Evaluation of canine adipose-derived multipotent stromal cell differentiation to ligamentoblasts on tensioned collagen type I templates in a custom bioreactor culture system

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OBJECTIVE
To evaluate differentiation of canine adipose-derived multipotent stromal cells (ASCs) into ligamentoblasts on tensioned collagen type I (Coll) templates in a perfusion culture system.

SAMPLES
Infrapatellar fat pad ASCs from healthy stifle joints of 6 female mixed-breed dogs.

PROCEDURES
Third-passage ASCs (6 X 10^6 cells/template) were loaded onto suture-augmented Coll templates under 15% static strain in perfusion bioreactors. Forty-eight ASC-Coll constructs were incubated with ligamentogenic (ligamentogenic constructs; n = 24) or stromal medium (stromal constructs; 24) for up to 21 days. Specimens were collected from each construct after 2 hours (day 0) and 7, 14, and 21 days of culture. Cell number, viability, distribution, and morphology; construct collagen content; culture medium procollagen-I-N-terminal peptide concentration; and gene expression were compared between ligamentogenic and stromal constructs.

RESULTS
ASCs adhered to collagen fibers. Cell numbers increased from days 0 to 7 and days 14 to 21 for both construct types. Relative to stromal constructs, cell morphology and extracellular matrix were more mature and collagen content on day 21 and procollagen-I-N-terminal peptide concentration on days 7 and 21 were greater for ligamentogenic constructs. Ligamentogenic constructs had increased expression of the genes biglycan on day 7, decorin throughout the culture period, and Coll, tenomodulin, fibronectin, and tenascin-c on day 21; expression of Coll, tenomodulin, and tenascin-c increased between days 7 and 21.

CONCLUSIONS AND CLINICAL RELEVANCE
Ligamentogenic medium was superior to stromal medium for differentiation of ASCs to ligamentoblasts on suture-augmented Coll scaffolds. Customized ligament neotissue may augment treatment options for dogs with cranial cruciate ligament rupture.

In dogs, most surgeries of the stifle joint involve repair of a ruptured cranial cruciate ligament (CrCL). Costs associated with CrCL rupture in dogs exceed $1 billion/y.1 In human patients with anterior cruciate ligament (ACL) rupture (analogous to CrCL rupture in dogs), ligament reconstruction is the standard of care because it improves knee kinematics and reduces cartilage and meniscus injury and osteoarthritic changes.2,3 In dogs with CrCL rupture, reconstruction of the ligament is rare owing, in part, from a shortage of allograft resources.4 Instead, contemporary treatments consist of periarticular joint stabilization or alteration of joint kinetics via osteotomy.5,6 Reconstruction of the original CrCL structure with customizable, suture-augmented neotissue to fit the stifle joint morphology of each patient may substantially augment current repair options for CrCL rupture in dogs.

In vitro engineering of functional tissue for surgical implantation to replace tissues lost to trauma or disease is rapidly becoming a reality with standardization of cell sources, biocompatible scaffold templates, and culture conditions that simulate in vivo environments.7 Adult multipotent stromal cells (MSCs) derived from adipose tissue, or adipose tissue-derived multipotent stromal cells (ASCs), are recognized as an prime resource for tissue engineering.8-10 Although ASCs are capable of differentiation into a wide spectrum of tissue types, evidence supports that cells from orthotopic tissues have advantages over those from nonorthotopic tissues for local tissue generation.10-12 This is attributed to many factors, including persistent embryonic epigenetic influences and local environmental signals that affect cell plasticity, maturity, and expansion capabilities.13-15 Progenitor cells
from the stifle joint infrapatellar fat pad of dogs have a higher percentage of MSC immunophenotypes, faster in vitro expansion, and greater plasticity, compared with those from the CrCL and joint capsule synovium, which makes them a promising choice for de novo generation of stifle joint tissues.10

Optimization of cell differentiation and de novo neotissue formation is the first step toward implantable, mature tissues customized for individual patients. Differentiation medium is distinct among species, tissue structures, and 2-D and 3-D culture mechanisms.16,17 Results of previous studies18–20 have established a framework for base medium, growth factors, and mechanical stimulation to guide ASC ligamentoblast differentiation protocols. Transforming growth factor-β and basic fibroblast growth factor (bFGF) enhance tenoblast tissue matrix production.21–23 Perfusion bioreactor culture permits creation of a stable 3-D environment with dynamic gas exchange, nutrient delivery and waste removal.24 Evaluation of a fairly long-term 3-D culture is necessary to assess ligamentoblast differentiation and potential for mature tissue production in a bioreactor culture system.

Templates composed of target tissue matrix components facilitate cell differentiation and extracellular matrix (ECM) production.9,25 Collagen type I is a major component of ligament tissue, and as such, is a popular and effective scaffold matrix for differentiation of ASCs into musculoskeletal cell lineages including ligamentoblasts.26–28 Current knowledge supports that effective ligament neogenesis requires mechanical stimulation of cell-matrix constructs for robust tissue organization.18,20 Static construct strain and shear forces of fluid flow within a perfusion bioreactor are baseline characteristics that promote ASC differentiation and matrix formation.29–32

The overarching goal for the study reported here was to establish a framework to achieve a customizable, suture-augmented canine ligament neotissue. The specific aim of the study was to induce canine ASCs to differentiate to ligamentoblasts on tensioned collagen type I (Col1) CrCL templates in a custom bioreactor culture system. Our hypothesis was that ligamentogenic medium perfusion culture would be more effective than stromal medium perfusion culture for promoting ASCs to differentiate to ligamentoblasts on tensioned CrCL ColI templates. Development and validation of a customizable protocol for production of viable canine ligament neotissue could substantially advance research and treatment of CrCL rupture in dogs.

Materials and Methods

ASC harvest and processing
All cell harvest procedures were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee (No. 10-004). Infrapatellar adipose tissue was harvested from the stifle joints of 6 university-owned mixed-breed sexually intact female dogs that were undergoing surgery for an unrelated stifle joint stabilization project.33 The dogs had a mean ± SEM age of 3.5 ± 0.4 years and body weight of 23.1 ± 0.9 kg. The stifle joints from which the infrapatellar adipose tissue specimens were obtained were considered clinically normal on the basis of the absence of radiographic evidence of joint disease prior to surgery and gross visual inspection of the joints during surgery.

Following harvest, the stromal vascular fraction was isolated from the infrapatellar adipose tissue as described.10 Then, 5 × 10^5 ASCs/cm^2 were seeded into 10-cm culture dishes (CellStar) containing stromal medium, and the dishes were incubated at 37 °C in an atmosphere with 5% CO\(_2\) and 90% humidity. The stromal medium was refreshed 4 hours after initiation of incubation and then every 2 to 3 days until the cell cultures achieved 80% confluence when they were detached with 0.25% trypsin (HyClone) and transferred to fresh culture dishes (denoted as P0 cells). Cells were cultured up to P1, detached, added to cryopreservation medium (80% fetal bovine serum [HyClone], 10% Dulbecco modified Eagle medium (DMEM)-Ham F12 [HyClone], and 10% DMSO [Thermo Fisher Scientific]) and stored in 1-mL aliquots (10^6 ASCs/mL) in cryovials. The cryovials were cooled to −80 °C in a freezing container (Thermo Fisher Scientific) and then stored in liquid nitrogen (−150 °C) for 6 to 8 months until use.

The P1 cells in the cryovials were thawed at 37 °C, and the vials were centrifuged at 260 × g for 5 minutes. The resulting cell pellet was rinsed 3 to 4 times with PBS solution (HyClone). Cells were subsequently cultured to the third passage (P3) as described above.

Canine CrCL harvest

Three intact CrCLs were obtained from client-owned dogs that were euthanized for reasons unrelated to musculoskeletal disease for comparison purposes with the neotissue constructs. The CrCLs were obtained with the owners’ consent immediately after death was confirmed.

Study design
Forty-eight constructs (2 constructs created with ASCs from each of the 6 donor dogs for each time point) were created by loading P3 cells onto suture-augmented, bovine Coll1 scaffold templates in custom perfusion bioreactors. Twenty-four constructs (1 construct created with ASCs from each of the 6 donor dogs for each time point) were cultured with stromal medium (DMEM-Ham F12, 10% fetal bovine serum, and 1% antibiotic-antimycotic solution [HyClone]), and the other 24 constructs were cultured with ligamentogenic medium (low-glucose DMEM [HyClone], 5% fetal bovine serum, 1% antibiotic-antimycotic solution, bFGF [Gibco; 1 ng/mL], transforming growth factor-β [Shenandoah Biotechnology Inc; 5 ng/mL], and platelet-derived growth factor [Gibco; 10 ng/mL]) for up to 21 days.
From each construct, a 6-mm-diameter skin biopsy punch tool was used to obtain 2 full-thickness specimens from the center of the high, middle, and low regions relative to the construct’s vertical position in the bioreactor after 2 hours (day 0) and 7, 14, and 21 days of culture. The cell number and distribution were assessed at all 4 sampling times, and the ligament-specific gene mRNA expression, microstructure, ultrastructure, and collagen content were assessed at 7, 14, and 21 days. A sample of culture medium was obtained from each construct every 7 days for quantification of procollagen-I-N-terminal peptide (PINP) concentration. All assays were performed with a minimum of 2 replicates for each specimen collected at each acquisition time.

**Perfusion bioreactor system**

The perfusion bioreactor system consisted of 2 chambers with a height of 50 mm and inner diameter of 20 mm (Figure 1). A frame with 15-mm-long, 6.3-mm-diameter crossbars on each end that were 45 mm apart and offset by 90° fit securely within each bioreactor chamber. Each chamber had a removable cap with a centrally located port (inner diameter, 3 mm) at the highest end. There was an identical port in the center of the lowest end of the chamber base. The lowest port of each bioreactor was attached to a port of a 10-mL medium reservoir (Synthecon) with tubing (Tygon; Saint-Gobain Performance Plastics; inner diameter, 4.8 mm) via a 3-way stopcock. The reservoir had an additional port (internal diameter, 3 mm) fitted with a microfilter (Millipore) for gas exchange. The port on each bioreactor cap was connected to a computer-controlled peristaltic pump (ISM404b; Ismatec) via 3-way stopcocks attached to 0.22-μm microfilters to which tubing (Tygon; Saint-Gobain Performance Plastics; inner diameter, 1.0 mm) between the bioreactor and pump was attached. System fluid flow rate and direction were controlled by a computer (LabView; National Instruments). All bioreactor system parts were sterilized with ethylene oxide prior to assembly and use.

**ASC-Coll construct culture**

The core of the scaffold template was size-2 monofilament absorbable polydioxanone sulfate suture (Ethicon) coiled into a 3-cm-long, 1-cm-wide loop consisting of 5 suture strands (Figure 1). One end of the suture loop was turned approximately 90° relative to the other to replicate the twisted bands of a natural canine CrCL. With the ends held in position by hemostats, a bovine Coll sponge (Avitene) rectangle measuring 3 × 2 × 0.3 cm was wrapped around the suture core and secured in place with size-0 braided absorbable suture in a finger trap pattern. Canine ASCs (6 × 10⁶ cells/scaffold; 3.3 × 10⁶ cells/cm³) were added to the template through the top port of a bioreactor chamber. G—Photograph of a canine ASC–Coll construct after 21 days of incubation in ligamentogenic medium.
braided absorbable polyglactin 910 suture (Ethicon) in a finger trap pattern. The polyglactin 910 suture tag at 1 end of the construct was affixed with a square knot to the lowermost bar of the bioreactor frame.

The Coll1 templates were saturated with PBS solution containing 10 μg of fibronectin/mL (R&D Systems), and the construct was elongated to 15% strain by tensioning the polyglactin 910 suture at the upper end around the top bar of the frame and securing it with a quick-release knot. Each frame was inserted into a bioreactor chamber and the system scaled. A total of 48 mL of the designated culture medium (stromal medium or ligamentogenic medium) was added through the stopcocks on the top of the chambers (24 mL/chamber) and equilibrated with fluid flow for approximately 60 minutes in an incubator (37 °C with 5% CO2 and 90% humidity). Fluid flow was then stopped, and ASC aliquots (6 × 10^6 cells/scaffold; 3.3 × 10^6 cells/cm^2) were added through the top port of reach bioreactor. Fluid flow was resumed 1 hour later. The constructs were cultured at a constant fluid flow rate (1 mL/min) in the incubator. The direction of the fluid flow was reversed when the medium subsided to the lowest end of the top frame bar within a bioreactor so that the constructs remained immersed throughout perfusion. The culture medium was refreshed every 7 days through the reservoir. The used culture medium (48 mL) was stored frozen at –80 °C until analysis for PINP concentration. The constructs were cultured for up to 21 days.

**Cell number quantification**

The relative number of cells within a tissue specimen was determined by use of resazurin reduction (alamarBlue; Invitrogen) performed in accordance with the manufacturer’s instructions. Tissue specimens were placed in PBS solution and incubated with 10% resazurin reduction solution in a 96-well plate for 4 hours at 37 °C. The fluorescence of aliquots from each sample (100 μL) in a black 96-well plate was measured with a microplate reader (Synergy HT; Biotek) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

**Cell distribution**

Confocal laser microscopy was used to assess the cell distribution within the constructs. Briefly, tissue specimens were treated with 5 μM calcein and 2 μM ethidium bromide (Sigma-Aldrich) in 200 μL of PBS solution. Twenty consecutive photomicrographs were obtained every 100 μm with the digital imaging system of a spectral confocal laser microscope (Leica). The micrographs were merged, and the relative numbers of viable and nonviable cells in each region (high, middle, and low portions) of the construct were determined.

**Construct microstructure and ultrastructure**

Tissue specimens designated for microstructure assessment were fixed in neutral-buffered 10% formalin. Subsequently, the specimens were serially dehydrated, embedded in paraffin, sectioned into 5-μm-thick slices, and stained with H&E stain. Digital photomicrographs (NanoZoomer; Hamamatsu Photonics) were obtained for each construct region.

Tissue specimens designated for ultrastructure assessment were fixed in 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1M sodium cacodylate (CAC) buffer (pH, 7.4) for 1 hour at room temperature (approx 22 °C), then transferred to buffer (3% glutaraldehyde in 0.1M CAC buffer) for 30 minutes. The specimens were rinsed with washing buffer (5% sucrose in 0.1M CAC buffer), postfixative buffer (1% osmium tetroxide in 0.1M CAC buffer), and water, then serially dehydrated, critical point dried, and sputter coated with gold. Digital images of the tissue specimens were obtained with a scanning electron microscope (Quanta 200; FEI Co) set at 15 kVp.

**Collagen deposition**

To assess collagen deposition, tissue specimens were digested with 500 μL of pepsin solution (concentration, 0.25 mg/mL) at 37 °C for 30 minutes. The soluble collagen content was quantified with a commercially available kit (Chondrex) in accordance with the manufacturer’s instructions. The assay was performed in a 96-well plate, and the tissue light absorbance was read with a microplate reader (Synergy HT; Biotek) set at a wavelength of 540 nm. The total collagen content in each specimen was extrapolated from a standard curve provided with the assay kit. All values were normalized to untreated Coll1 scaffold material.

**PINP synthesis**

Culture medium samples that were collected from the perfusion bioreactors system every 7 days and stored frozen at ~80 °C were thawed just prior to determination of the PINP concentration. Following thawing, each sample was thoroughly mixed, and 200-μL aliquots were extracted from the sample for PINP quantification, which was determined by use of a commercially available canine PINP ELISA (Biotang) in accordance with the manufacturer’s instructions. The assay was performed in 96-well plates, and the sample light absorbance was determined with a microplate reader (Synergy HT; Biotek) set at a wavelength of 450 nm. The PINP concentration was determined from the standard curve provided with the kit and normalized to that of unused medium. Thus, the measured PINP concentration reflected the PINP produced between culture medium changes.

**Determination of mRNA expression**

Each intact CrCL was snap frozen and pulverized (BioPulverizer; BioSpec Products Inc) in liquid nitrogen. Each CrCL and construct tissue specimen was then homogenized in a phenol-guanidinium thiocyanate solution (Sigma-Aldrich), and RNA was extracted in accordance with the manufacturer’s instructions.
Messenger RNA was reverse transcribed (Quantitect; Qiagen) into cDNA. Canine-specific primers for the genes Col1, collagen type III, biglycan, decorin, tenasin-c, fibronectin, and tenomodulin were designed from published gene sequences in accordance with National Center for Biology Information standards (Appendix). Relative gene expression was determined by use of SYBR green reagents (Quantitect; Qiagen) and a real-time PCR assay system (7900 Real-Time PCR System; SDS version 2.4; Applied Biosystems). Gene expression data from specimens was normalized to the reference gene (Glutaraldehyde phosphate dehydrogenase [GADPH]), and fold change with cycle threshold (Ct; 2ΔΔCt) in gene expression for specimens cultured in ligamentogenic medium relative to specimens cultured in stromal medium was determined as described. Gene expression data for the native CrCLs were likewise normalized to GADPH and reported as the ΔCt.

Statistical analysis
The Kolmogorov-Smirnov test was used to confirm normality of the data, and results were summarized as the mean ± SEM. The effect of construct sampling region (low, middle, or high) on each outcome of interest (number of cells, collagen content, PINP concentration, and gene expression) was assessed with a 1-way ANOVA. Region had no effect on any of the outcomes of interest; therefore, region data were combined for further analyses. The effects of culture time (0, 7, 14, and 21 days) and culture medium (ligamentogenic or stromal) on each outcome of interest were assessed with a 2-way ANOVA, with the Tukey test used for post hoc pairwise comparisons when necessary. The effect of culture time on mRNA expression was assessed by use of a 1-way ANOVA followed by the Tukey test for post hoc pairwise comparisons. All analyses were performed with statistical software (SAS version 9.4; SAS Institute), and values of P < 0.05 were considered significant.

Results
Cell numbers
The number of cells was determined for 20 ASC-Col1 constructs cultured in the ligamentogenic medium (ligamentogenic constructs) and 20 ASC-Col1 constructs cultured in the stromal medium (stromal constructs). The number of cells in the ligamentogenic constructs did not differ significantly from the number of cells in the stromal constructs at any culture time. For both ligamentogenic and stromal constructs, the mean number of cells at 7, 14, and 21 days was significantly greater than the mean number of cells on day 0, and the mean number of cells on day 21 was significantly greater than the mean number of cells at each of the other 3 culture times (Figure 2).

Cell distribution
The cell distribution was assessed for 12 ligamentogenic constructs and 12 stromal constructs. Most cells were viable and were uniformly distributed throughout the scaffold template within 2 hours (0 days) after loading. The number of viable cells increased with culture time in both ligamentogenic and stromal constructs (Figure 2).

Construct microstructure and ultrastructure
The microstructure and ultrastructure were evaluated for 18 ligamentogenic constructs and 18 stromal constructs. The cells in the constructs had distinct microstructural morphology and organization that was dependent on the type of medium in which they were cultured. After 14 days of culture, most cells in the stromal constructs had an ovoid shape and were present as single cells between the Col1 fibers of the template, whereas the cells in the ligamentogenic constructs had elongated fibroblast-like morphology and were arranged in linear clusters between parallel ECM fibers (Figure 3). Ultrastructurally, the attachment of cells to the ECM was evident after 7 days of culture in both the ligamentogenic and stromal constructs (Figure 3).
ter 14 days of culture, the amount of ECM was greater in the ligamentogenic constructs than in the stromal constructs. After 21 days of culture, the cells in the stromal constructs maintained a fibroblast-like morphology (ie, the cells had a prominent, spheroid cell body and elongated cell processes on each end). Conversely, the cells in the ligamentogenic constructs had a more ligamentoblast-like structure (ie, the cells had a complex polygonal shape with multiple cell processes).

Collagen content

Collagen content was determined for all 48 ASC-Col1 constructs. Compared with the collagen content in stromal constructs, the collagen content in the ligamentogenic constructs was numerically greater after 7 and 14 days of culture and significantly greater after 21 days of culture (Figure 4). The collagen content increased over time in both construct types. For the ligamentogenic constructs, the mean collagen content after 21 days of culture was significantly greater than the mean collagen content after 7 and 14 days of culture. For the stromal constructs, the mean collagen content after 21 days of culture was significantly greater than that after 7 days of culture.

Culture medium PINP concentration

The PINP concentration in used culture medium samples was determined for 12 ligamentogenic constructs and 12 stromal constructs. The mean PINP concentration in medium samples increased over time for both the ligamentogenic and stromal constructs. The mean PINP concentration in medium samples obtained from ligamentogenic constructs was consistently greater than the mean PINP concentration in medium samples obtained from stromal constructs throughout the culture period and was significantly greater after 7 and 21 days of culture (Figure 4). The mean PINP concentration in medium samples obtained from ligamentogenic constructs was significantly greater on day 21 of culture, compared with that on days 7 and 14. The mean PINP concentration...
in medium samples obtained from stromal constructs was significantly greater on days 14 and 21 of culture, compared with that on day 7 of culture.

**Target gene mRNA expression**

Target gene mRNA expression was determined for 12 ligamentogenic constructs and 12 stromal constructs. Gene mRNA expression in ligamentogenic constructs relative to stromal constructs increased with culture time (Figure 5). Specifically, relative to stromal constructs, mRNA expression in ligamentogenic constructs was significantly greater for the biglycan and decorin genes on day 7, for the decorin gene on day 14 and for the Col1, tenomodulin, fibronectin, tenascin-c, and decorin genes on day 21. For the ligamentogenic constructs, mean mRNA expression of the Col1, tenascin-c, and tenomodulin genes on day 21 was significantly greater than the respective mean mRNA expressions of those genes on day 7.

For the 3 native canine CrCL tissue specimens, mRNA expression was greatest for the decorin (mean ± SEM Ct, -7.1 ± 0.004) and fibronectin (-4.7 ± 0.03) genes. Other genes were expressed at low levels.

**Discussion**

Results of the present study indicated that canine ASCs derived from the infrapatellar fat pad attached to and proliferated on Col1 scaffold templates maintained in stromal or ligamentogenic medium for up to 21 days, that ligamentogenic medium was more effective than stromal medium for inducing collagen and procollagen synthesis and ligament-specific gene expression, and that ASCs cultured in ligamentogenic medium assumed a ligamentoblast-like morphology and produced an organized ECM characteristic of native CrCL tissue on suture-augmented Col1 scaffold templates. Hence, we accepted our hypothesis that ligamentogenic medium perfusion culture would be more effective than stromal medium perfusion culture for promoting differentiation of ASCs to ligamentoblasts on tensioned Col1 templates. The results of this study established the potential to produce viable implantable neoligament tissue with long-term perfusion culture, and the methods described create a basic platform that can be customized for individual patients and expanded to meet clinical demands.

It is well established that Col1 promotes cell adhesion and ECM production by MSCs in most species. However, sustained cell proliferation during long-term culture is less common. Lack of detectable cell proliferation has been reported to be a consequence of inadequate nutrient and oxygen exchange following pore collapse from Col1 template shrinkage, which is exacerbated by collagen deposition following MSC differentiation. In the present study, the application of tension to the Col1 scaffolds helped to maintain a surface for cell attachment, but that alone cannot explain the observed cell proliferation, which reportedly declines or ceases with cell differentiation and maturation. Results of other studies indicate that construct tension stimulates both ligamentoblast differentiation and proliferation, possibly by downregulating matrix metalloproteinase 1, which inhibits cell proliferation. Sustained cell proliferation during long-term culture is a positive attribute for a culture system because the initial cell inoculate is unlikely to survive for the duration needed for generation of viable graft tissue. The cell division observed in the ligamentogenic constructs of the present study was likely a balance of self-renewal and differentiation that may have led to cells at many different stages of differentiation. Additional characterization of cell division is necessary to determine whether cell proliferation is sufficient for maintenance of a progenitor cell population over longer (>21 days) culture periods.

During the process of graft ligamentization, the mechanical properties of the graft decline to their...
The use of polyglactin 910 suture material to place a finger trap pattern around the constructs of the present study was intended to conform to the Col1 to the polydioxanone frame and replicate an in vivo canine hamstring (biceps femoris, semitendinosus, and semimembranosus muscles) CrCL reconstruction graft. It also facilitated application of strain to the Col1 on the surface of the relatively rigid polydioxanone frame. Polyglactin 910 has a short half-life of 14 days, which will likely limit the strength of the construct following in vivo implantation. Coated polyglactin 910 might be more acceptable given that it has a longer half-life and causes less inflammation during suture breakdown than uncoated polyglactin 910. However, neither uncoated nor coated polyglactin 910 is associated with wear debris or foreign body reactions associated with nonabsorbable suture material. This preliminary information may help inform material selection and configuration for future 3-D printing of CrCL scaffold templates.

An important component of graft survival and function is soft tissue integration in bone tunnel fixation sites. The templates of the present study were designed to allow neotissue proliferation along the length of standard bone tunnels created for graft positioning and fixation. In an experimental model of lapin ACL reconstruction conducted in rabbits, viable cells including MSCs promoted integration of the graft within both femoral and tibial bone tunnels, suggesting that viable cells at every level of the graft may benefit graft integration. The graft templates created in the present study were designed to permit fixation of graft tissue to cortical bone in medium-sized dogs as described. However, the constructs were amenable to fixation with either cancellous or cortical bone as previously described in both dogs and human subjects. The structure of the grafts allow them to be customized and altered to fit patients of various sizes undergoing various surgical fixation techniques.

The ligamentogenic medium used in the present study contained bFGF and transforming growth factor-β to recapitulate the early phases of natural ligament healing and had a low serum content to up-regulate Col1 expression. The cell morphology, ECM organization, Col1 content, and culture medium PINP concentration for the ligamentogenic constructs were superior relative to those for the stromal constructs, which supported the theory that the ligamentogenic medium would be better than stromal medium for facilitation of ligamentogenesis. For the constructs of the present study, there appeared to be fewer cells on the Col1 constructs after fixation and processing for light microscopy than were apparent with resazurin reduction and cell viability staining. This is reported to be a consequence of low cell retention on the Col1 sponge following conventional processing for histologic evaluation.

In the present study, target gene expression in the ligamentogenic constructs was greater than that in the stromal constructs and was temporally consistent with ligamentogenesis. High expression of biglycan early, decorin throughout, and Col1, tenomodulin, fibronectin, and tenascin-c by day 21 in the ligamentogenic constructs resembled the typical stages of gene upregulation during ligament formation and early maturation. Biglycan and decorin are proteoglycans that regulate assembly of collagen fibrils during ligament repair and development, with biglycan responsible for early and decorin responsible for later stages of collagen fibril assembly. Tenomodulin and tenascin-c are biomarkers of early stage ligament repair and are necessary for cell proliferation and collagen fibril maturation. Collagen type I is a more mature form of collagen that appears later in the ligamentogenesis process, and fibronectin is a biomarker of early stage ligament repair that is abundantly expressed during the proliferation phase of ligamentogenesis. High expression of decorin and tenomodulin and low expression of Col1 and collagen type III in native CrCL tissue are consistent with mature tissue. As expected, in the present study, the gene expression profile for the neotissue constructs differed from that of the mature native CrCLs. Collectively, these results suggested that the neotissue generation observed in the present study was consistent with the proliferative phase of neoligamentogenesis that overlaps with early ligament maturation. It is possible that implantation of viable tissue protected by suture augmentation may obviate the necrotic phase of ligamentization required for mature tissue harvested elsewhere. This would facilitate implantation and reduce healing time required for graft reconstruction. Additional work is required to compare standard soft tissue graft material with custom neotissue graft material in vivo.
The results of the present study are limited to the described in vitro culture conditions and progenitor cell isolates. Further research is necessary to validate these results with ASCs obtained from a large, more diverse population of donor dogs. Additionally, the constructs of the present study were exposed to static tension, and results may differ for constructs exposed to dynamic tension. Current knowledge supports that musculoskeletal tissue grafts survive and support tissue formation in traumatized and diseased tissues including bone, cartilage and tendon.\textsuperscript{18,84,85} However, assessment of graft strength, elasticity, and behavior in an inflamed synovial joint environment was not performed in the present study and is vital before this technology can be used for clinical applications.

In the present study, we established that it was possible to generate custom, viable, ligament neotissue from canine ASCs. This tissue may augment current treatment options for repair of CrCL rupture in dogs. Additionally, the recapitulation of the proliferative phase of ligament repair in a controlled bioreactor culture system provides a mechanism to assess interventions designed to limit or reverse ligament degeneration and promote tissue repair. Further research is necessary to optimize culture conditions for accelerating the ligamentization process. Cryopreservation will likely be necessary for transport and storage of neotissue grafts, so pre- and post-cryopreservation properties must be established. The findings of this study represented an essential early step toward successful generation of canine ligament neotissue for treatment and study of ligament rupture in dogs.

**Acknowledgments**

Supported in part by the Department of Veterinary Clinical Sciences, Tynewald Foundation, and Sigma Xi Grants in Aid of Research program.

The authors declare that there were no conflicts of interest.

**References**


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**Appendix**

Canine-specific primer sequences used in reverse-transcription PCR assays to detect target gene mRNA expression in canine ASC-Col1 constructs and native canine CrCLs.

<table>
<thead>
<tr>
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<th>Primer</th>
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<td>GACCACCCACCTGCTTGT</td>
<td></td>
</tr>
</tbody>
</table>

NCBI = National Center for Biology Information.