Evaluation of Polycaprolactone Scaffold with Basic Fibroblast Growth Factor and Fibroblasts in an Athymic Rat Model for Anterior Cruciate Ligament Reconstruction

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Anterior cruciate ligament (ACL) rupture is a common ligamentous injury often necessitating surgery. Current surgical treatment options include ligament reconstruction with autograft or allograft, which have their inherent limitations. Thus, there is interest in a tissue-engineered substitute for use in ACL regeneration. However, there have been relatively few *in vivo* studies to date. In this study, an athymic rat model of ACL reconstruction was used to evaluate electrospun polycaprolactone (PCL) grafts, with and without the addition of basic fibroblast growth factor (bFGF) and human foreskin fibroblasts. We examined the regenerative potential of tissue-engineered ACL grafts using histology, immunohistochemistry, and mechanical testing up to 16 weeks post-operatively. Histology showed infiltration of the grafts with cells, and immunohistochemistry demonstrated aligned collagen deposition with minimal inflammatory reaction. Mechanical testing of the grafts demonstrated significantly higher mechanical properties than immediately postimplantation. Acellular grafts loaded with bFGF achieved 58.8% of the stiffness and 40.7% of the peak load of healthy native ACL. Grafts without bFGF achieved 31.3% of the stiffness and 28.2% of the peak load of healthy native ACL. In this *in vivo* rodent model study for ACL reconstruction, the histological and mechanical evaluation demonstrated excellent healing and regenerative potential of our electrospun PCL ligament graft.

Introduction

RUPTURE OF THE ANTERIOR cruciate ligament (ACL) is one of most common ligament injuries of the knee,¹ with over 200,000 patients diagnosed with ACL disruptions annually.² Due to the inherent inability of a ruptured ACL to heal, as many as 175,000 cases of diagnosed ACL injuries require surgery each year in the United States,² with an estimated cost of 1 billion dollars annually.³ Current treatment strategies employ autograft or allograft tendon for ligament reconstruction. Though high success rates can be achieved with both autograft and allograft replacement, serious complications are associated with these reconstruction options.⁴ Procurement of autograft tissue is associated with donor site morbidity including weakness, decreased range-of-motion, and chronic knee pain. Also, autograft supply is limited, particularly in cases of rerupture or multiligamentous injuries. Conversely, the use of allograft tissue increases the risk of pathogen transmission and adverse inflammatory response. Moreover, the supply of allograft tissue is limited by a finite donor pool.⁵ Synthetic nonde-

gradable grafts were developed in the 1970s and 1980s but were hampered by premature graft rupture, foreign body reactions, osteolysis, and synovitis.⁶ Because of these limitations, there are currently no synthetic grafts available for clinical use in the United States.

Due to the aforementioned limitations of existing graft options and to recent advances in biology, engineering, and regenerative medicine, there has been increasing interest in a tissue-engineered solution for ACL grafting. Current tissue engineering strategies employ synthetic materials that degrade in the body to allow host tissue ingrowth while avoiding the limitations associated with permanent synthetic material implantation.⁷ Polycaprolactone (PCL) is one such material that is currently utilized for a number of medical applications including adhesion barrier and wound dressing,⁸ which has been used in a wide variety of applications including vascular, bone, cartilage, nerve, skin, and esophageal tissue engineering.^{5,9–16} Favorable biocompatibility, relatively long *in vivo* half-life, degradation time, adequate mechanical strength, and high elasticity contribute to the popularity of this polymer in tissue engineering.⁷ In a rodent

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model of wound healing, implanted electrospun PCL was shown to be nonimmunogenic and to integrate into local tissue without adverse reactions.¹³

Our laboratory has developed a synthetic scaffold by electrospinning PCL to produce an aligned fiber scaffold for ACL reconstruction. Our previous study showed that this scaffold, when implanted into rat knees, was biocompatible, integrated with native tissue, and had increasing mechanical strength over time.¹⁷

As with other tissue engineering applications, cell source and growth factors are other important considerations for the design of tissue-engineered graft. Bone marrow-derived mesenchymal stem cells (BMSCs), ACL-derived fibroblasts, and skin fibroblasts have all been studied for ACL tissue engineering.¹⁸ Furthermore, there has been a great deal of investigation into determining the appropriate growth factors for ACL regeneration. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), growth and differentiation factor (GDF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF) have all been tested and have demonstrated increased cell proliferation, fibroblastic differentiation, or matrix production *in vitro*.^{19,20} In a previous *in vitro* study of a porous PCL scaffold with bFGF incorporated via collagen coating, we demonstrated controlled release of bFGF, and that bFGF released from the scaffold enhanced collagen type I, collagen type III, and tenascin-C gene expression.²¹

Although scaffold design, cell type, and growth factor combinations have been examined extensively for ACL tissue engineering, relatively few have been tested *in vivo*. And in many tissue engineering applications, the *in vitro* findings often do not translate to *in vivo* performance.^{22,23} Thus, in this study, we examined the efficacy of adding bFGF and human foreskin fibroblasts (HFFs) to our previously designed ACL graft to improve cellular proliferation and matrix synthesis.¹⁷ In this article, we will describe the comparison of electrospun PCL grafts, with and without growth factor and cells, using a modified version of a previously established rat model of ACL reconstruction.^{24,25} We hypothesized that scaffolds treated with bFGF and seeded with HFFs would have superior biologic and mechanical properties than scaffolds without bFGF and HFFs.

Materials and Methods

Graft design

Medical grade ester terminated poly (ϵ -caprolactone) in granule form (IV = 1.15 dL/g in chloroform, Mw = 140,000 g/mol; Lactel Absorbable Polymers, Birmingham, AL) was dissolved 10% w/w in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich, St. Louis, MO). The solution was electrospun around a lathe mandrel rotating at a speed of 3450 rpm, using a 20 kV voltage source and a constant infusion rate of 2.5 mL/h for a total of 0.5 mL per scaffold. Electrospun mats were laser cut using a VersaLaser Cutter 2.3 (Universal Laser Systems, Scottsdale, AZ) to dimensions of 1.5 mm $\times$ 35 mm $\times$ 150 μ m with 150 μ m diameter holes at 15% pore area. Holes were laser cut in the mats to increase porosity on a macroscopic level, to complement the porosity on the microscopic level, as this has been shown to promote vascularization and infiltration of cells vital to tissue regeneration.¹⁴ The scaffolds were then plasma etched (Harrick

Plasma PDC-001 plasma cleaner, Ithaca, NY) to induce hydrophilicity,²⁶ and bathed in 70% EtOH. Collagen coating was performed using a 8:1:2.5 sterile solution of Purecol (Advanced Biomatrix, San Diego, CA), 10 $\times$ phosphate-buffered saline (PBS), and 0.1 N NaOH diluted 1:9 in 1 $\times$ PBS at 4°C. For scaffolds in bFGF containing groups, bFGF (Sigma) was added to the collagen solution, vortexed, and then pipetted onto the electrospun mats, for a total quantity of 100 ng bFGF loaded per scaffold. After 24 h of drying, four scaffold layers were stacked and affixed to one another using 5-0 vicryl sutures. This resulted in a construct that was 0.6 mm thick (Fig. 1).

HFFs (p7; ATCC, Manassas, VA) were cultured in fully supplemented Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and 1% penicillin/streptomycin, in an incubator with 5% CO₂ under humidified conditions. To seed the scaffolds, cells were trypsinized, suspended in media, placed in 15 mL centrifuge tubes, and put in the Mini Labroller Rocker (Labnet International, Edison, NJ) in the incubator for 24 h before surgery to allow cell attachment. Cell concentration in the seeding suspension was set to allow a scaffold seeding density of 100,000 cells/cm². Cell seeding was confirmed using DAPI stain (Life Technologies, Camarillo, CA), and quantified using the Picogreen[®] assay (Life Technologies), following the manufacturer's suggested protocols.

Animal model and operative reconstruction

All animal procedures were approved by the Institutional Animal Use Committee. Forty-four 8–10-week-old male athymic rats (rnu; Charles River, Wilmington, MA) were

FIG. 1. Electrospun polycaprolactone scaffold. (A) SEM image of electrospun fibers. (B) Gross photographs of AP and lateral views of assembled 4-layer scaffold. The portions of the scaffold that will be cut off (C), in the bone tunnel (BT), and intra-articular (IA) once the scaffold is implanted are indicated. Color images available online at www.liebertpub.com/tea

used in this study. The rats were housed under the care of the Veterinary Medical Unit at our institution. Rats were housed two per cage preoperatively. Postoperatively, rats were housed individually, and they were not restricted in weight bearing or immobilized in any manner. Animals had *ad libitum* access to autoclaved food and water.

ACL reconstruction was performed using the electrospun polymer graft on the left hind limb, with 11 rats in each of the four graft groups: collagen-coated scaffolds (Col), collagen + bFGF-coated scaffolds (Col + bFGF), collagen-coated scaffolds seeded with cells (Col + HFF), and collagen-coated scaffolds with growth factor and cells (Col + HFF + bFGF). Of the 11 rats in each group, 3 were sacrificed for histology at 8 weeks, 3 were sacrificed for histology at 16 weeks, and 5 were sacrificed for biomechanical testing at week 16. At the time of sacrifice, the contralateral native ACLs were used as controls for 11 rats ($n=3$ for histology at weeks 8 and 16 and $n=5$ for mechanical testing at week 16). Additionally, the right knees of five rats sacrificed at week 16 were used for postmortem ACL reconstruction using the graft with immediate mechanical testing thereafter as an additional control, thus constituting the $t=0$ group without requiring the sacrifice of additional rats for this group. Thus, six groups were examined in total: (1) electrospun polymer grafts immediately postimplantation ($t=0$), (2) Col only grafts, (3) Col + bFGF grafts, (4) Col + HFF grafts, (5) Col + HFF + bFGF grafts, and (6) native ACLs.

An adaptation of a previously established rat ACL reconstruction model^{24,25} was performed. Anesthesia was induced by inhalation of 2% isoflurane with 2 L/min oxygen, delivered by inhalation chamber. Throughout the surgical procedure, the rats were maintained under anesthesia with 2% isoflurane in 2 L/min oxygen, delivered via flow-by with a nose cone. Rats were then injected with buprenorphine (0.03 mg/kg, Buprenex) and a single dose of ampicillin (25 mg/kg) subcutaneously. Fur was clipped from the surgical site, and the area was prepped with three alternating scrubs of chlorhexidine and 70% ethanol. The rats were then placed in a supine position, on a heated pad to prevent hypothermia. The left hind limb was placed in extension, and draped in a sterile manner. A longitudinal cutaneous incision was made along the medial aspect of the lower extremity, centered at the level of the knee. The fascia was incised, and the incision was retracted laterally to expose the anterior knee. The patella, along with the attached quadriceps and patellar tendons, was dislocated laterally, exposing the femoral condyle. The prepatellar fat pad was incised, and the cruciate ligaments were severed. The knee was flexed to ~ 60 degrees and a 1.6 mm Kirchner wire was used to drill a femoral tunnel from anteromedially to posterolaterally, through the femoral insertion and then a tibial tunnel was drilled anteromedially, through the tibial footprint. Both sides of the grafts were affixed to a 1.2 mm Keith needle used to pass the graft through the bone tunnels across the joint space, replacing the native ACL. With the graft under manual tension, 4-0 vicryl was used to suture the ends of the scaffold to the periosteum and soft tissue, and excess graft was trimmed from both ends. A layered closure was performed. Postoperatively, the rats were injected with 0.3 mg/kg buprenorphine every 12 h for 72 h. At the appropriate time postoperatively, rats were euthanized by carbon dioxide inhalation.

Histology/immunohistochemistry

For histological analysis, the knee of each animal was fixed in 4% paraformaldehyde at 25°C for 48 h. Fixed knee harvests were then decalcified with Immunocal reagent (Decal, Tallman, NY) and submitted to a core laboratory for sectioning and mounting. Representative sections were also stained with hematoxylin and eosin (H&E) and these slides were digitized by the core facility. Four intra-articular images at 10 $\times$ magnification were analyzed per animal, with an $n=3$ animals per group. These images were used for manual quantification of cells per high-powered field.

IHC. Colorimetric staining of expression of CD68 and type I collagen was performed on paraffin-embedded sections dewaxed with xylene washes and rehydrated. Ficin (Sigma-Aldrich) was applied for 20 min at room temperature for antigen retrieval. Sections were permeabilized in a 0.025% Triton X wash (Sigma) and were then blocked at 25°C for 1 h in a solution of 4% normal goat serum and 0.4% bovine serum albumin in tris-buffer saline/Tween20. Sections were incubated overnight at 4°C with primary antibody solutions of (1) 1:200 dilution of rabbit anti-rat type I collagen (Millipore, Billerica, MA), or (2) 1:300 dilution of rabbit anti-rat CD68 (Abcam, Cambridge, MA), followed by 30 min incubation at 25°C with biotinylated goat anti-rabbit IgG secondary antibody and conjugation of peroxidase substrate (VECTASTAIN Elite ABC Kit; Vector Laboratories, Burlingame, CA) for 30 min at 25°C. DAB peroxidase substrate (Vector Laboratories) was then added until desired stain intensity was visible under light microscopy.

For immunofluorescent staining for Ku80 expression, sections were dewaxed, permeabilized, and blocked as above. Sections were then incubated with a primary antibody solution of 1:250 dilution of rabbit anti-Ku80 (Abcam) for 1 h at 25°C, followed by incubation with a secondary antibody solution of 1:500 dilution of Alexa Fluor 488 goat anti-rabbit IgG (Abcam) for 30 min at 25°C. Sections were then mounted using ProLong Gold antifade reagent with DAPI (Life Technologies, Carlsbad, CA) before visualization and imaging using fluorescence microscopy.

Picrosirius red staining and quantification. Samples were sectioned and mounted by a core laboratory as described above. Paraffin-embedded slides were dewaxed with xylene, followed by rehydration. Slides were stained for fibrillar types I and III collagen using Picrosirius Red Stain Kit (Polysciences, Inc., Warrington, PA). Images were taken under polarized light with a DMLB Leica Microscope (McBain Systems, Chatsworth, CA). Image analysis was performed to quantify the area fraction of birefringence. Four intra-articular images at 20 $\times$ magnification were analyzed per animal, with an $n=3$ animals per group. The percent area of birefringent pixels was determined by converting images to black and white, where the threshold was set to convert all birefringent pixels to black and all non-birefringent pixels to white. The area fraction of black pixels was determined using ImageJ software (National Institutes of Health, Bethesda, MD) and reported as mean $\pm$ standard error per group.

Biomechanical testing

All soft tissue except the graft was removed from the harvested knees. The femur and tibia were secured with wires and potted in polymethylmethacrylate and tested with the knee in 20° flexion. The graft was kept moist during dissection and potting by regular and frequent spraying of normal saline. The femur-graft-tibia complex was mounted onto an Instron tensile tester (Model 5564; Instron, Norwood, MA) with a 1 kN load cell. The graft was pretensioned to 2N and then tested to failure at a strain rate of 0.5 mm/s. The data were used to generate load-displacement curves. The peak and slope of the linear portion of the curve were used to determine failure load and stiffness, respectively, for the six groups: (1) Col grafts immediately post-implantation ($t=0$), (2) Col grafts at 16 weeks postop, (3) Col+bFGF grafts at 16 weeks postop, (4) Col+HFF grafts at 16 weeks postop, (5) Col+HFF+bFGF grafts at 16 weeks postop, and (6) native ACLs, ($n=5$).

Statistical analysis

Mechanical testing values are reported as mean $\pm$ standard error of the mean. Two-way analysis of variance with Bonferroni correction was used to assess differences between groups. Differences between time points were verified using unpaired t -tests. Statistical significance was achieved if $p < 0.05$.

Results

Tolerance of surgical procedures

All rats tolerated the surgery well, and experienced no complications. Clinically, no gross wound dehiscence, erythema, swelling, hematoma, effusion, or drainage was observed postoperatively.

Histology

H&E staining demonstrated bony integration at both the femoral and tibial ends of the graft. The grafts were infil-

trated with abundant cells, and the neo-ligament appeared to be well aligned (Fig. 2). There was no remaining polymer observed, indicating that the scaffold had been fully resorbed by 16 weeks. The cell number was significantly higher in the Col+bFGF group than in the Col group at 16 weeks (Fig. 3A). Immunohistochemistry for Ku80, a marker of human cells, demonstrated no positive staining in the grafts by week 8 (Fig. 3B).

Immunohistochemistry for CD68 (or ED1), a glycoprotein specifically expressed on cells of the macrophage/monocyte lineage, demonstrated minimal staining across all samples, without grossly observable differences among the graft groups (data not shown). Qualitatively, it was noted that there was more staining in the bone tunnels than in the intra-articular region, and more positive staining at 8 weeks than at 16 weeks.

Collagen deposition

Immunohistochemistry for Type I Collagen demonstrated positive staining across all graft groups (Fig. 3A), without grossly observable differences among these groups. Qualitatively, it was noted that there was more intense positive staining in the graft than in the native ACL.

To further examine collagen deposition on the tissue-engineered grafts, picrosirius red staining was performed, and the percent area birefringence was calculated to quantify the amount of aligned collagen observed. Under polarized light, positive birefringent staining was observed in all graft groups, signifying the presence of aligned collagen fibers. Qualitatively, there was more staining in the native ACL than in the graft groups. Furthermore, under polarized light, colors of longer wavelengths (red and yellow) were observed more in the native ACL than in the grafts (Fig. 3B). Using image analysis, the percent area of intra-articular sections that were birefringent was quantified (Fig. 4). At week 16, it was found that $\sim 30\%$ of the implanted grafts were birefringent under polarized light, as compared to 91% in the native ACL (Fig. 5). There was no statistically significant difference among the four graft groups at week 16.

FIG. 2. Hematoxylin and eosin staining of electrospun Col+HFF graft (top), Col+bFGF graft (middle), and native ACL (bottom), at femoral insertion (left), mid-substance (center), and tibial insertion (right), 20 $\times$. Grafts were harvested at 16 weeks postoperatively. ACL, anterior cruciate ligament; bFGF, basic fibroblast growth factor; HFF, human foreskin fibroblast. Color images available online at www.liebertpub.com/tea

FIG. 3. (A) At 16 weeks postoperatively, Col+bFGF grafts had significantly more cells per high power field than Col grafts, $n=3$, $*p<0.05$. (B) At 8 weeks postoperatively, Col+HFF grafts demonstrated no positive immunostaining for Ku80 (*left*), a marker of human cells, compared to HFFs in monolayer (*right*). The cells were counterstained with DAPI, a nucleus stain. Color images available online at www.liebertpub.com/tea

Also, as a control, the birefringence of a collagen-coated graft stained with picrosirius red before implantation (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea) was quantified and found to be 0.7%.

Biomechanical analysis of PCL grafts

Biomechanical properties were assessed immediately after sacrifice. All tested samples failed at the mid-substance. Using load–displacement curves generated from tensile testing, failure load and stiffness were computed for each group (Table 1). At 16 weeks postimplantation, the elec-

trospun polymer graft groups (Col, Col+bFGF, Col+HFF, and Col+HFF+bFGF) had significantly increased peak load as compared with the immediate postoperative control (16.0 ± 3.4 , 23.1 ± 6.1 , 17 ± 6.9 , and 15.1 ± 4.9 N, respectively, vs. 7.7 ± 3.0 N) ($p<0.05$). Similarly, three out of the four graft groups (Col, Col+bFGF, and Col+bFGF+HFF) demonstrated significantly higher stiffness (12.4 ± 3.8 , 23.3 ± 8.1 , and 10.1 ± 2.1 N, respectively) compared with the immediate postoperative control (6.2 ± 3.9 N) ($p<0.05$). There were no statistically significant differences among the four graft groups at 16 weeks postoperatively in terms of mechanical properties. In comparison, the native ACL was found to have a peak load of 56.7 ± 1.6 N and a stiffness of

FIG. 4. (A) Colorimetric immunohistochemistry staining for Type I Collagen of Col+HFF graft (*top*), Col+bFGF graft (*middle*), and native ACL (*bottom*), $20\times$. Grafts were harvested at 16 weeks postoperatively. (B) Picrosirius red staining of Col+HFF graft (*top*), Col+bFGF graft (*middle*), and native ACL (*bottom*), as visualized under polarized light, $20\times$. Grafts were harvested at 16 weeks postoperatively. Color images available online at www.liebertpub.com/tea

TABLE 1. MECHANICAL TESTING RESULTS OF COLLAGEN-COATED, ELECTROSPUN POLYCAPROLACTONE GRAFTS AS COMPARED TO NATIVE RAT ANTERIOR CRUCIATE LIGAMENT

Group	Stiffness (N/mm)	Peak load (N)
Col graft, immediately postimplantation	6.2 ± 3.9	7.7 ± 3.0
Col graft, 16 weeks	12.4 ± 3.8 ^a	16.0 ± 3.4 ^a
Col + bFGF graft, 16 weeks	23.3 ± 8.1 ^a	23.1 ± 6.1 ^a
Col + HFF graft, 16 weeks	4.4 ± 1.2	17.0 ± 6.9 ^a
Col + HFF + bFGF graft, 16 weeks	10.1 ± 2.1 ^a	15.1 ± 4.9 ^a
Native ACL	39.6 ± 6.7 ^b	56.7 ± 1.6 ^b

^aSignificantly higher than Col graft immediately postimplantation.

^bSignificantly higher than all other groups, $n=5$, $p<0.05$.

ACL, anterior cruciate ligament; bFGF, basic fibroblast growth factor; Col, collagen coating; HFF, human foreskin fibroblast.

39.6 ± 6.7 N/mm, which was significantly higher than all other groups. By 16 weeks postoperatively, Col grafts achieved 31.3% of the stiffness and 28.2% of the peak load of healthy native ACL while Col + bFGF grafts achieved 58.8% of the stiffness and 40.7% of the peak load of healthy native ACL.

Discussion

In this study, we compared the *in vivo* efficacy of electrospun PCL grafts with and without the growth factor bFGF and HFFs in an athymic rat model. The rat model used in this study is a modification of a previously established model for autologous ACL reconstruction with flexor digitorum longus (FDL) tendon.^{24,25} Although many animal models exist for ligament tissue engineering, the rat presents a number of benefits compared to larger models. These advantages include fewer ethical considerations, greater sample size, easier husbandry and handling, and reduced

costs.^{27,28} In addition, the rat model has been used as a model for orthopedic tissue regeneration, including cartilage, tendon, and bone tissue engineering.^{29–31} Athymic nude rats were chosen for this study due to their impaired cell-mediated immune response,^{32,33} allowing for the implantation of xenogeneic human cells. Overall, our athymic rat model was employed successfully in our study, providing a reproducible method of evaluating tissue-engineered grafts for ACL reconstruction, without any clinical complications.

The electrospun polymer scaffold facilitated both cell and matrix alignment in the regenerated ACL. These grafts resulted in successful bony integration with increased strength over time; load to failure increased three-fold as compared with the reconstructed ACL immediately postoperatively. While none of the grafts were able to fully recapitulate the strength of the native ACL, it is important to recall that by 4 months postoperatively, even the gold standard autograft or allograft does not have the full strength of native ACL. In an ovine study, it was found that at 52 weeks, the autografts had 38 and 67% the peak load and stiffness, respectively, of control ligaments.³⁴ A goat study demonstrated that at 3 years postoperatively, a patellar tendon autograft had 44% of the strength and 49% of the stiffness of native ACL.³⁵ Our Col + bFGF grafts, achieved 58.8% of the stiffness and 40.7% of the peak load of a healthy native ACL at only 16 weeks, is very promising in light of this. In comparison to other tissue engineering attempts, cell-seeded collagen constructs implanted in a rabbit completely resorbed by 8 weeks, preventing mechanical testing,³⁶ a decellularized semitendinosus tendon graft that was repopulated with fibroblasts demonstrated 32.2% of the peak load of native ACL at 8 weeks postoperatively,³⁷ and a silk-based scaffold seeded with MSCs resulted in a peak load that was 18.7% that of native ACL at 24 weeks postoperatively.²⁸ Using a stacked bone and ligament monolayer construct, one group demonstrated 52% of the tangent modulus and 95% of the geometric stiffness of native ACL in an ovine model at 6 months.²⁵ Specific to rat models of ACL reconstruction, we reported a peak load of 13.3 N and stiffness of 16.0 N/mm

FIG. 5. Picrosirius red staining of neoligament for collagen. Brightfield (A) and polarized light (B) images of stained Col + bFGF graft at 16 weeks. (C). Quantification of percent area birefringence, signifying aligned collagen fibers. Col ($t=0$), collagen-coated scaffold before implantation; Col, collagen-coated scaffolds. Other than Col ($t=0$), all graft groups are at 16 weeks postimplantation. *Significantly lower than all other groups, $n=5$, $p<0.05$. **Significantly higher than all other groups, $p<0.05$. Color images available online at www.liebertpub.com/tea

for the Col grafts 12 weeks after implantation in Sprague-Dawley rats.³⁸ Packer *et al.* reported a peak load of ~ 7 N and a stiffness of ~ 3.5 N/mm 2 weeks after undergoing ACL reconstruction with FDL autograft.³⁹ Fu *et al.* found that Sprague-Dawley rats reconstructed with FDL autograft had ~ 25 – 30% the stiffness and peak load of native ACL at 6 weeks.⁴⁰

While our scaffold did facilitate the formation of aligned collagen fibers as evident from the positive birefringent staining of the grafts with picrosirius red, the quality of the elaborated collagen was histologically inferior to that of native ACL. When viewed under polarized light, smaller and less well-aligned collagen fibers stained with picrosirius red appear yellowish-green (shorter wavelength colors), as compared to larger, more tightly packed, and better aligned collagen fibers, which appear red and yellow (longer wavelength colors).⁴¹ Additionally, the immunohistochemistry for type I collagen stained darker in the graft than in native ACL; this can be explained by the fact that less aligned collagen has more epitopes exposed to the reagent, resulting in higher stain intensity.^{42,43} The difference in collagen quality, and quantity, likely contributes to the differences in mechanical properties that were observed between the grafts and native ACL.

While many polymers have the potential for use in ligament reconstruction, this study investigated PCL because it is biologically inert, nontoxic, degrades slowly *in vivo*, and is easily manufactured into a desired conformation.⁷ PCL is also mechanically robust and shows little plastic deformation under mechanical stress.⁴⁴ Its use has been established in the bone tissue engineering literature as a reliable reservoir for mineralization and type I collagen deposition due to its aligned nanofiber structure when electrospun.⁴⁵ Also, it has been shown that the high surface to volume and short diffusion length scale of the small diameter fibers in electrospun PCL mats are favorable for controlled drug delivery and use in tissue engineering.⁴⁶ Previously, the degradation rate of our electrospun mats was characterized *in vitro*, and it was found that when placed in isotonic solution at 37°C , 7% of the original mass was lost by 42 days.⁴⁷ However, the *in vivo* degradation rate is likely faster due to the presence of cells and enzymes that can help accelerate the degradation process. For this study, our time points were selected based on previous work in which acellular electrospun PCL grafts were implanted into rat knees and assessed at 6 and 12 weeks postoperatively. In that study, it was found that the regenerated ligament progressed between 6 and 12 weeks in terms of mechanical strength, but that longer time points were required to allow for the implanted graft to further mature and remodel.¹⁷ Thus, for this study, the 8 and 16 week time points were chosen to allow sufficient polymer degradation and graft remodeling.

It was found that the implanted grafts were biocompatible based on a lack of clinical or histological adverse reaction and facilitated native cell infiltration as seen in the graft at 16 weeks postoperatively. We observed minimal macrophage infiltration. While athymic rats are known to have a muted cell-mediated immune response due to an aplastic thymus and thus depleted T cells, they do have B cells and can produce antibodies and thus we would have expected some immune response^{32,48} if the grafts were not biocompatible. Thus, it is still relevant that we observed minimal

immune response in the rats. Furthermore, in earlier studies, we implanted acellular scaffolds in immune competent rats and observed that there was a small transient inflammatory response by 6 weeks that was resolved by 12 weeks, indicating that our polymer scaffold is unlikely to cause a chronic inflammatory state.¹⁷

We elected to investigate the use of HFFs, a readily available FDA-approved cell source, that have been shown to have similar proliferative capacity as compared to adult bone marrow stem cells.⁴⁹ In addition, HFFs have been shown to be sensitive to various stimuli including substrate.^{50–52} It has been shown that HFFs can respond to aligned fibers, and to growth factor gradients by increasing their collagen deposition.^{52–54} Another important consideration for choosing HFF is that FDA-approved cell banks already meet stringent regulatory standards and HFFs have already been extensively tested for viruses, retroviruses, tumorigenicity, endotoxins, and mycoplasma, making them suitable for clinical reconstructive procedures.⁷ In addition, HFFs were found to exert immune-modulatory properties comparable to those seen in BMSCs.⁵⁵ This is an important characteristic as the unabated inflammatory response to cell-scaffold constructs may promote disorganized scar and diminish the synthesis of functional collagen. In this study, minimal immune response was observed in our athymic rats. However, it did not appear that the implantation of HFFs on the scaffolds was beneficial in ligament regeneration. One possibility is that the HFFs had no beneficial effect because they did not persist *in vivo*; by week 8, we did not observe any HFFs in our scaffold. Given that HFFs were observed on the scaffold before implantation (Supplementary Fig. S2), but not by week 8, it can be hypothesized that the cells somehow did not survive postimplantation. This may have been due to apoptosis or detection by the host immune system.

As we saw abundant scaffold infiltration with cells in all groups, including those implanted with acellular scaffolds, it can be concluded that host cells populated the scaffold and were able to elaborate extracellular collagen. Furthermore, it is possible that the early presence of HFFs impaired scaffold infiltration by native cells. In the literature, it has been reported that the addition of adipose-derived stem cells to decellularized tendon grafts did not make a difference in terms of collagen production or mechanical properties.⁵⁶ Additionally, it has been shown that cells in ACL allografts do not persist for more than 4 weeks *in vivo*,⁵⁷ and that human cells implanted into immunodeficient mice were no longer present by 1 week.⁵⁸ Furthermore, cells can be removed from allografts to decrease antigenicity.^{37,59} Given our findings, future investigation will focus on further refining our scaffold without the use of cells, and movement to an immunocompetent animal model.

Growth factors have been shown to affect ECM synthesis in tissue-engineered grafts.¹⁹ bFGF has been shown to improve mechanical properties and increase collagen synthesis in an acellular poly-L-lactic scaffold placed in rabbits.⁶⁰ Also, increases in cellular proliferation and expression of ligament markers have been observed with bFGF.⁶¹ Treatment of fibroblasts with bFGF resulted in increases of matrix proteins such as fibronectin, GAG, and laminin.^{62,63} Specific to HFFs, bFGF has also been shown to stimulate their proliferation.⁵² Our group also has demonstrated that

the addition of bFGF to monolayer cultures of HFFs resulted in a qualitative increase in collagen as observed via picrosirius red staining (unpublished data). Additionally, prior *in vitro* data from our laboratory have demonstrated a sustained release from our collagen-coated PCL scaffolds, with 56% of loaded protein eluted over a period of 3 weeks.⁶⁴ The dose of 100 ng bFGF/scaffold was also selected based on data from previous work.²¹ In our study, addition of bFGF did not result in a statistically significant increase in collagen production or mechanical strength. However, the trend observed was that the peak load and stiffness of the grafts was higher in the col+bFGF grafts than in the col control grafts. Additionally, the observation that the Col+bFGF grafts had significantly more cellularity than the Col grafts at 16 weeks supports the idea that bFGF has a beneficial effect on the graft. Perhaps a higher dose of bFGF, also delivered over a sustained period of many weeks to months, may result in a more pronounced effect of growth factor incorporation into the scaffold. For a rat wound healing model, doses as high as 3 μ g/scaffold have been reported previously.⁶⁵ We are currently exploring new methods of sustaining more prolonged delivery of higher quantities of bFGF via the use of microaggregates.⁶⁶ Additionally, the use of other fibroblast-promoting growth factors, such as EGF, GDF, TGF- β , or PDGF, or combinations of growth factors may demonstrate a more pronounced effect when combined with our scaffold.

Limitations of this study include the choice of the animal model itself. The rat may have such a robust healing response that clinically significant differences were not observed among treatment groups that may be observed in a larger animal model. Also, the inherent differences in anatomy and gait in the quadrupedal rat compared to bipedal humans mean that the biomechanics of the ACL do vary and that translation of clinical parameters between models should be done with knowledge of these limitations. However, this issue is common in animal studies and does not negate the importance or translational potential of this research.

Furthermore, it may be considered a limitation that sham surgery was not performed on the contralateral side to control for the potential healing effects of performing the arthrotomy and drilling the bone tunnels in the process of ACL reconstruction. However, there would be animal use concerns regarding bilateral hind limb surgery on the rats, as this may cause significantly impaired mobility compared with unilateral surgery in the postoperative period. Thus, we felt that the most prudent use of the contralateral limb was for evaluation of the native ACLs as a positive control, and for the postmortem implantation of scaffolds to provide baseline mechanical testing data for our scaffold. However, in future iterations of our study, the use of animals solely for sham surgery may be considered.

Conclusions

In summary, this study evaluated an electrospun PCL graft with and without bFGF and/or HFFs in a reproducible rodent model of ACL reconstruction. The elaboration of an aligned collagen matrix with minimal inflammatory response was demonstrated, along with mechanical properties that improved from the time of implantation. Acellular

grafts loaded with bFGF achieved 58.8% of the stiffness and 40.7% of the peak load of healthy native ACL. While there were no statistically significant differences found with the addition of bFGF or HFFs, trends observed may indicate that the addition of HFFs had a detrimental effect on aligned collagen production and mechanical properties and that bFGF may have a beneficial effect on mechanical properties.

Future investigation is warranted to optimize the dose or delivery mechanism of bFGF and to study the use of a PCL scaffold fabricated with higher molecular weight polymer to achieve initial graft mechanical properties closer to that of native ACL.

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Disclosure Statement

No competing financial interests exist.

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