Determining the Efficacy of Insulin for use in Factor Delivery Device for Tendon Healing and Regeneration

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Determining the Efficacy of Insulin for use in Factor Delivery Device for Tendon Healing and Regeneration

Daisy Mamuad Ramos, PhD
University of Connecticut, 2019

Tendon injuries account for roughly half of the 33 million musculoskeletal injuries that occur every year. Of all patients seeking nonsurgical treatment, 29% eventually require surgery. The use of autografts, allografts, and xenografts have limitations associated with donor site morbidity, availability, and immunogenicity. Tissue engineering has emerged as a feasible approach to find treatments for injuries requiring tissue replacement through biodegradable scaffolds, stem cells, and biomolecules.

Tissue engineering scaffolds serve to provide a temporary, biomimetic template for cells, as well as act as delivery vehicles for biomolecules, such as growth factors. Growth factors have been widely popular in tissue engineering owing to their importance in embryonic development and healing. Insulin-like growth factor-1 (IGF-1) has been extensively researched for its ability to encourage cell proliferation, inhibition of cell apoptosis, and collagen formation. The homology of insulin and IGF-1, structurally, as well as biologically, has motivated various comparative studies, as well as investigations in the use of insulin for tissue engineering applications. This work explored insulin as a bioactive factor in promoting tendon regeneration and investigated insulin delivery through electrospun blend fibers made from synthetic polymer, polycaprolactone and natural material, cellulose acetate.

The treatment of human mesenchymal stem cells (MSCs) with concentration of 100ng/ml showed increased expression of tendon related genes and ECM proteins, suggesting phenotypic development of MSCs towards tendon lineage. Insulin functionalized scaffolds were observed to
support tendon differentiation of MSCs, in-vitro. The bioactive scaffolds were implanted in a rat Achilles tendon model and found to encourage better healing through greater collagen deposition and fiber organization. However, no significant findings were found in mechanical properties of the healed tissue when compared to control groups. Increase in collagen and matrix organization with no difference in mechanical property warrants further research in functional analysis of the tendon and exploration into possible early immunomodulation effects. For the first time, insulin has been investigated for use in tendon tissue engineering applications and shown to achieve better ECM organization with use of a bioactive insulin functionalized scaffold.
Determining the Efficacy of Insulin for use in Factor Delivery Device for Tendon Healing and Regeneration

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B.S., Trinity College, 2010

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Dedication

This thesis is dedicated to my parents, Angela and Mario Ramos, whose love and life lessons taught me the values of education, responsibility, humility, and compassion.
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List of Figures

Figure 1.1. Overview of tendon extracellular matrix-----------------------------------------------1
Figure 1.2. Development compartments of the embryo---------------------------------------------13
Figure 2.1. Cells were treated with varying concentrations of insulin proliferation, gene and collagen I staining-----------------------------------------------22
Figure 2.2. Proliferation and gene study at low serum concentrations-----------------------------23
Figure 2.3. Continuous treatment of tenocytes with insulin---------------------------------------25
Figure 2.4. Initial bioactivity studies of insulin functionalized scaffolds------------------------26
Figure 3.1. Insulin and IGF-1 amino acid sequence-----------------------------------------------31
Figure 3.2. The effect of fetal bovine serum concentrations on cellular proliferation-------------54
Figure 3.3. Proliferation of cells treated with various concentration of insulin-------------------55
Figure 3.4. Metabolic activity of cells treated with higher concentrations of insulin-----------56
Figure 3.5. Gene expression of cells treated with single dose of insulin------------------------57
Figure 3.6. Gene expression of cells treated with single dose of insulin or IGF-1-------------58
Figure 3.7. Gene expression of cells treated with continuous dose of insulin-------------------59
Figure 3.8. Gene expression of cells treated with continuous dose of insulin or IGF-1---------60
Figure 3.9 Collagen and GAG deposition with continuous dose of insulin------------------------61
Figure 4.1. Synthesis of PCL------------------------------------------------------------------71
Figure 4.2. Structure of cellulose and its derivative cellulose acetate--------------------------73
Figure 4.3. The electrospinning set up----------------------------------------------------------74
Figure 4.4. SEM images of various scaffold compositions------------------------------------------82
Figure 4.5. Tensile testing of scaffold----------------------------------------------------------83
Figure 4.6. DNA concentration on polymer compositions after 24 hours---------------------------84
Figure 4.7. Insulin-FITC was conjugated to fibers and viewed under confocal microscopy--------85
Figure 4.8. Quantification of immobilized insulin-----------------------------------------------86
Figure 4.9 DNA concentration on insulin treated scaffolds and neat scaffolds-----------------87
Figure 4.10. Insulin released into media----------------------------------------------------------87
Figure 4.11. Gene expression of tendon markers on insulin immobilized scaffolds----------------88
Figure 5.1. Schematic of surgical model----------------------------------------------------------99
Figure 5.2 Method to quantify cell number--------------------------------------------------------102
Figure 5.3 Method to quantify collagen expression using ImageJ analysis-------------------------103
Figure 5.4 Method to quantify second harmonic generation images-------------------------------104
Figure 5.5 Amount of cellular DNA present on scaffolds after seeding-----------------------------105
Figure 5.6 Surgical procedure------------------------------------------------------------------106
Figure 5.7 Glucose monitoring

Figure 5.8 Gross morphology of tendons at 4 & 8 weeks

Figure 5.9. H&E staining 4 & 8 weeks

Figure 5.10. Cell density

Figure 5.11. Relative expression of SRY at 4 weeks

Figure 5.12 Representative trichrome staining

Figure 5.13. Trichrome quantification

Figure 5.14. Collagen I quantification

Figure 5.15. Collagen III quantification

Figure 5.16. Collagen I: Collagen III ratio

Figure 5.17 Collagen alignment

Figure 5.18 Mechanical testing set up

Figure 5.19 Mechanical properties of healed tendon

Figure 5.20. Gene expression of inflammatory response

Figure 5.21 Gene expression of ECM markers
Table of Contents

Chapter 1: Tendon and Tissue Engineering Overview
1 Tendon Overview 1
1.1 Tendon structure and extracellular matrix composition 2
1.1.1 Ground Substance 3
1.1.2 Cells 4
1.1.3 Tendon Vascularity 5
1.2 Tendon Biomechanics 5
1.2.1 Response to Mechanical Loading 6
1.3 Tendon Injury and Healing 8
1.3.1 Injury 8
1.3.2 Healing 8
1.3.3 Treatment Modalities 9
2 Tissue Engineering Overview 10
2.1 Evolution of Biomaterials 10
2.2 Exogenous Cells 11
2.3 Mechanical Stimulation 12
2.4 Tendon Regulatory Factors 13
2.4.1 Embryonic Development 13
2.4.2 Transcription Factors 15
2.4.2.1 Scleraxis 16
2.4.2.2 Mohawk 16
2.4.2.3 Early Growth Response 17
2.5 Growth Factors 17
2.6 Challenges of Tendon Tissue Engineering 18

Chapter 2: Specific Aims
1 Objectives/Rationale 19
2 Selected Preliminary Data 21
2.1 Bolus Studies 21
2.2 Continuous Treatment Studies in Low Serum 21
2.3 Insulin effect on tenocytes 24
2.4 Scaffold Conjugation and Bioactivity Studies 26
3 Specific Aims and Hypotheses 27
3.1 Specific Aim I 27
3.2 Specific Aim II 27
3.3 Specific Aim III 28

Chapter 3: Application of Insulin and Insulin-Like Growth Factor-1 in Musculoskeletal Tissue Engineering
1 Introduction 29
1.1 Insulin Superfamily 29
2 Discovery and Clinical use 31
2.1 Discovery of Insulin 31
2.2 Metabolic Insulin Regulation 32
2.3 Insulin in the treatment of Diabetes 33
3 Homologous receptors and signal transduction 36
4 Applications in Musculoskeletal Tissue Engineering 38
Chapter 5: In-vivo Study of Insulin in Rat Achilles Tendon Defect

1 Introduction
1.1 Commercial Grafts
1.1.1 Biological
1.1.2 Synthetic Prosthesis
2 Animal Tendon Model
3 Specific Aim III
3.1 Materials and Methods
3.1.1 Cell Attachment
3.1.2 Donor Cell Harvest
3.1.3 Scaffold Preparation
3.1.4 Surgical Procedure
3.1.5 Blood Glucose Testing
3.1.6 Sample Collection
3.1.7 Histological evaluation
3.1.8 Second Harmonic Generation
3.1.9 Biomechanical Testing
3.2 Results
3.2.1 Cellular Attachment
3.2.2 Surgical Procedure
3.2.3 Blood Glucose Levels
3.2.4 Gross Morphology
3.2.5 Cell Infiltration
3.2.6 Trichrome Stain
3.2.7 Collagen Staining
3.2.8 Second Harmonic Generation
3.2.9 Biomechanics
3.2.10 Inflammation and Remodeling mRNA expression
4 Discussion

Chapter 6: Summary and Future Directions

References
Appendix A: List of Publications
Appendix B: About the Author
Chapter 1: Tendon and Tissue Engineering Overview

1 Tendon Overview

The human musculoskeletal system is a coordinated system of connective tissue that work in concert to bring stability to the body and allow for the myriad of daily human activities. Tendon is an important tissue that connects muscles to bones and whose primary function is to mediate movement and bring stability to joints. This chapter gives a brief overview of tendon structure, biomechanics, as well tendon injuries and current treatments.

![Figure 1.1. Overview of tendon extracellular matrix.](image-url)
1.1 Tendon structure and extracellular matrix composition

Tendon structure is greatly governed by its function, thus slight variations in morphology and organization exists between tendons in different locations. Tendons that are connected to muscles that generate strong, resistive forces, such as the quadriceps are short and broad, whereas flexor tendons, located in the fingers, are long and thin (1). Irrespective of their location, healthy tendons exhibit shiny white appearances and are composed of dense parallel bands of collagen fibers arranged in a hierarchical structure. It is this unique organizational structure that endows tendon tissues with high mechanical strength.

The major constituent of tendon is collagen fibers, which make up 85% of the total dry weight (2). The fundamental building blocks of collagen fibers is procollagen, which is made up three polypeptide chains that are arranged in a helical structure. Tendon fibroblast cells secret procollagen into the extracellular matrix, where they are cleaved to form tropocollagen (3). Tropocollagens are roughly 1.5nm in diameter and self-assemble to form aggregates of tropocollagen molecules called microfibrils, which then give rise to fibrils (4). Fibrils range from 100-500nm in diameters (5) and under scanning electron microscopy, can be seen as black and white striated bands (3). Fibrils combine to form tendon fibers that are 10µm in dimeter. These collagen fibers are visible under light microscopy and display a crimp like pattern when unloaded. The sinusoidal pattern facilitates 1-3% elongation of the tendon, which serves as a buffer to sudden mechanical loading (5). Collagen fibers are arranged in bundles to form tendon fascicles, which in turn make up the tendon body. Individual tendon fascicles are surrounded by connective tissue known as endotenon, which links to the epitenon, which surrounds the entire tendon body (4). The hierarchical structure of the tendon is displayed in Figure 1.
In addition to collagen I, other collagen types in smaller amounts are also present in tendon. In healthy tendon collagen III is found at the endotenon and epitenon, but is also present in higher numbers in aging and injured tendons. Compared to collagen I, collagen III forms smaller and less organized fibrils, with lower mechanical strength. Collagen V is intercalated into the core of collagen I and is involved in the regulation of fibril growth. Collagen II, VI, IX, X, and XI have also been found in trace amounts in tendon, mainly at the bony insertion site (6).

1.1.1 Ground Substance

The surrounding tissues that encase the collagen fibers are referred to as the ground substance. The ground substance is composed of proteoglycans, glycosaminoglycans, glycoproteins and other small molecules (1). Proteoglycans are large hydrophilic molecules, dispersed between within and between collagen fibrils, that are capable of absorbing water 50 times their weight. Proteoglycans are composed of a protein core with covalently bonded glycosaminoglycans (GAGs) (1). Higher concentration of GAGs are found in tendons subjected to higher compressive forces (7). Under SEM, proteoglycans are seen as filaments attached to collagen fibrils (8). Proteoglycans serve to aid in resisting high compressive and tensile forces. Additionally, they allow for cell migration and rapid diffusion of water soluble molecules. Aggrecan is known to retain water in the ECM, whereas decorin was observed to facilitate fibrillar slippage. The most predominant proteoglycan in tendon is decorin and may be involved in the regulation of fiber diameter (2). Animal models with mutations lacking decorin showed irregular diameters of collagen fibrils (8), whereas downregulation of decorin was associated with the development of collagen fibrils with larger diameters and higher tensile strengths in ligament scar (8, 9).
Glycoproteins in tendons include fibronectin and tenascin-C. Fibronectin is found on the surface of collagens and is involved in wound healing. Tenascin-C interacts with collagen fibrils to aid in mechanical stability of the ECM (6). Tenomodulin is a transmembrane glycoprotein observed to be involved in tendon maturation and is considered to be highly specific to developing tendons.

1.1.2 Cells

Tendon fibroblasts are the dominant cell type in tendons and are referred to as tenoblasts and tenocytes. These fibroblasts reside in rows parallel to and between collagen fibers. They are responsible for synthesizing ECM proteins such as collagen and proteoglycans. At early developmental stages, such as newborns, there is a high cell-to-matrix ratio in the tendons, with varying shapes and sizes. As tendon matures, the cell-to-matrix ratio decreases and cells become more elongated and transform to tenocytes. Mature tenocytes have long processes to maintain close contact with few surrounding cells and matrix components (1), with high nucleus-to-cytoplasm ratio and lower metabolic activity (7).

Other cells present in tendons include chondrocytes, synovial cells, and endothelial cells (6). Additionally, the presence of tendon stem cells has also been discovered. Bi et al. discovered a unique population of mesenchymal stem cells (10). These tendon-derived mesenchymal stem cells have been shown to self-renew and have the same tri-differentiation properties as that of other mesenchymal stem cells (11). It is believed these tendon stem cells play a critical role in the tendon maintenance and repair, but may also potentially play a role in the development of tendinopathy through aberrant differentiation (12).
1.1.3 Tendon Vascularity

Though the amount of vascularization and source vary depending on the tendon location and function. When compared to other tissues, overall tendon ECM has limited vascularity. Tendons thus derive nutrients from their attachments, at both the bony or muscle ends or from the surrounding paratenon, which is known to be the main source of vascularity (3). Vascularization decreases with maturation, with significantly more blood supply during development. Vascular endothelial growth factor, a known simulator of angiogenesis, is upregulated in tendon during development and the period following injury.

1.1.4 Tendon Biomechanics

The unique structure and composition of tendon endows tendon tissues with unique biomechanical properties such as high mechanical strength and viscoelasticity. As the connective tissue between muscles and bones, tendons undergo large amounts of tensile loading. Mechanical behavior of tendon can be described in stress strain diagram with 4 regions. Below 2% strain, is the toe region, where the crimped shaped collagen fibers, as previously described, are stretched and begin to straighten out. Below 4% strain, is the elastic region. The collagen fibers are oriented in the direction of the tensile load. If subjected to higher strains, the tendon is stretched beyond its physiological limit and microscopic tears in the fibers begin to appear. Beyond 8-10% strain, macroscopic tears occur, leading to tendon ruptures (12).

Tendons are can be described as having viscoelastic properties, which simply means tendons display both elastic and viscous behavior. Elastic materials will return to their original length if within the elastic region, whereas viscoelastic materials have a time dependent strain rate. Thus tendons display more deformation at low strain rates than at high strain rates. Slow strain
rates result in tendons absorbing more mechanical energy and becoming less effective in transmitting loads. At high strain rates, tendons become stiffer, absorb less mechanical energy and thus can more effectively transmit the mechanical force to the attached bone. This viscoelastic property of tendons is attributed to water, collagenous proteins, proteoglycans, and their interactions (12).

1.1.5 Response to Mechanical Loading

In the past most of our understanding of tendon adaption to mechanical loading, has been provided through animal models due to the difficulty of evaluating human tendons. Animal models subjected to physical activity compared to controls showed structural changes in tendon strength and appearance (6). The ultimate load and energy absorbed at failure of the brevis tendon (tendon in the foot) was increased in rabbits subjected to 40 weeks of exercise (13, 14). The adaptability of tendons to mechanical loading was observed in a mouse study. In mice subjected to daily treadmill exercise, changes in fibril diameter, number and cross sectional area were observed, increasing one-week post treatment and decreasing during the 3-7th week of the study. Long term effects showed increase in overall fibril number, but not in mean diameter, or cross sectional area (15). Other studies have shown the increase of collagen deposition following strenuous endurance training in the Achilles tendon of roosters (16).

On the opposite spectrum, the effects of immobilization on tendons have also been studied. In general, due to the lower metabolic rate and vascularity of tendons, atrophy due to immobilization is less pronounced and dramatic when compared to other musculoskeletal tissues (17). Following an Achilles tendon rupture, the healing of rabbits immobilized at the knee joint versus rabbits who were immobilized at both the knee and ankle joint were compared after 4 weeks
Rabbits immobilized at the knee and ankle joints had decreased ultimate stress and stiffness of the Achilles tendon, as well as the formation of irregular and uneven collagen fibers, dilated veins and capillaries. The authors hypothesized that immobilization retards the healing of the ruptured tendons due to congestion and tension deprivation. Dramatic changes in tendon cell shape, cell number, and collagen fiber alignment have also been observed in immobilized tendons.

Studies looking into the overuse of tendons look at the effects of chronic exposure to excessive loading. Advances in non-evasive imaging such as MRI and ultrasound, have made it possible to evaluate the effect of physical activity on human tendons. In humans, Rosager et al. evaluated MRI images of Achilles tendons in long distance runners, (defined as those who ran more than 80km a week) and compared them to age matched controls. Larger cross-sectional areas of the tendon was observed in runners. The increase in cross-sectional area is hypothesized to reduce the average stress caused by the repetitive loading of running. Tendinopathy is believed to be caused by repetitive mechanical loading that cause strains below the failure threshold, which in turn cause microinjuries and inflammation.

In general, animal models have shown that tensile strength, elastic stiffness, and total weight of the tendons tend to gradually increase with gradually increasing physical activity due to increased thickness of collagen fibrils and fibers. This results in larger and stronger tendons with increased resistance to injuries. However, if excessive loading is applied, the sum effect may be detrimental and cause inflammation. Despite the research that has been done, much is still unknown regarding tendon adaption to physical activity in humans.
1.1.6 Tendon Injury and Healing

1.1.6.1 Injury

It is estimated that 50% of the 33 million musculoskeletal injuries are related to tendon or ligament damage (5, 21). The most common tendinopathies pertain to the rotator cuff tendons, medial and lateral elbows, patellar, and Achilles tendon. These tendons are known to be more highly stressed than others, are subjected to repeated strains, and are less vascularized at their midsubstance (22).

Tendon injuries can be divided into two subclasses: 1) acute and 2) chronic degeneration. Though acute injuries can be caused by trauma, spontaneous ruptures may be a result of chronic injuries due to accumulation of microtears that generate an aberrant tendon matrix (23). Tendinopathies include inflammatory related injuries such as tendinitis as well as non-inflammatory conditions such as tendinosis and ruptures (21). Histology of injured tendons are amorphous, grey brown in appearance and display disorganized collagen, with increased cellularity, neovascularization, and proteoglycan ground substance (24). Though the etiology of tendinopathy remains elusive, Sharma et al. summarizes the possible causes to ischemia, hypoxia, tenocyte apoptosis, or presence of cytokines and inflammatory prostaglandins (25), all of which can be brought on by excessive mechanical loading and overuse.

1.1.6.2 Healing

Healing is limited due to lower vascularity and hypocellularity. Tendon healing is often described in three stages 1) inflammatory, 2) proliferative, and 3) remodeling. The first stage of healing can last for several days and is marked by the infiltration of erythrocytes and neutrophils. Macrophages and monocytes in the area remove necrotic material, while vasoactive and
chemotactic factors are released. Tenocytes migrate to the area and begin to produce collagen III. During the proliferative stage, more collagen III is synthesized and there is an upregulation of water and glycosaminoglycan content. This stage can last for a few weeks before the remodeling stage begins. The first half of the remodeling stage can be described as the consolidation stage. At this point, during 6-10 weeks post injury, the injured tissue changes from cellular to fibrous and alignment of cells and collagen fibers begin to orient along the direction of stress. Collagen I synthesis is highly upregulated. After 10 weeks, the tendon transitions to the maturation phase which may last for year and tendon scar tissue is developed. At the end of healing, tenocyte metabolism and vascularity start to decrease.

1.1.7 Treatment Modalities

Tendon injuries are clinically treated with either conservative or surgical interventions. Conservative treatments include physical therapy and injections of non-steroidal anti-inflammatory drugs (NSAIDS) or corticosteroids. Non-surgical treatments have been shown to be less successful with 29% of patients eventually needing surgical treatment (21, 26). Surgical treatment often involves debridement of injured tissues and reattachment of the tendon using an allograft, graft from cadaver, autograft, graft from secondary area of the body, or xenografts, grafts from animals. Information on the performance of commercial grafts is further discussed in Chapter 5. Current grafts on the market have been met with variable results and high retear rates, and observed to treat only pain with little functional gains (27, 28).
2 Tissue Engineering Overview

Regenerative capacity of the human body has long been speculated through ancient myths and stories. Today, science has shown that regenerative capacity is not only possible, but tissue regeneration is being exploited to its greatest extent. The discovery of stem cells, in parallel to the increasing knowledge of cell biology and the biomedical sciences, has contributed to the growing field of regenerative and tissue engineering. Various tissues and organs are being studied in hopes of finding solutions to human diseases and injuries. Tissue engineering, as defined by Dr. Langer and Dr. Vacanti, is an interdisciplinary field which applies the principles of engineering and life science toward the development of biological substitutes that restore, maintain, or improve tissue function (29). Strategies for tissue engineering often encompass the use of biomaterials, cells and biomolecules, separately or in combination to regenerate tissues or whole organs. What began as a science fiction is soon becoming a reality as scientists are developing new ways to incorporate biomaterials into the body to heal and regenerate various tissues and organs. The following sections will give an overview of tissue engineering principles as it relates to tendon regeneration.

2.1.1 Evolution of Biomaterials

Throughout history it was discovered that several metals such as silver, copper, and gold could stay in the body without causing an immunogenic reaction. Today, metal implants are made from stainless steel and cobalt base alloys and titanium. Though still widely used in a variety of biomedical applications such as hip replacements and orthopaedic fixation devices, stress shielding of metal implants is a problem as the mechanical mismatch of natural tissues versus metal implants proved to be detrimental to the surrounding tissue. By implanting structures that were significantly stronger than the surrounding body environment, the surrounding muscles atrophied and became
weaker. Initially, such implants were thought of as inert and served only as place holders. Additionally, problems with infections and necessary revision surgeries contribute to the limitations of metal implants.

Today the properties of the native tissue govern the design of implants or scaffolds in tissue engineering in hopes of regenerating new tissue. Scaffolds are designed to degrade in-situ, and to be eventually replaced with native tissue. Further details on materials for tendon tissue engineering applications are discussed in Chapter 4. To stimulate regeneration, tissue engineering strategies utilize cells, as well as biochemical and physical cues given by the scaffold and bioactive molecules.

2.1.2 Exogenous Cells

The exact role of exogenous cells in regeneration remains unanswered. Some studies suggest cells play a direct role in laying new extracellular matrix for the regenerate tissue, while other studies show that cells have a more paracrine effect, serving only to recruit native cells to the injury site. The answer may lie somewhere in the middle, with cells playing both direct and indirect roles in regeneration. Nevertheless, the addition of exogenous cells are observed to have a beneficial effect and to aid in tissue engineering (30, 31).

Several types of cells have been applied towards tendon tissue. Tendon fibroblast cells have been studied, but their low availability limits their use. As previously mentioned, tendon is hypocellular, thus obtaining the desired number of tenocytes poses to be a challenge. Embryonic stem cells may have great potential in regenerating tendon, as it has been shown that the fetal tendon maintains the ability to regenerate, even after being transplanted into an adult environment (32). This indicates the regenerative capacity of the tendon is intrinsic to the tissue itself and not the environment. However, the legal and moral controversies surrounding the use of embryonic
stem cells limits their wide spread use. The limitations of tendon fibroblasts and embryonic stem cells make adult progenitor cells attractive for use in tendon tissue engineering. Mesenchymal stem cells (MSCs) are adult stem cells that have the capacity to differentiate into cartilage, bone, adipose, neurons, muscles, tendon, ligaments, dermis and other connective tissues (33). These cells have been extracted from a variety of sources including bone marrow, adipose, and tendon. Induced pluripotent stem cells (iPSCs), terminally differentiated cells that have been dedifferentiated into back into stem cells, have also been explored for tendon applications. Though there is a growing interest in iPSCs, and much research has gone into their potential in tissue engineering, they are far from clinical use. By far, the most popular cell type and most clinically relevant is MSCs due to their versatility and relative abundance.

2.1.3 Mechanical Stimulation

Mechanical stimulation has been observed to play a critical role in tissue regeneration of load-bearing tissues, such as tendon. Custom bioreactors have been designed to provide mechanical stimulation to cell-seeded constructs to observe effects of mechanical stimulation, in-vitro. Shearn et al. developed a pneumatic mechanical stimulation system to provide mechanical strain to stem cell seeded collagen sponge constructs and observed increased stiffness in the stimulated constructs (34) with increased gene expression of collagen I and III (35). Xu et al. found greater proliferation and enhanced differentiation of tendon derived stem cells with scaffolds that received mechanical stimulation (36). Though its commonly accepted that mechanical stimulation can modulate cellular behavior, the mechanism of how cells sense mechanical forces and convert them to chemical signals remains elusive.


2.1.4 Tendon Regulatory Factors

When compared to other musculoskeletal tissues, the mechanistic drivers of tendon regulation remain largely unknown. For example, the absence of myogenic factors, Myf5, MyoD, and Mrf4 (40) leads to the loss of skeletal muscle in mice, whereas the absence of Sox9 leads to complete absence of cartilage (41, 42). To date the absence of a singular factor has not been shown to prevent the complete disappearance of tendon formation. However, several factors have been observed to play crucial roles in the formation of proper tendon. Thus it is hypothesized that it is a concert of regulatory factors that give rise to tendon development (43, 44).

2.1.5 Embryonic Development

Figure 1.2. Development compartments of the embryo.

Through studying tendon embryonic development, researchers can piece together the biochemical signals conducive for tendon formation. The discovery of scleraxis as a tendon transcription factor has allowed researchers to more easily identify tendon progenitor cells and trace their embryonic origins. The embryonic origin of tendon progenitor cells varies among trunk, craniofacial and limb tendons.
Trunk tendons or axial tendons connect muscles located along the spinal column to the vertebrate, providing stability to the spine and allows for extension, rotation and flexion. It was observed that the inception of axial tendon progenitor cells develops at the edge of sclerotome compartment adjacent to the myotome compartment of the somite. These scleraxis expressing cells are now described as a fourth compartment of the somite, called the syndetome. The syndetome is believed to be regulated from neighboring compartments (37). Tendon development of axial tendons have been observed to be largely dependent on muscle development, based on the studies showing the ablation of the dermomyotome before the formation of the myotome (embryonic origin of muscles) led to a lack of scleraxis expression in chick embryos. Thus tendon progenitor cells may derive from the sclerotome compartment, but require signals from the myotome (38). These signals are believed to be related to fibroblast growth factors (FGFs), whose absence resulted in loss of scleraxis expression. Cartilage precursor cells of the sclerotome appear to regulate the specification of cells that form the syndetome. Expression of scleraxis required the downregulation of Pax1. Overexpression of Pax1 resulted in the inhibition of scleraxis expression in chicken embryos (37), whereas the inhibition of mutant mice lacking cartilage, showed a slight increase in scleraxis expression. Thus, the differentiation of tendon from the somite appears to be highly dependent on the combination of activating and suppressing signals from neighboring compartments.

Craniofacial tendons originate from the neural crest, whereas limb tendons originate from the lateral plate region. Limb and craniofacial tendons have shown initial developments that are independent of muscle, however fail to progress in later stages, suggesting muscle is required for tendon maturation. It is hypothesized muscles provide the required mechanical forces that allow for tendon maturation. Results from studies looking parallel at bone and tendon embryonic
development have been mixed, suggesting tendon dependency on bone development may vary according to location and proximity. Further research into limb tendon development has shown the removal of the dorsal limb ectoderm resulted in the loss of scleraxis expression (39) suggesting the ectoderm plays a critical role in tendon development. Specific ectodermal signals have not yet been identified.

Transforming growth factor β (TGF-β) superfamily proteins may also be involved in tendon development. In TGF-β null mice, most tendons and ligaments in the limbs, trunk, tail and head were gone. Moreover, it is suggested TGF-β is critical to forming tendon attachments to bone and muscles (40). The bone morphogenetic protein (BMP) family, which include growth and differentiation factor (GDF) proteins have also been recognized for their role in tendon development. In GDF null mice, abnormalities in the structure and biomechanical properties of tendons were present. Moreover, the fate of limb tendon progenitor cells has been shown to be regulated by both BMP and FGF signaling. The inhibition of BMP signaling resulted in tendon differentiation, whereas the inhibition of FGF signaling resulted in chondrogenesis in developing chick embryos (39).

2.1.6 Transcription Factors

Transcription factors are proteins that help regulate the transcription of DNA to RNA. As a result, transcription factors play a critical role in the regulation of RNA and ultimately protein expression. Three transcription factors have been identified to affect tendon development: scleraxis (Scx), Mohawk (Mkx), and early growth response 1 (Egr1).
2.1.6.1 Scleraxis

Scleraxis is known as a basic helix-loop-helix (bHLH) transcription factor due to its protein structure, which consists of two alpha helices connected by a short loop. It is currently the most distinctive and earliest marker for tendon progenitor cells (41). Scleraxis was first discovered within mesenchymal cell lineages that give rise to connective tissues and found to be specific to tendons and ligaments (39, 42). Schweitzer et al. found scleraxis to be induced by signals from the ectoderm and restricted with BMP signaling. Though scleraxis appears to affect both cellular differentiation and ECM regulation (41) of tendon, removal of scleraxis does not result in the disappearance of tendons. Scx$^{-/-}$ mutant mice were viable albeit with severe tendon defects in force-transmitting and intermuscular tendons, suggesting other regulatory factors (43).

2.1.6.2 Mohawk

The Mohawk (Mkx) gene is part of the homeobox gene family, which include a large family of genes known to direct regulation of anatomical patterns and morphogenesis during embryonic development. A study examining Mkx null mice found that mice had hypoplastic tendons with reduced fiber diameters. Mice expressed scleraxis and the number of tendon cells remained the same as wild type mice, however there was a significant reduction in tendon mass. This suggests Mohawk may be related to tendon maturation and plays a significant role in the regulation of type 1 collagen formation in tendon cells (44). Studies looking into overexpression of Mkx in mesenchymal stem cells showed an increase of tendon-related markers and increased collagen fiber diameter (45, 46).
2.1.6.3 Early Growth Response

The early growth response (EGR) transcription factors have been also been linked to tendon development to some extent. Mutant mice without EGR do not display abnormal tendon morphology, but have decreased expression of scleraxis and collagen I (47). Null mice with Achilles tendon injuries showed decreased expression of EGR-1, scleraxis, and collagen, one week after injury, whereas wild type mice showed dramatic increase in the same markers. This suggests EGR-1 may be involved in tendon healing (48).

2.1.7 Growth Factors

Other regulatory factors identified in tendon regulation include growth factors, such as aforementioned TGF-β, FGF, GDF, as well as number of other growth factors such as insulin-like growth factor (IGF) and platelet derived growth factor (PDGF). Growth factors can be defined as “signal molecules that are involved in the control of cell growth and differentiation” (49). Growth factors, which are naturally occurring cytokines, have been shown to be upregulated during development and healing and play vital roles in cellular proliferation, vascularization and protein synthesis.

Growth factors that have been associated with increased cellular proliferation include epidermal growth factor (EGF), PDGF, and IGF (50-53). It is believed that the increase in cell proliferation results in increased collagen production, leading to more robust tendon healing. Discovery of new members of the TGF-β superfamily from the work of Storm et al. revealed GDF-5, 6, 7 (BMP 14, 13, 12, respectively) (54). Though initially thought to be involved in bone and cartilage development, the work of Wolfman et al. showed the induction of ectopic connective
tissue with the GDF growth factors (55). Since then various studies have looked at the effects of GDF on tendon differentiation (56, 57).

The suppression of growth factors is an alternate strategy in tissue engineering. The presence of growth factors during the inflammatory stage of healing, indicate the possible link between growth factors and scar tissue development. For example, the presence of TGF-β1 has been associated with increased scar formation and adhesions. The suppression of TGF-β1 resulted in increased range of motion in rabbits (58).

Though growth factors have been shown to be potent inducers of tendon differentiation, challenges associated with short biological half-life, high required effective doses, high costs and difficulty obtaining FDA approval, limit their clinical use (59).

2.1.8 Challenges of Tendon Tissue Engineering

Due to the limited current understanding of tendon biology and development, tendon tissue engineering also remains at an infant stage when compared to other musculoskeletal systems. Unlike other musculoskeletal tissues such as bone or cartilage, much of the tendon differentiation process remains undefined. As a consequence, there in a lack of standardized differentiation media, as well as a lack of standardized methods to assess tendon differentiation. Ongoing research in normal tendon development, as well as tendon pathology, will continue to improve our understanding of tendon tissue and help to develop new tendon tissue engineering strategies.

Another remaining challenge for tendon tissue engineering is the development of appropriate animal models and translation to clinical practice. Current animal models involve an acute injury followed by immediate repair. The current models in no way reflect chronic degenerative tendon injuries or their conditions. Thus, better animal models that can translate results from small animal to larger animal models will generate more clinically relevant data (60).
Chapter 2: Specific Aims

1 Objectives/Rationale

Tendons, as part of the human musculoskeletal system, help actuate movement through coordinated efforts between muscles and bones. The elastic properties of tendons allow them to mediate stress concentrations and act as buffer zones between soft and hard tissue. Any disturbance to the system can cause debilitating pain and loss of function. Tendon injuries are common with roughly 200,000 surgeries, with roughly 4.1 million physician visits attributed to rotator cuff injuries alone (61).

With the increasing rise in musculoskeletal injuries, new treatments must address the problems of current strategies with use of allografts and autografts. Tendon injuries cause the afflicted time away from work, decreased productivity and increases the economic health burden. Tissue engineering has emerged as a feasible approach to develop modern treatments for tendon injuries. Though the use of growth factors in tissue engineering is heavily research, commercial use of growth factors has not widely matriculated due in part to the low half-life and high costs. The high costs and instability of growth factors have prompted research towards growth factor alternatives, such as small molecules and other proteins (62).

The 1980s saw the production of recombinant human insulin, which made it easier and cheaper to mass produce insulin (63). Since then, insulin has been structurally characterized and though its most popular use continues to be for the treatment of type I diabetes, interests into other uses of insulin have grown. The discovery of insulin homologues, later named insulin-like growth factor (IGF) I and II, which were observed to stimulate cell proliferation and growth, ignited research in areas comparing the two molecules and into non metabolic functions of insulin (64, 65). Today, IGF is used in tissue engineering as a growth factor supplement to enhance
proliferation and differentiation of cells (51, 52). An alternative approach is to use commercially available insulin protein. Due to the homology of IGF and insulin, we propose insulin as a possible biological factor for tendon tissue engineering.

We aim to study the potential use of insulin protein towards tendon tissue engineering and to develop an insulin and cell delivery device to help improve and accelerate tendon healing and regeneration. The use of insulin to elicit tenogenic differentiation presents a more applicable use in a clinical setting than other biochemical factors, as it is widely available and has FDA clearance for other medical applications. If successful, the engineered scaffold can be used as a platform for other soft tissue engineering applications.
2 Selected Preliminary Data

2.1 Bolus Studies

Cells were seeded on PCL electropsun fibers and treated for 24 hours in insulin containing media at various concentrations (0, 0.01, 0.1, or 1nM). After 24 hours of treatment, treatment media was removed and replaced with basal media for the remaining of the study. At later time points, groups treated with higher concentrations of insulin had lower rate of proliferation (Fig. 1A), which may indicate for cells to be in a differentiation state, rather than a proliferative state [insert reference]. When the same groups were investigated for gene expression of tendon markers, such as collagen I, collagen III, scleraxis, and tenomodulin, higher expression was found with cells treated with 1nM of insulin (Fig.1B). When immunostained for collagen I and actin filaments, cells grown with 1nM insulin showed higher collagen formation and more organized ECM (Fig.1C).

2.2 Continuous Treatment Studies in Low Serum

The effect of continuous treatment with insulin was also evaluated on cells grown on PCL electrospun nanofibers. To remove the effect of interference from the serum, low serum conditions were used. In the presence of low serum, cell have compromised proliferation, as seen in Fig.2A. Cell numbers drop and remain stagnant from day 3 to day 14. However, cells treated with insulin 20 or 250ng/ml of insulin had increased proliferation by day 14. Thus, in the presence of low serum conditions, insulin may have a healing effect on cell growth. Gene analysis comparing cells grown in low and normal serum conditions, showed higher expression of tendon ECM markers, tenascin C and decorin, with cells grown with low serum (Fig 2). However, lower expression of collagen in observed in cells treated in low serum. The lack of nutrients may be contributing to a less robust growth of collagen I. Higher level of serum may be needed for collagen growth.
Figure 2.1. Cells were treated with varying concentrations of insulin. A) DNA content was measured indicating lower proliferation at later time points with groups treated with higher concentration of insulin. B) Gene expression of tendon markers showed increased expression with cells treated with 1nM insulin. C) Cells were stained for col I, actin and nuclei stain. Cells treated with 1nM insulin showed greater ECM organization.
Figure 2.2. A) Proliferation of cells continuously treated with insulin in low serum conditions had higher rates than the control groups at later time points. B) Gene expression of cells treated with control base media or with 20ng/ml (3.4nM) insulin in 1% or 10% serum. Cells treated with 1% serum showed equal or higher expression of ECM markers, TNC and DCN when compared to cells treated with 10% FBS. Addition of insulin increased expression. However, low serum conditions showed compromised expression of collagen I.

2.3 Insulin effect on tenocytes

To gain insight on the possible effects of delivered insulin on existing tendon cell populations, rat tenocytes were continuously treated with IGF-1 or insulin in media. In the low serum conditions, cells treated with IGF-1 or insulin had higher proliferation at day 10 than cells grown with basal media in low and normal serum conditions. These cells showed very high
expression of collagen I and mature tendon marker in low serum conditions when compared to basal media in normal conditions. Moreover, cells with IGF-1 or insulin treatment showed significantly higher collagen I expression. This was corroborated with confocal images of collagen immunostaining (Fig.3).
Figure 2.3. Continuous Treatment of insulin and IGF on rat tenocytes. A) Proliferation of cells treated continuously with insulin or IGF in low serum conditions compared to cells treated with normal or low serum controls. B) Expression of tendon markers of cells grown in low serum conditions with basal, IGF or insulin. Fold change is normalized to basal media with normal conditions with 10% FBS. C) Collagen I and nuclei staining of cells on scaffold with basal (left), IGF (middle), or insulin (right) treatment.
2.4 Scaffold Conjugation and Bioactivity Studies

Insulin was then conjugated to CA/PCL fibers (Fig. 4A) using carbodiimide conjugation. To confirm conjugation, insulin-FITC was conjugated to the fibers and visualized under confocal microscopy. As shown, Fig 4B, fluorescent signal is found throughout the scaffold and is well distributed. At early time point, day 3, there were significantly more cells on the insulin conjugated fiber than the control group (Fig. 4C). At day 7, higher expressions of scleraxis and collagen I are observed on cells grown on the insulin conjugated fibers (Fig 4D). This initial studies show that the insulin is remains bioactive after conjugation. However, longer time points will need to be evaluated to determine how long this effect last.

Figure 2.4. Bioactivity of insulin scaffolds tested. A) SEM image of scaffold made from CA/PCL blend (3:1). B) Conjugation of FITC-insulin on nanofibers. C) Proliferation of cells on control and insulin conjugated fibers. D) Gene expression on control and insulin conjugated scaffolds.
3 Specific Aims and Hypotheses

3.1 Specific Aim I: To evaluate and characterize the efficacy of insulin as a bioactive factor in promoting proliferation and tenogenic differentiation.

Rationale: Previous studies have shown the efficacy of IGF-1 in promoting cellular proliferation and tendon maturation. Insulin, a homologue of IGF-1, has shown capable of binding to IGF-1 receptors, and therefore may be a viable alternative as a tendon differentiation factor. A single bolus of insulin was shown to increase tendon markers in bone marrow mesenchymal stem cells(66).

Hypothesis: It is hypothesized the addition of insulin in culture media as a single or continuous treatment will provide appropriate chemical signals to elicit a tenogenic response. Cells are hypothesized to react similarly to cells treated with IGF-1, which have been shown in literature to increase cell proliferation and collagen production.

3.2 Specific Aim II: To develop and characterize a novel insulin delivery matrix system for tendon regeneration.

Rationale: Electrospun fibers provide the ideal topographic and elastic properties as tendon grafts and have been widely used as cell and protein delivery systems.

Hypothesis: It is hypothesized the electrospun fibers can be used successfully to deliver insulin with tunable properties to modulate insulin release. It is also hypothesized that the incorporation
of insulin in a polymeric fibrous matrix will promote and induce differentiation of mesenchymal stem cells towards a tendon lineage.

3.3 Specific Aim III: To conduct bioactive feasibility assessments of an insulin delivery matrix system in combination with bone marrow derived MSCs, *in-vivo*.

**Rationale:** The *in-vivo* model will provide information on the feasibility of insulin in a tendon injury model. The effectiveness of a one-time dose and continuous treatment will be assessed based on overall morphology and healing response. Moreover, donor cells will be tracked to determine if cells are playing a direct role in ECM development.

**Hypothesis:** It is hypothesized that the implantation of a bioactive matrix system to augment a tendon defect will aid in healing and regeneration of the injured tendon. It is also hypothesized that the addition of donor MSCs will further enhance the tendon regeneration process.
Chapter 3: Application of Insulin and Insulin-Like Growth Factor-1 in Musculoskeletal Tissue Engineering

1 Introduction

Since its formal conception in 1988 (67), the field of tissue engineering (TE) continues to make advancements towards finding solutions to existing medical dilemmas. The goals of tissue engineering aim to enhance the healing and regeneration of lost or damaged tissues and organs through the development of biomaterials and incorporation of biological factors such as cells and bioactive factors. Amongst these strategies, growth factors have been widely popular owing to the discovery of their importance in embryonic development and tissue healing. Various growth factors have been utilized to elicit cellular behaviors such as proliferation, migration, and differentiation. Insulin-like growth factor-1 (IGF-1) has been extensively researched for its ability to encourage cell proliferation, inhibition of cell apoptosis, and anabolic effects on musculoskeletal tissues (51, 52, 68-70). Insulin-like growth factor-1 is named as such due its homology to insulin protein. The homology between insulin and IGF-1 and their receptors in terms of structural composition has motivated various comparative studies (64, 69, 71), which have shown similar effects of insulin on tissue growth (72, 73). This review focuses on the function of insulin and IGF-1 and highlights the current knowledge of their effects on musculoskeletal tissue growth and subsequent use in tissue engineering applications.

1.2 Insulin Superfamily

The insulin superfamily is comprised of insulin, insulin-like growth factors, and the relaxin-like family, as listed in Table 1. Insulin like growth factors can further be divided into IGF-I and IGF-II. The relaxin-like family are also subdivided into H1, H2, H3 relaxin and insulin-like peptide 3, 4, 5, and 6 (74). Members of the insulin superfamily are considered an ancient class of
proteins and are thought to be highly conserved in unicellular eukaryotes, as well as invertebrates such as insects, mollusks, and chordates and vertebrates (75). Gene studies show the insulin gene to be highly conserved in all vertebrate species. It is believed insulin and IGF may have been derived from a common ancestral insulin type gene that split early on in vertebrate species (76).

All members of the insulin superfamily are initially expressed as pre-prohormones, with four distinct regions in a continuous chain consisting of a N-terminal signal peptide, B-chain, C-chain, and C-terminal A-chain. The removal of the signal sequence peptide on the N-terminal, as well as the formation of two disulfide bonds between the A and B chains, and a single bond within the A chain, signifies the transition from a pre-prohormone to prohormone. In the active hormones, with the exception of IGF, the C chain is cleaved off, leaving behind two peptide chains. For insulin like growth factors, the active hormone remains as a single peptide chain, as the C-chain is preserved. Insulin and the insulin-like growth factors bind and activate tyrosine kinase receptors (RTK), whereas the relaxin-like family of proteins bind and activate G-protein coupled receptors (74, 77). The difference in receptor types may indicate the divergent functions between the relaxin-like proteins and IGF and insulin. Insulin and IGFs play key roles in cell cycle, survival or apoptosis, migration, proliferation, differentiation, metabolism, reproduction, and longevity (78), whereas the relaxin-like family appear to have a more prominent role in reproduction (79).
Table 1. The Insulin Superfamily

<table>
<thead>
<tr>
<th>Insulin Superfamily</th>
<th>Subdivision</th>
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<tbody>
<tr>
<td>Insulin</td>
<td>▪ Insulin</td>
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<tr>
<td>Insulin-like growth factors</td>
<td>▪ IGF-1</td>
</tr>
<tr>
<td></td>
<td>▪ IGF-II</td>
</tr>
<tr>
<td>Relaxin-like family</td>
<td>▪ Gene 1(H1) relaxin</td>
</tr>
<tr>
<td></td>
<td>▪ Gene 2 (H2) relaxin</td>
</tr>
<tr>
<td></td>
<td>▪ Gene 3 (H3) relaxin</td>
</tr>
<tr>
<td></td>
<td>▪ Insulin-like peptide 3 (Leydig insulin-like peptide; relaxin-like factor)</td>
</tr>
<tr>
<td></td>
<td>▪ Insulin-like peptide 4 (placentin; early placenta insulin-like peptide)</td>
</tr>
<tr>
<td></td>
<td>▪ Insulin-like peptide 5</td>
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<td>▪ Insulin-like peptide 6</td>
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Figure 3.1. A and B chains of insulin and IGF-1. Boxes indicate identical residues

2 Discovery and Clinical use

2.1 Discovery of Insulin

The discovery of insulin in 1922, though somewhat controversial, can be credited to Canadian scientists, Banting, Best, Collip, and Macleod. Their research performed at the University of Toronto brought about revolutionary treatment for those suffering from diabetes mellitus, a condition caused by the body’s inability to regulate glucose levels. At the time, there
was an understanding of an antidiabetic substance produced in the pancreas and though several attempts were made by others to create extracts from the pancreas, most resulted in harmful side effects, negating any clinical benefits. The University of Toronto team was the first to produce a purified extract of what they later termed, insulin, which forever changed the treatment and prognosis of diabetic patients. Banting and Macleod were awarded the Nobel Prize in Physiology or Medicine in 1923 for their contributions. They later split the award money with Best and Collip. Details of the history and drama surrounding the discovery of insulin is given by Rosenfeld in his review, *Insulin: discovery and controversy* (80).

### 2.2 Metabolic Insulin Regulation

Insulin is an anabolic hormone produced in the pancreas and is the main regulator of intermediary metabolism through its acute effect in lowering blood glucose levels. Clusters of cells, known as islets of Langerhans, contain insulin secreting cells known as \( \beta \)-cells that make up approximately 50% of all cells in the islets. Preproinsulin is synthesized in the \( \beta \)-cells of the pancreas. Shortly after, preproinsulin is released into the endoplasmic reticulum where it undergoes proteolytic enzymatic cleavage into proinsulin. The proinsulin undergoes further cleavage to excise the C peptide, producing the mature form of insulin and the free C peptide is stored in vesicles in the Golgi apparatus. Human insulin contains 51 amino acids and has a molecular mass of 5.808kDa (81, 82). The peptide chains, A and B contain 21 and 30 amino acids, respectively.

The synthesis and secretion of insulin is highly dependent on glucose concentrations. When the glucose concentration is zero or less than 1nM, the membrane potentials of beta cells is approximately -70mV. At elevated glucose levels (6mM) the membrane undergoes depolarization and receives an influx of extracellular calcium. It is believed the influx of intracellular calcium
(occurs at potentials above -20mV) triggers the exocytosis of insulin granules, resulting in insulin release into the bloodstream (83, 84). The released insulin proteins bind to the extracellular domains of insulin receptors on skeletal muscle, as well as adipose and liver cells, triggering a signal transduction cascade that results in the uptake of glucose through glucose transporter GLUT-4 in fat and muscle cells.

**2.3 Insulin in the treatment of Diabetes**

In the early days of insulin production, insulin came from various animal sources such as canine, porcine, or bovine (85). Since 1977, with the advancement of biotechnology, the mass production of insulin has possible though the use of recombinant technology that allows the synthesis of human insulin through bacteria or yeast (82). Additionally, it is also now possible to create insulin analogs that have altered physicochemical, biological, and pharmacodynamic properties to help better regulate the glucose levels (86). Today, the human insulin market is predicted to be worth approximately 43 billion US dollars by the year 2021, according to Zion Market Research (https://www.zionmarketresearch.com/news/global-human-insulin-market). The demand for insulin and insulin products has been increasing due in part to the increasing number of diabetic patients. In 2015, the CDC reported that 9.4% of Americans have diabetes or prediabetes, which amounts to 30.3 million cases in the United States alone. The WHO reported an 8.5% global increase in the prevalence of diabetes among adults (18 year of age or older) in 2014, which amounts to 422 million affected people. Diabetes is the major cause of heart disease and stroke and is the seventh leading cause of death in the United States (87).
2.4 Discovery of IGF

The insulin-like growth factors were first identified in the latter half of the 20th century, decades after the discovery and purification of insulin. Throughout its discovery, IGF-1 has been called many things, which is reflective of its various biological activities (88). In 1957, Salmon and Daughaday observed that there was an undiscovered factor present in normal serum that stimulated the growth of costal cartilage, measured through sulfate uptake. They termed it “sulfation factor” (89). A few years later, in 1963 Froesch et al. described non-suppressible insulin-like activity (NSILA) components that were unaffected by anti-insulin antibodies (90, 91). By 1972, due to the molecule’s interaction with growth hormone (GH), the molecule was lumped with somatomedin, a group of hormones that inhibit and regulate GH (92). It was not until 1976, when the molecule was isolated and identified with structural homology to proinsulin, did the name insulin-like growth factor became the standard terminology (93, 94).

2.5 Role of IGF-1 in Development and Growth

The insulin-like growth factors are essential to embryonic development and growth. IGF-1 is mainly synthesized and secreted by the liver and can be transported via the endocrine system. Local synthesis of IGF-1 has also been observed and also believed to take part in paracrine and autocrine signaling.

Majority of circulatory IGF-1 is bound to IGF binding proteins. The IGF-binding proteins (IGF-BP) extend the half-life of IGF-1 and modulate its activity, as only free, unbound IGF-1 can activate its receptor. There are six IGF-binding proteins that have been identified (94, 95), with 75-90% of circulatory IGF-1 coupled to IGF-PB3, which forms a large ternary complex. Included
in the IGF-1, IGF-BP3 structure is acid labile subunit (ALS) which serves to provide structural stability to the unit (96).

Insulin-like growth factor-1 exists in its active form as single polypeptide chains with 70 amino acids with a molecular weight of 7.649kDa (94). The peptide chain contains 4 domains B, C, A, and D. Domains B and A are structurally analogous to the B and A chains of insulin, with 50% of matching amino acid sequences, shown in Figure 1. The C-peptide consists of 12 amino acids and does not share any homology to the C-peptide of proinsulin, which contains 35 amino acids (88). The D-domain is believed to interact with the IGF-binding proteins. Additionally, the pre-prohormone structure of IGF-1 contains a fifth domain known as the E-domain, whose function remains ambiguous (95).

Production of IGF-1 in the liver is modulated by growth hormone (GH). Thus GH or GH receptor deficiencies often result in compromised IGF production. For example, Laron Syndrome (LS) is a congenital condition that is characterized by the inability to produce IGF due to a genetic mutation in the GH receptor gene. Newborns with LS are slightly shorter and exhibit retarded skeletal maturation and organ growth throughout childhood (94, 97, 98). Further evidence for the importance of IGF-1 to normal growth and development is shown through studies with knockout mice with deleted IGF-1 or GF receptor gene, which display a 40-45% size reduction compared to healthy controls (99). The development of recombinant human IGF-1 has allowed its use for IGF-1 deficient disorders. In 2005, the US Food and Drug Administration approved the use of recombinant human IGF-1 for IGF-1 deficiency due to genetic GH resistance, defects in GH signaling pathway, GH receptor defects, or IGF-1 gene defects.

IGF-1 has also been used as a therapy for insulin resistance and for insulin dependent and non-insulin dependent diabetes mellitus with some level of success (100). However, insulin-like
growth factor-1, like other growth factors, have significant drawbacks that limit its use. Systemic injection of IGF-1 was used as a possible therapeutic for insulin resistant patients. Patients received IGF-1 injections of 120 and 160ug/kg twice a day. However adverse effects such as edema of the face and hands, mild weight gain, fatigue, tachycardia and local burning at the injection site, along with other negative effects caused the study to prematurely end (101).

3 Homologous receptors and signal transduction

The insulin receptor (IR) and IGF-1 receptor (IGF-1R) are part of a family of ligand-activated receptor tyrosine kinases (RTK), a class of transmembrane receptors with intrinsic enzymatic activity that regulate cell growth, differentiation and survival. The human genes for IGF-1R and IR also show similarities in the size and number of individual exons (102, 103). Insulin and IGF-1 have much higher affinities for their respected receptors, however in certain circumstances, such in the case of supraphysiological concentrations, have been observed to bind to each other’s receptors. Hybrids of IR and IGF-1R have also been documented. Two isoforms of the IR have been observed, IR isoform A (IR-A) and IR isoform B (IR-B). Subsequent studies showed subtle differences between IR-A and IR-B, specifically with the absence of exon11 in IR-A, which results in the absence of 12 amino acid residues in the extracellular subunit of IR-A and renders a greater affinity of IGF-II to IR-A (104, 105).

Unlike other RTKs, both the insulin receptor (IR) and IGF-1 receptor (IGF-1R) exists in dimeric form in the absence of ligand binding. The IR and IGF-1R contain two extracellular $\alpha$ subunits and two transmembrane $\beta$ subunits, with each $\alpha$ subunit linked to one $\beta$ subunit via disulfide bonds. The $\alpha$ subunits have a cysteine-rich domain similarly found with members of the epidermal growth factor (EGF) (106, 107). Here, IR and IGF-1R have the least homology (48%)
giving rise to ligand specificity. The tyrosine kinase domain within the intracellular β subunits of IR and IGF-1R share 84% homology (102, 108).

Due to the similarity of insulin and IGF-1 and their respective receptors, many have investigated their distinct and overlapping functions. Early studies suggested metabolic functions mediated through the insulin receptor, whereas growth promoting actions were stimulated via IGF receptors (64, 109). However, later studies found cross reactivity of both peptides to the other receptors (65) and binding of insulin to IGF-1 receptors to propagate signaling pathways for proliferation and collagen formation (71). Moreover, mitotic or growth promoting actions of insulin has been observed with certain cells including hepatocytes, endothelial cells (65), muscle cells (110), lung fibroblast cells (71), and umbilical cord derived MSCs (70). Likewise, metabolic functions of IGF-1 have been observed (111).

Insulin receptor substrate (IRS) and Shc are two major substrates of IR and IGF-1R (102, 112). For insulin, the P13K/Akt pathway has been mainly seen as responsible for acute metabolic actions. The Ras/ERK pathway is associated with insulin effects on proliferation and differentiation through gene transcription regulation (113). Differential roles of IRS and Shc proteins have also been found with IGF-1 signaling. Kim et al. observed in neuroblastoma cells that activation of Shc resulted in MAP/ERK pathway that resulted in neurite outgrowth, whereas activation of P13K via IRS was associated with membrane ruffling and growth cone extension (114).

Adding to the complexity of IR and IGF-1R signaling pathways is the presence of hybrid receptors or heterodimers of IR and IGF-1R. IGF was found to have higher ligand affinity to hybrid receptors, with no difference between the IR-A or IR-B (115). Other factors that can affect signal transduction include number of receptors, receptor distribution, and ligand binding kinetics. For
instance, it was observed that insulin analogues had greater mitotic effects than insulin, which was correlated to a slower dissociation rate of the ligand (116). More recently, Cai et al. have demonstrated specificity of insulin and IGF-1 signaling to be due to differences in the intracellular domains of IR and IGF-1R and to some extent, their extracellular domains as well. Using cells that express only IR, IGF-1R, or receptors with mutated intracellular or extracellular domains, they observed the intracellular domains of IGF-1R coupled more strongly with Shc and genes involved with proliferation, whereas IR more strongly coupled with IRS and genes related to metabolic pathways. The difference in action was attributed to differences in the juxtamembrane in the intracellular domains, specifically at position 973 of IR and 951 in IGF-1R, which greatly affected substrate phosphorylation (117).

4 Applications in Musculoskeletal Tissue Engineering

Insulin-like growth factor-1 has been widely researched for its growth promoting effects. Mitotic effects of IGF-1 have been repeatedly reported in various cell types, as well as its anabolic effect on tissue extracellular matrix. Comparable studies with insulin resulted in similar results, suggesting the potential use of insulin as a IGF-1 growth factor analog. The following sections highlight the current understanding on the effects of IGF-1 and insulin on various musculoskeletal tissues including bone, cartilage, muscle, and tendon.

2.1 Bone

It has been widely accepted that hepatic IGF-1 plays a critical role in skeletal development. For example, IGF-1 null mice have significant growth retardation and often do not survive (118). Moreover, congenic mice with low serum IGF-1 exhibit reduced trabecular bone density and reduced cortical thickness (119). However, findings by Yakar et al. question the role of hepatic IGF-1 in postnatal growth and development. Mice with IGF-1 gene deletion only within the liver
showed 75 percent reduction in circulating IGF-1, but showed similar body weight, body length, and femoral length to normal mice. This implies that growth was mediated by non-hepatic IGF-1 (120). A later study with mice with 85-90% reduction in circulatory IGF-1 showed reduction in linear growth, decrease in mineral density, and decrease in periosteal circumference and cortical thickness (121). Thus, it appears that a critical threshold of circulatory IGF-1 is needed to maintain normal bone growth.

As previously mentioned, circulatory IGF-1 is produced by the liver, however the expression of IGF-1 and its receptor in majority of cells indicate the presence of autocrine/paracrine signaling of IGF-1. To gain an understanding on the direct effects of IGF-1 on bone, Zhang et al. studied knockout mice with mutant osteoblasts, missing the IGF-1 receptor gene. Compared to normal controls, the mutant mice displayed a decrease in trabecular bone volume and connectivity, with significantly lower rates of bone mineralization (122). IGF-1 concentrations in bone is found to decrease with age, and is also significantly reduced in patients with osteoporosis (123). IGF-1 treatment on bone defects of aged rats showed improved mineralization and biomechanical properties (124), suggesting the importance of IGF-1 to bone health maintenance.

In recent years, the importance of insulin on bone has also been highlighted. A growing body of evidence shows a relationship between bone remodeling and metabolic homeostasis. Bone health of diabetic patients provides some insight. Type I diabetes mellitus patients, characterized by the inability to produce insulin, have an increased risk of fractures with low bone mineral density (125). Reduced bone formation and bone turnover were found to be associated with type I diabetes mellitus (126). On the other hand, type II diabetes mellitus patients who have insulin resistance, exhibit normal bone mineral density (127, 128), but still present similar risk of fractures
as Type I diabetes mellitus patients (129). It was hypothesized that insulin resistance could cause aberration in osteoblastic insulin signaling. This was found to be the case in insulin resistant rats who displayed decreased osteoblast proliferation (125, 130).

Closer examination of the effect of insulin on bone have been studied through cell studies involving osteoblast cells. To elucidate the effect of insulin signaling on bone and to eliminate possible confounding effects from IGF-R signaling, Fulzele et al. engineered mutant mice with osteoblast cells lacking insulin receptors. Much like the mutant mice with osteoblast specific IGF-1 receptor knockout, the insulin receptor mutant mice displayed decreased trabecular bone due to failure of osteoblast maturation and decreased bone formation. Additionally, with time the mice developed insulin resistance, along with peripheral adiposity. Further examination revealed decreased circulating undercarboxylated osteocalcin. Osteocalcin is a factor produced by osteoblasts. Prior to secretion, the osteocalcin undergoes carboxylation that endows the molecules with a higher affinity for bone matrix. A small percent of the osteocalcin remains undercarboxylated and is released into circulation. Treatment with exogenous osteocalcin was shown to improve the metabolic conditions of the mutant mice suggesting that insulin signaling in osteoblast stimulate osteocalcin production, which in turn can regulate pancreatic insulin secretion to control glucose homeostasis (131). Work from Ferron et al. showed that insulin signaling in osteoblast is necessary for whole glucose homeostasis through osteocalcin activity (132). Interestingly, the disruption of IGF-1 receptor in osteoblast cells enhanced insulin signaling and action. It is believed that the normal presence of IGF-1 receptors dampens insulin receptor signaling through the formation of heterodimer or hybrid receptors (133).

Anabolic effects of insulin on bone were demonstrated early on with studies involving bone organ culture systems. Studies conducted on fetal rat parietal bones showed increased collagen
synthesis (134, 135), as well as increased mineralization and bone matrix formation (136). Direct effect of insulin on bone healing have been conducted on rodent and rabbit models. Insulin injected locally into the intramedullary cavities of femur fractures in nondiabetic rats, immediately following injury, resulted in enhanced healing (137).

Several scaffolds have been used in combination with IGF-1 or insulin. Meinel et al. encapsulated IGF-1 in poly(lactic-co-glycolic) acid (PLGA) microspheres (138). The microspheres were later added to alginate hydrogels in combination with tricalciumphosphate for use in an in-situ delivery device. The release of IGF-1 remained stable for up to 7 days. Osteoblast-like cells, MG63 cultured on the IGF-1 loaded hydrogel scaffold showed a 7-fold increase in proliferation. Bone marrow stromal cells grown on IGF-1 incorporated mineralized PLGA scaffolds also demonstrated enhanced attachment and proliferation (139). Such studies demonstrate the mitotic effect of IGF-1 (140). Srouji and colleagues have researched the use of crosslinked gelatin hydrogel to deliver IGF-1 and transforming growth factor-β (TGF-β), alone and in combination. When used in a rat tibia injury model, growth factor hydrogels showed significantly more bone formation when compared to hydrogel controls (141). Similar findings were found when the same hydrogel system was used to heal a rat mandibular bone defect, supporting the osteoinductive activity of IGF-1, in combination with TGF-β (142).

The incorporation of insulin into delivery systems, minimize possible systemic effects of free insulin and help to achieve sustained release for bone healing. Gandhi and colleagues examined the effects of local insulin delivery on fracture healing. By utilizing diabetic mice, they show the presence of an intramedullary insulin delivery device did not affect the systemic glucose levels, but did however result in the complete restoration of the cortical bone at 8 weeks. Complete bridging of the facture was observed in non-diabetic rats (143). In other another system, insulin
was loaded into PLGA nanospheres. The insulin loaded nanospheres were incorporated into a nanohydroxyapatite/collagen scaffold and implanted into a critical sized defect in a rabbit mandible model. Significantly higher bone formation was observed on insulin loaded scaffolds when compared to controls (144).

2.1.1 Cartilage

Tissue engineering of cartilage is of great interest to researchers due to cartilage’s lack of intrinsic ability to heal and regenerate on its own. Degenerative diseases such as osteoarthritis (OA), as well as acute trauma can lead to the erosion of joint surfaces and loss of mobility, along with debilitating pain. Insulin-like growth factor-1 has been identified as a key factor in the maintenance of articular cartilage. Decline in circulatory IGF-1 has been associated with the development of OA (145). Moreover, decreasing response to IGF-1 has also been observed in aged and arthritic cartilage (146, 147), thus further exasperating the problem. Insulin-like growth factor is known to promote the production of proteoglycans (148, 149), which allows cartilage ECM to withstand high compressive forces. A decline of IGF-1, as well as a lowered response to IGF-1, results in lower proteoglycan synthesis and subsequently cartilage degeneration (149). Lower responsiveness to IGF-1 may be due to increase production of IGF binding proteins, which act to inhibit IGF-1 receptor binding (145).

Over the years, a number of studies have looked at the effects of insulin on ATDC5 cells, a cartilage cell line, derived and isolated from mouse teratocarcinoma AT805 (150-153), often used as model for in-vitro chondrocyte research. Initial work carried out by Atsumi and colleagues showed that ATDC5 cells treated with insulin responded with increased glycosaminoglycans (GAG) formation, which make up proteoglycan structures. Collagen II expression, a characteristic differentiation marker of cartilage, was also increased. Insulin concentrations as low as 100ng/ml
could elicit GAG formation, but was only significantly increased with a minimum dose of 10µg/ml (150). Since that initial study, several studies on the effect of insulin on ATDC5 cells have been undertaken. Hidaka et al. showed stimulation with insulin caused activation of the P13k pathway and resulted in the increase of collagen II and collagen X gene expression. They argue that the high concentration required to elicit a response (10µg/ml) indicates insulin effects may be mediated through IGF-1R (152). However, work from Phornphutkul et al. showed that concentrations as low as 300ng/ml was adequate to stimulate collagen X expression in ATDC5 cells. Furthermore, this concentration caused the phosphorylation of IR, but not IGF-1R, indicating insulin effects may in fact be mediated by its own receptor (153).

Kellner et al. were first to assess the in-vitro treatment of primary bovine chondrocytes with insulin supplemented media. Treatment with insulin increased growth rate, GAG content and lead to a more mature cartilage phenotype compared to the control group (154). Other studies confirm insulin to be essential in the in-vitro differentiation of human mesenchymal stem cells with a dose dependent effect capping off at 10µg/ml (73). In fact, insulin is a componenet in standardized chondrogenic differentiation media.

Several insulin delivery systems for chondrogenic regeneration have been investigated. Chitosan spheres that were used to encapsulate insulin at 5wt% were able to release approximately 100ug/ml of insulin throughout 28 days. The chitosan spheres promoted rounded morphology and increased GAG on cultured ATDC5 cells (155). Similar findings were found with subcutaneously implanted collagen hydrogels with insulin (156), as well as insulin loaded PLGA microspheres (157).
4.4 Muscle

The insulin-like growth factors have been observed to play keys roles in adult skeletal muscle regeneration and hypertrophy (158). Such effects are thought to be mediated through IGF effects on resident satellite cells, muscle-specific adult stem cells that reside between the basal lamina and sarcolemma of skeletal muscle fibers (159, 160). During increased loading or muscle injury, satellite cells, which are typically mitotically quiescent, become activated and become involved in muscle regeneration. The initial response of satellite cells is to first proliferate, then differentiate into myoblasts-like cell, with eventual fusion with existing myofibers or creation of new ones (160). IGF-1 has been shown to be involved in both the proliferation and differentiation of muscle tissue (158, 161-163).

Proliferative effect of IGF on satellite cells have been demonstrated from a number of researchers including Chakravarthy et al., who investigated the proliferative capacity of satellite cells after sessions of muscle immobilization. It was found muscle atrophy resulting from a single period of immobilization (10 days), dramatically decreased the proliferative potential of the residing satellite cells, which could not be recovered even after 9 weeks. However, cells isolated from animals receiving infusion of IGF-1 (1.9µg/day) for 14 days, had significantly higher proliferative capacity (164). IGF-1 has also been shown to stimulate muscle growth. Direct local infusion of IGF-1 into tibialis anterior muscle of adult rats for 14 days caused a 9% increase in muscle mass, as well as increase in muscle protein, when compared to control animals receiving saline infusions. Increase in muscle DNA content also occurred suggesting increase in resident cell numbers (165). Mechanisms in IGF induced muscle growth may be related to the suppression of protein breakdown, or proteolysis (166).
Early studies evaluating insulin effects on myoblasts indicate insulin to induce similar effects as IGF-1 (167, 168). Though there remains some controversy whether insulin is acting as an IGF-1 analog via IGF-1 receptors (169), there are reports that support insulin effects through its own cognate receptor (170). Interestingly, a study on muscle specific knockout mice showed mice with single receptor deletion, either insulin or IGF-1 receptor, displayed normal glucose homeostasis and muscle mass. However, the combined loss of both insulin and IGF-1 receptors, resulted in dramatic decrease in muscle mass and fiber size (171). These results suggest muscle growth to be mediated by both insulin and IGF-1 receptors and also indicate the insulin effect on muscle growth is separate from metabolic processes. It is worth noting that in clinical studies examining the effects of systemic insulin on muscle growth, an increase in muscle protein synthesis is not observed unless there is an increase in muscle blood flow and amino acid delivery and availability (172). In fact, it has been documented that insulin infusion into circulation lowers amino acid levels in the blood (173). Increased muscle protein synthesis is often only seen at supraphysiological insulin levels and when given locally.

Currently, tissue engineering studies looking into the use of insulin towards muscle regeneration have been limited. Though insulin effects on myoblasts were earlier demonstrated to induce both proliferation and myogenesis, it has not been widely applied in tissue engineering applications. A few recent works aim to elucidate the signaling pathways involved in insulin-induced myogenesis. Conejo et al. found myogenesis induction with insulin on C2C12 cells, a mouse skeletal muscle line, to involve the P13k/p70S6-kinase and p38-MAPK pathways, with inhibition of the p44/p42-MAPK (168). Activation of the P13k pathway by both insulin and IGF-1 are thought stimulate growth through suppression of proteolytic activity (174). Other insulin related cell studies in muscle have in current years mainly focused on the effects of glucose on muscle growth. In high
glucose environments, myogenesis of C2C12 cells was shown to be inhibited, with an increase in IGF binding proteins. Use of high insulin concentration resulted in the promotion of myogenesis (175). Thus, future work should continue to explore the potential of insulin in muscle regeneration. On the other hand, research into IGF-1 in tissue engineering applications is ongoing. For example, effect of IGF-1 on smooth muscle tissue has been explored in recent years for applications in bladder regenerative therapies. A mutant variant of IGF-1 was created to covalently attach to fibrin. The mutant variant IGF-1 was found to have equal bioactivity as wild type IGF-1 and has been successfully incorporated into functionalized scaffolds with therapeutic benefits towards bladder regeneration (176, 177).

4.5 Tendon

Similar to cartilage ECM, tendon tissue is limited in its ability to regenerate due to a low number of resident cells, as well as low vascularity. Insulin like growth factor-1 has been identified to be involved in all phases of tendon healing that include inflammatory, proliferative, and remodeling stages (178). During the inflammatory stage, RNA and protein levels of IGF-1 are upregulated indicating a role of IGF-1 during the early stages of healing (179, 180). Mitotic effects of insulin in tendon cells and tendon animal models have also been well documented (181, 182). In a full thickness supraspinatus defect in white rabbits, an increase of IGF-1 was observed on the fifth day post-surgery, during the proliferative stage. The growth factor was found present in blood cells and vascular endothelial cells near the proximal tendon (183). Insulin-like growth factor-1 has also been observed to increase collagen synthesis (51, 52, 184). Injection of IGF-1 was shown to increase collagen content near local areas within the injection site (52).

Despite the numerous studies conducted on IGF-1 for tendon applications, there has been minimal exploration of insulin towards tendon regeneration. Mazzocca et al. was the first to
investigate the effect of insulin treatment on bone marrow derived mesenchymal stem cells. It was shown that following a single bolus dose of insulin, cultured bone marrow derived mesenchymal stem cells showed increased phenotypic markers of tendon including increase collagen I, scleraxis, tendomodulin, and proteoglycans (66). In ongoing studies, insulin was conjugated to the surface of polycaprolactone/cellulose acetate blend fibers. Human mesenchymal stem cells grown on the insulin functionalized scaffolds also had increased expression of tendon mRNA. In these studies, no significant effect of insulin on proliferation was observed when compared to the control group. However, a recent study on insulin on umbilical cord derived mesenchymal stem cells found significant proliferation after treatment with insulin, but only at minimal concentrations of 1µg/ml.

Tendon tissue engineering presents its own unique challenges compared to other musculoskeletal tissues. Currently there does not exist a standardized differentiation media for tendon. Additionally, many markers of tendon phenotype development are common to other musculoskeletal tissues. For example, collagen I, the main constituent of tendon ECM is ubiquitous in bone, while GAG markers such as decorin and aggrecan, are also found in cartilage. The lack of unique markers and standardized assay poses challenges for tendon tissue engineering, however ongoing research from the fields of developmental biology and advances in biomedical science fields will provide better insights into tendon healing and regeneration.

3 Closing remarks

At its discovery insulin revolutionized the lives of diabetic patients and has played an integral part in advancing techniques used in protein characterization. Likewise, the discovery of a new insulin-like protein, IGF-1 has contributed greatly to the understanding of growth and development. Today, IGF-1, with its mitotic and growth promoting effects, is widely researched
in musculoskeletal tissue engineering across all tissues in the field. The influence of growth factors on tissue development and healing make them attractive biomolecules to stimulate and differentiate cells towards desired lineages. However, the limitations associated with growth factors have prompted researchers to look for growth factor alternatives, such as small molecules, peptides and other proteins. The analogous structures of insulin and IGF-1, as well the observed overlapping effects of insulin, makes insulin an attractive candidate as a substitute for IGF-1.

4 Specific Aim I: To evaluate and characterize the efficacy of insulin as a bioactive factor in promoting proliferation and tenogenic differentiation.

4.1 Materials and Methods

4.1.1 Cell Studies

Human mesenchymal stem cells, no more than passage 5, were used to for cell studies. Prior to the start of the study, cells were serum starved overnight to sync cells to the same cell cycle phase G0. Initially cells were seeded on tissue culture plates to determine the optimal concentration of insulin for single or continuous treatment (n=4). Once an optimal concentration was determined, cells were seeded on fibrous matrices and treated with the optimal concentration of insulin, as a single or continuous treatment.

4.1.2 Scaffold Fabrication

Electrospun fibers were fabricated using standard electrospinning techniques and parameters. Polycaprolactone (Mn~ 80kDa) was used to create electrospun fibers with diameters in the range of 1-3µm. A 12% weight/volume of PCL was solubilized in 85:15 ratio of methylene
chloride and ethanol. A volume of 8ml of PCL solution was placed into a syringe and electrosupn on 10x10cm of foil using the following parameters: flow rate: 2ml/hr, working distance: 1cm/kV, voltage: 22kV. The scaffold matrices were dried under vacuum and subsequently cut into 1x1cm squares. Prior to cell studies, scaffolds were sterilized via incubation in 70% EtOH and UV treatment for 30min on each side.

4.1.3 Proliferation

Cell proliferation was measured using PicoGreen assay or MTS colometric assay. Scaffolds were collected at early time points 6, 12, and 24 hours after seeding and later time points at day 3, 7, 14, and 21. At collection, samples (n=4) were washed with PBS and collected into 2ml tubes containing 500ul of 1% Triton-x buffer. Samples were stored at -20degC until all samples were collected. Samples then underwent three freeze-thaw cycles and vortexed to lyse cells and remove DNA content.

Quant-iT™ PicoGreen® dsDNA Assay kit was purchased from Thermo Fisher Scientific. The kit contains a reagent that is an ultrasensitive fluorescent nucleic acid stain, which allows quantification of double-stranded DNA (dsDNA) in solution. Fluorescence was measured using a plate reader with excitation and emission set to 480nm and 520nm, respectively, and with sensitivity set at 65. A standard using the supplied λDNA was created to correlate fluorescent values to known DNA concentrations. Scaffolds containing no cells were used for background and their readings were removed from all samples to remove any interference from the scaffold.

MTS assay was also used to determine metabolic activity as a measure of proliferation for short studies. MTS CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega. The MTS assay is a colorimetric assay that measures cell metabolic activity. The
MTS reagent is added directly to the cells. The cells interact with the reagent to by reducing it and creating a colored formazan product that can be quantified through absorbance at 490nm. At time point, cells were washed with PBS and 100µl of fresh media was added, along with 20µl of MTS reagent. Cells were incubated in the cell culture incubator (37degC, 5% CO₂) for 2 hours. After the incubation period, absorbance was measured at 490nm using a plate reader.

4.1.4 Gene Expression

4.1.4.1 RNA Extraction

At specific time points, day 3, 7, 14, 21, scaffolds were washed with PBS and placed in sterile Eppendorf tubes. A volume of 900µl of TRIzol reagent from Invitrogen was added to the tubes and incubated at room temperature for two minutes. TRIzol® is a solution of phenol and guanidinium isothiocyanate that serve to denature proteins, while simultaneously stabilizing biological material. A volume of 210µl of chloroform was added to the tubes. The tubes were vortexed and later centrifuged at 4degC for 20 minutes at 10,000rpm. The addition of chloroform causes phase separation of protein, DNA and RNA. The centrifugation step enhances the separation, causing protein to fall to the bottom, DNA to the land on the surface of the protein layer and RNA remains in the aqueous phase. The aqueous layer was carefully removed and placed into new sterile tubes. The RNA was then subjected to subsequent washing steps for purification. An equal volume of isopropanol and the tube was centrifuged for 30 minutes at 10,000rpm at 4degC. Centrifugation causes the RNA product to precipitate out of solution and form a pellet. The lysate was removed and a volume of 500µl of 80% ethanol was added to the tube followed by centrifugation at 4degC for 10 minutes at maximum speed. This step was repeated thrice. At the last washing, ethanol was removed and the tubes were incubated at room temperature with open
lids, to allow all remaining ethanol to evaporate. RNA was then eluted with 20µl of ultrapure water. The RNA was quantified using 1µl of RNA solution on a nanodrop reader. The 260/280 ratio given by the nanodrop indicated quality of the RNA product.

4.1.4.2 Complimentary DNA Synthesis

The collected RNA was reversed transcribed to complimentary DNA (cDNA) using a BioRad iScript cDNA Synthesis Kit. A total of one microgram of RNA was used for cDNA conversion. A BioRad CFX96 qPCR machine was used to monitor and implement temperature gradients for cDNA synthesis.

4.1.4.3 Qualitative Polymerase Chain Reaction

To determine the expression of specific tendon related genes Qualitative Polymerase Chain Reaction (qPCR) was conducted using SYBR green dye. The fluorescent dye binds double stranded DNA by intercalating between base pairs. The fluorescent signal is used to quantify the amount of amplified DNA. Primers were designed and ordered through IDT. Primer sequences used are listed in Table 1.

Table 1: Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCN (185)</td>
<td>CGCCTCATCTGAGGAGCTT</td>
<td>TACTGGACCGGGTTGCTGAA</td>
</tr>
<tr>
<td>TNMD (51)</td>
<td>GAA GCG GAA ATG GCA CTG ATG A</td>
<td>TGA AGA CCC ACG AAG TAG ATG CCA</td>
</tr>
<tr>
<td>SCX (51)</td>
<td>CAG CCC AAA CAG ATC TGC ACC TT</td>
<td>CTG TCT TTC TGT CGC GGT CCT T</td>
</tr>
<tr>
<td>COL III (186)</td>
<td>GATGTGCAGCTGGCATTTCC</td>
<td>CCACTGGCCTGATCCCATGTAT</td>
</tr>
<tr>
<td>COL I (186)</td>
<td>TGGTGCAGCTGTCTCTTCCA</td>
<td>CACGGACGCCATCTTTGC</td>
</tr>
</tbody>
</table>
4.1.4.4 Fold Change Calculations

To calculate fold change, delta-delta CT method was implemented. Housekeeping gene betaActin was used and the control group with no treatment was used for normalization.

4.1.5 Protein Expression

Total deposited collagen content was measured qualitatively and quantitatively using immunofluorescence and PicoSirius Red stain, respectively. Total GAG content was measured using dimethylmethylene (DMMB) blue assay.

4.1.5.1 Immunofluorescence

For immunofluorescence, scaffolds were washed with PBS and fixed with formalin for 15 minutes at room temperature. Scaffolds were then washed with cold PBS for 5 minutes, twice. Scaffolds were permeabilized with 0.25% Triton solution and incubated for 10 minutes. The Triton was removed and scaffolds were again washed with cold PBS for 5 minutes. The scaffolds were then blocked with 3% BSA for 30 minutes at room temperature. The BSA solution was then removed and primary antibody for collagen I (rabbit polyclonal purchased from Abcam) was added in 1%BSA solution for 1 hour. The primary antibody was then removed and the scaffold was washed thrice in PBS for 5 minutes. Secondary antibody was then added for 40 minutes, followed by subsequent PBS washing step, for 5 minutes, thrice. Dapi staining was added prior to viewing. All scaffolds were viewed under confocal microscopy.
4.1.5.2 PicoSirius Red

Direct Red 80, purchased from Sigma, was used to create a 1mg/ml PicoSirius Red solution in picric acid. Scaffolds were washed with PBS and fixed with 4% formalin for 15 minutes. Scaffolds were then washed with PBS, thrice. A volume of 500µl of PicoSirius Red solution was added to the scaffolds and allowed to incubate at room temperature for 1 hour. After the incubation period, all solution was removed from the scaffolds and the scaffolds were washed with DI water until the washing solution remained clear. Apparent stain on the scaffolds were then solubilized with 0.01N NaOH and the absorbance was measured at 550nm.

4.1.5.3 GAG

GAG content was measured using dimethylmethylene (DMMB) blue assay. At specific time points, scaffolds were washed with PBS and placed in tubes. A volume of 500µl of digestion buffer was added directly to the scaffolds. Digestion buffer composed of Tris/EDTA buffer, 18.5% iodacetamide, 1% pepstatin and proteinase K (1mg/ml). Scaffolds were incubated in digestion buffer overnight for at least 16 hours at 56degC. Standard was created using chondroitin sulfate. A volume of 50µl of standards and samples was added to 200µl of DMMB solution and absorbance was measured at 520nm using a microplate reader.

4.1.6 Statistical Analysis

All studies were analyzed using ANOVA and Bonferroni multiple comparisons post-test using GraphPad Prism software.
4.2 Results

4.2.1 Proliferation

![Figure 3.2. The effect of fetal bovine serum concentrations on cellular proliferation at day 1 and day 7.](image)

Cells were treated with varying concentrations of fetal bovine serum to determine the optimal concentration to use for further studies. Low serum conditions were desired to decrease the likelihood of interference from factors naturally present in the serum. However, if the serum conditions are too low, cells may not survive. Thus a minimum serum concentration level was needed that maintained cell population. A 2%FBS concentration was chosen to conduct all future studies as this was the minimum required to maintain cell population without negative effects or heightened response, as shown in Figure 3.2.
Figure 3.3. (Top) Proliferation of cells treated with 24 hours of treatment, evaluated at 6, 12, and 24 hours during treatment. (Bottom) Proliferation of cells treated continuously with insulin, evaluated at day 3, day 7 and day 14. No significant differences were found between insulin treatment groups and control group.
Figure 3.4. Metabolic activity of insulin treated cells after 24 hours at higher concentrations using MTS assay. (* p<0.05, **p<0.01, ***p<0.001).

Cells were cultured on electrospun PCL fibers and treated with DMEM media containing various concentrations of insulin and 1% penicillin/streptomycin. Cells were collected after 6, 12 and 24 hours in treatment (n=6). To determine long term effects of insulin on proliferation, cells were grown on electrospun PCL scaffolds (n=6) and treated continuously with various concentrations of insulin. DNA was collected at day 3, day 7 and day 14. There was no statistical difference between any of the treatment groups and the control group within the first 24 hours of treatment and subsequent days following treatment.

To determine if higher concentrations of insulin would have an effect on proliferation of human mesenchymal stem cells, cells were treated with higher concentrations of insulin supplemented on tissue culture plate and evaluated for metabolic activity after 24 hours. Metabolic
activity of cells treated with 1\(\mu\)g/ml insulin supplemented media or higher, showed significantly higher metabolic activity after 24 hours when compared to the control.

### 4.2.2 Single Bolus Gene Studies

![Gene expression graphs](image)

Figure 3.5. Gene expression of cells treated with a single 24-hour treatment of insulin and evaluated at day 7 and 14. Cells treated with 10ng/ml had significant increase in gene expression of all markers tested compared to the control group (* \(p<0.05\), **\(p<0.01\), ***\(p<0.001\), statistically significant from basal control group; # \(p<0.05\), ##\(p<0.01\), ###\(p<0.001\), statistically significant from indicated group).
Cells grown on TCP received a single dose of insulin of varying concentrations for 24 hours. After 24 hours, the insulin medium was removed and changed to basal medium. After 7 and 14 days, post treatment, RNA was collected from the samples (n=6) and evaluated for tendon gene expression, shown in Figure 3.6. Cells treated with 10ng/ml (1.7nM) of insulin had the highest expression of the markers evaluated, scx, tnmd, col I, col III, and dcn and were statistically significant from the control group at day 7. Cells treated with 100ng/ml and above had increased expression of col I, col III and dcn at day 7. At day 14, there was an overall decrease in the expression of markers when compared to day 7.

Figure 3.6. Gene expression of cells treated with single bolus treatment of 10ng/ml of insulin compared to cells treated with single dose treatment of 250ng/ml IGF-1 (* p<0.05, **p<0.01, ***p<0.001, statistically significant from basal control group; # p<0.05, ##p<0.01, ###p<0.001, statistically significant from indicated group).
To evaluate comparison to IGF-1, cells were grown on electrospun PCL fibers and received a single bolus treatment of 10ng/ml insulin, 250ng/ml IGF-1, or control medium. Gene expression was evaluated at day 3, 7, 14, and 21. Cells treated with insulin peaked significantly at day 7 for scx, col I and dcn. At day 3 and later time points, cells treated with a single 24-hour treatment of insulin were not statistically significant from the control group. Cells treated with single 24-hour treatment of IGF-1 had significant expression of scx at day 3, tnmd at day 21, and col III at day 3. In general, at later time points, day 14 and 21, expression of markers was decreased, with the exception of tnmd, which peaked at day 21 for both insulin and IGF-1 treated groups.

4.2.3 Continuous Gene Studies

Figure 3.7. Cells continuously treated with insulin and evaluated at day 7 and day 14, (* p<0.05, **p<0.01, ***p<0.001, statistically significant from basal control group; # p<0.05, ##p<0.01, ###p<0.001, statistically significant from indicated group).
In a separate study, cells were treated with various concentrations of insulin supplemented media up to 14 days. In general, cells treated with 50ng/ml insulin or below, had decreased expression of markers by day 14. However, cells treated with 100ng/ml or 200ng/ml insulin had increased or maintained elevated expression of markers by day 14. At day 14, there is a dose dependent expression of scleraxis, as well as collagen I.

### 4.2.4 Comparison to IGF-1

![Comparison to IGF-1](image)

Figure 3.8. Gene expression of cells treated continuously with 100ng/ml of insulin compared to cells treated 250ng/ml IGF-1 (* p<0.05, **p<0.01, ***p<0.001, statistically significant from basal control group; # p<0.05, ##p<0.01, ###p<0.001, statistically significant from indicated group).
A concentration of 100ng/ml was chosen to evaluate the temporal expression of continuous treatment of insulin since this was the minimal concentration required to maintain expression after 7 days. Samples were compared to 250ng/ml of IGF-1. With continuous treatment, there was no significant increase in scx expression on any days for both insulin and IGF-1 treated cells. Gene expression of col I and III were upregulated for both groups at all time points, peaking significantly higher on day 7 for both treatments. Decorin was slightly upregulated, but was not significantly higher than controls for both groups, except for IGF-1 treated groups on day 7.

4.2.5 Collagen and Glycosaminoglycan content

Figure 3.9. (Left) Collagen deposited on nanofiber scaffolds at day 21. No significant differences between any of the treatment groups with the basal control group. (Right) GAG content on nanofiber scaffolds collected at day 21. Groups treated continuously with IGF-1 or insulin had statistically significant increase in GAG content than cells treated with the basal control medium or cells treated with a one-time dose of IGF-1 or insulin. (* p<0.05, **p<0.01, ***p<0.001, statistically significant from basal control group).
Collagen and glycosaminoglycans deposited on nanofibers after 21 days, were evaluated from cells treated with one-time dose of insulin or IGF-1 or continuous treatment of insulin or IGF-1. There was no difference amongst the groups in collagen content, however cells treated continuously with IGF-1 or insulin showed significantly higher GAG content than cells with single dose treatment or control medium. At earlier time points, there were no significant differences between groups (data not shown).

5 Discussion

IGF-1 is shown to be upregulated during tissue development and healing and plays a role in proliferation and collagen synthesis (51, 181). Various studies show the benefits of IGF-1 in healing and tissue engineering applications, however, the high costs and associated complications with achieving FDA approval of growth factors has prompted an interest in finding growth factor alternatives. Insulin protein is analogous in structure to IGF-1, but is more readily accessible at lower costs. The discovery of insulin like growth factors prompted interested research in the overlapping functions of insulin. Several studies have shown the potential of either factor to bind to the other’s receptors when present above physiological conditions in endothelial (65) and fibroblasts cells (71).

The use of insulin as a bioactive factor in tissue engineering applications has previously been researched for chondrogenic differentiation. The potential use of insulin in tendon healing applications was first demonstrated by Mazzocca et al.(66). Mazzocca et. al. showed that a monolayer of MSCs exposed to a single bolus dose of insulin for 24 hours showed characteristics of tendon cells (66). Bone marrow derived MSCs treated with 0.1nM insulin for 24 hours had
increased gene expression and protein levels of tendon related markers. A single treatment application was emphasized for clinical relevancy, as treatment would be given concurrently during the time of surgery. This work further explored the use of insulin for tendon applications with MSCs grown on electrospun matrices. In a clinical setting, matrices can act as cell and biofactor delivery devices to maintain cells and treatments at targeted areas.

The use of low serum conditions in the study may have had a profound effect on the insulin concentrations required for proliferation and differentiation. Low serum was used to decrease possible interaction from growth factors naturally present in the media. Herchenhan et al., found the effect of IGF-1 to be greater in low serum conditions using 0.5% FBS. Additionally, preliminary data showed higher expression of tendon markers from samples treated with low serum. A concentration of 2% FBS was chosen for this study, as this allowed maintenance of cells without drastic decrease in cell population by day 7. Our studies suggest no effect of insulin on cell proliferation. Though some studies have found insulin to have mitotic effect on ATDC5 cells (72) and smooth muscle cells (110), others have not (155). Mitotic effects from insulin are more greatly associated with differentiated cells, whereas this effect does not seem to hold for stem cells. As there is the high likelihood that the effect of insulin may be cell dependent, it is difficult to assess whether greater insulin concentrations are needed to induce mitotic effects on MSCs. Additionally, due to the no serum conditions used during the 24-hour study, and low serum conditions during the weekly study, it may be that cells were nutrient deprived with minimal metabolic energy that was not adequate for proliferation. Conversely, cells may have been expending their energy towards differentiation, rather than proliferation. Moreover, increasing the insulin dosage may alternatively foster chondrogenic differentiation rather than tendon differentiation.
Insulin effect on proliferation was measured using DNA concentration collected from scaffolds at 6, 12, and 24 hours and 3, 7, and 14 days after cell seeding. All groups had slight increase in DNA content over time despite the low serum condition (Figure 1A). A 2% FBS concentration was previously found to maintain cell populations without dramatic increase or decrease in cell population (Figure S1). No significant differences were found in cell proliferation among cells treated with or without insulin supplemented media on PCL scaffolds at concentrations up to 200ng/ml insulin. The data suggests, in the presence of low serum conditions, at the concentrations tested, insulin did not have an effect on the proliferation rate. Mitotic effects of insulin appear to be highly dependent on cell type. Human lung fibroblast cells showed significant increase in proliferation after 24 hours post treatment with 2µg/ml insulin (71). In human hepatic stellate cells, significant increase in proliferation was observed with concentrations as low as 1nM insulin (~ 5.81ng/ml) (69). Insulin treatment on human umbilical cord matrix derived mesenchymal stem cells showed no effect on proliferation at insulin concentrations below 1µM (~5.81µg/ml), but significant increase in proliferation with concentrations above 2.5µM (~14.5µg/ml) (70).

To determine if higher insulin concentrations was needed to induce proliferative effect on human bone marrow derived mesenchymal stem cells, metabolic activity of cells grown on tissue culture plate was measured after 24 hours of treatment. Our findings showed significant increase in metabolic activity with cells treated with concentrations of 1µg/ml insulin or higher (Figure 1B). This corroborates with the findings of Li et. al. who observed increased proliferation in umbilical cord matrix derived mesenchymal stem cells. The concentration of insulin needed to stimulate proliferation may depend on the number of insulin receptors present on the cell. If there are lower number of insulin receptors present in the cell, higher dose of insulin may be needed to elicit a
proliferative effect (70). No studies to our knowledge have been conducted on the abundance of insulin or IGF-1 receptors on human mesenchymal stem cells. Though proliferation was observed at higher concentrations, microgram concentrations of insulin was not explored further as these concentrations levels have been shown to induce cartilage differentiation.

Our dose response studies for bolus treatment showed that a concentrations of 10ng/ml insulin was optimal for gene expression of tendon markers, which is greater than what was previously found. The increase in insulin concentration may have been required to overcome the low serum conditions used in these studies. The decreasing expression of tendon markers from both insulin and IGF-1 treated cells suggest that the effects of a single bolus treatment may become attenuated over time. Whether or not the earlier elevated expression of markers is enough to induce cytokine activity and improve tendon healing in an in-vivo model will need to be assessed.

Based on the dose dependent studies, a concentration of 100ng/ml insulin was chosen for the continuous treatment of insulin as this concentration was found to be the minimum required to increase collagen I after 14 days. When compared to continuous treatment with IGF-1, there were no major differences. However, when compared to the bolus study, it is apparent that continuous treatment of insulin was better in maintaining expression of tendon markers beyond day 7. This is supported with the GAG content data that showed significantly higher expression from cells treated continuously with insulin or IGF-1 than cells treated with control or one-time dose. There was no significant difference in collagen formation on the scaffold amongst the groups. When deposited collagen was quantified using Sirius red, there were no statistical differences between treatment groups.

From Aim I, it was shown that a bolus single dose of 10ng/ml given for 24 hours was able to increase expression of tendon markers, whereas continuous treatment of insulin increased GAG
formation. Glycosaminoglycans play a significant role in resisting tendon compression through their hydrophilic properties. In addition, proteoglycans, which GAGs are attached to, play a role in regulating tendon ECM in terms of regulating fiber diameter and subsequently mechanical properties. The upregulation of tendon markers and increased GAG formation are promising results supporting the use of insulin as a bioactive factor to promote tendon healing and regeneration.

Insulin’s potential as a differentiation factor has been widely explored for cartilage applications (72, 154-157), with only one known publication in the area of tendon regeneration, as previously described (66). However, insulin’s analogue IGF-1 has been widely researched for tendon regeneration (51, 52, 184) and shown to increase collagen production (69), the main constituent of tendon extracellular matrix (ECM). For this reason, insulin may have potential in tendon tissue engineering. Dose studies with cells seeded on tissue culture plates, showed higher expression of tendon markers with cells treated with insulin supplemented media when compared to the control group (Figure 2). Scleraxis, a transcription factor that is highly expressed by tendon progenitor cells (39, 42) at early time points, was significantly upregulated at day 7 for cells treated with insulin concentrations equal to and above 10ng/ml insulin. A dose dependent expression of scleraxis was found at day 14, with 100ng/ml (p≤0.01) and 200ng/ml (p≤0.001) insulin treated groups maintaining significant upregulation. Expression of collagen I was also dose dependent and significant increase was observed with cells treated with 100ng/ml insulin and 200 ng/ml concentrations at day 7 (p≤0.05) and day 14 (p≤0.001). No dose dependent pattern was found in the expression of ECM markers, collagen III and decorin. Tenomodulin, a mature marker for tendon (187), was found to be significantly increased at day 14 with cells treated with 200ng/ml insulin (p≤0.01). Since cells treated with 100ng/ml and 200ng/ml insulin tended to have the highest
expression of markers, with no statistical difference between the two, 100ng/ml insulin concentration was chosen as the optimal concentration to move forward in testing with cells on electrospun fibers.
Chapter 4: Developing the Extracellular Matrix

1 Introduction

All cells of connective tissue reside in an extracellular matrix (ECM) composed of fibers and ground substance. It is this extracellular matrix that provides a framework or scaffold which provides structural support to the cells that dwell in it. The importance of the ECM in the growth and development of cells, have inspired many tissue engineering scientists to mimic the natural ECM in fabricating tissue engineering scaffolds. The following sections highlight the fabrication methods of tendon scaffolds, materials used, and the dual function of scaffolds to serve as drug delivery devices. Further, these strategies are presented in relation to the delivery of insulin for tendon tissue engineering.

1.1 Mimicking the Extracellular Matrix

The extracellular matrix of tendon cells is composed of cells, collagen fibers and the ground substance which contains proteoglycans, as previously described in Chapter 1. Several strategies have been taken to mimic the natural environment of tenocytes, including the use of collagen based scaffolds, the use of nano- and microfibers sheets, and the use of decellularized ECM. Biodegradable materials are employed as tissue engineering scaffolds to provide an initial template for cells, with the hope that as the scaffold degrades, it will be replaced by the regenerate tissue. As such, tissue engineering scaffolds must support cellular attachment, proliferation and differentiation. The chemical, physical, and biological properties of the scaffold all contribute to overall effect of the scaffold on cell behavior. Considerations for tissue engineering scaffold materials and design include 1) biocompatibility with nontoxic degradation products; 2) adequate
porosity for cell infiltration, as well as nutrient and waste transport; 3) mechanical integrity; 4) topographical cues (188).

1.2 Biomaterials for scaffolds

Pertinent to the design of ECM mimicking scaffolds is material selection. The physical characteristics of the material determines the biocompatibility of the scaffold, as well as its mechanical properties and degradation. Metals, ceramics and polymeric materials are utilized for tissue engineering scaffolds, but mostly all tissue engineering scaffolds are made from polymeric materials due to their tunable mechanical properties and versatility. Polymers can be divided into two class of materials – 1) synthetic materials, which include polyesters based materials and 2) naturally derived materials, which includes collagen and other biopolymers, such as polysaccharides and silk.

1.2.1 Synthetic Polymers

Synthetic polymer based materials are commonly used in tissue engineering scaffolds due to their versatility and relatively easy manipulation of chemical and physical properties, which can be optimized for specific applications (188). Additionally, when compared to natural polymers, synthetic polymers have higher degree of processing flexibility, without concerns of immunogenicity (188, 189). Synthetic polymers used in tissue engineering include aliphatic polyesters, polyanhydrides, polyphosphazenes, and polyurethanes. Since polyesters represent the most widely used materials for tissue engineering scaffolds, they are reviewed in the following sections.
1.2.2 Aliphatic Polyesters

Aliphatic polyesters include polylactide (PLA), polyglycolide (PGA), their copolymer poly(lactide-co-glycolide) (PLGA), and polycaprolactone (PCL). These polymers are approved for human clinical applications by the Food and Drug Administration (FDA) for particular biomedical devices and sutures. Their FDA clearance has contributed to the increased use of these materials in tissue engineering applications. Synthesis of aliphatic polyesters can be done through polycondensation, ring opening polymerization, or enzymatic polymerization. Degradation of these polymers occurs through hydrolytic degradation of the main chain ester bonds. Degradation rate is highly dependent on the polymer characteristics such as crystallinity, hydrophilicity and molecular weight and results in monomers that can be easily metabolized (190). Polymer PLGA is one of most popular material for tissue engineering and drug delivery applications. However, accumulation of PLGA’s acidic byproducts, lactic acid or glycolic acid, can significantly lower the local pH creating a harmful environment for cells and accelerating polymer degradation (191). In an experiment comparing identical scaffolds of PLGA and PCL, lower cell viability and infiltration were observed on PLGA scaffolds, attributed to the acidic degradation of PLGA (192). Another disadvantage for PLGA is its high off-the-self cost. Polycaprolactone represents a slower degrading, biodegradable polymer with less economic costs.

1.2.3 Polycaprolactone

Polycaprolactone is a semi-crystalline polymer with glass transition temperature of 60°C and a melting temperature range of 59-64. It has been used in a variety of scaffold fabrication techniques due to its solubility in wide range of solvents and its versatility to be blended with various polymers (193). As previously mentioned, PCL possesses slower degradation kinetics
when compared to PLA, PGA or PLGA. This has deterred its use, however its slower degradation kinetics may be ideal for long term drug delivery applications, as well as regeneration of slower growing tissue (194).

![Figure 4.1. Synthesis of PCL](image)

1.2.4 Biopolymers

The desire to mimic and use natural bioinspired patterns and designs have propelled scientists to use the natural materials themselves. One of the main advantages of natural polymers over synthetic polymers is their innate biological functions that contribute to overall better bioactivity and biocompatibility. The presence of biologically functional moieties allows for greater interactions with cells and proteins. Natural polymers can be derived from microbial, animal, or plant sources and can be divided into protein-origin polymers and polysaccharides. Protein-origin polymers include collagen, gelatin, silk fibroin, elastin and soybean. Polysaccharides include chitosan, starch, alginate and cellulose (195). This work involves the use of cellulose and thus only cellulose will be reviewed here.

1.2.4.1 Cellulose Acetate

Cellulose is the most abundant organic biopolymer in the world and is thus readily available at low costs. Structural polysaccharides like cellulose are made up of linear chains of
monosaccharides linked together by glycosidic bonds. Majority sources of cellulose is derived from plants as it is present in plant cell walls, however cellulose can be produced by bacteria, algae, and few animal species (196). The structure of cellulose, includes three hydroxyl units per glucose molecule, which results in very strong hydrogen bonding between polymer chains or high intermolecular forces. Thus cellulose in its native form is insoluble in most solvents. By switching the hydroxyl side groups, cellulose derivatives can be synthesized that are easier to process and use. Cellulose acetate is a derivative of cellulose which has the hydroxyl groups replaced by acetic acid. Cellulose acetate is formed by processing cellulose with acetic anhydride or acetic acid, with sulfuric acid as a catalyst. The result is acetic side chain in place of hydroxyl groups. The amount of acetic substitution is given by the degree of acetylation. Cellulose acetate is dissolvable, lending itself more applicable to scaffold fabrication. The limitations of natural polymers include batch-to-batch variability and lower versatility in terms of processing.
1.3 Electrospinning

The electrospinning process is a versatile and simple method to fabricate fibers with diameters in the micro- and nanoscale range. In the last two decades, there has been renewed interest in the electrospinning process to create nanofibers due to the growing number of applications in filtration, protective clothing, tissue engineering scaffolds, and drug delivery devices (1).

Electrospinning involves the use of electrostatic forces to create nanofibers (in the range of 100-500 nm, (1). A typical electrospinning apparatus consist of a collector electrode, source electrode, high voltage supply and syringe. The experimental set up is shown in Figure 4.1. A high voltage is applied to a polymer solution or melt. The voltage polarizes the charged ions due to the electrostatic repulsion between like charges. In addition, there is an electrostatic attraction between the charged liquid and a collector at the other end. At equilibrium, the surface tension and electrostatic forces cause the liquid to form a cone like shaped, known at the “Taylor” cone. When the electrostatic forces overcome the surface tension, a jet is emitted from the Taylor cone towards the collector. As it travels the jet decreases in diameter and the solvent is evaporated such that a solid fiber is formed and collected at the collector plate (1).
Fiber diameter and morphology are the two most important considerations in processing nanofibers and are governed by process parameters and solution characteristics. Process parameters include the applied voltage, flow rate, and the distance between the capillary or needle and the target collector. Solution parameters include, but limited to, solution viscosity, which is related the solution concentration, and the conductivity of the solution. In addition, humidity and temperature of the surrounding environment may also play a role. Much research has been done on the effects of these process and solution parameters on fiber diameter and morphology.

The surface to volume ratio of electrospun scaffolds may have an effect on the overall biodegradation of the material when compared to the bulk material. Additionally, lower polymer crystallinity has been observed with electrospun scaffolds when compared to films of the same material, thus degradation may be accelerated (197). The addition of cells and the interaction between cells and the fibers may also contribute to the in-vivo degradation prolife.
Figure 4.3. The electrospinning set up involves a syringe pump that pushes the polymer melt at a constant rate. A high voltage source attached to the end of the needle opening, charges the polymer. When the electrostatic forces overcome the surface tension, polymer is ejected from the needle. The electrically charged polymer jet is attracted to the grounded collector. On its trajectory to the collector, the polymer undergoes a series of loops and turns, which thins out the polymer jet resulting in nanoscale sized fibers.

1.4 Drug delivery and considerations

Electrospun fibers can also be used to deliver biomolecules such as drugs, growth factors and other proteins. The simplest method is direct adsorption of the biomolecule to the surface of the scaffold. This method protects the bioactivity of the biomolecule, however interaction between the biomolecule and the scaffold relies on relatively weak forces that do not allow for great control of release. A burst release is often observed, with most of the biomolecule eluted once the scaffold is in a hydrated environment. To gain better control of release kinetics of the biomolecule, it can be directly added to the polymer solution prior to electrospinning. Furthermore, the biomolecule can be coaxially spun with the polymer, such that the biomolecule is encased in the middle of the fiber. Longer release profile can be obtained using these methods, however, the biomolecule is subjected to degradation from the solvent in the polymer solution. Direct tethering of the biomolecule to the surface of the molecule is another strategy that results in a chemical bond between the biomolecule and the surface of the scaffold. Such strategy avoids burst release effects and is often conducted in aqueous conditions which do not pose a risk to the bioactivity of the biomolecule.
Specific Aim II: To develop and characterize a novel insulin delivery matrix system for tendon regeneration.

2.1 Materials and Methods

2.1.1 Fabrication of Electrospun fibers

Scaffolds were fabricated using standard electrospinning techniques. Polycaprolactone (80,000 Mn) and cellulose acetate (CA) were weighed to create a 12.5 weight/volume percent solution in trifluorethanol. The ratio of PCL to cellulose acetate varied from 25, 50, and 75 percent CA. Polymer solutions were dissolved overnight at room temperature. Polymer solutions were electrospun at 1kv/cm, at a flow rate of 2ml/hour and collected on a grounded plate with a 10x10cm foil. Scaffolds were dried and stored under vacuum until use.

2.1.2 Characterization

2.1.2.1 Fiber Diameter

Scaffolds were sputter coated with gold/palladium and viewed under SEM at 500x magnification. Using Image J software, 50 fibers from 3 images were measured to give an average fiber diameter for each polymer composition.

2.1.2.2 Contact Angle

Contact angle was measured using a goniometer. A drop of water was placed on 1x1cm scaffold and the resulting contact angle was recorded. A total of 6 readings were averaged to obtain the average contact angle for each polymer composition.
2.1.2.3 Tensile Testing

Tensile mechanical testing was conducted using 10mmX20mm sheets, per ASTM standards on an Instron Tensile machine using a 50N load cell. Scaffolds were incubated in PBS overnight prior to testing.

2.2 Cellular Attachment

Scaffolds were cut into 1x1cm squares and were sterilized with immersion in 70% ethanol and exposure to UV on each side for 30 min. After sterilization, scaffolds were placed in 48-well plate dishes and incubated with standard DMEM cell culture media (10% FBS, 1% penicillin/streptomycin) overnight. Human mesenchymal stem cells, in passage 5, were seeded on the scaffolds, with an initial seeding density of 30,000. After 24 hours, scaffolds were collected in 2ml tubes and digested with proteinase K. A volume of 500ul of proteinase K solution was added to the scaffolds. The tubes were closed and kept at 56degC water bath for 16 hours. After digestion, scaffolds were frozen until assay was performed. Quant-iT™ PicoGreen™ dsDNA Assay Kit was used to measure DNA content. Readings from cell-free scaffolds served as scaffold negative controls and were subtracted from sample readings.

2.3 Insulin Conjugation

After sterilization, 1x1cm scaffolds were placed in 24-well plate dishes and treated with 500mM of sterile NaOH for 1 hour to cleave ester bonds present in PCL. This allowed for free carboxyl groups on the scaffolds to partake in conjugation. The scaffolds were then incubated in sterile 100mM MES buffer for 1 hour. The MES buffer was removed and fresh MES buffer containing coupling reagents EDC/NHS (500ug/ml) and insulin (10ug/ml) was added to the wells.
The well plates were wrapped with parafilm and placed on a rocker overnight. After conjugation, the MES conjugation buffer was removed and the scaffolds were washed with sterile water repeatedly 5x to remove unreacted products.

2.4 Insulin Quantification

The amount of insulin conjugated on the scaffolds was measured using bicinchoninic acid assay (BCA). Scaffold samples were incubated in MES buffer with insulin in the presence or absence of crosslinking agents EDC/NHS. Scaffolds in buffer with insulin without crosslinking agents gave the amount of insulin directly adsorbed onto the scaffold. This value was then subtracted from the total amount of insulin on the scaffold to render the amount of insulin chemically conjugated onto the scaffold.

2.5 Cell Studies

Cells studies were conducted on 1x1cm scaffolds using commercially available bone derived human mesenchymal stem cells from Lonza. Scaffolds were sterilized and conjugated as previously described. Cells were seeded with an initial seeding density of 50,000/scaffold. Cells were treated with DMEM media with low serum (2% FBS) and 1% penicillin/streptomycin. Media was exchanged every 2-3 days.

2.6 Proliferation

At day 3, 7, and 14, scaffolds were washed and collected into 2ml tubes. The tubes were frozen until analysis was performed. At the end of the study, all samples were digested with using papain digestion. A volume of 500ul of papain digestion buffer was added to the tubes and
incubated at 65degC overnight for 16 hours. After digestion, amount of DNA in the lysate was measured using Quant-iT™ PicoGreen™ dsDNA Assay. Cell-free scaffolds served as negative controls and readings were subtracted from sample values to remove interaction with scaffold material.

2.7 Gene Expression

At day 3, 7, and 14, scaffolds were washed and collected into 2ml tubes. The tubes were kept in -80degC until analysis. RNA was extracted using standard Trizol extraction methods. At the end of the study, Trizol was added directly to the tubes. The tubes were vortexed and incubated at room temperature for 5 minutes. Chloroform was then added to the tube at a ratio of 210:900 of chloroform to trizol. The samples were vortexed and centrifuged at 10,000xg for 30 minutes in 4degC. Centrifugation resulted in phase separation of RNA, DNA and protein. The top aqueous layer, which contains the RNA, was carefully pipetted into new autoclaved 2ml tubes. Equal volume of isopropanol was added to the RNA lysate and the samples were kept in -80degC overnight. The next day, the samples were centrifuged at 10,000xg for 30 minutes in 4degC causing the RNA to form a pellet. The tube was carefully decanted and 80% ethanol was added. The samples were centrifuged at maximum for 10 minutes in 4degC. The tube was decanted and fresh ethanol was added. The samples were centrifuged at maximum for 10 minutes in 4degC. The tubes were decanted to remove as much ethanol as possible. The samples were spun down and any remaining ethanol was removed using a clean pipette. Care was taken not to disturb the RNA pellet. The RNA pellet was then reconstituted in 20ul of water. The RNA was quantified using a NanoDrop instrument. A concentration of 1ug of RNA was used to synthesize cDNA using BioRad
iScript cDNA synthesis kit. To analyze the presence of tendon related genes, qPCR was conducted using BioRad iTaq™ Universal SYBR® Green Supermix.

2.8 DMMB

Using the same lysate from the papain digestion, amount of glycosaminoglycans (GAG) were measured by carrying out dimethylmethylene blue assay (DMMB).

3 Results

3.1 Material Selection

Polycaprolactone was chosen as a synthetic polymer for its mechanical strength, however due to its high degree of hydrophobicity and subsequent lower interaction with biological material, it was blended with natural polymer cellulose acetate (CA). Cellulose acetate is derivative of cellulose, one the most abundant natural material on earth. Biological materials have higher degree of interaction with CA due to greater hydrophilicity. To assess the optimal polymer composition, electrospun fibers were fabricated using the following weight ratios of PCL:CA, 100:0, 75:25, 50:50, 25:75. All fiber compositions had a polymer weight:volume ratio of 12.5% and were dissolved overnight in trifluorethanol. The polymers were electrospun at 1kv/cm, at a flow rate of 2ml/hour. Fibers were collected on a grounded plate.

3.1.1 Fiber Diameter

At all polymer concentrations, PCL, 25%CA, 50%CA, and 75%CA, bead-free fibers were produced. SEM microscopy was used to measure fiber diameter of the scaffolds. Overall, fiber
distributions ranged from nano- to micrometer range. Increasing CA concentration rendered decreasing fiber diameters.

3.1.2 Mechanical Properties

Mechanical testing was conducted using 10mmX20mm sheets, per ASTM standards on the Instron Tensile machine with a 50N load cell. Increasing stiffness was observed with increasing concentration of CA until 75%CA, where modulus is decreased. Moreover, 75%CA scaffolds had the lowest tensile strength, whereas there was no difference between 25%CA and 50%CA. As expected, PCL had the highest tensile strength and elasticity.
Figure 4.4. SEM images of various compositions. All fiber diameters were statistically significant, except for PCL and 50%CA, which were similar in diameter.
Water Contact Angle

The addition of CA did not significantly decrease the water contact angle of nonhydrolyzed scaffolds. However, after treatment with 500mM NaOH for 1 hour, water contact angle of all CA compositions were not measurable on the goniometer, as water was readily absorbed on the material as quickly as the droplet was placed on the sample.

Table 1: Water contact angle

<table>
<thead>
<tr>
<th>Scaffold Composition</th>
<th>PBS Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL</td>
<td>116°±9°</td>
</tr>
<tr>
<td>75% PCL- 25% CA</td>
<td>114°±6°</td>
</tr>
<tr>
<td>50% PCL- 50% CA</td>
<td>106°±2°</td>
</tr>
<tr>
<td>25% PCL- 75% CA</td>
<td>105°±1°</td>
</tr>
</tbody>
</table>
3.1.4 Cellular Attachment

To assess cellular attachment on the various composition of polymer, human MSCs were seeded statically on the fibers. After 24 hours, the scaffolds were collected and DNA was measured using PicoGreen Assay kit. All CA scaffold compositions had higher cellular attachment than the PCL scaffold after 24 hours. However, increasing CA concentration decreased the number of cell attachment.

![DNA Concentration](image)

**Figure 4.6**: DNA concentration on polymer compositions after 24 hours

3.1.5 Protein Immobilization

Scaffolds were first hydrolyzed in 500mM NaOH in order to cleave ester bonds on PCL and create free carboxyl groups on the scaffolds that could participate in amide bonding of insulin
onto the surface of the scaffold. The scaffolds were incubated overnight at room temperature in MES buffer (100mM) containing the coupling reagents EDC/NHS and insulin-FITC.

Figure 4.7. Insulin-FITC was conjugated to fibers and viewed under confocal microscopy.
Figure 4.8. The amount of insulin immobilized onto the scaffold decreased at higher concentrations of cellulose acetate

3.1.6 Biological Assessments

Cells seeded on insulin functionalized scaffolds showed decreased DNA concentration at later time points compared to cells grown on control scaffolds.
Figure 4.9 DNA concentration on insulin treated scaffolds and neat scaffolds.

Figure 4.10. Insulin released into media
Figure 4.1. Gene expression of tendon markers on insulin immobilized scaffolds. Results mimic those of cells treated with insulin supplemented media.

4 Discussion

A PCL-CA blend was chosen to combine the advantages of both synthetic and natural polymers. Polycaprolactone is widely used in tissue engineering applications mainly due to its strong mechanical properties and versatility, as well as its FDA approval in sutures and drug delivery systems (198). Moreover, PCL lends itself to be easily blended with other polymers. The addition of natural polymers like cellulose acetate have been shown to improve biocompatibility (199, 200). In recent years, the global market for cellulose acetate has been rapidly increasing with
a predicted value of approximately 7 billion dollars by the year 2021 (201). The relative abundance and flexibility has fueled interest in cellulose acetate in a myriad of areas including biomedical applications such as tissue engineering and drug delivery (202, 203).

SEM images of PCL-CA blend scaffolds showed bead free, continuous fibers (Figure 4A). Properties of scaffolds are listed in Supplementary Table 1. With increasing CA concentrations, there was a decrease in fiber diameter (Figure 4B). A similar trend was found with PCL-CA blend fibers fabricated by Farooq et al. (204). The addition of CA also lowered the tensile strength of the scaffolds, with significantly less maximum load associated with the blend fibers (Figure 4C). Similarly Bin et al. fabricated electrospun PVA-CA blend scaffolds and found increasing CA content in the blend scaffolds decreased overall mechanical strength (205).

With increasing CA concentrations, the water contact angle was decreased (Figure 4D). This was expected as PCL is highly hydrophobic, whereas CA is hydrophilic. Higher DNA concentration was found on the blend scaffolds when compared to PCL only group (Figure 4E). Significantly higher DNA content was found on the 25%CA group when compared to PCL (p<.05). This is attributed to the increase in hydrophilicity of the scaffold, which would have allowed for greater cellular attachment.

There are several methods that can be employed to deliver biomolecules on electrospun fibers including physical adsorption, co-electrospinning, and chemical conjugation. Physical adsorption, the simplest method, relies on relatively weaker interactions such as electrostatic forces, hydrogen bonding and van der Waals interactions that can be difficult to control (206). Biomolecules that are added to the polymer solution for co-electrospinning run the risks of degradation from solvents used in the solution (207). Chemical conjugation is a much milder process that is conducted in aqueous solutions, which helps preserve the bioactivity of the
biomolecule. Additionally, chemical conjugation of protein on the surface of scaffolds allows for greater control and longer activity of the immobilized biomolecule (206). Cells can directly interact with the immobilized insulin and modulate cellular behavior. Thus, chemical conjugation was implemented to functionalize the scaffolds with insulin.

The electrospun fibers were initially hydrolyzed to provide ester groups as sites for conjugation of insulin to the fibers. No significant difference was found in the amount of immobilized insulin between PCL and 25%CA scaffolds, which measured 67ng and 76ng, of insulin per scaffold respectively. However, significantly lower amounts of immobilized insulin were measured on 50%CA and 75%CA scaffolds, 26ng and 8ng, respectively. The amount of immobilized insulin on the PCL and 25%CA scaffolds are in range with other similar reactions on PCL fibers. Yoo et. al observed 49ng of neural growth factor conjugated to PCL/PEG electrospun fibers (208), whereas Cheng et. al found 120ng of collagen immobilized on PCL/chitosan fibers (207). Based on the findings with regards to tensile strength, cellular attachment, and amount of immobilized insulin, the scaffold composition with 25% cellulose acetate was chosen to conduct biological feasibility studies.

Clinically, insulin delivery must be dealt with cautiously as insulin is an important and integral part of normal homeostasis. The concentrations tested in these studies are far below the international unit of insulin (34.7µg/ IU) and thus are not expected to have systemic effects. Moreover, insulin was immobilized onto the scaffold, thus reducing a possible burst release effect. Insulin released into the media was measured during the duration of the study. By day 14, roughly 19% of the insulin was eluted into the media. This demonstrates a slow release of insulin and demonstrates the avoidance of a burst release effect commonly associated with biomolecules that are physically encapsulated.
At day 14, there was significant decrease in DNA content with the insulin conjugated scaffolds when compared to the control group (p ≤0.001). This may indicate that cells seeded on the insulin conjugated scaffolds are expending energy towards differentiation rather than proliferation, as evidence indicates an inverse relationship between the two cellular modalities (209). Earlier findings showed no difference in proliferation with cells on PCL scaffolds with insulin supplemented media. This may be due to experimental differences. For insulin immobilized scaffolds, a lower initial seeding density was used to ensure cells adequate space to proliferate. This resulted in overall less DNA content, but a visible and significant increase in DNA concentration at day 14 for cells treated on control scaffolds.

In terms of differentiation markers, there was an upregulation of scleraxis observed at day 7 (p ≤0.05) with a significant increase in tenomodulin at day 14 (p ≤0.05). When compared to cells treated directly with insulin supplemented media, cells grown on insulin immobilized scaffolds seem to have a slower progression as evidenced by later increase in tenomodulin at day 14, rather than day 7 as was seen with the former. What might account for this difference in the accessibility of cells to the insulin protein. Though chemical conjugation of biomolecules onto the surface of fibrous scaffolds allow for greater attachment to the fibers, the site of attachment may potentially decrease the bioactivity. Due to chemical modification, cells may not recognize the biological ligands of the biomolecule, as they may not be fully exposed (206). It is important to note however, that cells grown on the insulin immobilized scaffolds still had an upregulation of tendon markers. Thus the amount of immobilized insulin on the scaffolds was suffice to increase tendon markers and remained bioactive throughout the study.
Chapter 5: *In-vivo* Study of Insulin in Rat Achilles Tendon Defect

1 Introduction

Tendon injuries account for roughly half of the 33 million musculoskeletal injuries that occur annually (5). Treatment for tendon injuries include nonsurgical and surgical treatments, however nonsurgical treatments have shown to gain functional recovery in only 60% of cases (26). Additionally, 29% of patients seeking nonsurgical treatments eventually receive surgical intervention (21, 210, 211). Tendon surgical repairs will vary according to type of tendon rupture, however in general, tendons are repaired through debriding the torn area and suturing the tendons ends together or back to the insertion site, depending on the location of the tear. In cases where there is limited tissue left, grafts can be used to augment the tendon tissue. However, commercially available grafts have had variable clinical results (28), calling the need for more effective treatments. Tissue engineering has emerged with new solutions to tendon injuries by way of scaffolds and biomolecule and cell delivery strategies (212). Testing of tissue engineering scaffolds for their biocompatibility and effectiveness are first often conducted in small rodent models and rabbits before proceeding to larger animal studies such as ovine. These animal studies provide important preclinical data.

1.1 Commercial Grafts

1.1.1 Biological

The practice of tendon grafts to augment large tendon defects involves the use of autografts, allografts or xenografts. Autografts, taken from another site of the body, is limited in availability and requires a secondary site of injury that poses increased risk for complications. Thus,
transplantation of allografts and xenografts have gained increased popularity for rotator cuff and Achilles tendon repair (28).

The Graftjacket is a commercially available allograft taken from human cadaver skin. The tissue is decellularized using a patented technique that removes epidermal and dermal cells, and preserves ECM components such as collagen, elastin, and proteoglycans (213). It has been used successfully for skin lesions (214, 215) and abdominal repair (216, 217). Applications for Graftjacket in Achilles (218) and rotator cuff repair (219, 220) have also been tested and shown to improve clinical outcomes, however retear rates as high as 30% have been found with its use in rotator cuff repairs.

Xenografts, taken from animals, also make up a large portion of available biological grafts. Porcine small intestine submucosa (SIS) are the source for the Restore patch, as well as CuffPatch. The Restore patch was the first biological scaffold on the market (28) and is composed of ten individual layers of porcine SIS. Approximately 90% of the Restore patch is made up of collagen with the remaining 5-10% made up of lipids, carbohydrates and TGF-β(213, 221, 222), allowing it to be marketed as an acellular collagen scaffold with growth factors. Though animal studies (223, 224) and some clinical trials (225) showed favorable results with Restore, subsequent studies have shown high failure rates and limited success (226, 227). Moreover, Zheng et al. reported the presence of multiple layers of porcine cells within the patch (228), highlighting one of the limitations associated with biological scaffolds.

Other biological scaffolds include the Zimmer patch and TissueMend, which are sourced from porcine and fetal bovine dermis, respectively. The Zimmer patch, (formally known as Permacol) and TissueMend are composed of a single layer of acellular skin graft, composed mainly of collagen. Results of clinical trials of the Zimmer patch have been mixed. Some studies showed
great improvement was found in pain relief, range of motion and satisfaction in ten patients who underwent rotator cuff reconstruction with the Zimmer patch (229, 230). Conflicting results were found in another study with four patients who had recurrent tears and decreased range of movement after 3-6 months following repair with the Zimmer patch (231). No clinical data has been published on the TissueMend for tendon applications (28).

Due to their inherent biological properties, biological grafts have shown to be highly biocompatible. However, risks of disease transmission and rejection due to the presence of foreign DNA, requires better processing methods to remove cellular material. Biological grafts are also plagued with lower mechanical properties and undefined degradation rates (28). The performance of many commercial grafts have been mixed owing to the fact that many studies are retrospective without proper controls.

### 1.1.2 Synthetic Prosthesis

Synthetic, non-absorbable tendon grafts provide much higher mechanical properties than biological grafts, do not carry risks for disease transmission and have tunable material properties. Tendon prosthesis made from polyester, polypropylene, polyarylamide, dacron, carbon, silicone, and nylon fibers were popular in the 1980s and 1990s, however complications associated with biocompatibility have limited their use. The synthetic prosthesis had issues such as implant degradation, device failure, severe synovitis and inflammation. Due to these complications, many synthetic grafts have since been stopped for use in tendon applications. (28, 232-234). Research has now moved towards finding degradable synthetic materials, reviewed in Chapter 4.
2 Animal Tendon Model

*In-vivo* animal models provide vital information on the performance of scaffolds in a biological and dynamic environment that cannot be easily simulated in an *in-vitro* environment. Tendon models for tendon regeneration include the rotator cuff tendons (31, 235, 236) as well as the Achilles tendon (179, 237). The tendon model for this work will focus on the rat Achilles tendon model.

The Achilles tendon is one the largest tendons in the body, ranging from 11-26cm long, with an average length of 15cm (238). The Achilles is a conjoined tendon that connects the gastrocnemius and soleus muscles to the calcaneal tuberosity (239) that aids in walking, running, and jumping. The tendon is subjected to high loads reaching as high as 9kN during running activities, which equals up to 12.5 times body weight (238, 240). The blood supply of the tendon is derived from the musculotendinous and osteotendinous junctions, as well as vessels in the surrounding connective tissue, supplied from the posterior tibial artery and peroneal artery, respectively. Like most tendons, vascularity to the Achilles tendon is overall low, but the midsection of the tendon is marked with relatively higher hypovascularity. As a result, most injuries and ruptures occur at the midsection, 2-6cm proximal to the bony insertion (238).

Tendon ruptures often occur during strenuous physical activities that involve sudden forces caused by pivoting or rapid acceleration. In the United States, majority of Achilles injuries (68%) are attributed to sport injuries, specifically basketball, tennis, and American football. Middle-aged males, described as “weekend warriors,” who occasionally participate in athletic endeavors, were shown to have the greatest risk of injury (241). The incidence of Achilles tendon ruptures in North America range from 5.5 to 9.9 ruptures per 100,000 people (242). Surgical treatment of Achilles tendon rupture consists of reattaching the tendon ends using nonabsorbable sutures, such as fiber
wire, followed by several weeks of immobilization and physical therapy thereafter. In a two year follow up, patients repaired tendons were found to be structurally and mechanically different from the contralateral uninjured tendon, with reduced modulus and stiffness (243). Thus, there is room for better treatment outcomes for surgical Achilles tendon repairs.

Due to the high incidence of Achilles injuries, the rat Achilles tendon model has been frequently used to model tendinopathy, repair treatment modalities, and rehabilitation protocols (244). Due to the location and size of the tendon, the Achilles tendon is more easily accessible than other tendon models. Like all acute injuries, the model does not recapitulate the environment of chronic degeneration that is associated with many tendon injuries. Moreover, anatomy and kinematics of tendons in animals do not usually match those of humans. Despite these limitations, animal models provide useful information on cellular and tissue level principles in a living organism (244).

Once efficacy is established in a small animal model, the tissue engineering scaffold must be tested in larger animal models with the scaffold located in the correct anatomical location for which the device is designed for. If results remain favorable, the device must then be proven to be safe. Only then can human trials be conducted. The easiest route to obtaining FDA approval is to follow the path of an existing FDA approved device (245).
Specific Aim III: To conduct bioactive feasibility assessments of an insulin delivery matrix system in combination with bone marrow derived MSCs, *in-vivo*.

3.1 Materials and Methods

3.1.1 Cell Attachment

The effectiveness of cell loading on the scaffold was tested using both dynamic and static conditions. After sterilization, 1x1 cm scaffolds undergoing static seeding, were placed in 48-well plates. A volume of 200 ul of media containing approximately 500,000 cells were added to the top of the scaffolds. The scaffolds were incubated at 37 degrees C and collected at 1, 2, 3, 4, 6, 12, and 24 hours after seeding. Scaffolds receiving dynamic seeding, were placed in sterile 2 ml tubes with 200 ul of media containing approximately 500,000 cells and were gently rocked. Scaffolds were collected at 1, 2, 3, 4, 6, 12, and 24 hours after seeding. At collection time point all scaffolds washed and processed for DNA content as previously described.

3.1.2 Donor Cell Harvest

Male Sprague dawley rats, approximately 8 weeks old, were euthanized using CO₂ asphyxiation. The long bones of all limbs were dissected and collected from the animal. All muscles and other tissues were cleared off from the bone. The bone was then dipped in 70% ethanol. In a biological sterile hood, the ends of the long bones were cut using bone scissors. An 18-gauge needle was used to flush the bone canal with DMEM. The cell media was filtered through a 20 μm filter and collected in a 50 ml conical tube and underwent centrifugation. Centrifugation resulted in the separation of blood red cells and bone marrow stromal cells. The stromal cells were
isolated and plated onto 10mm tissue culture plates and allowed to proliferate. Cells were passaged up to P2 and used as donor cells for cell seeded scaffolds.

### 3.1.3 Scaffold Preparation

Scaffolds were cut into 3x3mm squares and sterilized with 70% ethanol and UV light, as previously described. Scaffolds with insulin functionalization underwent EDC conjugation, as previously described, 48 hours prior to implantation. In brief, sterilized scaffolds underwent EDC conjugation with insulin using filtered aqueous solutions. A cell suspension of 1 million cell density were added to the scaffold and allowed to attach to the scaffold for 24 hours prior to surgery. All scaffolds were incubated in DMEM prior to implantation.

### 3.1.4 Surgical Procedure

Female Sprague dawley rats, (9-12-week old) were used in the study. Animals were housed in the university animal tower for a minimum of 48 hours prior to operation to allow animals to acclimate to the new surroundings. All operations were conducted in the UConn Health small OR room. Prior to surgery, the animal was anesthetized with isoflurane, (level 2) using a chamber box. The animal was then transferred to a nose cone connected to a scavenger system. The left ankle was shaved and cleaned with alternating chlorhexidine and povidone-iodine, 3x. The animal was given a half dose of buprenorphine and transferred to the operating table. A 3cm incision was made over the left Achilles tendon. Once exposed, the Achilles tendon was isolated from the surrounding fascia. At 5mm away from the calcaneus, the tendon was fully ruptured with a scalpel. The tendon was then repaired according to treatment group 1) defect only (no repair) 2) suture repair 3) neat scaffold repair with PCL/CA blend scaffold 4) Insulin functionalized scaffold 5) Neat scaffold
with cells 6) Insulin functionalized scaffold with cells. A 6-0 prolene sutures using a Kessler stitch technique was first used to augment the tear for repair groups. The scaffold groups were augmented with a 3mmx3mm scaffold placed over the repair using 7-0 prolene sutures. The skin was sutured closed with 5-0 sutures and surgical glue. The animal was given pure oxygen and the remaining half dose of buprenorphine. The animal was given two doses a day, 8-14 hours apart, of buprenorphine for two days after the surgery. Animals were housed individually post-surgery for two weeks. After two weeks, animals were group housed.

Figure 5.1. Schematic of surgical model. The tendon was cut 5mm away from the bone calcaneus. The tendon was either left with no repair, sutured only, or sutured with the scaffold overlaying the tendon repair.
3.1.5 **Blood Glucose Testing**

Due to the potential of insulin to affect blood glucose levels, blood from the rat tail vein were collected weekly for four weeks. The animals were first anesthetized with isoflurane. A 25-gauge needle was used to prick the lateral tail vein. A droplet of blood was then collected on a glucose monitor strip and read using a glucose reader. Readings above 60mg/dL were considered normal.

3.1.6 **Sample Collection**

At 4 and 8 weeks, animals were euthanized using CO₂ asphyxiation. The repaired tendon was exposed and isolated. Tendon samples for histological evaluations were cut at the calcaneus and muscle attachments, washed with PBS, and placed in formalin. Samples used for biomechanical testing, were removed with the muscle and foot attached. The tissue was wrapped in sterile gauze soaked in PBS and immediately stored in -80°C until analysis.

3.1.7 **Histological evaluation**

The samples were fixed at -20°C for 3 days, washed with copious PBS and stored in 70% ethanol. Samples were embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin and Trichrome (Gomori). Separate slides were used for immunohistochemistry staining of collagen I and collagen III. All slides were scanned and digitized using an Aperio CS2 slide scanner, with 20x magnification. A total of 12 fields starting from 2cm away from the calcaneous to 8cm (2 fields at each 1cm increment) from 3 separate specimens were evaluated for all image quantifications.
Hematoxylin and eosin stain was used to count the number of cells in the tissue, using ImageJ plugin. Hematoxylin stains cell nuclei a deep blue allowing demarcation of the nuclei. Images were converted to 8bit and threshold was adjusted to select only nuclei. To count cells, the particles were analyzed, using a 30 square pixel size minimum, as shown in Figure 5.2.

The trichrome stain differentiates between muscle, collagen, and nuclei, where the components are dyed red, blue and black, respectively. Dyes were separated using ImageJ plugin, color deconvolution. The relative percent area of blue and red dye were recorded.

Collagen staining was quantified using ImageJ analysis, as previously described (31). A total of 12 fields per sample (n=3), at 20x magnification were assessed for total area of collagen I and collagen III. The same areas evaluated on a single sample were kept constant for collagen I and collagen III. The image was separated using ImageJ color deconvolution plugin for HDAB (see Figure 5.3). The amount of brown stain was evaluated by selecting areas at a maximum threshold. Threshold values were kept constant for all samples. The ratio was obtained by the total area of collagen I staining divided by the total area of collagen III staining.
Figure 5.2. Protocol schematic for determining cell number using ImageJ analysis. The H&E image is converted to an 8bit image. Cells are chosen by thresholding the nuclei and analyzed using particle analysis. All particles with at least 30 square pixel area are counted.
Figure 5.3 Method to quantify collagen expression using ImageJ analysis, involves color deconvolution plugin and measuring the area at a common threshold value.

3.1.8 Second Harmonic Generation

A ZEISS 780 confocal laser scanning microscope was used to generate second harmonic generation (SHG) images of unstained 5µm thick tendon sections using 900nm Ti:Sapphire laser. Tiled images were obtained covering a 1.2x1.2mm area. For each sample, 6 images were obtained along the length of the tendon, covering 6mm area (Figure 5.4A). Image analysis was conducted as previously described (246, 247). Briefly, ImageJ was used to process images to FFT plots. A circular projection was drawn at the center of the FFT plot and ImageJ Oval Profile plugin was used to calculate pixel intensity along the radius of the circular projection. The summed pixel
intensities were plotted against the angle of acquisition producing a FFT alignment plot. FFT peak was used a measure of collagen alignment (Figure 5.4.B).

Figure 5.4. A) Protocol schematic for processing SHG images, using Fast Fourier Transformation analysis. B) Fast Fourier Transformation of SHG images to create FFT alignment graphs.

Transformation analysis.
3.1.9 Biomechanical Testing

Biomechanical testing was conducted using an Instron tensile 5869 machine. The foot of the animal was encased in block of orthodontic resin. In one set of studies, the muscle attachment was gripped by a custom device that was directly attached to the load cell. The device was fabricated using designs modified from Wieloch et al. (248). The original design contained compartments for liquid nitrogen. The modified design contained compartments to hold dry ice instead of liquid nitrogen as shown in Figure 5.2. The dry ice freezes the muscle attachment to prevent slippage. In another set of testing, the muscle was glued between sand paper. No differences were found between the set of test used with the custom cryoclamp or the sandpaper method. The tensile tests were conducted with ramp speed of 0.1mm/s using a rectangular geometry. Values for tensile strength and modulus were obtained.

3.2 Results

3.2.1 Cellular Attachment

Figure 5.5. Amount of cellular DNA present on scaffolds after seeding. Scaffolds dynamically seeded, after 24 hours showed highest amount of DNA concentration.
Dynamic seeding was shown to be superior in terms of DNA concentration when compared to static seeding thus indicating higher cellular attachment. The largest jump in the amount of DNA concentration was from 1 hour to 2 hours in static seeding and from 6 to 24 hours in dynamic seeding, as shown in Figure 5.5. Thus, to allow for the greatest amount of cell attachment, all scaffolds with donor cells were dynamically seeded for 24 hours prior to implantation.

3.2.2 Surgical Procedure

The study consisted of a total of 9 animals per group per time point. Surgical procedure is depicted in Figure 5.6. All animals survived surgery with no complications for the remainder of the study.

Figure 5.6. (i) Surgical area prepped for surgery (ii) Exposed Achilles tendon (iii) Plantaris tendon is separated and excised (iv) Achilles tendon is separated from surrounding tissue (v) A suture across the distal end of the tendon is placed prior to cutting the tendon (vi) A distance
of 5mm is measured distal to the calcaneous (vii) The tendon is cut (viii) Gap displayed between the two tendon ends (ix) Using a Kessler knot, the tendon ends are approximated (x) PCL/CA scaffold measuring 3x3mm (xi) The scaffold is sutured over the repaired area (xii) The scaffold over the repaired tendon (view 1) (xiii) The scaffold is over the repaired tendon (view 2) (xiv) The incision is closed (xv) Animal is mobile immediately after surgery.

3.2.3 Blood Glucose Levels

Figure 5.7 Glucose monitoring of rats showed no difference in glucose measurement between rats treated with and without insulin.

Glucose blood was collected from the rat tail vein weekly for 4 weeks, post-surgery. The blood was placed on a glucometer strip and the blood glucose measurement was taken. There was no difference in the glucose blood levels of animals treated with scaffolds with or without insulin. This indicates the implanted insulin did not have an effect on the overall blood glucose levels of the animals.
3.2.4 Gross Morphology

Figure 5.8. Gross morphology of tendons at 4 and 8 weeks.

The morphology of repaired tendons at 4 and 8 weeks showed increased scar tissue when compared to native uninjured tissue (Figure 5.8). All groups had overall increased tendon size when compared to the native tissue. At 8 weeks, the scaffold is still visible for all scaffold groups.

3.2.5 Cell Infiltration

Overall, higher cells number were observed on the scaffold areas when compared to nonscaffolds areas for all scaffold treatment groups (Figure 5.9). At 4 weeks, there was significant increase in cell infiltration in the cell seeded groups when compared to defect and suture repair group. At 8 weeks, insulin scaffold group had the highest number of cells and was statistically significant from the defect group (Figure 5.10).
Figure 5.9. Representative images of H&E stains at 4 and 8 weeks from treatment groups.

High cell infiltration into scaffold area can be seen in scaffold treatment groups.
Figure 5.10. Cell density tissues collected at 4 and 8 weeks. Note: native tendon has cell density of approximately 130 cells/.24mm$^2$. 
Figure 5.11. Relative expression of SRY on cell seeded scaffold groups. Results indicate minimal presence of donor cells after 4 weeks, post-surgery.

Donor cells contained male sex gene, sex determining region Y, (SRY). To see the amount of cells containing SRY, DNA collected from the tendon samples were collected and analyzed using qPCR. Within the DNA sample, at most, there was approximately 0.04% of SRY gene found from the tendons treated with insulin functionalized scaffolds. Though this was statically significant from the other scaffolds with donor cells, the very low percent indicates a very low chance of the presence of donor cells.

3.2.6 Trichrome Stain

At 4 weeks, all treatment groups displayed a statistically significant difference in the percent area of red (fuchsin) and blue (aniline) dye when compared to native Achilles tendon. Native tendon exhibited a strong red dye. At 8 weeks, an increase in red dye was observed with
scaffold treated groups. Defect and suture group maintained higher percent of blue dye, whereas, Ins scaffold and cell seeded groups had higher percent areas of red dye (Figure 5.11 & 5.12).

Figure 5.12. Representative trichrome stain of treatment groups showing whole tendon specimens.
### B

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<th>Suture Repair</th>
<th>Scaffold</th>
<th>Ins Scaffold</th>
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### C

#### Week 4

- **Average Percent Area**
- **Aniline**
- **Fussin**

#### Week 8

- **Average Percent Area**
- **Aniline**
- **Fussin**
Figure 5.13. A) A total of 12 FOV were analyzed to determine the relative percent of blue and red dye in the tissues. B) Representative trichrome stain FOV with corresponding H&E stain. C) Quantification of relative red (fuscin) to blue (aniline) stain.

3.2.7 Collagen Staining

At 4 and 8 weeks, there was an overall stronger staining for collagen III than collagen I for all groups. At 4 weeks, defect and suture repair groups showed the lowest staining of collagen I, when compared to all scaffold groups. Tendons repaired with insulin scaffold with cells had the greatest amount of collagen I staining, which was statistically significantly from non-scaffold groups and all other groups at 4 and 8 weeks, respectively (Figure 5.13). At 8 weeks, collagen III levels remained overall constant for all groups from 4 to 8 weeks. However, cell seeded groups exhibited statically significant collagen III staining when compared to all other groups at both 4 and 8 weeks (Figure 5.14). A higher ratio of collagen I to collagen III is observed on all scaffold groups with cells and insulin scaffold when compared to no repair, suture, or scaffold groups at 4 weeks. At 8 weeks, no repair and suture groups remain low in collagen I to III ratio. The insulin scaffold with cells group had the highest ratio at 8 weeks (Figure 5.15). Native tendon was found to have a collagen I to collagen III ratio of approximately 2.0.
Figure 5.14. A) 12 FOV for the measurement of Collagen I. B) Representative images for each group at 4 and 8 weeks. C) Quantification of collagen I staining. Markings indicate statistical significance compared to Ins Scaffold+ cells, unless otherwise indicated. *p<0.05, **p<0.01, ***p<0.001
Figure 5.15. A) 12 FOV for the measurement of Collagen III. B) Representative images for each group at 4 and 8 weeks. C) Quantification of collagen III staining. Markings indicate statistical significance compared to Ins Scaffold+ cells, unless otherwise indicated. *p<0.05, **p<0.01, ***p<0.001
Figure 5.16. A) Representative images of collagen I and collagen III of the same area. B) Quantification of the ratio of collagen I to collagen III.
3.2.8 Second Harmonic Generation

Second harmonic generation images were used to quantify the overall alignment of collagen fibers of the healing tendons at 4 and 8 weeks. Lower alignment was found with insulin scaffold and cell seeded scaffolds at early time point, 4 weeks. However, at 8 weeks significantly greater alignment was associated with cell seeded scaffold groups.

Figure 5.17. Quantification of SHG images indicating overall collagen alignment.

3.2.9 Biomechanics

At 8 weeks all groups displayed tendons that were thicker in width when compared to native uninjured tendon (data not shown). The thickening of repaired tendons is common phenomenon observed in injured tendons (249-251). At 8 weeks, treatment groups demonstrated a significant decrease in ultimate tensile stress and modulus when compared to native Achilles tendon. All treatment groups showed approximately 10% of the total tensile strength of uninjured tendon. However, no differences were found amongst the treatment groups in tensile strength,
indicating insulin scaffold and insulin scaffold with cells had no detrimental, or beneficial effect on the biomechanical properties of the tendon at 8 weeks. All tendon failed at the bone, where the tendon was removed from its bony attachment.

Figure 5.18. Mechanical testing set up of tensile testing. Bone end was encased in orthodontic resin and the muscle end was held by parallel plates with sand paper. Breakage for all groups occurred at the bony end, where the tendon detached from the bone.
Figure 5.19. A) Stress vs. elongation graph of treatment groups after 8 weeks of healing. B) Quantification of tensile stress and modulus of treated tendons showed no significant difference between groups.
Figure 5.20. Gene expression of pro-inflammatory and anti-inflammatory markers from tissues collected at 4 and 8 weeks.
Figure 5.21. Gene expression of matrix markers from animal tissues collected at 4 and 8 weeks.
3.2.10 Inflammation and Remodeling mRNA Expression

At 4 weeks, there was an upregulation of inflammatory markers IL-1β for all groups, with a decrease at 8 weeks. All other inflammatory markers were relatively more highly expressed at 8 weeks when compared to 4 weeks. There was a significant increase of Ly6c in the suture repair group at 8 weeks when compared to all others. The marker CCL2 was significantly higher in the scaffold group at 8 weeks compared to others and CD68 was significantly expressed by insulin scaffold+cell group when compared to defect, insulin scaffold, and scaffold+cell groups. The suture group expressed the highest expression of anti-inflammatory markers IL10 and CD168 at 8 weeks when compared to all other groups.

No statistical differences were found in the mRNA expression of tendon matrix markers between groups. However, expression of all matrix markers were elevated at 8 weeks when compared to 4 weeks.

4 Discussion

The goal of these studies was to evaluate the feasibility of insulin as a bioactive factor in promoting tendon healing. Our prior in vitro studies indicated that mesenchymal stem cells treated with insulin had increased expression of tendon markers as well as collagen synthesis (66). The results from the previous experiments have shown a positive effect of insulin in supporting the expression of tendon markers in mesenchymal stem cells (Chapter 3). Furthermore, the bioactivity of insulin was shown to be preserved in an insulin functionalized scaffold (Chapter 4). To determine the performance of the insulin functionalized scaffold in an in-vivo environment, the scaffolds were implanted into a rat tendon defect model, along with bone marrow derived mesenchymal stem cells. Prior to implantation, studies showed that dynamic seeding technique
resulted in higher scaffold cellular attachment when compared to static seeding. Moreover, an incubation period of 24 hours allowed for the greatest number of cell attachment (Figure 5.5). Due to these findings, all cell seeded scaffolds were dynamic seeded for 24 hours prior to implantation. During the study, animals who received treatment with insulin scaffolds did not show decreased glucose levels during a 4-week study period (Figure 5.6). This indicates the implanted insulin did not have a systemic effect on the animals.

At 4 and 8 weeks all groups displayed tendons that were thicker in width when compared to native uninjured tendon, as shown in Figure 5.7. The thickening of repaired tendons is common phenomenon observed in injured tendons (39-41). From the gross morphology, insulin scaffolds showed better tissue integration when compared to the plain scaffolds. Since the insulin scaffolds and plain scaffolds were made from the same material composition and handled the same way, it leads one to believe that the immobilized insulin may be creating a more biocompatible environment. A major factor in determining the biocompatible of an implant is the binding of nonspecific proteins to the surface of the scaffold (42). The rapid adsorption of proteins to an implant occurs immediately after implantation and can trigger an immune response (43). The presence of the immobilized insulin on the surface of the scaffold may have contributed to a lower binding of nonspecific proteins. There does not appear to be any differences in gross morphology between cell-seeded scaffolds and non-seeded scaffolds.

Within the scaffolds, cells were found to infiltrate throughout the entirety of the scaffold with vascularization present. This indicates the porosity of the scaffold was adequate for cell and vascular infiltration. A large body of evidence exists indicating the presence of donor cells or exogenous cells, promotes better healing and regeneration (30, 39, 45-47). However, the exact role of these donor cells remains elusive. Some studies have shown the continued presence of donor
cells weeks after implantation. Lee et al. observed fluorescently labeled human donor cells in a rat Achilles tendon defect after 4 weeks, along with human specific collagen type I and tenascin C (48). This indicates the implanted human mesenchymal stem cells contributed to the building of the extracellular matrix, which resulted in better healing. Others have suggested a paracrine role for donor cells (49-51). Caplain et al. describes the indirect effects of mesenchymal stem cells as trophic activity through release of growth factors and cytokines. Greater resolution of tendon repair was observed on rotator cuff supraspinatus tendons treated with exogenous mesenchymal stem cells, despite low presence of the donor cells, at six weeks (30). In the present study, at 4 weeks, less than 0.05% of DNA was evidenced to contain the male sex gene SRY (Figure 5.13). This indicates that by 4 weeks, the presence of donor cells was very low. However, repair groups that contained donor cells, showed higher increase of cellular infiltration within the scaffold at 4 weeks (Figure 5.14). Cell infiltration into the scaffold may indicate a higher efficacy of donor cells in encouraging cellular attachment and migration (40). The donor cells may have released factors that caused host cells to migrate to the area, resulting in greater cellular infiltration to the scaffold (52). Our studies did not include an evaluation of cells types, so it remains a question as to what types of cells infiltrated the scaffold. Future studies will need to be done to elucidate the cell populations present on the scaffold.

The expression of collagen is ubiquitous in tendon, as it makes up the majority of tendon tissue. Traditionally, trichrome stains collagen blue/green, whereas muscle fibers and erythrocytes stain red. However, it was observed that native tendons, with no injury stained brightly red. Similar findings were found in Martinello et al. where Masson Trichrome staining is suggested to be a useful measure of collagen spatial arrangement of tendon lesions (252). Healthy tendon was found to have a high percentage of fuchsin, red dye and low percent of aniline, blue dye. After injection
of collagenase, blue dye was observed, peaking at 30 days-post injection, with gradual decrease for the remainder of the study (120 days). This observed phenomenon of collagen fibers staining red in healthy tissue may be related to the affinity of the dyes to bind to the collagen fibers. Fuchsin dye binds to all tissues, whereas aniline dye preferentially dyes collagen fibers more readily. Thus collagen blue/green staining is the result of the displacement of fuchsin dye by aniline dye (253). Craik and McNeil were the first to report a change in dye affinity between stretched and relaxed collagen during trichrome staining, observing that stretched collagen had greater affinity to the red dye (254). Flint et al. attempted to elucidate the reason for this phenomenon (253). They postulate that in a relaxed tendon state, there is lower availability of positively charged binding sites for the anionic dyes to bind to, due to internal electrostatic pairing with adjacent negatively charged groups. However, in stretched or tensioned tendons, the ionic groups are sufficiently widely separated, such that extensive interactions within the tissue do not occur. Thus, there are more available active dye bindings sites that retain the red dye and would require significantly more time for color displacement to occur (253). If such is the case, trichrome staining may perhaps give an indication of the tensioned state of the tendon and subsequently it’s functional use. This is in alignment with the findings of Martinello et al. who found blue dye evident in degenerated tendons, as these tendons would presumably be experiencing lower loads than healthy tendons, which stained red. As the tendon heals, the animal may gradually be putting more force onto the tendon, thus resulting in a greater affinity for the red dye. Likewise, in this study, it may be the case that at 4 weeks, less tension was applied to the tendon at the earlier stages of healing resulting in majority blue staining for all groups. At 8 weeks, with greater healing, animals may have been able to apply more weight onto the leg, thus increasing tension in the tendon. Thus, greater fuchsin
staining in the scaffold treated groups may be an indication of increased tension and utilization of the tendon, compared to defect and suture repair groups.

Immunostaining of collagen I and collagen III provide more details of the ECM of the healing tendon. The deposition of collagen III is one of the initial steps in tendon healing and represents an unorganized collagen matrix. Collagen III is subsequently replaced by collagen I as the tendon heals and remodels resulting in a more organized and aligned matrix. Thus, the ratio of collagen I: collagen III is used as a measure of collagen organization (31). In our studies, native tendon had a ratio of 2. For all groups, at both time points, greater staining of collagen III was observed. Thus, collagen ratios remained below 1. At 8 weeks, higher ratios of collagen I to collagen III were found with all scaffold groups when compared to defect and suture repair. Moreover, insulin scaffold with cells had the highest ratio at 8 weeks. However, this trend was not significant owing to large standard deviations, which may be a consequence of error propagation that can result from calculated values. Nevertheless, the cell seeded insulin scaffold group demonstrated the highest ratio of collagen I, suggesting that the combination of insulin functionalized scaffold and cells may contribute to a higher organization of the ECM during the remodeling phase. Analysis of SHG images of the tendons corroborate these findings as greater collagen alignment was statistically higher with the insulin scaffolds with cells. However, neat scaffold with cells also demonstrated higher alignment, suggesting organization may be a result of donor MSCs, rather than insulin.

Cartilage cells were present in all groups. Cartilage cells were identified through rounded cell shape with large cytoplasmic area arranged in characteristic whorls (251). At four weeks, whorls were observed in samples and by 8 weeks, large cartilage nodule formations, along with bone were present in some areas of the tendons. The occurrence of cartilage formation was
independent of treatment and appeared at random. Endochondral ossification is a well-documented phenomenon that occurs with tendon tenotomy (251). Rooney et al. suggests tendon cells undergo metaplasia following injury and produce regions of fibrocartilage. Future studies will need to address this issue. Future studies will need to address this issue.

Disorganization the ECM has been linked to scar tissue with inferior mechanical properties. Conversely, higher organization of the ECM generally indicate better tissue healing and improved mechanical properties. However, though histological data suggested greater organization of the tendon ECM associated with the insulin+cell group, this was not translated to an increase in biomechanical strength. All treatment groups showed significantly lower mechanical properties than native tendon in both tensile stress and elastic modulus at 8 weeks. Several studies have reported positive histology data with no biomechanical gains at late time points (179, 255, 256). Significant gains in biomechanical properties are often found at early time points up to two weeks (256). Early gains in mechanical strength, but not at later time points may be due to several reasons including waning effects of treatment. Since the scaffold was functionalized with insulin, it may be possible that an increase in cells and ECM matrix made it more difficult for cells to gain access to the insulin protein, thus not being beneficial at later time points. Furthermore, 8 weeks may have been a long enough period where any differences between groups disappeared due to the normal course of healing. Mechanical testing at an earlier time point may produce differences among the groups. Additionally, it may be the case that histological gains from insulin+cells group were not substantial enough to make a difference in the biomechanical properties of the tendon. Though scaffolds were found to be adequate in promoting collagen in-vitro environment, the in-vivo environment presents a completely different dynamic and may warrant separate dosing optimization experiments in an in-vivo setting.
Pro-inflammatory and anti-inflammatory markers were evaluated to render immunomodulatory information regarding insulin and BMSCs (Figure 5.19). In evaluating the mRNA expression of tendon samples, due to large standard deviations, there were few significant differences within the treatment groups. However, trends indicate temporal expression of markers. There was a higher expression of IL-1β, a classical pro-inflammatory marker at 4 weeks when compared to 8 weeks. All other markers tended to be upregulated at 8 weeks. This includes the pro-inflammatory markers, Ly6c, CCL2, CD68, CCR7, and IL-6 and anti-inflammatory markers, IL-10 and CD168. The upregulation of both pro- and anti-inflammatory markers may indicate that there is a heterogeneous population of cells and macrophages in the healed tissues. Evaluation of ECM markers also showed upregulation at 8 weeks for all groups (Figure 5.20). The large standard deviations prevented any statistical differences between the groups. A higher sample number in future studies will help clarify and elucidate any immunomodulatory effects of insulin and BMSCs.

To the best of our knowledge, this is the first studies to evaluate the feasibility of insulin for tendon healing and regeneration in an *in-vivo* model. In general, it was observed that tendons healed with insulin in combination with mesenchymal stem cells showed increased expression of collagen I and overall greater organization of the ECM through collagen alignment. Though the present study provided some evidence for the potential use of insulin functionalized scaffolds in a tendon injury, future studies should focus on optimizing the *in-vivo* insulin dosage, as well as the immunomodulatory effects of insulin and BMSCs.
Chapter 6: Summary and Future Directions

Surgical treatments for tendon injuries are prone to retears and have shown to be limited in functional recovery (28). New advances in the field of tissue engineering opens new possibilities in the management and treatment of tendon injuries through the use of biomimetic scaffolds, stem cells, and biomolecules. To date, no standardized media has been confirmed for the differentiation of stem cells to tendon lineage, however studies have shown the use of various growth factors to induce tendon cell phenotype, as well as collagen synthesis. Insulin-like growth factor-1 (IGF-1) has consistently been shown to induce collagen synthesis and to improve tendon healing (51, 52). However, the clinical use of growth factors is limited due to the difficulty in obtaining FDA approval, short half-life, and potential risks such as cancer (257). Thus, the overall investigative focus of this thesis was to determine the efficacy of insulin, a homologous molecule to IGF-1, in tendon differentiation and tendon healing.

In Aim I, mesenchymal stem cells were treated with insulin supplemented media, as a one-time dose or continuous dose. Data showed mitotic effects of insulin to be minimal at concentrations below 1ug/mL. Preliminary data showed increased metabolic activity of cells treated with 1ug/mL or higher of insulin after 24 hours. In terms of differentiation, continuous treatment of insulin at a minimum of dose of 100ng/ml concentration increased and maintained expression of tendon cell and ECM markers. The data supports that insulin treatment can be used to enhance the differentiation of mesenchymal stem cells towards tendon phenotypic development.

In Aim 2, PCL/cellulose acetate blend scaffolds were fabricated and characterized for their ability to act as a cell and insulin delivery vehicle. A composition of 75:25 ratio of PCL to cellulose acetate was found to be optimal amongst the groups tested for maximum cell and protein delivery, as well as mechanical property. Insulin was chemically linked to the scaffold surface via amide
bonding. In this way, insulin is tethered to the scaffold and is not readily released to avoid burst release of insulin into the body. Biological studies showed mesenchymal stem cells seeded on insulin functionalized scaffolds expressed tendon markers similarly to findings found in Aim 1, using insulin supplemented media. Thus, the insulin protein on the functionalized scaffold remained bioactive.

Lastly, in Aim 3, the performance of the insulin functionalized scaffold was evaluated \textit{in-vivo}, in a rat Achilles tendon model. The results of the animal studies showed significantly more collagen staining and greater ECM organization with tendons treated with the insulin scaffold in combination with mesenchymal stem cells. Disorganization the ECM has been linked scar tissue with inferior mechanical properties. Conversely, higher organization of the ECM generally indicate better tissue healing and improved mechanical properties. However, no significant differences were found in terms of tensile strength or modulus among the treatment groups at 8 weeks. Several studies have reported positive histological data, with minimal mechanical gains at later time points (ref). A limitation of the study includes the lack biomechanical testing at earlier time points. Differences in biomechanical properties have been reported at 2- and 4-week post injury. Future studies should look at performing biomechanical testing at earlier time points.

The combination of insulin at the wound site and donor mesenchymal stem cells did appear to improve tendon healing through greater organization of the ECM. The presence of donor cells is hypothesized to have a paracrine effect in recruiting native cells to the wound site, whereas insulin is hypothesized to increase collagen synthesis, similar to the effects of IGF-1. The role of exogenous or donor cells are not yet fully understood. Several studies suggest donor cells take an active role through direct ECM protein synthesis, while others support a more indirect role through paracrine signaling. The role of macrophages in tendon healing have also been investigated and
found to be involved during the inflammatory and remodeling stages. Monocytes, along with neutrophils quickly infiltrate the tendon injury within the first 24 hours and after several days, the monocytes differentiate into macrophages. Two phenotypes of macrophages have been observed. Macrophages in the early stages are needed to digest necrotic materials. These macrophages are designated as M1 and are considered pro-inflammatory as they promote the expression of inflammatory factors such as IL-1, TNF-α, IL-6, and nitric oxide. The other macrophage phenotype, known as M2 is considered to be anti-inflammatory and helps to coordinate ECM deposition and tissue repair. Recently published work by Zheng et al. studied the effect of bone marrow mesenchymal stem cells on the polarization of macrophages (258) and reported that co-culture with MSCs reduced macrophage differentiation into M1 phenotype and reduced inflammation. Sugg and colleagues noted that the expression of M1 markers peaked at 2 weeks post Achilles tendon transection, whereas expression of M2 markers appeared at 4 weeks (259). Thus, future studies looking to gain mechanistic insight into the role of insulin, donor MSCs, and their combination on tendon healing, should look at early gene and protein expressions at 1-4 week post-surgery.

Collectively, the data supports the potential use of insulin in tendon repair and regeneration. More studies should be done to elucidate the mechanism in which insulin imparts its beneficial effects. There are three possible scenarios in which the insulin can be activating cellular mechanisms. In one scenario, insulin is binding to the IGF-1 receptor and propagating the IGF-1 pathway. As previously mentioned, in the excess of insulin, insulin has a slight affinity to the IGF-1 receptor. It has been proposed that therapeutic effects of IGF-1 on tendon healing is due to its activation of the P13k/Akt pathway, which promotes MSCs growth, mobilization and survival. Additionally, the possibility that insulin is binding to its own receptor cannot be ruled out. There
is evidence for anabolic actions of the insulin receptor for other musculoskeletal tissues such as bone and cartilage (153, 260). Thus, it is likely that the insulin receptor may also promote tendon ECM growth. Lastly, and perhaps the most complicated, would be the scenario in which the insulin molecule is binding to a hybrid receptor of insulin and IGF-1. Hybrid receptors of insulin and IGF-1 are known to occur, but to what degree is still unknown. Interestingly, when IGF-1 receptors were removed from osteoblastic cells, enhanced signaling through the insulin receptor was observed. Authors predict that the increase in insulin receptor activity is due the lack of hybrid receptors that work to dilute the presence of insulin receptor homodimers (133). Mechanistic studies can be conducted through inhibition of receptors and seeing if the effects of insulin are preserved. Similar studies have been conducted in attempts to elucidate the separate effects of insulin and IGF-1. Additionally, since many mechanistic studies on the insulin family have been conducted mainly on terminally differentiated cells such as hepatocytes (69), cartilage (153), and osteoblast cells (144), mechanistic studies on mesenchymal stem cells would provide novel information. Few studies have been conducted with insulin treatment on MSCs for tendon applications. For example, the relative abundance of insulin receptors and IGF-1 receptors on mesenchymal stem cells have not yet been investigated, but may provide better understanding of the interaction of insulin on mesenchymal stem cells.

Current work in the lab is investigating the potential of exendin-4, a novel antidiabetic agent which shares 53% homology with human glucagon-like peptide-1 (GLP-1). Exendin-4 was originally developed to improve glucose control, however recent studies have shown that it may also have a proliferative effect on cells (261-263). Ongoing studies in the lab are looking at the effects of exendin-4 as bioactive factor to aid in tendon regeneration. Current studies show positive results from exendin-4 treated cells with increased expression of tendon markers at the gene and
protein levels. Further studies can also look at insulin analogues as bioactive molecules. Structural modification of the insulin molecule has the potential to change its binding affinity to the IGF-1 receptor. Modifications to the B-chain of insulin molecules increases its structural homology to IGF-1 and thus its affinity to the IGF-1 receptor (264, 265). Various insulin analogues has shown to have increased mitotic effects (266, 267). Interestingly, Hansen et al. reports the increased mitogenic potency of insulin analogues may not be due to a greater affinity to the IGF-1 receptor, but rather due to slower dissociation from the insulin receptor. The sustained activation of the insulin receptor tyrosine kinase results in the sustained phosphorylation of intracellular Shc protein, which is believed to be the cause of increased mitogenic activity (266).

Another interesting avenue to explore is the efficacy of insulin on healing tendon injuries in diabetic conditions. Tendon injury studies on diabetic rats show compromised tissue healing when compared to healthy animals. Lower healing may be attributed to altered expression of collagen and MMPs (268), as well as lower fibroblast proliferation and decreased lymphocyte infiltration (269). It would be interesting to investigate if the application of insulin functioned scaffold on a tendon injury of a diabetic rat, improves tendon healing and biomechanics. Our findings have shown the feasibility of insulin to positively affect tendon healing and regeneration. Future in-vivo studies should also evaluate the effectiveness of different insulin loadings. The current work did not evaluate the optimal in-vivo dosing. It may be possible to achieve increased benefits of insulin at higher loading concentrations. However, care should be taken to limit the amount of insulin delivered to the animal within the range that does not affect the metabolic system of the animal.

Another direction to explore is the possibility for insulin to be used for tissue engineering of interfacial tissues. Previous work has supported the use of insulin for bone, cartilage, and muscle
growth (reviewed in Chapter 3). The current work showed evidence for insulin for tendon regeneration. The wide availability and relatively cheaper costs of insulin compared to traditional growth factors makes insulin an attractive bioactive molecule. Relative concentrations seem to modulate its effect on cells. Higher concentrations have been used for bone applications, while concentrations for tendon tend to be lower. Insulin concentrations for cartilage differentiation are intermediate of those used in bone and tendon. Thus functionalizing a scaffold with an insulin gradient may promote better healing of interfacial tissues such as at the bony attachments of tendons and ligaments or at the interface cartilage and bone tissues.

Further work on improving the scaffold design for mechanical strength should also be considered for clinical translation. The current study focused on the feasibility and delivery of insulin in tendon healing, thus the scaffold was placed over the repaired tendon injury to act as a patch, rather than as a bridge, augmenting two tendon ends. Thus, the current study did not take into consideration the required mechanical strength needed to augment a tendon segmental defect. To improve the mechanical strength of the scaffold, multiple layers of electropsun sheets can be fabricated together into one unit. The layers may be altering sheets of micro- and nanofibers so as to impart mechanical strength on the scaffold from the microfibers and increased surface area and bioactivity from the nanofibers.

Since its discovery in the 1920s, insulin has been extensively researched in terms of its protein structure and metabolic actions. The advent of biotechnology to synthesize recombinant insulin has made a dramatic impact in the lives of diabetic patients, as well as its availability. Despite the wealth of knowledge of this protein, new discoveries of its anabolic effects on tissues have just come to light in recent past. This seemingly humble protein may have undiscovered potential in tissue engineering.
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Appendix A: List of Publications

Research Articles:


Appendix A: List of Publications


Book Chapters:


Oral Presentations:


Appendix A: List of Publications

**Posters Presentations:**

19. Abdumalik SR, D ; Rudraiah, S ; Mazzocca, AD ; Kumbar, SG., editor. Evaluation of GLP-1 Receptor Agonist Exendin-4 on Tendon Regeneration. Biomedical Engineering Society 2017; Phoenix, AZ


Appendix A: List of Publications


Daisy Ramos is a strong advocate for STEM and STEM education. As a first generation college attendee, she has experienced many of the challenges students face in pursuit of higher education. She is fortunate to have had a number of mentors throughout her educational career who have supported and advised her on her interests in STEM.

At an early age, Daisy was exposed to the field of biotechnology and how it relates to the agricultural scenery of her home state of Hawaii. Dr. Kabi Neupane, from the local community college, served as her advisor on her high school research project that looked to create a testing tool to evaluate the extent of transgenic contamination of non-GMO papaya crops in Hawaii. This early research experience, along with her experiences in the Upward Bound Math and Science Program at Leeward Community College, inspired Daisy to pursue education in the sciences.

During her last year in high school, Daisy applied to the QuestBridge College Match Program, a non-profit organization aimed at bridging low-income students with scholarship opportunities at US colleges and universities. Through this, she received a scholarship to attend Trinity College in Hartford, CT. At Trinity College, Daisy was invited to enroll in the Interdisciplinary Science Program headed by the Science Director, Dr. Alison Draper. It was through this program, Daisy had the opportunity to conduct electrophysiology research in Dr. Harry Blaise’s lab, where she studied the effects of high electrical brain stimulation on memory formation in rats. It was there where Daisy’s interest in biomedical research grew.

After receiving her Bachelors of Science degree in Biomedical Engineering, Daisy was recruited to the Materials Science and Engineering Program at the University of Connecticut through the Graduate Assistance in Areas of National Need (GAANN) fellowship. She was accepted into the labs of Dr. Sangamesh Kumbar and Dr. Cato Laurencin, where she went on to conduct her doctoral research on which this thesis is based on.

Throughout her doctoral studies Daisy mentored a number of high school and undergraduate students, as well as engaged in a number of local activities promoting and supporting STEM education. She believes early awareness and exposure, as her own personal experiences, are essential to increasing the number of women and minorities in STEM.

Following the completion of her dissertation, Daisy plans on taking some time to reacquaint herself to the white, sandy beaches of home and enjoy some of her hobbies like baking, and eating local cuisines, before heading back to the Mainland.