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Title: Osteointegration of soft tissue grafts within the bone tunnels in Anterior Cruciate Ligament reconstruction can be enhanced

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Osteointegration of soft tissue grafts within the bone tunnels in Anterior Cruciate Ligament reconstruction can be enhanced

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Abstract

Anterior cruciate ligament reconstruction with a soft tissue autograft (hamstring autograft) has grown in popularity in the last 10 years. However, the issues of a relatively long healing time and an inferior histological healing result in term of Sharpey-like fibers connection in soft tissue grafts are still unsolved. To obtain a promising outcome in the long run, prompt osteointegration of the tendon graft within the bone tunnel is essential. In recent decades, numerous methods have been reported to enhance osteointegration of soft tissue graft in the bone tunnel. In this article, we review the current literature in this research area, mainly focusing on strategies applied to the local bone tunnel environment. Biologic strategies such as stem cell and gene transfer technology, as well as the local application of specific growth factors have been reported to yield exciting results. The use of biological bone substitute and physical stimulation also obtained promising results. Artificial engineered tissue has promise as a solution to the problem of donor site morbidity. Despite these encouraging results, the current available evidence is still experimental. Further clinical studies in terms of randomized control trial in the future should be conducted to extrapolate these basic science study findings into clinical practice.

Anterior cruciate ligament (ACL) reconstruction is one of the most common operations in the field of Sports Medicine. It is estimated that there are more than 100,000 ACL reconstructions performed in the United States each year [18]. The graft used in the majority of ACL reconstructions is a biological graft [4]. The initial improved laxity is provided by the fixation strength of the implant, and the long term improved laxity depends on the healing of the graft within the host bone tunnel. The success rate is typically expected to be greater than 90%. However, patients are required to abstain from pivoting motion sports activity for a period of three to nine months to minimize early failure. Such failure is usually secondary to a pulling out of the graft from the bone tunnel before osteointegration occurs [12].

Soft tissue autograft (hamstring autograft) for ACL reconstruction has grown in popularity in the past 10 years [4, 32, 70]. The reported clinical results in terms of post-operative laxity, return to previous activity level and patient satisfaction are comparable with bone-patellar tendon-bone (BPTB) autograft, but the donor site morbidities are significantly less [3, 5-7, 13, 23, 29]. The commonly cited donor site problems of BPTB autograft include anterior knee pain, patellar tendonitis, difficulty in kneeling, extension loss, and potentially significant complications such as patellar tendon rupture and patella fracture [10, 33, 38]. The commonly reported disadvantages of hamstring autograft are knee flexion weakness, internal rotation weakness and decreased flexion range of the knee joint. These problems are due to the degraded function of the hamstrings and all of them are mild [1]. Thus, many surgeons prefer to use the hamstring autograft as the choice of graft in performing ACL reconstruction.

Despite the high success rate of ACL reconstruction, it is noted that the number of revision ACL reconstruction procedures is approximately 3,000 to 10,000 in the United States annually [14]. Repeated trauma, incorrect tunnel positioning, implant failure and delayed healing of the graft within the bone tunnel are potential reasons for this outcome [12]. The anticipated graft incorporation within the bone tunnel is expected to be longer in ACL reconstruction performed with soft tissue biological grafts [16, 45]. Thus, many investigators are looking for methods for enhancing the biological healing of soft tissue graft within the bone tunnel to allow predictable osteointegration to occur within a certain minimal period of time.

It is reported in previous studies that there are two types of healing patterns when a tendon is placed into a bone tunnel. One is a direct healing characterized by a fibrocartilage transition zone between the tendon and bone, resembling normal ACL insertion [20, 45, 62]. This healing pattern, however, can only be observed at the tunnel entrance [45, 47, 79] or at a later time point of the healing process [64]. Instead, in most cases, an indirect type of tendon healing is observable, and which is characterized by perforating collagen fibers that continue from the tendon into bone, and these fibers are named "Sharpey's fibers" [16, 17, 26, 46, 47, 49, 55, 56, 71]. However, there is still no evidence that these Sharpey-like fibers are appropriate in returning function to normal.

Besides the inferior quality of the tendon-to-bone healing, another principal obstacle to tendon graft ACL reconstruction is the long healing time. The complete healing of the tendon graft takes several months [15, 56, 71], but the rehabilitation should start as soon as possible after the operation [63]. In the early healing period, the "weak site" is the tendon-bone interface [58]. Although instruments or suture techniques can provide temporary fixation, the risk for graft slippage and failure still raises concerns for surgeons. Hence, the patients are required to restrain from pivoting activities for a period of three to nine months to protect this interface before complete osteointegration into the tendon. Therefore, further investigation is needed to enhance the rate and quality of osteointegration of the tendon in bone tunnel.

In recent decades, numerous methods have been reported to enhance tendon healing in the bone tunnel. In this article, we review the current literature in this research area, mainly focusing on the strategies applied to the local bone tunnel environment, and have made an attempt to identify the most appropriate and promising methods in this area. The related articles were obtained from the PubMed database using various combinations of the following key words: anterior cruciate ligament, reconstruction, tendon, graft, healing and osteointegration. Once an article was found, the "related articles" option of PubMed was used to further expand the search. We identified 31 articles which directly concerned the methodologies on enhancing tendon healing in bone tunnel in animal models (5 on bone substitutes; 6 on periosteum autograft; 13 on growth factors and gene therapy; 3 on stem cells; 2 on physical stimulation and 2 on artificial tissue engineering). As well, review of these articles yielded additional 52 relevant articles and hence totally 83 articles were included in our review paper. Even though this search was carried out with great rigor, it may not be complete.

Natural tendon healing in bone tunnel

The mechanisms of healing are complex and incompletely understood. At the beginning of the healing process, as early as 3~7 days after the tendon is placed into the bone tunnel, the gap between the tendon graft and bone tunnel is infiltrated with loose and poorly organized fibrovascular tissue. An inflammation reaction characterized by an influx of cells, such as fibroblasts, vascular endothelia, neutrophils and macrophages, is obvious within the tendon-bone interface with the expression of various growth factors and cytokines, such as type-III collagen, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), bone morphogenetic proteins (BMPs) [20, 24, 28, 35, 56, 58]. Afterwards, at around 1~2 weeks, the interface tissue increases in cellularity and vascularity, with an increased number of osteoblasts, chondroid cells and fibroblasts around the bone tunnel wall. Bone growth factors, such as BMPs, are aggressively expressed and newly formed trabecular bone can be seen sparsely near the bone wall [20]. At approximately 4 weeks after the operation, the fibrovascular interface tissue become denser and better organized, and is gradually degraded by the chondroid cells from the bone into the tendon graft with a deposition of type-II collagen into the matrix, resulting in newly formed lamellar bone and early fibrocartilage progressively growing into the interface tissue. The bone wall surrounding

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the tendon also has become thicker as a result of the new bone formation. However, during this stage, the collagen fibers are still poorly organized and the continuity between the tendon and bone can just occasionally be seen at this period. Later, at 6 weeks, the collagen fibers become more mature, and the continuity between the tendon and bone is reestablished. At around 8 weeks after the operation, perpendicular oriented fibers resembling Sharpey fibers can be seen along the axis of the tendon in the interface. These Sharpey-like fibers are composed of type-III collagen and connect the bone and the tendon graft, and are regarded as the earliest sign of osteointegration into the tendon [35, 56, 58]. As a result, the biomechanical outcomes of tendon healing at the early stage are essentially dependent on collagen fiber continuity and tendon osteointegration. ( Table 1)

Tendon healing in this manner may be mostly attributable to the local biologic and biomechanical environment in the bone tunnel. The inflammatory reactions at the early stage lead to fibrosis rather than tissue regeneration [20, 26]. Moreover, insufficiency of undifferentiated cells in the tendon-bone interface makes tissue regeneration impossible [34]. Finally, the micromotion of the tendon continually taking place in the bone tunnel during the healing process results in chronic inflammation which impairs the osteointegration process [56-58, 60].

## Bone substitutes

Bone substitute materials have long been used in orthopaedics for bone defects and/or cavity filling. To fill the gap between the tendon and bone with bone substitute materials would seem to be clinically applicable. The bonding capability may lead to an instant improved laxity of the tendon graft in the bone tunnel [22, 54]. Moreover, with the capabilities of osteoinductivity and osteoconductivity, some materials can promote bone formation and therefore replace the defect with newly formed bone. To date, most studies have focused on calcium phosphate cements (CPC), with encouraging results.

In 2004, Tien et al. [69] filled the tendon-bone interface with CPC in a rabbit ACL reconstruction model. As early as 3 weeks after the operation, they observed many growing bone islands within the CPC, and this new bone had formed directly on the surface of the bone and the tendon. By 6 weeks, the interface was almost completely filled by new bone with matured and well organized collagen fibers anchoring it. The interface appeared to have healed completely at 24 weeks, as bone had integrated into the tendon. (Table 2 ) They did not see new bone formation in the control group until 12 weeks after the operation. Furthermore, better biomechanical properties were seen in the CPC augmented groups concerning early pullout strength in one week ($6.51\pm1.33N$ compared with $2.05\pm0.95$; P=0.03) and two weeks ($11.49 \pm 2.87N$ compared with $5.45 \pm 3.96N$; P=0.03) post operation.

Mutsuzaki et al. [41] reported a similar finding in a rabbit ACL reconstruction model. Before inserting the tendon into the bone tunnel, they soaked it in a $Ca^{2+}$ solution and then a $NaHPO_4$ solution in turn so as to yield calcium phosphate crystal deposition on and between tendon collagen fibrils. They named the tendon treated in this manner the CaP-hybridized tendon. From the histological findings, they found the healing process was enhanced in the treated groups, with

newly formed bone seen almost all the way around the tendon as early as 2 weeks after surgery. According to their description, by CaP hybridization, the surface of the tendon became more bioactive. Both the osteoblasts and osteoclasts recognized this surface and their activity indicated it was a good place for interaction with each other. Therefore, new bone tissue formed on the tendon surface by the osteoblasts following CaP resorption by osteoclasts, and finally the tendon bonded to the bone tunnel directly. However, the authors did not test the mechanical effects.

A more recent study, conducted by Huangfu and Zhao [22], used an injectable tricalcium phosphate(TCP) cement to fill the outer part of the bone tunnel after inserting the tendon graft in a dog ACL reconstruction model. From histologic examination, they discovered Sharpey-like fibers as early as 4 weeks after the operation on the experimental side, while only loose connective tissue on the control side. By 6 weeks after the operation, new bone and regular collagen fibers had filled the tendon-bone interface and the Sharpey-like fibers had became dense and more regular in the shape of bundles anchoring the new bone. However, on the control side, these Sharpey-like fibers could not be seen until 8 weeks after the operation. At 12 weeks, fibrocartilage and calcified bone were seen in the interface, demonstrating a direct ACL insertion appearance on the experimental side, while there were only dense fibers in the controls. (Table 2 ) Mechanical examination showed that the pullout strength was higher on the experimental side at 2 weeks(11.49±2.87N compared with 5.45±3.96N; P<0.05) and 4 weeks (62.90±7.62N, compared with 33.60±5.87N; P<.001) after the operation.

In a biomechanical study, Tien et al. [69], in a rabbit ACL reconstruction model, found that at one week post operation, the CPC group had a mean maximal tensile strength of 6.51±1.33N, while the non-CPC group had 2.05±0.95N(P=0.03). In a study by Huangfu et al. [22], in a dog ACL reconstruction model, the ultimate pullout load at 2 weeks post-operation was found to be 29.13±2.89N in the tricalcium phosphate group, compared to 14.43±4.20N on the control side(P<.001). Recently, Robertson et al. [54], in an in vitro study, used α-BSM calcium phosphate cement to fix the tendon graft within porcine femoral and tibial bone tunnels. All the specimens were underwent uniaxial tensile testing to failure after 4 hours storage in the incubator at 37°C. Their study showed that the mean load to failure for the tendon grafts in the femoral and tibial tunnels reached 71.7N and 90.62N respectively. Although all of the above mentioned studies showed significantly better tensile strength in treated group than in the non-treated group, the maximal pullout strength in these animal studies is extremely low. The superiority in the treated group may not be clinically significant if the results is extrapolated to human clinical studies.

Another kind of injectbale bone substitute, magnesium-based bone adhesive, has been reported recently. Studies have shown that this kind of adhesive has better biomechanical properties comparing with calcium-based cement and can increase new bone formation in bone defects [78]. Gulotta et al. [19] injected this kind of material in the bone tunnel around the semitendinosus graft

in a rabbit ACL reconstruction model, and the histomorphometric analysis showed more fibrocartilage and less fibrous tissue in the treated interface at 6 weeks after the operation, with increased osteointegration also observed in the treated groups. The load-to-failure at 6 weeks after operation was up to 71.8±31.8N in the experimental limbs compared with 43.4±14.8N in the controls (P=.04). No significant inflammatory response or tissue necrosis observed in this study. However, the degradation characteristics of this magnesium-based bone adhesive were not well examined in this study. It is possible that nonabsorbable or slowly absorbed materials might interfere with the tendon-bone healing when applied to the tendon-bone interface.

Bone substitute materials are easily available on the clinical market. Furthermore, they are not difficult to handle and can be quite convenient for filling the bone-tendon interface in the bone tunnel. However, there are some practical problems associated with the use of bone materials. These include the setting time, the potential dispersion on early contact with blood or aqueous media. Besides, the injectable characteristics (e.g. viscosity, injectable time and cohesion) of the cement are essential in ACL reconstruction by arthroscopy techniques.

## Periosteum autograft

Numerous experimental studies have demonstrated that the periosteum contains mesenchymal or multipotent stem cells which can induce bone and cartilage formation [21, 42, 52]. Some authors believe that filling the gap between the tendon and bone with periosteum autografts may provide undifferentiated cells to the tendon-bone interface and this results in better tendon healing results.

In 2000, Ohtera, et al. [43] evaluated the effects of two types of periosteum on tendon-to-bone healing in the rabbit. In their study, a fresh periosteum was used in the left tibia and a frozen one was used in the right to wrap the long digital extensor tendon before insertion into the bone tunnel in the proximal tibial metaphysis. 4 weeks after the operation, the fresh specimen group produced a premature form of fibrocartilagenous attachment in the bone tunnel, while the frozen periosteal graft demonstrated simple fibrous tissue. Bone remodeling around the tendon graft could be seen in both groups, but was seen earlier in the fresh group. Chen et al. [8], also in a rabbit extra-articular model, sutured a proximal tibial periosteum on the surface of a long digitorum tendon and then placed it into a bone tunnel in the proximal tibia. They found a fibrovascular interface tissue formed by periosteal tissue between the tendon and bone in the early period of 4 weeks post-operation. New bone was also seen interdigitated with this interface tissue. By 8 weeks, new bone was seen growing into this fibrous layer, it become integrated and mixed together with tendon and bone surface by 12 weeks after the operation. The failure load and the interface strength both progressively increased with time. However, no statistically significant difference was seen in failure strength between the experimental group and controls until 8 weeks post operation.

Kyung et al. [30] reported a similar study in a rabbit model. They wrapped the long digital extensor tendon with periosteum before they transplanted it into a bone tunnel. From the

histological findings, they found collagen fibers in terms of granulation tissue in the experiment group at 3 weeks post-operation. These fibers perpendicularly connected tendon and bone, resembling Sharpey fibers. However, only poorly organized fibers were seen in the controls. At 6 weeks after the operation, fibrous interface tissues were still seen in the control group, with less new bone formation and without any cartilaginous components. In contrast, the cartilaginous components increased in the periosteum treated group. Furthermore, the continuity of collagen fibers was increased and Sharpey-like fibers obviously crossed the tissue junction perpendicularly. The biomechanical test showed that the treated group had better strength and failure load at ether 3 or 6 weeks post-operation. A more recent study, a rabbit model reported by Youn et al. [82], showed that periosteal augmentation of the tendon graft could enhance the structural integrity of the tendon-bone interface. (Table 3) In addition, when the fresh inner layer of periosteum was facing toward the bone tunnel, the significant new bone formation was highly organized around the bone tunnel and better mechanical strength was achieved in pullout test at 6 weeks after operation (11.1±3.1 kg compared with 6.9±3.1kg; P<0.05).

In clinical practice, autogeneous fresh periosteum is readily available and appears to be more applicable and promising than other biologic methods. Therefore, this technique may be feasible to apply to ACL reconstruction for the enhancement of tendon graft healing within the tunnel. Recently, the use of periosteum was further evidenced by a clinical study. In a prospective randomized study performed by Robert et al. [53], they found that the use of a periosteal flap can significantly reduce the tunnel enlargement at the outlet to the articular side. This study is a good start toward clinical application of the periosteum. However, whether application of periosteum to soft tissue grafts in ALC reconstruction will lead to a superior clinical outcome requires further randomized clinical trials preceded by a power analysis.

## Growth factors and gene therapy

There is increasing interest in the use of biological agents to enhance tendon-bone osteointegration within the bony tunnel. A number of growth factors are known to have osteoinductive activity or activity that may modulate bone formation, such as bone morphogenetic proteins (BMPs), transforming growth factors (TGF) and fibroblast growth factors (FGF). It is possible that adding exogenous bone-growth factors to the local interface would result in a more favorable outcome. The most widely investigated bone-growth factors appear to be the BMPs. Both clinical and animal studies have shown that they can effectively induce new bone, cartilage, ligament, and tendon formation at both heterotopic and orthotopic sites [25, 40, 80].

Rodeo et al. [58] first used two different doses of recombinant human BMP-2(rhBMP-2) carried by an absorbable collagen sponge to fill the tendon-bone interface in the dog. As early as 7 days after the operation, extensive new bone formation was seen in the high dose treated group, which effect, however, was accompanied by bone resorption. At 2 weeks, both doses of the BMP treated groups showed significant bone formation, but the low dose group also exhibited minor bone resorption. At 8 weeks in both of the treated groups, bone became closer to maturation and

the collagen fiber continuity was seen to have increased. In addition, the tendon treated with rh-BMP-2 exhibited greater adherence to bone at the early time point, 2 weeks after the operation, but not at four, six, or eight weeks. (Table 4) Later, Ma et al. [36], using a variety of doses rhBMP-2 in a rabbit ACL reconstruction model, found that new bone formation and integration to the tendon graft takes place in a dose-dependent manner when treated with rhBMP-2. Furthermore, the BMP treated groups exhibited a faster tendon healing process and greater stiffness at the tendon-bone junction than controls. These authors still found that bone resorption is not distinctly evident when using a slow delivering BMP carrier. Anderson et al. [2] reported a study on the effects of the product "Bone Protein", which contains various bone-growth factors, on tendon-bone healing in a rabbit ACL reconstruction model. From the histologic results, there was extensive formation of new bone trabeculae and cartilage in the tendon-bone interface in the treated groups at 2 weeks after the operation, while only fibrovascular tissue had formed in controls. By 4 and 8 weeks after operation, the treated groups showed more consistent and denser tissue, with more cartilage and new bone formation in the interface. The new bone was in closer apposition to the tendon compared to the control groups. (Table 4) By application of bone growth factor into the healing interface, greater average ultimate load to failure was observed in the treated specimen at the individual 2 weeks (54.69N compared with 37.27N; P=0.04), 4 weeks(65.82N compared with 39.44N; P=0.01), and 8-week(70.74N compared with 39.37N; P<0.001) after operation.

Besides BMP-2, BMP-7 has also been investigated. In the study of Mihelic et al. [39], low dose BMP-7 (25μg) was applied to the bone-tendon interface in a sheep ACL reconstruction model. The histologic analysis showed that in the BMP treated group more extensive new bone had formed around the tendon, resulting in a distinct dense trabecular network connecting the bone and tendon. At three weeks post operation, the treated group had a mean pull-out force of 350 ± 40N, while the control group had 212±28N (P<0.1). At six weeks after operation, the mean pull-out force reached 380±33N in the treated group and 215±44N in control group (P<0.1). More recently, Yamazaki et al. [81], in a beagle dog ACL reconstruction model, found that local administration of TGF-β1 enhanced not only perpendicular collagen fiber synthesis, but also new bone formation in the tunnel wall. Hence, the bonding strength of the tendon graft to the bony tunnel wall was observed to have been significantly increased at 3 weeks after the surgery. Another growth factor, Granulocyte colony-stimulating factor (G-CSF) was also shown to be able to enhance osteointegration of the tendon-bone interface by increased angiogenesis and osteogenesis [61]. In this study, Sharpey-like fibers were found as early as 4 weeks, compared to 12 weeks in the natural healing process.

As shown by the evidence provided above, the administration of growth factors at the time of surgery has been suggested to enhance the incorporation of a soft tissue graft into the bone tunnel.

However, to date, there are still certain limitations to the use of growth factors, one of which is the determination of the dose. Although some study demonstrated that healing outcomes might  be dose-dependent when a specific growth factor applied, the optimal dose for multiple factors is still unknown. Furthermore, the growth factor method is a one-time application to improve the healing results. The biological half-lives of these factors are typically in the range of minutes to hours. The long time effects on tendon healing are therefore questionable. Thus, choosing an acceptable delivery system to maintain the factors' vitality while providing a stable delivery environment is required. One approach is to transfer the specific genes to the host cells, rather than to directly carry out local delivery of the growth factors themselves. Martinek et al. [37], in a rabbit reconstruction model, used gene transfer techniques to infect tendon grafts with the BMP-2 gene and evaluated the effects on bone integration into the tendon in the bone tunnel. At 2 weeks after the operation, a large number of osteoblasts with increased activity were observed filling in the interface in the treated specimens, and a broad, newly formed matrix showed a transition from fibrous to cartilageginous tissue to new bone along with the process of mineralization by the timepoint of 4 to 8 weeks after operation. On the other hand, only dense connective tissue formed in the control specimens. (Table 4) The biomechanical properties were significantly enhanced in the treated specimens in terms of stiffness ($29.0 \pm 7.1$ N/mm compared with $16.7 \pm 8.3$ N/mm; $p<0.05$) and the ultimate load to failure ($108.8 \pm 50.8$N compared with $45.0 \pm 18.0$N; $p<0.05$) compared with controls in eight weeks after operation. Two years later, a study by Lattermann et al. [31] further tested the feasibility of gene delivery to the ligament insertion site in the rabbit. They demonstrated sustained gene expression in the tendon-bone interface for up to 4 weeks.

Gene transfer techniques appear to be favorable for altering cell behavior during the healing process, and provide a sustained delivery of growth factor throughout the early healing stage, which is a period of 4-6 weeks after operation. However, the appropriate length of availability of the growth factor in the local tendon-bone interface is still unknown. Moreover, for transferring genes to cells, specific recombinant viruses must be used. It is unknown how patients would tolerate such a virus. Furthermore, it is still unclear whether the cells of tendon or bone would be the better target. In the study of Lattermann et al. [31], they found that, the bone canal provides a more efficient target for direct adenoviral gene delivery than the tendon in the rabbit. Moreover, Kobayashi et al. [27] found that it is the host cells which contribute to the bone-tendon healing process rather than the graft cells. Taken together with the findings, it appears the cells from the bone tunnel would be a promising target for gene transfer. However, further studies are still required to make a strict comparison between these two targets.


## Stem Cell transplantation

Stem cell technology rapidly developed over the recent decades. One of the most popular stem cell types, bone mesenchymal stem cells (MSC), have been extensively investigated and have

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been shown to facilitate the formation of various structural and connective tissues, such as bone, cartilage, fat, tendon, and muscle [50, 51, 83]. Therefore, it may be reasonable to expect that bone MSCs would promote the regeneration of bone and fibrocartilage-like tissue under the specific environmental conditions of the tendon-bone interface.

Ouyang et al. [44], in a rabbit model, used a fibrin glue to immobilize bone marrow stromal cells and then injected them into the bone tunnel after a hallucis longus tendon had been implanted. At 6 weeks post operation, greater perpendicular collagen fiber formation was seen in the experimental group    than the controls. In addition, cartilage-like cells proliferation, fibrocartilage-like tissue formation and collagen type II were seen only in the stem cell-treated specimens. In the control group, the soft tissue between the tendon and bone was mainly fibrous tissue parallel to the load axis, and direct collagen fibers similar to Sharpey's fibers were observed to be only a slight amount after 6 week. Lim et al. [34] reported a similar finding in their study in the same year. They carried out bilateral ACL reconstruction in a rabbit model using hamstring autografts. The grafts were coated with fibrin glue before being inserted into the bone tunnel. Bone marrow mesenchymal stem cells (MSCs) were seeded in the fibrin glue in the experimental groups, while the fibrin glue remained blank in the controls. At 2 weeks after the operation, the interface in the control group consisted of only irregular vascular tissue and fibroblasts, whereas large crops of mature cartilage cells were seen filling in the interface in the experimental group. By 8 weeks, a mature and distinct fibrocartilage transitional zone from bone into the tendon grafts was seen in the MSC treated tendon. This zone stained strongly for type II collagen. In the controls, only mature scar tissue with some Sharpey's-like fibers spanning the tendon-bone interface was observed. Biomechanical study revealed that MSC-enhanced grafts had significantly higher mean failure load (84.7N compared with 38.1N, $P$=0.02) and stiffness(32.6N/mm compared with 17.2N/mm, $P$=0.05) at 8 weeks post-operation. It was evident that MSC coating improves the healing process and results in better mechanical properties. More recently, the same research group [65] conducted a study to analyze the effects of BMSCs on enhancing osteointegration into an allograft tendon. They used similar techniques to create an ACL reconstruction rabbit model and coated the allograft tendon with autogeneous BMSCs in a fibrin glue carrier before insertion into the bone tunnel. They found that in the treated groups chondrocyte-like cells infiltrated around the interface by as early as 2 weeks after the operation, and these cells became more orderly at 4 weeks with a laying down of chondrosteoid matrix. By 8 weeks, a transition zone demonstrating osteointegration could be seen in the treated groups which resulted in better biomechanical properties. In contrast, in the control group, the interface between tendon and bone was still distinct, with few collagen fibers but no collagen type II even as late as 8 weeks after the operation. The histological results from this allograft study were very similar to that of the autograft study by the same research team [34]. (Table 5)

Although exciting early results have been obtained, one of the mains limitations regarding stem cell technology is that it is inconvenient to clinical application because of the need for complicated

processes, such as bone marrow harvesting, stem cell culture, and cell differentiation and purification. The patient needs to wait about one month such processes before the reconstruction operation. Secondly, the stem cells used in the experiment are not labeled. As yet it has not been possible to determine the extent to which the stem cells play a role in fibrocartilage formation in the interface. Therefore, more studies are still required to demonstrate the specific role of the stem cells in the tendon-bone interface and also to develop a more convenient way to apply stem cells to the host.

## Inflammation modulatation techniques

As discussed above, inflammation during the healing process may result in fibrosis rather than regenerated tissue. Hence, using biological techniques to moderate inflammation may improve the healing outcome. The macrophage is one of the most important inflammatory cells accumulating around the tendon graft from the earliest healing stage, and plays a critical role in the initiation and regulation of tendon healing [26]. Altering macrophage behavior might result in changes in the tendon-bone healing pattern. This was tested by the same group as described above more recently [20]. In a rat ACL reconstruction model, the experimental group was treated with clodronate to disable the macrophages. As a result, it was found that the healing outcome in the treated group was significantly improved in terms of enhanced new bone integration and better collagen continuity in the tendon-bone interface, but with less fibrosis tissue. The width of the interface was significantly reduced compared to the controls. Furthermore, better biomechanical properties were demonstrated in the treated group with respect to load to failure (13.5±4.2 N compared with 9.7±3.9 N; $p < 0.05$) and stiffness (11.5±5.0 N/mm compared with 7.5±3.2 N/mm; $p < 0.05$) in forty-two days after operation. It is known that macrophages produce matrix metalloproteinases(MMPs) which play a central role in extracellular matrix remodeling during the healing process. Demrirage et al. [11] used alpha-2-macroglobulin to block MMP activity in a rabbit ACL reconstruction model. In the treated specimens, there a greater number of areas of denser connective-tissue ingrowth with less cellularity and vascularity. The interface tissue was more mature and contained numerous perpendicular collagen bundles (Sharpey-like fibers). The ultimate load to failure was significantly greater in the treated specimens than in the controls at both two and five weeks after the operation.

The biologic interactions between various cells within the tendon-bone interface during the healing process are complex. The role of the numerous cytokines involved in the healing process are still not well understood. It is also still unknown which is the best cell or cytokine target should be modified. The main purpose of biologic modulation is to avoid fibrosis scar healing and facilitate osteointegration. Further studies are required to improve our understanding of the tendon-bone-healing mechanism.

## Artificial tissue engineering

Using artificially engineered tissue to fill the gap between the tendon and bone is a novel approach to promoting tissue regeneration in the interface [48]. Recently, Chen et al. [9], used poly(ethylene glycol) diacrylate(PEGDA)-based polymers as the scaffold, periosteal progenitor cells(PPCs) as the seeded cells and BMP-2 as the growth factors. By photoencapsulation techniques, they created an injectabel hydrogel consisting of PEGDA, PPCs and BMP-2. Subsequently, the photopolymerized hydrogel was injected into the bone tunnel in the proximal tibial metaphysis after the tendon had been inserted in a rabbit model. Better histologic results were found in the treated groups in terms of a greater number of perpendicular fibers at the insertion, the promotion of fibrocartilagenous attachment and new bone formation in the interface, along with significantly increased tendon pullout strength in either 3 weeks (37.3±7.3N compared with 27.1±3.8N; P<0.05)or 6 weeks (56.1±7.8N compared with 40.1±5.2N; P<0.05) after operation respectively. Spalazzi et al. [66] reported a study on an engineered multiphase scaffold for tendon integration into bone. In order to mimic the tendon-bone interface, they developed a novel triphased scaffold seeded with fibroblasts, chondrogytes and osteoblasts in the first, middle and last phase, respectively. Through an *in vivo* study in an athymic rat model, they found these three types of cells reacted well in the scaffold, with distinct mineral and fibrocartilage-like tissue formed. The host tissue infiltrated successfully into the engineered tissue. This tissue engineering product appears to help provide a functional interface between soft tissue and bone, and it may facilitate bone integration into the tendon when applied to the bone tendon gap in the bone tunnel in ACL reconstruction.

## Mechanical and physical stimulation

Studies have shown that physical shock wave treatment can induce neovascularization and improve blood supply at the bone-tendon junction [74, 75]. It has also shown the ability to effectively stimulate new bone formation in acute or chronic non-union bone fracture [73, 77]. Therefore, this physical method has been used in an attempt to enhance the tendon-bone healing. Wang et al. [76] used a rabbit ACL reconstruction model to show a time-dependent effect of shock waves on the tendon-bone interface. New trabecular bone was seen to be significantly increased around the tendon graft at each time interval after 4 weeks of treatment. Furthermore, in the treated groups, the tendon grafts were largely surrounded by trabecular bone, while there was only fibrous tissue in the controls. The degree of contact between tendon and bone was significantly better in the treated groups. Another physical stimulation method is Low-intensity pulsed ultrasound (LIPUS). This method has been shown to have positive effects on bone and ligament healing processes including angiogenic, chondrogenic and osteogenic activities [59, 67, 68]. Walsh et al. [72] used an intra-articular sheep model of ACL reconstruction to examine the LIPUS effects on tendon-bone healing. After the operation, LIPUS was applied daily for 20 minutes on

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the lateral aspect of the femur in the treated groups. 3 weeks after the operation, new bone had formed at the margin of the bone tunnel in the experimental group, while there was only connective tissue in the controls. Sharpey-like fibers could be seen as early as 6 weeks in the treated group, but were not seen in the controls. The LIPUS treated specimens were significantly stiffer than controls in mechanical pull-out test at 3 weeks (P<0.02) and at 26 weeks (P<0.05) after operation. However, the difference of peak load between the treated ones and controls was only found statistically significant at 26 weeks (P<0.05).

## Conclusion

There are many recent attempts which have been made to enhance the healing of soft tissue grafts within the host bone tunnel in ACL reconstruction. The stem cell technology utilized in recent rabbit models seems to be an approach to recreating the normal fibrocartilage tendon bone junction within a reasonably short period of time. However, the requirement of a separate operation for harvesting the stem cells and the time lag in multiplying the cell number may be obstacles to its use in clinical practice. Local application of specific growth factors on the graft during the time of ACL reconstruction is another attractive way to enhance osteointegration. However, the issues of the carrying vehicle, the optimal dosage and the effective duration of the release of the growth factor in human are still unsolved problems. The use of a biological bone substitute (e.g. tricalcium phosphate) is convenient, despite the fact that the time required for osteointegration is still suboptimal. Moreover, the injectable characteristics of the bone substitutes are not well examined in the animal studies at present moment. We do not know whether these materials are appropriate for ACL reconstruction by arthroscopy techniques. The development of an artificial biological graft (with the mechanical property of a native anterior cruciate ligament and prompt and anticipated osteointegration within the bone tunnel) using a tissue engineering technique on an artificial scaffold would be of great help in ACL reconstruction, as this would help eliminating the problem of donor site morbidity. However, the current research in this area at present is still limited to the promotion of graft-host bone interface healing.

Despite these encouraging results, the current available evidence is still experimental. Although a number of studies provided statistically significant improved biomechanical results in the treated group, a practical question is that there is no data on the mechanical failure of the normal ACL in the animal model they studied. Thus, even though the tendon healing was enhanced in terms of improved biomechanical properties as treated with the specific intervention, we don't know if there is any clinical relevance. The current results in animal studies  still not evidence  more aggressive or earlier rehabilitation after ACL reconstruction.

In general, these recent efforts have remarkably improved the understanding of the healing of soft tissue graft with the host bone tunnel in the animal model of ACL. However, whether the results of these basic science studies can be extrapolated to clinical practice remains unanswered at the present moment. Future works in the form of well designed randomized controlled trial

should be conducted.

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**Table 1**

Table 1: Histological Characteristics Of Natural Tendon Healing Within Bone Tunnel At Different Time Intervals

| | ≤1 week | 1~2 weeks | 2~4 weeks | 4~6 weeks | 8~12 weeks |
|---|---|---|---|---|---|
| Cells | Obvious inflammation reaction characterized by macrophages, neutrophils, fibroblasts and mononuclear cells dominant[20, 22, 26] Osteoblasts were seen [20, 41] | Osteoblasts increased and more active[37] | Higher cellularity but less inflammation reaction[17, 22, 37, 56] Osteoblasts activated[37] | Activated osteoblasts decreased [37] | Adipose cells accumulation[69] |
| Fibrous tissue | Fibrous tissue 1oose and irregular [20, 22] | Fibrovascular tissue loose and poorly organized but highly cellularity and vascularity [20, 37, 56] | Denser, better organized and more mature fibrous tissue [20, 37, 41] | Fibrous tissue become tense and remodeling[20, 22] | Dense fibrous tissue connecting tendon to bone[37] |
| Collagen fibers | | | Collagen fibers irregularly organized and continuity between tendon and to bone occasionally seen [20, 41, 56, 69] | Collagen fibers connecting tendon to bone[20] | Sparse Sharpey-like fibers linking tendon and bone[17, 22, 56] |
| Bone formation | | Newly formed trabecular bone seen at 10 days[20] | Early fibrocartilage and more trabecular bone formed [20] | | Osteoporotic change with thinner native trabecular bone [69] |

Table 2 Histological Changes In Tendon-bone Interface With Bone Substitutes

| Method | | Authors | Time intervals | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ≤1 week | 2 weeks | 3 weeks | 4 weeks | 6 weeks | 8 weeks | 10 weeks | 12 weeks | 24 weeks |
| Bone substitutes | CPC | Tien 2004[69] | CPC infiltrated the interface Thin layer of fibrous tissues | | bone islands within CPC | | New bone infiltrated Collagen fibers matured and well organized | | | New bone infiltrated Collagen fibers continuity | Bone ingrowth into the tendon Collagen fibers continuity between tendon and bone |
| | CaP-hybridized | Mutsuzaki 2004[41] | Red blood cells or fibrin clots in 3 days Sparse fibrous tissue and new bone trabeculae at 5 days | Osteoblasts and osteoclasts around the tendon Abundant esteoids, bone and cartilage-like tissue | Osteoblasts and osteoclasts around the tendon New formed bone directly bound to tendon | New formed bone increased and directly bound to tendon | | | | | |
| | TCP | Huangfu 2007 [22] | | TCP island surrounding with osteoblasts and fibrous tissue | | Loose fibrous tissue New bone islands Sharpey-like fibers | New formed bone Sharpey-like fibers | New bone replaced TCP Sharpey-like fibers abundant and connecting tendon and bone | Uncalcified fibrocartilage between tendon and new formed bone | Normal ACL insertion appearance Sharpey-like fibers linking tendon and bone | |

**Table 3**

Table 3    Histological Changes In Tendon-bone Interface With Periosteum Autografts

| Method | Authors | Time intervals | | | | |
|---|---|---|---|---|---|---|
| | | 3 weeks | 4 weeks | 5 weeks | 6 weeks | 12 weeks |
| Periosteum autografts | Ohtera 2000[43] | | premature fibrocartilagenous in fresh grafts simple fibrous tissue in frozen grafts | | | |
| | Chen 2003 [8] | | Fibrovascular interface tissue New bone formation | Bone mineralization and maturation Bone integration into tendon | | Bone integration into tendon Collagen fiber remodeling Fibrocartilage formation |
| | Kyung 2003 [30] | | Sharpey's fibers connects the tendon to the bony wall Highly cellular, fibrovascular interface tissue Cartilaginous cells | | Cartilaginous components Sharpey's fibers crossing the tissue junction. Increased collagen fiber continuity | |
| | Youn, 2004 [82] | New woven bone formatted Unorganized fibrous structure Chondrocytes at the cambium layer-bone interface. | | | Significant new bone formation with a well-organized lamellar structure Tight interdigitation between the tendon graft at the cambium layer-bone interface | |

**Table 4**

Table 4    Histological Changes In Tendon-bone Interface With Grow Factors and Gene Therapy

| Method | | Authors | Time intervals | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 3 days | 7 days | 2 weeks | 4 weeks | 6 weeks | 8 weeks |
| Grow factors | 680μg rhBMP-2 | Rodeo 1999[58] | Highly cellular granulation tissue Fibroblasts, Mesenchymal cells, Mononuclear cells | Osteoclast-mediated resorption of the lamellar bone Extensive formation of new bone trabeculae | More cellular fibrovascular interface tissue Bone resorption with extensive new bone formation | New bone progressive mineralization Extracellular matrix and collagen fiber continuity Increased Cellularity and vascularity decreased | | New bone progressive mineralization Collagen fiber continuity increased |
| | 180μg rhBMP-2 | | | | Highly cellular fibrovascular interface tissue Minimal bone resorption with extensive new bone formation | New bone progressive mineralization Extracellular matrix and collagen fiber continuity increased Cellularity and vascularity decreased | | New bone progressive mineralization Collagen fiber continuity increased |
| | Bone protein | Anderson 2001[2] | | | Extensive formation of new bone trabeculae and cartilage with closer apposition to tendon | Cartilage and fibrovascular tissue more mature Better collagen-fiber continuity | | More cartilage and new bone formation |
| | BMP-2 gene transfer | Martinek 2002[37] | | | Loose granulation-type tissue A large number of activated osteoblasts | A broad layer of matrix formed resembling chondroosteoid depostion | Transition zone from fibrous to cartilageginous tissue to new bone | Transition zone like direct insertion more pronounced |

**Table 5**

Table 5　Histological Changes In Tendon-bone Interface With Inflammation Modulation and Stem Cells

| Method | | Authors | Time intervals | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | ≤1 week | 2 weeks | 4 weeks | 5 weeks | 6 weeks | 8 weeks |
| Inflammation modulation | Macrophage apoptosis | Hays 2008[20] | Poorly organized collagen fibers Loose fibrovascular tissue Trabecu lar bone and Osteoid | Distinct fibers continuity Dense fibrovascular tissue Fibrocartilage Osteoid increased | Four-zone insertion site resemble direct healing Sharpey-like fibers increased | | Osteoid formation | |
| | MMPs blocking | Demirag 2005[11] | | Fibrous tissue with less cellularity and vascularity Sharpey-like fibers limited Collagen network longitudinally oriented | | Denser connective tissue ingrowth Abundant perpendicular collagen fibers | | |
| Stem cells | BMSCs | Ouyang 2004[44] | | Perpendicular collagen fibers Cartilage- like cells Fibrocartilage-like tissue | Perpendicular collagen fibers Cartilage- like cells Fibrocartilage-like tissue Type I, II and III collagen | | Perpendicular collagen fibers Cartilage- like cells Fibrocartilage-like tissue Type I, II and III collagen | |
| | MSCs | Lim 2004[34] | | Large crops of mature cartilage cells were seen filling in the interface | Cartilage cells more organized Cartilage zone blended into the tendon substance | | | Cartilage cells matured Fibrocartilage transitional zone distinct |
| | BMSCs allografts | Soon 2007[65] | | Large area of immatured Chondrocyte-like cells | Chondrocyte-like cells more matured and orderly Chondroosteoid matrix laying down, | | | Fibrocartilage transitional zone Abundant amount of collagen II |