

Interface Tissue Engineering and the Formulation of Multiple-Tissue Systems

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Abstract Interface tissue engineering is an exciting field which focuses on the development of tissue engineered grafts capable of promoting integration between different types of tissue and between the implant and surrounding tissue. Focusing on interface tissue engineering, and using the insertion site between the anterior cruciate ligament and bone as an example, this chapter discusses strategies in soft tissue to bone integration as well as current tissue engineering efforts in this area. This review begins with the clinical significance of this problem, followed by a review of existing fixation methods, and tissue engineering efforts aimed at addressing this critical issue. The development of multi-phased scaffolds designed for the replacement of more than one type of tissue, as well as novel in vitro co-culture systems will be introduced. Future directions in the field of interface tissue engineering will also be discussed.

Keywords Co-culture · Interface tissue engineering · Ligament–bone insertion · Scaffolds

Abbreviations

3-D	Three-dimensional
ACL	Anterior crucial ligament
BG	45S5 bioactive glass
hBMSCs	Human bone marrow stromal cells
GAG	Glycosaminoglycan
PLAGA	Poly(lactide-co-glycolide)
SEM	Scanning electron microscopy

1**Introduction**

A significant challenge in orthopedic tissue engineering lies in the integration of soft tissue with bone tissue. The establishment of a continuous interface is critical to the long-term success of implant systems intended for the replacement and regeneration of cartilage, ligaments and tendons. The interface or insertion connects bone and soft tissue, and its primary function is to redistribute the complex load and strains between the two types of tissue. It is also believed to act as a conduit for nutrients and cells for otherwise poorly vascularized soft tissues such as ligament or cartilage. After surgical repair or reconstruction of soft tissue and during the initial healing period, the interface between the graft and bone is mechanically the weakest point of the graft. Unfortunately the existing soft-tissue grafting systems are unable to restore both the structural and functional characteristics of the interface between bone and soft tissue.

In the past decade, tissue engineering has emerged as an alternative approach to implant design and tissue regeneration. Significant advancements have been achieved in the development of tissue engineering technologies, and several prototypes of these grafts have successfully undergone both animal and clinical trials. Design methodologies developed from current tissue engineering efforts can be readily applied to regenerate the interface between tissue types. In this review, interface tissue engineering is defined as the application of tissue engineering principles to develop scaffold systems capable of facilitating the integration between different tissue types, as well as between the biomaterial and surrounding tissue. In this chapter, both research strategies and current tissue engineering efforts in facilitating bone and soft-tissue integration will be discussed. Focusing on the regeneration of the ligament–bone interface, this chapter will describe the clinical significance of this problem, existing fixation methods, and tissue engineering efforts aimed at addressing this challenge. The tissue engineering strategies outlined in this chapter may be applied to a variety of tissue–tissue systems, with clinical relevance in the regeneration of cartilage-to-bone, tendon-to-bone, and ligament-to-bone insertions for both orthopedic and dental applications.

2 Background

2.1 Anterior Cruciate Ligament (ACL) Injuries and Reconstruction Grafts

The anterior cruciate ligament (ACL) consists of a band of regularly oriented, dense connective tissue that spans the junction between the femur and the tibia. It participates in knee motion control, acts as a joint stabilizer, and serves as the primary restraint to anterior tibial translation. The ACL is the most frequently injured knee ligament [1], and approximately 75 000 ligament repair and reconstruction procedures are performed annually in the United States [2]. Due to its intrinsically poor repair potential, the ACL does not heal upon injury and surgical intervention is often required. If untreated, injuries to the ACL will lead to functional impairment, secondary meniscus tear and the development of joint arthrosis [3, 4]. Clinically, autogenous graft based on either bone-patellar tendon-bone or hamstring tendon graft is the preferred system for ACL reconstruction. This is primarily due to a lack of alternative solutions. Synthetic ACL grafts include carbon fibers [5], Leeds-Keio ligament (polyethylene terephthalate) [6], the Gore-Tex prosthesis (polytetrafluoroethylene) [7], the Stryker-Dacron ligament prosthesis, which is made of Dacron tapes wrapped in a Dacron sleeve [8], and the Gore-Tex ligament augmentation device made from polypropylene [9]. These grafts have exhibited good short-term results but encounter clinical failure in the long term, as they are unable to replicate the mechanical strength and structural properties of human ACL tissue [10–12]. Limitations associated with long-term ligament repair include plastic deformation of the replacement material, weakened mechanical strength compared to the original structure and fragmentation of the replacement material due to wear [12].

Although autografts are superior to allografts, xenografts, and synthetic alternatives, ACL reconstruction based on these grafts has resulted in the loss of functional strength from the initial implantation time, followed by a gradual increase in strength that never reaches the original magnitude [13–16]. Despite its clinical success, the long-term performance of autogenous ligament substitutes is dependent on a variety of factors including the structural and material properties of the graft, the initial graft tension [17–20], the intra-articular position of the graft [21, 22], as well as graft fixation [23, 24]. Side effects such as tendonitis, arthritis, muscle atrophy and donor-site morbidity often occur. Moreover, there is often a lack of hamstring tendon graft integration with host tissue, in particular at the bony tunnels, which contributes to the suboptimal clinical outcome of these grafts [10, 11, 14]. The fixation sites at the tibial and femoral tunnels, instead of the isolated strength of the hamstring tendon graft, have been identified as the mechanically weak points in the reconstructed ACL [23, 24]. Poor interfacial integration may lead

to the enlargement of the bone tunnels, and in turn compromise the long-term stability of the graft.

There is a steady rise in reported ACL injuries due to an aging and increasingly active population, exacerbated by the higher number of failures associated with current treatment modalities that require revision surgeries to correct. A disproportional number of ACL injuries occur in the teen- to middle-aged (15–35 years old) segments of the general population [2]. The relatively high level of physical activity required and desired by the active lifestyle of these individuals places extensive demands on ACL grafts, especially in terms of their fixation strength and healing potential, both immediately after surgery and during intensive rehabilitation. Furthermore, the number of revision surgeries has increased significantly in the past few years [25], and no surgical procedure has been shown to restore knee function completely without associated side-effects, such as long recovery periods, muscle atrophy, tendonitis, and arthritis.

It is clear that graft fixation is a critical weakness that severely limits the initial mechanical properties of the ligament substitutes utilized in the clinical setting. The long-term success of the reconstructed ACL is a function of the type and integrity of the initial graft fixation to host bone tissue. Thus optimized functional treatment and fixation modalities in ACL reconstruction must be developed to meet the demands of an aging yet still active population.

2.2

Current Fixation Methods Used in ACL Reconstruction

Increased emphasis has been placed on graft fixation, as the post-surgery rehabilitation protocols require the immediate ability to exercise full range of motion and reestablish neuromuscular function and weight bearing [26]. During ACL reconstruction, the bone-patellar tendon-bone or hamstring tendon graft is fixed into the tibial and femoral tunnel using a variety of fixation techniques. Fixation devices range from staples, screw and washer, press fit Endobutton®, to interference screws, accompanied by a myriad of surgical techniques for utilizing these devices. Traditionally, the bony or soft tissue is fixed within the bone tunnel or on the periosteum at a distance from the normal ligament-insertion site. The femoral fixation differs from the fixation methods utilized in the tibial insertions. The EndoButton®, and the Mitek®, anchor are utilized for the fixation of the femoral insertions, and staples, interference screws, or interferences screws combined with washers are used to fix the graft to the tibial region. The integration of quadruple semitendinosus-gracilis tendon grafts with bone is critical to the success of the indirect and direct fixation methods practised in the clinical setting.

In the past few years, the interference screw has emerged as the standard method for graft fixation. The interference screw, about 9 mm in diameter

and at least 20 mm in length, is used routinely to secure tendon to bone and bone to bone in ligament reconstruction. Both metallic and polymeric interference screws have been utilized in ACL reconstruction. Surgically, the knee is flexed and the screw is inserted from the para-patellar incision into the tibial socket, and the tibial screw is screwed just underneath the joint surface. After tension is applied to the femoral graft and the knee is fully bent, the femoral tunnel screw is inserted via the anteromedial arthroscopy portal. This procedure has been reported to result in stiffness and fixation strength levels adequate for daily activities and progressive rehabilitation programs [27]. A large factor in the reported success levels associated with interference fixation can be attributed to the fact that implant fixation is now possible near the normal insertion zone [26].

While the use of interference screws has improved the fixation of ACL grafts, mechanical considerations and biomaterial-related problems associated with existing screw systems have limited the long-term functionality of the ligament substitutes [28]. Screw-related laceration of either the ligament substitute or bone plug suture has been reported [29]. In certain cases (3%), tibial screw removal was necessary to reduce the pain suffered by the patient [30]. Stress relaxation, distortion of magnetic resonance imaging, and corrosion of the metallic screws have led to the development of biodegradable screws based on poly- α -hydroxy acids [31, 32]. A second surgery may be required to remove the metallic screws [33]. While lower incidence of graft laceration was reported for biodegradable screws [29], the highest interference fixation strength of the grafts to the tibia and femur tunnels is reported to be 475 N [34], which is significantly lower than the attachment strength of ACL to bone. When tendon-to-bone fixation with polylactic-acid-based interference screws was examined in a sheep model, intraligamentous failure was reported by six weeks [35]. Fixation strength was also found to be dependent on the quality of bone (mineral density) and bone compression. Moreover, while most biodegradable screws provide similar fixation strength as that of titanium interference screws in the fixation of bone-tendon-bone grafts [36–38], the fixation strength of degradable polymers during soft-tissue-to-bone fixation have not been fully characterized. Large-scale immune responses have also been observed for polyglycolide-based interference screws [39]. Clearly, optimal fixation of ACL grafts remains a significant clinical challenge.

2.3

Tendon-to-Bone Healing After ACL Reconstruction Surgery

Understanding the biology of tendon-to-bone healing is essential for developing an optimal rehabilitation protocol for patients who undergo ACL reconstruction surgery, as well as for the design of new fixation devices for soft-tissue-to-bone incorporation. The biochemical composition and func-

tion of the interface between tendon and bone during ACL reconstruction is poorly understood. In a study by Panni et al. [40], a persistent fibrocartilage region was only seen in the fast-healing group, suggesting that this layer may contribute to the eventual formation of direct insertions from tendon to bone. Thomopoulos et al. [41] examined matrix gene expression during healing in a rat rotator cuff-injury model. In situ hybridization studies revealed that there exists a zonal-dependent change in gene expression patterns at the insertion site. The expression levels of type I and XII collagen and aggrecan remained above normal, while the expression of collagen type X and decorin decreased over time. In the natural tendon-to-bone insertion, as in the case of the supraspinatus tendon, the zonal distribution is similar to those found in ACL to bone insertions. However, the biochemical content of the four regions may be significantly different, particularly relating to the type of collagen and matrix molecules present at the interface. The biochemical difference arises from the fact that tendons differ from ligaments in both structural and mechanical properties.

For bone-patellar tendon-bone grafts, bone-to-bone integration with the aid of interference screws is the primary mechanism facilitating graft fixation. Several groups have examined the process of tendon-to-bone healing for hamstring tendon-based ACL grafts [35, 40, 42–48]. Blickenstaff et al. evaluated the histological and biomechanical changes during the healing of a semitendinosus autograft for ACL reconstruction in a rabbit model [48]. Graft integration occurred by the formation of an indirect tendon-to-bone insertion at 26 weeks. However, large differences in graft strength and stiffness remained between the normal semitendinous tendon and ACL after 52 weeks of implantation. In a similar model, Grana et al. [47] reported that graft integration within the bone tunnel occurred by an intertwining of graft and connective tissue and anchoring of connective tissue to bone by collagenous fibers and bone formation in the tunnels. The collagenous fibers had the appearance of the Sharpey's fibers seen in an indirect tendon insertion. Rodeo et al. examined tendon-to-bone healing in a canine model by transplanting digital extensor tendon into a bone tunnel within the proximal tibial metaphysis. A layer of cellular fibrous tissue was found between the tendon and bone, and this fibrous layer matured and reorganized during the healing process. As the tendon integrated with bone through Sharpey-like fibers, the strength of the interface increased between the second and the twelfth week after surgery. The progressive increase in strength was correlated with the degree of bone ingrowth, mineralization, and maturation of the healing tissue [42].

The majority of the tendon-to-bone healing studies examined extra-articular models or fixation far away from the joint line. Panni et al. reported a dependence in the rate of graft healing (the formation of direct collagen-fiber-mediated bone-tendon junction) to the site of graft placement [40]. To approximate the original anatomy of the ACL, it is believed that fixation should be as close to the joint line as possible [49]. Recently, Weiler and as-

sociates examined tendon-to-bone healing when the graft was fixed anatomically using biodegradable poly(D,L-lactide) interference screws in a sheep model. A fibrous interface between the graft tissue and the bone tunnel was only partially developed, which was in contrast to studies in which non-anatomic fixation was used. It was reported that hamstring tendon to bone healing during compressive interference screw fixation led to partial reestablishment of the transitional zones or mineralized cartilage between soft tissue and bone at 24 weeks. Direct contact healing of the implant between the graft and the bone surface may be possible when compression is applied during healing.

The above studies have provided valuable insight into the process of tendon-to-bone healing, and have demonstrated that in-depth examination of the insertion zone is needed. It is important to note that, in most cases, tendon-to-bone healing with and without interference fixation does not result in the complete reestablishment of the normal transition zones of the native ACL–bone insertions. This inability to fully reproduce these structurally and functionally distinct regions at the junction between graft and bone is detrimental to the ability of the graft to transfer mechanical stress across the graft proper and will lead to sites of stress concentration at the junction between soft tissue and bone. A systematic characterization of the ACL–bone insertion zone will not only provide a much needed reference frame to compare tendon–bone healing, but will also facilitate the design of novel fixation devices aimed at promoting soft-tissue-to-bone healing.

3

Strategies for Interface Tissue Engineering

As discussed above, ACL injuries do not heal effectively and surgical intervention is required. There is an increase in clinical utilization of hamstring tendon-based ACL graft due to the donor site morbidity associated with bone-tendon-bone grafts. Despite their distinct advantages over synthetic substitutes, autologous soft tissue grafts have a relatively high failure rate. The primary cause for the high failure rate of these grafts is the lack of consistent graft integration with the subchondral bone within the tibial and femoral tunnels. The site of tendon contact in the femoral or tibial tunnels represent the weakest point mechanically, in the early postoperative healing period [50], causing the success of ACL reconstructive surgeries to be heavily dependent on the extent of soft-tissue fixation to bone.

There has been increasing interest in finding tissue engineering solutions to soft-tissue graft to bone fixation. To develop a functional interface, several factors must be taken into consideration. First, the structural and mechanical properties of the insertion zone must be characterized. In the functional tissue engineering paradigm outlined by Butler et al. [51], the first two critical

parameters determining the success of any tissue engineering effort are the determination of the material properties of the tissue to be replaced, followed by the measurement of *in vivo* stresses and strains in the native tissue. Neither the structural nor the mechanical properties of the insertion zone has been fully characterized. Compositional and structural distributions in the native tissue are likely correlated with functionality. Therefore, both qualitative and quantitative examinations of the interface will permit the identification and selection of the critical design parameters for scaffold design, as well as providing insight into the structure–function relationship at the interface. The ideal scaffold for the interface should be able to support the growth and differentiation of relevant cell types, while promoting the formation of multiple tissue types. The scaffold system should exhibit a gradient of structural and functional properties mimicking those of the native insertion zone. Finally, the tissue engineered graft has to be incorporated into the current design of ligament scaffolds or aid the integration of existing grafting systems for ACL repair. The following sections will review current knowledge of the structure and material properties of the ACL to bone insertion zone, as well as tissue engineering efforts focused on regenerating the soft-tissue-to-bone interface.

3.1

Structure and Biochemical Properties of the Insertion Site

Two insertion zones can be found in the human ACL: one at the femoral end and another located at the tibial attachment site. The ACL is attached to mineralized tissue through the insertion of collagen fibrils and there exists a gradual transition from soft tissue to bone. The tibial insertion zone differs structurally from the femoral insertion site, and the femoral attachment exhibits a more direct insertion of the collagen bundle into the cartilage and subchondral bone matrix. The long axis of the femoral attachment is tilted slightly forward from the vertical, and the posterior convexity is parallel to the posterior articular margin of the lateral femoral condyle. The attachment of ACL to the tibial plateau is wider than its femoral counterpart, and the ligament is inserted to the front of and lateral to the anterior tibial spine. The femoral attachment area in the human ACL was measured to be $113 \pm 27 \text{ mm}^2$ and $136 \pm 33 \text{ mm}^2$ for the tibia insertion [52].

Examination of morphological changes and distribution of types I and II collagen at the ACL–bone insertion sites during development will guide any reconstructive approach of the interface *in vitro* [53]. During development of ligament insertions in the rat knee, highly cellular ligaments insert to epiphyseal cartilage, which is in turn inserted to subchondral bone. Ossification of the epiphyseal cartilage occurs and hypertrophic chondrocytes are found near the insertion to bone. Subchondral bone formation becomes more compact and the thickness of fibrocartilage regions increases. Thus, epiphyseal cartilage resorption occurs simultaneously with osteogenesis. In addition, lig-

ament metaplasia occurs as cartilage resorption and osteogenesis progresses, contributing to the increasing thickness of fibrocartilage during the development of ligament insertions [53, 54].

Unlike the insertion between tendon and bone, the interface between ACL and bone has not been examined in detail. It is known that, structurally, the transition from ACL to bone consists of four distinct zones: ligament, fibrocartilage, mineralized fibrocartilage, and bone [53, 55–59]. As seen in Fig. 1, the first zone, which is the ligament proper (L), is composed of solitary spindle-shaped fibroblasts aligned in rows, and is embedded in parallel collagen fibril bundles of 70–150 μm in diameter. Primarily type I collagen makes up the extracellular matrix, and type III collagen, which is composed of small reticular fibers, is located between the collagen I fibril bundles. As shown in both Figs. 1 and 2, the second zone is composed of ovoid-shaped chondrocyte-like cells. The cells do not lie solitarily, but are aligned with 3–15 cells per row. Collagen fibril bundles are not strictly parallel and are much larger than those found in zone 1. Type II collagen is found within the pericellular matrix of the chondrocytes, with the matrix still predominantly consisting of type I collagen. This zone is primarily avascular and the primary sulfated proteoglycan is aggrecan. The next zone is mineralized fibrocartilage. For this region, chondrocytes appear more circular and hypertrophic, surrounded by larger pericellular matrix distal from the ACL [56]. Type X collagen, a specific marker for hypertrophic chondrocytes and subsequent mineralization, is detected and found only within this zone [55]. The inter-

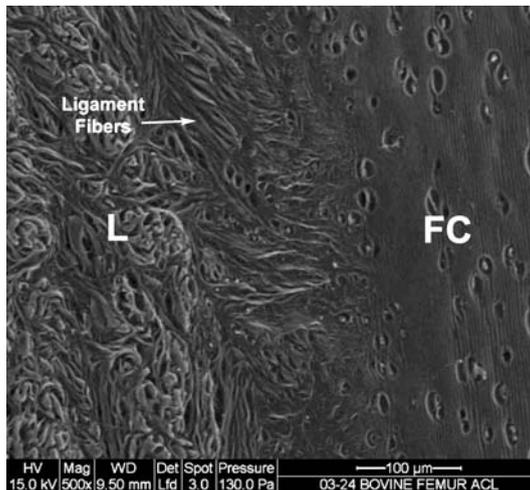


Fig. 1 Scanning electron micrograph of a sample cross section of the bovine ACL–femur insertion site, focusing on the insertion between ligament (L) to the fibrocartilage region (FC). Note the presence of collagen fibers, which directly insert into the FC region, and the ovoid chondrocytes in the fibrocartilage zone to the right. (500 \times)

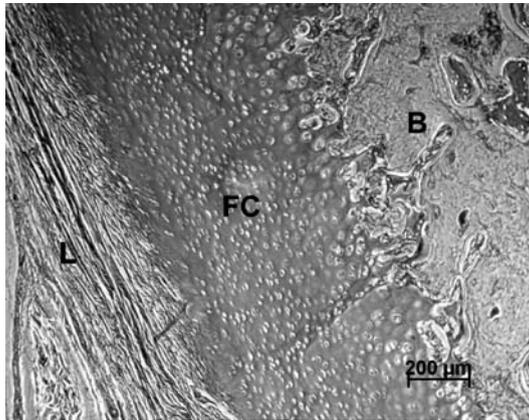


Fig. 2 Histological analyses of the bovine ligament-to-bone insertion site reveal the presence of the multiple-tissue zone and various cell types. This is an image using the modified Goldner's Masson trichrome stain of a cross section of the ACL-bone femoral insertion site. The nucleus is in black, the bone region is stained red while the soft tissue is stained green. The ligament (L), fibrocartilage (FC), and bone (B) regions can be seen. (5 \times)

face between mineralized fibrocartilage and subjacent bone is characterized by deep interdigitations. Increasing number of deep interdigitations is positively correlated to increased resistance to shear and tensile forces during development of rabbit ligament insertions. The last zone is subjacent bone and the cells present are osteoblasts, osteocytes and osteoclasts. The predominant collagen is type I, and fibrocartilage-specific markers such as type II collagen are no longer present.

A limited number of studies have examined the biochemical or mechanical properties and the development of each zone at the ACL-bone interface [53, 55–58, 60]. Types II, IX, and X collagen were detected within the fibrocartilaginous zone at the bovine medial collateral ligament and the ACL femoral insertion zones [55]. Type I collagen staining was reported to be lower in the insertion zone compared to the ligament proper and the bone [55]. Moreover, the distribution of type II collagen was dependent on proximity to bony areas, with type II found primarily away from the mineralized ends of the interface. Variations in collagen content in the ligament bundles are believed to be related to the differences in mechanical properties and forces experienced by these tissues [61]. While the role of fibrocartilage at this interfacial zone is not yet well understood, it may promote the integration of ligamentous tissue with bone, while responding to functional loads specific to the interface. This will be explored further in the next section.

These microscopic and qualitative examinations of the insertion zone have shed unique insight on the structural and biochemical organization of the interface. There is, however, a lack of quantitative understanding of the struc-

tural variations existing in the insertion zone, in particular in terms of the collagen distribution, collagen ratios (types I/III, I/II, I/X), fibril diameters, and cellular distribution. A systematic characterization of the interface from nanoscale to macroscale levels can yield the much needed design parameters, based on which a new generation of graft-to-bone fixation devices can be engineered. This new understanding will in turn aid the design of new ACL grafts and contribute on a broader scale to current efforts in promoting graft fixation.

3.2

Mechanical Properties of the ACL–Bone Interface

There is limited knowledge regarding the material properties of the bone–ACL insertion zones, and the specific factors determining their repair and regeneration. The above described zonal variations from soft to hard tissue at the interface are believed to facilitate a gradual change in stiffness and may prevent the build up of stress concentrations at the attachment sites [62]. However, direct measurement of the stress and strain behavior at the insertion zones has been difficult, as these regions are less than 500 μm to 1 mm in length. Consequently, there is limited data available in the literature which describes the material properties of the interface between ACL and bone. Inferred differences in material properties have been reported. Butler et al. evaluated the strain distribution within the ACL by performing failure tests of human ACL sub-bundles [63]. A spatial variation in strain was observed along the length of the ACL, with the largest strains measured at the insertion sites. In addition, it was shown that the anterior ACL sub-bundles have a significantly larger strain-energy density and failure stress values compared to the posterior ACL bundles [63]. These observations lead the authors to suggest that inhomogeneity should be introduced into the design of ligament replacement grafts.

It is well known that the weakest region between two materials of different mechanical properties is located at their interface, where the development of stress concentrations can lead to failure. When the mechanical properties of ACL were examined in a bone–ligament–bone complex, Woo et al. reported that the highest deformation occurred near or at the insertion zones [62]. The presence of a transition region comprised of fibrocartilage and mineralized fibrocartilage instead of an abrupt change from ligamentous tissue to bone in the native ACL would minimize the formation of stress concentrations in the region. In the study by Butler et al. which examined location-dependent variations in ACL mechanical properties, most of the ligaments were reported to fail at the insertion during failure tests [63]. Gao et al. reported that histological analysis revealed that avulsion fracture at or near the cement line of the subchondral bone was the most commonly observed mode of failure for ACL–bone complexes [64].

It is interesting to note that the insertion zone is dominated by non-mineralized and mineralized fibrocartilage, which are tissues adept at transmitting compressive loads. Mechanical factors may be responsible for the development and maintenance of the fibrocartilaginous zone found at many of the interfaces between soft tissue and bone [65]. The fibrocartilage zone, with its expected gradual increase in stiffness, seems to be less prone to failure [64]. It has been suggested that the fibrocartilage zone balances out the bending that otherwise would have resulted in fatigue failure [66–68]. Benjamin et al. suggested that the amount of calcified tissue in the insertion zone may be positively correlated to the force transmitted across the calcified zone [67]. Using simple histomorphometry techniques, Gao et al. determined that the thickness of the calcified fibrocartilage zone was 0.22 ± 0.7 mm and that this was not statistically different from the tibial insertion zone [54].

These observations suggest that the structure of the ACL–bone interface may be correlated with the mechanical properties and related functionality of the insertion zone. Therefore, reproducing the non-calcified and calcified fibrocartilage-rich interface in vitro on an ACL graft-fixation device may promote its integration with bone in vivo. It is expected that there will be a regional dependence of mechanical properties, varying from the ligament proper to the trabecular bone.

3.3

Design Parameters for an Interface Tissue Engineered Graft

In the past decade, tissue engineering has emerged as a possible solution to the problems associated with existing grafts for ACL reconstruction. It has the potential to provide improved clinical options through the in vitro generation of biologically based functional tissues for transplantation at the time of injury or disease. Tissue engineered ACL grafts are attractive as they exhibit the many advantages of autogenous grafts, without the associated limitations. With the advent of tissue engineering, several groups have reported on potential ACL constructs using collagen fibers, biodegradable polymers and composites. Brody et al. [69] examined the effects of canine fibroblasts seeded on knitted Dacron ligament prostheses prior to implantation. These modified prostheses demonstrated a more uniform and abundant encapsulation with connective tissue than unseeded prostheses. Dunn et al. developed skin fibroblast-seeded collagen scaffolds for ACL reconstruction [70, 71], and in vivo studies found that the tissue engineered scaffolds were viable after reimplantation into the donor rabbit. Altman et al. seeded human bone marrow stromal cells (hBMSCs) on modified silk-fiber-based scaffolds with predesigned mechanical properties similar to those of human ACL [72, 73]. It was reported that the hBMSCs readily differentiated in fibroblast-like cells and gene expression for type I and III collagen were

up-regulated. The fiber-based scaffold geometry promoted the alignment and growth of these stem cells, and the resultant silk construct supported the growth and differentiation of bone marrow stromal cells into ligament fibroblast-like cells.

As discussed above, the interface between graft and bone is the weakest point during the initial healing period, recent efforts in ACL tissue engineering have begun to take into account the need to promote graft integration. Goulet et al. [74] developed a bioengineered ligament model, where ACL fibroblasts were added to the structure and bone plugs were used to anchor the bioengineered tissue. Fibroblasts isolated from human ACL were grown on bovine type I collagen, and the bony plugs were used to promote the anchoring of the implant within the bone tunnels. Cooper et al. [75] and Lu et al. [76] developed a tissue engineered ACL scaffold using biodegradable polymer fibers braided into a 3-D scaffold. The scaffold is comprised of three regions, one middle section with higher porosity for ligament ingrowth, and two bony attachment regions with smaller pore size and lower porosity. This scaffold has been shown to promote the attachment and growth of rabbit ACL cells *in vitro* and *in vivo* [75–77].

The identification of relevant design parameters for ligament–bone interface tissue engineering is hindered by the lack of physiologic design parameters related to the native femoral and tibial insertion zones. In-depth understanding of the structural and material properties of the native insertion zone at the nanoscale, microscale, and macroscale level is a prerequisite to formulating design parameters for a tissue engineered interface. Based on known structure–function relationships, it will be critical to mimic the architecture, as well as chemical and biological compositions of the insertion zone.

3.4

Multi-Phased Scaffold System for Interface Tissue Engineering

Scaffold design is critical in interface tissue engineering, since a supporting substrate is essential for maintaining mechanical strength, structural support, and for providing the optimal growth environment for tissue formation during the early stages of the repair process. While there are currently no reported studies directly examining the potential of multi-phased scaffolds for interface tissue engineering, our laboratory has begun to develop interfacial scaffolds aimed at regenerating the insertion site. With the native ligament–bone insertion zone as a reference point, we have formulated a multi-phased scaffold system with a gradient of chemical compositions, structural properties, and mechanical properties. Similar to the four transition zones found at the insertion site and in contrast to a homogenous scaffold, a scaffold with predesigned inhomogeneity may be able to sustain the distribution of complex stress and strain across the interfacial zones. By emulating the structural

distribution of the native ligament–bone insertion zone, functional interfaces based on multi-phased scaffolds may be able to support the growth and integration of multiple-tissue systems.

Our approach is to combine a novel composite system which will support bone formation and osteointegration, with a fiber-based scaffold system which will facilitate the growth of ligamentous tissue and the formation of an interface. This composite system will be based on a 3-D composite scaffold of ceramic and biodegradable polymers. Lu et al. [78] combined poly-lactide-co-glycolide 50:50 (PLAGA) and bioactive glass (BG) to engineer a degradable, three-dimensional composite (PLAGA-BG) scaffold with improved mechanical properties. This composite is selected as the bony phase of the multi-phased scaffold proposed here (Phase C) as it has unique properties as a bone graft. The PLAGA-BG composite integrates the advantages of the parent phases, while minimizing known limitations associated with each component. A significant advantage of the composite is that it is osteointegrative. No such calcium phosphate layer is detected on PLAGA alone, and currently, osteointegration is deemed a critical factor in facilitating the chemical fixation of a biomaterial to bone tissue. Another advantage of the scaffold is that the addition of bioactive glass granules to the PLAGA matrix results in a structure with a higher compressive modulus than PLAGA alone. The compressive properties of the composite approach those of trabecular bone. Therefore, in addition to being bioactive, the PLAGA-BG would lend greater functionality *in vivo* compared to the PLAGA matrix alone. Moreover, the combination of the two phases serves to neutralize both the acidic byproducts produced during polymer degradation and the alkalinity due to the formation of the calcium phosphate layer. Through hydrolysis reactions, PLAGA degrades into glycolic and lactic acids, the release of which can induce a biologically significant decrease in local pH. BG releases alkaline ions which produce an elevated local pH. By forming a composite of PLAGA and BG, the acidic and basic degradation products may be neutralized, and a physiological level pH can be maintained. The composite has also been shown to support the growth and differentiation of human osteoblast-like cells *in vitro* [78].

This interfacial scaffold has a layered structure, with one phase optimal for ligament tissue formation and the other optimal for bone formation. The intermediate region is the region where an interfacial zone may be developed through the interaction of ACL fibroblasts and osteoblasts. We believe that a multiple-tissue system is more relevant physiologically than a scaffold with homogenous properties and may in turn promote the fixation of soft tissue to bone. By implementing the appropriate zonal-dependent variations in cell type, density, and collagen distribution into the design of multi-phased scaffolds, functional biomimetic scaffolds may be developed. By co-culturing osteoblasts and ligament fibroblasts on a multi-phased scaffold system with a gradient of material properties, we can form graft systems comprised of

multiple tissues instead of just a single type of tissue. This novel scaffold system is currently been evaluated *in vitro* and *in vivo*, with promising initial results.

3.5

Development of In Vitro Co-Culture Models

Progressing through the four distinct zones which make up the native ACL insertion, several cell types can be identified, including ligament fibroblasts, chondrocytes, hypertrophic chondrocytes, osteoblasts, osteoclasts, and osteocytes. Cell-to-cell interactions may be critical in the development of a functional interface. Introduction of multiple cell types and novel co-culture systems will also be critical in facilitating the formation of the transition zones observed at the interface. To this end, the development of an *in vitro* multi-cell-type culture system will aid current efforts in interface tissue engineering. In addition, these model systems will augment our understanding of the developmental process of the insertion zones.

There are very few studies published in the literature describing co-culturing systems, especially in musculoskeletal systems. We first reported on an *in vitro* co-culture system of osteoblasts and chondrocytes, combining an osteoblast monolayer culture with a condensed micromass culture of chondrocytes [79]. It was hypothesized that osteoblast–chondrocyte interactions would lead to the development of an interfacial zone. The co-culture model permits immediate interaction between osteoblasts and chondrocytes, while maintaining the chondrogenic phenotype within the micromass. As shown in Fig. 3A, chondrocytes within the micromass exhibit spherical morphology while cells at both the surface (osteoblasts) as well as surrounding monolayer have spread. It was found that co-culture had no effect on type I collagen production by osteoblasts, but did delay mineralization. The effects of co-culture on chondrocytes were evident at the surface interaction zone, particularly in terms of glycosaminoglycan (GAG) and collagen production. Figure 3B shows that, after 14 days of culture, characteristic pericellular distribution of GAG was evident within the chondrocyte micromass region. No GAG production was observed in the osteoblastic monolayer. Co-culture and/or interactions with chondrocytes may have delayed osteoblast-mediated mineralization. The expression of specific interfacial markers such as type X collagen was confirmed in the co-cultured samples, which are preliminary confirmations of our hypothesis that co-culture may lead to the development of an interfacial zone between these cells.

Currently, there are no reported studies in the literature on neither the co-culture of ligament fibroblasts with osteoblasts, nor on the *in vitro* regeneration of the bone–ligament interface. Lu et al. [80] reported on initial observations of an osteoblast–ligament fibroblast co-culture. As seen in Fig. 4, after 14 days of co-culture, both human ligament fibroblasts and osteoblasts

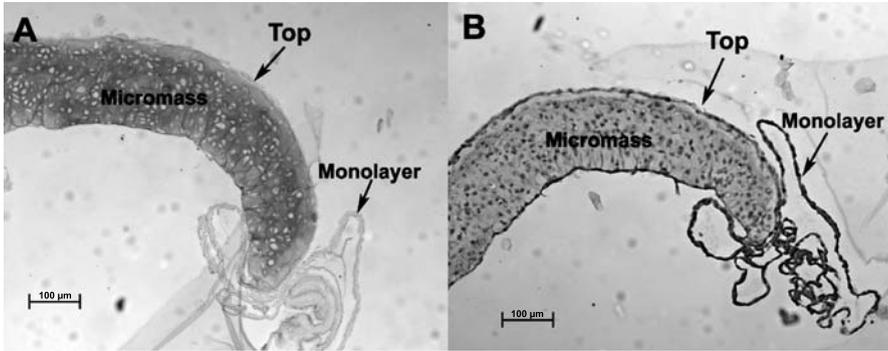


Fig. 3 Co-culture model of osteoblasts and chondrocytes. An osteoblastic monolayer is cultured on a micromass monolayer. Hematoxylin and Eosin stain (**B**) of a co-cultured micromass at day 14. Cells within the micromass exhibits spherical morphology while cells at both the surface (osteoblasts) as well as surrounding monolayer have flattened. Alcian Blue stain (**A**) of the cross section of a co-cultured micromass after 14 days of culture revealed the characteristic pericellular distribution of glycosaminoglycans (GAG) within the micromass region. No GAG production was observed in the osteoblastic monolayer. (10 \times)

proliferated and expanded beyond the initial seeding areas. These cells continued to grow into the interfacial zone, and eventually a contiguous and confluent culture was observed at the interface [81]. These studies demonstrate the potential of in vitro co-culture systems as a model for examining the development of an interface between ligament and fibroblasts. Results from these studies will also aid in the formulation of co-culture systems on multi-phased scaffolds.

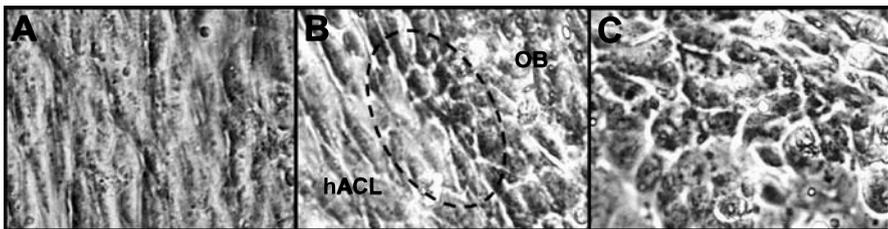


Fig. 4 Co-culture model of osteoblasts and fibroblasts. Human osteoblast-like cells and primary human ACL fibroblasts were separated by an anti-cell adhesion spacer in the culture well. The spacer was removed after 7 days and the cells were allowed to interact. It was observed that both human ACL fibroblasts (*left*) and osteoblasts (*right*) proliferated and expanded beyond the initial seeding areas after 14 days (**B**). These cells continued to grow into the interfacial zone, and eventually a contiguous and confluent culture was observed at the interface. Human ACL fibroblast (**A**) and osteoblast cultured alone (**C**) served as control groups. (32 \times)

3.6 In Vitro Model for Interface Tissue Engineering

Once the appropriate scaffold has been designed, it must be tested *in vitro* using the optimized co-culture model. By exploring the co-culture of osteoblasts, chondrocytes, and ligament fibroblasts on a multi-phased scaffold system with a gradient of material properties, graft systems comprised of multiple tissues instead of a single type of tissue may be developed. The effects of mechanical loading, growth factor and alternate cell types can be readily evaluated using this *in vitro* model. To this end, methodologies developed from *in vitro* cell co-culture models must be successfully translated onto co-cultures on biologically relevant substrates in both 2-D and 3-D forms. Recently, Spalazzi et al. [82] evaluated the interaction of bovine osteoblasts and chondrocytes on 2-D and 3-D polymer ceramic composites. As shown in Fig. 5, when a preformed osteoblast-containing matrix was present on the polymer-ceramic substrate, chondrocytes readily formed cell-matrix extensions which were absent in cultures without osteoblasts. In addition, the presence of a preformed osteoblastic layer promoted the maintenance of the spherical shape of chondrocytes, suggesting that this co-culture sys-

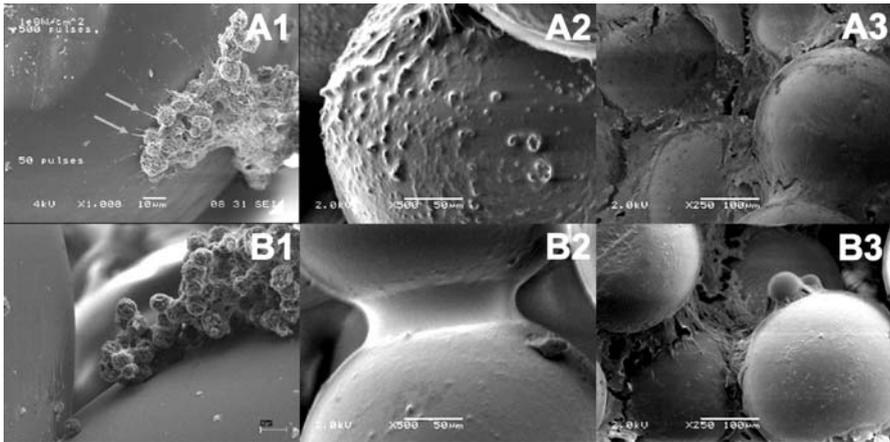


Fig. 5 Osteoblast and chondrocyte co-culture on a 3-D scaffold. Scanning electron micrographs of chondrocytes seeded on 3-D composite scaffolds in the presence (A) or absence (B) of osteoblast preformed matrix for: 1) 30 mins, 2) 24 hours, and 3) 7 days are shown here. Note that, compared to the control group (A2, 1000 \times), cell matrix adhesions (*arrows*) were only found on the osteoblast-chondrocyte co-culture group (A1, 1000 \times). Chondrocytes maintain a semi-spherical morphology on the pre-seeded scaffolds at 24 hours, as seen in A2 (500 \times), but are almost completely spread at the same time on the control scaffold, as seen in B2 (500 \times). Long-term cultures of osteoblasts-chondrocytes (A3, 250 \times) and chondrocytes alone (B3, 250 \times) revealed extensive matrix formation and coverage of the microspheres

tem on a 3-D scaffold may promote the chondrogenic phenotype *in vitro*. In long-term culture, it was found that more extensive cell growth and matrix elaboration were observed on the co-cultured scaffolds as compared to the chondrocyte control. It is clear that in-depth examination of cell–cell interactions during co-culture on tissue engineered scaffolds is needed, and will yield valuable information which can be utilized to optimize the interface scaffold prior to *in vivo* studies.

3.7

In Vivo Model for Interface Tissue Engineering

In vivo animal models for interfacial grafts will be essential for determining the healing potential of the scaffold system. Existing animal models for ligament replacement grafts are usually based on rabbit [45, 48, 66, 71, 83, 84], canine [85, 86], sheep or goat [7, 14, 87–89] systems, and they can be modified and rendered more relevant for interface tissue engineering. The development of *in vivo* models has not been addressed, thus significant research efforts in interface tissue engineering should be focused in this area.

4

Summary and Future Directions

Interface tissue engineering is a relatively new and exciting field with tremendous potential. The review of both background literature and current interface tissue engineering efforts presented here demonstrates that there is a pressing need for functional fixation devices capable of integrating soft-tissue grafts with bone. The clinical motivation for interface tissue engineering stems from the suboptimal performance of existing ACL reconstruction grafts and the absence of graft integration at the junction between graft and bone. There is currently a lack of in-depth understanding of the structural and mechanical properties of the ligament-to-bone insertion zones. A systematic characterization of the interface from nanoscale to macroscale levels can yield the much needed design parameters critical to the development of a new generation of graft-to-bone fixation devices. This new understanding will in turn aid in the design of new ACL grafts and contribute on a broader scale to current efforts in promoting biological graft fixation.

Moreover, the development of both co-culture systems and multi-phased scaffold systems will significantly advance existing efforts in interface tissue engineering and functional fixation devices for ACL reconstruction. This novel approach is potentially more effective since it takes into consideration the nature of the native ACL–bone insertion zone, which is comprised of distinctly ordered tissue regions including bone, mineralized and non-mineralized fibrocartilage, and ligament. By implementing the appropriate

zonal-dependent variations in cell type, density, and collagen distribution into the design of multi-phased scaffolds, functional biomimetic scaffolds may be developed. The development of both in vitro and in vivo models to test the efficacy of the interfacial scaffolds will be critical for the success of the interfacial grafts. In addition, the interface tissue engineering strategies delineated here may be applied to the regeneration of cartilage-to-bone, tendon-to-bone, and ligament-to-bone insertions, with potential impact on orthopedic and dental as well as other clinical applications.

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