newtons (N), and linear stiffness (N/mm) were characterized as a function of extraction temperature up to 90 °C for a 60-minute processing period by SEM and single-pull-to-failure mechanical analysis.

The geometric hierarchy of the matrix that best mirrored the ACL was as follows: 1 ACL prosthesis = 6 parallel cords; 1 cord = 3 twisted strands (3 twists/cm counterclockwise); 1 strand = 6 twisted bundles (3 twists/cm clockwise); 1 bundle = 30 parallel extracted fibers. The resulting ACL matrix contained 3240 preextracted fibers with a combined cross-sectional area of 3.67 mm^2 and could tightly fit within a 4-mm-diameter bone tunnel. The selected silk matrix exhibited mechanical properties comparable to those of the native human ACL: UTS of ~2100 N, stiffness of ~240 N/mm, yield point of ~1200 N, and 33% elongation at break. Regression analysis of matrix fatigue data, when extrapolated to physiological load levels (400 N) [Chen and Black, 1980] to predict number of cycles to failure in vivo, indicated a matrix life of 3.3 million cycles.

To test the mechanical integrity of silk fibroin in physiologic cell culture conditions, bundles of 30 extracted silk fibers 3 cm between sutures were prepared. Bundles were seeded as described above (See Protocol 8.4), using a 1×10^6 cells/ml cell suspension and cultured for 1, 7, 14, and 21 days ($n = .8$ per time point); otherwise identical nonseeded matrices were generated for use as controls. Single pull-to-failure mechanical analysis was performed as described above by clamping directly on the knotted sutures, eliminating the need for molded epoxy ends; also, matrices were saturated with cell culture medium throughout the testing procedure. The silk fibers retained mechanical tensile strength over 21 days in tissue culture conditions, and no statistically significant changes in UTS were observed when comparing seeded and nonseeded matrices.

Human BMSCs readily adhered, spread, and grew on the silk fiber matrix after 1 day in culture and formed cellular extensions to bridge neighboring fibers. A

uniform cell sheet covering the fibrous construct was observed by 14 days of culture. Measures of total DNA, from 2 cords per time point, confirmed BMSC proliferation on the silk, with the highest amount of DNA measured after 14 days in culture. Real-time RT-PCR assessment of 3 seeded cords cultured for 14 days indicated ligament-specific marker expression (e.g., collagen types I and III, tenascin-C) by the cultured BMSCs. Collagen types II and bone sialoprotein, as markers of cartilage- and bone-specific differentiation, respectively, were negligibly expressed. Furthermore, the ratio of collagen type I expression to collagen type III was 8.9:1, consistent with that of cruciate ligaments [Amiel et al., 1984] and not indicative of a wound-healing response characterized by excessive collagen type III expression. In comparison to human BMSCs grown in collagen gels for 14 days in a static environment [Altman et al., 2002b], baseline levels of collagen types I and III and tenascin-C expression on the silk matrix were 6.1-fold, 8.2-fold, and 7.6-fold greater, respectively.

9.1.2. Silk Matrix Surface Modification

In ligament tissue engineering the appropriate matrix, when combined with cells and environmental stimuli, must support cell attachment, spreading, growth and collagen matrix production. We have shown that a silk matrix can support BMSC attachment and differentiation. As a first step in in vitro ligament optimization, surface modification of the silk fiber matrix with RGD peptide was selected to enhance early stages of cell-matrix interactions. RGD-containing peptide is the main integrin-mediated cell attachment domain found in many extracellular matrix proteins; however, RGD binding domains are not present in the native *B. mori* silk. We hypothesize that enhanced initial cell attachment, spreading, and migration resulting from RGD coupling will increase cell density and ECM production, leading to higher rates of ligament development in vitro. This hypothesis is derived from related studies in which high initial cell densities led to better gap junction communication. The effects of silk matrix-RGD peptide coupling on BMSC and ACL fibroblast (as a positive control) behavior over 14 days of culture were explored.

RGD modification increased the rates of both BMSC matrix adhesion and spreading within 1 h of seeding; BMSC and ACLF matrix density was significantly increased by 1.6- to 2.1-fold (MTT assay) 1 day after seeding. It is known that RGD surface modification can improve cell mobility and, in turn, dramatically increase proliferation [Sottile et al., 1998]; however RGD modification of the fibroin matrix did not affect cell growth rates from 7 to 14 days of culture. Thus the effect of RGD coupling was only evident at initial seeding, resulting in an increase in cell attachment and spreading. SEMs of both cell types on modified and nonmodified matrices indicated the development of a cell sheet and possible ECM formation over 14 days in culture; however, the cell/ECM on RGD-modified matrices was thicker and more continuous than on nonmodified matrices to such an extent that silk fibroin fibers could not be seen in the micrographs at 14 days.

Collagen type I, the predominant protein of most connective tissues and the main constituent of the ACL, occupying >86% of its dry tissue weight, was chosen as

a representative marker for cell matrix production. RGD coupling, through the enhancement of initial cell density, induced changes in collagen type I production over time as determined by mRNA transcript (monitored via semiquantitative RT-PCR) levels at day 7 and protein concentration at day 14. The significant increases observed in collagen type I mRNA levels from BMSCs and ACLFs grown on RGD-modified matrices are likely the result of an increase in cell density; enhanced cell-cell interactions likely induced a switch from cell proliferation to matrix production (differentiation). The increase in collagen type I transcript levels from BMSCs was fivefold greater than the increase observed from ACLFs (i.e., 180% compared with 30%).

ELISA against collagen type I at day 14 confirmed the trends observed in transcription levels at day 7. By 14 days of culture, a 410% increase in BMSC collagen type I production on RGD-modified compared with nonmodified matrices was observed, supporting the notion of a switch from a proliferative nondifferentiated state at low cell density (e.g., 7 days) to a matrix-producing state at high cell densities (14 days). Furthermore, although the effects on collagen type I matrix production may simply be the result of an increase in cell density, the introduction of additional cell binding sites through RGD modification may also induce subsequent intracellular signaling events to promote cell matrix production and differentiation [Grzesiak et al., 1997].

For tissue engineering, BMSCs should attach firmly on silk matrices, and, appropriately, most BMSCs remained on the RGD-modified silk film after trypsinization for 15 min, whereas most of the cells seeded on tissue culture plastic and EDC/NHS-treated and nonmodified silk films detached. These data are consistent with several studies that indicated strong adhesion of cells to RGD peptides on surfaces [Massia and Hubbell, 1990; Neff et al., 1999].

The present study has begun to address these issues by demonstrating a role for RGD-modified silk fibers in accelerating the attachment, coverage, and differentiation of BMSCs. These studies extend our prior work [Altman et al., 2002a, b] and suggest a path forward for continued optimization of the process.

9.2. Mechanical Stimulation as the Ligament Differentiation Inducer

Mechanical stimuli are known to be able to trigger a number of signaling pathways (protein kinase: FAK, ILK, Src; adaptor proteins: Shc, Grb-2, Crk; small GTPases: Rho, Ras) through cell surface receptors, $\alpha_5\beta_1$-integrin for example, [Giancotti, 1997]. The receptors, in turn, may activate the mitogen-activated protein kinase (MAPK) pathway, which counteracts apoptosis and promotes cell survival, thus increasing cell density. Mechanical stimulation could also activate MAPK via autocrine release of growth factors [Chiquet, 1999; Kim et al., 1999; MacKenna et al., 2000; Ruoslahti, 1997]. The identification of the signal transduction pathways involved in the present BMSC-silk-bioreactor system may lead future research in interesting directions.

Previously we proved that mechanical stimulation alone, without the addition of growth factors, can induce BMSC differentiation into ligament lineage [Altman

et al., 2002b]. BMSCs seeded in a collagen gel system aligned along the direction of mechanical stimulation and produced collagen types I and III and tenascin-C, the typical markers of ligament differentiation.

To investigate the impact of mechanical stimulation upon the cells cultured on silk fibers, matrices were exposed to multidimensional cyclic stresses (torsion and tension). SEM analysis showed that BMSCs seeded on the RGD-modified silk matrices remained attached after 6 days of stimulation and aligned along the direction of mechanical stimuli, whereas most of the cells seeded on the nontreated silk fibers detached [Chen et al., 2003]. Ongoing experiments focus on optimizing the parameters of mechanical regime and dynamic medium flow to support BMSC growth and differentiation into ligament fibroblasts.

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SOURCES OF MATERIALS

Item	*Supplier*	*Storage Condition*
ABI Prism 7000 real time system	Applied Biosystems	
Axiovert S100 light microscope	Zeiss	
Basic fibroblast growth factor (bFGF)	Invitrogen	−20 °C
Bombyx mori silkworm silk	Rudolph-Desco	
CCD color video camera (DXC-390)	Sony	
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)	Sigma	4 °C
DMSO	Sigma	Room temperature
Donkey anti-rabbit IgG	Jackson ImmunoResearch	4 °C
Dulbecco's modified Eagle's medium (DMEM)	Invitrogen	4 °C
ELISA kit for collagen type I	Chondrex	−20 °C
ELISA kit, human collagen type I	Chondrex	
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)	Pierce	
Fast-Track software Wavemaker32 version 6.6	Instron	
Fetal bovine serum (FBS)	Invitrogen	−20 °C
Frame grabber card (CG-7 RGB)	Scion	
Glycine-arginine-glycine-aspartate-serine (GRGDS) peptide	Sigma	
Human bone marrow stromal cells	Clonetic-Poietics	

Item	*Supplier*	*Storage Condition*
N-hydroxysuccinimide (NHS)	Pierce	
Instron 8511 servohydraulic tension/compression system	Instron	
L-Ascorbic acid 2-phosphate	Sigma	Room temperature
Microplate reader	Molecular Devices	
MTT	Sigma	4 °C
Pancreatic elastase	Sigma	−20 °C
Penicillin-streptomycin (P/S)	Invitrogen	−20 °C
Pepsin	Sigma	−20 °C
Phosphate-buffered solution (PBS)	Invitrogen	Room temperature
PicoGreen	Molecular Probes	−20 °C
Polaron SC502 Sputter Coater	Fison	
Polyolefin heat shrink tubing	Appleton Electronics	
Primers and probes, markers of ligament formation (Assay on Demand)	Applied Biosciences	
QuiAmp DNA mini kit	Quiagen	−20 °C
Rabbit anti-human collagen type I antibody	Biodesign	−20 °C
Scanning Electron Microscope JEOL JSM-840A	JEOL	
Scion Image software version 1.9.1	Scion	
Spectra Max 250 microplate spectrophotometer	Molecular Devices	
Sputter coater	Fison	
Superscript Preamplification System	Invitrogen	
1,1,2-Trichlorotrifluoroethane	Aldrich	Room temperature
Trypsin-EDTA	Invitrogen	−20 °C
Wavemaker32 version 6.6	Instron	

REFERENCES

Altman, G.H., Diaz, F., Jakuba, C., Calabro, T., Horan, R.L., Chen, J., Lu, H., Richmond, J., Kaplan, D.L. (2003) Silk-based biomaterials. *Biomaterials* 24: 401–416.

Altman, G.H., Horan, R.L., Lu, H.H., Moreau, J., Martin, I., Richmond, J.C., Kaplan, D.L. (2002a) Silk matrix for tissue engineered anterior cruciate ligaments. *Biomaterials* 23: 4131–4141.

Altman, G.H., Horan, R.L., Martin, I., Farhadi, J., Stark, P.R., Volloch, V., Richmond, J.C., Vunjak-Novakovic, G., Kaplan, D.L. (2002b) Cell differentiation by mechanical stress. *FASEB J.* 16: 270–272.

Amiel, D., Frank, C., Harwood, F., Fronek, J., Akeson, W. (1984) Tendons and ligaments: a morphological and biochemical comparison. *J. Orthop. Res.* 1: 257–265.

Bramono, D.S., Richmond, J.C., Weitzel, P.P., Kaplan DL, Altman GH. (2004) Matrix metalloproteinases and their clinical applications in orthopaedics. *Clin Orthop Relat Res.* 2004 Nov;(428): 272–85. Chen, E.H., Black, J. (1980) Materials design analysis of the prosthetic anterior cruciate ligament. *J. Biomed. Mater. Res.* 14: 567–586.

Chen, J., Altman, G.H., Karageorgiou, V., Horan, R., Collette, A., Volloch, V., Colabro, T., Kaplan, D.L. (2003) Human bone marrow stromal cell and ligament fibroblast responses on RGD-modified silk fibers. *J. Biomed. Mater. Res.* 67A: 559–570.

Chiquet, M. (1999) Regulation of extracellular matrix gene expression by mechanical stress. *Matrix Biol.* 18: 417–426.

Dunn, M.G., Maxian, S.H., Zawadsky, J.P. (1994) Intraosseous incorporation of composite collagen prostheses designed for ligament reconstruction. *J. Orthop. Res.* 12: 128–137.
Dunn, M.G., Tria, A.J., Kato, Y.P., Bechler, J.R., Ochner, R.S., Zawadsky, J.P., Silver, F.H. (1992) Anterior cruciate ligament reconstruction using a composite collagenous prosthesis. A biomechanical and histologic study in rabbits. *Am. J. Sports. Med.* 20: 507–515.
Frank, C.B., Hart, D.A., Shrive, N.G. (1999) Molecular biology and biomechanics of normal and healing ligaments—a review. *Osteoarthritis Cartilage* 7: 130–140.
Giancotti, F.G. (1997) Integrin signaling: specificity and control of cell survival and cell cycle progression. *Curr. Opin. Cell. Biol.* 9: 691–700.
Greenwald, D., Shumway, S., Albear, P., Gottlieb, L. (1994) Mechanical comparison of 10 suture materials before and after in vivo incubation. *J. Surg. Res.* 56: 372–377.
Grzesiak, J.J., Pierschbacher, M.D., Amodeo, M.F., Malaney, T.I., Glass, J.R. (1997) Enhancement of cell interactions with collagen/glycosaminoglycan matrices by RGD derivatization. *Biomaterials* 18: 1625–1632.
Inouye, K., Kurokawa, M., Nishikawa, S., Tsukada, M. (1998) Use of *Bombyx mori* silk fibroin as a substratum for cultivation of animal cells. *J. Biochem. Biophys. Methods* 37: 159–164.
Kim, B.S., Nikolovski, J., Bonadio, J., Mooney, D.J. (1999) Cyclic mechanical strain regulates the development of engineered smooth muscle tissue. *Nat. Biotechnol.* 17: 979–983.
Lo, I.K., Marchuk, L., Hart, D.A., Frank, C.B. (2003) Messenger ribonucleic acid levels in disrupted human anterior cruciate ligaments. *Clin. Orthop. Relat. Res.* 407: 249–258.
Lo, I.K., Marchuk, L.L., Hart, D.A., Frank, C.B. (1998) Comparison of mRNA levels for matrix molecules in normal and disrupted human anterior cruciate ligaments using reverse transcription-polymerase chain reaction. *J. Orthop. Res.* 16: 421–428.
MacKenna, D., Summerour, S.R., Villarreal, F.J. (2000) Role of mechanical factors in modulating cardiac fibroblast function and extracellular matrix synthesis. *Cardiovasc. Res.* 46: 257–263.
Massia, S.P., and Hubbell, J.A. (1990) Covalent surface immobilization of Arg-Gly-Asp- and Tyr-Ile-Gly-Ser-Arg-containing peptides to obtain well-defined cell-adhesive substrates. *Anal. Biochem.* 187: 292–301.
Minoura, N., Tsukada, M., Nagura, M. (1990) Physico-chemical properties of silk fibroin membrane as a biomaterial. *Biomaterials* 11: 430–434.
Neff, J.A., Tresco, P.A., Caldwell, K.D. (1999) Surface modification for controlled studies of cell-ligand interactions. *Biomaterials* 20: 2377–2393.
Ruoslahti, E. (1997) Stretching is good for a cell. *Science* 276: 1345–1346.
Santin, M., Motta, A., Freddi, G., Cannas, M. (1999) In vitro evaluation of the inflammatory potential of the silk fibroin. *J. Biomed. Mater. Res.* 46: 382–389.
Sofia, S., McCarthy, M.B., Gronowicz, G., Kaplan, D.L. (2001) Functionalized silk-based biomaterials for bone formation. *J. Biomed. Mater. Res.* 54: 139–148.
Sottile, J., Hocking, D.C., Swiatek, P.J. (1998) Fibronectin matrix assembly enhances adhesion-dependent cell growth. *J. Cell Sci.* 111 (19): 2933–2943.
Weitzel, P.P., Richmond, J.C., Altman, G.H., Calabro, T., Kaplan, D.L. (2002) Future direction of the treatment of ACL ruptures. *Orthop. Clin. North. Am.* 33: 653–661.