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TISSUE ENGINEERING OF LIGAMENTS

G. Vunjak-Novakovic,[1] Gregory Altman,[2,3]
Rebecca Horan,[2] and David L. Kaplan[2]

[1]*Massachusetts Institute of Technology, Harvard-MIT Division of Health Sciences and Technology, Cambridge, Massachusetts 02139; email: gordana@mit.edu*
[2]*Department of Biomedical Engineering, Tufts University, Medford, Massachusetts 02155; email: gregory.altman@tufts.edu, david.kaplan@tufts.edu, rebecca.horan@tufts.edu*
[3]*Tissue Regeneration, Inc., Medford, Massachusetts 02155*

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■ **Abstract** Tissue engineering is emerging as a significant clinical option to address tissue and organ failure by implanting biological substitutes for the compromised tissues. As compared to the transplantation of cells alone, engineered tissues offer the potential advantage of immediate functionality. Engineered tissues can also serve as physiologically relevant models for controlled studies of cells and tissues designed to distinguish the effects of specific signals from the complex milieu of factors present in vivo. A high number of ligament failures and the lack of adequate options to fully restore joint functions have prompted the need to develop new tissue engineering strategies. We discuss the requirements for ligament reconstruction, the available treatment options and their limitations, and then focus on the tissue engineering of ligaments. One representative tissue engineering system involving the integrated use of adult human stem cells, custom-designed scaffolds, and advanced bioreactors with dynamic loading is described.

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INTRODUCTION

The incidence of tissue failures and repairs is already high and continues to increase owing to increased life spans and the improved quality of life. The risk for transmission of diseases has imposed additional restrictions on the already limited availability of human transplants. The anterior cruciate ligament (ACL), which serves as the primary stabilizer of the knee motion, is susceptible to ruptures or tears that can cause pain and discomfort, joint instability, and eventually degenerative joint diseases (1). An estimated 200,000 Americans required reconstructive surgery of ligaments in 2002 with a price tag exceeding five billion dollars (2, 3).

Three options have been utilized for the repair or replacement of damaged ligaments using biological substitutes: autografts, allografts, and xenografts. *Autografts* (patellar tendon with bony attachments, or two of four hamstring tendons harvested from the patient at the time of surgery) have produced the most satisfactory long-term results and are referred to as the "gold standard" (4). Donor site morbidity remains the limiting factor of patellar tendon grafts, because it is often associated with pain, muscle atrophy, and tendonitis, resulting in prolonged rehabilitation periods (5). Frozen *allografts* of ligaments with bony attachments frequently result in an immunological foreign-body response (6) that hinders tissue remodeling (7, 8). The risks of disease transmission, a lack of donors, and donor-recipient compatibility are significant problems. Bovine *xenografts* cross-linked with glutaraldehyde have been used with an initial success, but more recent studies have not reproduced these early findings (8). Bovine xenografts failed to obtain Food and Drug Administration (FDA) approval owing to occasional recurrent effusion, graft failure, and synovitis. Synthetic materials were popular in the 1980s, but their implantation frequently led to implant degeneration and failure (9).

In addition, a variety of synthetic materials have been used for ligament replacement (e.g., Versigraft, Dacron, Gore-Tex, Leeds-Keio polyester, polypropylene-based Kennedy Ligament-Augmentation Device), but with limited success (10–12) (Table 1). Of 855 prosthetic ligaments tracked for 15 years, 40% to 78% failed owing to wear debris, tissue reactions, and mechanical limitations (13). Experience with these synthetic ligament grafts replacement are particularly instructive from the perspective of tissue engineering. The first generation of ACL prostheses included polytetrafluorethylene (Gore-Tex®), polyester (Dacron®), carbon fibers, and ligament-augmentation devices (LAD) made of polypropylene. True (permanent) prostheses were designed to have high initial and sustained strength characteristics as well as an increased resistance to fatigue failure in order to withstand forces associated with continuous cyclic joint loading. In contrast, scaffolds for tissue-engineered ligaments must have high initial strength, but they must be designed to gradually transfer the load-bearing responsibilities to the maturing

TABLE 1 Synthetic materials historically utilized for ligament replacement (5)

	Advantages	Disadvantages	Ultimate tensile strength (N)	Stiffness (N/mm)	Elongation at break (%)
Gore-Tex®	High strength and fatigue life, limited particulate debris	Lack of tissue ingrowth, fraying at bone tunnels, chronic effusions, ultimate longevity	5300	322	9
Dacron®	High strength, supported collagenous ingrowth	Stress-shielding of collagenous in-growth, rupture of the femoral or tibial insertion, rupture of the central body, elongation	3631	420	18.7
Carbon fiber	Synthetic material	Particulate matter, foreign body response in synovium	660	230×10^9	1
LAD	Protects graft during maturation	Inflammatory reaction, high complication rate	1730	56	22

tissue while allowing for autogenous cell and tissue ingrowth, which in turn contributes to the required mechanical properties. Augmentation devices (stents) are temporary prostheses designed to increase the immediate strength of the graft and augment the graft-host tissue fixation (14, 15). The Gore-Tex® ACL is made of a single strand of expanded polytetrafluorethylene that is wound into multiple loops. The prosthesis has been implanted in more than 18,000 patients worldwide (14). This prosthesis was designed to give immediate fixation with early load-bearing capabilities, thus promising early mobilization and return to activity (16). The Gore-Tex® graft ultimately failed from material fatigue owing to the lack of tissue ingrowth, likely the result of both the graft design and material properties; fraying at the bone tunnels and chronic effusions were observed (14). The graft eventually fatigued because it was unable to withstand the cyclic loads in active individuals without reinforcement of the in vivo environment; it was approved by the FDA for use only in previously failed autogenous reconstructions. The Gore-Tex II® prosthesis is currently being tested in Canada.

The Dacron® ligament was designed as a hybrid prosthesis to solve the problems of stiffness (i.e., stress shielding) that led to high failure rates in previous devices (10). The Dacron® prosthesis consists of a core of four tightly woven Dacron® tapes that are encased by a sleeve of loosely woven Dacron® velour, which promotes abundant tissue ingrowth. Complications resulting from the Dacron® prosthesis included rupture of the femoral or tibial insertion of the ligament, rupture of the central body of the prosthesis, and elongation of the ligament, possibly resulting from the permeation of synovial fluid and connective tissue (17). Although tissue ingrowth was significant, the graft did not provide knee stability because organized collagenous ingrowth was not observed, likely owing to stress shielding and the nondirectionality of the sheath covering.

The carbon fiber device was designed as a scaffold that would support rapid ingrowth of fibroblastic tissue and produce new collagen (14). The carbon fiber prosthesis is formed from carbon fiber (Plastafil®) bundles that are coated with biodegradable medical grade gelatin. The predominant complication associated with carbon fibers was the presence of particulates due to the choice of biomaterials. An immediate foreign-body response was observed in the synovium (18), and free carbon fibers were found within the joint and the regional lymph nodes (19). To reduce shedding of particulates, the carbon fiber implants were coated with polylactic acid, but this modification never received an approval by the FDA.

The Kennedy Ligament-Augmentation Device® was designed to provide protection to a weak portion of the quadriceps patellar tendon autograft using an over-the-top reconstruction as well as to the primary repair of the (e.g., partially torn) ACL. The LAD is composed of a ribbon of polypropylene woven from eight braids, and it is mostly of historic interest because the primary repair of the ACL is not routinely performed and because the over-the-top reconstruction has been displaced by newer techniques. LADs had high rate of complications in primary ACL reconstructions (up to 63%) and experienced a delay in maturation because of stress shielding (20).

ANTERIOR CRUCIATE LIGAMENT

Structure-Function Relationships

Ligaments have a unique combination of molecular, structural, and mechanical properties, but there is no single unique marker that can be used to distinguish between ligaments and other tissues. In addition, ligament properties vary with the anatomic location, age, and many factors associated with an injury or disease. On the structure-function level, the unique helical organization of collagen fiber bundles is thought to be essential for ligaments to perform their stabilizing function. During the rotation of the human knee ($\sim$140° in extension/flexion), the mode of attachment results in a 90° twist of the ACL and the peripheral fiber bundles. During flexion, ACL fibers remain isometric in length, allowing equal distribution in load to all fiber bundles, and thereby maximize its strength. It is this particular feature that allows the ACL to sustain high loading through all degrees of knee joint motion. A prosthesis that does not account for the isometric nature of the ACL fibers would result in a large increase in the length of fibers and the high risk for rupture. The opposite is observed for a fiber bundle attached posterior-posterior, which would become lax and unable to stabilize the load.

The complex ACL geometry and function are in part responsible for the difficulty encountered in developing suitable surgical replacements. Prosthesis design must consider the ultimate tensile strength and the stress-strain behavior of native ligaments (21). The biomechanical basis for many of the synthetic graft failures lies in the mismatches between the mechanical properties of synthetic materials and native tissues. Of particular importance is the high linear stiffness of the prosthesis that can lead to stress shielding of host tissue that tends to place the majority of the physiological load on the prosthesis alone. For example, the lack of collagen alignment can shift the dynamics of continuous ligament remodeling toward degradation and reduce the load-bearing capacity of the newly formed tissue.

The rigors of the intra-articular environment also include a rather unique biochemical environment. The cruciate ligaments (anterior and posterior) are encapsulated by synovium-containing fluid designed to lubricate the joint and prevent clots. As a result, the intra-articular environment has very low capacity for healing, leaving a torn ACL incapable of self-repair. A vascular network extending from the femoral and tibial bone attachment sites through the ligament provides long-term nourishment of the cruciate ligaments. Immediately following ACL reconstruction and prior to angiogenesis [occurring within $\sim$12 weeks of implantation (22)], the graft is dependent on the synovial fluid for nutrient and metabolite exchange. Because of the diffusional limitations of transport, the interior of the tissue is often necrotic, leading to alterations in mechanical properties. Jackson and coworkers (6) showed that even after a prolonged healing time of 6 months autologous patellar tendon grafts regained only 61% of their initial strength.

Human ACLs range in length from 27–32 mm. The fiber crimp pattern allows for 7% to 16% of creep prior to permanent deformation and ligament damage. The

ACL withstands cyclic loads of approximately 300 N about 1–2 million times per year, and it is regularly exposed to tensile forces ranging from 67 N (for ascending stairs) to 630 N (for jogging) (23). The mechanical and viscoelastic properties of the human ACL are well known (7, 21, 24–27). The established standards for ACL grafts are 1730 N for the tensile strength (26), 182 N/mm for linear stiffness (26), and 12.8 N-m for energy absorbed at failure (21).

Molecular Markers

There is no unique marker for ACL fibroblasts. Ligaments (native and engineered, normal, and disrupted) have been evaluated on the presence of a combination of factors (Table 2):

- Extracellular matrix components (e.g., collagen types I and III, elastin, fibronectin, decorin, and biglycan) (12, 28–30)
- The relative ratio of collagen type I to type III (∼88% Type I: 12% Type III) (28, 29, 31)
- Types and amounts of reducible cross-links (29)
- Cell morphology within distinct regions of the ACL (29, 32)
- The ultrastructure of collagen network (e.g., crimp pattern, collagen fibril diameter) (29, 30).

These specific features are used to distinguish the ACL from other ligaments, tendons, and tissues in general. Available data on ACL repair, embryonic development, and composition indicate that collagen types I and III, fibronectin, tenascin-C, elastin, laminin, and tissue inhibitor metalloproteinase-1 (TIMP-1) are present within the ACL (28, 29, 33–35). Furthermore, the study of the mechanical stimulation of cultured ACL fibroblasts suggests that the type I and type III collagen, tenascin-C, and vimentin all respond to mechanical loading (36–39).

TABLE 2 Phenotypic differences between some ligaments and tendons based on biochemical analyses (29)

	Percent total collagen (mg collagen/g dry tissue)	**Ratio collagen Type I to collagen Type III proteins**	**Total glycosaminoclycans (mg hexosamine/g dry tissue)**
Cruciate ligaments (ACL and PCL combined)	80.3 +/− 1.0	7.3 +/− 1.4	9.89 +/− 0.56
Medial collateral ligament	79.7 +/− 1.1	10.1 +/− 2.6	4.56 +/− 0.26
Patellar tendon	86.7 +/− 0.9	>19	3.92 +/− 0.16
Achilles tendon	86.8 +/− 1.0	>19	2.75 +/− 0.20

Recent work quantifies eight specific proteins in torn human ACLs: collagen type I and III, fibronectin, tenascin-C, elastin, laminin, vimentin, and TIMP-1. In order to better understand the mechanisms of ACL healing, the expression of matrix metalloproteinases (MMPs) and the amount of the extracellular matrix (ECM) in ruptured human ACLs has been measured and a genetic baseline characterizing ACL tissue and behavior has been generated (40). Marker expression correlates to several predictors, such as age, gender, postinjury period, and the site of tissue scarring. A concise quantitative baseline of genotypic selected markers in ACL is essential for assessing mesenchymal stem cells (MSC) differentiation toward the ligament lineage in cell-based restoration strategies, such as tissue engineering. Importantly, establishment of such a baseline would allow the design of biomimetic culture environments for tissue engineering of functional ligaments.

LIGAMENT TISSUE ENGINEERING

Functional Tissue Engineering

Cells, biomaterial scaffolds, and biochemical and physical regulatory signals can be utilized in a variety of ways to engineer tissues in vitro and in vivo. In most cases, the goal is to replicate some aspects of the environment present in vivo during normal tissue development. Tissue engineering generally involves the presence of reparative cells, a structural template, facilitated transport of nutrients and metabolites, and a provision of molecular and mechanical regulatory factors. An envisioned scenario of clinically relevant tissue engineering involves the use of autologous cells, biodegradable scaffolds (designed to serve as a temporary structural and logistic template of tissue development), and bioreactors (designed to control the cellular environment).

Scaffold structure, mechanical properties, and the rate of degradation largely determine the mass transport and mechanotransduction at the cellular and tissue levels. For engineering of load-bearing tissues, the maintenance of mechanical properties of the scaffold until the new tissue becomes mechanically competent is an important requirement for scaffold design.

Tissue *engineering bioreactors* are generally designed to support physiologic concentrations of attached cells within clinically sized scaffolds and to control the environmental conditions via enhanced mass transfer (41). The provision of mechanical regulatory signals that are normally present in native tissues (e.g., a combination of dynamic strain and torsion for ligaments) is thought to be essential for the proper development of functional tissue properties (42, 43).

Despite the great potential of tissue engineering, human clinical trials with tissue-engineered ligaments have not been completed, perhaps owing to the lack of:

- Readily available autologous cell sources that do not result in donor site morbidity

- Biomaterial scaffolds with adequate composition, structure, and mechanical properties, capable of supporting ligament development in vitro (in a bioreactor) and remodeling in vivo (in a joint)
- Advanced bioreactor systems capable of mechanical conditioning of the cultured tissues, such that the engineered constructs are already functional to some extent at the time of implantation.

One approach to the engineering of ligaments, tissues that serve a predominantly biomechanical function, is based on the paradigm of functional tissue engineering. The set of principles recently defined by Butler et al. (43) stressed the importance of the mechanical properties of skeletal tissues and recognized the need to define mechanical requirements for tissue-engineered grafts and to utilize physical signals to direct cellular activity and phenotype toward functional tissue assembly, and thereby to achieve mechanically competent tissue repair.

Approaches to Ligament Tissue Engineering

The shift from synthetic to biologically based ACL replacements was seen in early studies in which reconstituted type I collagen fibers were embedded in a type I collagen matrix, combined with polymethylmethacrylate bone fixation plugs, and used as ACL replacement tissues in rabbits (44). Subsequent studies utilized ligament analogs consisting of ligament fibroblasts seeded onto cross-linked collagen scaffolds (45, 46). Implantation of prestressed collagen sutures seeded with MSC repaired critical-size tendon defects (47). The collagen-fibroblast system was subsequently modified by using ligament fibroblasts seeded in noncross-linked collagen that was prestressed between the bone anchors (48). Passive tension within the engineered ligament induced fibroblast elongation and alignment and induced structural reorganization of the extracellular matrix (48). The application of cyclic mechanical loading during cultivation promoted the proliferation of smooth muscle cells and the assembly of newly synthesized ECM (49) as well as the synthesis of type I collagen by ACL fibroblasts (37). The cultivation of human ACL fibroblasts in collagen gel attached to two bone anchors and exposed to cyclic stretching resulted in ligament-like tissue architectures that depended on the application of mechanical stimuli (50, 51).

One advanced model system that has been used to engineer functional autologous ACLs in vitro involves an integrated use of three components:

- Adult MSC (isolated from bone marrow)
- Tissue formation template (collagen or silk fiber matrix designed to mimic structural and mechanical properties of a native ACL)
- Advanced bioreactors (providing environmental control and multidimensional dynamic loading).

The approach uses the directed differentiation and functional assembly of cultured MSC into engineered tissues that have the architecture and mechanical properties resembling those of native ligaments, in an environment designed to

resemble some aspects of that in developing native ligaments (e.g., 42, 52). Engineered ligaments can be monitored in culture (magnetic resonance imaging), and characterized in vitro (molecular, structural, and mechanical properties) and in vivo (phenotype stability, capacity for survival, and vascularization). The ultimate goal of functional tissue engineering is to generate mechanically competent grafts for improved tissue repair.

Key Requirements—Clinical Aspects

Tissue-engineered ligaments with appropriate biological and mechanical properties already established at the time of implantation would eliminate the limitations associated with present day surgical techniques. The following requirements for clinically relevant tissue engineering of ligaments have been identified (5):

- No additional surgical procedure for tissue harvest
- Minimal patient morbidity: no requirement for tendon graft harvest for use in ACL reconstruction
- Simple surgical technique for implantation with reliable fixation methods, enabling the graft to withstand aggressive rehabilitation
- Immediate stabilization of the knee to allow patients to rapidly return to their preinjury functions
- Minimal risk for infection or disease transmission
- Biocompatibility of the graft material
- Support of host tissue ingrowth without causing stress shielding (through the design of matrix geometry, porosity, and mechanical properties)
- Biodegradation at a rate providing adequate mechanical stability following implantation and during the transition to functional replacement tissue during remodeling in vivo
- Mechanical integrity for a duration sufficient to allow host tissue ingrowth

Key Requirements—Tissue Engineering Aspects

The above requirements for clinically relevant tissue engineering can be translated into the following set of in vitro culture requirements:

- Autologous cell source (e.g., MSCs from adult human bone marrow that can be obtained by needle biopsy, selected, and expanded in vitro)
- Biomaterial scaffold (e.g., fibrous silk matrix custom designed for ligament tissue engineering, wire-rope structure, mechanical properties matching those of native ligaments, high porosity to allow cell infiltration, and biodegradation at a rate matching that of tissue formation)
- Bioreactor system (e.g., with medium perfusion to control the cellular microenvironment and with mechanical loading to induce/enhance functional tissue assembly)

- Biochemical regulation of MSC differentiation (e.g., by supplemental growth factors, bioinductive matrices, or at the genetic level)
- Biophysical regulation of MSC differentiation (e.g., by mechanical loading that is physiologic for ligaments: dynamic axial tension and torsion with periods of loading and rest)
- Quantitative methods of characterization in vitro (molecular, structural, and mechanical properties) and in vivo (phenotype stability, survival, vascularization, and mechanical function).

Cell Source

Ideally, the patient should be treated with her/his own cells to eliminate concerns associated with the use of allograft cells (e.g., immunorejection and transmission of disease). ACL fibroblasts were shown to respond to specific growth factors, dynamic mechanical stimulation, and static tension (37, 48, 53). However, functional integration of allogeneic grafts has been delayed by a foreign-body response of the host (6), whereas a suitable source of autologous ACL fibroblasts is not yet available.

One candidate source of cells for engineering skeletal tissues is adult human bone marrow, which maintains a pool of MSCs capable both of some degree of self-renewal and of differentiation into cells of various mesenchymal lineages. MSCs have several distinct features that make them an obvious choice for engineering a variety of tissues:

- Marrow cells are routinely collected from adults using a needle biopsy and do not pose ethical concerns inherent in using embryonic or fetal tissue.
- Mesenchymal cells form discrete colonies of fibroblasts that can be isolated on the basis of their adherence to a solid surface and expanded to achieve large total cell numbers (54–58).
- The number of MSCs in bone marrow decreases as a function of age (54), but the cell potential to proliferate and differentiate is comparable in newborns and older individuals (59, 60).
- Culture conditions can be designed to direct MSC differentiation into a desired mesenchymal phenotype, including bone-forming osteoblasts, cartilage-forming chondrocytes, fat-storing adipocytes, and tendon/ligament-forming cells (47, 61–64).
- Engineered ligaments formed from MSCs could serve as suitable autografts without the need for additional surgery (involved in conventional autografts) and without the risk of immune reaction (seen with allografts).

Regulatory Factors

Biochemical factors are essential for inducing and/or supporting the manifestation of specific mesenchymal cell lineages. Oxygen can markedly affect ECM synthesis

rates and the in vitro development of engineered tissues (65, 66). Little is known about the effects of oxygen on stem cell differentiation into ligament fibroblasts. Fermor et al. (67) showed that high oxygen tensions (21%) supported optimal ACL fibroblast proliferation, whereas lower tensions (10%) enhanced ECM collagen synthesis. Ascorbate-2-phosphate, a long-acting derivative of vitamin C, enhanced cell growth in vitro and supported the maintenance of connective tissues (67, 68). Growth factors, such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-II (IGF-II), and transforming growth factor-beta (TGF-β) have the capacity to increase cell proliferation (31, 69–74). Additionally, insulin, TGF-β, and IGF-II promote protein expression and the generation of ECM in soft connective tissues (72, 73, 75, 76). TGF-β and EGF, as well as TGF-β and insulin, act in concert to stimulate normal proliferation of fibroblasts and mesenchymal cells (73, 77, 78). Serum contains potent stimulators of in vitro cell growth and differentiation, including amino acids, growth factors, vitamins, proteins, hormones, lipids, and minerals, as well as protein inhibitors of cell differentiation.

Mechanical signals that affect the growth and development of native ligaments in vivo are likely to play similar roles during the in vitro cultivation of engineered ligaments. The application of loading can affect tissue development in at least two ways, by (*a*) enhanced mass transport, and (*b*) direct stimulation of the cells. Mechanical stimulation of cultured ligament fibroblasts upregulated the expression of collagens type I and type III, fibronectin, and tenascin-C (37–39, 79). Human MSC, in the absence of specific ligament growth and regulatory factors, could be directed to differentiate into ligament-like cells through the application of physiologically relevant cyclic strain (42, 80). Loading can cause changes to the extracellular environment both in native and in engineered ligaments by direct effects on cell shape and interfibrillar spacing or by fluid flow that can enhance the rate of mass transport to and from the cells.

Our knowledge about the effects of mechanical factors applied during cultivation (e.g., torsion and tension; frequency, amplitude, and regime of stimulation) on tissue structure and function at various hierarchical levels is limited. Controlled studies of mechanical factors affecting tissue-engineered ligaments can greatly benefit from the use of controllable physiologically relevant tissue models that can be studied with the application of dynamic loading in situ. Experimental studies of mechanotransduction are further complicated by the interactions between physical signals (associated with joint loading) and molecular factors (genetic expression, soluble and matrix-immobilized regulatory molecules). Experiments conducted more than 40 years ago suggested that mechanical factors and regulatory molecules acting in concert can determine the type of connective tissue that forms in a culture of MSCs (81).

Biomaterial Matrix

Biomaterial scaffolds provide a structural and logistic template for cell attachment and tissue development, and they biodegrade in parallel with the accumulation of

tissue components. Most studies suggest that a scaffold is essential for promoting orderly tissue repair (82–86). Scaffold structure determines the transport of nutrients, metabolites, and regulatory molecules to and from the cells, whereas its mechanical properties determine mechanotransduction at cellular and tissue levels. Ideally, a scaffold should be made of biocompatible, biodegradable material and degrade at a rate matching the rate of new tissue deposition (87). Biomaterials have been explored for use in skeletal tissue engineering (88–93), in some cases in conjunction with the delivery of regulatory molecules (56, 94–101). Regulatory molecules are usually supplemented to culture medium (102–106); they have also been incorporated into biomaterial scaffolds to provide localized delivery (107–114). Tissue engineering can greatly benefit from the new generation of bioinductive biomaterial scaffolds, for example those capable of delivering multiple growth factors (115) or capable of releasing growth factors in response to mechanical loading (116).

Collagen and silk have been used clinically for decades (e.g., hemostats and surgical sutures) and more recently for tissue engineering (52, 117–121). Collagen type I gel provided a three-dimensional environment for seeded ligament fibroblasts to proliferate and organize in response to mechanical stimulation (42, 48). However, the utility of collagen gels was limited by their inferior mechanical properties. Fibrous silk and collagen scaffolds were subsequently adopted in order to provide the necessary mechanical competence of the developing ligaments throughout the in vitro cultivation and following implantation in vivo.

COLLAGEN SCAFFOLDS Commercially available highly purified type I collagen has been used in form of gel and bundles of parallel fibers. Collagen was selected on the basis of its demonstrated ability to support the growth of ligament fibroblasts under static tension (48). Cross-linked collagen fibers were seeded with human MSCs. Fibrous collagen supported cell attachment, spreading, and fiber coverage with extracellular matrix deposition.

After one day of culture, the cells formed focal contacts, adopted a spindle-like morphology, and aligned along the scaffold fibers. After three days of culture, a smooth cell sheet was formed, and the number of cells increased with time in culture. Unfortunately, the capacity of the engineered construct to support mechanical loading decreased with time in culture, and as a result, the scaffold fibers could not impart repeatable stresses to the attached MSCs as previously observed (44–46). The poor mechanical integrity of the fibers created further concerns regarding anchoring of the fibers both in vitro (in the bioreactor) and in vivo (in the knee). The mechanical deficiencies, the bioburden associated with the use of bovine collagen products and leaching of chemical cross-linking agents, combined with the poor mechanical integrity of the collagen fibers prompted the search for improved biomaterials.

SILK SCAFFOLDS Silk has been used clinically for decades and has gained renewed interest as a biomaterial for tissue engineering (52, 80, 118–121). Initially, two

problems were associated with the use of silk fibers: (*a*) native silkworm silk fibers were allergenic because of the glue-like protein coating, sericin (119), whereas spider silk fibers that are not coated with sericin are not abundant enough for use, and (*b*) silk fibers have high linear stiffness and, if organized in a parallel geometry, would fail to mirror the tensile strength and linear stiffness of native ACLs.

We have extensively explored the potential of native silk fibers for ligament tissue engineering from both the mechanical and biological perspective (52, 117). The processed silk does not have bio-burden risks associated with bovine-derived collagen; it is inexpensive and biocompatible, biodegrades at a slow rate in vitro and in vivo, and has outstanding and tailorable mechanical properties. Virgin silk is processed to extract sericin, the potential allergen (122–124). For the fabrication of surgical sutures, the fibers are coated with wax or silicone to obtain black braided silk (e.g., Perma-Hand^{TM}). To obtain highly purified silk, we developed a specialized extraction process for the treatment of silk fibers under static tension (117). Figure 1*A* and 1*B*, respectively, show silk fibers before and after the sericin coat is removed.

Silk undergoes proteolytic degradation (125–128) at a rate that depends on the environmental conditions (125, 128–131). Silk fibers lose their tensile strength within one year in vivo and degrade completely within two years. Degradation half-lives of six weeks were reported for subcutaneous implantation in rats (128, 129). In vitro, silk can be degraded by protease (121) and chymotrypsin (132). Importantly, for silk to degrade, proteolytic attack that typically coincides with the onset of angiogenesis is needed. Overall, silk is a slowly degrading biomaterial with biocompatibility comparable to that of most materials in clinical use (e.g., polyglycolic acid (PGA) used to make degradable sutures, collagen).

Silk fibroin, when organized into an appropriate wire-rope geometry, exhibits mechanical properties similar to a functional ACL (80). In particular, the scaffold stiffness could be decreased without impacting the tensile strength of scaffold fibers by specific modifications of scaffold architecture. The six-cord wire-rope silk fiber matrix (Figure 1*C*) was designed to meet four important requirements of ligament tissue engineering:

- Enhance tissue infiltration (>90% void volumes)
- Minimize mass-transport limitations
- Increase surface area for cell attachment and extracellular matrix deposition
- Decrease the linear stiffness to match that of a native ligament and thereby avoid stress shielding.

Mechanical testing of a series of single cords from the wire-rope scaffold showed consistent load-elongation behavior (Figure 1*F*). Fatigue testing revealed a scaffold life in excess of 10 million cycles at a physiological load. As a result of silk's high tensile strength and the wire-rope design, the matrix provided significant void volume for cell seeding in vitro and tissue ingrowth in vivo. Compared to a native human ACL (per equivalent length), the silk matrix occupies only ∼12%

of the volume of a human ACL (determined for an ACL 27 mm in length and 8 mm in diameter).

Silk scaffolds also support cell attachment and spreading by providing an appropriate 3-D culture environment. Cells readily attached to scaffolds, as evidenced by scanning electron micrographs (SEM) taken 30 min post seeding (Figure 1*D*). The cells spread after 1 h of culture, formed confluent cell sheets by day 7, and accumulated a ligament tissue matrix by day 14 (Figure 1*E*).

Bioreactor System

Recent studies support the notion that bioreactors providing dynamic loading are essential for meeting the complex requirements of in vitro engineering of functional skeletal tissues (Tables 3 and 4). Various commercial bioreactor systems are available. However, the applicability of these systems to ligament engineering is limited owing to either the generality of the device, which lacks the specificity needed to mimic an in vivo ligament environment, or the design of the device tailored to meet the requirements of another tissue type other than ligaments.

An advanced bioreactor system with medium perfusion and mechanical loading was used to engineer ligaments starting from MSCs (52). The bioreactor was specifically designed for ligament tissue engineering to provide the application of multidimensional mechanical strains (axial tension/compression and torsion) to MSC cultured in a hydrogel or on a fibrous scaffold (Figure 2, see color insert).

TABLE 3 Overview of selected commercial bioreactor systems

Commercially Available Systems			
Manufacturer	**Product**	**Defining features**	**Limiting factors**
Synthecon	Rotary Culture Max	Perfusion, rotating wall vessel	Incubator required, one vessel, no environmental or mechanical control
Synthecon	RCCS	Rotating wall vessel	Incubator required, one vessel, no environmental or mechanical control
Synthecon	RCCS-4	Four rotating wall vessels	Incubator required, no environmental or mechanical control
FlexCell International	FX-4000T FX-4000C	Tension, 24 dishes, vacuum driven Compression, 24 dishes, pump driven	Incubator required, no environmental, compression or perfusion control, limiting actuation and force monitoring systems, limited number of tissue geometries and sizes able for culture
FlexCell International	FlexFlow	Shear stress applied via fluid flow	Incubator required, no environmental or mechanical control

TABLE 4 Overview of selected bioreactor systems for which results have been published

Group (Reference)	Tissue	Defining features	Constraints
Georgia Institute of Technology (138)	Blood vessel constructs	Dynamic conditioning through inflation and deflation of silicon tubing	Incubator required, no environmental control, limited mechanical control
Department of Anesthesiology and Biomedical Engineering, Duke University (139)	Arteries	Precise pulsatile radial stress and luminal flow	Incubator required, no environmental or mechanical control
M.E. for Biomechanics, University of Bern, Switzerland (140)	Cartilage	Samples compressed with sinusoidal dynamic displacement	Incubator required, no environmental or perfusion control
Lab for Tissue Engineering, German Heart Institute, Berlin (141)	Cardiovascular structures	Perfusion system, shear stress from .2–10 dyne/cm^2	Incubator required, no environmental or mechanical control
University of Ferrara, Italy, School of Medicine (142)	Liver	Constant perfusion from 2 ml/min to 4 ml/min	Incubator required, no environmental or mechanical control
Department of Chemical Engineering, Zurich, Switzerland (143)	Epidermal autografts	Computer-controlled medium and gas exchange	Each chamber is 1-cm thick, no environmental or mechanical control
Division of Bioengineering, Massachusetts Institute of Technology (144)	Cartilage	Dynamic control of displacement, load, shear angle, or torque	Incubator required, no individual cassettes

The bioreactor is computer controlled and consists of (*a*) 12 individual cartridges containing one engineered ligament apiece, each within a separate perfusion loop; (*b*) a gas exchanger to control medium pH and oxygen levels, (*c*) a 12-channel peristaltic pump, and (*d*) a strain-control system for mechanical stimulation of cultured constructs. Two bioreactors are operated in parallel, allowing concurrent cultivation of 24 ligaments, e.g., 12 with loading and 12 with unloaded controls (Figure 2*A*).

Each bioreactor cartridge is comprised of top and bottom mounts, a tube (an inside diameter of 0.75″, length sized to accommodate various ligaments, e.g., for knee joint or a temporomandibular joint), and two anchor shafts (Figure 2*B*,*C*). Cartridges are designed to allow for quick assembly and disassembly, and all parts can be steam sterilized. Each cartridge is operated within a separate flow loop with medium recirculation via one channel of a multichannel peristaltic pump and with an environmental chamber for oxygen and carbon dioxide exchange (52). Medium flow minimized diffusional distances around the growing ligament (Figure 2*D*). Axial strain (1%–10%) and torsional strain (up to 90°) could be applied via the top platen at frequencies of 0.01–1 Hz, with preset periods of loading and rest (52). Key functions of the bioreactor system include

- precise control of the applied mechanical strain (axial: <0.1 μm, torsion: $<0.01°$);
- control of medium flow rate (0.17–17 ml/min per cartridge) and pathway (perfusion through and around the constructs);
- precise control over oxygen concentration in culture medium (0% to 95% saturation, $\pm 0.5\%$) and pH (± 0.05); and
- bench-top design that eliminates the need for housing within an incubator.

Mechanical stimulation induced cell alignment in the direction of the resulting force (concurrent axial and torsional strain over 21 days of culture) (Figure 3*A*), in contrast to random cell orientation in unstimulated controls (Figure 3*B*) (42), and fostered the expression of collagen type I and type III and tenascin C (Figure 4). At the same time, neither upregulation of bone nor cartilage-specific cell markers was observed. Importantly, tension and torsion, without specific inducers of ligament differentiation, directed MSC differentiation into a ligament cell lineage in preference to alternative paths (i.e., bone or cartilage).

Cell function (e.g., proliferation rate, differentiation), ligament structure (e.g., amounts and distributions of tissue components, ultrastructural organization), mechanical behavior, and expression of ligament-specific markers are expected to change over the course of tissue cultivation. These changes are likely to depend on the developmental stage of the tissue and the environmental conditions (i.e., biochemical and mechanical signals) during cultivation. The model system allows systematic variation and quantitative assessment of environmental conditions and cell/tissue responses, thus enabling quantitative studies of in vitro tissue development.

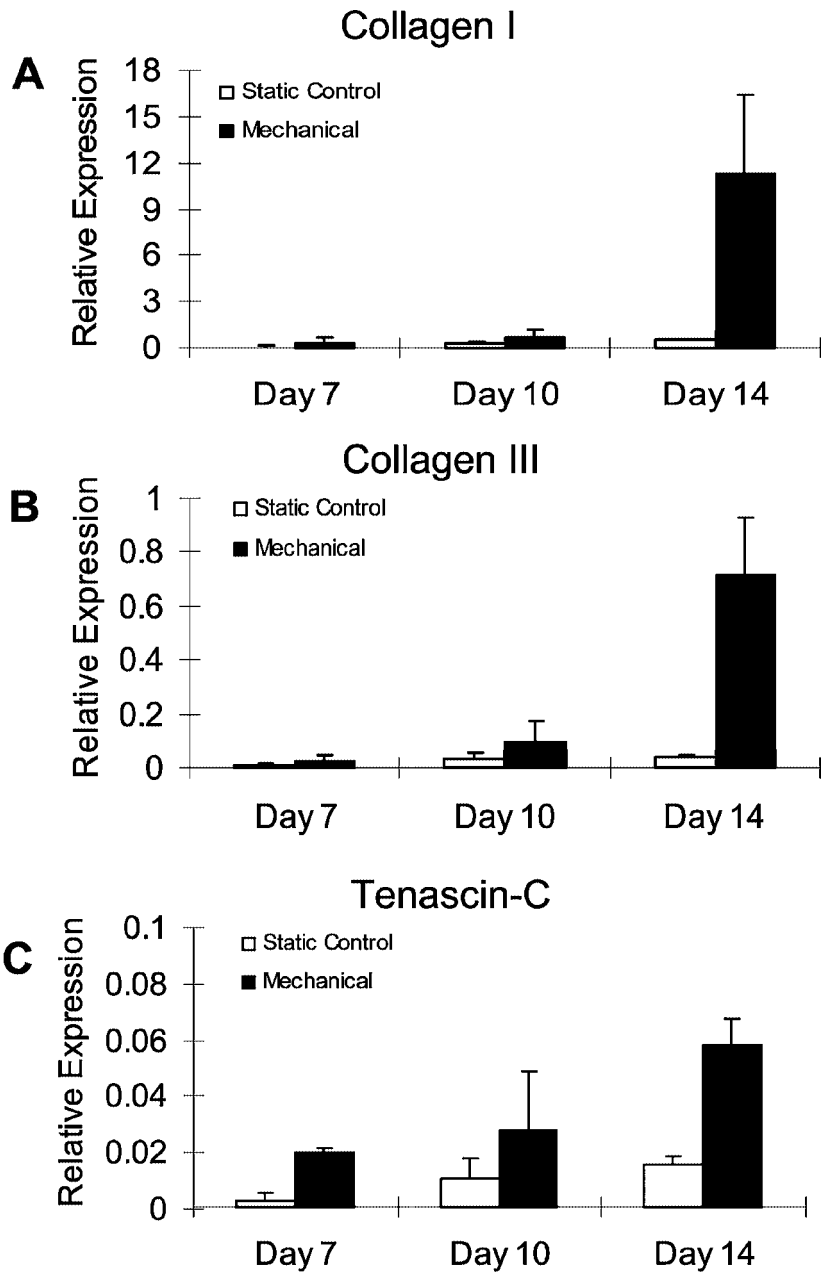

Figure 4 Mechanical stimulation fostered cell differentiation: reverse transcription–polymerase chain reaction (RT-PCR) marker transcripts for (*A*) collagen I, (*B*) collagen III, and (*C*) tenascin C increased with time in culture and were higher for stimulated than nonstimulated ligaments.

Ligament-specific marker transcripts (collagen types I and III, tenascin C) increased with time in culture and were at all times higher in mechanically stimulated than nonstimulated ligaments, as determined by real-time RT-PCR (Figure 4). Maximum cell density was achieved 10 days post seeding, whereas maximum ligament-specific matrix transcription was observed after 14 days of culture, suggesting a transition from a proliferative into a differentiation state. Importantly, cells and extracellular matrix of mechanically stimulated ligaments aligned in the direction of loading and infiltrated the scaffold forming a multilayered tissue. Quantitative real-time RT-PCR of collagen types I and III and tenascin-C normalized to glyceraldehyde-3-phosphate dehydrogenase-indicated ligament-specific responses of MSC cultured on the six-cord matrix for 14 days (42). Expression of collagen type I was approximately one third of that in torn human ACLs. Furthermore, the ratio of collagen I to III exceeded that of normal and torn human ACLs, indicating a ligament-specific response and not that of scar tissue (e.g., high levels of collagen type III transcription).

The application of mechanical stimuli prior to uniform matrix coverage inhibited cell growth and matrix deposition. In contrast, dynamic axial and torsional strains applied after five days of static culture increased cell density and enhanced the alignment of cells and extracellular matrix along the scaffold fibers. This is consistent with previous results for other engineered tissues. For example, mechanical compression enhanced the synthesis of cartilaginous tissue matrix by chondrocytes cultured in agarose gels by amounts that increased as more matrix deposits occurred around the cells (133, 134). The effects of the same load can thus differ for different developmental stages of engineered ligaments and different regions within the same tissue sample.

In Femor et al. (67), a transition of cultured ACL fibroblasts from proliferation to collagen matrix synthesis occurred at days 7–9. Because of these findings and the data presented in Figure 4, in which we observed a 20-fold increase in collagen type I production in 14 days as well as upregulation of ligament specific markers, the development of structural and functional properties of engineered ligaments should be studied at 7-, 14-, and 28-day periods to gauge the time-dependent influences on proliferation (1 week), marker expression and differentiation (2 weeks), and the development of ligament integrity, structure, and function (4 weeks).

SUMMARY

Tissue engineering is emerging as a significant clinical option to address tissue and organ failure by implanting biological substitutes of the compromised tissues. When compared to the transplantation of cells alone, engineered tissues offer the potential advantage of immediate functionality. The high incidence of ACL failures, lack of capacity for self-repair, and limitations of current treatment options have driven the research into ligament tissue engineering as a new option. An envisioned scenario of clinically relevant tissue engineering of ligaments involves

the use of autologous cells, biodegradable scaffolds designed to serve as structural and logistic templates of tissue development, and bioreactors designed to control the cellular environment and provide the necessary biophysical regulatory signals. Engineered tissues can also serve as high-fidelity models for controlled studies of cell function and tissue development under normal and pathological conditions. We discussed the requirements for the ACL reconstruction, the available treatment options and their limitations, and focused on one advanced cell-scaffold-bioreactor system for tissue engineering of ligaments. When cultured on biomaterial scaffolds with architectural and mechanical properties resembling those of native ligaments, and with the application of dynamic loading, human bone marrow–derived mesenchymal stem cells were induced to assemble ligament-like tissue structures. The possibility of using biophysical regulatory signals to direct and modulate cell differentiation and tissue assembly can form a basis for tissue engineering of custom-designed, functional ligaments. However, a lot remains to be learned, and several risks and uncertainties surround the development of an autologous human tissue-engineered ACL. It is expected that coordinated in vitro and in vivo studies, involving the use of systems similar to those described in this article, will help define the exact conditions and duration of ligament cultivation for a clinically optimal outcome.

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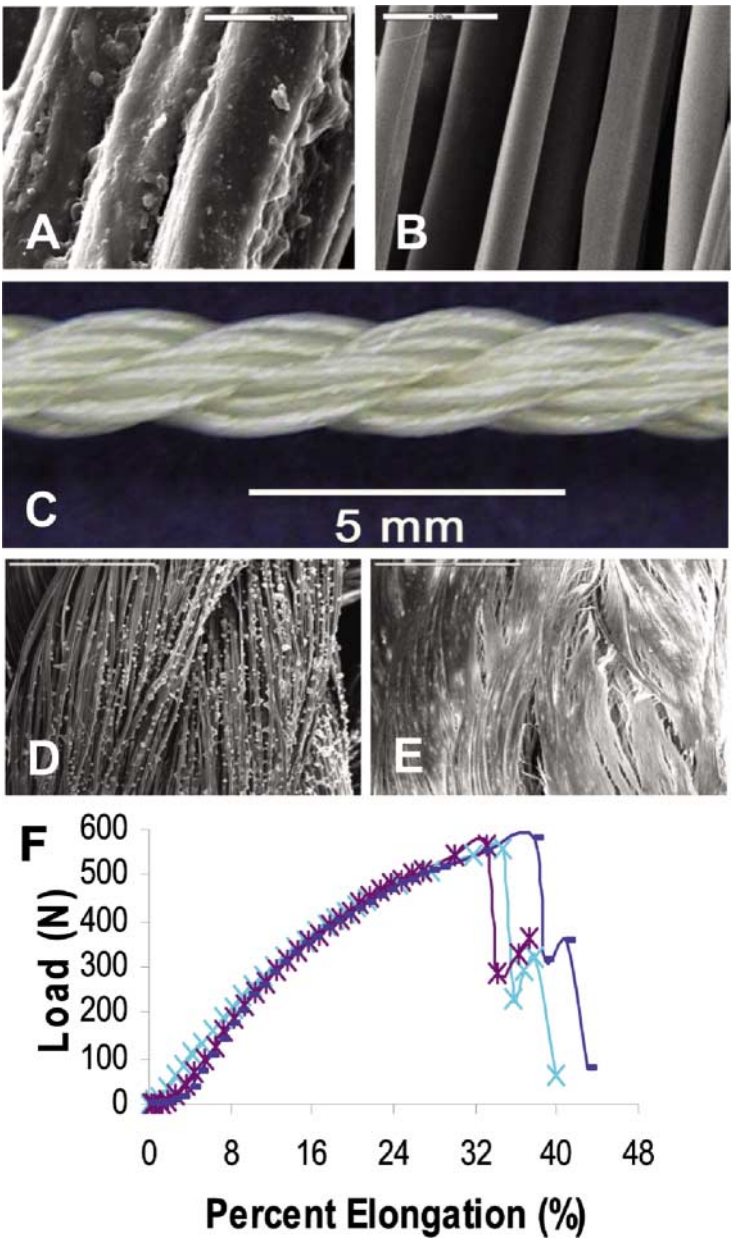

Figure 1 Custom-designed silk scaffolds. (*A*) Virgin silk containing a sericine coat, (*B*) processed silk fibers, (*C*) 6-cord wire-rope silk scaffold, (*D*,*E*) scanning electron micrographs of human MSC (*D*) after 30 min of seeding on scaffolds and (*E*) after 14 days of cultivation. (*F*) Load-elongation curves for three independently made scaffolds [from Altman et al. (80), with permission of Elsevier Ltd.].

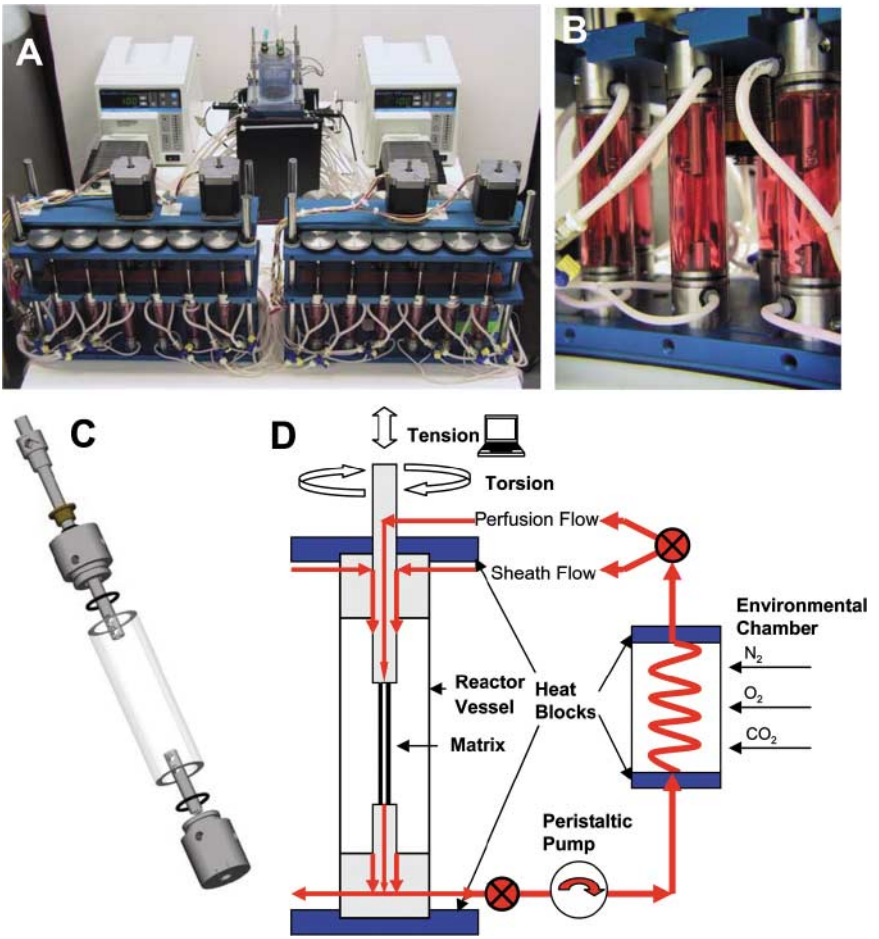

Figure 2 Bioreactor system for ligament tissue engineering. (*A*) Functioning bioreactor system consisting of two units, each with 12 cartridges that can be used concurrently (e.g., 12 with loading, 12 without loading). (*B*) Cartridges containing an anchored silk scaffold during culture with human MSC. (*C*) Exploded view of the cartridge assembly. (*D*) Schematic illustration of one cartridge with its flow loop and anchors for the transmission of mechanical forces (from Altman et al. (52), with permission of ASME).

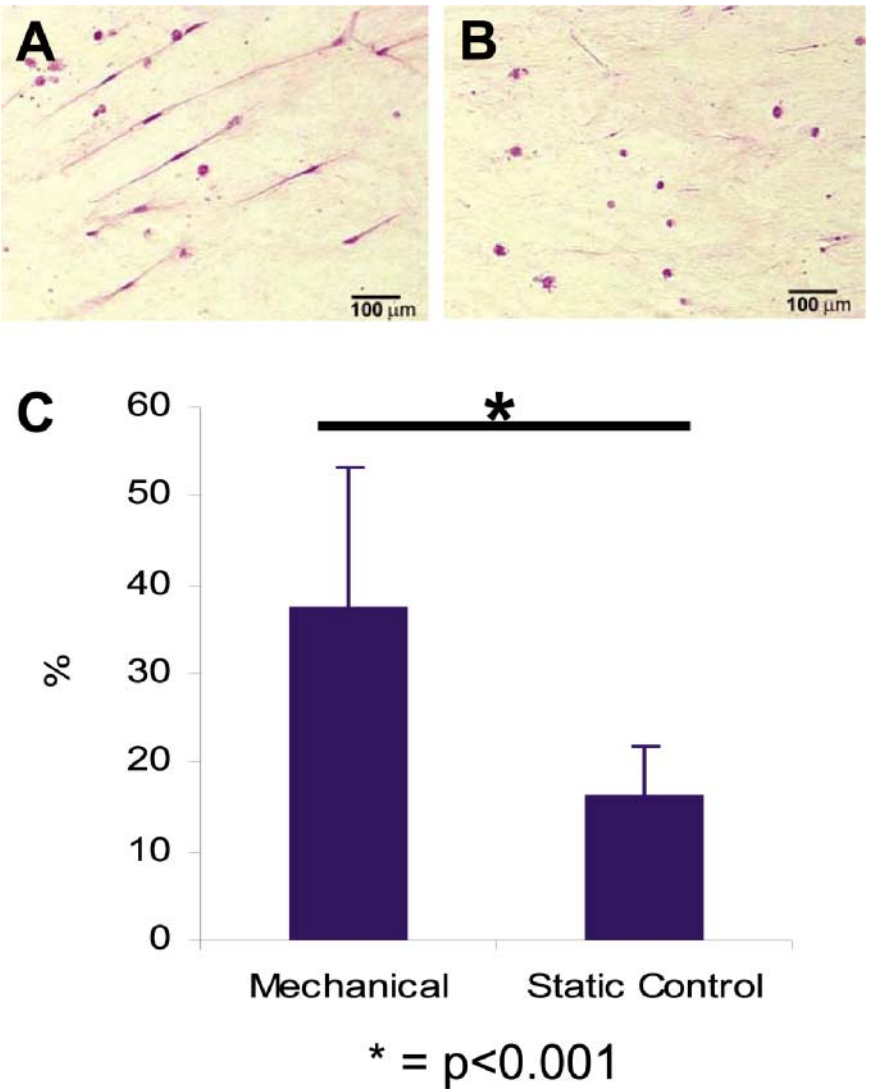

Figure 3 Mechanical stimulation fostered cell alignment. Dynamic tension and torsion applied over 21 days of culture induced MSC in collagen gel to align in the direction of force. (*A*,*B*) H&E of stimulated and nonstimulated constructs, respectively. (*C*) Mechanical stimulation caused a 2.5-fold increase ($p < 0.001$) in cell alignment. From Reference 42.

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