Bridge-Enhanced ACL Repair: A Review of the Science and the Pathway Through FDA Investigational Device Approval

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Abstract—Injuries to the anterior cruciate ligament (ACL) are currently treated with replacement of the torn ligament with a graft of tendon harvested from elsewhere in the knee. This procedure, called "ACL reconstruction," is excellent for restoring gross stability to the knee; however, there are relatively high graft failure rates in adolescent patients (Barber *et al.* in Arthroscopy 30(4):483–491, [2014]; Engelman *et al.* in Am J Sports Med, [2014]; Webster *et al.* in Am J Sports Med 42(3):641–647, [2014]), and the ACL reconstruction procedure does not prevent the premature osteoarthritis seen in patients after an ACL injury (Ajuied *et al.* in Am J Sports Med, [2013]; Song *et al.* in J Sports Med 41(10):2340–2346, [2013]; Tourville *et al.* Am J Sports Med 41(4):769–778, [2013]). Thus, new solutions are needed for ACL injuries. Researchers have been investigating the use of scaffolds, growth factors and cells to supplement a suture repair of the ACL (bridge-enhanced repair; also called bio-enhanced repair in prior publications). In this paper, we will review the varied approaches which have been investigated for stimulating ACL healing and repair in preclinical models and how one of these technologies was able to move from promising preclinical results to FDA acceptance of an investigational device exemption application for a first-in-human study.

Keywords—Bench-to-bedside, FDA, IDE, Anterior cruciate ligament, Bridge-enhanced ACL repair.

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A REVIEW OF THE SCIENCE BEHIND BRIDGE-ENHANCED ACL REPAIR

In Vitro and In Vivo Studies of Bridge-enhanced ACL Repair

Augmentation of soft tissue repair can be approached in several different ways. Cells, growth factors and scaffolds can be used individually or in combination to provide a protected space for ACL healing. In this section, we will review the efforts to enhance ACL repair in each of these areas.

Cell Seeding

The reasons behind the failure of the anterior cruciate ligament to heal are not completely known. One hypothesis is that there is a dearth of viable cells in the ACL that are able to participate in the wound repair process. To overcome that potential problem, researchers have worked to add exogenous cells to a scaffold in the ACL wound site in an effort to stimulate ACL healing. By delivering cells that can participate in ligament healing, such as fibroblasts, the ACL may heal better and faster. *In vitro* studies have provided evidence that such an approach might be useful, particularly when the cells are placed on a collagen scaffold. Huang *et al.* showed that fibroblasts strengthen collagen scaffolds *in vitro*.[24] Dunn *et al.* found that fibroblasts synthesized tenfold more collagen when cultured on collagen scaffolds instead of culture plates.[11] *In vitro* studies have also shown promise. Bellincampi *et al.* implanted fibroblast-seeded collagen scaffolds in rabbit knees. The seeded fibroblasts remained attached to the scaffold and were viable 6 weeks after the implantation.[5]

Bone marrow stromal cells (BMSCs) have also been studied in trials. Van Eijk et al. compared BMSCs and fibroblasts cultured on resorbable suture material. After 12 days, BMSCs had expanded to a greater extent than the fibroblasts and had a higher degree of collagen gene expression.[56] Applying mechanical forces has also been found to induce the production of collagen and ECM proteins by the BMSCs, as well as realign the collagen fibers.[2] Fan et al. used silk scaffolds seeded with BMSCs as a graft to replace the torn ACL in a rabbit model. Compared to the controls with no BMSCs, the ACLs with BMSCs healed better as measured by biomechanical studies and histology.[15] When they used this same technique in the porcine model, abundant cells and ECM were seen at 24 weeks post-operatively.[14] Soon et al. reconstructed rabbit ACLs using Achilles tendon allografts with and without BMSCs. Grafts with BMSCs had significantly higher load-to-failure rates and better healing seen on histology.[47]

While cell seeding of scaffolds prior to implantation seems a logical way to get cells into the volume of tissue trying to be engineered, this technique does have several downsides. The first downside is the fact that most cell-seeding techniques are designed to occur in stages—first, a procedure to harvest the desired cells, then a period of time where the cells are expanded in culture to get greater numbers of cells and then a second procedure to implant the cell seeded scaffolds. While two procedures might be tolerated by the patient, this staged technique also confers additional regulatory hurdles—the company growing the cells outside the body needs to prove it can do so safely and effectively, and that the cells will not become contaminated with bacteria, virus or harmful chemicals during the in vitro expansion. The company also has to prove it has systems in place to make sure one patient's cells are never contaminated by another patient's cells and that the correct cells are delivered to the correct patient for the second procedure. In addition to the extra regulatory and patient burden, once the cells are implanted, they die at an accelerated rate, typically with only a few percentage of the implanted cells found to survive more than a few weeks in the body.[41,44,55] This may be due to a relatively rapid change in environment for these cells, from the carefully monitored and controlled in vitro environment to an in vivo environment which may have significantly less nutrition or oxygen.

Growth Factors

In vitro studies with ligament fibroblasts have shown that epidermal growth factor (EGF),[9,10,43] fibroblast growth factor (FGF),[28,29] insulin-like growth factor,[43] platelet-derived growth factor (PDGF),[10,32,43] and transforming growth factor (TGF)[9,32] improve collagen synthesis and/or cell proliferation. In vivo, Kobayashi et al. reported that FGF improves healing and neovascularization of partially lacerated ACL in canines.[26] Spindler et al. found increased collagen expression and maximum load when recombinant human TGF was added, but they used MCL in rabbits, not an ACL model.[48] Letson et al. showed that rat ligaments increased their stiffness when PDGF was added, but this study was also done using an MCL model.[28] Several authors have found that combinations of growth factors have synergistic effects on cellular proliferation and matrix production in vitro.[10,23,28,31] This would make it seem logical that adding these types of factors to a scaffold would have the same effect in vivo. However, often when growth factors which have been found to have a significant effect in vitro are tested in vivo, functional benefit is not noted.[48,49] This may be due to problems with delivery or with the interaction of the implanted growth factors with the complex in vivo environment. In vivo, endogenous growth factors are typically secreted over time by host cells in response to various cues [17,38]and in association with a host of other factors.[18,61] In tissue engineering, growth factors are often administered as a one-time dose,[25,30] sometimes in a carrier [22] or transduced cell [53] to result in longer secretion of the growth factor. However, even with these modifications, many exogenous growth factors are cleared relatively rapidly, within hours, from the joint or tissue in vivo.[8,13].

Platelet-rich plasma (PRP) has also been investigated as a source of growth factors to stimulate healing of the ACL and ACL grafts. The results of clinical trials using PRP are quite variable. Nin et al. conducted a randomized control trial comparing the outcomes of patients undergoing an ACL reconstruction with an allograft supplemented with PRP and those undergoing allograft reconstruction alone. No difference was seen in inflammatory parameters, appearance on MRI, or clinical outcomes between groups with and without PRP.[37] Vogrin et al. found no difference on MRI for vascularization of the intra-articular part of the graft when PRP was added to a hamstring autograft. However, they did see an increase in early vascularization of graft at the interface of the graft and bone.[59] In contrast, Orrego et al. reported that patients who had received PRP supplementation of their graft were significantly more likely to have low-intensity signal on MRI, which is a sign of graft maturation.[39] Silva et al. found that PRP did not affect healing at the bone-graft interface.[45] In that prospective study, 40 patients had their ACL reconstructed with hamstring tendon autograft with or without injection of PRP in the graft tunnel or in the joint. No difference in MRI

signal intensity was found 3 months after surgery.[45] A recent systematic review of PRP use for ACL surgery suggested there may be some benefit on graft maturation but limited effect on graft-bone interface healing.[58] However, the major conclusion of that review was that additional studies are needed to determine the true effect of PRP on ACL healing.

One of the reasons for the mixed results of PRP may be premature dissolution of PRP and fibrin based products in the synovial joint. Harrold *et al.* have demonstrated that fibrinolysis is relatively robust in the intra-articular environment, preventing blood clotting within the joint.[19] This active fibrinolysis system may also affect the stability of PRP, which, like blood, has its protein base as fibrin. Thus, the use of PRP in the joint may be somewhat limited by its relatively fast breakdown in the intra-articular environment, particularly by intra-articular plasmin. However, Kroon *et al.* noted that when fibrin is combined with collagen, a copolymer forms which is resistant to degradation by plasmin.[27] Thus, one could consider combining fibrin-based PRP with a collagen scaffold—combining the desirable biologic properties of PRP with the stability of the collagen and fibrin-collagen copolymer.

Recent reports of this combination of collagen scaffolds and platelets, called ''bridge-enhanced ACL repair'' have detailed beneficial effects on ACL healing in large animal models of ACL grafts and healing ACLs. Fleming *et al.* showed that supplementation of an ACL graft with a collagen-platelet composite improves the biomechanical properties of ACL allografts in pigs.[16] Murray *et al.* showed that supplementation of a primary repair of ACL in pigs with collagen-PRP hydrogel improves the biomechanical properties of the healing ACL after 4 weeks *in vivo*.[35] The PRP-collagen hydrogel also increased healing of a central wound defect in ACL of canines.[36] Finally, while ACL is known not to heal after primary repair with suture, primary repair with collagen-platelet composite bridge healed just as well as ACL reconstruction, the current standard of treatment, when measured by the results of biochemical testing in a large animal model at 3, 6, and 12 months after surgery.[34,57] In addition, the use of a bridge-enhanced repair using a collagen-platelet composite resulted in significantly less osteoarthritis of the knee at 1 year after surgery in the porcine model (Fig. 1).[34]

The chondroprotective function of the bridge-enhanced ACL repair in the pig model is not yet understood. One potential mechanism could be the anti-inflammatory effect of the blood cells which are added to the scaffold. Prior studies have demonstrated that the cytokines and growth factors released from platelets increase chondrocyte proliferation and matrix production,[50] and inhibit the pro-inflammatoric cyto-kine IL-1ß,[29,54] and thus the platelets in the injected blood may have had a chondroprotective effect. A second hypothesis is that the repair of the ligament may preserve some of its proprioceptive function. A bridge-enhanced repair preserves the torn ends of the ACL (which contain proprioceptive nerve fibers). With healing of the ligament, it is possible that small stresses on the ligament tissue may trigger those proprioceptive fibers to activate the hamstring musculature and dynamically stabilize the knee. It may be that these autonomic microcorrections help restore normal dynamics and kinematics of the knee, which ACL reconstruction does not.[3,6,42,51] Lastly, the addition of exogenous collagen may simply provide a substrate for the collagenase enzymes released in the joint after injury,[20] thus preventing these degradative enzymes from acting on the articular cartilage. Further studies are required to determine whether any of these hypotheses are plausible or if another mechanism is responsible for the chondroprotective effect of the bridge-enhanced repair.

The porcine model has several limitations. The most obvious one is that the pig is a quadruped, and thus the porcine gait is dissimilar to human gait. In addition, post-surgical rehabilitation in the porcine model is relatively uncontrolled when compared with post-surgical rehabilitation for the majority of human patients. However, there are also some strengths of the porcine model. The porcine haematologic profile and wound healing mechanism are very similar to that in humans compared to other animals [33,40] and anatomical and biomechanical features are comparable to humans as well.[7,40,62]

In summary, cells, growth factors and scaffolds can all be used to stimulate ligament repair. The ACL, which lives in the intra-articular environment of the knee, fails to heal after injury because of premature dissolution of the provisional scaffold that naturally forms from blood in the wound site of other tissues, like the medial collateral ligament. A substitute provisional scaffold, consisting of a collagen device and added autologous blood, can be surgically implanted in the wound site of the ACL to stimulate ACL healing. This technique of bridge-enhanced ACL repair may someday provide a valuable therapeutic option for patients with an ACL tear. The combination of a stable provisional scaffold and whole blood that provides proteins and growth factors can be a useful and translatable tissue engineering solution for ACL repair, and potentially the repair of other soft tissues. However, even with its strengths, a technology that has good results in an animal model may have unanticipated problems when evaluated in human patients. To minimize the risk to human patients, additional work assessing the potential safety, sterility

(ACLT)   (ACLR)   (BE-Repair)   (BE-ACLR)
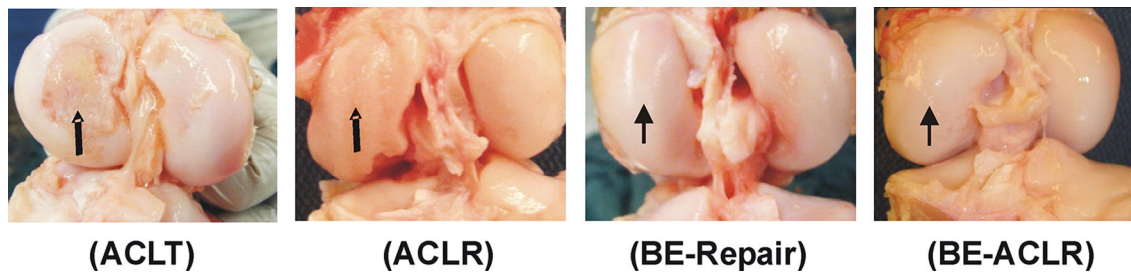
FIGURE 1. The distal femur for four porcine knees at 1 year after ACL transection and either no further treatment (ACLT), ACL reconstruction with a bone-patellar tendon-bone allograft (ACLR), bridge-enhanced repair using a scaffold loaded with autologous blood (BE-Repair) and an ACL reconstruction enhanced with a scaffold loaded with autologous blood (BE-ACLR). The medial femoral condyle in the ACLT and ACLR groups have breakdown of the articular surface consistent with post-traumatic osteoar-thritis (black arrows). These changes are seen in the same location as seen in human patients after ACL injury, even with reconstruction. In the bridge-enhanced groups, less damage is noted, suggesting a potential beneficial effect of adding a scaffold loaded with autologous blood to an ACL repair or reconstruction (adapted from Murray and Fleming, AJSM 2013).

and biocompatibility of the technology must be done prior to translating a technology to the bedside. The steps in that translation are detailed in the next section.

THE PATHWAY THROUGH FDA INVESTIGATIONAL DEVICE APPROVAL

Bridge-enhanced repair of the ACL using collagen-platelet scaffolds has demonstrated promising biome-chanical properties and a significant reduction in post-traumatic osteoarthritis in the large animal models. One next logical step, knowing these results, is to progress to a first in human trial for bridge-enhanced ACL repair. However, clinical use of a new technol-ogy rightfully requires additional validations of the scaffold prior to human use, including demonstrations of safety, consistency, sterility, and biocompatibility of the manufactured product. The Food and Drug Administration (FDA) provides excellent guidelines for many of these processes; however, in the past, the majority of translations of these types of technologies have been performed in industry and behind closed doors. Furthermore, each technology is different and not all guidelines can be applied in the same way to each device. As a NIH-funded academic laboratory, we recently had the opportunity to go through the processes required by the FDA to obtain an investi-gational device exemption (IDE) in preparation for a first in human trial (Flow chart see Fig. 2). In the next section, we will review our recent experience moving from bench to beside in an academic laboratory for bridge-enhanced ACL repair. We hope that our experience will begin to clarify some of these issues for other academic laboratories looking to translate tissue engineering technologies to a first in human study.

Device or Biologic?

One of the first major forks in the road to human trial is to determine if the technology is a drug, device or a combination of both. For devices, the pathway toward an IDE is taken in concert with the Center for Devices and Radiologic Health (CDRH) of the FDA. This route consists primarily of demonstrating safety, consistency, sterility and biocompatibility of the manufactured product. However, for products which also incorporate growth factors or cells, the pathway is often guided by both CDRH and the Center for Bi-ologics Evaluation and Research (CBER) as well as the Office of Combination Products (OCP). For scaffolds incorporating biologic elements, additional concerns must be addressed, including methods for minimizing potential contamination during manufacturing (par-ticularly for products that cannot be terminally steril-ized), proof that implanted cells will remain in place, and reasonable data to support the assurance that growth factors will be delivered in such a way as to only affect the target cells. Thus, determining the pri-mary review center for a new technology has a signif-icant impact on the regulatory pathway to clinical trial. For the academic investigator, early interaction with the FDA can be particularly helpful. Obtaining clarity from CDRH, CBER or OCP as to the designation of the technology as a device, biologic or combination product can expedite the processes in proceeding through an IDE or Investigational New Drug (IND) application. A request for designation can be submit-ted to the OCP when the investigator is not clear on the correct designation to obtain guidance from the FDA for the specific technology.

Once it is determined which application (IDE or IND) and which Center(s) of the FDA need to be involved (CDRH, or CBER), the next step is to pre-pare a pre-submission application (formerly known as a pre-IDE) and request pre-submission feedback from

Device or Biologic?	•**Early contact with CDRH, CBER, OCP key (Request for designation)** •**Device (IDE** Application) --> contact Center for Devices and Radiologic Health (CDRH) (demonstrate safety, consistency, sterility and biocompatibility) •**Biologic or Combo (IND** Application) --> contact CDRH and Center for Biologics Evaluation and Research(CBER)/Office of Combination Products (OCP)
Pre-Submission Application	•Investigator prepares outline of experiments/information to be included in eventual IDE/IND application based on FDA guidance documents •Submit pre-Submission document to FDA. FDA will set date for pre-submission meeting and review pre-submission application prior to the meeting. •Guidance documents at http://www.fda.gov/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm080202.htm
Pre-Submission Meeting	•Feedback as email, teleconference or in-person presentation with FDA review team •Modifications/additions/deletions to the application (manufacturing, sterility, biocompatibility) are discussed •**Valuable for establishing connection with FDA for future questions**
Creation of Design History File	•Product Specification (Product Title, Performance Characteristics, Material Requirements, Test Methods, Certifications, Quality Controls, Product Rejection Criteria, Responsible Personnel) •Hazard Analysis, failure mode, effect analysis (FMEA) •Design verification, validation activities (Release Test Descriptions) •Labeling •Design review meeting minutes
Manufacture where?	•Internal Manufacturing --> Check if Design Control Requirements can be met. Need regulatory consultant and internal team. OR •Contract Manufacturer --> Identify GLP/GMP facility with experience in FDA approval for similar devices.
Manufacture	•Follow GLP regulations (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRsearch.cfm?CFRPart=58) •Set up Process Specifications Docs (details of each manufacturing step, Figure 4) •Set up Design Control Docs (detailing the process of design and validation) •Set up Quality Control Documents (Material Safety Data Sheets, Calibration Records, Training Documents, Certificates of Analysis, Production Logs (Figure 5), and Production Routers (Figure 6)) •Traceability from final product back to raw material source must be possible.
Packaging	•Contact medical device packaging companies/consultant for expertise in packaging •Packaging has to pass burst/bubble test •High quality materials and sealing equipment may prevent expensive packaging failures
Sterility	•Evaluate Sterilization method •Method should not interfere with the release criteria of the product •Bacteria: lot testing or sterility validation both options for the early stage product 　•Lot testing requires the greater of 20% of the lot samples or 8 samples (whichever number is greater) per lot be tested 　•For batches between 100 and 500 scaffolds, 20 scaffolds have to be tested for each lot 　•Sterilization validation requires more extensive testing but once it is completed, less loss from each lot for this testing •Viral contamination 　•Lot testing and literature review may be possible; may also require viral inactivation studies. 　•Need to check with the FDA for your product.
Other testing	•After establish DESIGN FREEZE (final manufacturing, sterilization, packaging protocols), need biocompatibility and stability studies •Cytotoxicity, Maximization Sensitization, Intracutaneous Reactivity, Systemic Toxicity, Genotoxicity (Bacterial Reverse Mutation Study), Genotoxicity (in vitro Chromosomal Aberration in Mammalian Cells), Genotoxicity (Mouse Peripheral Blood Micronucleus), Muscle Implantation Study, 6 week Systemic Toxicity Study in Rats Following Subcutaneous Implantation. Particularly for the intracutaneous and implantation studies, it may be helpful to include an FDA-approved material as a control. •Stability studies to establish shelf life for final product.

FIGURE 2. Flow chart listing steps to successfully get through an IDE application for a medical device at the FDA.

the FDA. A pre-submission is a document prepared by the investigative team, which outlines the experiments to be performed and information to be included in the actual IDE application. The pre-submission is sent to the FDA, assigned an identification number and then sent to a lead reviewer and review team. The review team reviews the pre-submission and may have questions for the investigative team which can initially be addressed by email or phone in order to modify the pre-submission. Feedback from the FDA can be requested through email, teleconference, or through an in-person pre-submission conference. Through the pre-submission process, the investigative team has the opportunity to present their technology and proposed testing and receive feedback from the FDA review team as to whether the proposed work to be done to

document manufacturing, sterility and biocompatibility appear acceptable, or what modifications or additions may be required. This feedback serves as a valuable starting point for discussions with the FDA and development and implementation of a creditable IDE application.

A guidance document for the preparation of a pre-submission is available on the FDA website: (http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationand Guidance/GuidanceDocuments/UCM311176.pdf)

Likewise, a guidance document for the preparation of an IDE document is also available on the FDA website: (http://www.fda.gov/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm080202.htm).

In our experience, the key data required for our IDE application fell into three major categories: consistency of the manufactured product, sterility and biocompatibility. This may vary depending on the intended use of the device, technology of the device, and/or the thoroughness of the existing preclinical testing provided in the pre-submission or the rest of the IDE (for example, bench or animal functional testing). The ramifications are that an equivalent focus may be required by the investigator in these other content areas (or others beyond these examples).

Control of the Manufactured Product

The control and consistency of the manufactured product is supported and demonstrated by the generation of a design history file which is kept as a controlled document at the laboratory with sections sent to the FDA as part of the IDE application. The documents which make up a design history file include the product specification, risk analysis methods such as hazard analysis, failure modes and effects analysis (FMEAs), design verification and validation activities, labeling, design review meeting minutes and the results of testing performed such as biocompatibility and sterility. The product specification is a document that details the explicit set of requirements for a material, design, product, or service. This document typically contains details such as a descriptive title of the product, the performance characteristics or material requirements of the device, the test methods used to measure any of the specific characteristics, the required certifications, quality control requirements, provisions for rejecting a specific product or batch of product, and the person(s) responsible for questions on the specification.

The risk analysis is a structured tool for the evaluation of potential problems which could be encountered when using a medical device and is based on ISO 14971 Annex E. FMEA is often used as a tool as part of the hazard analysis. FMEA requires generating a list of possible defects in the product, assesses the effect of such a potential defect, and identifies potential solutions. It should be conducted at the beginning of the design effort and as part of each design review to identify potential design weaknesses. Such analysis are performed to allow for the early identification of potential design inadequacies that may adversely affect the safety and performance of the medical product. These potential problems can then be eliminated or their effect minimized through design correction or other means. FMEA documents can include risks associated with the design (DFMEA), processing (PFMEA) and end-use application (AFMEA) of the product. A sample PFMEA for a tissue engineering scaffold is found in Fig. 3.

The design verification and validation protocols and reports include a series of documents that define the testing which will be performed on the product before its release into clinical use. These protocols can define the specific testing and number of products to be tested prior to clinical use.

In addition to this specific quality documentation for the IDE, the process requires that the Laboratory meet certain parts of the FDA Quality System Requirements (21 CFR Parts 820), and more specifically 820.30, Design Control. Some of the requirements of this regulation are challenging to meet in an academic laboratory—the regulatory burden is significant in terms of time and cost. Therefore, an academic laboratory moving to first in human trials has at least two paths forward: (1) identify a independent contract manufacturing company that can provide GMP/GLP facility and is willing to work with the laboratory to develop the required procedures or (2) proceed with manufacturing internally ensuring that adequate laboratory controls are in place and that Design Control requirements are fully met. Use of a contract manufacturer can be valuable. Typically, these organizations have experience with preparing the documentation for the IDE application as well as GMP capabilities. However, use of a contract manufacturer may not always be possible for the academic investigator. If a product is truly novel, the contract manufacturer may not be willing to take on the risk of an entirely new manufacturing procedure. If the product is too similar to what a contract manufacturer is already producing, the new product may be seen as competition. In addition, for devices with associated intellectual property, there may be difficulties with the academic institution agreeing on terms of ownership of the intellectual property that might arise from transitioning the manufacturing process from laboratory to larger scale manufacturing. Lastly, hiring a contract manufacturer to perform GMP and the regulatory work to bring a product to clinical trial is expensive and may require a level of funding that can be challenging for the academic investigator to obtain.

						Severity	Occurrence	Rating	Rating Level	Risk Reduction Rqd?	Design Change	Design Val	Design Ver	Ship Testing	Storage Stability	Sterilization Val	Biocompatibility	Inspection	F.U. / Labeling	Lot Analysis	Process Val	Other	Results, Notes	Severity	Occurrence	Rating	Rating Level	Residual Risk Acceptable?
ID#	Process Step	Cause of Failure	Failure Mode	Effect of Failure																								
1	Sterilization	Sterilization damages scaffold	Scaffold breaks apart	Ineffective procedure		6	4	24	ALARP	Yes	x		x									x						
2		Sterilization damages scaffold	Scaffold becomes brittle	Ineffective procedure		6	4	24	ALARP	Yes	x					x												
3		Sterilization dose insufficient	Product not sterile	Patient contracts infection		8	2	16	ALARP	Yes					x	x												
4		Product inadvertently not sterilized	Product not sterile	Patient contracts infection		8	1	8	BAR	No						x							Lot release requires review of sterilization processing documentation					

FIGURE 3. Example of a section of a production failure mode effect analysis (PFMEA). This document serves to record all the potential causes of failure the design and manufacturing team can think of and to record the methods that will be used to reduce this risk. BAR, broadly acceptable region; ALARP, as low as reasonably practicable; INT, intolerable. Severity score ranges from 1 to 10, with 1 being the least severe and 10 being the most severe. Occurrence ranges from 1 to 10 as well, with 1 being infrequent risk of occurring and 10 being frequent likelihood of failure to occur. Rating is the product of severity and occurrence and is assigned a classification of either BAR, ALARP, or INT. Higher ratings between 40 and 100 are not considered acceptable and classified as INT. Risks rated between 16 and 36 may be acceptable, providing there is adequate justification regarding risk/benefit ratio and are classified as ALARP. Risks rated below 16 are rated as BAR.
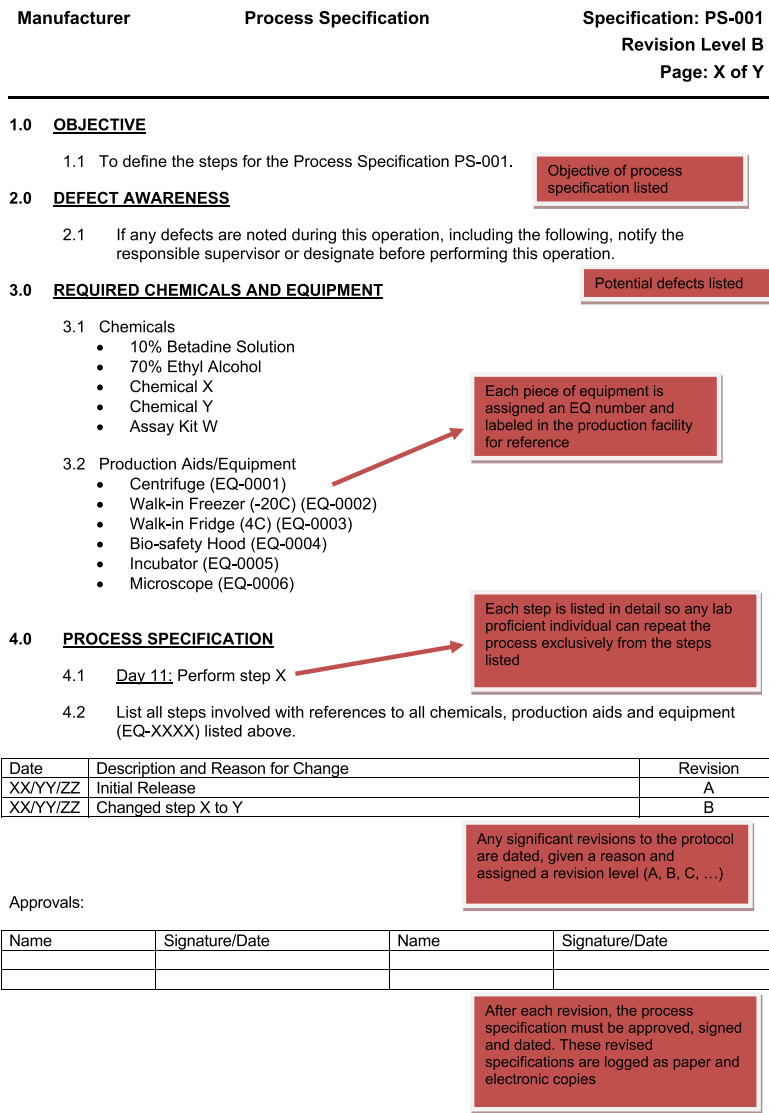
In our experience with bringing our collagen-based scaffold to the clinic, we were unsuccessful in finding a contract manufacturer to produce our scaffold. After discussing this with the FDA, we elected to proceed with manufacturing the scaffold for the first clinical study in our laboratory. We hired a regulatory consultant to guide us through the Good Laboratory Practice (GLP) standard and to generate the required design control documents required for the IDE application. In the end, this approach provided flexibility in designing experiments as the data was gathered and allowed the opportunity for independent decision-making, which is inherently more satisfying for any investigator. The pace of the work was dictated by the availability of funding. In addition, being in an academic setting, there was a continuing incentive to improve the product until the testing met the standards that clinicians and scientists hold in developing a product for patients.

The GLP regulations are found in 21 CFR Part 58: GLP for Nonclinical Laboratory Studies (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRsearch.cfm?CFRPart=58). The standards for GLP production with design control required the generation of three major sets of documents which assured consistency in manufacturing: process specifications (the details of each manufacturing step, Fig. 4), design control documents (detailing the process of design and validation), and quality control documents including material safety data sheets, calibration records, training documents, certificates of analysis, production logs (Fig. 5), and production routers (Fig. 6). As our material was sourced from bovine tissue, we also needed to generate documentation as to the source of the tissue and the steps taken to minimize the risk of bovine infectious agents (in particular, bovine spongiform encephalopathy).

The process specifications (Fig. 4) are essentially a detailed protocol of each manufacturing step. They should be written so that any person with basic laboratory skills could reproduce the manufacturing process. The basic format is much like that of any *in vitro* research protocol and include three sections: the objective, the materials and the methods. Our in-house documentation required that process specification must be signed by three people: the project sponsor (typically the principal investigator), the manufacturing lead (in our lab, one of our post-doctoral students, Dr. Proffen, served as our manufacturing supervisor), and the person in charge of quality, in our case the regulatory consultant, Gordon Roberts. An example of a process specification is found in Fig. 4. For our production of an extracellular matrix scaffold, we found it easiest to break the process down into very simple steps, separating tissue collection, washing steps, enzyme treatment, lyophilization, etc. into individual protocols. If we needed to modify a step, we could modify only that particular process specification rather than a large document.

Lastly, for each scaffold, there must exist an easily traceable path back through the manufacturing procedure all the way to each individual animal from which the source tissue was taken. The production router (Fig. 6) specifies a unique scaffold identifier for each scaffold lot, as well as the reagents, materials and equipment used to create the scaffold lot. This is important so that if there is any problem with the performance of a scaffold, potential sources of the problem

Manufacturer	Process Specification	Specification: PS-001
		Revision Level B
		Page: X of Y

1.0   OBJECTIVE

　　1.1   To define the steps for the Process Specification PS-001.

> Objective of process specification listed

2.0   DEFECT AWARENESS

　　2.1   If any defects are noted during this operation, including the following, notify the
　　　　responsible supervisor or designate before performing this operation.

> Potential defects listed

3.0   REQUIRED CHEMICALS AND EQUIPMENT

　　3.1   Chemicals
- 10% Betadine Solution
- 70% Ethyl Alcohol
- Chemical X
- Chemical Y
- Assay Kit W

> Each piece of equipment is assigned an EQ number and labeled in the production facility for reference

　　3.2   Production Aids/Equipment
- Centrifuge (EQ-0001)
- Walk-in Freezer (-20C) (EQ-0002)
- Walk-in Fridge (4C) (EQ-0003)
- Bio-safety Hood (EQ-0004)
- Incubator (EQ-0005)
- Microscope (EQ-0006)

> Each step is listed in detail so any lab proficient individual can repeat the process exclusively from the steps listed

4.0   PROCESS SPECIFICATION

　　4.1   Day 11: Perform step X

　　4.2   List all steps involved with references to all chemicals, production aids and equipment
　　　　(EQ-XXXX) listed above.

Date	Description and Reason for Change	Revision
XX/YY/ZZ	Initial Release	A
XX/YY/ZZ	Changed step X to Y	B

> Any significant revisions to the protocol are dated, given a reason and assigned a revision level (A, B, C, …)

Approvals:

Name	Signature/Date	Name	Signature/Date

> After each revision, the process specification must be approved, signed and dated. These revised specifications are logged as paper and electronic copies

FIGURE 4.   Sample template for a process specification document. These documents are used for each step of the processing—most manufacturing procedures will have several of these documents for each manufacturing process. At the top, the process specification number and revision level are listed. The document contains the objective, defect awareness, required materials and specific process steps, as well as associated signatures once the process specification is defined.

can be identified, studied, traced, and rectified. An example of a production router is found in Fig. 6.

The assembly of process specifications, design control documents, quality control documents and production routers enables the investigative team to standardize the production process, helping to assure that each lot of scaffolds produced are similar and meet the standards determined during the design control process.

Sterility Testing

After establishing a repeatable manufacturing protocol, sterility concerns should be addressed. In our case, our extracellular matrix-based scaffold was originally manufactured and tested using "aseptic" processing. These procedures are identical to how tissue culture is performed in a laboratory. All instruments, glassware, and reagents were sterilized prior to contact with the material being processed. Sterilization of instruments and glassware was performed using steam sterilization in an autoclave. In addition, all instruments and containers used during the manufacturing procedure were depyrogenized to remove endotoxin. Bacterial endotoxins are lipopolysaccharides from the cell wall of Gram-negative bacteria. Endotoxins get into the environment as a result of autolytic disintegration of the bacterial cells. Even when the bacteria

Manufacturer	Assay W	Specification: LOG-0001
	Lot XXYYZZ	Revision Level: B
		Page: X of Y

Assay W - Date Performed - Lot XXYYZZ

Sample #	Insert Sample #

Prepare Chemical X + Y solution
Describe all steps needed to make the solution

Making the solutions:

Solution	Need (ul)	Make (ul)	Instructions	Amounts	
Chemical X + Y solution	950	1000	Add amounts of chemical X and Y and vortex	500	Chemical X (uL)
				500	Chemical Y (uL)

Assay W:
Briefly describe all steps necessary to perform the assay including making of standard curves, measuring results
This should not be as detailed as the process specifications; this is simply a reference guide

Results:
Provide calculations and results of assay in readable format that can be inserted as attachment in the production router

Date	Description and Reason for Change	Revision
XX/YY/ZZ	Initial Release	A
XX/YY/ZZ	Added Chemical Y	B

Any significant revisions to the protocol are dated, given a reason and assigned a revision level (A, B, C, …)

Approvals:

Name	Signature/Date

After each revision, the process specification must be approved, signed and dated. These revised specifications are logged as paper and electronic copies

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FIGURE 5.   Sample template for production log document. These documents are used to document the performance of any step in the process, be it manufacturing or device testing. At the top, the number of samples tested and the specific numbers are listed. The solutions used in the testing are then specified in the next section, followed by the method for the assay or production step. The results of the assay or production step are then recorded in the results section. The production log template is signed before use.

are killed, endotoxin can be left behind that may elicit a strong immune response.[21] Sterile, endotoxin-free plastic ware is available, and glassware and metal instruments can be rendered endotoxin free by exposing them to temperatures of 200 °C for at least 4 h.[21] Under aseptic processing conditions without terminal sterilization, the scaffolds produced in our laboratory were implanted in over 500 animal knees, with only one infection seen after surgery. This suggests that aseptic processing was reasonably effective in minimizing contamination of the scaffold. However, when the stricter sterility testing required for clinical devices was performed (Microtest), five out of five scaffolds failed the testing, suggesting there remained small numbers of bacteria contaminating the scaffolds. Therefore, we evaluated multiple terminal sterilization techniques for our extracellular matrix scaffold. Terminal sterilization is performed on a device after it is placed in its final packaging. The entire package is sterilized using gas, steam, radiation or other techniques and thus no more "processing" of the device can be done after terminal sterilization. This ensures that no contamination can be introduced into the scaffold prior to final use in a patient. Examples of

terminal sterilization methods include radiation (gamma, ultraviolet light or electron beam), steam, gas (ethylene oxide, supercritical CO_2) or chemical (alcohol or peracetic acid).

After finalization of the manufacturing protocol and selection of the optimal terminal sterilization method, we produced a "clinical grade" batch of scaffolds, that were packaged and terminally sterilized. From this lot, samples were sent for sterility testing for bacteria and fungus (MICROTEST, Agawam, MA), and additional samples sent for viral testing (Charles River Laboratories, Malvern, PA). The bacterial and fungal testing first required a feasibility study, where the scaffolds were tested for any antibacterial or antifungal property that might interfere with the sterility testing. Once the scaffolds passed the initial feasibility test, lot sterility testing was performed on a subset of scaffolds from each lot. As stated in $USP < 71 >$, for batch sizes smaller than 100, the greater of 10% of the lot samples or 4 samples (whichever number is greater) should be tested for each medium. Since two media are tested, one for aerobic and one for anaerobic bacteria, a total 20% of the lots in the scaffold or a minimum of 8 are tested. For batches between 100 and 500 scaf-

Manufacturer

Production Router
Lot XXYYZZ

Process Specification	Equipment	ID	Check	Chemicals	Serial	Lot	Amount used	Comments, etc.
PS-001 - Name of Process Specification Rev: ____	Centrifuge	EQ-0001	✓			**12345ABC**	**10 ml**	Attach print-out of LOG-0001; List major results from LOG-0001
	Walk-in Freezer (-20 C)	EQ-0002		10% Betadine Solution	67618015017			
	Walk-in Fridge (4°C)	EQ-0003		70% Ethyl Alcohol	1423H16			
	Bio-safety Hood	EQ-0004		Chemical X	1658216			
	Incubator	EQ-0005		Chemical Y	2651ZA1AZ			
	Microscope	EQ-0006		Assay Kit W	123456			

Record the lot number and amount used (if relevant) of each chemical

| Date: |
| Technician's Name: |
| Technician's Signature: |

Fill in revision level at time process specification is performed

Check each piece of equipment before use for proper function and date of calibration. If satisfactory, place a check mark next to the piece of equipment

Technician performing the process specification must date, print and sign their name

Date	Description and Reason for Change	Revision
XX/YY/ZZ	Initial Release	A
XX/YY/ZZ	Added Chemical Y	B

Any significant revisions to the protocol are dated, given a reason and assigned a revision level (A, B, C, …)

Approvals:

Name	Signature/Date

After each revision, the process specification must be approved, signed and dated. These revised specifications are logged as paper and electronic copies

FIGURE 6. **Sample production router document. In the production router, the equipment used in each step is identified and inspected for date of calibration and proper function. The lot number of each chemical used in the processing is recorded in the next section and the technician performing the step signs and dates the form each time a step is completed.**

folds, not more than 10 scaffolds have to be tested for each medium. To evaluate potential viral contamination of the scaffolds, we first performed a literature review detailing the effect each of our major processing steps was likely to have on the reduction of any bovine viral load of the scaffold. As our processing steps were similar to those previously demonstrated to result in a 10^{-6} reduction in viral contamination (the standard set per ISO 11137), we were able to combine the literature review with viral lot testing for our IDE. For viral lot testing, tests for nine bovine viruses were performed. Viruses can be classified four ways: enveloped DNA-based, non-enveloped and DNA-based, enveloped RNA-based and non-enveloped RNA-based. Scaffolds were tested for contamination with the most likely bovine viruses from each class. This data was added to the "lot release" data for this batch and for subsequent batches designed for the biocompatibility studies, preclinical animal trials and first in human trial.

In terms of sterility of the scaffold itself, there are two major routes to take when producing a sterile and virus free scaffold lot: lot release testing and validation studies. The methods described above fall under lot release testing. For academic laboratories likely to produce only a few lots of clinical grade material, lot testing may be the preferred route for assuring sterility of the medical device. Lot testing is less expensive and data on the effectiveness of the sterilization method is more easily available. However, lot release testing requires use of a significant number of scaffolds per batch (20% of each batch smaller than 100 scaffolds), causing a significant loss of scaffolds produced. Therefore, if the investigative team envisions making larger numbers of lots of scaffolds, sterilization validation for both bacteria and viruses could be performed. Depending on the sterilization method, there are specific ISO guidelines that have to be followed. For radiation techniques, the bioburden level of ten scaffolds from three consecutive lots each is determined before sterilization. Based on this bioburden, a minimum validation dosage is chosen as dictated by ANSI/AAMI/ISO guidelines (11137 or TIR33). Then 10 scaffolds from another lot are sterilized using the minimum validation dose and the dose is verified if 0 or 1 of the 10 pieces is positive for bacteria. Dose audits are performed regularly by conducting periodic microbial load tests of 10 pieces of a lot every 3 months and then subsequently sterilizing an additional 10 scaffolds with the validation dose and repeating the sterility testing. For the viral validation, the source of the bovine virus is the source tissue itself, therefore individual

processing/sterilization steps are tested for their potential to reduce the source virus burden. For this step, tissue or scaffolds are processed up to the process/sterilization step to be tested and shipped out to the testing facility where they are spiked with a specific virus load. Then the process/sterilization step is performed and the reduction in virus burden is measured. After testing all potential virus reducing processing/sterilization steps the individual reductions in viral loads are combined. The virus validation is verified if a total viral reduction of 10^{-6} during the processing/sterilization steps is achieved. This type of validation of the manufacturing process is more expensive in the short run than lot testing, but is still valuable to do relatively early in the process to ensure the process results in at least a 10^{-6} reduction in virus. If it is found later that the manufacturing steps do not result in a cumulative reduction of 10^{-6}, then additional manufacturing steps may need to be added which can change the final product and necessitate repeating the terminal sterilization, biocompatibility and any other critical preclinical studies prior to proceeding.

Biocompatibility Testing

Once we had established manufacturing protocols and a terminal sterilization technique, biocompatibility testing was performed. This was also performed at an outside facility with experience in running these tests to ISO 10993-1 specifications (NAMSA, Northwood, Ohio). The standard biocompatibility tests for medical devices are outlined in (http://www.iso.org/iso/catalogue_detail.htm?csnumber = 44908). These tests are typically run on either an extraction of the device (the device is placed into an extraction solution for a specified period of time, and then the extraction solution is tested for biocompatibility) or if the device is soluble, it may be dissolved in a fluid and then the solution tested. For devices including collagen which are semi-soluble, the decision between testing as an extraction or a solution can be unclear and preliminary studies to determine which route is appropriate are well worth doing.

The following nine biocompatibility tests are typically performed for implantable medical devices: Cytotoxicity, Maximization Sensitization, Intracutaneous Reactivity, Systemic Toxicity, Genotoxicity (Bacterial Reverse Mutation Study), Genotoxicity (*in vitro* Chromosomal Aberration in Mammalian Cells), Genotoxicity (Mouse Peripheral Blood Micronucleus), Muscle Implantation Study, 6 week Systemic Toxicity Study in Rats Following Subcutaneous Implantation. Particularly for the intracutaneous and implantation studies, it may be helpful to include an FDA-approved material as a control.

Preparation of an Investigational Device Exemption Application (IDE)

The guidelines for preparing an IDE can be found at (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/HowtoMarketYourDevice/InvestigationalDeviceExemptionIDE/ucm046706.htm). In essence the investigative team needs to prepare all the documents in a specified order and submit two identical copies, as well as an electronic copy, to the FDA. In addition to the manufacturing documentation noted above, the IDE application also requires specification of an "Investigational Plan"—essentially the details of the proposed clinical trial—as well as a copy of the proposed informed consent document to be used when recruiting patients into the trial. Other components include copies of all labeling to be used for the device as well as the instructions for use to be provided with each device. All of these materials should typically be reviewed by the Institutional Review Board (IRB) of the investigator's institution prior to inclusion in the IDE, although the IRB may prefer to review the material only after the IDE has been approved by the FDA.

After IDE Submission

Once the FDA has received the IDE application, they will notify you in writing of the receipt date and the IDE number the application has been assigned. At 30 days after receipt, the IDE application is considered approved, unless the FDA otherwise informs the sponsor that the IDE is approved, approved with conditions, or disapproved. In cases of disapproval, you have the opportunity to respond to the deficiencies and/or to request a regulatory hearing under 21 CFR Part 16. Additional information on the IDE approval process can be found at on the FDA website (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/HowtoMarketYourDevice/InvestigationalDeviceExemptionIDE/ucm046164.htm). In our case, there were several questions about the proposed first in human study that we needed to clarify which we were able to do within the time window of approval, and subsequently, approval of the IDE was granted.

Summary

Bridge-enhanced ACL repair in its current form may provide a valuable therapeutic option for patients with ACL tear. It takes advantage of the biologic basis of wound healing in other tissues by providing a stable provisional scaffold which can immobilize autologous blood in the otherwise fluidic wound site. This technique has shown promise in preclinical models—with ACL repair strengths similar to that of ACL recon-

struction at 3, 6, and 12 months after surgery, as well as demonstrating the potential to lower the risk of premature osteoarthritis after ACL injury. Bringing a tissue engineering technology to the bedside is enabled by good science, regulatory expertise and early communication with the FDA. Building a team to approach the regulatory process, including experts in regulatory requirements, a project manager, a dedicated scientific team, experts in biocompatibility, sterility and packaging evaluation and the FDA staff, are all important for a successful IDE application. In addition, the cost of bringing a tissue engineered product through to the bedside is expensive and time-consuming, with little academic recognition for this type of work. However, this type of work can be instrumental in moving a product from bench to bedside and it can be done in the framework of an academic laboratory.

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CONFLICT OF INTEREST

Dr. Murray reports grants from NIH, during the conduct of the study; In addition, Dr. Murray is an inventor listed on patents held by Boston Children's Hospital in the area of ligament repair.

REFERENCES

[1]Ajuied A., F. Wong, C. Smith, *et al.* Anterior cruciate ligament injury and radiologic progression of knee osteoarthritis: a systematic review and meta-analysis. *Am J Sports Med.* 42(9):2242–2252, 2013.

[2]Altman, G. H., R. L. Horan, I. Martin, *et al.* Cell differentiation by mechanical stress. *FASEB J.* 16(2):270–272, 2002.

[3]Andriacchi, T. P., P. L. Briant, S. L. Bevill, and S. Koo. Rotational changes at the knee after ACL injury cause cartilage thinning. *Clin. Orthop. Relat. Res.* 442:39–44, 2006.

[4]Barber, F. A., C. H. Cowden, 3rd, and E. J. Sanders. Revision rates after anterior cruciate ligament reconstruction using bone-patellar tendon-bone allograft or autograft in a population 25 years old and younger. *Arthroscopy.* 30(4):483–491, 2014.

[5]Bellincampi, L. D., R. F. Closkey, R. Prasad, J. P. Zawadsky, and M. G. Dunn. Viability of fibroblast-seeded ligament analogs after autogenous implantation. *J. Orthop. Res.* 16(4):414–420, 1998.

[6]Beynnon, B. D., R. J. Johnson, S. Naud, *et al.* Accelerated versus nonaccelerated rehabilitation after anterior cruciate ligament reconstruction: a prospective, randomized, double-blind investigation evaluating knee joint laxity using roentgen stereophotogrammetric analysis. *Am. J. Sports Med.* 39(12):2536–2548, 2011.

[7]Boguszewski, D. V., J. T. Shearn, C. T. Wagner, and D. L. Butler. Investigating the effects of anterior tibial translation on anterior knee force in the porcine model: is the porcine knee ACL dependent? *J. Orthop. Res.* 29(5):641–646, 2011.

[8]Byun, S., M. D. Tortorella, A. M. Malfait, K. Fok, E. H. Frank, and A. J. Grodzinsky. Transport and equilibrium uptake of a peptide inhibitor of PACE4 into articular cartilage is dominated by electrostatic interactions. *Arch. Biochem. Biophys.* 499(1–2):32–39, 2010.

[9]Deie, M., T. Marui, C. R. Allen, *et al.* The effects of age on rabbit MCL fibroblast matrix synthesis in response to TGF-beta 1 or EGF. *Mech. Ageing Dev.* 97(2):121–130, 1997.

[10]DesRosiers, E. A., L. Yahia, and C. H. Rivard. Proliferative and matrix synthesis response of canine anterior cruciate ligament fibroblasts submitted to combined growth factors. *J. Orthop. Res.* 14(2):200–208, 1996.

[11]Dunn, M. G., J. B. Liesch, M. L. Tiku, and J. P. Zawadsky. Development of fibroblast-seeded ligament analogs for ACL reconstruction. *J. Biomed. Mater. Res.* 29(11):1363–1371, 1995.

[12]Engelman, G. H., P. M. Carry, K. G. Hitt, J. D. Polousky, A. F. Vidal. Comparison of allograft versus autograft anterior cruciate ligament reconstruction graft survival in an active adolescent cohort. *Am J Sports Med.* 42(10):2311–2318, 2014.

[13]Evans, C. H. Novel biological approaches to the intra-articular treatment of osteoarthritis. *BioDrugs.* 19(6):355–362, 2005.

[14]Fan, H., H. Liu, S. L. Toh, and J. C. Goh. Anterior cruciate ligament regeneration using mesenchymal stem cells and silk scaffold in large animal model. *Biomaterials.* 30(28):4967–4977, 2009.

[15]Fan, H., H. Liu, E. J. Wong, S. L. Toh, and J. C. Goh. *In vivo* study of anterior cruciate ligament regeneration using mesenchymal stem cells and silk scaffold. *Biomaterials.* 29(23):3324–3337, 2008.

[16]Fleming, B. C., K. P. Spindler, M. P. Palmer, E. M. Magarian, and M. M. Murray. Collagen-platelet composites improve the biomechanical properties of healing anterior cruciate ligament grafts in a porcine model. *Am. J. Sports Med.* 37(8):1554–1563, 2009.

[17]Frank, S., M. Madlener, and S. Werner. Transforming growth factors beta1, beta2, and beta3 and their receptors are differentially regulated during normal and impaired wound healing. *J. Biol. Chem.* 271(17):10188–10193, 1996.

[18]Harrison, S., P. Vavken, S. Kevy, M. Jacobson, D. Zurakowski, and M. M. Murray. Platelet activation by collagen provides sustained release of anabolic cytokines. *Am. J. Sports Med.* 39(4):729–734, 2011.

[19]Harrold, A. J. Fibrinogenolysis in joints. *Nature.* 186:1057, 1960.

[20]Haslauer, C. M., K. A. Elsaid, B. C. Fleming, B. L. Proffen, V. M. Johnson, and M. M. Murray. Loss of extracellular matrix from articular cartilage is mediated by the synovium and ligament after anterior cruciate ligament injury. *Osteoarthr. Cartil.* 21(12):1950–1957, 2013.

[21]Hecker, W., D. Witthauer, and A. Staerk. Validation of dry heat inactivation of bacterial endotoxins. *PDA J. Pharm. Sci. Technol.* 48(4):197–204, 1994.

[22]Hee, C. K., J. S. Dines, D. M. Dines, *et al.* Augmentation of a rotator cuff suture repair using rhPDGF-BB and a type I bovine collagen matrix in an ovine model. *Am. J. Sports Med.* 39(8):1630–1639, 2011.

[23]Hildebrand, K. A., S. L. Woo, D. W. Smith, *et al.* The effects of platelet-derived growth factor-BB on healing of the rabbit medial collateral ligament. An *in vivo* study. *Am. J. Sports Med.* 26(4):549–554, 1998.

[24]Huang, D., T. R. Chang, A. Aggarwal, R. C. Lee, and H. P. Ehrlich. Mechanisms and dynamics of mechanical strengthening in ligament-equivalent fibroblast-populated collagen matrices. *Ann. Biomed. Eng.* 21(3):289–305, 1993.

[25]Kaux, J. F., L. Janssen, P. Drion, *et al.* Vascular Endothelial Growth Factor-111 (VEGF-111) and tendon healing: preliminary results in a rat model of tendon injury. *Muscles Ligaments Tendons J.* 4(1):24–28, 2014.

[26]Kobayashi, D., M. Kurosaka, S. Yoshiya, and K. Mizuno. Effect of basic fibroblast growth factor on the healing of defects in the canine anterior cruciate ligament. *Knee Surg. Sports Traumatol. Arthrosc.* 5(3):189–194, 1997.

[27]Kroon, M. E., M. L. van Schie, B. van der Vecht, V. W. van Hinsbergh, and P. Koolwijk. Collagen type 1 retards tube formation by human microvascular endothelial cells in a fibrin matrix. *Angiogenesis.* 5(4):257–265, 2002.

[28]Letson, A. K., and L. E. Dahners. The effect of combinations of growth factors on ligament healing. *Clin. Orthop. Relat. Res.* 308:207–212, 1994.

[29]Lippross, S., B. Moeller, H. Haas, *et al.* Intraarticular injection of platelet-rich plasma reduces inflammation in a pig model of rheumatoid arthritis of the knee joint. *Arthritis Rheumatol.* 63(11):3344–3353, 2011.

[30]Lohmander, L. S., S. Hellot, D. Dreher, *et al.* Intraarticular sprifermin (recombinant human fibroblast growth factor 18) in knee osteoarthritis: a randomized, double-blind, placebo-controlled trial. *Arthritis Rheumatol.* 66(7):1820–1831, 2014.

[31]Marui, T., C. Niyibizi, H. I. Georgescu, *et al.* Effect of growth factors on matrix synthesis by ligament fibroblasts. *J. Orthop. Res.* 15(1):18–23, 1997.

[32]Meaney Murray, M., K. Rice, R. J. Wright, and M. Spector. The effect of selected growth factors on human anterior cruciate ligament cell interactions with a three-dimensional collagen-GAG scaffold. *J. Orthop. Res.* 21(2):238–244, 2003.

[33]Mueller, X. M., H. T. Tevaearai, D. Jegger, O. Tucker, and L. K. von Segesser. Are standard human coagulation tests suitable in pigs and calves during extracorporeal circulation? *Artif. Organs.* 25(7):579–584, 2001.

[34]Murray, M. M., and B. C. Fleming. Use of a bioactive scaffold to stimulate anterior cruciate ligament healing also minimizes posttraumatic osteoarthritis after surgery. *Am. J. Sports Med.* 41(8):1762–1770, 2013.

[35]Murray, M. M., K. P. Spindler, E. Abreu, *et al.* Collagen-platelet rich plasma hydrogel enhances primary repair of the porcine anterior cruciate ligament. *J. Orthop. Res.* 25(1):81–91, 2007.

[36]Murray, M. M., K. P. Spindler, P. Ballard, T. P. Welch, D. Zurakowski, and L. B. Nanney. Enhanced histologic repair in a central wound in the anterior cruciate ligament with a collagen-platelet-rich plasma scaffold. *J. Orthop. Res.* 25(8):1007–1017, 2007.

[37]Nin, J. R., M. Leyes, and D. Schweitzer. Anterior cruciate ligament reconstruction with fresh-frozen patellar tendon allografts: sixty cases with 2 years' minimum follow-up. *Knee Surg. Sports Traumatol. Arthrosc.* 4(3):137–142, 1996.

[38]Nissen, N. N., P. J. Polverini, A. E. Koch, M. V. Volin, R. L. Gamelli, and L. A. DiPietro. Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. *Am. J. Pathol.* 152(6):1445–1452, 1998.

[39]Orrego, M., C. Larrain, J. Rosales, *et al.* Effects of platelet concentrate and a bone plug on the healing of hamstring tendons in a bone tunnel. *Arthroscopy.* 24(12):1373–1380, 2008.

[40]Proffen, B. L., M. McElfresh, B. C. Fleming, and M. M. Murray. A comparative anatomical study of the human knee and six animal species. *Knee.* 19(4):493–499, 2012.

[41]Proffen B. L., P. Vavken, B. C. Fleming, C. M. Haslauer, C. Harris, J. T. Machan, M. M. Murray. Addition of autologous mesenchymal stem cells to whole blood for bio-enhanced ACL repair has no benefit in the porcine model. *Am J Sports Med.* 2014. doi:10.1177/0363546514559826.

[42]Scanlan, S. F., A. M. Chaudhari, C. O. Dyrby, and T. P. Andriacchi. Differences in tibial rotation during walking in ACL reconstructed and healthy contralateral knees. *J. Biomech.* 43(9):1817–1822, 2010.

[43]Schmidt, C. C., H. I. Georgescu, C. K. Kwoh, *et al.* Effect of growth factors on the proliferation of fibroblasts from the medial collateral and anterior cruciate ligaments. *J. Orthop. Res.* 13(2):184–190, 1995.

[44]Shen, W., X. Chen, Y. Hu, *et al.* Long-term effects of knitted silk-collagen sponge scaffold on anterior cruciate ligament reconstruction and osteoarthritis prevention. *Biomaterials.* 35(28):8154–8163, 2014.

[45]Silva, A., and R. Sampaio. Anatomic ACL reconstruction: does the platelet-rich plasma accelerate tendon healing? *Knee Surg. Sports Traumatol. Arthrosc.* 17(6):676–682, 2009.

[46]Song, E. K., J. K. Seon, J. H. Yim, S. H. Woo, H. Y. Seo, and K. B. Lee. Progression of osteoarthritis after double- and single-bundle anterior cruciate ligament reconstruction. *Am. J. Sports Med.* 41(10):2340–2346, 2013.

[47]Soon, M. Y., A. Hassan, J. H. Hui, J. C. Goh, and E. H. Lee. An analysis of soft tissue allograft anterior cruciate ligament reconstruction in a rabbit model: a short-term study of the use of mesenchymal stem cells to enhance tendon osteointegration. *Am. J. Sports Med.* 35(6):962–971, 2007.

[48]Spindler, K. P., J. M. Dawson, G. C. Stahlman, J. M. Davidson, and L. B. Nanney. Collagen expression and biomechanical response to human recombinant transforming growth factor beta (rhTGF-beta2) in the healing rabbit MCL. *J. Orthop. Res.* 20(2):318–324, 2002.

[49]Spindler, K. P., M. M. Murray, K. B. Detwiler, *et al.* The biomechanical response to doses of TGF-beta 2 in the healing rabbit medial collateral ligament. *J. Orthop. Res.* 21(2):245–249, 2003.

[50]Spreafico, A., F. Chellini, B. Frediani, *et al.* Biochemical investigation of the effects of human platelet releasates on human articular chondrocytes. *J. Cell Biochem.* 108(5):1153–1165, 2009.

[51]Tashman, S., P. Kolowich, D. Collon, K. Anderson, and W. Anderst. Dynamic function of the ACL-reconstructed knee during running. *Clin. Orthop. Relat. Res.* 454:66–73, 2007.

[52]Tourville, T. W., R. J. Johnson, J. R. Slauterbeck, S. Naud, and B. D. Beynnon. Assessment of early tibiofemoral joint space width changes after anterior cruciate ligament injury and reconstruction: a matched case-control study. *Am. J. Sports Med.* 41(4):769–778, 2013.

[53]Uggen, J. C., J. Dines, C. W. Uggen, *et al.* Tendon gene therapy modulates the local repair environment in the shoulder. *J. Am. Osteopath. Assoc.* 105(1):20–21, 2005.

[54]van Buul, G. M., W. L. Koevoet, N. Kops, *et al.* Platelet-rich plasma releasate inhibits inflammatory processes in osteoarthritic chondrocytes. *Am. J. Sports Med.* 39(11): 2362–2370, 2011.

[55]van Buul, G. M., M. Siebelt, M. J. Leijs, *et al.* Mesenchymal stem cells reduce pain but not degenerative changes in a mono-iodoacetate rat model of osteoarthritis. *J. Orthop. Res.* 32(9):1167–1174, 2014.

[56]Van Eijk, F., D. B. Saris, J. Riesle, *et al.* Tissue engineering of ligaments: a comparison of bone marrow stromal cells, anterior cruciate ligament, and skin fibroblasts as cell source. *Tissue Eng.* 10(5–6):893–903, 2004.

[57]Vavken, P., B. C. Fleming, A. N. Mastrangelo, J. T. Machan, and M. M. Murray. Biomechanical outcomes after bioenhanced anterior cruciate ligament repair and anterior cruciate ligament reconstruction are equal in a porcine model. *Arthroscopy.* 28(5):672–680, 2012.

[58]Vavken, P., P. Sadoghi, and M. M. Murray. The effect of platelet concentrates on graft maturation and graft-bone interface healing in anterior cruciate ligament reconstruction in human patients: a systematic review of controlled trials. *Arthroscopy.* 27(11):1573–1583, 2011.

[59]Vogrin, M., M. Rupreht, D. Dinevski, *et al.* Effects of a platelet gel on early graft revascularization after anterior cruciate ligament reconstruction: a prospective, randomized, double-blind, clinical trial. *Eur. Surg. Res.* 45(2):77–85, 2010.

[60]Webster, K. E., J. A. Feller, W. B. Leigh, and A. K. Richmond. Younger patients are at increased risk for graft rupture and contralateral injury after anterior cruciate ligament reconstruction. *Am. J. Sports Med.* 42(3):641–647, 2014.

[61]Werner, S., and R. Grose. Regulation of wound healing by growth factors and cytokines. *Physiol. Rev.* 83(3):835–870, 2003.

[62]Xerogeanes, J. W., R. J. Fox, Y. Takeda, *et al.* A functional comparison of animal anterior cruciate ligament models to the human anterior cruciate ligament. *Ann. Biomed. Eng.* 26(3):345–352, 1998.