5-2008

Tendon and ligament repair: regeneration and maturation

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ABSTRACT

The thesis constitutes the studies about the two aspects of tendon and ligament tissue engineering: regeneration and maturation. Injuries to tendon and ligament are among the most common injuries to the body, particularly in the young and physically active population. Associated with the problems of incomplete healing and recurrent injury, these injuries are not only responsible for large health care cost, but also result in lost work time and individual morbidity. Tissue engineering holds promise in treating these conditions by replacing the injured tissue with engineered tissue exhibited similar mechanical and functional characteristics. Collagen plays a central role in tendon and ligament regeneration, as collagen type I is responsible for more than 60% and 80% of the dry weigh of tendon and ligament structures, respectively. The hierarchical organization of collagen type I in bundles confers most of the mechanical properties of tendons and ligaments. Consequently, tendon or ligament tissue engineering studies are mainly focused on seeding cells into collagen gels. However, up to now, no cell-collagen constructs have been able to achieve sufficient mechanical properties and the complex architecture of the tendon and ligament is never fully reproduced.

A major cause for low mechanical property of regenerating tendon or ligament is the slow maturation. The maturation of the engineered tissue is dominated by the maturation degree of extracellular matrix, such as collagen crosslink density. To
overcome the slow growth and maturation of tissue engineering grafts, one goal of this project is to accelerate the tissue maturation using gene therapeutics approach.

Therefore, in the first part of this thesis, two different genes, lysyl oxidase (LOX) and decorin, were transfected into fibroblasts by retrovirus infection. LOX initiates the covalent cross-linking of collagen and elastin in the extracellular space by oxidizing specific lysine residues in these proteins to peptidyl α-amino adipic-δ-semialdehyde (AAS). These aldehyde residues can spontaneously condense with vicinal peptidyl aldehydes or with ε-amino groups of peptidyl lysine to generate the covalent crosslinkages, which stabilizes and insolubilizes polymeric collagen or elastin fibers in the extracellular matrix. Decorin is considered a key regulator of matrix assembly because it limits collagen fibril formation and thus directs tendon and ligament remodeling due to tensile forces. In this study, we found that the mechanical property of the tissue-engineered grafts was significantly increased by over-expressing LOX or decorin gene. And decorin over-expression uniformed the mean diameter of the collagen fibers.

In the second part of this thesis, ECM-based hydrogel system was used to control the delivery of chemotaxic growth factors, such as hepatocyte growth factor (HGF), for recruiting endogenous stem cells. This approach was used to attract endogenous stem cells to the lesion site for tendon or ligament regeneration. In this study, we found that stem cells could be recruited effectively to the local site where HGF were released by chemically modified hyaluronic acid (HA) and gelatin (Gtn) based hydrogels both in vitro and in vivo.
DEDICATION

This thesis is dedicated to my family and friends for their generous support.
ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. I thank Dr. Xuejun Wen for his continuous guidance and support. I thank my other committee members, Dr. Zhi Gao and Dr. Ken Webb for their helpful suggestions and comments throughout my studies. I thank Dr. Ning Zhang, Dr. Hai Yao, Dr. Qian Kang, Jane Jourdan, and Yuhua Zhang for their help in my experiments. I thank the Clemson-MUSC Bioengineering department’s faculty and students for their support, especially our lab members, Xiaowei Li, Yongzhi Qiu, Vince Beachely, and Xiaoyan Liu.
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CHAPTER 1

THESIS ROADMAP

The thesis constitutes the studies about the two aspects of tendon and ligament tissue engineering: regeneration and maturation. Injuries to tendon and ligament are among the most common injuries to the body, particularly in the young and physically active population. Associated with the problems of incomplete healing and recurrent injury, these injuries are not only responsible for large health care cost, but also result in lost work time and individual morbidity. Tissue engineering holds promise in treating these conditions by replacing the injured tissue with engineered tissue exhibited similar mechanical and functional characteristics. Collagen plays a central role in tendon and ligament regeneration, as collagen type I is responsible for more than 60% and 80% of the dry weigh of tendon and ligament structures, respectively. The hierarchical organization of collagen type I in bundles confers most of the mechanical properties of tendons and ligaments. Consequently, tendon or ligament tissue engineering studies are mainly focused on seeding cells into collagen gels. However, up to now, no cell-collagen constructs have been able to achieve sufficient mechanical properties and the complex architecture of the tendon and ligament is never fully reproduced.

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CHAPTER 2

BACKGROUND

Tendon Structure

Tendons are anatomic structures that connect muscles to bones and transmit the forces created in the muscles to bones making joint movements possible. Healthy tendons, which exhibit high mechanical strength and good flexibility, are brilliant white in color and fibro-elastic in texture. Tendons vary in form. They can be rounded cords, strap-like bands or flattened ribbons [1]. Tendons consist of collagens, proteoglycans, glycoproteins, water and cells.

Tendon cells

Tendon fibroblasts, including tenoblasts and tenocytes, comprise about 90-95% of the cellular elements of the tendon. The other 5-10% includes the chondrocytes at the pressure and insertion sites, the synovial cells of the tendon sheath on the tendon surface, the vascular cells, such as capillary endothelial cells and smooth muscle cells of the arterioles [2, 3].

The newborn tendon has a very high cell-to-matrix ratio. Tenoblasts are immature tendon cells, and they are spindle-shaped, with numerous cytoplasmic organelles reflecting their high metabolic activity [1]. When the cell-to-matrix ratio gradually decreases with aging, the tenoblasts become elongated and transform into tenocytes. Tenoblasts and tenocytes align in rows between collagen fiber bundles, and
they are responsible for synthesizing extracellular matrix proteins, producing an organized collagen matrix and remodeling it during tendon healing [4].

Tendon extracellular matrix

Tendons are rich in collagens, with the most abundant tendon component being type I collagen, which constitutes about 60% of the dry mass of the tendon and about 95% of the total collagen [4-6]. The remaining 5% consists of type III and V collagen. Type III collagen has major role in tendon early repair [7] and type V collagen copolymerizes with type I collagen to regulate tendon fibril diameter during fibrillogenesis [8] and aging [9].

Collagen is arranged in hierarchical levels of increasing complexity, beginning with tropocollagen (a triple-helix polypeptide chain), which unites into fibrils; fibers (primary bundles); fascicles (secondary bundles); tertiary bundles; and the tendon itself [10] (Figure 2.1). Soluble tropocollagen molecules form cross-links to create insoluble collagen molecules, which aggregate to form collagen fibrils. A bunch of collagen fibrils form a collagen fiber, which is the basic unit of a tendon. Collagen fibers are bound by endotenons, a thin layer of connective tissue that contains blood vessels, lymphatics and nerves [11]. Fascicles are composed of fiber bundles, and bundles of fascicles are enclosed by the epitenon, a fine, loose connective-tissue sheath containing the vascular, lymphatic, and nerve supply to the tendon [11].
Besides collagens, tendons also contain proteoglycans and glycoproteins. There are many proteoglycans, including aggregan and decorin [12]. Aggrecan holds water within the fibrocartilage and resists compression [13]. Decorin, which located in the tropocollagen interstices, is believed to regulate fibril diameter and to provide fibril-to-fibril and fibril-to-proteoglycan binding[14]. Glycoproteins present in tendon extracellular matrix include tenascin-C and fibronectin. Tenascin-C contributes to the mechanical stability of the extracellular matrix through its interaction with collagen fibrils [15]. Fibronectin has role in wound healing, its synthesis increases at wound healing [16].

Elastin, which composes about 2% of the dry weight of the tendon, is another component of tendon extracellular matrix. Elastic fibers are scarcely present in human tendons, and it is reported that elastic fibers were actually demonstrable in only 10%
of healthy human tendons [17]. The function of elastic fiber is not entirely clear, but they may contribute primarily to the recovery of the wavy collagen fiber configuration after tendinous stretch [18].

**Tendon mechanical properties**

Tendons transmit force generated by muscle to bone, and act as a buffer by absorbing external forces to limit muscle damage. Tendons exhibit high mechanical strength, good flexibility, and an optimal level of elasticity to perform their unique role [19]. Tendons are viscoelastic tissues, and they display stress relaxation and creep [1].

A typical tendon stress-strain curve is shown to demonstrate the behavior of tendon (Figure 2.2). The mechanical behavior of tendon collagen is dependent on the number and types of intra- and inter- molecular bonds [20]. At rest, collagen fibers and fibrils display a crimped configuration. The initial concave portion of the curve is called toe region. This toe region, where tendon is strained up to 2%, represents the stretching-out the crimp pattern [18]. In the linear region of the stress-strain curve, where the tendon is stretched less than 4%, collagen fibers lose their crimp pattern and the fibers become more parallel [21]. In this region, the tendon behaves in an elastic fashion, and returns to its original length when unloaded. The slope of this linear region is referred to as the Young’s module of the tendon [4]. When the tendon is stretched over 4%, microscopic failure occurs. When the tendon is stretched over 8-10%, macroscopic failure occurs. And further stretch causes tendon rupture [18].
Figure 2.2 Tendon stress-strain curve showing the initial non-linear toe region followed by a linear region up to a failure region.

Tendons are viscoelastic and sensitive to different strain rates. Tendons are more deformable at low strain rates. Therefore, the tendons absorb more energy, but are less effective in transferring loads. At high strain rates, tendons become less deformable with a high degree of stiffness and are more effective in moving large loads [10]. Furthermore, tendons are at the highest risk for rupture if tension is applied quickly and obliquely, and highest forces are seen during eccentric muscle contraction [20].

Ligament Structure

Ligament is fibrous tissue that connects bones to other bones. Ligament is composed of closely packed collagen fiber bundles organized mostly in a parallel
configuration along the length of the tissue to resist tensile loads [22]. So, ligament and tendon have a similar hierarchical structure that affects their mechanical behavior.

**Ligament cells**

Fibroblast is the major cell type of ligament. The cells are relatively few in number and represent a small percentage of the total ligament volume. Fibroblasts are not only responsible for collagen synthesis but also for collagen renewal process, which includes enzymatically breaking down and removing old collagen [23].

**Ligament extracellular matrix**

Ligaments are approximately two-third water and one-third solid. Water is responsible for contributing to cellular function and viscoelastic behavior. The solid components of ligaments are principally collagen. The most abundant ligament component is type I collagen, which constitutes about 80% of the dry mass of the ligament and about 85% of the total collagen [24]. The rest collagens in ligament include collagen type III, V, VI, XI, and XIV. Type III and V collagen make up 8% of and 12% of the dry weight of the ligament, respectively, and they have been shown to be involved in regulating collagen fibril diameter [25]. Besides collagen, ligaments also contain proteoglycans, elastin, glycoproteins such as actin, laminin and integrins [24].

Collagen is arranged in hierarchical levels in ligament, which is similar as in tendon. Briefly, collagen molecules assemble sequentially into microfibrils, subfibrils,
and fibrils (20-150nm in diameter) before forming fibers, which are 1-20μm in diameter [23]. Crosslinked fibers further make up a subfascicular unit, which is surrounded by a loose band of connective tissue called endotenon. The diameter of subfascicular units is 100-250μm. Three to twenty subfasciculi subsequently form a fasciculus (from 250μm to several millimeters in diameter)[23]. The fasciculus are surrounded by epitenon, which supports the neurovascular elements of the ligament [23].

**Tendon and ligament injuries**

Tendon injuries are among the most common injuries to the body, particularly in the young and physically active population. The most common tendon disorders are observed in the Achilles tendon (100,000 per year) [26], in the rotator cuff in the shoulder (51,000 per year) [27], and patellar tendon donor autograft injuries in the knee (42,000 per year) [22].

Tendon injuries can be acute or chronic, and are caused by intrinsic or extrinsic factors, either alone or in combination. Acute injuries typically arise from trauma, whereas chronic injuries often occur after repetitive subfailure mechanical events followed by inflammation [28, 29]. Chronic injuries can be associated with inflammation (tendonitis), without inflammation (tendinosis), or involve the surrounding tissue (peritendinitis) [19].
Acute tears of rotator cuff tendons commonly occur in athletes participating in overhead sports [30], whereas chronic degeneration and tearing tend to occur in older more sedentary subjects [27]. The Achilles tendon is often injured traumatically, but it is also frequently involved in chronic injuries. Acute Achilles tendon injury and pain usually occur in active individuals, when the tendon is subjected to high or unusual loads. In contrast to acute injuries, Achilles tendinopathies are caused by repeated microinjuries coupled with a degenerative or failed healing response [31].

Ligaments are most often torn in traumatic joint injuries that can result in either partial or complete ligament discontinuities. For example, over 100,000 anterior cruciate ligament (ACL) ruptures estimated to occur each year in the United States[32]. All of these tendon and ligament disorders can bring significant morbidity and decrease the quality of life.

Tendon and ligament healing

When tendons or ligaments are injured, the body initiates a process of healing. Tendon and ligament healing can be largely divided into three overlapping phases: the inflammatory, repairing, and remodeling phases [33].

The initial inflammatory phase lasts about 24 hours. In this phase, erythrocytes, platelets and inflammatory cells (neutrophils, monocytes, macrophages) migrate to the wound site and clean the site of necrotic materials by phagocytosis. In the mean time,
these cells release vasoactive and chemotactic factors, which recruit fibroblasts to begin collagen synthesis and deposition [4]. During this phase, there is an increase in fibronectin, glycosaminoglycan, water, and collagen type III content, which collectively stabilize the newly formed extracellular matrix [10]. A few days after the injury, the repairing phase begins. This phase lasts a few weeks. In this phase, the predominant cell types are fibroblasts along with a smaller number of macrophages and mast cells [34]. Fibroblasts synthesize abundant collagen and other extracellular matrix components such as proteoglycans and deposit them to the wound site. Synthesis of type III collagen peaks during this stage, water content and glycosaminoglycan concentrations remain high [1]. About 6 weeks later, the remodeling phase begins. In this phase, fibroblasts decrease in size and slow their matrix synthesis, and collagen fibers begin to orient themselves longitudinally along the long axis of the tendon or ligament. The repair tissue changes from cellular to fibrous, which again changes to scar-like tendon tissue after 10 weeks [1]. When the scar enters maturation, type III to type I collagen ratio, collagen crosslinks, and glycosaminoglycan, and water concentrations approach normal levels. During the later remodeling phase, covalent bonding between collagen fibers increases, which results in repaired tissue with higher stiffness and tensile strength [4]. Although the tensile strength of the healing tissue improves over time, it does not reach the levels of uninjured, normal tissue (Figure 2.3) [34]. Despite remodeling, the biochemical and mechanical properties of healed tissue never match those of intact tendon. In normal tendon or ligament, the collagen fibers are all arranged in a vertical alignment to best
withstand the force that the tendon or ligament would be loaded; while in repaired
scar tissue, the collagen fibers are randomly arranged in many directions. Also the
healed tissue is unable to recover normal crosslink density, it may be because of the
alteration in the proportion of collagen types, proteoglycans and other unknown
factors within the wound environment [33]. Mechanically, healed tendons or ligament
do not achieve normal tissue failure force in the long term. Butler et al reviewed that
1.5 months after creating a defect injury in the patellar tendon, the repair tissue
achieves 7-9% of normal failure force and by 3 and 6 months after injury, repair tissue
failure force changed to 11% and 8% of normal respectively [31].

Figure 2.3 Tensile strength of the injured tendons during wound healing process.
Traditional Treatments for tendon and ligament injuries

Because tendons and ligaments heal poorly, there are many different types of treatment used in tendon and ligament disorders, such as physical therapy, reduction of inflammation, restoration of flexibility and surgical repair [27, 35-37]. Some current treatments and some therapies in development will be introduced in the following paragraphs.

Clinical used treatments

Sutures There are a variety of different suture techniques used clinically, each with their preferred suture material that may be employed at the discretion of the surgeon. Some of these techniques include Lin locking [38], Savage [39], Kessler [40], modified Kessler [39], and epitenon suture [41] method. It has been shown that the repair strength is directly proportional to the number of strands crossing a repair site, ruptures usually occur at the knots in the suture, and epitenon sutures increase repair strength over core sutures alone [34, 41].

Eccentric exercise therapy Eccentric strength training is particularly effective in treating tendinopathies and helps promote the formation of new collagen [42]. Eccentric contraction involves the lengthening of muscle fibers as the muscle contracts, preferentially loading the tendon [43]. Eccentric exercise has proved beneficial in Achilles tendinosus and patellar tendinosus and may be helpful in other tendinopathies [43].
NSAIDs Nonsteroidal anti-inflammatory drugs (NSAIDs) effectively relieve tendinopathy pain and may offer additional benefit in acute inflammatory tendonitis because of their anti-inflammatory properties. However, because the majority of chronic tendinopathies are not inflammatory, few data exist to support the use of NSAIDs over analgesics without anti-inflammatory effects [43].

Other therapies include cryotherapy (reduction of acute inflammation and decrease in cell metabolism [44]), heat treatment (stimulation of cell activity and increase of blood flow [44]), and physiotherapy (same as heat treatment).

Treatments under development

Laser treatment Low-level laser therapy (LLLT) is using of low-power lasers and superluminous diodes for the treatment of a variety of medical conditions, and it now has been known as a possible treatment for tendon and ligament injuries. LLLT can enhance ATP production, enhance cell function and increase protein synthesis [45]. LLLT has also been shown to have positive effects on reduction of inflammation, increase of collagen synthesis, and angiogenesis [45]. There have been a few studies regarding effects of LLLT on tendon and ligament repair [45, 46].

Corticosteroid injection Corticosteroids may reduce inflammation and inhibit protein synthesis [44]. Injected corticosteroids may be more effective than oral NSAIDs for relief in the acute phase of tendon pain, but they do not tend to alter long-term outcomes. The effects of peritendinous corticosteroid injections are unknown, but they should be used with some caution [43]. Because the role of inflammation in
tendinopathies is unclear, corticosteroids may serve only to inhibit healing and reduce
the tensile strength of the tissue, predisposing to spontaneous rupture [43].

Other treatments under development include shock-wave therapy (stimulate
tenocytes for repair), nitroglycerin patches (enhance collagen synthesis), and
sclerosant injections (block tendon blood flow) [44].

There are many drawbacks of existing treatments. One principal drawback is
that the characteristic response to injury is for fibroplasias to occur, which inevitable
leads to scar tissue in the tendon or ligament. Although remodeling of the scar tissue
occurs over time, the subsequent tissue is not normal and, in particular, has less
compliance and functionality than the original tendon and ligament matrix [47].

As a result of the deficiencies of current treatment, there is great interest in
investigating the potential for tissue engineering in tendon and ligament injuries.

Tissue Engineering

Many people suffer from tendon and ligament diseases each year. For example,
100,000 people suffer from Achilles tendon disorders every year, 51,000 people suffer
from rotator cuff tendon disorder each year, and over 100,000 anterior cruciate
ligament (ACL) ruptures estimated to occur each year in the United States [32].
Associated with the problems of incomplete healing and recurrent injury, these
injuries are not only responsible for large health care costs, but also result in lost work
time and individual morbidity [48]. The rate at which these injuries have increased and the degree to which costs have as well. For example, between 1992 and 1999, total knee replacement increased over 77.8%, at a compounded annual rate of 15.5% [49]. Surgical repair, artificial prostheses, mechanical devices, and both autographs and allografts continue to be important in medical treatment of certain tendon disorders. However, there are drawbacks of these traditional treatments for tendon repair. For example, prosthetic devices have complications such as durability and poor long-term performance, and allografts have drawbacks such as donor scarcity, donor-site morbidity, tissue rejection, and disease transmission. Tissue engineering holds promise in treating these conditions by replacing the injured tissue with engineered tissue with similar mechanical and functional characteristics [50].

A commonly applied definition of tissue engineering, as stated by Langer and Vacanti, is “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ” [50]. In other words, tissue engineering is the use of a combination of cells, engineering and materials methods, and suitable biochemical and physio-chemical factors to improve or replace biological functions.

The basic concept of tissue engineering includes a scaffold that provides an architecture on which seeded cells can organize and develop into the desired organ or tissue prior to implantation. The scaffold provides an initial biomechanical profile for the replacement tissue until the cells produce an adequate extracellular matrix. During the formation, deposition, and organization of the newly generated matrix, the
scaffold is either degraded or metabolized, eventually leaving a vital organ or tissue that restores, maintains, or improves tissue function [51] (Figure 2.4).

Figure 2.4 Concept of tissue engineering.

a, Cells are harvested from autologous; b, cells are harvested from allogenic or xenogenic sources; c, cell proliferation in vitro; d, cells are seeded to a scaffold; e, expanded cells reinjected to the patient without scaffold material; f, expanded cells reinjected to the patient with scaffold; g, cells are cultured in bioreactor; h, cultured cells reinjected to the patient.

Tissue Engineering Treatment For Tendon and Ligament Repair

Biomaterials, cell sources, growth factors and bioreactors are four components for tissue engineering. In this part, these four components are reviewed for tendon and ligament tissue engineering.
Biomaterial and Scaffold

There are several requirements govern the choice of materials for tendon and ligament tissue engineering. Firstly, the material must be biocompatible and non-toxicity [52]. The material must not cause abnormal responses in local tissues surrounding the implant and should not produce toxic or carcinogenic effects. Secondly, the material should avoid infection, so that the material should be sterile-able before seeding the cells onto the scaffold, and should remain sterile prior to surgical implantation. The sterilization technique should be carefully chosen to not alter the mechanical and physical properties of the material. Finally, the scaffold material should provide some mechanical integrity initially to replace the function of the lost tendon or ligament before the scaffold degrades and is replaced with new extracellular matrix. Importantly, tendon and ligament have highly hierarchal organized collagen structure, so the further requirement of the scaffolds for tendon and ligament repair is that the scaffolds need the similar micro and macro morphology as tendon and ligament.

As mentioned above, collagen type I constitutes about 60% of the dry mass of the tendon and 80% of the dry weight of ligament, its hierarchical organization in bundles confers most of the tendon and ligament mechanical properties, collagen plays a central role in tendon and ligament tissue engineering. Collagen gels have been used as model system for in vitro tissue engineering [53, 54], and could be used to assess the effects of different stimuli on cell proliferation. Also, the collagen gels have been used as a vehicle to deliver mesenchymal stem cells in defects of the
rabbits in vivo [55]. It is also reported that preliminary alignment of the cells in the gel increases the efficiency of the method [56]. But the collagen constructs are not able to achieve sufficient mechanical properties, and the complex architecture of the tendon is never fully reproduced.

Although collagen type I play a central role in tendon and ligament tissue engineering, collagen type I does not account for all the tissue properties. The proteoglycans and small leucine rich protein (SLRP) also play important roles in the organization and mechanical properties of the tendon and ligament structure [57]. Collagen cross-linking has been proposed to enhance early mechanical properties, and this will be discussed in detail later. Besides collagen, chitin-based [58] and chitosan-based [59, 60] scaffolds also have been reported used for tendon tissue engineering.

Except the materials from nature, some synthetic materials have been reported used for tendon repair. Knitted poly (D,L-lactide-co-glycolide) (PLGA) scaffolds have been shown to possess good mechanical strength and internal communicating spaces and have been effectively used for rabbit Achilles tendon repair [61]. Recently, electrospin technique has been employed to modify the polymers to facilitate cell attachment, new ECM deposition, and tissue formation for better tendon repair [62].

Cell

Fibroblast: There are two types of tendon fibroblast cells in tendon, tenocytes and tenoblasts. And fibroblast is major cell type in ligament. Some studies have been done using fibroblasts for tendon and ligament repair in animal models [63, 64].
Stem cells: Stem cells are very attractive cell sources for tendon tissue engineering. Stem cells are undifferentiated cells and have the ability to self-renew and differentiate to one or more types of specialized cells. Stem cells can be classed to two types: embryonic stem (ES) cells and adult stem cells. ES cells are from blastocysts and fetal tissue. And they have potential in the field of tissue engineering and regenerative medicine because they have the potential to produce most types of cells in the body [65-67]. Although the potential of ES cells in tissue engineering is vast, there are many problems must be overcome. For example, human ES cells may be contaminated by animal cells or proteins. It is reported that nonhuman protein expressed by human ES cell lines grown on animal feeder layers [68]. Other problems such as immunorejection and tumorigenesis are also obstacles for the clinical applications of ES cells. Currently, no embryonic stem cells-base therapies are approved for human use.

Adult stem cells are undifferentiated cells, which are found among differentiated cells in a tissue or organ. Adult stem cells can renew themselves and they can give rise to mature cell types that have characteristic morphologies and specialized functions. Adult stem cells are rare, but they are located in various tissue niches throughout the body, including bone marrow, brain, liver, skin, blood, etc. [69].

Mesenchymal stem cells (MSCs) have attracted much attention because of their multipotential properties with regard to differentiation, and their possible use for cell and gene therapy. MSCs reside in diverse host tissues, bone marrow was considered to be the most accessible and enriched source of MSCs [70]. Also, MSCs have being
isolated from various tissues, such as cartilage [71], peristium [72], synovium [73], synovial fluid [74], muscle [75] and tendons [76]. Fetal tissue [77], placenta, umbilical blood and vasculature [78] also have been reported to contain MSCs. MSCs retain the ability to differentiate into a variety of cell types, including adipocytes [79], chondrocytes [80], osteoblasts [79], skeletal muscle cells [81], and so on. Several methods are currently available for isolation of MSC based on their physical and physicochemical characteristics, for example, adherence to plastic or to other extracellular matrix components. Their wide extend resources, multipotency of differentiation, ease of isolation and their highly reduced immunoreactivity after allogenic transfer make MSCs an ideal cell type for tissue repair and regeneration in cell therapy applications. Several studies have demonstrated the possible use of MSCs in transplantation for systemic diseases, or implantation to repair local tissue defects. Several in vivo and in vitro experiments have demonstrated the potential of MSCs isolated from bone marrow [82] or from adipose tissue[83] for tendon engineering. Under mechanical stimulation, MSCs differentiated in fibroblasts, aligned themselves in collagen gel, and helped in vivo tendon regeneration.

Although using MSCs for tissue repair is very promising, there are still issues related to the application of MSCs for human use. For example, the pluripotency of MSCs would decrease during in vitro propagation [84, 85]. The cost of in vitro process for cell propagation and transplantation is high. To overcome this problem, we are developing a new strategy to use endogenous stem cells for tissue repair. The detail will be discussed later.
Growth Factor

The main growth factors that affect growth and differentiation of ligament and tendon tissue include basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF)-1, and members of the family of TGF-β/bone morphogenetic proteins (TGF-β/BMPs).

bFGF is the major mitogenic agent for fibroblasts, and it increases cell proliferation of rat tenocytes [86]. Injection of bFGF results in a dose-dependent increase of cell proliferation and collagen type III production [87]. PDGF is also an important mitogenic agent for fibroblasts [86]. Researches found that enhanced expression of PDGF-BB in healing ligament led to an initial promotion of angiogenesis and subsequent enhanced and accelerated matrix deposition[88]. And it is also reported that PDGF-BB could augment tendon repair in a rotator cuff injury model [89]. IGF-1 provides some anti-inflammatory functions and also acts as a chemotactic attractant for endothelial cells[90]. It was reported that via a possible anti-inflammatory mechanism, IGF-1 could reduce maximum functional deficit and accelerate recovery after Achilles tendon injury[90].

The growth factors can be delivered locally, but they are easy to be over loaded. It is important to understand the right time for the administration of growth factors and their dosage for effective therapies.

Bioreactor

Bioreactors, which provide dynamic loading, are essential for meeting the complex requirements of in vitro engineering of functional skeletal tissues. It is
reported that an increase in cell proliferation, migration and collagen synthesis has been observed in lacerated chicken tendon cultured in vitro undergoing cyclic tension after 14 days [91]. Some studies demonstrated that cyclic strain (5%, 1Hz, for 15 or minutes) could increase TGF-β, bFGF, and PDGF production in human tendon fibroblasts [92], cyclic mechanical stretching (8%, 0.5HZ, for 4 hours) slightly but significantly increases tendon proliferation on a microgrooved substrate, and increases collagen type I and TGF-β expression [93]. And there are some bioreactors designed specifically for ligament tissue engineering. For example, Altman and his colleagues designed an advanced bioreactor, which provided the application of multidimensional mechanical strains (axial tension/compression and torsion) to MSC cultured in a hydrogel or on a fibrous scaffold [94]. The mechanical stimulation induced cell alignment in the direction of the resulting force, and fostered the expression of collagen type I and type III and tenascin C [94].

In conclusion, well combination of the four components (cell, scaffold, growth factor and bioreactor) is very important for the success of tendon and ligament tissue engineering.

Two major strategies may be applied for tendon tissue engineering. One is the in vivo approach of tendon engineering involving in situ delivery of cells, biomolecules, hydrogels or scaffolds to boost the regeneration and healing process to deal with the repair of small defects and the induction of tissue self-regeneration [88]. The other is the in vitro tissue engineering involving the growth of tendon-like tissue
structure outside of the body and the implantation into the defect. This strategy is more concerned with larger defects or tissue replacement.

Tendon and Ligament Maturation

The overall shape and function, in terms of flexibility and locomotion, of the human skeletal system depend on a basic framework of collagen fibers [95]. The collagen fibers are essentially in extensible and therefore provide mechanical strength and through that strength confer and maintain form whilst allowing flexibility between various organs of the body. The collagen fibers of tendons are aligned in parallel and therefore loaded instantly, permitting maximum transfer of the energy of muscle contraction to the bone.

Collagen

The collagen molecule, also called tropocollagen, is composed of three intertwined polypeptide chains. Each of the polypeptides that constitute the collagen molecule is designated as an α-chain. They intertwine, forming a right-handed triple helix. Except at the ends of the α-chain, every third amino acid in the chain is a glycine molecule because glycine is the smallest amino acid. A hydroxyproline frequently precedes each glycine in the chain, and a proline frequently follows each glycine in the chain. The glycine, along with proline and hydroxyproline, is essential
for the triple-helix conformation. Sugar groups, which are joined to hydroxylysyl residues, are associated with the helix. Because of these sugar groups, collagen is properly described as a glycoprotein [96]. The $\alpha$-chains that constitute the helix are not all alike, and according to differences in $\alpha$-chains and differences in molecules organization and arrangement, as many as 28 different types of collagen have been described in literature. Tendon and ligament are predominantly fibrous Type I collagen.

The production of fibrillar collagen involves a series of events within the cells that leads to the production of procollagen, the precursor of the collagen molecule. The production of the actual fibril occurs outside of the cell and involves enzymatic activity at the plasma membrane to produce the collagen molecule, followed by the assembly of the molecules into fibrils under guidance of the cell. Most of the synthesis of procollagen is in the rough endoplasmic reticulum (RE). Soluble procollagens are “packaged” in the Golgi apparatus to be excreted to outside the cell. Then the procollagen stays close to the cell surface where most of maturation occurs. The procollagen moves to the exterior of the cell by means of exocytosis of secretory vesicles. Then the procollagen, on secretion from the cell, is converted to collagen molecules by procollagen peptidase, which cleaves the uncoiled ends of the molecule. The aggregated collagen molecules then align to form the final collagen fibrils (Figure 2.5).
Figure 2.5 Schematic diagram showing the different posttranslational modifications and assembly of type I collagen into fibrils [96]. Procollagen consists of a 300-nm-long triple-helical domain flanked by a trimeric globular C-propeptide domain and a trimeric N-propeptide domain. Procollagen is secreted from cells and is converted into collagen by the removal of the N- and C-propeptides by procollagen N-proteinase and procollagen C-proteinase respectively. The collagen generated in the reaction spontaneously self-assembles into cross-striated fibrils that occur in the extracellular matrix of connective tissues. The fibrils are stabilized by covalent cross-linking, which is initiated by oxidative deamination of specific lysine and hydroxylysine residues in collagen by lysyl oxidase.

Collagen maturation

Collagen maturation occurs outside the cell. Once outside the cell, an enzyme-mediated cross-linking takes place and the triple helical collagen molecules line up and begin to form fibrils and then fibers. The cross-linking mechanism in the fibrillar collagens is based upon aldehyde formation from the single telopeptide lysine or hydroxylysine residue. This step is initiated by a specialized enzyme called lysyl
oxidase (LOX), which oxidatively deaminates these residues to promote crosslink formation. Once LOX deaminates the residues to initiate the cross-linking, the subsequent reactions of the aldehydes are spontaneous and governed by post-translational modifications and the structural organization of the collagen. Two major cross-links are aldimines which are formed by telopeptide lysine-aldehydes condense with either lysine or hydroxylysine residues in the conserved sequence of the triple helix and keto-imines which are formed by hydroxylated telopeptide lysine-aldehyde reacts with the ε-amino group of a helical hydroxylysine [97]. Other forms of cross-links such as aldol-derived cross-link that is formed by reaction of the intramolecular aldol condensation product with histidine also exist. The formation of cross-links in mature collagen creates stability and gives definition to the structure. The majority of tendons and ligaments contain both the aldimine and the keto-imine cross-links. The ratio depends upon the particular function of the tissue, in particular whether there is tension on the tissue.

Besides aldehyde formation, another kind of intermolecular cross-linking of collagen which will increase with age is glycation. Glycation can affect the properties of collagen in a number of ways, for example, its ability to form precise supramolecular aggregates, the alteration of its charge profile and hence its interaction with cells, and, additionally, glycated collagen can act as an oxidizing agent [97].

Increasing collagen cross-linking of engineered tissue may enhance mechanical stability. Some strategies have been investigated to increase collagen cross-linking. Besides enzymatic cross-linking (initiated by LOX) and glycation,
which mimic the physiological cross-linking, chemical processing, is another method for cross-linking. But the chemically cross-linked engineered tissue may subject to calcification in vivo. And glycation increases a tissue’s platelet aggregation potency and increases the potential for thrombosis formation. And glycation is widely known because of the side effect of diabetes when too much glycation occurs [98, 99]. Collagen cross-links formed by the lysyl oxidase (LOX) have no known negative side effects. In contrast, too few LOX-generated cross-links can lead to decreased tissue strength.

Lysyl Oxidase

The LOX family consists of five members: LOX and four known family members called Lysyl oxidase-like (LOXL [100], LOXL2 [101], LOXL3 [102], and LOXL4 [103]). The LOXLs are isoenzymes: enzymes that have the same catalytic activity as LOX but different construction. They have related by different functions including cell growth control, tumor suppression, senescence and chemotaxis [104]. LOXL2, LOXL3, LOXL4 create a subfamily characterized by the presence of four scavenger receptor cysteine-rich (SRCR) domains [103].

Protein-lysine 6-oxidase (lysyl oxidase, LOX; EC 1.4.3.13), which is expressed and secreted by fibrogenic cells, is a copper amine oxidase. LOX initiates the covalent cross-linking of collagen and elastin in the extracellular space by oxidizing specific lysine residues in these proteins to peptidyl \( \alpha \)-amino adipic-\( \delta \)-semialdehyde (AAS). These aldehyde residues can spontaneously
condense with vicinal peptidyl aldehydes or with ε-amino groups of peptidyl lysine to generate the covalent crosslinkages, which stabilize and insolubilize polymeric collagen or elastin fibers in the extracellular matrix [105]. LOX plays a central role in the morphogenesis and repair of connective tissues of the cardiovascular, respiratory, skeletal, and other systems of the body.

Lysyl oxidase is synthesized in a precursor form as the 50kDa, N-glycosylated prolysyl oxidase (pro-LOX). Pro-LOX is secreted to the extracellular space where it undergoes activation to the 32 kDa, functional catalyst by proteolytic cleavage at a specific Gly-Asp bond [106].

Lysyl oxidase contains two cofactors, Cu (II) and a peptide-linked carbonyl residue, at its active site essential to its catalytic function. The carbonyl cofactor contains an ortho-quinone function and has been identified as lysine tyrosylquinone (LTQ), derived by oxidative post-translational modification of a specific tyrosine residue (tyrosine 349 in rat pro-LOX) to a dihydroxyphenylalanine (DOPA) quinone residue. Subsequently, the amino group of a specific lysine residue (lysine 314 in rat pro-LOX) covalently bonds to a ring carbon of peptidely DOPA quinone [107] (Figure 2.6). Copper in lysyl oxidase appears to be involved in the transfer of electrons to and from oxygen to facilitate the oxidative deamination of targeted peptidyl lysyl groups in tropocollagen or tropoelastin and to internally catalyze quinone cofactor formation [108]. LOX catalyzes primary amine oxidation through a ping pong \textit{bi ter} kinetic mechanism (Figure 2.7) [109].
Figure 2.6 Structure of the carbonyl cofactor of lysyl oxidase, lysyl tyrosine quinone[105].

Figure 2.7 Mechanism of action of lysyl oxidase[105].
LOX for many years was unable to be studied because LOX aggregates in most solutions. When it was found that when urea is added to the solution LOX became soluble and that urea can be easily separated, LOX studies began in earnest. It was found that LOX is essential in the functioning of embryonic cardiovascular system [110], and lungs [110]. It also is important in wound healing and has possible chemotaxic effects especially to monocytes. LOX has also been shown to enhance mechanical properties in vascular tissue scaffolds [112].

Decorin

Proteoglycans (PGs) play a crucial role in collagen fibrillogenesis, and therefore in tendon function. PGs are composed of a core protein to which one or more GAG chains are covalently attached.

Decorin is the most abundant tendon PG. Decorin is a member of the small leucine-rich proteoglycan (SLRP) family, a group of secreted proteins that includes biglycan, fibromodulin, lumican, and keratocan, among others [113-115]. SLRPs play major roles in collagen fibrillogenesis, growth factor modulation, and direct regulation of cellular growth [116-118].

Fibroblast, chondrocytes, endothelial cells and smooth muscle cells are decorin-producing cells. Decorin is composed of three domains: an N-terminal region which possesses a single chondroitin/dermatan sulfate side chain and a distinct pattern of Cys residues (CX$_3$CXC$_6$C), a central region composed of ten leucine-rich repeats which are believed to be the prime sites of interaction with other proteins, and another
Cys-rich C-terminal region [119]. Positioning of collagen in the model is consistent with other evidence that the central leucine-rich repeats comprise the high-affinity collagen-binding site [120, 121]. It is believed that decorin likely maps to a narrow region near the C-terminus of collagen type I, very close to none of its major intermolecular cross-linking sites [119]. Decorin is considered a key regulator of matrix assembly because it limits collagen fibril formation and thus directs tendon remodeling due to tensile forces [122, 123]. The assumed location of decorin on collagen fibrils in vivo and its ability to retard collagen fibrillogenesis in vitro suggest that it has a role in the collagen network organization and in the maintenance of tissue integrity [124]. Thus absence of decorin might affect not only fibril formation but also fibril stability within tissues.

Gene Therapy

Although the direct application of human recombinant proteins can result in beneficial effects on the healing process, high dosages and repeated injections of these proteins are often required due to their relatively short biological half-lives [125]. In addition, the use of growth factor proteins to promote healing is severely hindered by the difficulty of ensuring their delivery to a specific injured site [125]. Combined tissue engineering, gene therapy is a very promising strategy to overcome the problems of protein therapy. For example, cell isolated from the patients could be
genetically engineered to express a given therapeutic gene, and then seeded into a particular scaffold. After in vitro proliferation, the cells can be transplanted back to the patient to promote tissue regeneration.

Gene therapy is the science of the transfer of genetic material into individuals for therapeutic purposes by altering cellular function or structure at the molecular level. By employing these techniques, genes can be used therapeutically to produce proteins to treat and potentially cure diseases [126].

Vectors

In order for target cells to produce the protein products of the introduced gene, the exogenous genetic material must be delivered to the cell’s nucleus. This process of transfection exists in two classes of vectors: viral and non-viral.

The non-viral vectors are usually easier to produce and result in lower toxicity and immunogenicity. The use of non-viral vectors can be in the form of injections of naked DNA (usually plasmids), liposomes, or particle-mediated gene transfer. The genetic material can be placed in liposomes in order to increase DNA uptake in tissue culture. But non-viral gene delivery efficiency is hindered by a low transfection rate, the success associated with non-viral vector usage remains limited.

Currently, viral vectors represent a more efficient method of gene delivery [127, 128]. The most commonly used viruses are adenovirus, retrovirus, adeno-associated virus, and herpes simplex virus.
Retroviruses are RNA viruses that carry a gene for a reverse transcriptase that transcribes the viral genetic material into a double-stranded DNA intermediate. This DNA intermediate is then incorporated into the host DNA allowing the host cell machinery to produce all the necessary viral components. Because the viral genome is stably integrated into the host DNA, any modification that has been made will be passed to all daughter cells that are derived from the transfected cells [129].

In contrast to retroviruses, adenovirus does not integrate its genome into the host genome. Instead, the adenoviral genome remains in the nucleus as an episomal element after infection of the host cell. Adenoviral vectors are ease of purification and concentration, and they could infect various cell types, dividing or non-dividing, at high efficiency rate [129].

Adeno-associated virus requires a helper virus such as adenovirus or herpes simplex virus for replication. Adeno-associated virus is actually a member of the parvovirus family of single-stranded DNA viruses. The small size of the genome allows for easy manipulation such that shuttle vectors carrying the entire genome have been constructed. This virus is non-pathogenic and not associated with any known disease but can infect a wide variety of cells, dividing or non-dividing, although with varying levels of efficiency [129].

Herpes simplex virus vectors can infect most cells including non-dividing cells, and they have large carrying capacity and ability to insert expression cassettes into specific loci of the genome that allows for the construction of vectors able to express multiple transgenes [130].
Strategies

There are two general ways that gene therapy can be performed: a direct in vivo method and an indirect ex vivo method. The direct method involves transferring the genetic material into the target somatic cells in vivo. The indirect technique involves removal of cells from the patient followed by genetic modification of the cells ex vivo and return of the cells to the patient. The direct method is technically simpler, while the indirect gene delivery technique is safer because the gene manipulation takes place under controlled conditions outside the body [32]. Furthermore, this method allows for selection of the cells that express the therapeutic gene at higher levels [131]. The choice of the strategy depends on the disease to be treated, the gene to be delivered to treat the disease and the vector used to deliver the gene.

In the study presented in this thesis, LOX or decorin transfected retrovirus were used to infect mouse embryonic fibroblasts (MEF) to accelerate collagen maturation for tendon or ligament regeneration through an indirect ex vivo strategy.

Stem Cells Recruitment

A classic concept of tissue repair holds that inflammatory cells enter the damaged tissue or damaged tissue secret signaling molecules and signal resident cells mitosis. Several studies suggest that multipotent stem cells can also contribute to
tissue repair after mobilization, migration and engraftment into the damaged tissue [132]. In addition, circulating immature cells seem to participate in regeneration of many different tissues [133, 134]. Some models show the role of stem cells in tissue remodeling, such as for hepatic regeneration [135, 136], muscle regeneration [137, 138] and infarcted myocardium [139, 140]. It is not surprising that using endogenous stem cells have attracted lots of attention because they hold great therapeutic potential for endogenous tissue repair and tissue engineering.

**Stem cell mobilization**

Stem cell mobilization is to release of stem cells from their local niche into the circulation in response to chemotherapy or cytokine stimulation, which was first documented in the late 1970s and 1980s [141]. Mobilizing cells is a preferable strategy for clinical use since this may allow higher yield of these cells, faster engraftment and lower procedural risks compared with traditional stem cell transplantation approach. Several molecules, such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), and IL-8, IL-12, and stromal cell derived factor-1 (SDF-1)[142] are shown to be able to mobilize endogenous stem cells into circulation. These molecules differ in their time frame to achieve mobilization, the type of cells mobilized and efficiency. G-CSF is the most commonly used agent, usually administrated daily at a dose of 5-10μg/kg for 5-10 days [141]. It has been shown that G-CSF could mobilize more CD34^+ cells and have less toxicity than any other single agent. GM-CSF as a
single agent is used less often today for mobilization than G-CSF, because it mobilizes somewhat less well than G-CSF and because of relatively higher incidence of both mild and severe side effects [143]. SDF-1 is an α-chemokine that strongly chemoattracts hematopoietic stem/progenitor cells (HSPCs) through interaction with their unique receptor CXCR4. It has become evident that the SDF-1-CXCR4 signaling axis plays an important role in the homing and engraftment of HSPCs in the bone marrow [144].

Stem cells also migrate to nonhematopoietic organs, such as the liver, especially during liver injury/inflammation which creates an alarm situation and transmitting stress signals that mobilize and recruit stem cells as part of organ repair.

Hepatocyte growth factor (HGF)

Hepatocyte growth factor (HGF) is a pleiotropic cytokine of mesenchymal origin, promoting motility, proliferation, invasion, morphogenesis and survival of a wide spectrum of cells, namely epithelial and endothelial cells [145]. The coordinated integration of these processes plays a pivotal role in organ formation during embryogenesis and tissue homeostasis in adults. In adults, HGF displays in vivo cytoprotective activity in different cell types of injured organs. It is reported that HGF is able to enhance mesenchymal stem cells engraftment in injured heart [146]. Many researches have demonstrated that HGF could activate mesenchymal stem cells migration in vitro [144, 147, 148].
In the study presented in this thesis, HGF was loaded into extracellular matrix based hydrogels to attract mesenchymal stem cells to the local site for tissue repair.

References


CHAPTER 3

RESEARCH OBJECTIVES

The preceding literature review has identified the necessity of new strategies to treat tendon or ligament injuries. Majority of research on tendon and ligament tissue engineering recently were focused on the cell seeded collagen gels. However, up to now, no cell-collagen construct has been able to achieve sufficient mechanical properties. On the other hand, a better cell source need to be identified for tendon and ligament tissue engineering, due to taking tenocytes out would cause donor-site morbidity.

According to the current problems in tendon and ligament tissue engineering, our goal in this study is to enhance tissue-engineered grafts maturation and use endogenous stem cells for de novo tendon or ligament regeneration and repair. The specific objectives of this study are:

(1) To engineer a functional graft with the similar histological structures and mechanical properties as native tendon or ligament tissue. Gene therapy would be used as model approach, by which cells can over-express genes, such as LOX or decorin, to increase collagen cross-linking, which can enhance collagen maturation, and optimize collagen fibril formation.
To attract endogenous stem cells to the lesion site for tendon regeneration. ECM-based hydrogel system would be used to control the delivery of chemotactic growth factors, such as hepatocyte growth factor (HGF), for recruiting mesenchymal stem cells into the lesion site.
PART I: CHAPTER 4
ACCELERATION OF THE MATURATION OF TISSUE ENGINEERED GRAFTS BY GENE THERAPY FOR TENDON OR LIGAMENT REPAIR

Abstract

Despite significant progress of tissue engineering technologies has been achieved both in academic labs and in industries, a number of difficulties are still present which slow down the commercial process of tissue engineered products. For tendon and ligament repair by means of tissue engineering, one major difficulty is how to gain functional grafts with the similar mechanical properties as the native tissues. To overcome the poor performance of engineered tendon tissue grafts on histological structures and mechanical properties, we try to accelerate the maturation of engineered grafts and optimize the graft structure using gene therapy. The maturation of tendon tissue is dominated by the amount and the crosslinkage of the extracellular matrices, such as collagen, elastin, glycosaminoglycans, and so on. Maturation of collagen and elastin is controlled by the degree of crosslinking in these molecules. Crosslinking can occur in various ways, but in vivo most crosslinkings are mediated by lysyl oxidase (LOX), which recognizes different binding sites that are similar on the type I, II, and III collagens and elastin. On the other hand, decorin is considered a key regulator of matrix assembly, and it plays a major role in the collagen network organization and in the maintenance of tissue integrity. In this study, infected MEF cells that expressed LOX or decorin were utilized as a model system to
test our strategies. The results demonstrated that LOX and decorin significantly promote tissue maturation indicated by showing the significant increase in the strength of the engineered grafts in vitro and in vivo.

Introduction

Tendons and ligaments are anatomic structures that connect muscles to bones or bone to bones and transmit the forces to bones making joint movements possible. Healthy tendons, which exhibit high mechanical strength and good flexibility, are brilliant white in color and fibro-elastic in texture. Tendon and ligament injuries are among the most common injuries to the body, particularly in the young and physically active population. The most common tendon disorders are observed in the Achilles tendon (100,000 per year) [1], in the rotator cuff in the shoulder (51,000 per year) [2], and patellar tendon donor autograft injuries in the knee (42,000 per year) [3]. Ligaments are most often torn in traumatic joint injuries that can result in either partial or complete ligament discontinuities. For example, over 100,000 anterior cruciate ligament (ACL) ruptures estimated to occur each year in the United States [4]. All of these tendon and ligament disorders can bring significant morbidity and decrease the quality of life.

Treatment of tendon and ligament disorders has traditionally involved reduction of inflammation, restoration of flexibility and surgical repair. When inflammation (tendinitis) and degeneration (teninosis) cannot be resolved, primary
repairs, autografts [5], allografts [6], and xenografts [7], and resorbable synthetic biomaterials [7-9] have been attempted, but with varying success. Recently, tissue engineering approaches have been evaluated to treat tendon disorders, based on the concept that an improved repair outcome will achieved by combing principles of engineering and biology Thus the focus of developing new treatment strategies for tissue repair has shifted from transplantation and replacement to biological regeneration.

Recent advances in sophisticated scaffold fabrication, controlled release of biomolecules, stem cell biology, and bioreactor development, have raised extensive interests in tissue engineering and regenerative medicine-based tissue regeneration strategies in academic researchers, clinicians, and biotechnology industries. To date, fundamental techniques for tissue engineering both in vitro and in vivo, such as scaffold fabrication, controlled release of growth factors and genes, manipulation of stem cells and precursors cells, and dynamic bioreactors, have been established for clinical translation of tissue engineering strategies.

Despite significant progress has been achieved both in academic research and in industry, a number of difficulties slow down the commercial process. One major difficulty is how to gain a functional graft the similar mechanical properties as native tissues. To overcome the poor performance of engineered tendon or ligament tissue grafts on histological structures and mechanical properties, we try to accelerate the maturation of engineered grafts and optimize the graft structure using gene therapy. The maturation of tendon tissue is dominated by the maturation degree of
extracellular matrix. The maturation of extracellular matrix is proceeded by the
cross-linking of various components in extracellular matrix, such as collagen, elastin,
glycosaminoglycans, and so on. Cross-linking of ECM is also an effective approach to
improve the strength of constructs for tissue-engineering applications. Since collagen,
which constitutes more than 60% of the dry mass of tendon, is the major component
of ECM of tendon. The most abundant ligament component is type I collagen as well,
which constitutes about 80% of the dry mass of the ligament and about 85% of the
total collagen [10]. The rest collagens in ligament include collagen type III, V, VI, XI,
and XIV. Type III and V collagen make up 8% of and 12% of the dry weight of the
ligament, respectively, and they have been shown to be involved in regulating
collagen fibril diameter [11]. In our study, for tendon or ligament tissue engineering,
the maturation of collagen type I was focused on.

The collagen fibers of tendons and ligaments are aligned in a parallel and
therefore loaded instantly, permitting maximum transfer of the energy of muscle
contraction to the bone or bone to bone. Collagen has triple-helix structure. Collagen
synthesis occurs in the endoplasmic reticulum, packaged in the Golgi apparatus and
excreted to extracellular environment. Maturation/cross-linking of collagen occurs
outside the cell. The formation of cross-links in mature collagen creates stability and
gives definition to the structure. The major ways to form cross-links in vitro are
nonenzymatic cross-linking and enzyme cross-linking. Nonenzymatic cross-linking
includes glutaraldehyde fixation, sugar-mediated cross-linking and so on. The main
drawback to glutaraldehyde derived crosslinks is that glutaraldehyde-fixed tissues are
subject to calcification in vivo. Sugar-mediated cross-link formation, also called glycation can form covalent bonds between collagen molecules and increase tissue stiffness. Glycation increases a tissue’s platelet aggregation potency and increase the potential for thrombosis formation. Collagen cross-links can be mediated by secretory enzymes such as lysyl oxidase (LOX). In fact, too few LOX-generated cross-links is reported to associate with decreased tissue strength. Protein-lysine 6-oxidase (lysyl oxidase, LOX; EC 1.4.3.13), which is expressed and secreted by fibrogenic cells, is a copper amine oxidase. LOX initiates the covalent cross-linking of collagen and elastin in the extracellular space by oxidizing specific lysine residues in these proteins to peptidyl α-aminoadipic-δ-semialdehyde (AAS). These aldehyde residues can spontaneously condense with vicinal peptidyl aldehydes or with ε-amino groups of peptidyl lysine to generate the covalent crosslinkages, which stabilize and insolubilize polymeric collagen or elastin fibres in the extracellular matrix [12]. LOX plays a central role in the morphogenesis and repair of connective tissues of the cardiovascular, respiratory, skeletal, and other systems of the body.

Besides collagen, proteoglycans (PGs) play a crucial role in collagen fibrillogenesis, and therefore in tendon function. Decorin is the most abundant tendon PG. Decorin is considered a key regulator of matrix assembly because it limits collagen fibril formation and thus directs tendon remodeling due to tensile forces [13, 14]. The assumed location of decorin on collagen fibrils in vivo and its ability to retard collagen fibrillogenesis in vitro suggest that it has a role in the collagen network organization and in the maintenance of tissue integrity[15]. Thus absence of
decorin might affect not only fibril formation but also fibril stability within tissues.

In our study, we used LOX or decorin transfected retrovirus to infect mouse embryonic fibroblasts (MEF) to accelerate collagen maturation for tendon repair.

Materials and Methods

Materials

LOX plasmid was a gift from Dr. Ben Fogelgren. Decorin plasmid was purchased from ATCC. DMEM media, FBS, L-glutamine solution, non-essential amino acids solution were purchased from Invitrogen. Restriction endonucleases used for plasmid construction were purchased from New England Biolabs.

Derivation of mouse embryonic fibroblasts (MEF)

CF-1 strain mice at 12.5 days gestation were anesthetized. Mouse was placed belly up in sterile tissue culture hood. The belly skin was cut using sterilized instruments to expose the peritoneum. And then the peritoneal wall was cut and the uterine horns were exposed. Uterine horns were removed and put to a new petri dish after washing. Then embryonic sacs were open and embryos were released using forceps and scissors. Visceral tissue was separated from embryos, and the embryos were put in a new plate. Mince tissue with dissecting scissors into grain sized pieces. Add 2ml trypsin, and mince for an additional few minutes until pieces are further reduced in size. Add an additional 5ml trypsin and place dish into incubator for 20-30
minutes. And then vigorously pipet mixture up and down. Add about 20ml MEF culture media (DMEM media with 10% FBS, 1% 200mM L-glutamine solution and 1% non-essential amino acids solution) and transfer contents to a sterile 50ml plastic conical tube. Divide the mixture to several T75 flasks. Incubate the flasks containing the minced tissue mixture in a 37°C tissue culture incubator overnight. Continue to culture the flask until at least 90% of the flask surface is covered with cells.

**Plasmid construction**

Plasmid pENTR 1A (Invitrogen) was used to get the entry clones containing LOX or Decorin genes. To construct pENTR-LOX plasmid, EcoRI/PmeI double digested LOX fragment was ligated with EcoRI/EcoRV digested pENTR 1A fragment. To construct pENTR-Decorin plasmid, BamH1/BglII digested Decorin fragment was ligated with BamH1/EcoRV digested pENTR 1A fragment. Recombination reactions were then carried out to transfer the LOX or Decorin gene into expression vector. Briefly, entry clone was mixed with MSCV-IRES-GFP-GATEWAY expression vector and LR clonase II enzyme mix (Invitrogen). The mixture was incubated at 25°C for 1 hour, and proteinase K was then added to the reaction and incubated for 10 min at 37°C. The recombination product could be used to transform competent cells. (As show in Figure 4.1)
Retrovirus production

HEK 293 cells were transfected with effectene transfection system (Qiagen) with the expression plasmids MSCV-LOX-IRES-GFP or MSCV-DECORIN-IRES-GFP together with packaging plasmid EcoPAC. The amount of DNA used for transfection was 4.5μg expression plasmid and 5.5 packaging plasmid (molecular ratio 1:1) per 150mm dish. 24 hours after transfection, medium was discarded and 10ml fresh medium was added. Medium containing viral particles was collected at 48 hours after transfection. Medium was then centrifuged at 3000rpm for 15min at 4°C to pellet debris. Supernatant was stored at -80°C until use.
Cell infection

MEF cells were trypsinized and seeded into 6-well plate to grow overnight at 37°C in a humidified 5% CO₂ incubator. 1.5ml viral stock was mixed with 1.5ml fresh medium and then added to cells with 30-50% confluence after old medium was removed. Polybrene was added to each well to a final concentration of 10 μg/ml. Cells were incubated for 24 hours at 37°C in a humidified 5% CO₂ incubator. The following day, medium was removed and 2ml fresh medium was added to incubate for another 24 hours. Cells were checked for the efficiency of infection under a fluorescence microscope by the expression of the GFP protein.
Generate viral expression constructs containing genes of interest (LOX or Decorin)

Cotransfect the 293T cell line with viral expression construct and packaging plasmid

Harvest viral supernatant

Add the viral supernatant to MEF cells

Assay for protein expression of interest

Figure 4.2 Retrovirus production and MEF cells infection.
Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted using Versagene RNA kit (Invitrogen). Reverse transcription was accomplished with 5μg of total RNA using the Transcript™ kit (BioRad). PCR was carried out under the following conditions: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min (30 cycles), and a final extension at 72°C for 10 min. PCR production were analyzed by electrophoresis on a 1% agarose gel and visualized with ethidium bromide. The sequences of oligonucleotide primers were as follows:

LOX:
Forward sequence:
5’-GACCTGGGGGCAGGTACCGG-3’
Reverse sequence:
5’-TGGGTGTGTTGCATCAAGCAG-3’

Decorin:
Forward sequence:
5’-CTTAACCTCGAGATGAAGGCCACTATCATCCTCCT-3’
Reverse sequence:
5’-AATATGGATCCTTACTATAGTTTCGAGTTGAATGGC-3’
Western blotting

MEF cells infected with LOX or Decorin expressing retrovirus were lysed with lysis buffer, and protein concentration was determined using the BCA kit. 10μg of protein lysate and protein marker were run in 10% SDS-PAGE followed by transfer to a PVDF membrane. Western analysis was carried out using the following primary antibodies: goat polyclonal anti-LOX (E-19) antibody (Santa Cruz), and goat polyclonal anti-human decorin antibody (R&D systems). After extensive washing, immunocomplexes were detected with horseradish peroxidase-conjugated appropriate secondary antibodies followed by enhanced chemiluminescence reaction (ECL™, Amersham).

Preparation of MEF-collagen constructs

First, collagen type I (rat tail collagen type I, BD Biosciences) gel was prepared. Briefly, determine the final volume of collagen solution to be used and the desired final collagen concentration (1.6mg/ml). 10X PBS with 1/10 final volume was added to the collagen type I. Then 0.023 final volume of 1N NaOH was added into the solution. Then appropriate DMEM culture media was added to the mixture to adjust the collagen type I to final concentration 1.6mg/ml. Left the mixture on ice until ready for use.

10^5 cells were mixed with collagen type I gel. Then the cell and collagen gel mixture were pipetted into specially designed silicone dishes that permitted contraction around posts (Figure 4.3). For each acellular sample, only the collagen
(1.6mg/ml) was pipetted into the silicone dish. All constructs remained in an incubator (37°C, 5% CO₂) for four weeks and were fed high glucose DMEM with ascorbic acid and 10% FBS twice weekly. 4 weeks after cell culture, some of the samples were for the mechanical test and the rest of them were for histology study.

Figure 4.3 Silicon mode. Each silicone dish consists of eight wells into which cells and collagen gels were pipetted. The cells contracted the gels around two posts located in the base of each well.

Aligned Polyurethane fibers fabrication (electrospinning)

Fibers were electrospun from polymer solutions containing a degradable PU (synthesized in our lab) based on lysine diisocyanate (LDI, Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan). 5-7 wt/v% and degradable PU was dissolved in dichloromethane:dimethylformamide (3:1) (DCM:DMF, Sigma) at concentrations of 13-17 wt/v%. Polymer solution was feed by a syringe pump (Medfusion 2010i; Medex Inc.) at adjustable feed rates of 0.010- 0.020 ml/min through 1/16”
polyethylene tubing into a 30blunt tipped needle (Smallparts). A voltage of 10 kV was applied to the needle tip with a high voltage power supply (Gamma High Voltage ES40P-10W). The needle tip was held at 10 cm above the level of the collecting device. The collecting devices were grounded. Polymer fibers deposited across the parallel plates were collected with a home-made polycarbonate frame.

Cell culture in aligned nano-polyurethane fibers

The aligned polyurethane fiber frames were sterilized using 1N HCl and washed with 1X PBS for several times. $10^6$ normal MEF cells were seeded on the nanofibers first. Two days after seeding, the MEF cells were confluent on the fibers, and then the frames were turn over for the second time cell seeding. LOX or Decorin expressing cells were seeded on the other side of the frames. The cells were cultured with high glucose DMEM culture media with ascorbic acid and 10% FBS in 37°C, 5% CO$_2$ incubator. One week after second time cell seeding, the frames were taken out and the nano-fibers with cells were rolled over for animal surgery. One of the frames was fixed with 4% Paraformaldehyde, and then stained with Alexa 546-phalloidin.

Briefly, the sample was blocked in TBSA-BSAT (10mM Tris-HCl, 150mM NaCl, 0.02% sodium azide, 1% Triton X-100) for 1 hour at room temperature, and then washed with wash buffer (PBS, with 0.05% sodium azide, 0.05% Triton X-100) for 3 times, 10 minutes each. Then the sample was incubated in appropriate diluted 546-phalloidin solution for 2 hours at room temperature. The sample could be stored
in the wash buffer at 4°C and for later use. The stained sample was taken pictures under Confocal microscope.

**In vivo animal surgery**

Nude rats were anesthetized and shaved to facilitate the incision. Incisions were made on the back with scissors, and pockets adjacent to the incision site were created with the aids of curved forceps. The nano-polyruethane fibers cultured with different cells were implanted and the incision was closed. 4 weeks after surgery, the samples were taken out from the pockets of rats, and some of them were for the mechanical test, and some of them were fixed for histology study.

**Histology**

For histology study, the samples were fixed in 10% neutral buffered formalin. All samples were then processed through a gradient of alcohols embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin (HE staining). For Sirius red collagen staining, the sections were deparaffinized and rehydrated first. Then the sections were dipped in saturated picro-sirius red solution for one hour and washed with 0.01 N HCL solution twice for 0.5 minute each. Dehydrate the sample with 100% ethanol and clear in xylene. The sections were mounted and ready for microscope analysis.
Mechanical test

The retrieved samples were tested for mechanical properties using tensile test (Bose Inc). The stress strain curves were obtained through a mechanical testing software provided by the manufacturer.

Statistics analysis

Data will be presented as the mean +/- the standard error of mean for each group. One-way analysis of variance (ANOVA) will be performed to determine the effect of LOX or Decorin on the properties of regenerating tissue. Statistical significance is accepted at p< 0.05.

Results and Discussion

LOX and decorin expression in MEF

Our goal in this study was to implement gene therapy as a tool to optimize the performance of mechanically challenged engineered tissue. Mouse embryonic fibroblasts were infected with LOX or decorin-expressing retrovirus. RT-PCR confirmed the transcription of LOX or decorin genes compared to control group that infected with retrovirus with empty vector (Figure 4.4A). LOX or decorin protein expression was confirmed by western blotting (Figure 4.4B).
Figure 4.4 LOX and decorin expression in MEF. A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of RNA isolated from control MEF cells and LOX or Decorin expressing retrovirus infected MEF cells. B) Western immunoblotting analysis of LOX or Decorin expression in MEF.

**Mechanical test for in vitro experiment**

After four weeks cell culture, there was significant difference in the strength among the groups (Figure 4.5). The maximum load for the control MEF group was 0.2 ±0.04 N, and the maximum load for the LOX and decorin group was 0.8 ±0.13 N and 0.5±0.09N, respectively. The maximum load for the collagen-cell graft significantly increased after the cells transfected with LOX or decorin. The collagen-cell constructs were 1mm in width and 20mm in length. Young’s modulus of the control grafts was 780 ±80KPa, young’s modulus of decorin-expressing group and LOX-expressing group was 1980 ± 21.8KPa and 2110 ± 23.2KPa, respectively (Figure 4.6).
Figure 4.5 Force-displacement curves of the collagen-cell grafts. (A) Control group. (B) LOX transfected group. (C) decorin transfected group.

Figure 4.6 Young’s Modulus of collagen-MEF constructs.
Collagen plays a central role in tendon and ligament regeneration, as collagen type I is responsible for major composition of the tissue, and its hierarchical organization in bundles confers most of the tendon mechanical properties. Consequently, tendon engineering studies mainly cell seeded collagen gels. Contraction of the gel is related to the cell seeding density, generally followed by alignment and reorganization of the matrix [16, 17]. But, up to now, no tenocytes-collagen constructs have been able to achieve sufficient mechanical properties, and the complex architecture of the tendon is never fully reproduced [18]. Also, collagen type I does not account for all the tissue properties. The role of proteoglycans and small leucine rich protein (SLRP) in the organization and mechanical properties of the tissue structure has been recently outlined [19]. In our study, in order to enhance mechanical property of cell-collagen grafts, cross-linking strategy was used by over-expressing LOX gene in MEF cells. Meanwhile, over-expressing decorin gene in MEF cells was also used to enhance the mechanical property of the tissue-engineered grafts. Our data showed that the LOX or decorin expressing MEF-collagen grafts had much stronger mechanical property than the control grafts.

TEM result for in vitro cell culture

TEM images of collagen fibril diameters are shown in Figure 4.7. Majority collagen fibrils in the control cells (blank vector transfected) were 15 ~ 20 nm, in LOX-expressing group were 30-35 nm, and in decorin-expressing group were 10~ 15
nm. Mean diameters of collagen fibrils in the decorin-expressing group were smaller than control group and LOX-expressing group.

![TEM result for in vitro cell culture](image)

**Figure 4.7** TEM result for in vitro cell culture. A) Control group, B) LOX-expressing group, C) decorin-expressing group, and D) The diameter distribution of collagen fibrils.

The major proteoglycan in the tendon or ligament is decorin, which anchors to d-bands with several collagen fibrils by non-covalent binding [20]. Decorin is composed of dermatan sulfate, which is a glycosaminoglycan that affects the
morphology and formation of collagen fibrils and core protein by covalent binding [21]. It is reported that tendons of animals lacking decorin had collagen fibrils with irregular diameters [14]. Down-regulation of decorin has also been shown to be correlated with the development of collagen fibrils with large diameters [22]. Our data showed that there were significant differences among the diameters of the control group, decorin-expressing group, and LOX-expressing group.

Cells aligned after culture in nano-polyurethane fibers

Cells in all three groups are aligned well with the direction of oriented nanofibers as shown in Figure 4.8. Mouse embryonic fibroblasts attached to scaffolds over a 24-h period, after 3 days culture, the cells were aligned well on the oriented nanofibers compared to the cells seeded on the normal culture flask (data not shown). One of the considerable challenges remaining in the field of tendon and ligament tissue engineering is to produce a construct with an anatomically correct architectural framework, both in terms of cell morphology and matrix deposition. No cell-collagen constructs are able to achieve sufficient mechanical properties, and the complex architecture of the tendon is never fully reproduced. It is therefore hypothesized that by creating an initial architecture, newly formed ECM will be deposited along the prescribed fiber direction in these nanofibrous scaffolds, expediting the formation of a neo-tissue with enhanced functional characteristics.
Mechanical test for in vivo experiment

Four weeks after surgery, the grafts were taken out for mechanical test. Our data showed that there was significant difference in linear stiffness between the groups (Figure 4.9). The maximum load for the control group was 1.8 ± 0.3 N, the maximum load for the LOX group and decorin group were 3.5 ± 0.6 N and 3.1 ± 0.5 N, respectively. The scaffolds were 1mm in diameter and 20mm in length. Young’s modulus of the control grafts was 3200 ± 36.1KPa, young’s modulus of decorin-expressing group and LOX-expressing group was 4670 ± 52.8KPa and 6130 ± 69KPa, respectively (Figure 4.10). The strength of the graft was significantly increased when the cells over-express LOX or decorin.
Figure 4.9 Force-displacement curves of the nanofiber-cell grafts. (A) Control MEF cells. (B) Infected MEF cells with LOX-expressing retrovirus. (C) Infected MEF cells with decorin-expressing retrovirus.

Figure 4.10 Young’s Modulus of the grafts retrieved from the animals.
There are some inconsistent results about the effect of decorin on collagen fibrillogenesis and its biomechanical properties. Decorin-deficient mice were reported to have fragile skin that is not able to withstand sudden tensile strain. Electron microscopic examination of the skin in decorin knockout mice showed coarser, irregular and haphazardly arranged collagen fibrils compared with wild type mice[14]. Overexpression of decorin by rat arterial smooth muscle cells enhances contraction of type I collagen in vitro[23]. In our study, overexpression of decorin in MEF cells was also proved to increase the mechanical property of MEF-collagen graft although the mean diameter of collagen fibrils was reduced. While Nakamura et al demonstrated that in vivo treatment of injured rabbit ligament by antisense decorin oligodeoxynucleotides led to an increased development of larger collagen fibrils in early scar and a significant improvement in both scar failure strength and scar creep elongation under loading which suggested that down-regulation of decorin helps to improve the healing of various soft tissues including tendon and ligament[22]. One explanation for that paradox is that decorin uniforms the collagen fibrils leading to better-organized collagen structure that has improved mechanical property. It is noticeable that the mean diameter of collagen fibrils in decorin-expressing group (10-15nm) only showed slight decrease compared with control group (15-20nm). And the distribution of diameters of collagen fibrils is more confined in decorin-expressing group than in control group. We hypothesize that moderate overexpression of decorin can help well organize collagen structure and improve its mechanical property but too much decorin may induce the opposite effects by inhibiting collagen fibril formation.
In later situation, down-regulation of decorin may be necessary to improve the collagen structure. Another explanation is that in our study decorin, which is essential for the regulation of newly synthesized collagen, was secreted to regulate the fibril formation of soluble collagen molecules while in Nakamura’s work decorin worked on existing mature tendon. And the difference of animals used in the experiments might also be counted.

Histology

Sirius red staining was used to examine the maturation of collagen fibrils. When examined through crossed polars, the matured collagen fibers are bright yellow or light red, and the immature collagen fibers are green. Figure 4.11 shows the Sirius red staining of the grafts retrieved from the animals. Immature collagen fibrils were dominated in control group (Figure 4.11 A) and decorin-expressing group (Figure 4.11 C) with overall green staining profile. Significant more mature collagen fibrils were found in LOX-expressing group (Figure 4.11 B) with more yellow and light red staining profile.
Conclusions

Our in vitro and in vivo experiments both showed that the mechanical property of tissue-engineered graft could be significantly increased by over-expressing LOX or decorin gene in fibroblast cells. The maturity of the collagen fibrils could be improved or accelerated by over expressing LOX. The electrospinning technique can help cells aligned on the nanofibers.

References


Abstract

The traditional concept of stem cell therapy envisions the isolation of stem cells from patients, propagation and differentiation in vitro, and subsequent re-injection of autologous cells to the patient. There are many problems associated with this paradigm, particularly during the in vitro manipulation process and the delivery and local retention of re-injected cells. An alternative paradigm that could be easier, safer, and more efficient, would involve attracting endogenous stem cells and precursor cells to the defect site for de novo tissue regeneration. Hepatocyte growth factor (HGF), a pleiotropic cytokine of mesenchymal origin, exerts strong chemoattractive effect on mesenchymal stem cells (MSCs) and neural stem cells (NSCs), inducing migration of MSCs in vitro. However, HGF undergoes rapid proteolysis in vivo, resulting in a very short lifetime of the bioactive cytokine. To maintain the therapeutic level of HGF at the defect site necessary for endogenous stem cell recruitment, sustained, long-term, and localized delivery of HGF is required. Thiol-modified glycosaminoglycans hyaluronan (HA) and heparin (HP), combined with modified gelatin (Gtn), were crosslinked with poly (ethylene glycol) diacrylate (PEGDA) afford semi-synthetic ECM-like (sECM) hydrogels that can both provide controlled growth factor release and permit cell infiltration and proliferation. Herein
we compare the use of different sECM compositions, for controlled release of HGF and concomitant recruitment of human bone marrow MSCs into the scaffold \textit{in vitro}. Also, the stem cells recruitment ability by HGF-loaded scaffolds was demonstrated in a subcutaneous implantation model in mouse.

Introduction

The traditional concept of cell therapy is based upon several basic steps. The first step is cell sourcing and cells may be isolated from autologous, allogenic, or xenogenic sources. Second, the isolated cells are expanded \textit{in vitro} to a cell population sufficient for effective treatment. The expanded cells can also be seeded on a scaffold and cultured in a bioreactor. Finally, the expanded cells are re-implanted into the patient. However, this final process is associated with ethical, economic, regulatory and clinical problems.

Clinically, allogenic and xenogenic sources face the greatest likelihood if immune rejection by the patient. Ethical and regulatory issues must also be resolved for this to be a routine clinical treatment. Thus, autologous cells would seem to be the best choice, but cell isolation from patients in need of treatment can cause additional normal tissue morbidity. In order to obtain sufficient number for transplantation, \textit{in vitro} proliferation is essential, which may cause undesirable phenotype change [1]. Pluripotency of stem cells may decrease during in vitro culture [2, 3]. Allogenic and xenogenic components used in culture may cause host immune rejection. In addition,
the cost for in vitro expansion of stem cells is very high, since a battery of growth factors is needed for the propagation procedures. The economic and multi-week expansion period present important challenges to these clinical procedures. Finally, while mesenchymal stem cells attract much attention due to their pluripotency, the pluripotency of mesenchymal stem cells decreases during in vitro culture [2, 3] using conventional 2-D culture conditions.

An alternate cell source could be endogenous stem cells. Indeed, a regenerative medicine approach for tissue repair focused on the direct manipulation of endogenous adult stem cells is very appealing. There are several advantages to the use of endogenous stem cells for tissue repair. First, using endogenous stem cells avoids the immunocompatibility issues that accompany the use of allogenic and xenogenic cells. Second, it is easier, safer, and more efficient to use endogenous stem cells for tissue repair to expand and re-implant autologous cells. Third, only a single surgical intervention is required, rather than two surgeries several weeks apart. Finally, the process of recruiting endogenous stem cells offers both regulatory and economic advantages relative to ex vivo approaches.

The utilization of endogenous stem cells may be enhanced in two ways. One strategy is to mobilize the endogenous stem cells into the circulation. For example, it is reported that granulocyte colony-stimulating factor (G-CSF) mobilizes stem and progenitor cells from the bone marrow into the peripheral blood, from which they can “home” into the lesion site in the brain and have a protective or restorative effect [4]. Also, the mobilized endogenous stem cells are showing promising outcome for
cardiac repair [5]. A second strategy is to enhance the recruitment of endogenous stem cells into the lesion site for tissue regeneration. Several factors, such as hepatocyte growth factor (HGF) [6] and stromal cell-derived factor-1 (SDF-1) [7] have shown chemotaxic effects on stem cells. In this study, we used HGF as a model factor to attract bone marrow mesenchymal stem cells into an ECM-like hydrogel in vitro.

Hepatocyte growth factor (HGF) has been shown to be a strong chemotactic factor for the mobilization and migration of mesenchymal stem cells (MSCs) [6]. Exposure of MSCs to HGF induced migration of MSCs in vitro. However, like most proteins, HGF undergoes rapid proteolysis in vivo, resulting in a very short lifetime of the bioactive growth factor. The half-life of HGF that was delivered in a soluble form in vivo is 3-5 minutes. In contrast, the time required to recruit a sufficient number of stem cells for tissue repair is usually days to weeks. Therefore, direct injection of growth factors to the repair site has limited success.

To maintain the therapeutic level of HGF at the repair site necessary for endogenous stem cell recruitment, sustained, long-term, and localized delivery of HGF is essential. Several polymer delivery systems are being developed for proteins and growth factors delivery. Reservoir devices, solid implants, polymeric micro- and nano-particles, and hydrogels are the most commonly used. Polymer systems have many advantages; for example, they can stabilize proteins, provide localized delivery, and produce diffusion-limited concentration gradients in tissues. ECM-derived polymers with a wide array of physiological functions represent ideal substrates for HGF delivery and stem cell recruitment, since ECM based materials may provide
adhesion sites for migrating stem cells to grow in. Recently, an injectable, in situ-crosslinkable semi-synthetic ECM-like hydrogel, or sECM, was created by crosslinking thiol-modified hyaluronan (HA) and gelatin (Gtn) using poly(ethylene glycol) diacrylate (PEGDA) [8]. This material offers a flexible composition and compliance, simplicity of use, and was developed for ease of translation from in vitro to preclinical in vivo as well as clinical uses [9]. In this study, we compared different compositions of the sECM hydrogel for controlled release of HGF to recruit human bone marrow mesenchymal stem cells (hMSCs) to the scaffold in vitro. Also, the stem cells recruitment ability by HGF-loaded scaffold was evaluated in a subcutaneous implantation model in mouse.

Materials and Methods

Materials

Thiolated chemically-modified HA (CMHA-S), thiol-modified gelatin (Gtn-DTPH), thiol-modified heparin (HP-DTPH) and PEGDA were kindly provided by Glycosan BioSystems Inc. (Salt Lake City, UT). Bovine serum albumin (BSA), 4’,6-diamidino-2-phenylindole (DAPI) and heparin were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human hepatocyte growth factor (HuHGF) was purchased from PeproTech (Rock Hill, NJ). Draq 5 was purchased from Alexis Corporation. Alexa 546-phalloidin was purchased from Molecular Probes Inc. (Eugene, OR). Recombinant mouse hepatocyte growth factor (mHGF) and
Recombinant mouse granulocyte colony stimulating factor (mG-CSF) were purchased from R&D Systems, Inc. (Minneapolis, MN). Human HGF ELISA kit was purchased from Biosource International, Inc. (Camarillo, CA). 8μm pore size transwells were purchased from Corning Costar Inc. (Coring, NY). Rabbit polyclonal CD34 antibody and monoclonal Stro-1 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rat anti-mouse Sca-1/Ly6 antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Secondary antibodies, Texas Red F(ab’) Fragment Goat Anti-Rat IgG, Cy2 Goat anti-rabbit IgG (H+L) and Cy2 Goat anti-mouse IgG (H+L), were purchased from Jackson ImmunoResearch Laboratories, Inc.(West Grove, PA).

Hydrogel preparation for in vitro experiments

Two different sets of hydrogels were prepared. For HA:Gtn gels, CMHA-S and Gtn-DTPH in DI water solution (pH 7.4) were mixed in a volume ratio of 1:1, and then HA:Gtn solution was cross-linked by mixing it with 2 wt% PEGDA in DI water in a volume ration of 4:1. For HA:Gtn:HP gels, HP-DTPH in DI water solution (pH 7.4) were mixed with the HA:Gtn solution mentioned above: the concentration of HP was 0.3%. Hepatocyte growth factor (HGF) was incorporated non-covalently in the gels by pre-mixing 500 ng HGF with 200 μL of the modified HA and gelatin solution prior to cross-linking. Gels formed within 20 minutes.
In vitro release kinetics of hHGF

HGF was allowed to release from these hydrogels at 37 °C into a PBS buffer solution supplemented with 1% BSA, 1 mM EDTA, and 10 μg/mL heparin. At days 1, 2, 3, 5, 9, 14, 20, and 26, 100μl of conditioned medium was sampled, and an equal volume of fresh release medium was added back to maintain the total volume. Samples were then immediately frozen at -20°C until measurement. Each release condition was performed in triplicate.

HGF measurement with enzyme-linked immunosorbent assay (ELISA)

Released amount of HGF was quantified with sandwich enzymelinked immunosorbent assay (ELISA) using an assay from a commercially available kit (Biosource). Briefly, 100 μL of the sample was added into the designated ELISA wells and incubated at 4 °C overnight. After the incubation, the plate was washed 4 times with a wash buffer. Then, 100 μL of biotin antibody was added into each well, and the plate was incubated at room temperature for 1h. Then, the plate was washed 4 times, and 100 μL Streptavidin solution was added and incubated for 45 min at room temperature. Then, the substrate was added to the wells and the absorbance was measured at 450 nm. Total accumulated released HGF was calculated by integration of the individual measurements over the cumulative time of the experiment.
Human bone marrow derived mesenchymal stem cells culture

Human bone marrow–derived mesenchymal stem cells (hMSCs) were obtained from Lonza (Allendale, NJ). The expression of CD105 and CD 44 characterized before use for this study. The expression of CD 105 and CD 44 are more than 92%. hMSCs were maintained in culture in MSCGM™ - Mesenchymal Stem Cell Medium (Lonza, Allendale, NJ).

In vitro experimental procedure for stem cell recruitment

The migration capacity of hMSCs was analyzed using Costar Transwell invasion chambers with polycarbonate membrane filters of 6.5 mm diameter and 8μm pore size to form dual compartments in a 24-well tissue culture plate. Samples each containing 10⁴ cells in 200 μL of media were added to the upper compartments. The lower compartments were covered with 200 μL hydrogels, with or without HGF as a source of chemoattractants, and 400 μL culture media were added. The migration chambers were incubated for 8 hours at 37 °C and 5% CO₂. After incubation, cells on the top surface of the filters were wiped off with cotton swabs. Cells that had migrated into the lower compartment and attached to the lower surface of the filter were counted after being stained with Alexa 546-phalloidin and Draq 5. Briefly, the samples were blocked in TBSA-BSAT (10mM Tris-HCl, 150mM NaCl, 0.02% sodium azide, 1% Triton X-100) for 1 hour at room temperature, and then washed with wash buffer (PBS, with 0.05% sodium azide, 0.05% Triton X-100) for 3 times, 10 minutes each. Then the samples were incubated in appropriate diluted
546-phalloidin solution for 2 hours at room temperature. After 3 times of wash in wash buffer, samples were incubated in appropriate diluted Draq 5 for 15 minutes at room temperature. Finally, after 2 times of wash in wash buffer, samples were incubated in appropriate diluted DAPI solution for 15 minutes at room temperature. The samples could be stored in wash buffer at 4°C for later use. The stained samples were taken pictures under Confocal microscope.

Polyurethane (PU) scaffolds fabrication

Three-dimensional, porous, scaffolds were fabricated using a particulate leaching technique. Briefly, 16 wt% of polyurethane (SG80-A, Noveon Inc, Cleveland, OH, USA) was dissolved in dimethylacetamide (DMAC, Sigma-Aldrich Inc., St. Louis, MO, USA) at 60°C for 12 hours. Grounded sodium chloride particles at 100 ~ 150 μm in diameter were then added into the SG80-A in DMAC solution at 45 wt% and mixed thoroughly. This was then poured into stainless steel molds, which were placed in a dry ice/ethanol bath for 15 minutes. Finally, the molds were dropped into distilled water until the scaffolds released from the molds. The scaffolds were then placed into another beaker of distilled for one week to allow the salt to dissolve. The scaffolds were frozen at -20 °C for several hours before they were cut into rectangular slices (5 mm×3 mm×3 mm). The slices were then washed in distilled water for 12 hours before sterilized using ethylene oxide (EtO). The scaffolds were immersed into HA-Gtn gel with or without mHGF at 4°C overnight. The next day, the scaffolds, which were loaded gel, were picked out and ready for animal surgery.
Morphological analysis of the scaffolds was conducted using scanning electron microscopy (SEM, JEOL 5410). Scaffolds were allowed to vacuum dry for 24 hours prior to SEM analysis. The samples were sputter coated with gold at a thickness of 50-70 nm using a Cressington 108 AUTO sputter-coater with a current of 30 mA for 2 minutes. The coated scaffolds were analyzed on a SEM with an acceleration voltage of 10 kV. Representative images of the scaffolds were obtained.

**Scaffold implantation**

The stem cells recruitment ability was evaluated in a mouse subcutaneous implantation model. Male DBA/2 mice aged 6-8 weeks were injected with MG-CSF (5μg/100g body weight) for 5 consecutive days through intraperitoneal route. The mice were anesthetized and shaved to facilitate the incision. Incisions were made on the back with surgical blade, and pockets adjacent to the incision site were created with the aids of curved forceps. Six scaffolds (with or without mHGF) were implanted per mouse.

**Histology**

Seven days after surgery, the mice were anesthetized and perfused. The scaffolds were fixed in 10% formalin. The scaffolds were then embedded in paraffin, section and stained with stem cell markers (Stro-1), DAPI and Draq 5. Briefly, after embedded in paraffin and cut into 10μm sections using microtome. The sections were washed with xylene for 5 minutes each, and were incubated in two washes of 100%,

90
95%, 85%, 75%, and 50% ethanol for 10 minutes each. The sections were rinsed twice in DI water for 5 minutes each. Incubated the sections in 0.03% H₂O₂, 80% methanol for 30 minutes and rinsed twice in DI water for 5 minutes each. Sections were incubated in 10mM sodium citrate buffer pH6.0 at room temperature for 1 hour and then boiled using water bath or microwave and maintain at 95-99°C for 10 minutes. Sections were cooled to room temperature, rinsed in DI water three times for 5 minutes each, and rinsed in PBS for 5 minutes. Sections were blocked in 4% goat serum in PBS for 1 hour. Primary antibodies (Stro-1) were prepared as indicated on manufacturer datasheet in 1XPBS with 4% goat serum. After blocking, primary antibody was applied. Sections were incubated at 4°C overnight. The specimens were rinsed three times in 1XPBS with 0.3% Triton X-100 for 5 minutes each. Then the specimens were incubated in appropriate secondary antibodies for 2 hours. After 3 times washing in wash buffer, samples were incubated in 1:10,000 diluted Draq 5 for 15 minutes at room temperature. Finally, after 2 times washing in 1XPBS, sections were incubated in appropriate diluted DAPI solution for 15 minutes at room temperature. The sections were mounted for visualization using confocal laser microscope.

**Statistical analysis**

Data will be presented as the mean +/- the standard error of mean for each group. One-way analysis of variance (ANOVA) will be performed to determine the effect of HGF loading on the recruitment of stem cells. Statistical significance is
accepted at p< 0.05.

Results

Cumulative in vitro HGF release from ECM-based hydrogel

HGF release profiles from the hydrogel with or without heparin were in vitro. Immobilization of heparin in HA-Gtn based hydrogel may protect growth factors from enzymatic degradation and thermal denaturation [10]. These crosslinked heparin-containing hydrogel behave in an analogous fashion to a heparan sulfate proteoglycan, extending the release times of growth factors in vitro and in vivo while retaining their bioactivity [11, 12]. Figure 5.1 presents the time course of total cumulative release for HGF. Covalently cross-linked heparin slows down the HGF release significantly in HA: Gtn based gels, but also reduces the net quantity released at a given time point. Without heparin, HA: Gtn gels released a total of 35% of the initially loaded HGF in 26 days. In contrast, HA: Gtn: HP gels released a total of 18% of their loaded HGF at the end of 26 days.
Figure 5.1 Cumulative in vitro HGF release from HA: Gtn and HA: Gtn: HP hydrogels. (A) Release amount in nano grams and (B) Percentage of release.
Bone marrow mesenchymal stem cells migration in vitro

In this experiment, four groups were assigned. In the negative control group, no HGF was present in either the lower or upper compartment of Transwells. In the positive control group, 20 ng/mL HGF was present in the culture media of the lower compartment of Transwell. In the third group, 500 ng of HGF encapsulated in an HA: Gtn gel was placed in each lower compartment. In the fourth group, 500 ng of HGF encapsulated in HA: Gtn: HP gel was placed in each lower compartment.

In order to analyze the migration ability of hMSCs in response to released HGF, two locations were observed under a confocal microscope: the lower surface of the filter and the hydrogel in each lower compartment (Figure 5.2). Figure 5.3 shows the number of hMSCs that migrated to the lower surface of the filter. All four groups (Figure 5.3B, 5.3C, 5.3D) showed significantly greater number of MSCs on the lower surface of the filter than the negative control group (Figure 5.3A). Figure 5.4 presents the cell density in cells per mm² on the lower surface of the filter. Significantly more MSCs migrated across the filter than for each group relative to the negative control.

The numbers of cells recruited into the hydrogels in the lower compartments were also determined. Large numbers of cells were found inside the HA: Gtn gel loaded with 500 ng of HGF (Figure 5.5C). Indeed, significantly more cells were found in the HA: Gtn gel loaded with 500 ng of HGF (Figure 5.5C) than in any of the other three groups: negative control group without HGF loading (Figure 5.5A), positive control group with 20 ng/mL of soluble HGF in medium (Figure 5.5B), and the HA: Gtn: HP hydrogel loaded with 500 ng of HGF (Figure 5.5D). Paradoxically, no cells
were found in HA: Gtn: HP gel loaded with 500 ng HGF Figure (5.5D), similar to the cell density in the negative control group Figure 5.5A.
Figure 5.2: 8μm pore size transwell. The device includes two compartments: the lower compartment and the upper compartment. The insert has a polycarbonate membrane with 8.0μm Pores. The cells were seeded on the membrane and the hydrogels were coated on the surface of the lower compartment.

Figure 5.3: MSCs across the 8 μm pores to the lower surface of the filter. (A) Negative control (no HGF), (B) positive control (20 ng/mL HGF in culture media), (C) 500 ng HGF encapsulated in HA: Gtn hydrogel, and (D) 500 ng HGF encapsulated in HA: Gtn: HP hydrogel.
Figure 5.4: The number of MSCs across the 8 μm pores to the lower surface of the filter.
Figure 5.5: MSCs migrated into the hydrogels on the lower compartment. (A) Negative control (no HGF), (B) positive control (20 ng/ml HGF in culture media), (C) 500 ng HGF encapsulated in HA: Gtn hydrogel, and (D) 500 ng HGF encapsulated in HA: Gtn: HP hydrogel.
Figure 5.6: The number of MSCs migrated into the hydrogels on the lower compartment.

SEM for porous polyurethane (PU) scaffolds and hydrogel scaffolds

Figure 5.7 shows the scanning electron microscopy (SEM) images of Polyurethane (PU) scaffold. Leaching is one popular approach used in tissue engineering to fabricate porous scaffolds. Pores or channels are created using porogens, such as salt (NaCl), wax, or sugar. In this study, NaCl was used for pores making. For particle leaching, pores are regulated by the salt particles and 100μm-150μm salts were used, so that the pores would be around 100μm-200μm and larger than
the cells size (about 15\( \mu m \)). After the solvent (DMAC) was removed and the salt was washed, pores can be seen in the interior and on the surface of the scaffold under SEM (figure 5.7), and the pores are well interconnected throughout the scaffold. There is enough space for cell migration into the hydrogel.

![SEM images showing morphology of porous scaffold with pore sizes 100-200 \( \mu m \) in diameter.](image)

**Figure 5.7:** SEM images showing morphology of porous scaffold with pore sizes 100-200 \( \mu m \) in diameter.

Mesenchymal stem cells recruitment in a mouse subcutaneous implantation model

In order to evaluate the stem cell recruitment potential of HGF loaded scaffolds in vivo, a subcutaneous implantation mouse model was used. According to the profile of the cumulative in vitro HGF release, HA:Gtn hydrogel was chosen for the in vivo study. In order to identify the implant at the time of scaffold harvesting,
HA:Gtn hydrogels were infiltrated into nondegradable polyurethane porous scaffolds. The scaffolds with or without mHGF loading were implanted to the back of the mice, and 1 week after surgery, the scaffolds were processed for immuno-staining and visualized under confocal laser microscope.

First, the number of cells infiltrated into the porous scaffold was quantified and compared among groups (Figure 5.8 A, D, and G). Significantly more cells were detected in the mHGF releasing scaffolds (Figure 5.8 D) than the control scaffolds (Figure 5.8 A) without mHGF loading. This data demonstrated that more cells could be recruited to the mHGF releasing scaffolds. In order to identify whether there are stem cells among the recruited cells, mesenchymal stem cell marker, Stro-1, was used to identify non-hematopoietic stromal stem cells [31]. Our data show that about 14.2±3.6 % of recruited cells expressed mesenchymal stem cell marker (Stro-1) in the scaffolds loaded with mHGF. While in the non-mHGF releasing scaffolds, the percentage of the cells expressed Stro-1 is significantly lower at 7.9±1.3 %. This demonstrated that scaffolds loaded with HGF may recruit more stem cells to the local implantation site (Figure 5.8 H).
Figure 5.8 Cells migrated into the scaffoldss after 1-week implantation. A-C) Scaffold without mHGF release, D-F) scaffold with mHGF release, A, D) Nuclei staining in blue, B and E) Stro-1 Staining in green. C and F) The overlay images of nuclei staining and Stro-1 staining. G) The number of cells in the scaffolds. And (H) The number of stro-1 positive mesenchymal stem cells.

Discussion

Thiolated hyaluronan are biocompatible and can be adapted to produce a variety of desirable biologic effects [13]. Gelatin is a partially hydrolyzed collagen product,
which is an excellent substrate for cell attachment, proliferation, and differentiation. Both thiolated HA [14, 15] and thiolated gelatin [16, 17] have been used in a number of tissue engineering applications, including the delivery of basic fibroblast growth factor (bFGF) for cutaneous wound repair [18] and human demineralized bone matrix for bone repair [19]. The hydrogels created by co-crosslinking thiol-modified HA with thiol-modified gelatin produces mechanically robust, bioresorbable scaffolds that can be implanted to achieve tissue growth in vivo[13, 20-22]. The HA-Gtn hydrogel is also an effective vehicle for cell delivery and retention in vivo, including the repair of osteochondral defects using encapsulated autologous MSCs [23]. Growth factors delivered locally using the HA-Gtn hydrogels elicit a strong angiogenic response, even when the growth factors are delivered in low, nanogram doses [11-13, 24]. In addition, it is reported that incorporation of small amounts of heparin in the gels can prolong and spatiotemporally regulate the rate of growth factor release in vitro [11, 12]. In this study, we demonstrated that through delivery of HGF from heparin-containing HA: Gtn hydrogels or HA: Gtn hydrogel only, the biologic activity of HGF can be maintained, and the human mesenchymal stem cells are most effectively recruited into the HGF releasing hydrogels when the release rate is maintained to certain threshold.

As commonly occurs with highly bioactive polypeptides in vivo, HGF undergoes rapid proteolysis and has a half-life in vivo of 3-5 minutes. Figure 5.1 illustrated that release of HGF from crosslinked thiol-modified HA and gelatin hydrogel in vitro could be sustained for up to 3 to 6 months based on the extrapolated value from a
26-day study. This is a dramatic increase in time of availability compared to the short half-life of free HGF in vivo. Furthermore, the rate of HGF release could be controlled with heparin. In the absence of heparin, HGF released twice as fast from HA:Gtn hydrogel than the HA:Gtn:HP hydrogel. This result is consistent with extended release of bioactive keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), angiopoetin-1 (Ang-1), and bFGF from similar heparan-sulfate mimetic hydrogels [10-12]

The biological activity of the HGF released from the hydrogels was confirmed by in vitro hMSCs migration experiments. On the lower surface of each filter, all groups except the negative controls showed an obvious accumulation of cells (Figures 5.3 and 5.4). In the hydrogels in the lower compartments, many cells migrated into the HA:Gtn gel loaded with 500ng HGF, while there were few cells attached on the negative control group or the HA:Gtn:HP gel loaded with 500ng HGF (Figures 5.5 and 5.6). This seemingly paradoxical result seems to indicate that the amount of loading and the release rate of HGF from the gel are both crucial parameters affecting the migration of hMSCs. If the release rate or amount of HGF is lower than the threshold for hMSC to response, there will be no control of hMSCs to migrate, since when we double the amount of HGF loading into the HA-Gtn-HP gel to 1000ng, significant amount of hMSCs migrated into the HA-Gtn-HP hydrogels. According to cumulative release of hHGF from two types of hydrogels (Figure 5.1), HA:Gtn hydrogel released about 10% of total stored hHGF (about 50ng) at the first two days, while HA:Gtn:HP hydrogel released less than 5% of total loaded hHGF (less than
25ng) at the first two days. In the literature, the minimum effect dosage for HGF activating mesenchymal stem cell migration was 20ng/ml [13, 27]. In our experiment, the incubation time for the mesenchymal stem cells migration was short (8 hours), hHGF did not have enough time to release to the amount that can attract stem cell migration efficiently, especially the HA-Gtn_HP hydrogel.

The other interesting indication is the importance of HGF gradient in the control of MSC migration. If we compare the 20 ng/mL HGF group (Figure 5B) with the 500ng/mL group (Figure 5C), fewer MSCs are migrated to the hydrogel in 20ng/ml group (Group II) than that in slow releasing group (Group III). There are sufficient amount of HGF in group II for MSC to migrate, however, the difference between group II and III is the establishment of HGF gradient. HGF gradient is well established in group III due to the constant release of HGF from the HA-Gtn hydrogel.

In vivo study further verified the observation in vitro. There were significant more cells observed in the mHGF-loaded scaffolds than that in control scaffolds (Figure 5.8 G). The total numbers of recruited stro-1 positive mesenchymal stem cells were also significantly higher in mHGF group (Figure 5.8 H).

Conclusions

The release characteristics of HGF and stem cell recruitment capacity of a series of HA-Gtn hydrogels loaded with HGF have been investigated in vitro and in
vivo. These hydrogels provide sustained release of biologically active HGF in vitro and in vivo, with HGF release sustained for over three weeks. This is a dramatic increase in time of availability compared to the short half-life of free HGF in vivo. HGF released from HA: Gtn hydrogel attracted human bone marrow MSCs to migrate into the hydrogel following the HGF gradient established by HGF loaded HA-Gtn hydrogels. To evaluate the stem cell recruitment ability in vivo, subcutaneous implantation mouse model was used. Significantly more cells were detected in the mHGF releasing scaffolds than the control scaffolds without mHGF loading. Mesenchymal stem cell marker, Stro-1, can be detected in the recruited cells. Our in vitro and in vivo studies demonstrated that stem cells could be recruited to the local site where HGF was released by chemically modified HA and modified gelatin based hydrogels. And this strategy is promising for tendon and ligament tissue regeneration applications.

References


CHAPTER 6

FUTURE DIRECTIONS

In the tissue maturation part of this thesis, we figured out that over-expression of LOX or decorin genes in cells could increase the stiffness of tissue-engineered grafts respectively by different mechanism. LOX cross-links collagen molecules to enhance collagen maturation, while decorin increases the stiffness of the grafts by uniforming the diameter of collagen fibers and regulating collagen fibrogenesis. In our future study, we are going to over-express both LOX and decorin to figure out whether they can further increase the stiffness of the graft, and to design a graft that has similar mechanical and functional characteristics of the native tissue in a short period of time.

The second part of this thesis is to use endogenous stem cells for tendon and ligament regeneration. Our in vitro and in vivo studies demonstrated that stem cells could be recruited to the local site where HGF were released by chemically modified HA and gelatin based hydrogels. In the future work, we plan to optimize heparin concentration in the hydrogel for growth factor releasing profile that best promotes stem cell recruitment. Furthermore, we are going to control the differentiation of the recruited stem cells into desirable cell types by controlling release of differentiation factors.