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Mechanical Stimulation toward Tissue Engineering of the Knee Meniscus

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Abstract—Current clinical practices do not adequately regenerate the meniscus of the knee secondary to a tear. Complete or partial meniscus removal leads to degenerative changes within the joint. Tissue engineering of the meniscus promises a potent solution. Before embarking on tissue engineering of the meniscus, it is crucial to have a thorough comprehension of the biomechanical role that this tissue fulfills and how the structure of meniscus is uniquely suited to that purpose. To better understand this, we have examined the meniscus, as well as associated tissues, within the body. For the first time, the knee meniscus is rigorously compared to ligament, tendon, and cartilage, and inferences are drawn on how mechanical stimulation may be used to channel growth in the meniscus. We have examined in detail the loading conditions that these tissues experience *in vivo* and how each is uniquely adapted to its loading environment. These tissues are capable of achieving some degree of remodeling because of mechanical stimuli. By understanding the mechanisms that can stimulate and promote regeneration in related tissues, we hope to harness that knowledge to achieve the goal of meniscal regeneration.

Keywords—Tissue engineering, Meniscus, Mechanical Stimulation, Fibrochondrogytes.

INTRODUCTION

The menisci of the knee are two semilunar fibrocartilaginous tissue structures that are responsible for shock absorption, load transmission, and stability within the knee joint.[1,66,103] According to the National Center for Health Statistics, there were 455,000 surgeries to excise the semilunar cartilage of the knee in 1996. As the population ages, these numbers are only likely to increase. The peripheral portion of the meniscus is vascularized and has the intrinsic ability to heal itself.[90] The inner portion of the meniscus is avascular and like articular cartilage cannot heal as can other tissues such as bone and skin. As a result, numerous meniscal injuries and early degeneration of the inner one-third of the meniscus tend to be permanent. Injuries to the knee menisci can cause significant discomfort and can lead to cartilage injury on the articular surfaces of the femur and the tibia, leading to the later development of osteoarthritis.[52]

Meniscal injuries occur in both young and older persons. Causes of meniscal injuries can range from sports injuries and car accidents to general degeneration of the joint associated with aging. Before the importance of this tissue was appreciated, treatments for meniscal injuries often involved the complete removal of the meniscus, a procedure known as meniscectomy. By removing the meniscus, the average stress in the joint can be increased nearly threefold, while peak stresses can be increased to an even greater magnitude.[52] The increased stress was found to lead to osteoarthritic changes in the joint.[17] This finding, which suggested that only the torn portions of the meniscus should be removed, led to the procedure of partial meniscectomy through an arthroscopic intervention. However, even partial meniscectomy has resulted in osteoarthritic changes such as osteophyte formation, articular cartilage degeneration, joint space narrowing, and symptomatic osteoarthritis.[51] The use of allografts has been suggested as a solution, but the usual shortcomings of transplanted tissues such as limited supply, the possibility of disease transmission, and the potential of host rejection prevent this from being a widely embraced approach.

Tissue engineering of the meniscus could provide a potential solution for regeneration of the meniscus in the avascular region as well as repair of overall tissue degeneration. There are many questions to be answered about the development of a tissue-engineered meniscus. These include the appropriate cell types, scaffolding material, necessary growth factors, type of bioreactor, and appropriate mechanical stresses to promote and channel growth. While there is a dearth of research dealing with meniscal response to mechanical stimulation, some work has been performed on structures that share similarities to the meniscus. Before implementing a biomechanics-based strategy to achieve regeneration of the meniscus, it is our belief that knowledge related to hyaline articular cartilage and tensile structures, such as tendons, needs to be understood and applied. This paper deals with mechanical stimuli of tissues that exhibit

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similarities to meniscal tissue for the purpose of tissue regeneration.

MENISCUS ANATOMY

Each knee contains two semicircular sections of fibrocartilaginous tissue, the menisci (Fig. 1), which are wedge-shaped in cross section and are in the medial and lateral aspects of the tibiofemoral articulated surface. The outer portion of the meniscus, which is vascularized and has the intrinsic ability to heal itself, is frequently referred to as the red zone. This area of the meniscus also has large nerve bundles running circumferentially and smaller nerves running radially.[65] The inner portion of the meniscus is avascular, has no innervation,[65,105] and is also referred to as the white zone. The areas of most innervation are the horns of the menisci, which are attached to bone through insertional ligaments.[33,65]

The medial and lateral menisci differ in shape and connections to the surrounding tissues. The medial meniscus is semicircular in shape while the lateral meniscus is more nearly circular in arrangement. The two menisci are connected by the transverse ligament that runs from the anterior medial meniscus to the anterior lateral meniscus. The menisci are attached to the tibial plate by anterior and posterior horns. Other attachment points are the medial collateral ligament, the meniscofemoral ligaments, and the joint capsule.[2] The meniscofemoral ligaments attach the lateral meniscus at the posterior horn to the lateral aspect of the medial femoral condyle.[53] There are two meniscofemoral ligaments, the posterior Wrisberg ligament and the anterior Humphry ligament. The occurrence of these ligaments varies by patient. Either or both may be present or absent.[53]

ULTRASTRUCTURE OF MENISCUS AND SIMILARITIES TO ARTICULAR CARTILAGE AND TENDON

The properties of the meniscus are determined largely by the architecture of its ultrastructure. The extracellular matrix of the meniscus, though arranged in a unique manner, shares similarities to both articular cartilage and tendon. The exterior region of the meniscus on the superior and inferior surfaces is arranged in a random meshwork and referred to as the superficial layer. The next region within the meniscus is a thin section termed the *lamellar region* and is composed of mostly randomly oriented fibers except at the outer periphery. In this latter region, the fibers are predominantly oriented in the radial direction. Lamellar fibers are 20- to 50-μm wide[72] and composed of individual fibers of collagen 120-nm thick. The deep zone of the meniscus is composed primarily of large circumferential fibers (50- to 150-μm diameter[26]), although radial fibers are present as

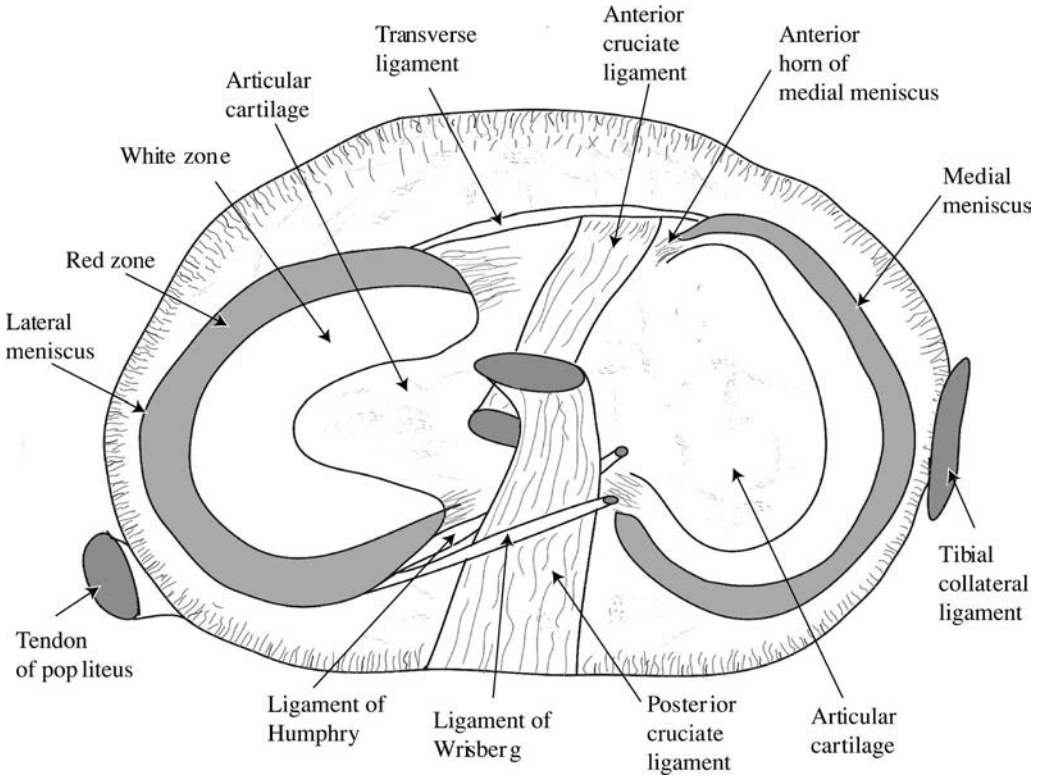

FIGURE 1. Meniscus anatomy.

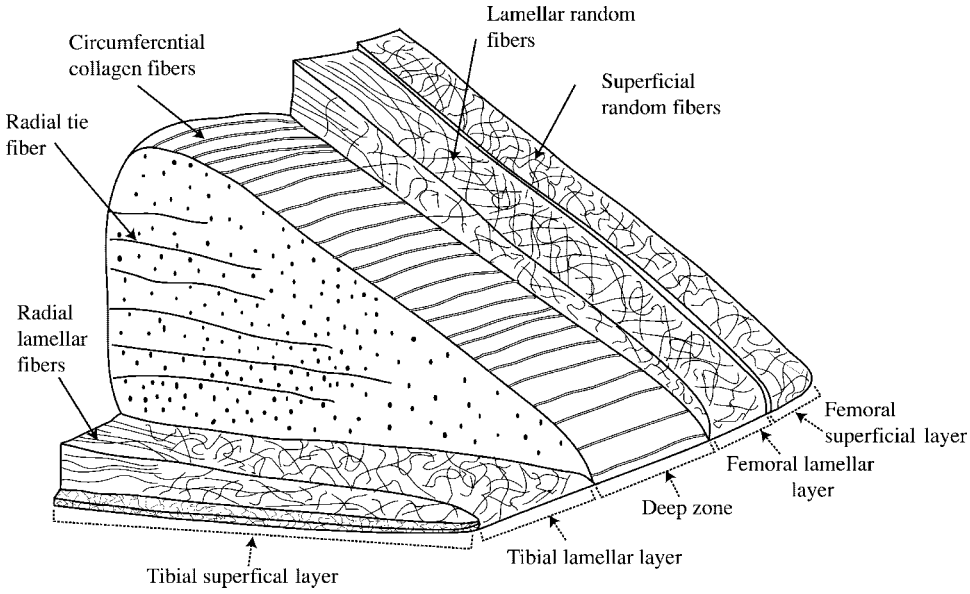

FIGURE 2. Zones and collagen structures of the meniscus.

well.[72] By comparison, the upper limit of collagen fibers of human tendon is around 300 μm.[46]

By wet weight, normal human meniscal tissue has been shown to be 70–75% water, 20–25% collagen, 0.6–0.8% glycosaminoglycans (GAGs), and the remainder DNA and adhesion molecules.[37] Age, disease, injury, and location can affect the relative numbers.[37,62,73] The two primary fibrillar components are collagen and elastin. Collagen accounts for 60–70% of the dry weight (Fig. 2), while elastin content is significantly smaller (0.6%).[62] Total GAG content of the human meniscus is around 2–3% dry weight (Table 1).[26,66]

The inner one-third of the meniscus is under a predominantly compressive load. Interestingly, this region of the meniscus most resembles the archetypical articular cartilage model. Sixty percent of collagen in this region is type II, and glycosaminoglycans (GAG) are five to six times the amount in the outer one-third.[15,69] Comparatively, 95% of articular cartilage is type II collagen which makes up 50–73% of the dry weight (Fig. 3(A)).[4,40] Cartilage is 15–25% GAG dry weight.[4,40] The superficial zone also has a two to three times greater relative amount of collagen type II as compared to the deep zone of the meniscus.[24] The small proteoglycans decorin and biglycan are also found within the inner one-third of the meniscus, with biglycan in greater abundance.[82] Biglycan is thought to have a role in protecting cells and found predominantly in the pericellular matrix of compressed cartilaginous tissues.[78,82]

The outer portion of the meniscus is composed of large, circumferentially oriented collagen type I fibers. These large parallel fibers resemble those of tendon in many aspects. Like the circumferential fibers of menisci, tendon is composed of collagen type I fibers organized in a parallel fashion. Unlike the meniscus, the tendon fibrils have a characteristic crimp or wave pattern (Fig. 3(B)).[46] The predominant forms of proteoglycan in both tissues are small proteoglycans such as decorin, a leucine-rich proteoglycan located between collagen fibers, which is thought to play a roll in organizing collagen fibrillogenesis.[47,68]

CELLS OF THE MENISCUS

Meniscal cells exhibit a unique combination of attributes of both fibroblasts and chondrocytes. Like chondrocytes (Fig. 4(A)), they present in a round or oval form with relatively large nuclei and situated in territorial matrix.[30] However, they produce predominantly collagen

TABLE 1. Properties of the meniscus and related tissues.

	Predominant collagen type	Collagen amount (% dry weight)	Water content	GAG content (% dry weight)	Cell type	Predominant mechanical function
Cartilage	Type II	50–73%[4,40]	75–80%[22]	15–25%[4,40]	Chondrocyte	Compression
Meniscus	Type I and II	60–70%[62]	72%[37]	2–3%[24,66]	Fibrochondrocyte	Compression and tension
Tendon (tensile region)	Type I[44]	65–75%[46]	70%[43]	0.2%[46]	Fibroblast	Tension

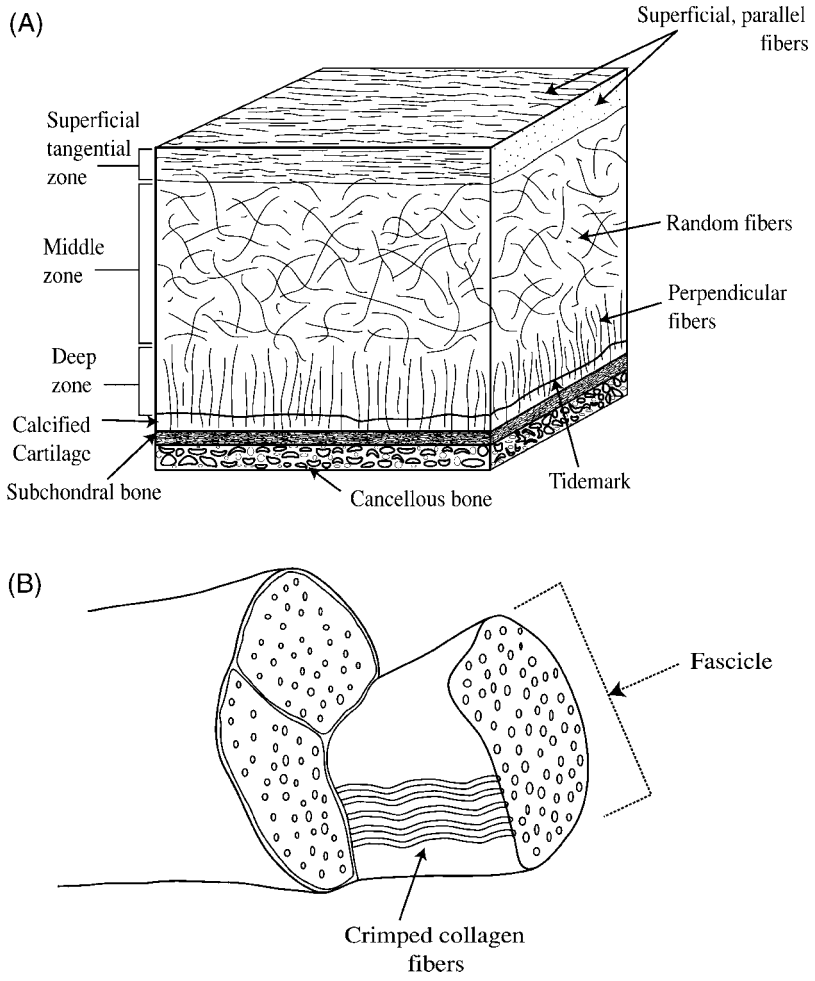

FIGURE 3. Zones and collagen fiber organization of (A) articular cartilage and (B) tendon.

type I, as fibroblasts do (Fig. 4(D)), and are thus termed *fibrochondrocytes*.[104]

Fibrochondrocyte appearance varies within the tissue as well. The cells of the meniscus are generally grouped into separate subpopulations based on their approximate location within the tissue. Cells of the superficial zone are usually either oval or fusiform and contain few processes (Fig. 3(B)).[91] The cells of the deep red zone are usually

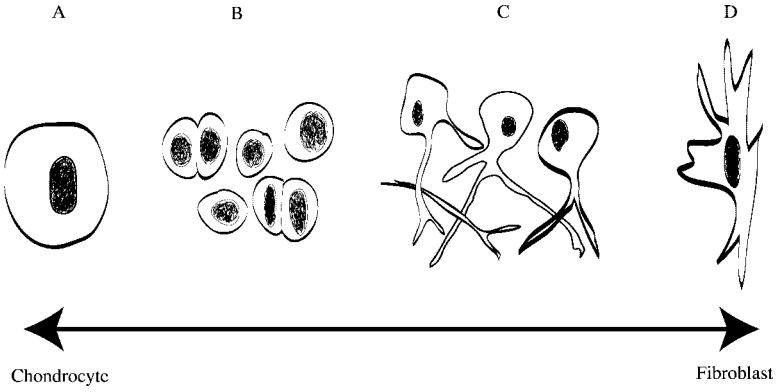

FIGURE 4. Fibrochondrocytes and related cells. (A) Typical chondrocytes of articular cartilage. (B) Oval and fusiform fibrochondrocytes from the white zone of the meniscus. (C) Red zone fibrochondrocytes. (D) Typical fibroblast type cell.

rounded and contain many processes that extend through the pericellular zone (Fig. 3(C)).[29] These processes, as well as gap junctions, allow communication between cells. It has been proposed that this communication aids in the orientation of collagen fibers within the deep zone.[36] Cells of the inner one-third, hyaline-like region, of the meniscus are rounded and lack projections.[36] In the inner one-third of the meniscus, the cells produce both collagen types I and II.[90]

BIOMECHANICS OF THE MENISCUS

The menisci play a crucial role in shock absorption, stability, and load transfer within the knee. When the menisci are loaded, they are compressed by the axial force; however, because of their wedge-shaped cross section, the menisci are pushed out radially as well. Because the menisci are anchored by anterior and posterior horn attachments, this leads to a circumferential stress in the tissue. Thus, because of their unique shape and properties the menisci take an axial compressive load and convert it to a circumferential tensile load. Shear force is also induced between the collagen fibrils within the tissue as the meniscus is forced out radially. There is negligible shear on the tissues from the tibial plateau and femoral condyle due to the almost frictionless contact surfaces.[28]

The material properties of the tissue allow the meniscus to deform so as to increase the congruency within the joint which has the effect of improving stability as well as minimizing stress. During ordinary walking, the menisci carry about 50% of the load transmitted through the knee, which can be five to six times the body weight.[74] Without the menisci, the cartilage on cartilage contact would be predominantly at the center of the tibial plateau and this would decrease overall joint stability.[103] When the knee bends, the femoral condyles rotate and slide across the tibial plateau.[28] During this movement, the menisci deform toward the posterior tibial plateau to maintain their function. It has been shown that the posterior excursion of the lateral meniscus is about twice that of the medial meniscus during full-range motion.[93]

The menisci contribute to lubrication of the joint by releasing fluid as they are loaded.[26] The viscoelastic properties of the meniscus aid in nutrient supply to the articular cartilage as fluid is exuded and taken up through repeated compression and relaxation of the tissue. This creates a circulation which is essential to nourishing cells.[26]

MATERIAL PROPERTIES

The menisci are generally described as a biphasic tissue with the liquid phase being water and hydrolytes and the solid phase being the extracellular matrix and GAGs. This composition leads to the tissue behaving in a viscoelastic manner, meaning that stresses and strains developed within the tissue are dependent not only on load applied but also on rate of loading.[66] When compared to articular cartilage, another biphasic tissue that also exhibits viscoelastic response to loading, it has been shown that articular cartilage has a dynamic shear modulus approximately 10 times that of meniscus.[108] The human meniscus has an aggregate modulus between 0.09 and 0.15 MPa[90] while human articular cartilage is in the range of 0.53–1.34 MPa.[40] The meniscus is also approximately five times less permeable to fluid flow than articular cartilage.[25] This means that water cannot as readily leave the tissue when it is subjected to a load, and thus the meniscus is an excellent shock absorber.

The menisci are highly anisotropic materials so that when discussing mechanical properties of the tissues, it is important to note load direction (i.e., circumferential, radial, etc.) and which portion of the meniscus is described. Tissakht and Ahmed[94] performed a tensile test study and found that the human meniscus' elastic modulus in the circumferential direction (72.85–131.42 MPa) is about 10 times higher than in the radial direction (3.74–16.21 MPa). By comparison, human patellar tendon has an elastic modulus of around 450 MPa.[89] As can be seen, the material properties of the tissue which makes up the meniscus are uniquely structured to serve its biomechanical role within the knee.

MECHANICAL STIMULATION OF ARTICULAR CARTILAGE

The mechanical environment in which a tissue is placed has a profound effect on that tissue's biology.[83,84,97] It has long been known that bone remodels to adapt to the biomechanical environment it experiences according to Wolff's law.[106] More recently it has been observed that mechanical stimulation of cartilage has resulted in an upregulation of cellular synthesis, proliferation, and tissue properties.[16,18,81,84] Although there is a dearth of research on mechanical stimulation with meniscal tissue engineering, some inroads have been made toward engineering of related tissues. Above we have seen the many similarities that the meniscus shares with both tendon and articular cartilage. By employing strategies that have been shown to be beneficial in engineering these tissues, we hope to demonstrate a similar potential in the regeneration of the meniscus.

Direct Compression of Articular Cartilage

Articular cartilage experiences direct compression *in vivo* as one articulating surface contacts another. The properties of the tissue allow it to be deformed hundreds of times a day without damage. In fact, by immobilizing the joint, unloaded articular cartilage begins to degenerate, while tissue that is actively loaded improves its mechanical characteristics.[32] There are two primary methods for investigating chondrocyte response to loading *in vitro*. The first is to use explants in their native ECM. The second is to use cells seeded on a scaffold such as agarose or collagen

sponges. Hydrogels such as agarose force the chondrocytes to maintain their rounded native morphology. It has been well established that static compression decreases synthesis in a dose-dependent manner. For example, Ragan *et al.*[75] determined that when a free swelling explant (1.15-mm thick) was compressed back to the level of cut thickness (1.0 mm), no significant difference occurred in mRNA levels for aggrecan or type II collagen. However, strains of greater than 50% decreased mRNA to approximately 35% of controls.

Dynamic compression of articular cartilage is the most thoroughly examined area of mechanical stimulation of a tissue similar to meniscal tissue. It is also the area of most debate, because of the varying degrees of success of upregulating positive markers of healthy articular cartilage (proteoglycan synthesis, type II collagen, etc.). In studies that have demonstrated positive effects, frequency of loading is one of the most important factors. In a study examining a wide range of frequencies, stimulation below 0.01 Hz exhibited little response.[79] At 0.01 Hz, strains above 1% yielded an increase in radiolabeled proline and sulfate incorporation by approximately 30 and 40%, respectively. Frequencies of 0.1–1.0 Hz stimulated uptake of both proline and sulfate by approximately 20–40%. Lee *et al.*[56] examined frequencies between 0.3 and 3 Hz with chondrocytes seeded in agarose. At 15% strain, it was found that 1 Hz stimulated GAG production approximately 40%. Other studies in the range of 0.1–3 Hz have also demonstrated an increase in cellular production.[10,11,55]

Not all dynamic loading studies have been observed to elicit a positive response.[54,95] The effect of loading may to be tied to platen type and steady state offset strain in the tissue. Steady state offset strain is when specimens retain some residual strain from one cycle to the next. Thus, the loading regimen can be viewed as a static compression overlaid with a small dynamic component. It has been suggested that the static portion of this trend dominates tissue response.[95] In a study using load controlled dynamic stimulation, the static offset ranged from 29 to 60%.[57] Bovine fetal and calf DNA synthesis decreased to less than 50% that of free swelling controls, while radiolabeled sulphate was approximately 75% of controls. Static compression in the same range of strain decreased radiolabeled sulfate and proline incorporation to approximately 55 and 40%.[41] Another reason that some dynamic studies may have failed to elicit a positive response is the use of a porous platen, which may more readily exude water. With the loss of water pressure, a greater strain is attained for a given force. The removal of the subchondral bone, as well as the surrounding tissue, is also likely to result in greater strains than occur *in vivo* because of less constrained fluid exudation. However, not all porous platen experiments show reduced synthesis. Sauerland *et al.*[80] demonstrated a positive effect, using 0.5-Hz sinusoidal loading for 5 s at 0.5 MPa followed by rest periods. It is possible that this relatively light load, coupled with a resting period to allow tissue recovery, circumvents the effect of residual strain summation and potential negative effects of porous platens.

Growth factors are another potent stimulator of articular cartilage. Chondrocytes *in vitro* respond readily to cell signaling from osteoarthritic synovial fluid.[96] Bonnassar *et al.*[10] showed that IGF-1 and dynamic compression (0.1 Hz, 3% strain) increased protein and proteoglycan production 180 and 290%, respectively, greater than either stimulus alone. Thus, dynamic compression can augment the effects of administered growth factors. Differentiation of cells may also be tied to dynamic loading. Elder *et al.*[20] demonstrated that chick limb bud mesenchymal cells were readily differentiated along a path of chondrogenesis when subjected to a dynamic loading at 0.15 Hz, and this effect was increased at 0.33 Hz.

Dynamic compression on cell seeded constructs can have a pronounced effect on material properties as well. In a study where calf articular chondrocytes were dynamically loaded for 4 h a day for 4 weeks in agarose, the aggregate modulus of the constructs increased to six times that of controls.[61] At 100 kPa, this put the constructs' aggregate modulus in the same order of magnitude as native tissue. A similar effect was shown by Waldman *et al.*,[102] but interestingly, collagen and proteoglycan content was not significantly different from that of controls. An important implication of this is that structuring of ECM components is as important as quantity produced, and also highlights the importance of mechanical testing of tissues.

Hydrostatic Pressure Stimulation of Articular Cartilage

Hydrostatic pressure culturing, like direct compression, is an attempt to utilize an *in vivo* force for the purpose of *in vitro* culturing. The cartilage within the knee is hydrostatically pressurized whenever the tissue is compressed. This is caused by the inability of interstitial fluid, drawn into the tissue by the proteoglycans, to rapidly exit the tissue as it is compressed. During dynamic loading much of the stress within the cartilage is taken up by the fluid component of the tissue shielding the extracellular matrix.[5] While fluid is exuded, large pressures are experienced within the extracellular space. It has been estimated that normal daily living provides 7–10 MPa of hydrostatic pressure while contact stresses on cartilage in the hip joint can range from 3 to 18 MPa.[38,87]

Static hydrostatic pressure loading has been shown to be either stimulatory or inhibitory, depending on duration and pressure applied. For instance, Hall *et al.*[34] demonstrated that a short 20-s stimulus of pressure between 7.5 and 20 MPa followed by 2 h in static culture increased GAG production in bovine explants. Pressures from 2.5 to 10 MPa with the same 20 s/2 h culturing regimen upregulated collagen production. Pressures outside this range (up to 50 MPa) did not alter the expression of collagen or proline beyond unloaded controls. Interestingly, when

pressures were applied for the full 2 h, there was an increase in the production of collagen and GAG at 10 MPa. However, there was a dose-dependent decrease in cellular production from 20 to 50 MPa.

There are two primary methods to apply dynamic hydrostatic loads to tissues. The first is to only place the tissues into the pressurizing device for the period of loading and otherwise culture the tissue elsewhere (i.e., static culture or rotary flask) when not pressure stimulated. The second method is to use an instrument capable of providing both nutrients and stimulation, thus eliminating the need to remove the constructs. Both systems have advantages and disadvantages. Stand-alone hydrostatic pressure systems are easier to maintain, currently capable of higher frequencies, and lend themselves to culturing regimens including additional forms of stimulation, such as direct compression or direct shear. The downside is that they require time to set up prior to every pressurization run; sterility is also another issue during loading. This can be quite labor intensive for a long-term experiment. Perfusion integrated hydrostatic pressure chambers run at least semiautonomously, supplying nutrients and pressure as programmed. Currently, no perfusion integrated system for cartilage constructs appears to be capable of frequencies greater than 0.1 Hz, a serious limitation as indicated by the studies below.

Stand-Alone Hydrostatic Pressure

The frequency at which the load is applied plays a critical role in the effect hydrostatic pressure has on articular cartilage explants. Parkinnen *et al.*[70] tested bovine cartilage explants with 5 MPa cyclic hydrostatic loading at 0.0167, 0.05, 0.25, and 0.5 Hz for 1.5 h. Only in the 0.5-Hz explants were GAG quantities greater than controls (17%). Studies examining higher frequencies on explants and constructs may prove beneficial. In a monolayer study, it was demonstrated that 10 MPa applied at 1 Hz increased collagen type II and aggrecan mRNA levels, 36 and 31%, respectively, over a 4-h period.[88]

Duration of loading is also important to the outcome of hydrostatic pressure loading. Smith *et al.*[87] used a 10 MPa peak pressure cyclic (1 Hz) loading regimen for 2, 4, 8, 12, and 24 h. Also examined was the effect of an intermittent loading regimen on adult bovine cells for only 4 h per day for four continuous days. Collagen production was maximized in the 4-h cyclic loading, while aggrecan production continued to increase over 24 h. The intermittent loading protocol produced a ninefold increase in collagen II mRNA signal and a 20-fold increase in aggrecan mRNA.

Hydrostatic pressure has also been shown to aid in the differentiation of mesenchymal progenitor cells along a chondrogenic pathway. Cells produced a 94.5% increase in GAG and 76.8% increase in collagen when cultured in cyclic hydrostatic pressure for 7 days and then static culture for 14 days.[3] These results support the idea that mechanical environment is critical for proper cell development *in vitro*.

Perfusion-Integrated System

Load durations of longer than 48 h without removing the samples from the hydrostatic pressure chamber are possible when using an apparatus capable of supplying both dynamic pressure and nutrients. To achieve this, Carver and Heath[14] developed such a system that provided loading every 4 h for 20 min. For these experiments, loading pressures were either 3.44 or 6.87 MPa at 0.05 Hz and culture medium was perfused through the culturing chambers prior to loading.[13,14] Another interesting aspect of this study was the comparison of the response of adult and juvenile equine cells seeded on PGA scaffolds. It was found that the lower pressure had no effect on collagen production but the higher pressure did increase collagen synthesis in both adult and juvenile cells. The juvenile cells produced GAG in higher quantities than the adult cells at either pressure.[13] One final augmentation to this study was performed. Prior to placement in the hydrostatic pressure chamber, cells were cultured in mixed systems for various times.[101] The culturing conditions that produced the maximum amount of GAG (2 weeks spinner flask/4 weeks hydrostatic pressure) were different from those which produced the most collagen (4 weeks spinner flask/2 weeks hydrostatic pressure). It was a combination of sequential culturing techniques, rather than intermittent pressure or spinner flask alone, that led to the highest values. Future studies may show that better quality tissue is produced by using different culturing techniques during different stages of tissue culture.

The idea that culturing techniques can be combined concurrently as well as sequentially has been explored. Hansen *et al.*[35] used reduced oxygen tension combined with intermittent hydrostatic pressure loading to achieve an additive effect. It was found that monolayer bovine chondrocytes proliferated at the highest rate when cultured with 5% O_2 combined with a 2-min pressure/30-min no-pressure cycle (18% greater than 21% O_2 static controls). The highest expression of collagen type II was with a 5% O_2 culture and a 30-min pressure/2-min no-pressure cycle (approximately 62–66% greater than controls). Thus, loading regimens can be tailored for proliferation or matrix synthesis at different stages of culture.

Shear Stimulation of Articular Cartilage

Shear is also a potent stimulator for inducing cartilage tissue response. There are two primary methods that have been used experimentally to induce shear on tissue or chondrocyte cultures. The first is by fluid induced shear. The second is by direct mechanical contact and is termed *direct shear*.

Fluid Shear

A common method of inducing fluid shear on cell monolayers is by use of a cone viscometer.[12] Smith *et al*.[86] employed such a system to apply 1.6 Pa of shear to both bovine and human chondrocytes. It was noted that cells elongated so that the major axis of the cell was parallel to the direction of fluid flow. Shear also mediated cell metabolism. GAG production was upregulated approximately twofold, but the core proteoglycan was larger than in control cells as were the individual GAG chains on the proteoglycan. Prostaglandin E_2, which is an indicator of proinflammatory mediators, increased 20 times that of controls. Also increased was mRNA for tissue inhibitor of metalloproteinase-1 (TIMP-1), which suggested that the balance between matrix secretion and destruction had also been changed by shear.[86] The theory that fluid shear could induce an inflammatory response was supported by the finding that 1.6 Pa of fluid shear increased matrix metalloproteinase-9 expression, another indicator of osteoarthritis, approximately 300% above static controls.[43] Smith *et al*.[87] expanded upon the previous study and noted that under the same culturing conditions, nitric oxide (NO), a proinflammatory molecule, production increased 9-fold while Interleukin-6 mRNA increased 10-fold.

Fluid shear is useful for studying the effects of shear on cells without intact extracellular matrix. However, the current practice of engineering tissue most often requires a scaffold to seed the cells on. Using fluid shear on a matrix with cells mixed in becomes a perfusion system. Direct perfusion systems are designed so that the scaffold is tightly fit in a tube with media flowing through it, forcing all the flow to pass through the scaffold. This allows for tight control of nutrient delivery, oxygen content, and other parameters. Flow enters on one side of a construct and exits on the other, thus one face is stimulated by a flow of higher oncoming pressure. Also, as the fluid travels through the scaffold, cells align parallel to the direction of shear. This would be disadvantageous because in different zones of the meniscus cells lie in different orientations. There is also the possibility of washing away cellular products that are necessary for the development of a mechanically functional tissue (collagen, proteoglycans, etc.), or for intercellular signaling (cytokines, interleukins). A method of circumventing this problem is recycling part of the medium that has already passed through the scaffold with new media.

Perhaps the greatest concern with direct perfusion is the shear that the cells within the construct experience. When cells proliferate and produce ECM, this decreases the permeability of the construct to media perfusion. To supply the greater number of cells with the same amount of nutrients per cell, a larger volume of media must be forced through a less permeable matrix resulting in higher shear stress. It has been demonstrated that shear of as little as 0.092 Pa can damage cells.[31] Chondrocytes have been shown to upregulate proinflammatory markers at 1.6 Pa.[43] Studies using direct perfusion systems have demonstrated an increase in proliferation, GAG, and collagen production, but have not examined mechanical properties of the constructs or incidence of proinflammatory markers.[19,71]

Direct Shear

Direct shear of explants and tissue engineered constructs is designed as an attempt to separate the effects of strain on the cell from those induced by fluid flow and hydrostatic pressure.[32] In pure shear, such as torque applied through the central axis of a rod, there is no volumetric change, and thus minimal pressure gradient or fluid flow. In simple shear, such as that induced by antiparallel forces on opposite sides of a cube, there can be low levels of fluid flow due to bending effects on the leading and trailing edge of the constructs.[27]

To apply direct shear to cartilage bovine explants, Frank *et al*.[27] constructed an apparatus capable of applying both dynamic compression and shear. After culturing tissues for 24 h under 0.1-Hz frequency and 1% shear deformation, it was determined that direct shear of the explants upregulated radiolabeled sulfate and proline incorporation by 25 and 41% when compared to controls. Further studies with this same apparatus were undertaken for the purpose of examining the effect of frequency. Jin *et al*.[45] examined frequencies of 0.01, 0.1, and 1 Hz and strains of 1 and 3% for 24 h. It was found that all frequencies examined with a strain of 3% stimulated protein production by approximately 50% and proteoglycan production by 25%.

Long-term studies of intermittently applied direct shear have been performed to study the effect of shear on mechanical properties of tissue constructs.[102] Constructs were composed of porous calcium polyphosphate loaded with immature bovine articular cartilage cells. Constructs were cultured for 400 cycles followed by a 2-day rest period, for a total culture duration of 4 weeks. Proteoglycan was approximately 35% greater than controls while collagen had increased 40%. Mechanical tests were also performed. It was determined that the aggregate modulus of this tissue was 112 kPa, approximately six times that of controls.

Shear has also been utilized in conjunction with growth factors. Jin *et al*.[44] combined IGF-1 and shear stimulation and determined there was an additive, though not synergistic, effect of costimulation on immature bovine explants. IGF-1 alone increased both proteoglycan and protein synthesis approximately twofold at a dosage of 150 ng/ml. By adding shear (6%, 0.1 Hz), proteoglycan and protein synthesis was increased a further 25–35%.

From these studies, it can be seen that fluid shear applied on chondrocytes seems to be detrimental by increasing osteoarthritic markers and resulting in incorrect cell orientations. However, application of direct shear seems to be largely beneficial, producing constructs with increased mechanical properties.

MECHANICAL STIMULATION OF LIGAMENTS AND TENDONS

Ligaments connect bone to bone, providing joint stability. The purpose of tendons is to transmit tensile force generated in muscle to bone. Tendons are well suited to this task, having a high tensile strength of between 50 and 100 MPa. This is due to the large parallel bundles of collagen type I oriented in the same direction as the tendon.

Tendon cells, sometimes termed *tenocytes* or tendon fibroblasts, tend to be grouped in elongated rows parallel to and interspersed within the collagen fibrils.[8] The fibroblasts have numerous processes that extend from the cell and toward like projections on fibroblasts of other rows. These form an elaborate network through which cells communicate via gap junctions.[63]

Besides collagen and elastin, which account for 65–75% and around 2%[46] of the tissue's dry weight, respectively, tendon contains proteoglycans. One such proteoglycan, decorin, has been demonstrated to play a role in formation and orientation of collagen fibrils.[42] In a study comparing normal and decorin gene knockout mice, the elastic modulus of the knockout mice tail tendon fascicles was only 60% that of normal.[21] In studies comparing the tensile and compressive regions of bovine tendon, it was determined that in the tensile region, small proteoglycans composed 90% of the total proteoglycan content.[98] In the pressure zone, the ratio changed to approximately equal, the rest being composed of large proteoglycan molecules similar to aggrecan.[98] In areas where tendon wraps around bone, it is subject to compression and shear as well as tension.[23] Tendon responds to compression by forming a fibrocartilaginous tissue.[8] This same effect can be observed in ligament.[59] The compressed regions of these tissues share many characteristics with both articular cartilage and the meniscus; these include rounded cells,[7,8,23] expression of aggrecan and biglycan,[22] and type II collagen in areas of compression.[7,8,64] Tenascin-C, a molecule that aids in the round morphology characteristic of chondrocytes, also has a threefold greater occurrence in areas of compressed bovine tendon compared with tensile tendon.[60] In humans, the GAG content of compressed tibialis posterior tendon was three times that of regions under tensile load within the same tendon.[100] The assumption is that these changes are a response to compressive loading.[99] Further evidence for this has been demonstrated by removing the load from compressed tendon in a rabbit model and noting a 40% decrease in GAG content as well as decreased antibody reactivity with collagen type II.[59] It is important to note the similarities of these related tissues to the meniscus. From these comparisons of ligament and tendon to meniscus, it is evident that these tissues are highly related in terms of mechanical functions, cell population, and ultrastructural components. By examining the methods shown to stimulate regeneration in these tissues, we hope to achieve regeneration in the meniscus.

Tensile Loading Stimulation of Ligaments and Tendons

The number of mechanical stimulation experiments performed on ligaments and tendons is not nearly as extensive as those of articular cartilage, however some factors have been elucidated. It now seems apparent that the response of ligament and tendon is directly related to their mechanical environment. Stress deprivation leads to a detrimental change in their biomechanical and biochemical properties,[9] while overuse can also bring about deleterious effects.[76] Static tension appears to orient fibers in the direction of stress as well as upregulate specific genes such as TIMP-1, TIMP-3, and collagen $\alpha 1$.[48] In fact, static tension inhibits collagenase degradation of devitalized tendon explants.[67] Cyclic loading has also been demonstrated to improve cellular migration into areas of tendon laceration,[92] as well as increased gene expression for type I and type III collagen in ligament (163 and 269% increase respectively).[49]

Like other tissues thus far examined, duration of loading affects the tissue response. Zeichen *et al.*[107] examined fibroblasts stimulated for 15, 30, and 60 min, and measured the rate of proliferation at 6, 12, and 24 h after the simulation. In both the 15- and 60-min stimulation groups, proliferation was increased only at the 6- and 24-h time points while proliferation had decreased for all times measured in the 30-min stimulation group. The authors speculated that certain durations of loading may be beneficial while others are detrimental.

Cyclic strain has been shown to increase cellular production of TGF-β, bFGF, and PDGF after both 15 and 60 min of culture time.[85] Growth factors also modulate fibroblast behavior in tendon. When cultured in the presence of 100 pM of PDGF and cyclic load (5% strain, 1 Hz), the rate of DNA synthesis was increased 10.5-fold compared to nonloaded, non-PDGF stimulated controls and 5.4 times that of nonloaded controls.[6]

Age of cells in the tissue appears relevant as well. Berry *et al.*[9] cyclically stretched fibroblasts (5% strain, 1 Hz) from "young" and "adult" cell lines (neonatal human foreskin fibroblasts and adult human dermal fibroblasts). While both sources responded by an increase in proliferation of about 75%, the young cells increased collagen synthesis while adult cells decreased synthesis.

Fibrocartilaginous responses to tension are not limited to tendon. The rat ACL fibrocartilage responds to a static load by increasing type I collagen mRNA production at both 0.5- and 1-h time points, 40 and 32%, respectively, but decreasing mRNA to only 44% of controls after 2 h.[39] It would seem that like cartilage, fibrocartilage matrix production is inhibited by static loads. Majima *et al.*[58] demonstrated that rabbit medial collateral ligament scar tissue responds to 0.5-Hz tensile stress of 1 MPa for 1 min followed by

14-min rest repeated 16 times.[58] It was found that collagenase mRNA decreased to 66% of controls while aggrecan increased 458%. Tension did not appear to have an effect on the expression of mRNA for collagen I; the authors hypothesized that mRNA for this molecule was already significantly expressed because of the use of recently formed scar tissue.

Histologically, as well as biochemically, loading appears to aid in tissue generation. However, much more work needs to be done in this area on the effects of loading explants and cell seeded scaffolds to determine the biomechanical effects of loading.

Compressive Loading of Ligaments and Tendons

Fibrocartilage from ligament and tendons responds to dynamic compressive loading *in vitro*. Koob *et al*.[50] demonstrated that fibrocartilage from bovine tendon continued to produce large proteoglycans (40% greater than controls) after 2 weeks in culture (0.17 Hz, 20 min/day, 544 kPa peak compressive stress, 5–25% strain). In a concurrent experiment, it was demonstrated that dynamic compression could restore large proteoglycan synthesis after 3 weeks in static culture to the same level as those maintained under dynamic compression. However, all groups had a lower amount of large proteoglycan than freshly harvested tendon.

The response of fetal tissue to compression was also examined by Evanko and Vogel.[23] This experiment demonstrated that dynamic compression of fetal bovine tendon increased radiolabeled uptake of sulfate into large proteoglycan and biglycan 300 and 50–100%, respectively. Age of the subject of the source of tissue thus appears to affect the response of compressed tendon *in vitro*, although exact comparisons are difficult because the loading regime differed from the above experiment in that strain varied from 35% initially to 12% because of limited recovery and the culturing period was 3 days continuous loading. The same group compared the effects of stimulating fetal bovine tendon with dynamic compression and the effect of 1 ng/ml TGF-β.[77] The two modes of stimulation had similar effects on decorin synthesis and aggrecan synthesis. However, TGF-β doubled the rate of biglycan expression, while stimulated only half the mRNA level of aggrecan that dynamic loading did.[77] It is unfortunate that the two stimuli were not introduced simultaneously, as an additive effect has been demonstrated with mechanical stimuli and some growth factors.[10,44]

It seems that dynamic compression of fibrocartilage may have a direct effect on cellular phenotypes. By cyclically loading these predominantly tensile tissues, cells can be channeled to a more chondrocyte-like state. This would be particularly useful in the white zone of the meniscus.

Hydrostatic Pressure Loading of Ligaments

Ligament scar tissue has also undergone hydrostatic pressure loading *in vitro*. Majima *et al*.[58] applied hydrostatic pressure to ligament scar tissue (0.5 Hz, 1 MPa). Hydrostatic pressure increased mRNA expression of aggrecan and collagen II 313 and 171%, respectively. This loading also decreased collagenase mRNA levels to 35% that of unloaded controls. More work in examining the effects of hydrostatic pressure on compressed regions of ligament and tendon needs to be performed.

SUMMARY OF GOALS FOR TISSUE ENGINEERING OF THE MENISCUS

The meniscus of the knee aids in load bearing, stability, and lubrication within the knee. Frequently, the meniscus is damaged by sports injuries or by overall degeneration associated with aging. Removal of the meniscus, or even partial removal, leads to degenerative changes within the joint associated with osteoarthritis. We believe that utilizing tissue engineering methods will be highly successful in regenerating the meniscus.

Before embarking on tissue engineering of the meniscus, it is crucial to have a thorough comprehension of the biomechanical role that this tissue fulfills within the body and how the structure of the meniscus is uniquely suited to that purpose. To better understand this, we have examined the meniscus, as well as associated tissues, within the body. We understand that the red zone of the meniscus is similar to tendon, which is a dense connective tissue composed primarily of collagen type I. In examining the inner white zone of the meniscus, we note that this tissue has a strong resemblance to both articular cartilage and compressed regions of ligament and tendon. We have examined in detail the loading conditions that these tissues experience *in vivo* and how each is uniquely composed to deal with its loading environment. All of these tissues are capable of some degree of remodeling because of mechanical stimuli. We wish to stimulate our construct in such a way as to form the properties we desire in our regenerated meniscus.

It is interesting to note that there are many variables to be explored when utilizing mechanical stimulation for tissue regeneration. It is also apparent that these variables seem to have "windows" of maximal effect, that is, too little and the tissue does not respond, too much and the tissue responds negatively. Time of stimulation is important, as is rest between loading cycles. Additionally, growth factors may increase the effect of mechanical stimulation. Further studies need to be performed using growth factors. The synergism between growth factors and the nonlinear cellular response to growth factor concentration is not yet well understood. As the above studies have demonstrated, some culturing conditions favor proliferation, others GAG or collagen production. More can be done than simply optimizing culturing conditions to grow one aspect of the tissue. For instance, it might be possible to utilize cyclic tensile strain to upregulate collagen I in the red zone. In the white zone, direct shear would be applied to synthesize a greater amount of

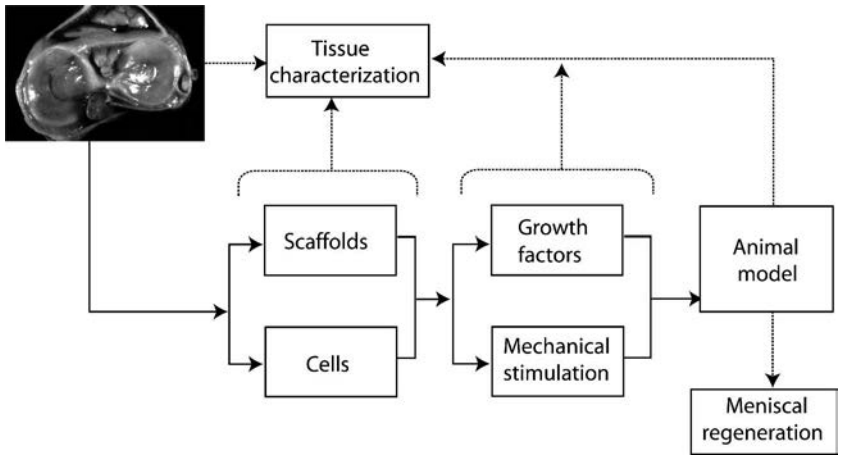

FIGURE 5. Regeneration strategy.

collagen II during the early stages of culture and then hydrostatic pressure to initiate GAG production.

It has been postulated that there might be a continuous spectrum of fibrocartilage between articular cartilage and dense connective tissue. And indeed, the meniscus would seem to be such a tissue. The cells of the meniscus are described as fibrochondrocytes because they appear to possess properties of both fibroblasts and chondrocytes. Thus, by understanding the mechanisms that can stimulate and promote regeneration in related tissues, we hope to harness that knowledge to regenerate the meniscus, which is our research effort's ultimate goal. The clinical implications of a tissue-engineered meniscus are many. Primary among these is the ability to replace part or all of a patient's meniscus post-meniscectomy. This advance would ameliorate pain, return function, and arrest the later development of osteoarthritis. However, much work needs to be performed before such a goal is realized.

Many hurdles need to be overcome before the goal of tissue engineering the meniscus is achieved. Success in a small animal model must be achieved before larger animals and clinical trials could be approved. There is no universally agreed-upon biomaterial to utilize as a scaffold and much work is devoted to optimizing current materials as well as developing new ones. Other questions such as ideal methods of cell expansion and growth factor optimization have yet to be fully addressed. Our strategy is illustrated in Fig. 5. The meniscus of our small animal model, the rabbit, will be extensively characterized biologically and biomechanically. This will serve as our "gold-standard" for our tissue-engineering project. Cells will be harvested from the native meniscus and expanded in culture. To choose our scaffold, fibrochondrocytes and scaffold biomaterials will be combined and the quality of the cultured construct will be evaluated against our gold standard in terms of biomechanical and biochemical properties. We believe that combining mechanical stimulation as well as growth factors will further improve our tissue-engineered construct. When our cultured construct performs adequately compared to our gold standard, we will implant the construct into our animal model to determine *in vivo* performance.

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