Tissue Engineering of Ligaments

Sarah Rathbone and Sarah Cartmell
School of Materials, Materials Science Centre, The University of Manchester
United Kingdom

1. Introduction

The main function of a ligament is to connect one bone to another bone across a joint, keeping them aligned to prevent abnormal motions and dislocations. The typical magnitude of force a ligament may experience during day-to-day activities varies. For example the anterior cruciate ligament of the knee can be exposed to daily tensile forces ranging between 67N for ascending stairs to 630N for jogging (Vunjak-Novakovic, Altman et al. 2004), whereas large loads, exceeding 1800N, can cause rupturing. Depending upon anatomical location and the extent of vascularisation, the ligament may or may not be capable of self-healing after a rupture.

Some of the most frequently ruptured ligaments occur in the knee joint, often through sporting activities such as skiing, football and basketball and the number of injuries are increasing each year (Cooper, Lu et al. 2005). Ninety percent of knee ligament injuries involve the anterior cruciate ligament (ACL) and medial collateral ligament (MCL) (Woo, Abramowitch et al. 2006). The MCL can self-heal, but the ACL cannot due to poor vascularisation. Because of this, alternative methods such as regenerative medicine have focused heavily upon the ACL with the aim of producing a fully functional tissue in vitro.

Figures indicate that approximately 250,000 people are diagnosed with ACL injuries each year in the USA (Doroski and Brink 2007), and approximately 150,000 need to undergo surgical treatment, known as an ACL reconstruction (Cooper, Lu et al. 2005). If the rupture is not treated it can cause loss of function of the associated joint which can then lead to early development of osteoarthritis (Cooper and Bailey 2006; Gentleman, Livesay et al. 2006).

The current gold standard procedure for an ACL reconstruction is surgical autografting. This involves using part of the patients own patellar tendon, hamstring or quadriceps to replace the ruptured ACL (Beasley, Weiland et al. 2005). However, these techniques cause donor site morbidity (Goulet and Germain 1997; Van Eijk, Saris et al. 2004; Cooper and Bailey 2006; Hairfield-Stein, England et al. 2007) which is associated with pain and a recovery period for the donor tissue site (Cooper, Lu et al. 2005; Hairfield-Stein, England et al. 2007). Generally 75-90% of patients have good or excellent long term success rates from these current grafting techniques (regarding functional stability and symptomatic relief upon return to normal activities) but unfortunately a substantial number of patients exist who have unsatisfactory results which could be attributed to graft failure (Vergis and Gillquist 1995). Some of these patients continue to endure pain, suffer from loss of motion secondary to the operative procedure and continue with recurrent instability (Vergis and Gillquist 1995), while others suffer from degenerative joint disease such as arthritis or

experience re-injury (Hairfield-Stein, England et al. 2007). Alternatively, allografts can be used where the donor tendon is taken from a cadaver, but the disadvantages associated with this include donor scarcity, the risk of the recipient contracting a disease from the donor, or tissue rejection (Ahmed, Collins et al. 2004; Vunjak-Novakovic, Altman et al. 2004). Prosthetic replacements (synthetic grafts) have previously been used, but these have shown to be inadequate due to wear and degeneration (Mascarenhas and MacDonald 2008). It is evident that surgical ACL reconstructions have limitations and do not always give completely satisfactory long-term results in a high proportion of patients, which consequently affects their quality of life (Vergis and Gillquist 1995; Lanza, Langer et al. 2007). Because of this dilemma, regenerative medicine could be an option, where in vitro tissue engineering of ligaments can offer a solution to the problems associated with the current surgical methods (Van Eijk, Saris et al. 2004; Hairfield-Stein, England et al. 2007). Tissue engineered ligaments could provide better performance in the long run by improved biocompatibility, integration into host tissue and the ability to remodel their own extracellular matrix (Nesic, Whiteside et al. 2006).

Tissue engineering is a method which combines knowledge from material science, engineering, molecular biology and medicine (Nesic, Whiteside et al. 2006). The basic procedure normally involves using scaffolds to act as structural supports for cell growth and maturation in-vitro, where a stimulus (chemical or mechanical) may also be applied to promote the formation of a functional tissue. This concept was originally developed to repair skin and cartilage, but is now being considered as a possible option to produce neoligament tissue. To date, many different types of material have been investigated as potentially suitable scaffolds for ligament tissue engineering, focusing upon their biocompatibility, degradability, surface properties for cell attachment and overall mechanical properties. These include polymers (such as polyurethane, polylactic acid, polyglycolic acid, polycaprolactone, polyhydroxyalkanoates and alginates), silk fibroin, glasses, hydrogels and biological materials such as de-cellularised tissues.

There has been much research into the application of chemical stimulus upon cell culture in vitro. It is well documented that specific growth and differentiation factors can trigger various cellular responses such as cell differentiation, cell division and matrix remodelling (Evans 1999), making them useful in tissue engineering to influence cell behaviour. Some of the most commonly studied growth factors include transforming growth factor beta-1 (TGF-β1), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Bioreactors also have applications in tissue engineering, where they can be used to optimise the cell culturing conditions; for example they can improve the mass transfer of nutrients to cells in a 3D scaffold (perfusion), improve cell seeding onto a scaffold (rotation), or provide a mechanical stimulus (in tension or compression) to influence cell behaviour. For ligament tissue engineering, the bioreactor is normally used to apply tensile straining forces within physiological ranges to promote differentiation and extracellular matrix (ECM) synthesis. From the literature, the mechanical loading regimes investigated have varied from 1-10% strain, 0.01-1Hz frequency, from ½ hour – 24 hours/day over a period ranging from 1 day to six weeks. Achieving the optimal culturing conditions for a ligament tissue engineered construct can be complex, where small changes can have large affects upon cell behaviour and their final product. This chapter will review in detail the different biomaterials, loading regimes and growth factors that have been currently investigated for this purpose.

2. Anatomy of the ligament

2.1 Structure and function of a ligament

A ligament is a capsule of connective tissue made of fibres joining one bone to another across a joint where they help to guide joint motions and prevent abnormal displacement of bones relative to each other (Einhorn, O'Keefe et al. 2007). They are very strong compared to other connective tissues, such as skin, because of the high tensile loads they need to withstand (Einhorn, O'Keefe et al. 2007). Although there are several hundred ligaments in the body, many of the examples given have focused upon the ACL because it is the most frequently injured knee ligament. Figure 1 indicates where the main knee ligaments are located around and within the knee joint.
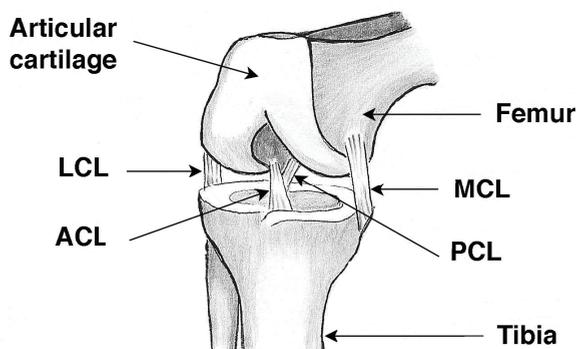

Fig. 1. The diagram illustrates where the fours main knee ligaments are located; the anterior cruciate ligament (ACL), the posterior cruciate ligament (PCL), the medial collateral ligament (MCL), and the lateral collateral ligament (LCL)

There are three main types of connective tissue within the human body, connective tissue proper (loose and dense regular connective tissue), fluid connective tissue (transports substances in blood) and supporting connective tissue (cartilage and bone). Skeletal ligament is a dense regular connective tissue, which is comprised of fibroblasts (connective tissue cells), and extracellular matrix (proteins and water making up the connective tissue). The periodical change in direction of collagen fibres gives the connective tissue a distinct undulating pattern. The fibroblasts (located within in the ECM) are responsible for producing the ECM components to maintain and repair the connective tissue. After an injury, these cells become mobile, migrating to the wounded tissue to increase the synthesis of specific proteins to aid tissue repair (Rogers 1983; Alberts, Johnson et al. 2000). The ECM is composed of two main classes of macromolecules; polysaccharide chains of glycosaminoglycans (which have adhesion functions and attract water), and fibrous proteins such as collagen, elastin and reticular fibres which give structural support to the tissue (Alberts, Johnson et al. 2000). The ground substance of the ECM is a hydrophilic water-like gel containing the polysaccharides and fibrous proteins, allowing diffusion of waste products and nutrients between the tissue cells and capillaries (Hansen, Masouros et al. 2006).

Ligament connective tissue is classed as dense regular tissue because the closely packed collagen fibres are aligned in an ordered regular, way, giving tensile strength and support to

the tissue. The basic structure of collagen is very similar in all collagen types, where its formation starts with the synthesis of polypeptide chains on the ribosome which are composed mainly of glycine, hydroxylysine and hydroxyproline repeats (Carpenter and Hankenson 2004). Inside the cell, three polypeptide α-chains coil together into a right-hand twist to make a triple helix (super helix) forming the procollagen molecule which then becomes exocytosed from the cell. The procollagen molecules polymerize in the extracellular space firstly by aggregating together into a microfibrils, then aggregating into fibrils, where finally, the fibres become stabilized by covalent cross-links which form within and between the tropocollagen molecules (Alberts, Johnson et al. 2000). It is this extensive cross-linking, particularly in collagen I, which gives the collagen fibrils their stability and great tensile strength, which in turn makes the tissue very strong so that the ligament can resist deformation from stretching forces (Doroski, Brink et al. 2007). Collagen type I, III and V are all structural components of ligament fibrils (Posthemus, September et al. 2009), where type I and III provide tensile strength and type V regulates fibre assembly and diameter (September, Schwellnus et al. 2007). Collagen type X is present where the ligament integrates into the bone. Tenascin-c, another type of protein found in ligament ECM, regulates the tissues response to mechanical loading (September, Schwellnus et al. 2007). The individual collagen fibrils are randomly orientated, but as they aggregate into fibres they gain a more parallel orientation with the longitudinal axis of the ligament, giving the tissue a crimping pattern (wavy appearance) (Goulet, Germain et al. 1997).

2.2 Bone attachment

Ligaments attach to the bone surfaces as an aggregation of collagen fibre bundles (Ellenbecker 2000), either by direct insertion, or both direct and indirect insertion. The ACL inserts into the bone by direct insertion, the most common ligament insertion type, where its collagenous fibres attach directly to the bone tissue. The collagenous fibres blend into the fibrocartilaginous layer, interweave through the fibrocartilage zone, through the mineralized fibrocartilage zone, then enter the bone (Beasley, Weiland et al. 2005; Woo, Abramowitch et al. 2006). The calcified collagenous fibres which anchor the ligament firmly to the bone are known as Sharpey's fibres (Einhorn, O'Keefe et al. 2007). The medial collateral ligament (MCL), which is also a knee ligament, is inserted into the bone by both direct and indirect insertion, where the superficial fibres (near to the surface) merge with the periosteum (the connective tissue surrounding the bone), while other fibres penetrate the bone deeper and attach to the bone directly at acute angles (Woo, Abramowitch et al. 2006).

2.3 Characteristic components of ligament tissue

It is the variation in ratio between collagen types and other ECM components which gives each ligament type its diversity and characteristic mechanical behaviour (Woo, Abramowitch et al. 2006). Due to the absence of specific markers, ligaments can only be distinguished from other ligaments and other tissue types (eg tendon) by structural, molecular and mechanical properties. Although there is no single specific marker in the ligament, tenscin-c has been considered to be a marker due to its characteristically high amounts in the ligament (Doroski, Brink et al. 2007), where the presence of collagen types I and III, tenomodulin, biglycan, decorin, elastin and fibronectin are also characteristic of ligament tissue (Vunjak-Novakovic, Altman et al. 2004; Chen, Huang et al. 2008). The total amounts and specific ratios of the ECM components, ground substance and cells are

characteristic properties unique for each type of connective tissue which relate to its anatomical location and function (Vunjak-Novakovic, Altman et al. 2004). Connective tissue types can be differentiated by the presence and total quantities of collagen, tenascin-c, elastin, fibronectin, decorin, biglycan, ratios of collagen types, crimping pattern and collagen fibril diameter. Table 1 lists the main components of a typical ligament as a percentage of their wet weights, whereas those in table 2 are the dry weights of the main collagen types and ratio of collagen type I : III which are unique to the ACL, as reported by various authors.

Tissue type (wet weight)	Collagen type I (%)	Other collagens such as type III, V, VI (%)	Elastin (%)	Fibronectin and other glycoproteins (%)	Proteoglycans (%)	Water (%)	Author
Ligament (general)	20	3-5	1-2	1-2	<1	70	(Einhorn, O'Keefe et al. 2007)

Table 1. The biochemical constituents of wet ligament tissue

Tissue type (dry weight)	Collagen type I (%)	Collagen type III (%)	Collagen type V (%)	Ratio of collagen I : III	Author
ACL	70-80	8-10	10-12	9:1	(Woo, Abramowitch et al. 2006; Doroski, Brink et al. 2007)

Table 2. The collagen content of the ACL in dry tissue

3. Cell adherence

It is essential for fibroblasts to attach to a substrate and spread out to enable them to grow, proliferate, mature and produce functional tissue. In order for these cells to adhere to their substrate, ECM adhesion proteins such as fibronectin, vitronectin or collagen are required to adsorb to the substrate first, where the cells will then subsequently adhere to the adhesion proteins. Fibronectin can exist in two major forms; (1) soluble plasma fibronectin, a constituent of plasma, and (2) insoluble cellular fibronectin, a component of the ECM (Pankov and Yamada 2002). Cellular fibronectin can be expressed by different cell types including fibroblasts (Pankov and Yamada 2002), where it is found in ligaments and other connective tissues. Studies have found it be up-regulated during ligament formation in embryogenesis where it guides the migrating cells (Laurencin and Freeman 2005).
Integrin-mediated binding enables the cell to become connected to its surroundings by linking the interior of the cell to the ECM proteins (outside the cell). The contact made with the ECM can generate intercellular signals which can affect gene expression, morphology, cell survival (Johansson, Svineng et al. 1997), control cell adherence, cell migration,

cytoskeletal organization (Sechler, Corbett et al. 1997) regulate growth, proliferation, differentiation, and subsequently affect development or maintenance of the ligament tissue (Alberts, Johnson et al. 2000; Vunjak-Novakovic, Altman et al. 2004). Two known intracellular pathways involved upon integrin-fibronectin binding are the Ras-MAPK (Ras-mitogen activated protein kinase) and the FAK (focal adhesion kinase) pathways.

Fibronectin mediated cell adhesion studies have been conducted by various researchers as a technique for improving cell attachment in vitro. Research has shown that cell retention of rat MSC's on fibronectin-coated surfaces was improved (Dennis and Caplan 1993), whilst other studies indicate that it increases the adhesive strength of cells, suggesting it occurred due to the increased number of bonds between fibroblasts and fibronectin-coated glass slides (Athanassiou and Deligianni 2001).

4. Response of fibroblasts to mechanical stimulus

4.1 Mechanical transduction

Mechanical forces play a major role in the formation and architecture of native tissues in vivo, but also help maintain healthy tissue (homeostasis) in adult tissue. During daily activities, human body tissues are subjected to mechanical forces of various magnitudes, depending upon the activity and posture during these movements. The ACL has been shown to withstand forces of up to 1730N in people aged 16-26 years, but much less in people aged 48-86 years, with a mean average of approximately 734N (Noyes and Grood 1976). However, the forces which can be tolerated become significantly reduced when they are perpendicular to the bone insertion sites (Einhorn, O'Keefe et al. 2007). The externally applied forces can alter the cells structure, mechanical properties, behaviour, and function (Miyazaki, Hasegawa et al. 2000) which are required for tissue homeostasis (Fulton 1984; Altman, Lu et al. 2002). Tissue homeostasis occurs through ECM remodelling (re-organization) which involves ECM degradation by apoptosis (programmed cell death) and the formation of new tissue by cell proliferation (multiplication). An equilibrium between proliferation and apoptosis is essential during ligament growth, healing, tissue homeostasis and adaptation to exercise (Chuen, Chuk et al. 2004).

The cells are believed to sense mechanical forces either through their cell surface integrin receptors or through ion channels in the cell membrane, and respond accordingly. These external forces can alter the cell structure, its mechanical properties, behaviour, and function (Miyazaki, Hasegawa et al. 2000), where, for example they may increase or decrease ECM production, or regulate proliferate and differentiate. Transducing (converting) external physical forces into cellular signals across the membrane is known as mechanical transduction (Ingber 1999). Although the mechanisms of transduction are not well understood, many investigations have been carried out upon integrin receptors and the cell cytoskeleton that support the theory that the cell senses external mechanical forces from the ECM via the integrin receptors (subsequently causing deformation and reorganisation of the cell cytoskeleton) (Pertigliano, McAllister et al. 2006) and activates specific cellular pathways.

4.1.1 Cytoskeletal tension

Upon cell-substrate binding via the integrin receptors, this exerts a force upon the cell cytoskeleton, which generates an intracellular tension. In effect, this links the cell membrane to the nucleus which influences gene expression, by relaying signals from the plasma

membrane to the nucleus (Matyas, Edwards et al. 1994). The cytoskeleton is composed of a network of protein filaments within the cytoplasm, which maintains cell shape, giving it structure and support, and allowing the cell to bear stress without splitting (Alberts, Johnson et al. 2000). The cytoskeleton has a number of other functions including connecting each fibroblast to other cells and to the substrate, generating tension within the cell to produce stress fibres, and also assisting the cell in locomotion (Fulton 1984). There are three proteins in the cytoskeletal network, actin, tubulin and vimentin, which assemble to form the three main structural filaments of the network system (actin filaments, microtubules, intermediate filaments respectively) (Portner, Bagel-Heyer et al. 2005). Both actin and the intermediate filaments are involved in connecting the fibroblasts' internal structure to other cells and to the ECM. When the fibroblast encounters a suitable substrate, it extends its projections (filopodia), which then attach to the substrate allowing the rest of the cell to adhere. This generates a small tension, where the cell subsequently spreads (Fulton 1984; Alberts, Johnson et al. 2000), promoting formation of stress fibres within the cell. This enables the cell to withstand the tension generated from the cell-ECM contact and make connections with the nucleus to modulate cell behaviour. The effects include activating specific genes (Fulton 1984; Altman, Lu et al. 2002) and generating key proteins (including degrading enzymes and ECM components) to remodel the ECM for promoting new tissue formation (Vunjak-Novakovic, Altman et al. 2004; Portner, Bagel-Heyer et al. 2005).

4.1.2 Intracellular cell signalling

The most widely accepted mechanism for mechanical transduction is that involving intracellular signalling pathways. Once the cell binds its substrate, the integrin receptors act as mechanoreceptors, receiving then relaying mechanical signals from the ECM through the cell (as biochemical signals) to the nucleus via intracellular pathways. This can either promote gene expression, regulate growth, proliferation or differentiation, subsequently affecting development or maintenance of the connective tissue (Alberts, Johnson et al. 2000; Vunjak-Novakovic, Altman et al. 2004). The most studied intracellular pathway is the Ras-mitogen-activated protein kinase (Ras-MAPK) pathway, which is considered to be the one which acts as a general, but unspecific signal transducer, converting the signal from the applied mechanical stress and relaying it through the cell interior (Chiquet, Sarasa-Renedo et al. 2003). The Ras-MAPK pathway becomes activated once the receptor receives an extracellular mechanical signal. Briefly, the received signal causes a kinase on the receptor to become activated which then activates a GTP-binding protein (Ras protein). The Ras protein causes downstream phosphorylations by activating the first MAP kinase (Raf) in the chain to phosphorylate the next MAP kinase (Mek), which phoshporylates and activates the next MAP kinase (Erk), which subsequently activates other proteins. Eventually the signal reaches the gene regulatory proteins in the nucleus which interact with transcription factors and promoters to regulate gene expression and protein activity (Alberts, Johnson et al. 2000).

4.1.3 Ion channels

Besides mechanical transduction via integrin receptors, external forces (stress) can also be conveyed into the cell through stretch-induced ion channels which open and close in response to cell membrane deformation (Matyas, Edwards et al. 1994). Ion channels are cation specific channels located in the cell plasma membrane, which allows rapid diffusion of the ions down their electro-chemical gradients across the lipid bilayer. The channels are

gated, only opening briefly to allow specific cations (such as calcium, sodium or potassium) to pass through, and then close again. The channel gates open in response to several types of specific stimulus, one those being mechanical stress which operates mechanically-gated channels (Alberts, Johnson et al. 2000), subsequently affecting the cell behaviour. Cyclic stretching has been reported to stimulate Ca^{2+} influx into osteoblasts, and it is thought that mechanical stretch-induced Ca^{2+} signal transmission may involve the actin filaments (Wang 2006).

5. Injuries sustained and healing potential

Ligaments in skeletally mature people are very strong and stiff at high loads, however, there can be some variation in the mechanical properties of each ligament type depending upon the individuals' gender and age. Strength and stiffness of the ACL has been found to significantly reduce as age increases in adults (Noyes and Grood 1976) and can be significantly lower in adult females compared to their male counterparts (Chandrashekar, Mansouri et al. 2006). Ligaments reach their maximum strength when the loading forces are aligned with the ligament fibres and aligned with the direction of bone insertion, becoming three times as strong as when the force acts perpendicular to the bone insertion sites (Einhorn, O'Keefe et al. 2007). They rupture when the load (externally applied force) becomes too excessive to withstand and the collagen fibres tear apart. The ACL, for example, normally ruptures in the mid-substance (middle region) when the knee joint experiences too much force, but it can also tear at the bone insertion sites. The position of the tibia relative to the femur can increase the magnitude of the stress placed onto the knee joint. The stress becomes greatest when the tibia is fully extended and internally rotated simultaneously, increasing the tension upon specific fibre bundles which are trying to resist deformation and abnormal motion. A rupture of the mid-substance occurs when the cross-links between the collagen fibrils slip allowing the tropocollagen helix to over-stretch, allowing the tissue to tear (Laurencin and Freeman 2005).

Generally, the blood supply to ligaments is sparse when compared to other tissue types (such as the skin), affecting their healing potential (Einhorn, O'Keefe et al. 2007) which can be limited further by anatomical location, age and gender. The healing capacity of the mature ACL is very low due to its anatomical location. It is encapsulated within the knee joint (intrasynovial), being surrounded by the lubricating synovial fluid, and is poorly vascularised (Amiel, Frank et al. 1984; Ahmed, Collins et al. 2004; Cooper, Lu et al. 2005). As a result it cannot self repair (Cooper, Lu et al. 2005; Lu, Cooper et al. 2005), therefore medical treatment is necessary. There is no direct blood supply from the fibrocartilage zone of the bone to the ACL, so the ACL relies mainly upon diffusion of nutrients and waste through the joint fluid from and to the blood vessels of the surrounding synovial tissue respectively (Beasley, Weiland et al. 2005). The surrounding synovial tissue is vascularized by the medial genicular artery, and the lateral inferior genicular artery, forming a vascular plexus (network of vessels) around the knee. It is the small vessels from the plexus which supply the ligament with the essential nutrients by diffusion (Zantop, Patterson et al. 2005). It possible that a few of these small blood vessels may actually penetrate the ACL and directly supply it with nutrients (Arnoczky 1983). As a result of poor vascularization to the midsubstance, the ACL has a low healing capacity and can not self repair (Carpenter and Hankenson 2004). In contrast to the ACL, the MCL which is extrasynovial can self heal

spontaneously because it has a greater vascularisation and receives more blood (Carpenter and Hankenson 2004).

After injury, those ligaments which are well vascularized have three stages of healing; inflammation, cellular proliferation and migration, ECM repair and finally ECM remodelling (Laurencin and Freeman 2005). Generally, these stages promote fibroblast proliferation. Fibroblasts and macrophages then migrate to the injured site and granulation tissue forms (stroma). GAG, elastin and collagen are synthesised to form new ECM, and finally the ECM is remodelled, where it initially forms into a disordered tissue but later becomes more organized (Alberts, Johnson et al. 2000; Laurencin and Freeman 2005). With avascular, or poorly vascularised ligaments, this process is not carried out, or only in a limited way, which prevents them from self-healing spontaneously and surgery may be needed to repair them. With the case of the ACL, if left untreated, this could eventually cause osteoarthritis in the knee because the ACL has failed to maintain correct bone alignment and control normal motion across the knee joint (Foster, Butcher et al. 2005; Utukuri, Somayaji et al. 2006).

6. Surgical treatment

After rupture, the ligament is normally repaired by surgery, which can be by suturing or grafting. Based upon clinical investigations, surgical grafting has become the gold standard for ligament repair (Einhorn, O'Keefe et al. 2007). In the case of the ACL, a surgical reconstruction is performed which is the only method shown to at least partially restore function, helping to improve the patient's quality of life. This method involves implanting a graft to replace the damaged ligament.

Three main types of grafts can be used; autografts, allografts or synthetic grafts. The current gold standard procedure for reconstructing an ACL is autografting, which involves using a ligament or tendon from another part of the patient's body and using it to replace the damaged ACL. This can be a section from the patients patellar tendon (joining knee cap to tibia) or the hamstring tendon (joining calf muscle to bone in heal) (Beasley, Weiland et al. 2005). Patellar tendon is often used because its strength and mechanical properties are similar to or exceed that of normal native ACL (Fenwick, Hazleman et al. 2002). The central third of the patellar tendon is removed with a piece of knee cap (bone plug) attached to one end, and a section of the tibia attached at the other end (Beasley, Weiland et al. 2005). The damaged ACL is removed, a bone tunnel (channel) is drilled out from the femur and tibia, and the graft is threaded through and screwed into leg bones. This reconstruction operation takes approximately 2 ½ hours. Allografting involves using a ligament or tendon from a different human donor, normally a cadaver (corpse). The procedure is the same as autografting, and may give the same results, but disadvantages include donor scarcity, the risk of the recipient contracting a disease from the donor, or tissue rejection (Vunjak-Novakovic, Altman et al. 2004). Clinical outcomes in the short term can be good, with 80% success rate in restoring knee stability (Einhorn, O'Keefe et al. 2007). Unfortunately, both autografting and allografting can be unsatisfactory methods for long term performance in some patients, where they suffer from instability (Woo, Abramowitch et al. 2006) due to mechanical failure, fatigue or creeping, (gradual stretching of the tissue under constant load). This occurs due to a slight mismatch in mechanical properties between the graft and native ligament tissue, where the injury may then recur at a later date (Lu, Cooper et al. 2005). It has been suggested that even two years after the implantation the tendon graft

remains structurally and mechanically different to normal native ligament and never actually becomes "ligamentised" (Fenwick, Hazleman et al. 2002). Another disadvantage of autografting is donor site morbidity which can cause pain, swelling, local nerve damage, scarring, stiffness weakness or infection (Einhorn, O'Keefe et al. 2007). Synthetic grafts have also been used such as carbon, the Gortex prosthesis, the Stryker-Dacon ligament, the Leeds-Keio artificial ligament, LARS ligament and Kennedy ligament augmentation devices, but creeping, fatigue and limited integration between host tissue and the graft have occurred several years after implantation (Ahmed, Collins et al. 2004; Cooper, Lu et al. 2005). The advantages and disadvantages of the current grafting methods are summarized in table 3.

	Autograft	**Allograft**	**Synthetic graft**
Advantages	No rejection. No disease transmission. No donor scarcity.	No donor site morbidity.	No donor site morbidity. No tissue disease transmission. No donor scarcity.
Disadvantages	Donor site morbidity. Patellar fracture. Quadriceps weakness. Limited bone integration. Mismatch in different tissue properties, causing mechanical failure, creeping, fatigue. Recurring injury.	Donor scarcity. Limited bone integration. Tissue rejection. Mismatch in different tissue properties, causing mechanical failure, creeping, fatigue. Recurring injury.	Limited bone integration (weak graft-host tissue interface). Mismatch in different tissue properties causing mechanical failure. Creeping (stretching & loosening). Poor long-term instability. Fatigue. Recurring injury.

Table 3. The advantages and disadvantages of three types of graft

7. Tissue engineering as regenerative medicine

7.1 Background

Tissue engineering is a rapidly developing area in regenerative medicine which uses knowledge of biological, chemical and engineering techniques to regenerate new tissue in-vitro (Cooper, Lu et al. 2005). Some tissues in the body are capable of self repair after injury, while others are not, and tissue engineering is a relatively new technique which could offer alternative methods to restore tissues and organ functions (Quaglia 2008). The techniques enable various biophysiological parameters to be controlled in order to develop a functional tissue ready for implantation (Lanza, Langer et al. 2007). The procedure involves using a scaffold to act as a structural support for cell growth and maturation in vitro to eventually produce a functional tissue to repair or replace damaged tissue. This concept was originally developed to repair skin, cartilage and bone, but is now being considered as a possible option to produce neo-ligament tissue rather than using the traditional surgical grafting approach. Although the current methods of treatment may help to fully restore the

ligaments in some patients, its long-term success in others is unsatisfactory, indicating that there is a need to find more successful, alternative methods of treatment for full restoration of ligaments. Unlike synthetic grafts which can degrade and lose strength over time, tissue engineered implants could perform better in the long term with their biocompatibility, improved integration into surrounding host tissue, and their ability to remodel the ECM as and when required to (Nesic and Whiteside 2006). Tissue engineering also has the advantage of producing an immediately functional tissue (Vunjak-Novakovic, Altman et al. 2004), but the successful incorporation of the soft tissue implant into bone could be a challenge.

For applications in ligament tissue engineering, a scaffold is required to be biocompatible, biodegradable, allow cell adherence, have sufficient surface area and volume for cell in-growth, be sufficiently strong to withstand mechanical loading forces in vitro and in vivo, and posses a similar stiffness to the native ligament tissue (if it is to be implanted before it degrades) (Christian, Jones et al. 2001 ; Cooper and Lu 2005; Probhakar, Brocchini et al. 2005; Gentleman, Livesay et al. 2006; Sahoo, Ouyang et al. 2006). These points are summarized in table 4. Often, three-dimensional (3-D) scaffolds are preferred to the two-dimensional (2-D) scaffolds because they not only allow cell in-growth, but can also retain cells in their differentiated state. From the literature, it has been reported that fibroblasts cultured in 2-D monolayers have de-differentiated (reverted back to their undifferentiated state) during cell culture (Schulze-Tanzil, Mobasheri et al. 2004), which may not be desirable for tissue engineering purposes.

Scaffold requirements	The purpose of this feature
Biocompatible	Avoids immunorejection (a cytotoxic response could kill the cells)
Biodegradable	To degrade at the same rate at which neotissue forms to avoid the need for surgical removal
Enable cell adherence	To allow cells attachment for growth and proliferation to occur
Provide sufficient surface area/volume	To provide sufficient space for cell spreading and growth
Possess comparable strength/stiffness	To withstand cyclic mechanical loading forces with magnitudes and strains similar to those found in vivo
Surgical implantation	Ease of fixing/bonding to bone (bio-active)

Table 4. The main requirements of a scaffold with respect to their application

7.2 Suitable cell types for ligament tissue engineering

Fibroblasts and mesenchymal stem cells (MSC's) have been considered to be the preferred cell type for seeding onto scaffolds in tissue engineering (Doroski, Brink et al. 2007). Some

reports suggest that MSC's are a potentially better source for ligament tissue engineering than ligament fibroblasts due to their higher expression of collagen type I and III (Ge, Goh et al. 2005). Other reports however, feel that ACL fibroblasts are more appropriate because of the characteristic ratios of collagen types produced during tissue repair (Fu, Harner et al. 1993). In one particular study MSC's were isolated from a human ACL and the results demonstrated that both ACL-derived MSC's and bone marrow MSC's expressed marker genes for ligament fibroblasts, but mRNA expression levels for collagen I and III were higher in the ACL-derived MSC's. It was concluded from this study that ACL-derived MSC's have an increased potential to form ligament fibroblasts in comparison to bone marrow MSC's (Huang, Chen et al. 2008). Co-culturing MSC's with ligament fibroblast has been shown to successfully induce MSC differentiation into fibroblasts, where this conclusion was based upon the mRNA expression of key ligament genes (collagen I collagen III, and tenascin-c) and synthesis of these key ligament proteins (Fan, Liu et al. 2008). This feature makes them an attractive cell choice for ligament tissue engineering.

7.2.1 Mesenchymal stem cells (MSC's)

Mesenchymal stem cells (MSC's) are multipotent progenitor cells, meaning they can differentiate into specific cell types of various cell lineages. They are found in multiple adult tissue types including bone marrow, muscle, synovial tissue and adipose tissue (Centeno, Busse et al. 2008), where they have the potential to produce cartilage, bone, muscle, tendon, ligament or fat (Papathanasopoulos and Gaiannoudis 2008) in response to the appropriate stimuli (Lanza, Langer et al. 2007). MSC's can be encouraged to move down specific cell lineages by using media which contains hormones such as dexamethasone, hydrocortisone, or growth factors such as transforming growth factor β (TGF-β) (Papathanasopoulos and Gaiannoudis 2008), cytokines, transcription factors (Lanza, Langer et al. 2007) or using purely mechanical stimulus (Altman, Horan et al. 2001). MSC's have been used successfully to regenerate articular cartilage in animal models and to regenerate bone in humans (Papathanasopoulos and Gaiannoudis 2008). Because they can be easily isolated and expanded (Papathanasopoulos and Gaiannoudis 2008; Yu, Chen et al. 2008), with the capacity to differentiate, this makes them desirable for tissue engineering applications (Papathanasopoulos and Gaiannoudis 2008). One of the first areas in which they were applied was in tendon and ligament tissue engineering (Lanza, Langer et al. 2007).

Another appealing feature of MSC's is their immunosuppressive and anti-inflammatory effects. They express low levels of major histocompatibility complex (MHC) class I molecules on their surface (preventing natural killer cells deleting them), and no class II MHC, allowing them to escape recognition by alloreactive T helper cells (Zhao, Liao et al. 2004; Lanza, Langer et al. 2007; Popp, Eggenhofer et al. 2008; Swart, Martens et al. 2008). However, it has been reported that MSC's infused into allogeneic MHC-mismatched mice have been rejected (Swart, Martens et al. 2008). In contrast, this was not the case when genetically modified MSC's were injected into a baboon (Zhao, Liao et al. 2004).

7.3 Biomaterials suitable for ligament tissue engineering

To date, many different materials in their various physical forms have been investigated as substrates for tissue engineering applications in general. These include synthetic polymers, natural polymers, glasses, silk, hydrogels, composites and many more. Only those related to ligament tissue engineering, are covered in this chapter (summarized in table 5).

Synthetic polymers such as polylactic acid, polyglycolic acid and polylactide-co-glycolide (PLA, PGA and PLAGA respectively) are approved by the USA food and drugs agency (FDA) for a variety of clinical applications (Cooper, Lu et al. 2005). These polymers degrade by hydrolysis of ester bonds (water breaks up the molecule), and the components are removed by the natural pathways of the body (Rezwan, Ghen et al. 2006), making them biocompatible. Some of these have been produced into cell scaffolds and tested for their suitability as substrates. PLAGA fibres have been used to make 3-D braided scaffolds, which consisted of 3 regions – the attachment site for the femur bone at one end, the main ligament region in the middle, and the attachment site for the tibia bone at the other end. The results indicated that the scaffold was biocompatible by the observed attachment, spreading and growth of the ACL fibroblasts initially seeded onto it (Cooper, Lu et al. 2005). PLAGA has also been produced into nano-fibres which were electrospun into a knitted PLAGA scaffold to increase the surface area for cell attachment. It significantly improved MSC attachment and proliferation, but also demonstrated that cell function had improved due to the increased mRNA expression of type I collagen and decorin (Sahoo, Ouyang et al. 2006). Braided PLA, PGA and PLAGA have also been found to enhanced rabbit ACL fibroblast attachment and support high cell numbers, being highest on PLA (Lu, Cooper et al. 2005).

Alginates are a natural linear polysaccharide copolymers extracted from brown algae belonging to the phaeophyceae (Hua and Wang 2009). They are currently used in the food, cosmetic and agricultural industries (Hua and Wang 2009). Because they are easy to process, with good biocompatibility and low toxicity, they have been studied in drug stabilization and drug delivery (Lee and Mooney 2001; Drury and Mooney 2003) and for tissue engineering purposes (Sakai, Masuhara et al. 2005), where bone marrow cells have been successfully cultured on them (Wang, Shelton et al. 2003). Other report have also confirmed there suitability by enabling cell adhesion, migration, proliferation and differentiation to take place (Zhao, Deng et al. 2003).

Polyhydroxyalkanoates (PHA's) are currently under investigation for their uses in tissue engineering. They are naturally derived biocompatible polyesters which are produced by microorganisms as carbon and energy stores in unbalanced growing conditions become (Chen and Wu 2005). They are known to be biocompatible because they are found naturally occurring in mammal blood and tissues, where their purity can be increased by removing long-chain fatty acids and the endotoxin lipopolysaccharide, preventing any adverse reactions (Zhao, Deng et al. 2003; Chen and Wu 2005). They range from hard and brittle to soft and elastomeric, but can also be blended (combined) with other types of PHA or modified at the surface to alter their mechanical properties and biocompatibility, and produced either as a film or a foam (Rezwan, Ghen et al. 2006). So far, PHA's have been used for a number of different applications including tissue regeneration, repair devices, sutures and bone marrow scaffolds (Shishataskaya, Volova et al. 2004). One report compared the mechanical and surface properties of several modified PHA's and it was concluded that hydrophilicity and a low tackiness were found to be more important than the surface roughness for cell attachment and growth. Substrate stiffness also appeared to influence cell attachment, where the stiffer, more brittle PHA's, retained a significant number of viable cells on their surfaces (Rathbone, Furrer et al. 2009). Because of their diversity in surface texture, flexibility and their biocompatibility, PHA's show potential as cell substrates in tissue engineering.

Two types of glasses that have been used for medical research are bioglass (developed in the 1969 by Larry Hench) and controlled release glass (CRG) which is a phosphate-based glass, developed in the 1970's. Bioglass does not dissolve completely in fluids, but changes chemically upon its partial degradation, and is currently regarded as the most biocompatible material for bone regeneration due to its bioactivity and osteoconductivity (Wu, Hsu et al. 2009). Unlike bioglass, CRG dissolves completely in fluids at a predetermined rate, leaving no solid residues because phosphorous pentoxide is a main component within its formulation. The metal ions in CRG are found naturally occurring within the body (Probhakar, Brocchini et al. 2005) and upon glass degradation (dissolution) the released ions become removed by the bodies own metabolic system without causing a toxic response, avoiding the need for surgical removal if implanted into the body. Because CRG has a controllable solubility in body fluids and do not need surgical removal, this makes them an ideal scaffold material for promoting neotissue formation in vivo (Ahmed, Lewis et al. 2003). Very little work has been carried out with phosphate based glasses (CRG) for soft tissue engineering, however Bitar and colleagues cultured several cell types, including tendon fibroblasts, upon glass disks with various dissolution rates, where they successfully attached, proliferated while maintaining their phenotype, and it was concluded that the glass (of specific composition) would be ideal scaffold materials for engineering of both hard and soft tissues (Bitar, Salih et al. 2004). In fibrous form, phosphate based glasses have high tensile strength, making them useful for tensile applications, but it is also possible to produce them with dimensions similar to ligament collagen fibres in vivo, particularly the diameter, which can potentially assist cell attachment and spreading. Bitar et al suggested that the fibre diameter of the phosphate based glass which they tested influenced cell attachment (Bitar, Salih et al. 2008).

Many other materials have been investigated. Silk fibroin scaffolds have supported and enhanced ligament- specific differentiation of human MSC's. The silk was cabled into 6-cord wire rope matrices, improving its elasticity. The authors suggested that the silk matrix had similar a hierarchical structure to the collagen fibres in native ACL, making the mechanical properties comparable to ACL in stiffness and strength (Wang, Kim et al. 2006). Silk fibroin has also been produced as a microporous silk sponge and incorporated into a knitted silk mesh. After seeding them with rabbit MSC's, the constructs were implanted into rabbits, where at 24 weeks the cells were well distributed throughout a regenerating ACL, and producing key ligament proteins (collagen I and III, and tenacin-c). Also a direct ligament–bone insertion was achieved resembling native ACL-bone insertion (Fan, Liu et al. 2008). Collagen hydrogels have been shown to successfully promote higher production of type I and type III collagen from the cells, where the tissue formation was improved with a ligament-like organisation (Noth, Schupp et al. 2005; Gentleman, Livesay et al. 2006). Poly desamino-tyrosine ethyl carbonate scaffolds have proved to be successful in supporting fibroblast growth while possessing the necessary strength for use as an ACL graft (Laurencin and Freeman 2005).

Composites (consisting of more than one type of material) have also been constructed and analysed. Gelatin with silk fibroin has been produced into microporous sponges around Silk cables, where Fan and colleagues co-cultured rabbit MSC's with ACL fibroblasts on the scaffold, which enabled MSC differentiation into ligament fibroblasts. They detected mRNA expression of collagen type I and III, and tenascin-c with the corresponding protein production (Fan, Liu et al. 2008).

Material	Physical form	Affect on cells/material properties	Author
Synthetic polymers			
PLAGA	Braided	Improved fibroblast attachment, spreading & growth.	(Cooper, Lu et al. 2005)
PLAGA	Electrospun PLAGA nano-fibres onto knitted PLAGA scaffold.	Improved porcine MSC attachment & proliferation. Cells gave higher expression of type I collagen, decorin and biglycan genes in comparison to cells on just a knitted PLAGA scaffold.	(Sahoo, Ouyang et al. 2006)
PGA coated with BioGlass	Mesh coated with Bioglass.	Increased fibroblast proliferation (208f cell line).	(Day, Boccaccini et al. 2004)
PLA, PGA, PLAGA coated with fibronectin	Braided	Enhanced rabbit ACL fibroblast adhesion and supported high cell numbers, (highest for PLA).	(Lu, Cooper et al. 2005)
Degrapol^R PU	Fibre-fleece	Supported fibroblast adhesion & proliferation.	(Milleret, Simonet et al. 2009)
Natural polymers			
Collagen hydrogel	Hydrogel + collagen fibres	Increased production of type I & III collagen fibres, giving better tissue formation, and ligament-like organization in the tissue.	(Noth, Schupp et al. 2005; Gentleman, Livesay et al. 2006)
Silk fibroin	Rope matrix	Enhanced ligament- specific differentiation of human MSC's.	(Wang, Kim et al. 2006)
Silk fibroin	Microporous silk mesh rolled around braided silk cord	MSC seeded construct was implanted into pigs. At 24 weeks MSC's differentiated into fibroblast-like cells, expressing collagen I and III, tenascin-c.	(Fan, Liu et al. 2009)
Silk fibroin	Microporous silk sponge incorporated into knitted silk mesh	Rabbit MSC seeded constructs implanted into rabbits. At 24 weeks cells were well distributed throughout the regenerating ACL, producing key ligament proteins (coll I & III, Tenascin-c), direct ligament –bone insertion with 4 zones was reconstructed resembling native ACL-bone insertion.	(Fan, Liu et al. 2008)

Silk fibroin	Silk fibroin electrospun onto knitted silk base (random/aligned)	Aligned fibres showed improved cell proliferation and collagen production compared to random orientation.	(Teh, Goh et al. 2008)
Composites			
Gelatin + silk fibroin	Microporous sponge around Silk cables	Co-cultured rabbit MSC + ACL fibroblasts on the scaffold allowing MSC differentiation into ligament fibroblasts (mRNA expression of Coll 1 & 3, Tenascin-c and corresponding protein production).	(Fan, Liu et al. 2008)
Glass			
Phosphate-based glass	Disks	Increased adhesion & proliferation of fibroblasts when CaO content was 46-48 mol%.	(Bitar, Salih et al. 2004)
Phosphate-based glass	Fibres	Increased adhesion & proliferation of fibroblasts when CaO content was 46mol%.	(Bitar, Knowles et al. 2005)
Bioactive glass	Disks	Supported rabbit MSC adherence and proliferation.	(Meseguer-Olmo, Bernabeu-Esclapez et al. 2008)
Collagen fibres	Braided/plied (cross-linked)	Implanted into goats, analysed over 11 months post implantation, concluded they were loosing strength, therefore not suitable as ACL substitute.	(Chvapil, Speer et al. 1993)

Table 5. Some of the various different materials previously used as scaffolds (for ligament tissue engineering) their physical forms, and their suitability for cell cultures

7.4 Material surface modifications using fibronectin

Because fibronectin is known to function as a cell adhesion protein in vivo, it has been studied in vitro as a method for improving cell attachment. It has been used to modify the surface of various biomaterials to improve cell attachment to the surface, making it useful for tissue engineering. Reports have shown that fibronectin has improved cell retention of rat MSC's on fibronectin-coated surfaces (Dennis and Caplan 1993), increased the adhesive strength of cells, which was probably due to the increased number of bonds between fibroblasts and fibronectin-coated glass slides (Athanassiou and Deligianni 2001). Other studies concluded that braided PLLA and PLAGA polymers coated with fibronectin (10µg/ml) improved attachment of rabbit ACL cells and effected long term matrix production (Lu, Cooper et al. 2005). From the work carried out by Garcia and colleagues, their results indicated that cell adhesion strength (in osteosarcoma cells) increased on glass surfaces in a concentration-dependent fashion as fibronectin concentrations increased from

0.1-1µg/ml. Plates coated with a concentration of 20µg/ml have been shown to improve cell adhesion of human MSC's in comparison to uncoated plates (Salasznyk, Williams et al. 2004), being more affective than coatings of collagen I or IV. However, in contrast to Salasznyk's findings, Vohra and colleagues who also used a fibronectin concentration of 20µg/ml (Vohra, Hennessy et al. 2008), suggested that although the fibronectin coating improved cell attachment compared to the negative control, MSC's preferred to attach to a collagen I coating in comparison to the fibronectin and negative control.

7.5 Bioreactor culture of tissue engineered ligaments

A bioreactor is a vessel designed to contain cultures, where the environmental conditions can be optimised and carefully controlled to encourage certain biological and biochemical processes to take place (Martin, Wendt et al. 2004). Currently, many different types of bioreactors exist. They can be used to improve mass transfer of nutrients, waste products and oxygen through the culture medium, improve cell attachment, cell growth and proliferation. Bioreactors have been used in tissue engineering to apply mechanical forces to cell constructs (mechanical loading), and reported to promote differentiation of mesenchymal stem cells (MSC) into ligament fibroblasts (Zhang, Wang et al. 2004; Meyer, Buchter et al. 2005), induce alignment of fibroblasts with the direction of the applied force, upregulate mRNA expression of key ligament genes and produce helically organized collagen fibres (Altman, Horan et al. 2001). Mechanical conditioning can also be used to improve the structural and functional properties of a tissue once it has been engineered. Only those bioreactors related to ligament tissue engineering will be discussed here.

When forces are applied to cells, the magnitude of the applied force, the way in which it is applied (constantly or alternating), the duration of time and the direction of forces (translational or rotational) will have a specific effect upon cell behaviour. These complex forces are experienced by the native tissue under physiological conditions. A specific combination or sequence of any of these can influence the cell to give a positive or negative response. Therefore variations in mechanical loading regimens can affect and alter the gene expression, and hence protein production and regulate tissue formation (Nesic, Whiteside et al. 2006).

8. Response of cells to mechanical stimuli in vitro

Many in vitro studies investigating the affects of mechanical loading have been performed with bioreactors. Kaplan and colleagues used a bioreactor to apply mechanical stimulation to mesenchymal progenitor cells seeded into a collagen 3-D gel matrix. The bioreactor applied multidimensional forces concurrently - translational (2mm) + rotational (90°) - at a frequency of 0.0167 Hz (one complete cycle of stress and relaxation per minute) constantly for 21 days, which was chosen to mimic the unique combination of forces experienced by the ligament under physiological conditions in vivo (Altman, Horan et al. 2001). Their results induced cell alignment parallel to the direction of the stretching force with an elongated cell morphology, mRNA expression of specific genes (type I and III collagen, tenascin-c and fibronectin), helically organized type I collagen fibres orientated in the direction of force, with the selective differentiation of human MSC's into a ligament cell lineage rather than towards alternative lineages. The controls (non- loaded cells), showed few of these features. They concluded that "the mechanical forces could play a role in differentiation and not just promote formation of specific tissue types from the already

Frequency (Hz)	Translational stretching (cyclic)	Rotational	Period of time	Type of bioreactor	Effect upon cells
1	Stretched by 5% of its initial length. Cyclic, uniaxial	No	24 hours	Custom built tensile bioreactor	Tendon fasicles showed up-regulation of collagen
0.0167	10% Increased by 2mm in length when stretched. Cyclic, biaxial	25% Twisted it by 90°	21 days	Custom built tensile/ compression bioreactor system	Human MSC's acquired spindle-shaped morphology & cell alignment, expression of collagen I & III, tenascin-c, fibronectin, helically organized collagen fibres, differentiation into ligament cell lineage.
	12% Cyclic, uniaxial	No	14 days	Custom built bioreactor	Human MSC's showed increased expression of collagen 1 & 3, Fn, elastin in loaded samples. Observed elongated fibroblasts embedded in a wavy orientated collagenous tissue with ligament-like ECM.

Frequency (Hz)	Translational stretching (cyclic)	Rotational	Period of time	Type of bioreactor	Effect upo...
0.5	Flexion	No	0.5, 2. 4 hrs	Flexible Silicone coated films	Osteoblasts (MG63... 2, BMP-4, proto-on... upregulation at 0.5... Alk-3 upregulation...
0.167	Flexion cyclic	No	24 & 48 hrs continuous	Flexercell (flexible substratum)	Human ACL cells... for coll I at 48hrs
0.5	Stretched by 4% & 8% Cyclic, uniaxial	No	4 hrs	Microgrooved silicon dishes	Osteoblast (MG63)... upregulation in ge... MMP-1
10 cycles /min (0.167Hz)	Stretched by 10% Cyclic, uniaxial	No	24 hrs	Silicon dishes, membrane coated with collagen I	Human ACL cells... expression in Coll...
1	5% Cyclic, uniaxial	No	1 hr/day for 15 days	-	Human ACL cells... regulation in mRN... of coll I & III, tenas... fibronectin.

Frequency (Hz)	Translational stretching (cyclic)	Rotational	Period of time	Type of bioreactor	Effect up
0.25	10% Cyclic, uniaxial	No	8hrs/day for 7dasy	Custom built tensile machine	Tracheal fibroblast of procollagen 1 a1
1	3% & 10% Cyclic, uniaxial	No	8 hrs & 48 hrs	Flexcell plates coated with collagen I	Human MSC's Up reg of coll I & I with 10% 48 hrs
	2% in centre of well, 17% at periphery of well. Cyclic, uniaxial	No	24 hrs	Culture plates with flexible membrane	Rabbit ACL cells Became spindle sh perpendicular to fo synthesis in coll I o periphery).
1	0.05 (5%) and 0.075 (7.5%) strains. Cyclic, uniaxial	No	0.5, 1, 2, 4, 16, 24 hrs	Flexercell membrane coated with collagen I	Human ACL fibro upregulation in ml for Coll I & III betw (0.05 strain). There was up reg o and at 24hrs, a dec (0.075 strain).
0.5	7%	No	2 hrs	ST150 STREX	Human ACL fibro upregulation in ml for collagen 1 (6 fol stress fibre formati distribution of inte ($\alpha_5\beta_3$) to peripheral cells.

Frequency (Hz)	Translational stretching (cyclic)	Rotational	Period of time	Type of bioreactor	Effect up
0.5	5% Cyclic, uniaxia	No	Ranging 15 mins – 24 hrs	-	Human fibroblasts stretching + 10ng/ enhanced synthesi (mRNA express).
0.5	4% 8% Cyclic, uniaxial	No	4 hrs/day for 2 days. Then rest for 24 hrs.	A modified Flexcell silicone substrate	Human ACL fibro higher proliferatio more cells were ali perpendicular to s than 4% or the stat
0.5	5% Cyclic, uniaxial	No	24 hrs	Bioflex culture plates	Pig ACL fibroblast upregulated collag expression, but no biglycan. Estrogen inhibited mRNA e genes, but estrogen upregulated collag
1	5% Or 4 different amplitudes ranging 0.5 – 5% Cyclic, uniaxial	No	4hrs OR 1 hr/day for 5 days/week over 9 weeks.	Custom built multi-test-station apparatus.	Bovine synoviocyt showed proliferati 24%. The 9 week tests sh fibrils aligned in d in the strained scar random on unstrai The larger the amp the more cells & EC scaffold.

Table 6. The mechanical loading regimens which have been applied to tissue engineered constructs in vitro and the effects they have had upon various cell types

Frequency (Hz)	Translational stretching (cyclic)	Rotational	Period of time	Type of bioreactor	Effect upo
1	Preload with 2 & 10mN. Then 10% strain. Cyclic, uniaxial	No	24 hrs	Custom built cell straining system	Preloading human der collagen gels influence catabolic processes. Pre then cyclic loading incr levels for latent MMP-1 Preloading to 2mN the increased stiffness of co
0.1	10% Cyclic, uniaxial	No	1hr/day for 6 weeks	Custom built bioreactor for dynamic stretching	Human extensor tenoc PGA fibres formed tend structure after in vitro l further matured by in v stimulation when impl showing better collager more mature fibril stru mechanical properties.
0.0125 0.125	0.06% 0.6% 3% 6%	No	24 hrs	Custom built bioreactor for dynamic stretching	NIH 3T3 mouse fibrobl 0.6% showed increased contours of scaffold wh frequencies) in compar controls. No significant seen between the 2 freq strain variation. The ad 10, 100ng/ml) had no a spreading, suggesting c adaption may be affect term mechanical loadir

Cell type	TGF concentration	Combined with other GF's	Effects	Author
MSC	hr TGF-β1 10ng/ml Static culture		10ng/ml increased GAG expression & proliferation after 14days, whereas 0.1-1ng/ml did not.	(Chen, Tsai et al. 2005)
h ACL explants	TGF-β1 0.6ng/ml Static culture	Ascorbic acid 25ug/ml	Cell number increased. Collagen production increased by 3 times.	(Meaney Murray, Rice et al. 2003)
h MSC	TGF-β 5ng/ml Static culture	TGF+Insulin Adding ascorbic acid to TGF promoted greatest ratio of collagen: total protein production	TGF alone encouraged differentiation into fibroblasts (regarding morphology & alignment). Collagen 1 & 3 express increased. Negligible expression of BSP & OSP.	(Moreau, Chen et al. 2005)
h MSC	TGF-β 0.1, 0.5, 1, 5 ng/ml Static culture		Proliferation rate increased as concentration increased (0.1-5ng/ml). 1ng/ml TGF + FCS increased matrix production.	(Locklin, Oreffo et al. 1998)
Rabbit ACL fibroblasts	TGF-β1 0.01, 0.1, 1ng/ml Static culture		Increased collagen & non-collagenous protein synthesis as concentration increased, highest being at 1ng/ml. The increase was mostly for collagen 1 which increased by 1.5 times.	(Marui, Niyibizi et al. 1997)
Sheep ACL explants	hr TGF-β1 10, 50, 100ng/ml Static culture		Increased proliferation (seen at 96 hrs).	(Spindler, Imro et al. 1996)
Human and rabbit mesenchymal progenitor cells	hr TGF-β1 Static culture		Chondrogenic	(Chen, Tsai et al. 2005)

Table 7. The effects of TGF-β1 upon fibroblasts and MSC's

9. Response of cells to chemical stimuli in vitro

During a cells life cycle, the cell needs to receive the appropriate signals at specific time points to instruct it to grow, proliferate, differentiate or synthesise ECM. These chemical signals are often provided by growth and differentiation factors. They bind the cell surface receptors on target cells to activate specific intracellular signalling pathways, controlling cell growth, proliferation, migration and differentiation (Quaglia 2008). The effect that a differentiation or growth factor has upon a cell can vary depending on the cell type, the stage in the cells life cycle and its environmental conditions. Such an example is transforming growth factor-β (TGF-β) which can be an inhibitor or stimulator of inflammation, or ECM remodelling by inducing mRNA expression of integrins, collagen and fibronectin (Evans 1999).

In order for tissue engineering to be successful, it is necessary to create an artificial environment for the cells, and possibly mimic the in vivo environment, to promote formation of new tissue. Such an environment can be created by using, not only a suitable scaffold or mechanical stimulus, but also a chemical stimulus by incorporating growth factors into the culture media (Tabata 2003). From published research work, many authors report using various different growth factors such as basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), transforming growth factor-β1 (TGF- β1), epidermal growth factor (EGF) and insulin-like growth factor (IGF) to encourage cells to proliferate, differentiate or increase the production of collagen (Schmidt, Georgescu et al. 1995; Spindler, Imro et al. 1996; Scherping, Schmidt et al. 1997; Murray, Rice et al. 2003; Pertigliano, McAllister et al. 2006). In vitro cell culture studies which used bFGF, PDGF, EGF or TGF-β1 individually, have shown them to increase the proliferation of ACL fibroblasts (Schmidt, Georgescu et al. 1995; Spindler, Imro et al. 1996; Scherping, Schmidt et al. 1997; Murray, Rice et al. 2003), whereas a combination of TGF with bFGF or EGF has promoted MSC differentiation into fibroblasts (Pertigliano, McAllister et al. 2006). Growth factors have also been identified for their roles in regulating healing and repair of connective tissue after injury (Spindler, Imro et al. 1996). It is thought that the response of musculoskeletal tissues to injury or mechanical stress is modulated by growth factors such as PDGF and TGF-β (Spindler, Imro et al. 1996).

9.1 In vitro studies with TGF-β1

TGF-β1 has been used in various studies ranging in concentration from 0.1-15ng/ml. Specific concentrations have been shown to increase fibroblast proliferation, increase collagen production, glycosaminoglycan expression, and encourage MSC's to differentiate into fibroblasts (Spindler, Imro et al. 1996; Locklin, Oreffo et al. 1998; Meaney Murray, Rice et al. 2003; Chen, Tsai et al. 2004; Chen, Tsai et al. 2005; Moreau, Chen et al. 2005; Giannouli and Kletsas 2006; Marenzara, Wilson-Jones et al. 2006). Table 7 summarises the effects that different concentrations of TGF-β1 have had on fibroblasts and MSC's.

10. Characterisation of a tissue engineered ligament

When creating a tissue-engineered ligament, it is important to be aware of the characteristic components and properties found in native ligament as a comparing standard for functionality (shown in table 1). The ACL characteristics are demonstrated below in table 8.

The variation in tensile strength has been correlated to age and gender, where strength and stiffness of the ACL has been found to be lower in adult females (Chandrashekar, Mansouri et al. 2006), and can also significantly reduce as age increases, being 2-3 times higher in younger people, aged 16-26 years, compared to those aged at approximately 60 years old (Noyes and Grood 1976).

Collagen fibre arrangement	Fibroblast distribution and orientation	Tensile strength - maximum force upon ACL at failure (N)	Maximum elongation of ACL at failure (mm)	Stiffness - Young's modulus of elasticity (MPa)	Author
Aligned in a fairly parallel orientation with the longitudinal axis of the ligament	Sparsely distributed throughout the ECM (approximately 20% if the tissue volume), aligned on collagen fibre bundles	556-1730	8-12	9-13	(Noyes and Grood 1976; Laurencin, Ambrosio, et al. 1999; Azangwe, Mathias et al. 2001; Chandrashekar, Mansouri et al. 2006; Doroski, Brink et al. 2007)

Table 8. Fibre organisation and mechanical properties of the ACL

11. Conclusions

Tissue engineering of ligaments is still in its early stages, but its prospects have great potential. Tissue engineering has the ability to overcome the limitations of autografts and allografts by generating a tissue with the correct structural and biomechanical properties for a more successful transplant, hopefully giving a better long-term mechanical performance. The benefits of using autologous cells from the patient reduces the risk of tissue rejection or transmission of infectious diseases associated with allografts, and also avoids donor site morbidity associated with allografts. Advances in research in this area continue to optimise a combination of the factors discussed in this chapter such as choice of biomaterial, cell type and stimuli for potential synergistic effects.

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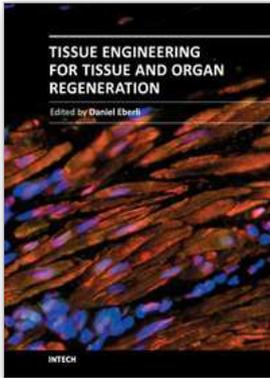
Tissue Engineering for Tissue and Organ Regeneration
Edited by Prof. Daniel Eberli

Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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