Endothelial Progenitor Cells for Rotator Cuff Healing

by

Hui Tung Tony Lin

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Abstract

The avascular environment at the tendon-bone interface is a major concern for rotator cuff healing, contributing to the high re-tear rate. We hypothesized that better tendon-bone healing would occur with transplantation of endothelial progenitor cells, a population of angiogenic cells, during rotator cuff repair in rats. The surgical procedure involved a unilateral detachment and repair of the supraspinatus tendon in a syngeneic population of Fischer 344 rats. The EPC group received one $10^6$ EPCs and the Fibrin (negative control) group received none. Biomechanical and histological analyses were performed at two, four, and six weeks post mortem. At 6 weeks, there was a statistically significant difference in the tendon attachment strength. While no significant difference was found in histology, the EPC groups showed a trend of increased vascularity. The EPCs provide improvements in tendon-bone attachment 6 weeks after the repair. However, such biomechanical improvement was not detectable via conventional histological methods.
Acknowledgments

I owe my deepest gratitude to my supervisors and mentors, Dr Emil Schemitsch and Dr Aaron Nauth, for their guidance, encouragement, and support. The years in the Musculoskeletal (MSK) Research Laboratory at St Michael’s Hospital (SMH) have been a wonderful learning experience. My graduate study has been challenging at times, but Dr Schemitsch and Dr Nauth were always positively optimistic. Their dedication to research and scholarship is nothing but inspirational. Without their supervision and mentorship, a suffix “MSc” after my name would not have been possible.

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The F344-Tg(UBC-EGFP)F455Rrrc (RRRC#: 307) rats were provided by the Rat Resource and Research Center P40OD011062, Columbia, MO, USA.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>AcLDL</td>
<td>Acetylated-low density lipoprotein</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblastic growth factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCN</td>
<td>Connective tissue growth factor/cysteine-rich 61/nephroblastoma over-expressed protein</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3'-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DiI-AcLDL</td>
<td>1,1'-dioctadecyl -3,3,3',3' -tetramethyl-indocarbocyanine perchlorate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGM2-MV</td>
<td>Endothelial Growth Medium 2-MV</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Flk-1</td>
<td>Fetal liver kinase-1</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescent minus one</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent protein</td>
</tr>
<tr>
<td>GH joint</td>
<td>Glenohumeral joint</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>Membrane type 1 matrix metalloproteinase</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule 1</td>
</tr>
<tr>
<td>PRFM</td>
<td>Platelet-rich fibrin matrix</td>
</tr>
<tr>
<td>PROM-1</td>
<td>Prominin 1</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>Scx</td>
<td>Scleraxis gene</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIMMP</td>
<td>tissue inhibitors of matrix metalloproteinase</td>
</tr>
<tr>
<td>UEA-1</td>
<td>Ulex europaeus 1 agglutinin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR-2</td>
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1 Introduction

Rotator cuff pathology can cause serious disability in occupational and athletic settings as well as a major functional impairment for those performing their daily activities. Rotator cuff pathology is the most common soft tissue injury of the shoulder, and is ranked only second to back and neck pain in workplace related injuries \(^8^6\). Over stressing of rotator cuff muscles by excessive load during repetitive overhead motion or as a result of acute trauma can cause a rotator cuff tear. Depending on the severity of the tear, surgical repair may be necessary to restore rotator cuff function.

With more than 75,000 surgical repairs of the rotator cuff performed annually at an average cost of $10,605 per operation \(^1^9^5\), the health care utility cost of rotator cuff treatment is estimated to be over 800 million US dollars alone. The overall economic burden would be much higher as rotator cuff disability limits physical movement and can have significant impact on work productivity and sick days. It also compromises daily living activities and severely reduces the quality of life.

1.1 The Rotator Cuff

The range of motion of the glenohumeral (GH) joint is second to none in the human body. Ligaments and capsule at the joint provide passive resistance to instability. Active stabilization is
primarily provided by a group of four muscles and their tendons, collectively termed the rotator cuff. From anterior to posterior, these muscles and tendons are the subscapularis, supraspinatus, infraspinatus, and teres minor. Each of the four muscles and tendons are responsible for the fine control and the dynamic stability of humeral movements. They centre the humeral head to the glenoid and prevent excessive glenohumeral translation in addition to rotating the humeral head about the GH joint. Weak or damaged rotator cuff muscles often lead to limited shoulder range of motion, poor GH joint stability, and compromised function at the shoulder.

1.1.1 Anatomy and Function

The rotator cuff originates from various bony surfaces of the scapula and inserts into the proximal region of the humerus. The subscapularis muscle originates from the medial two-thirds of anterior scapula, which is commonly termed the subscapular fossa. It inserts onto the lesser tuberosity of proximal humerus. As the sole anterior component of the rotator cuff, the subscapularis muscle serves to internally rotate and flex the humerus at the GH joint as well as limit anterior translation of the humeral head. On the posterior surface of the scapula, the infraspinatus and the teres minor muscles are external rotators and extenders of the humerus. The two muscles originate from the medial aspect of the infraspinatus fossa and insert into the middle and lower facet of the greater tuberosity of proximal humerus.

The most superiorly situated muscle of the rotator cuff is the supraspinatus muscle. Originating from the postero-superior surface of the scapular that is superior to the scapular spine, the supraspinatus muscle inserts onto the superior facet of the greater tuberosity. The tendinous
insertion at the greater tuberosity is not completely separate from infraspinatus and teres minor. It primarily participates in the abduction of the humerus as well as limits, to some extent, inferior translation of the humeral head. In addition, the supraspinatus muscle compresses the humeral head against the glenoid fossa when the arm is in an adducted position; the inferiorly directed component force serves to depress the humeral head. Due to the complex nature of the bony structures at the shoulder, the supraspinatus muscle/tendon passes through the subacromial space. The distal portion of the supraspinatus tendon is wrapped around the contour of the articular surface of the humeral head, where the articular surface of the tendon is subjected to compressive forces in addition to tension during the initial phase of humeral abduction.

The subacromial space is defined by the acromion, the acromioclavicular joint, the clavicle, and the coracoid. Situated between the acromion process and the rotator cuff tendons are the subacromial and the subdeltoid bursae that help to reduce friction between the structures. In addition to padding, the bursae also take part in the inflammatory response and provide a potential vascular source during rotator cuff healing. However, adhesion of the subacromial bursa to the rotator cuff tendons can effectively narrow the subacromial space and cause impingement of the tendons.
Figure 1-1. Cross-section (coronal plane) of the shoulder illustrating the relationships of the glenohumeral joint, the joint capsule, the subacromial bursa, and the rotator cuff (supraspinatus tendon). Figure adapted from http://what-when-how.com/rheumatology/periarticular-disorders-of-the-extremities-disorders-of-the-joints-and-adjacent-tissues-rheumatology/}

1.1.2 Healthy Tendon Insertion

A typical healthy tendon consists roughly of 20% cellular material and 80% extracellular matrix by volume. It is a regular dense connective tissue with relatively few resident cells known as
tenocytes. Tenocytes are responsible for producing the extracellular components of the tendon matter.

A healthy tendon is 55 to 70% water by weight\textsuperscript{96,162} and 95% of its dry mass is type I collagen. Type III collagen accounts for less than 5% of the tendon dry mass in a normal cadaver rotator cuff tendon. Together, the collagen molecules comprise the majority of solid substance in the extracellular matrix. The ratio of type III to type I collagen, however, increases with the severity of degeneration, tendon injury, and age\textsuperscript{102}.

In addition to the collagen molecules, the extracellular matrix contains a number of various macromolecules, termed ground substances, which help stabilize the structural and material properties. These major ground substances can be classified into two large molecular families, proteoglycans and glycoproteins. Proteoglycans are shaped like a bottle brush: a core protein with various glycosaminoglycan chains stemming from it. Glycosaminoglycans are linear polysaccharides containing repeating units of disaccharides. The negatively charged proteoglycan molecules are hydrophilic, and thus help retain water in the tissue. Of the proteoglycan family, decorin and aggrecan are in the tendon and the fibrocartilage\textsuperscript{144}.

The glycoproteins are branched molecules which are responsible in maintaining and repairing the tendon structure. These structural glycoproteins facilitate interaction between cells and their neighbours as well as cell adhesion to the specific tissues. For instance, fibronectin provides binding sites for fibroblasts and collagen, and chondronectin is essential for adhesion of chondrocytes to type II collagen.

Macroscopically, as the rotator cuff tendons approach their skeletal attachment, their extracellular matrix composition changes. The skeletal attachment of the rotator cuff tendons are fibrocartilaginous in nature. At a mature fibrocartilaginous enthesis, there are four distinctive,
but continuous zones of specialized tissues: dense fibrous connective tendinous tissue, unmineralized fibrocartilage, mineralized fibrocartilage, and bone. This transition zone is commonly referred to as the enthesis, footprint, osteotendinuous junction, or tendon-bone interface. Microscopically, the biochemical composition in the extracellular matrix at the tendon insertion reflects mechanical demands present at the soft and hard tissue interface. The interface facilitates the force transmission from tendon to the bone while resisting bending forces when the line of pull is not parallel to the insertion.

The tendon-bone insertion is where the two type I collagen-rich tissues, the bone and the tendon, join. At this interface, the calcified and uncalcified fibrocartilage is rich in type II collagen, and thus presenting a gap phenomenon with immunohistochemical staining of collagen molecules. Other ground substances such as glycosaminoglycans and proteoglycans help to dissipate the bending forces away from the insertion to the more flexible tendon body.
Figure 1-2. Pictorial representation of the tendon-bone transition (safranin O staining). At a mature fibrocartilaginous enthesis, there are four distinctive, but continuous zones of specialized tissues: dense fibrous connective tendinous tissue, unmineralized fibrocartilage, mineralized fibrocartilage, and bone.
1.1.3 Vascular Supply to the Rotator Cuff

The head of humerus receives its blood supply mainly from the axillary artery. Anatomic studies have demonstrated that the axillary artery branches into six vessels, with the suprascapular and the anterior and posterior humeral circumflex arteries being the largest contributor of blood supply to the head of humerus $^{148}$. The ascending arcuate branch of anterior humeral artery provides the major source of proximal humeral vascularity and the greater tuberosity and the insertion of the cuff tendons. Immediately proximal to the supraspinatus tendon insertion, there is an identified anastomotic area known as the critical zone where vascular deficiency is observed. Most degenerative changes and tears occur in this area. It is believed that the avascularity at this region compromises the tendon’s metabolic activity and its ability to heal after micro-trauma. Studies have identified that this critical zone in the supraspinatus tendon renders it prone to tear $^{43, 100, 142, 148}$. A possible decrease in vascularity was observed in patients up to six months after surgery $^{50}$. 
Figure 1-3. The vascular anatomy of the rotator cuff insertions (adapted from Rothman. Clin Orthop Relat Res. 1965 Jul-Aug;41:176-86)
1.2 Rotator Cuff Pathology

While the etiology of pathological changes are still debated, the current proposed causes for rotator cuff diseases fall under two categories, intrinsic and extrinsic factors. Intrinsic changes suggest the alteration of tendon chemical environment which compromises the structural integrity. Extrinsic insults, on the other hand, describe the mechanical influences that result in structural wear and tear. These factors, in combination, contribute to the pathological progression in rotator cuff degeneration.

1.2.1 Clinical Spectrum of Disease

The major clinical presentations of rotator cuff diseases include impingement, chronic tears, and acute tears. Primary impingement implies a decrease in the subacromial space in which the cuff tendons passes through. Increased friction with the surrounding tissues causes accelerated wear and tear. The symptoms show characteristics of Neer’s three-stage classification for impingement: (I) edema and hemorrhage, (II) fibrosis and tendinitis, and (III) degeneration, bony changes, and tendon ruptures. Instability at the glenohumeral joint capsule or lagging of scapular stabilizers can result in unfavourable humeral head translations, and consequently, secondary impingement of the cuff tendons. Excessive contact with the bony structures such as the postero-
superior glenoid rim frequently causes partial tearing of the supraspinatus and infraspinatus tendons.

Long term repetitive use in overhead motions combined with the inherent poor blood supply to the rotator cuff tendons can lead to degenerative changes and ultimately chronic tears. The disease here manifests in poor tendon property and compromised healing capacity, commonly seen in overhead workers and the elderly population. In contrast, acute tears of the rotator cuff tendons are often the result of traumatic incidences such as glenohumeral joint dislocation.

Incidences of rotator cuff tears have been observed in cadaveric specimens over the age of 50 to be between 39 and 60% \(^{40}\). It was estimated that the prevalence of rotator cuff tears may be up to 40% of asymptomatic individuals over the age of 50 \(^{202}\). Recent magnetic resonance imaging studies of rotator cuff tears in populations reported that 37% of the patients had tears of the supraspinatus and infraspinatus tendons \(^{154}\), and found that the majority of these tears (70%) were at the rotator cuff insertion \(^{194}\).

Clinically rotator cuff tendon tears are categorized into either partial-thickness or full-thickness (complete) tears. The Ellman classification \(^{47}\) of partial-thickness tears indicates the location (articular, bursal surfaces, or intrasubstance) of the tear. Then according to severity, the tears are sub-classified into Grade I tear (tears less than 3 mm deep), Grade II lesion (tears 3 to 6 mm deep), and Grade III lesion (tears greater 6 mm deep). Here the normal cuff tendon thickness is considered to be between 10 to 12 mm. If, however, the tears are full-thickness, a separate but similar classification system, the Cofield classification \(^{41}\), is used. A full-thickness tear suggests a complete tear involving one or more cuff tendons. A small full-thickness tear is less than 1 cm in size, a medium tear between 1 to 3 cm in size, a large tear between 3 to 5 cm, and a massive tear greater than 5 cm.
1.2.2 Pathophysiology of Rotator Cuff Disease

Intrinsic Factors of Rotator Cuff Disease

From a histolopathological point of view, rotator cuff tendinopathy often manifests in degenerative changes, and is widely believed to be the major factor leading to rotator cuff tendon tears. Structural and chemical alterations can be detected using biochemical and histological analyses. It is uncertain, however, if degeneration is the initiating pathology or secondary to other conditions such as impingement or chronic overuse.

The proposed intrinsic factors include the cuff quality reflected by the collagen composition and the presence of fatty infiltration, cytokines and inflammatory markers, tendon remodeling molecules the matrix metalloproteinases and their inhibitors, and genetics, diet, and lifestyle factors.

i. Rotator cuff quality

To determine the pathological progression of rotator cuff tendinopathy, markers of degeneration are used to assess the tendon quality. Signs of degeneration can manifest in the absence of inflammatory cells, lack of fibril structural organization, atrophy of fibrils, changes in cellularity, cell apoptosis, glycosaminoglycan accumulation, and fatty infiltration. With no changes in the expression of cytokines and receptors, however, inflammation is absent in the degenerative state. It is not known whether inflammatory response has taken place in earlier stages. Evidence
suggests that the extracellular matrix turnover in chronic tendinopathy reflects the cell-mediated remodeling process, but with prolonged impaired remodeling and gradual deterioration. Those tendons suffering from injury or degeneration have a higher ratio of type III to type I collagen. High levels of type III collagen, along with increased hydroxylysine content and excessive cross-links in collagen fibers, are characteristic features of mechanically weaker scar tissue. In addition, changes in water content can be indicative of tendon pathology. Injured tendon will synthesize more extracellular matrix proteins in an attempt to maintain structural integrity. Consequently, these proteins draw water into the tendon, thereby altering the mechanical property.

Torn rotator cuff tendons may lead to degenerative fat accumulation in the corresponding muscles. Fatty infiltration reduces tendon quality and predisposes repaired tendon to re-tear. The degree of fatty infiltration is associated with the severity of the tear, the type of tendon lesion, the age of the patient, and the chronicity of the tear. While accumulation of fat can be arrested upon successful repair and restoration of tendon function, the existing fatty content is not always reversible.

ii. Cytokines and inflammation

In the early phase of inflammatory response, the key modulators are the signaling molecules – cytokines. They act as molecular agents to promote cellular proliferation, metabolism, apoptosis, and extracellular matrix protein turnover in tendon tissue. The degree of inflammation and angiogenesis, however, is inversely proportional to the age of the patient, particularly on the articular side. In degenerative tendinopathy, imbalances of cytokine levels and inflammatory responses fails to trigger repair in the tendon, resulting in poor tendon quality. Chronic
overuse and repetitive stress significantly reduces the number of resident tendon cells, causing impairment to the tendinous repair process. Specifically, oxidative stress, inflammatory cytokines and regulators, and apoptotic mediators are potential factors for stress-induced apoptosis of fibroblast-like cells. The inflammatory response is a double edged sword which may initially be detrimental to the healing of tendon and triggering tendon cell apoptosis, but the process is essential for the repair of tendon tissue. The absence of acute inflammation results in poor clinical outcome in pathologic supraspinatus tendons. The relations between tendon degeneration, tendinopathy, and inflammatory responses have yet to be clearly delineated.

Growth factors are biochemical molecules which stimulate cellular activities, namely in proliferation and differentiation. Researchers have been trying to identify the roles of the various growth factors that contribute to the process of tendon-to-bone healing. Well-defined spatial and temporal expression of relevant growth factors will aid the progress in targeted treatment modalities. The early phase temporal expression of growth factors and tendon-associated proteins were examined at the tendon-bone interface in a rat supraspinatus repair model. These proteins include basic fibroblastic growth factor (bFGF), bone morphogenetic proteins (BMP) -12, -13, and -14, cartilage oligomeric matrix protein, connective tissue growth factor, platelet-derived growth factor-β (PDGF- β), and transforming growth factor-β (TGF- β). Their temporal profiles are thought to correspond to the various stages and the associated responses of acute tendon-bone healing. Increased levels of these proteins were found in the first week post-operation, but the levels returned to the normal values by four months. These growth factors have been independently investigated in many studies as well (see Biology of Rotator Cuff Healing section).
iii. Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases

Tendons undergo continuous remodeling, and the tendons that experience high mechanical stress such as the supraspinatus tendon will exhibit a greater extracellular matrix protein turnover rate than those subjected to lesser stress, such as the distal biceps tendon. Extracellular matrix metabolism is a balance between catabolic and anabolic activities. The major class of enzyme in tendon degradation is the matrix metalloproteinases (MMPs). Their activity is rivaled by tissue inhibitors of matrix metalloproteinases (TIMPs). MMPs are found at low levels in normal tendon tissue but high amounts are seen in injured tissues. Elevated MMP-1 synthesis in tendon cells is associated with up-regulation of cytokines and inflammatory modulators. The expression of TIMMMPs acts in a reciprocal manner to MMPs in torn tendons. The imbalance between these extracellular matrix enzymes and inhibitors reduces tendon quality and results in the progression of degeneration.

iv. Genetics, lifestyle, and diet

Other possible factors which lead to degenerative changes in the rotator cuff tendons have been investigated. Studies have proposed genetic predisposition to the pathogenesis of rotator cuff disease. For instance, Harvie and co-workers found that familial influence doubled the risk of developing rotator cuff tears and increased the chance to experience symptoms five folds between patients and their siblings. Not surprisingly, lifestyle choice such as smoking increases tearing risk in a dose-dependent manner even after adjustment for age. Longo et al investigated rotator cuff tears in association to patient lipid and glucose profiles. In both the
prospective serum lipid level study and the case control plasma glucose study, there seemed to be a trend towards a correlation between high values and the prevalence of rotator cuff tears \(^{101}\). While no direct histological or pathological correlation was made, the studies alluded to macro-circulatory and micro-circulatory impairment mediated tendon degeneration. However, atherosclerosis, a macro-circulatory disease, did not correlate with rotator cuff tears \(^{44}\). Lastly, hypertension is believed to be a potential factor for rotator cuff tear and its severity \(^{67}\). It was postulated that circulatory impairment may initiate degenerative changes in the footprint, and the hypoxic condition in the tendon was exacerbated by the administration of antihypertensive drugs.

**Extrinsic factors of Rotator Cuff Disease**

Intrinsic factors play a role in the degenerative changes of the tendons. However, the other major contributor to rotator cuff disorders are the extrinsic factors that contribute to tendon damage. Structural wear and tear often results from mechanical compression of the supraspinatus tendon against the coracoacromial ligament and the anterior acromion. Neer observed bony impingement in rotator cuff tears and this mechanical characteristic has been described as an extrinsic factor potentially contributing to rotator cuff pathology. Subsequent classification of acromial morphology showed correlation between hooked and curved acromions with rotator cuff tendon tears \(^{125}\). The shape of the acromion can be congenital or acquired through age-related changes. Excessive mechanical stress between the anatomical structures aided by pathophysiological conditions at the acromial region may encourage the pathogenic progression to a hooked acromion as well as the growth of acromial bony spurs.
Repetitive injuries and chronic overuse are recognized associations with rotator cuff pathology. The micro-trauma theory proposed by Nho and colleagues suggested that small injuries accumulate over time to result in significant structural deterioration, predisposing the tendon to acute tear. The overuse injury is frequently observed in the shoulder of the dominant arm in patients. In one study, about 75% of the patients presented full-thickness tear on their dominant side, and 36% to 50% of the patients had bilateral tears.

### 1.2.3 Treatment Options

Non-operative treatment is usually the first-line of treatment for most of the tears, with the exception of large, acute tear in young and active patients. When non-operative management fails, surgical intervention may be considered in hopes to reduce pain and restore function. The standard surgical repair of rotator cuff tears involves approximating the remains of the ruptured tendon to the humeral head and inserting them into a bony trough on the greater tuberosity. Within recent decades, surgical methods have evolved from open, to mini-open, to arthroscopic repairs. The attachment techniques have changed over the years as well, from transosseous suture repairs to single rows and double rows of suture anchors, and recently the suture bridge technique.

Generally, strenuous overhead activities are avoided for at least four to six weeks post-surgery for most of the repairs. Passive range of motion exercise is prescribed for six weeks followed by active motion in physiotherapy. As the shoulders approaches full range of motion, a more aggressive rehabilitative program is prescribed. At least a six month recovery prior to return to
sports is recommended. Despite all the precautionary and rehabilitative strategies, functional recovery to original strength is, however, is infrequent.

**Re-tear**

Despite the evolution of surgical techniques and improved repair methods, structural healing of the rotator cuff tendon to the humeral head still remains a challenge. While studies have found the tendons to heal after the rotator cuff repair, the incidence of re-tear is a major concern for current repair methods. A recent systematic review of post-operative outcomes indicates recurrence rates from 13% in small tear less than 1 cm to 61% in massive tears over 5 cm.\(^{45}\)

The causes of re-tearing after repair have been proposed: mechanical failure of repair construct and biological failure of healing at tendon-bone interface.\(^{117}\) Re-tears that occur within three months after the repair are attributed to mechanical failure; recurrences after the initiation of physical therapy are more reflective of the failure of biological healing to provide adequate mechanical strength.

The success of surgical repair relies heavily on the functional healing of the injury site, the maintenance of contact between the separated structures, and the regeneration of the enthesis.\(^{92, 181, 182}\) The hypovascular and hypocellular nature of tendons may be the primary reason for poor healing and lack of regeneration of a specialized tissue to connect tendon and bone.\(^{180}\)

Insufficient blood supply hinders reparative activities at the injury site and the lack of resident cells impedes healing and remodeling processes. Immature scar tissues are not replaced by the strong, mature collagen types appropriate at the tendon-bone interface. This predisposes the
repair to failure in the future as the presence of fibrovascular scar tissue renders the interface mechanically weaker than the native fibrocartilaginous attachment.

1.2.4 Biology of Rotator Cuff Healing

The process of tendon healing follows closely the general sequence for soft tissue healing: inflammation, proliferation, and remodeling. Hematoma usually occurs immediately after the initial trauma. While the platelets clot the damaged vessels to contain erythrocytes within the circulatory system, the platelets release chemotactic factors to promote the infiltration of inflammatory cells, mainly the monocytes and the macrophages. Angiogenesis and residential tenocyte proliferation are initiated at this stage as well as fibroblast recruitment and collagen synthesis. A few days after the initial trauma, the injured site enters the proliferative stage in which fibroblasts and tenocytes are rapidly recruited with type III collagen synthesis reaching its peak. During this stage, neovascularization reaches its peak as the metabolic demand is high. At this point the wound becomes scar-like. The remodeling stage occurs weeks after the injury as the overall cellularity and type III collagen decrease. Type III collagen is progressively replaced by the structurally organized type I collagen to yield superior mechanical strength. The water content and extracellular matrix components approach the normal levels of a tendon tissue. This remodeling and maturation process may take up to four months $^{162}$.

Although the healing process is slow, it is essential that the tendon remodeling achieves a structure and content which is similar to the uninjured counterpart. Unfortunately, in many cases,
the healed tendon is unlikely to reach its original biomechanical capacity as scar tissue is inferior to the native tendon tissue.

**Growth Factors**

Growth factors are biochemical molecules which stimulate cellular activities, namely in proliferation and differentiation. Researchers have been trying to identify the roles of the various growth factors that contribute to the process of tendon-to-bone healing. Well-defined spatial and temporal expression of relevant growth factors will aid the progress in targeted treatment modalities. The temporal expressions of eight early-phase growth factors in supraspinatus tendon-to-bone healing were characterized by Würgler-Hauri et al. In addition, Oliva et al. has recently compiled a list of known growth factors expressed in the three defined healing stages.

**i. Basic fibroblastic growth factor**

Consisting of a single chain polypeptide of 146 amino acids, basic fibroblastic growth factor (bFGF) has been shown to promote wound healing via its angiogenic activity and induction of a proliferative response. The proliferative effect on tenocytes was confirmed by Takahashi et al., but the administration of bFGF suppressed collagen synthesis in vitro. The delivery of exogenous bFGF gene into tenocytes, however, significantly increased the expression of type I and III collagen gene in vitro. In vivo studies of rotator cuff healing showed the peak of bFGF expression at week one, and another increase at week eight.
Recent in vivo investigation of bFGF application in a rat supraspinatus model demonstrated accelerated tendon-to-bone repair \(^{79, 80}\).

ii. Bone morphogenetic proteins

The bone morphogenetic proteins (BMPs) were first identified in bone extracts, and named according to their ability to directly induce ectopic bone formation, although they have also been found to participate in the growth and development of other tissues. BMPs constitute the largest subfamily of the transforming growth factor beta (TGF-\(\beta\)) superfamily, with over twenty identified members to date \(^{23, 32}\). With respect to their sequential similarity and functions, BMPs are typically divided in four smaller groups: BMP-2/ -4, BMP-5/ -6/ -7/ -8a/ -8b, BMP-9/ -10, and BMP-12/ -13/ -14. Within the smaller groups, BMP-12/ -13/ -14 are believed to be involved in tendon regeneration \(^{52, 122, 200}\). Acute increase of these BMPs at the supraspinatus tendon-to-bone attachment site within the first 2 weeks post-operation was reported by Würgler-Hauri et al, with the changes of BMP-12 being the most significant \(^{203}\). BMP-2 and BMP-7 induced moderate anabolic activities in rotator cuff tendon cells in vitro which were favourable for the healing of the tendon \(^{133}\). In a sheep rotator cuff repair model, BMP-12 was found to improve tissue healing and biomechanical properties \(^{160}\). Adenovirus-mediated BMP13 delivery to rotator cuff tendon repair in rats resulted in up-regulation of the tendon matrix gene expression and improved biomechanical strength \(^{211}\).
iii. Cartilage oligomeric matrix protein

Cartilage oligomeric matrix protein (COMP) is the fifth member of the thrombospondin protein family, and is therefore also known as thrombospondin-5. It is a non-collagenous pentameric glycoprotein present in the extra-cellular matrix of both tendon and cartilage. It binds to both type I and II collagen, the major components of fibrocartilaginous tissues. Södersten et al observed a positive correlation between the ultra-structural distribution of COMP in horse digital flexor tendons with respect to age, maturation, and training. A similar correlation was found in the expression of COMP and the exercise load in rat Achilles tendons. Although the function of COMP has yet to be clearly defined, current evidences suggest its role in supporting structural integrity of tissues. The expression of COMP is found in cultured monolayer chondrocyte, tendon and ligament cells. In the study of temporal expression of growth factors, Würgler-Hauri et al reported a significant increase of COMP level the first week immediate to the surgery.

iv. Connective tissue growth factor

Connective tissue growth factor (CTGF) belongs to the connective tissue growth factor/cysteine-rich 61/nephroblastoma over-expressed protein (CCN) family. While CNN proteins have similar structures, their biological functions vary greatly and are very dependent on the local cellular context. Their participation in angiogenesis, chondrogenesis, wound healing and fibrosis has been implicated in many studies. CTGF is a promoting factor for anabolic activity of the extracellular matrix, including fibroblast and endothelial cell proliferation, migration, adhesion, and survival. The expression of CTGF in mesenchymal stem cells (MSCs) was able to direct the MSCs down the fibroblastic differentiating pathway. Elevated
expression of CTFG was found in tendons after acute exercise or injury. The observations were consistent with the report of an initial increase of CTGF in the supraspinatus tendon insertion site at one week post-surgery.

v. Platelet-derived growth factors

Platelet-derived growth factors (PDGFs) are a group of mitogens and chemo-attractants for cells of mesenchymal origin. The PDGF family is composed of four structurally and functionally similar polypeptides, designated PDGF-A/-B/-C/-D, and the polypeptides can be joined by disulphide-bonded to form dimers. The expression of PDGF-BB has been observed in tendon healing models in close association with other growth factors such as bFGF, TGF-β, and IGF in vitro and in vivo. Genetic modification in tenocytes to express PDGF-B or the addition of PDGF-BB to fibroblasts enhanced collagen gene expression in vitro. Thomopoulos et al investigated flexor tendon repair and found that PDGF-BB promoted cell proliferation and collagen remodeling as well as biomechanical properties. The efficacy of PDGF on rotator cuff tendon healing has been tested by two groups. Uggen et al evaluated both in vitro and in vivo application of genetically transduced tendon fibroblasts, and reported a significant increase in collagen synthesis in cell culture and accelerated rotator cuff repair histomorphologically in rats. The other study by Hee et al demonstrated superior biomechanical and histological properties at the infraspinatus tendon insertion.
vi. Transforming growth factors

As their names suggest, the transforming growth factor-βs (TGF-βs) gained their reputation from their ability to induce and transform the growth of fibroblasts \(^{151, 170}\). With the discovery of more structurally and functionally related proteins, the TGF-βs now represent one of the four major subfamilies of the big TGF-β family of over 40 members \(^{151}\). Three isoforms of TGF-βs have been identified in mammals: TGF-β1, TGF-β2, and TGF-β3. The TGF-βs play important roles in the regulation of collagen synthesis during wound healing, tissue synthesis, fibrosis, and scarring as well as many other cellular processes.

In vitro studies provided evidence for the fibrogenic potential of TGF-β1 in tendons \(^{29, 88, 89}\). TGF-β1 has been widely associated with aggressive inflammation and wound healing responses \(^{30, 151}\). The response of collagen formation, however, varies with cells isolated from different parts of a tendon, as shown by Klass et al with rabbit flexor tendon cells \(^{88, 89}\). There is a general consensus that over-expression of TGF-β1 may have adverse effect by increasing type III to type I collagen ratio and tendon adhesion \(^{30, 87-89}\). Many studies of the supraspinatus tendon model confirmed transient and localized increase of TGF-β1 one week after surgery or injury \(^{54, 203}\), suggesting the role of TGF-β1 in the initial phase of tendon healing. Peak cell proliferation and cellularity at the injury site coincided with the increased level of TGF-β1 \(^{90}\).

Studies have noted the phenomenon that fetal wound healing is achieved in a regenerative manner, often termed scarless healing, while post-natal healing is achieved in a scar-mediated process \(^{161, 168}\). This difference in fetal and adult healing property can be attributed to expression of growth factors unique to the different stages of development \(^{20, 161}\). Manning et al attempted to mimic the fetal development and scarless healing stimulus via exogenous addition of TGF-β3.
They hypothesized the sustained delivery of TGF-β3 would stimulate regeneration of fibrocartilage at the adult tendon-to-bone insertion rather than proliferation of scar tissue at the region. The administration of TGF-β3, however, was insufficient to create a biochemical milieu which promotes scarless healing in mature rat rotator cuff enthesis. In fact, Manning et al found increased disorganized scar fibres at the fibrocartilaginous transition zone. The authors speculated the importance of the role of tissue environment and cellular composition which affected the healing property in the presence of TGF-β3. Nonetheless, early structural and biomechanical advantages were observed. Compiling their observations, the data suggested an accelerated response of all phases in the healing process with the addition of TGF-β3. Kim et al, however, reported that the application of exogenous TGF-β3 and neutralizing antibodies to TGF-β1 and -β2 did not improve supraspinatus tendon-to-bone healing. It is important to better define the healing properties of the TGF-βs and the other key factors to modulate and augment early tendon healing.

vii. Insulin-like growth factor

Insulin-like growth factors (IGFs) are liver-made proteins that participate in growth, development, and metabolic activity of various tissues. The stimulatory effects of IGF-I have been identified in musculoskeletal tissues. IGF-I has been implicated as a mediator in phases of wound healing of various tissues. Analyses of temporal expression of growth factors revealed that IGF-I expression occurred early on during the inflammatory phase, and then peaked again at four weeks. Other studies have found increased extra-cellular matrix synthesis and protein turnover in vitro, and accelerated tendon healing in rat models in vivo. To date,
studies on IGF-I applications have yet to examine their efficacy on the healing at the tendon-to-bone interface.

viii. Matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of zinc endopeptidases found in a range of multicellular organisms. MMPs are responsible for timely degradation of extracellular matrix in development, morphogenesis, tissue repair, and remodeling. A compilation of the MMPs and their association to orthopaedic applications was provided by Pasternak and Aspenberg, and Garofalo et al. It is commonly believed that general inhibition of MMPs will impair tendon healing. Modulating the levels of MMPs present at the injury may facilitate tendon healing. Bedi and coworkers reported a distinct histomorphologic difference at the supraspinatus tendon-to-bone insertion with local delivery of a universal MMP inhibitor, α-2-macroglobulin protein. They found greater fibrocartilage formation and collagen organization, and a significant reduction in collagen degradation within four weeks while no biomechanical advantage was detected at the insertion. Gulotta et al took a different approach by introducing the developmental gene MT1-MMP into mesenchymal stem cells before transplanting them to the tendon-bone insertion in a rat supraspinatus tendon. At four weeks, they obtained a higher presence of fibrocartilage and an improved biomechanical property at the tendon-bone insertion site. Future investigations are necessary to better characterize the effects of the different MMPs to the structural integrity of the repair. Modulation of MMP activity in the perioperative period may offer a novel biologic pathway to augment tendon-bone healing after rotator cuff repair.
Platelet-Rich Plasma

Platelet-rich plasma (PRP) is a natural storage vehicle of growth factors. The current working definition of PRP is a volume of autologous plasma with a platelet concentration of more than $10^6$ platelets/μL. It is obtained from whole blood by separating red and white blood cells from the plasma and platelets, and subsequently concentrating the plasma contents and platelets. Taylor et al compiled a list of GFs and bioactive proteins found in PRP: TGF-β1, PDGF, bFGF, VEGF, IGF-1, epithelial growth factor, and hepatocyte growth factor. Many clinical studies have been done with the application of PRP on musculoskeletal injuries, but only a handful have focused on rotator cuff tendon repair. The most recent study which investigated the efficacy of PRP found that PRP application during arthroscopic repair of supraspinatus tendon did not result in accelerated recovery. These clinical studies and randomized controlled trials have failed to demonstrate improved healing with PRP application at the time of rotator cuff repair.

A variation of PRP, termed platelet-rich fibrin matrix (PRFM), is also being used in attempt to augment rotator cuff repair. The safety and efficacy of the use of PRFM has been evaluated in randomized controlled trials as well as in other clinical investigations. The randomized trials by Castricini et al and Rodeo et al, and the clinical trial by Bergeson et al found that PRFM did not improve shoulder functions while Barber and colleagues showed lowered incidents of re-tear rates. However, no significant adverse effect was observed in any of the studies. It should be noted that there are considerable difference exist amongst various commercial PRP constructs available for use. Variability may present in the density of platelets and growth factors, the presence of anticoagulants, and the leukocyte infusion.
1.2.5 Vascularity of the Rotator Cuff

Similar to other tissues of the body, vascular changes are indicative of tissue health and condition. Normal tendon is relatively hypovascular when compared to its adjacent musculoskeletal structures, the bone and the muscle. The vascular supply to the supraspinatus tendon and the presence of its critical hypoxic zone has been the spotlight of debate. Rotator cuff tears often involve the supraspinatus tendon, and some speculate the presence of a hypoxic region which contributes to the progression of tendon degeneration in a similar fashion to hypoxic degenerative tendinopathy in the Achilles tendon.

However, the theories of hypovascularity and predisposition to degeneration and injury of the rotator cuff tendon in this region remain a source of controversy. The traditional description of the critical zone corresponds to a region of anastomoses between skeletal and tendinous blood supplies at the supraspinatus tendon. The area of hypovascularity at the supraspinatus tendon is between 5 mm proximal to the humeral insertion and the musculotendinous junction. The critical zone was more evident at the articular side of the tendon where almost no vessels were present distally in comparison to the bursal side. It was suggested that this deficient vascularization predisposes the supraspinatus to degenerative pathologies. Furthermore, full adduction of the arm compresses the supraspinatus tendon against the humeral head and increased pressure on the supraspinatus tendon effectively reduces perfusion to the articular side of the tendon.\textsuperscript{107, 142}

The concept of enhancing blood flow to injured regions has been applied to therapeutic treatments of chronic Achilles tendinopathy\textsuperscript{130, 131}, patellar tendinopathy\textsuperscript{171}, and lateral
epicondylitis. Glyceryl trinitrate, a topical drug, was used in various studies to induce local vasodilation and hence increase blood flow to the applied area. A double-blinded randomized controlled trial examined the effect of glycercyl trinitrate for chronic rotator cuff tendinopathy, and found positive effects including force and functional measures in patient outcomes.

Recently, the use of contrast-enhanced ultrasonography allowed a better visualization of the vascular dynamics of the rotator cuff tendon in human subjects, confirming a hypovascular zone in the supraspinatus tendon. Studies have evaluated the spatial distribution and age-related changes to circulation of the supraspinatus tendon in healthy adults as well as alternations after tendon tear or repair. There was a consistent region of decreased vascularity at the articular medial margin of the asymptomatic rotator cuff tendons. While all regions experienced an enhanced blood flow after exercise, the response at the articular medial region was considerably lower in comparison to the articular, lateral and bursal aspects of the tendon.

Funakoshi et al did a longitudinal characterization of the vascular pattern at the supraspinatus tendon and its associated anatomy and found unique temporal vascular responses in the investigated structures. The vascular response of the bursal aspect of the supraspinatus tendon peaked one month after the surgery. The peak response for the articular aspect, however, occurred at two months. The vascular activity decreased globally by three months after the surgery in the supraspinatus tendon. In contrast, the vascularity at the anchor hole which was at the region of tendon and bone contact showed elevated responses over the period of the study.

Proper blood supply is a pre-requisite for maintaining tendon quality. Hence, blood supply has becoming an emphasized therapeutic target for preventing degeneration and promoting healing of the rotator cuff tendon. Understanding the vascular dynamics of the rotator cuff will allow researchers and physicians to gain deeper insights to the pathophysiology of degenerative
changes and potentially lead to novel therapeutic strategies. However, at the present time there are no therapeutic options available for increasing vascularity in rotator cuff repair.
1.3 Rotator Cuff Repair Model

An appropriate rotator cuff model that is representative of the human rotator cuff consists of the following criteria: the shoulder musculature of the rotator cuff and its surrounding muscles (the biceps and deltoid muscles), the shoulder bony structures (the acromion, clavicle, coracoids, and humerus), the articulations of the bony structures, and the motions of the joints.

1.3.1 Animal Models and the Rat Model

Various animal models have been explored in attempt to recapitulate the disease and healing progression of the rotator cuff. Some of these animal models include the rabbit, the sheep, the canine, the primate, and the most popularized – the rat.

The rabbit rotator cuff model was employed to examine muscular fatty infiltration and degenerative changes, tendon-bone enthesis regeneration, and rotator cuff biologic augmentation. The sheep rotator cuff was large enough in size to introduce large tears and monitor its effect and healing progression comparable to the timeline for humans. Additionally, sheep rotator cuff muscles experience degenerative changes similar to that of chronic tears in humans. This model allows for temporal molecular analysis of pathological mechanisms after rotator cuff tear. The canine rotator cuff model provided a functional assessment of the scaffold augmentations as the animals place load to the rotator cuff which reflects the stress experience by human
While many of the quadrupedal models have similar shoulder architecture as the human shoulder, their rotator cuff tendons insert onto the humeral head separately, and therefore do not form a “true cuff” structure as that of the human rotator cuff. Non-human primates have the shoulder anatomy and physiology most similar to that of the human shoulder. The cost, complexity of facility management, and ethical considerations comprise the reasons for the lack of use of this model.

While the rats are also quadrupeds, their unique shoulder anatomy makes them a suitable animal model for rotator cuff studies. The rat shoulder shares similar morphology with a human shoulder, with the supraspinatus tendon passing through the subacromial space. Expanding from the rat supraspinatus model, an acute surgical repair model has been developed to specifically examine the healing at the insertion. The model has been extensively used to examine the various factors in and strategies for tendon healing.

The use of biological approaches to enhance tendon-bone integration has been a popular theme for the rat supraspinatus acute repair model. The surgery involves detachment of the supraspinatus from its humeral insertion immediately followed by a bone tunnel suture repair. Interventions are usually administered during the surgeries: growth factors and bioactive molecules in healthy or diseased-condition, pharmacological drugs, and cell therapies. Outcome measurements have included tendon biomechanical properties and histological examinations.

The characterization of early phase of supraspinatus tendon-bone healing in this model allowed the characterization of the temporal expression profiles of growth factors. Several studies have investigated the application of fibroblastic growth factor, bone morphogenetic protein-13, insulin-like growth factor and platelet-derived growth factor-β, and transforming...
growth factor-β\textsuperscript{111} in this model. All but the bone morphogenetic protein-13 study found that the growth factors were moderately beneficial for tendon healing. However, platelet-rich plasma, which contains a variety of growth factors that stimulates anabolic activities in tissues, was found to have no effect on the tendon-bone strength after surgery\textsuperscript{14}. Bioactive molecules such as the recombinant human parathyroid hormone \textsuperscript{73} (a hormone that increases osteogenesis and chondrogenesis) and alpha-2-macroglobulin \textsuperscript{17} (a large generic protease inhibitor) yielded superior macroscopic healing at the tendon-bone junction, but failed to increase strength, suggesting the formation of bone and fibrocartilage at the enthesis was not sufficient to translate into biomechanical improvements.

Diabetic and osteopenic conditions for the rat supraspinatus model have been developed. Diabetes mellitus significantly compromises healing at the tendon-bone interface, resulting in lower biomechanical load to failure and inferior tissue characteristics\textsuperscript{16}. Although the study by Cadet and co-workers did not perform surgical detachments of the rotator cuff tendons, estrogen deficiency induced osteopenia at the greater tuberosity resulted in negative changes at the insertion histomorphologically\textsuperscript{26}. The failure stress, however, did not show statistical significance.

Pharmacological agents such as bisphosphonate and non-steroidal anti-inflammatory drugs (NSAIDs) were administered to examine their effects on tendon-bone healing. In the same study, Cadet and co-workers also used zoledronic acid (bisphosphonate) to improve bone density at the insertion of the supraspinatus tendon\textsuperscript{26}. They found stronger mechanical strength at the tendon-bone junction with increased bone density at the supraspinatus footprint. It is important, however, to note that their study did not involve detachment and repair of the supraspinatus tendon. Further investigation to address the efficacy of bisphosphonate drugs in tendon-bone
healing is necessary to delineate the relation. Cohen et al conducted an experiment to examine the effect of NSAIDs on tendon-bone healing in the rat supraspinatus repair model \(^{34}\). Both of the anti-inflammatory drugs indomethacin and celecoxib compromised soft tissue healing, including some that failed to heal. The study confirmed the links between tendon-bone healing and the production of cyclooxygenase-2.

As mentioned in the previous chapter, cell therapy is an attractive therapeutic for regenerative medicine, especially when the cells themselves are both renewable and differentiable. The following section will discuss the application of cell therapies to the rat supraspinatus repair model.

### 1.3.2 Cell Therapy in the Rat Rotator Cuff Repair Model

Gulotta and colleagues investigated the use of mesenchymal stem cells (MSCs) during rotator cuff repair to improve tendon-bone healing \(^{62}\). MSCs are speculated to differentiate into cells associated with the tendon-bone interface and augment the tissue quality at the injury site. However, they found that MSCs showed no benefit over the control groups. Gulotta and colleagues then conducted a series of three subsequent studies with rotator cuff repair by genetically modifying the MSCs: MSCs transduced with the membrane type 1 matrix metalloproteinase gene (MSC-MT1-MMP) \(^{64}\), MSCs transduced with the scleraxis gene (MSC-Scx) \(^{65}\), and MSCs transduced with the bone morphogenetic protein-13 gene (MSC-BMP13) \(^{66}\). MSC-MT1-MMP and MSC-Scx demonstrated stronger interfaces, while MSC-BMP13 had no improvement.
It was found that MSCs alone were insufficient to improve the attachment strength of the supraspinatus tendon at 2 weeks and 4 weeks after repair. The following table provides a summary of the biomechanical results of these studies.

Table 1-1. Biomechanical results of rotator cuff studies using mesenchymal stem cells

<table>
<thead>
<tr>
<th></th>
<th>2 Weeks</th>
<th>4 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control(^63)</td>
<td>MSC(^{63-66})</td>
</tr>
<tr>
<td>Load (N)</td>
<td>11.2 ± 2.3</td>
<td>10.5 ± 2.4</td>
</tr>
<tr>
<td>Stress (MPa)</td>
<td>1.9 ± 0.6</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>5.7 ± 2.3</td>
<td>4.9 ± 1.8</td>
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<tr>
<td>Load (N)</td>
<td>22.1 ± 3.5</td>
<td>20.8 ± 4.4</td>
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<tr>
<td>Stress (MPa)</td>
<td>3.5 ± 1.0</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>9.8 ± 4.7</td>
<td>9.3 ± 3.3</td>
</tr>
</tbody>
</table>

* significant compared to the control group/MSC group (MSC group was the baseline value for subsequent genetic modifications)
Figure 1-4. The ultimate load to failure at the insertion. The mesenchymal stem cells transduced with MT1-MMP gene and Scx gene increase insertion strength at 4 weeks after the repair compared to the control group/MSC group (MSC group was the baseline value for subsequent genetic modifications). Mesenchymal stem cells alone were not sufficient to improve healing.

Histologically, the MSCs were tracked using Ad-LacZ transduction prior to transplantation. Their presence was evaluated by the activity of β-galactosidase post-mortem. Exposure to β-galactosidase turned X-Gal solution from colourless to blue, indicating the presence of metabolically active MSC-LacZ. The amount of blue was significantly more in the specimens treated with MSC-LacZ than MSC-Null, and the β-galactosidase activity was lower at 4 weeks comparing to that at 2 weeks.
At 4 weeks, the tendon-bone interface tissue was more robust than those at 2 weeks even under gross observation. The continuity between the repaired tendon and the bone was observed and the natural healing progression without intervention was noted.

The collagen fibre organization and the area of fibrocartilaginous tissue were quantified using birefringence represented in gray scale and Safranin O/Fast Green FCF staining respectively.

The following table provides a summary of the histological results from these studies.
Table 1-2. Histological results of rotator cuff studies using mesenchymal stem cells

<table>
<thead>
<tr>
<th></th>
<th>2 Weeks</th>
<th>4 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control&lt;sup&gt;63&lt;/sup&gt;</td>
<td>MSC&lt;sup&gt;63-66&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Organization</strong></td>
<td><strong>(gray scale)</strong></td>
<td><strong>(gray scale)</strong></td>
</tr>
<tr>
<td></td>
<td>16.5 ± 5.5</td>
<td>20.3 ± 5.9</td>
</tr>
<tr>
<td><strong>Fibrocartilage</strong></td>
<td><strong>(mm&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
<td><strong>(mm&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
</tr>
<tr>
<td></td>
<td>0.342 ± 0.201</td>
<td>0.596 ± 0.192</td>
</tr>
<tr>
<td></td>
<td>24.8 ± 2.5</td>
<td>26.5 ± 5.5</td>
</tr>
<tr>
<td><strong>Fibrocartilage</strong></td>
<td><strong>(mm&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
<td><strong>(mm&lt;sup&gt;2&lt;/sup&gt;)</strong></td>
</tr>
<tr>
<td></td>
<td>0.438 ± 0.115</td>
<td>0.343 ± 0.217</td>
</tr>
</tbody>
</table>

* significant compared to the control group/MSC group (MSC group was the baseline value for subsequent genetic modifications)

The mesenchymal stem cells alone did not statistically improve the biomechanical strength of the repaired tendons. The hypoxic environment leading to compromised cell activities was likely a factor for the result. Alternative cell types were considered and it was hypothesized that the issue of poor vascularity should be addressed.
1.4 Angiogenesis and Endothelial Progenitor Cells

1.4.1 Capillaries

The microvasculature of the body is the site of interchange between the circulation and the body tissues. These specialized vessels are called capillaries. The total diameter of the capillaries in a human body is 800 times longer than that of the aorta, and their total surface area is roughly 6000 metres squared. Accordingly, the flow speed in the capillaries is 0.3 millimetres per second whereas the average flow speed is 320 millimetres per second in the aorta. Their dimensions are 7 to 9 micrometres in diameter and 0.25 to 1 millimetres in length. Structural variations of capillaries in different tissues reflect a variety of complex metabolic demand and function of the associated tissues and organs.

The general structure of capillaries is composed of a layer of endothelium and a basement membrane. The endothelium consists of a single layer of endothelial cells of mesenchymal origin. Endothelial cells are polygonal in shape and elongated in the direction of blood flow. They rest on basal lamina, are held together by zonula occludens (tight junctions), and are in contact with neighbours through gap junctions. On the luminal membrane of endothelial cells is surface proteins which are immunogenic and coagulating modulators. Present along some locations surrounding the capillary endothelial cells are pericytes. These mesenchymal cells possess the capacity to transform into cells associated with blood vessels.
The structure of the capillaries dictates the function of the capillaries: the macromolecule permeability, the metabolic activities, and the anti-thrombogenic role. Accordingly, the various types of capillaries include the continuous (somatic) capillary, the fenestrated (visceral) capillary, renal fenestrated capillary, and the discontinuous sinusoidal capillary. Continuous capillary is the major capillary type in muscles, connective tissues, and nervous tissues. The prominent characteristic of the continuous capillary is the absence of fenestrae in the endothelium. Here, exchanges between the blood and tissues rely heavily on pinocytotic vesicles in the endothelial cells. Fenestrated, or visceral, capillaries are often seen in organs that allow rapid interchange of substances such as in kidneys and intestinal organs. From 60 to 80 nanometres in diameter, fenestrations closed by a thin diaphragm in the endothelium enable small molecules to cross freely. The renal fenestrated capillary is a specialized version of fenestrated capillary, which is encapsulated by the glomerulus. Lastly, the discontinuous sinusoidal capillary is marked by tortuous path, fenestration lacking diaphragms, enlarged lumen from 30 to 40 micrometres in diameter, and presence of macrophages along the endothelium. These capillaries have discontinued endothelium and basement membrane that allow mobilization of cells and higher levels material exchange, especially mid-sized plasma proteins such as albumin. Discontinued sinusoidal capillaries are present in the liver and hematopoietic tissues and organs such as the bone marrow and the spleen.

The second component of the capillary, the basement membrane, is a connective tissue which is by large composed of the extra-cellular matrix. The membrane provides structural integrity, acts as a filtration barrier, and regulates cell attachment and chemotaxis. The membrane is rich in collagen fibrils: type I and type III collagen.
1.4.2 Angiogenesis

The growth of vessels via pre-existing capillaries is termed angiogenesis. It is a complex process that involves proliferation, migration, and remodeling of endothelial cells into functional vasculature, and the tight balances between pro-angiogenic and angiostatic molecules. Angiogenesis is regulated by cytokines and chemokines, the extracellular matrix as well as physical forces of the vascular environment.

The most potent angiogenic cytokines are the vascular endothelial growth factors (VEGFs). VEGFs can induce vasodilation, increased permeability of and fenestration in the endothelium, activation of endothelial cells to a proliferative state \(^\text{190}\). On the surfaces of endothelial cells are VEGF tyrosine kinase receptors, specifically, the VEGF receptor-2 which binds to the VEGF-A isoform. Some of the major bioactive molecules involved in angiogenesis include fibroblastic growth factors, transforming growth factor-β, insulin-like growth factor-1, platelet-derived growth factors, angiopoietin, erythropoietin, and tumor necrosis factor-α. Each drives the angiogenic activity through various signaling pathways and downstream effects. The actions of the molecules are not independent of each other, but work in concert to facilitate microvascular expansion.

Successful migration and invasion of endothelial cells into the perivascular tissue is dependent on the proteolysis and remodeling of the extracellular matrix. The extracellular matrix offers molecular directional cues for angiogenesis as well as binding sites that provides spatial information to the cells \(^\text{190}\). Present on the cell surface are transmembrane protein integrins which modulate the motility and affinity of the cells through the extracellular matrices.
Lastly, hemodynamic forces in the vessels can affect the angiogenesis and subsequent remodeling processes. Shear stress is essential for the maintenance of endothelial integrity and responses; forms of shear stress can induce different angiogenic responses via distinct signaling pathways. Local inflammatory response alters the hemodynamics of the tissues, and the capillary network remodels to accommodate the environment. It is observed that once the inflammation subsides, the network remolds again to its original hemostatic architecture.

**Mechanisms of Angiogenesis**

Once the endothelial cells have been stimulated for angiogenesis, a cascade of events follows as the vessels prepare for neovascularization. There are two identified physiological angiogenic processes: sprouting and intussusceptive angiogenesis. Both types of microvascular growth have been observed in vivo.

Sprouting angiogenesis, as the name suggests, involves paracrine induced migration of a tip cell supported by lateral stalks cells into the perivascular tissue. Upon activation of the tip cell by angiogenic stimulation, the adjacent basement membrane is degraded by proteases. The direction of sprouting is guided toward higher gradient of growth factors until cellular contact is made with other capillary sprouts. Trailing the tip cell, the stalk cells form the lumen of the vessel and line the endothelium. Pericytes are recruited as the vessel architecture undergoes remodeling and maturation. The tip and stalk cell phenotypes are transient and highly regulated by notch signaling; the process of angiogenesis requires dynamic transitions between the endothelial, tip, and stalk cell phenotypes.
The other process of angiogenesis, intussusceptive angiogenesis, occurs via splitting the pre-existing vascular network or capillary plexus. The characteristic hallmark of intussusceptive angiogenesis is the formation of multiple intraluminal pillars from invaginations of opposing vessel walls in its initial stages. When the inter-endothelial junctions are re-organized, the core of each pillar is perforated to allow mural cell invasion and growth factor trafficking. As the pillar expands with increasing extracellular matrix deposition, it fuses with nearby pillars to create bifurcation in a vessel.

Both sprouting and intussusceptive angiogenesis rely on pre-existing vasculature. While intussusceptive angiogenesis is more limited in a sense that it can only occur in capillary plexuses developed from vasculogenesis or sprouting angiogenesis, its process is much faster and energy efficient. Capillary splitting does not require the disruption of basement membrane or extensive proliferation of endothelial cells to line the endothelium. Microvascular growth studies have identified the predominance of capillary sprouting in the early stages of vascular growth, followed by extensive hierarchical splitting of vessel during remodeling. The novel evidence suggests the temporal dependence of the angiogenic mechanisms.

It is plausible that during tendon healing in which metabolic resource is scarce, there is a tendency for the fibrovascular tissue to derive vessels via intussusceptive angiogenesis. However, the tendon is hypovascular, leaving the bone and the bursa as the major sources for pre-existing vasculature. The hypovascular nature of the tendon itself may impair its ability to heal.
1.4.3 Endothelial Progenitor Cells

Cell therapy is an attractive therapeutic avenue for regenerative medicine, especially when the cells themselves have the capacity to renew and differentiate. Stem cells and progenitor cells are populations of cells which possess such capabilities. Much excitement was generated when endothelial progenitor cells were identified. The first published detailed description for endothelial progenitor cell isolation is by Asahara and colleagues in 1997. It sparked a new paradigm in utilizing endothelial progenitor cells as a cellular strategy for therapeutic neovascularization. It is believed that these cells can induce angiogenesis via the secretion of angiogenic factors or by directly participating in the lining of endothelium. Endothelial progenitor cells are a rare but normal component of the elements that make up the circulating blood cells.

There are several sources from which these cells can be isolated. These include the peripheral blood, the bone marrow, and the umbilical cord blood. In addition, endothelial progenitor cells have been found to reside in tissues and organs such as fat and vascular tissues, the fetal liver, and the heart.

Currently, there is not a well-defined set of surface immune-phenotyping or functional assays to identify putative populations of endothelial progenitor cells. Various combinations of cell surface antigens or markers were used in attempt to characterize the cells. Most of these markers are not endothelial lineage specific; the marker profile reflects the hematopoietic lineage. Nonetheless, the conventional positive markers for detection are CD 34, CD 133, and vascular endothelial growth factor receptor-2 (VEGFR2) or fetal liver kinase-1 (Flk-1).
positivity indicates that the putative population is of the hematopoietic stem/progenitor cell population. The second marker, CD 133, also known as Prominin-1 or AC 133, is a strict character for early hematopoietic stem/progenitor cell that reside in the bone marrow, fetal liver, or in circulation. Lastly, the presence of vascular endothelial growth factor receptor-2 on the membrane warrants the ability for the cells to respond to vascular endothelial growth factor, a potent signaling molecule for angiogenesis.
Endothelial Progenitor Cells

Figure 1-5. Surface antigens and possible angiogenic mechanisms of endothelial progenitor cells. The conventional surface markers are CD 34, CD 133, and Flk-1 (VEGFR2). The mechanisms for angiogenesis are endothelial differentiation and angiogenic signaling.

In addition to surface immune-phenotyping, assays are often performed to demonstrate functional characteristics of putative cells of the endothelial lineage. The uptake of acetylated-low density lipoprotein (AcLDL) labeled with fluorescent probe 1,1'-dioctadecyl -3,3,3',3' - tetramethyl-indocarbocyanine perchlorate (DiI-AcLDL), the binding of lectin Ulex europaeus 1 agglutinin (UEA-1), and forming tubes in 3 dimensional matrices are frequently used in vitro behaviours. In contrast to the surface markers which correlate more to that of the hematopoietic stem cell phenotypes, the functional assays are indicative of endothelial properties. AcLDL uptake occurs through the scavenger cell pathway of lipid metabolism. While
internalizing AcLDL is a property of endothelial cells, monocytes and macrophages are also know to scavenge AcLDL macromolecules. Likewise, UEA-1 binds to glycoproteins that are found predominately on the endothelial cell surface, but are present on epithelial cells and other blood-derived cells. Accordingly, either of the functional assays alone is sufficient to distinguish endothelial progenitor cells from other hematopoietic cells. In vitro tubulogenesis assay is considered to be a very informative test; other cells do not typically form tubes in fibrous matrices. Endothelial progenitor cells, when in contact with extracellular matrix components, have the capacity to assemble and form a network of tubes that resembles the microvasculature in vivo.

Under the conventional isolation protocols, two distinct populations of endothelial progenitor cells have been identified. Early and late endothelial progenitor cells, named according to their time-dependent appearance in vitro, share common endothelial surface phenotypes but display different functional potentials. Early endothelial progenitor cells have a shorter life span (about 4 weeks) and higher levels of cytokine secretion. They are, however, poor in forming tubes in vitro. Late endothelial progenitor cells, also known as late outgrowth cells, have better endothelial incorporating and tubulogenic capacities; they are representative of mature endothelial cells yet still maintain proliferative angiogenic characteristics. The contrasting angiogenic profiles suggest the different roles during neovascularization. The paracrine and autocrine effects of the early cells augment the proliferation, migration, and incorporation of the late cells. This synergistic interaction of the mixed populations has been demonstrated in vivo. The transplantation of both types of progenitor cells together led to better perfusion than either of the cells alone.
The exact origin of endothelial progenitor cells is still uncertain. The cells are believed to be derived from the bone marrow, as observed in gender-mismatched marrow transplant patients. There are two proposed sources, the mesenchymal and the hematopoietic differentiations. The cells from the mesenchymal lineage lacked the early hematopoietic markers and were able to differentiate in vitro into osteocytes and adipocytes. When they were cultured for endothelial differentiation, the cells expressed endothelial antigens and formed vascular tubes when plated on extracellular matrix. In addition to the mesenchymal origin, the hematopoietic origin has been indicated as an alternative source for endothelial progenitor cells. A wealth of literature has described the hematopoietic stem cell-myeloid progenitor-monocyte pathway for endothelial precursor differentiation. Surface markers and angiogenic assays confirmed the plasticity of monocytes to give rise to vasculogenic cells. Myeloid progenitors are also found to contribute to endothelial progenitor cell differentiation and contribute to the vascular endothelium. The close association between the two cell lineages in the bone marrow could explain the phenotypic overlap of their progenies. A recent study highlighted the trans-differentiating potential from the mesenchymal lineage precursor (mesenchymoangioblast) to the hematopoietic lineage precursor (hemangioblast) given the appropriate stimulation.
1.4.4 Applications of Endothelial Progenitor Cells

Bone marrow transplant experiment showed that endothelial progenitor cells have 0.2 to 0.4 % engraftment rates in the vascular endothelium after four months \(^{36}\). Tissue specific vascular progenitor cells may result in different cell responses and fates during neovascularization \(^{108}\).

Various animal models have been used for pre-clinical investigations of endothelial progenitor cell therapy in ischemic conditions. Increased micro-vasculature in the myocardium, improved myocardial remodeling and function, and reduced scarring were observed with the addition of endothelial progenitor cells to ischemic heart condition \(^{84, 85, 91, 157}\). Similarly, anti-fibrogenesis, remodeling, and hepatic regeneration were associated with the transplantation of endothelial progenitor cells to the cirrhotic liver model \(^{120, 175, 191}\). The authors speculated that the secretion of angiogenic growth factors and enhanced vascularization lead to the improvement in liver function and animal survival. Additionally, extensive studies have examined the effects of delivering endothelial progenitor cells in ischemic hind limb models. Capillary density and limb blood flow increased, and the rate of limb loss was reduced \(^{83, 155, 193}\).

The pre-clinical animal studies done using endothelial progenitor cells have revolved around the theme of re-vascularizing injured tissues to enhance the tissue’s innate reparative ability. Increased vascular density and blood supply to target tissues along with ameliorated tissue quality were the results. Indeed, the endothelial progenitor cell population was expected to provide a source of angiogenic growth factors that stimulates endothelial proliferation. While these studies have confirmed the angiogenic effects of introducing endothelial progenitor cells to
these tissues, more studies are needed to delineate the specific mechanisms by which the cells promote neovascularization.

i. Clinical trials

Vascular progenitor cells have been investigated in clinical trials for critical limb ischemia and heart diseases. Randomized controlled trials have been conducted to evaluate angiogenic cell therapy in critical limb ischemia. Two sources of mononuclear cells were used in the studies: bone marrow mononuclear cells \(^{176}\) and peripheral blood mononuclear cells \(^{76}\). Both trials demonstrated feasibility and safety of treatment.

Clinical trials for angiogenic cell therapy in heart diseases have been divided into two categories \(^{165}\): acute myocardial infarction and chronic heart disease. Major trials for acute myocardial infarction include TOPCARE-AMI \(^{8,152}\), BOOST \(^{114,115,201}\), ASTAMI \(^{18,105}\), and REPAIR-AMI \(^{49,153}\). On the other hand, for chronic ischemic heart diseases, there are TOPCARE-CHD \(^{7}\) and FOCUS-CCTRN \(^{18,105,136}\). All trials but ASTAMI and FOCUS-CCTRN showed positive functional outcomes for the heart.

Two trials, TOPCARE-AMI and TOPCARE-CHD, employed both the bone marrow and the peripheral blood endothelial progenitor cells, and both found favourable improvements in the left ventricular function. Interestingly, the TOPCARE-CHD study compared circulating progenitor cells with bone marrow progenitor cells and found better outcome in left ventricular ejection fraction with the bone marrow progenitor cells \(^{7}\). The long term results of TOPCARE-AMI demonstrated safety and efficacy with progenitor cell restored cardiac function \(^{95}\).
ii. Orthopaedic applications

Vascular strategies have received much attention in the field of orthopaedics recently. The use of vascular progenitor cells to promote neovascularization in tissues with poor or deficient vascularity has been a major focus of recent investigations. This includes osteonecrosis of the femoral head, segmental bone defects, and transected ligament.

While the etiology of osteonecrosis is multifaceted and not yet fully understood, circulatory impediment is a widely accepted theory of pathogenesis. The addition of endothelial progenitor cells has shown beneficial effects on osteonecrosis of the femoral head in animal models. Both osteogenic and angiogenic responses have been reported. Clinically, bone marrow mononuclear cells containing endothelial progenitor cells have been continuously investigated over the years. While endothelial progenitor cell transplantations have showed promises, more studies are required to ensure efficacy and safety.

Endothelial progenitor cell therapy has demonstrated great potential to heal segmental bone defects in the rat femur. The addition of endothelial progenitor cells aided the healing of the bone defect, while the control group without these cells did not heal. A subsequent study showed that endothelial progenitor cell therapy enhanced expression of various VEGF isoforms in a bone defect model. The findings of these studies suggested that the endothelial progenitor cells encouraged early osteogenesis via the facilitation of angiogenesis.

Ligaments are known for their hypovascularity under normal physiological conditions. Healing of the medial collateral ligament was augmented by administering an endothelial progenitor cell-enriched population. The local transplantation of the CD 34-positive cells aided ligament
healing by increasing angiogenic activities and improving macroscopic, histological, and biomechanical results. Real-time reverse transcription polymerase chain reaction also revealed higher expressions of ligament-specific markers, collagen1A2 and tenomodulin, in the treatment group. The addition of CD 34-positive cells could alter the local environment to one that is favourable for ligament healing.

Gomes and colleagues investigated the supplement of bone marrow mononuclear cells during conventional transosseous rotator cuff repair in a clinical trial \(^{46}\). The idea of using these mononuclear cells was that they could revitalize tendons that have undergone avascular degeneration after the repair. The patients demonstrated functional improvements and tendon integrity after a twelve month follow up. The magnetic resonance imaging (MRI) results also suggested the formation of new tissue at the footprint in some of the patients. Although the preliminary results indicated positive clinical outcomes and MRI findings, the study did not include a control group and were unable to provide definitive evidence for tissue quality.

To our knowledge, the use of endothelial progenitor cells to augment rotator cuff repair has not been investigated. In vivo studies are necessary to assess the effectiveness of endothelial progenitor cell transplantation during rotator cuff repair.
1.5 Rationale, Hypothesis, and Objectives

1.5.1 Rationale

Biological strategies for the augmentation of rotator cuff tendon repair have been a popular topic of pre-clinical and clinical investigations, many of which employ growth factor supplementation, extra-cellular matrices, and/or anabolic progenitor cells. Due to their absence of immunogenic responses, autologous cell-based therapies have received much attention. With recent investigations of mesenchymal stem cells (MSCs) strategies yielding disappointing results, potentially due to impaired vascularization at the site of tendon-bone healing, alternative cell lines warrant consideration.

Endothelial progenitor cells are a population of heterogeneous cells that can be isolated from the bone marrow and the peripheral blood. Endothelial progenitor cells are believed to promote angiogenic activities through secretion of angiogenic factors (paracrine) and differentiation into vascular endothelial cells. This cell population has become a popular vehicle for supplementation of angiogenic growth factors. Studies in animal models of ischemia have shown their efficacy to induce vascular growth in tissues.

Recent orthopaedic application of endothelial progenitor cells showed superior bone healing compared to using mesenchymal stem cells. The endothelial progenitor cells increased vascularity in the animals (on the basis of Laser Doppler) and demonstrated superior healing of
bone. Given the relevant literature, it stands to reason that the addition of endothelial progenitor cells at the tendon-bone reattachment site in rotator cuff repair could enhance the vascular response and improve healing. Endothelial progenitor cell therapy could have a therapeutic effect on the hypovascular condition of the tendon and aid osteogenesis on the bone end to improve tendon-bone integration. However, the use of endothelial progenitor cells for the biological augmentation of rotator cuff repair has not previously been investigated. Our study aimed to investigate the augmentation rotator cuff repair and vascularity with the therapeutic application of endothelial progenitor cells.

1.5.2 Hypothesis

Our hypothesis was that the application of endothelial progenitor cells during rotator cuff repair would increase the biomechanical strength at the tendon-bone interface and increase angiogenesis during the early phase of rotator cuff healing.

1.5.3 Objectives

It was postulated that by promoting angiogenesis at the tendon-bone site, the endothelial progenitor cell-treated subjects would yield better tendon repair in a rotator cuff model. The study addressed the efficacy of endothelial progenitor cells to augment surgical rotator cuff
repair and also sought to investigate the fate of the transplanted endothelial progenitor cells. Our first objective was to confirm the endothelial progenitor cell population from our isolation using flow cytometry and functional assays. Our second objective was to assess the effect of endothelial progenitor cell transplantation on the biomechanical strength and histology of the healing rotator cuff tendon after surgical repair in a rat model. Our third objective was to evaluate the effect of endothelial progenitor cell transplantation on the vascularity at the repair site using immunohistochemistry. Our final objective was to examine the fate of the transplanted endothelial progenitor cells using genetically labeled cells.
2 Methods

2.1 Study Design

The study aimed to complete three objectives: evaluate the effect of endothelial progenitor cell transplantation on the strength and quality of healing tissue, quantify the angiogenic response, and monitor cell survival at the injury site. Accordingly, the study was designed to address these objectives.

Figure 2-1. The study design.
A population of syngeneic Fischer 344 rats was used to allow the transplantation of endothelial progenitor cells between specimens. They were mature healthy rats between 250 to 300 grams when ordered which roughly translates to three months of age. The animals were housed in pairs upon arrival from Charles River, and were allowed to acclimatize for at least two days prior to their surgery. All rats were allowed normal weight bearing and cage activities, standard diet and water ad libitum. The study was approved by the Animal Care Committee and the Research Vivarium, St. Michael’s Hospital.

The rats were divided into three groups. The first group (experimental group) received endothelial progenitor cells delivered in fibrin sealant during the surgical repair of the supraspinatus tendon. The second group (control group) received only the fibrin sealant. These two groups were allocated for biomechanical testing and histomorphometric analysis to answer the first two study questions. The third group, which was used to track cells, received endothelial progenitor cells which expressed green fluorescent protein delivered in fibrin sealant. This group was dedicated only for histology examination for the presence of the fluorescent protein.
2.1.1 Statistical Significance of the Study

The primary outcome measure for the study was biomechanical strength of the rotator cuff repair, specifically the ultimate load to failure at 4 weeks. Previous studies have set a 20% increase in biomechanical strength as the threshold for significance, and this was felt to be a clinically significant difference. Accordingly, the statistical significance for this study was set at 20% increase in biomechanical strength at 95% confidence ($\alpha = 0.05$) and 80% power ($\beta = 0.2$).
2.1.2 Sample Size Calculation

The calculation for sample size with respect to estimated standard deviation and expected differences was the following:

\[
n = \frac{2 \times \sigma^2 \times (Z_{1-\alpha/2} + Z_{1-\beta})^2}{\delta^2}
\]

where \( \alpha = \) the confidence interval; \( \beta = \) power; \( \sigma = \) the standard deviation; and \( \delta = \) the expected difference.

The pre-determined level of significance is 95% with a power at 80% gives

\[Z_{0.975} = 1.96\]

\[Z_{0.2} = 0.84\]

The same size variables were derived from previous data with regards to supraspinatus tendon strength at 4 weeks \(^{63}\).

\[\sigma = 3.5\]

\[\delta = 22.1 \times 0.2 = 4.42\]

\[n = \frac{2 \times 3.5^2 \times (1.96 + 0.84)^2}{4.42^2} = 9.83\]

Therefore, the determined sample size for each group (the experimental and fibrin groups) was ten.
2.2 Cell Isolation

2.2.1 Endothelial progenitor cell isolation

The endothelial progenitor cell isolation was performed following the developed protocol in the Musculoskeletal Research Laboratory as previously described\textsuperscript{9,124}. Syngeneic Fischer 344 rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were used for cell isolation to allow for transplantation without immunogenic complications. Endothelial progenitor cells were isolated from rat long bone marrow. Rats were anaesthetized with isoflurane and euthanized via cervical dislocation. Bilateral femurs and tibiae were carefully disarticulated and soft tissues were sharply removed post-mortem under sterile conditions. Dissected femurs and tibiae were immediately immersed in sterile phosphate buffered solution (Dulbecco’s Phosphate-Buffered Saline, Gibco, Carlsbad, California, USA) and transferred to a culture hood.

Bone marrow cells were obtained by flushing the intramedullary canals of the long bones with PBS. The collected solution was centrifuged at 220 relative centrifugal force (RCF) for 5 minutes at room temperature. The supernatant was discarded and the pellet was suspended in Endothelial Growth Medium 2-MV (EGM2-MV, Gibco, Carlsbad, California, USA), and transferred into a tube containing Ficoll (Ficoll-Paque Plus, GE Healthcare, Little Chalfont, United Kingdom) without disturbing the interface between the two solutions. The tube was centrifuged at 400 RCF, 30 minutes, room temperature. The middleuffy layer containing
mononuclear cells was extracted, rinsed in EGM-2-MV, and centrifuged at 100 RCF for 10 minutes, room temperature. The pellet was re-suspended and rinsed once more, and centrifuged at 100 RCF for 10 minutes, room temperature.

The mononuclear cells were re-suspended in EGM-2-MV and seeded on to human fibronectin-coated (Discovery Labware, Billerica, Massachusetts, USA) culture flasks and incubated at 37°C for four days to allow selection of endothelial progenitor cells via fibronectin adhesion and endothelial cell-specific medium before washing non-adhering cells. Medium was changed every two days after the initial four days. The cells were cultured for 9 to 10 days before transplantation, by which time they reach 80 % confluence.

2.2.2 Flow cytometry

Once the endothelial progenitors cells were cultured in the flask for 9 to 10 days, they were trypsinized (Trypsin-EDTA 1x, Mediatech, Manasass, Virginia, USA) for 5 minutes, harvested, and rinsed. They were then transferred into microcentrifuge tubes (200 000 cells/tube). The cells were pelleted and the supernatant was removed. A volume of 100 microlitres of 1 % bovine serum albumin (Sigma-Aldrich, St Louis, Missouri, USA) in phosphate buffered saline (BSA/PBS) was used to suspend the cells for each tube. The four antibodies were added accordingly. The tubes were incubated at 4°C in the dark for 20 minutes to allow antibodies to bind. The cell suspensions were transferred to flow tubes for analysis.
The cells were analyzed with MACSQuant flow cytometer. The event readings were selected for single cells only, and their fluorescence was gated using the fluorescent minus one (FMO) groups and the single stains. The surface antigens chosen for flow cytometry were CD 31, CD 34, CD 133, and Flk-1. The excitation formats for CD 31, CD 34, CD 133, and Flk-1 were fluorescein isothiocyanate (FITC), AlexaFluor 647, phycoerythrin (PE), and VioBlue (v450) respectively.

i. CD 31

Platelet endothelial cell adhesion molecule 1 (PECAM-1), designated CD 31, is a common endothelial marker. It is a type I integral membrane glycoprotein (130 kDa) and a member of the immunoglobulin family that is expressed at high concentrations on the surfaces and between cell junctions. CD 31 mediates homophilic and heterophilic cell-cell adhesion. CD 31 mediated endothelial cell-cell interactions are involved in angiogenesis in rats and mice. Accordingly, CD 31 antibodies are often used to measure angiogenesis in association with physiological processes such as tumor growth. However, in addition to endothelial cells, CD 31 is also expressed on platelets and subsets of leukocytes.

ii. CD 34

CD 34 is a type I transmembrane glycoprotein (110-120 kDa) of the CD 34/Podocalyxin family of sialomucin proteins. It is recognized as a highly glycosylated hematopoietic stem/progenitor cell antigen. The surface glycoprotein functions as a cell-cell adhesion factor, which mediates the
attachment of stems cells to the bone marrow extracellular matrix or directly to stromal cells. CD 34 is also expressed on the endothelium of capillaries.

**iii. CD 133**

CD 133 (117 kDa), or prominin 1 (PROM-1), is a 5-transmembrane glycoprotein specifically localized to cellular protrusions. Originally known as AC 133, CD 133 expression is restricted to a subset of CD34 stem/progenitor cells in the hematopoietic system in liver, bone marrow, cord blood, and peripheral blood. Only a small portion of CD 34− CD 133+ cells are found in these tissues. CD 133 is present on circulating endothelial progenitor cells, fetal neural stem cells, and other tissue specific stem cells.

**iv. Fetal liver kinase-1/Vascular endothelial growth factor receptor-2**

Fetal liver kinase-1 (Flk-1), also known as vascular endothelial growth factor receptor-2 (VEGFR-2), is a receptor protein tyrosine kinase of the immunoglobulin family. It is closely related to CD 177 (c-kit) and CD 140a (PDGF receptor α chain). As the name VEGFR-2 suggests, Flk-1 is a receptor for vascular endothelial growth factor (VEGF). It is expressed on endothelial cells, and in embryonic and adult tissues. In vivo and in vitro studies indicate that Flk-1 is required for embryonic development of hematopoietic and vascular endothelial cells as VEGF is an important signaling protein involved in vasculogenesis and angiogenesis.
Table 2-1. Staining Protocol for flow cytometry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CD 31</th>
<th>CD 34</th>
<th>CD 133</th>
<th>Flk-1</th>
</tr>
</thead>
<tbody>
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<td>Miltenyi Biotech</td>
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<td>Stock concentration</td>
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<td>0.2 mg/mL</td>
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<td>Format</td>
<td>FITC</td>
<td>AlexaFluor 647</td>
<td>PE</td>
<td>VioBlue</td>
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<tr>
<td>Aliquoted stock per tube (µL)</td>
<td>5</td>
<td>4</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Aliquoted BSA/PBS per tube (µL)</td>
<td>15</td>
<td>16</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Aliquoted volume (µL)</td>
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<td>20</td>
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</tr>
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<tr>
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<th>CD 133</th>
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</table>
2.2.3 Functional assays

Functional assays were performed to evaluate the putative endothelial progenitor cell populations. Both AcLDL uptake and lectin binding assays were carried out on the same slides.

i. AcLDL uptake and lectin binding assays

The isolated endothelial progenitor cell population was evaluated for the uptake of acetylated-low density lipoprotein labeled with fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-AcLDL, Invitrogen, Carlsbad, California, USA) and the binding of lectin GS II (Lectin GS II Alexa Fluor 488 Conjugate, Invitrogen, Carlsbad, California, USA). Two-well chamber slides were coated with human fibronectin (10 µg/mL) and cells were seeded at a density of 8000 cells per square centimetre with endothelial growth medium at 37°C overnight.

The next morning, stock DiI-AcLDL was diluted to 10 µg/mL with culture medium, and 500 µL of the solution was added to the appropriate chambers. The control chambers received 500 µL of endothelial growth medium. They were all incubated at 37°C for four hours followed by three phosphate buffered saline (PBS) rinses. The cells were then fixed with 2 % paraformaldehyde for ten minutes, and washed three times with PBS.

The lectin molecule, GS II, was used for lectin binding. Stock GS II was diluted to 20 µg/mL using PBS with calcium and magnesium (Dulbecco’s Phosphate-Buffered Saline with calcium and magnesium, Gibco, Carlsbad, California, USA). The solution was added to the appropriate
chambers (500 µL/well) and the control chambers received only PBS, and were kept at 4°C overnight on a shaker. The next morning, the chambers were washed three times with PBS. The chambers were disassembled and the slide was allowed to air dry in the dark. 4', 6-diamidino-2-phenylindole in permount (ProLong Gold with DAPI, Invitrogen, Carlsbad, California, USA) was added to each slide (80 µL), and cover slips were placed on. When the slides were imaged under fluorescent microscopy, the DiI-AcLDL appeared red, the GS II appeared green, and the nucleus appeared blue.

ii. **In vitro tube formation assay**

The isolated population of endothelial progenitor cells was plated on basement membrane Matrigel (BD Sciences, Bedford, Massachusetts, USA) for tube formation assay. Stock Matrigel was diluted 1:1 with sterile cold PBS. Diluted Matrigel was aliquoted into a 24-well plate (300 µL/well) and was incubated for 30 minutes to allow polymerization. Endothelial progenitor cells were seeded at 48 000 cells per well (cell density of approximately 25 000 cells/cm²) with 500 µL of endothelial growth medium. They were incubated for 24 hours and imaged with a microscope. Mesenchymal stem cells were used as the negative control. The number of tubes formed was counted and the total length was calculated.
2.2.4 Cell survival in fibrin sealant

Fibrin sealant has been widely used as cell delivery agent, namely with mesenchymal stem cells. Endothelial progenitor cells, however, have yet to be delivered via this method. To ensure endothelial progenitor cell survival in this delivery agent, the fibrin sealant, the following procedure was performed.

Commercial fibrin sealant (Evicel, Johnson & Johnson, Somerville, New Jersey, USA) is composed of two ingredients: (1) human clottable protein (often termed fibrinogen), and (2) human thrombin. Human clottable protein is activated upon exposure to thrombin in the presence of calcium ions, causing the mixture to clot.

The endothelial progenitor cells were trypsinized and counted. One million cells were re-suspended with 12.5 microlitres of thrombin, as it is the less viscous of the 2 components of fibrin sealant. The same volume of fibrinogen was added to the thrombin-cell suspension and incubated at 37°C without endothelial growth medium for 24 hours. Five millilitres of endothelial growth medium was added after 24 hours and the cells were incubated for another day. The cell survival was assessed at 24 hours and 48 hours by examining cell morphology (normal healthy cell appearance – adherence to flask, flat monolayer).
2.3 Surgery

Endothelial progenitor cells were prepared immediately prior to surgeries. After 9 to 10 days of in vitro expansion, the cells were harvested by trypsinization, and washed in endothelial basal medium without fetal bovine serum. For each rat, one million endothelial progenitor cells were mixed in 12.5 microlitres of thrombin solution of fibrin sealant and kept on ice. The delivery of the cells involved pipetting the 12.5 microlitres of thrombin and cell mixture with 12.5 microlitres of viscous fibrinogen solution to the central hole made on the greater tuberosity during surgery.

The surgical procedure involves a unilateral detachment of the supraspinatus tendon from its humeral insertion followed by a transosseous suture repair. The surgical model has been developed by Thomopoulos and colleagues to examine tendon-bone healing at the insertion. During the surgery, 10 day-old endothelial progenitor cells expanded in vitro were delivered in fibrin sealant. All surgical procedures were approved by the Animal Care Committee and the Research Vivarium, St. Michael’s Hospital.

Prior to the surgeries, endothelial progenitors were harvested from the culture flasks and suspended in thrombin, the less viscous solution of the fibrin sealant components. They were then kept on ice until use.

All the surgeries were performed in the same manner. In each surgery, once the rat had been anaesthetized with isoflurane (5 %) in combination with oxygen (2 L/min) in the induction chamber, it was weighted and given a pre-operation dose of buprenorphine (0.05 mg/kg) via
intraperitoneal injection. The rat was shaved from the elbow to the neck to expose the skin, disinfected with Betadine (povidone-iodine) and alcohol. The rat was transferred to the operating table and was maintained anesthetized with isoflurane (2 %) in combination with oxygen (2 L/min) through the nose cone.

A three centimetre skin incision was made with a scalpel blade at the anterolateral aspect of the rat’s right shoulder to expose the deltoid-acromion structure. The humerus of the rat was kept in adduction and external rotation, and a longitudinal incision was made on the deltoid muscle, splitting the muscle without detaching the deltoid from its acromion origin. Access to the supraspinatus tendon was created. The supraspinatus tendon insertion on the greater tuberosity of the proximal humerus could be effectively visualized by placing the humerus in adduction and internal rotation at the glenohumeral joint.

Before detaching the supraspinatus tendon from it insertion, a Mason-Allen stitch to the distal tendon was made using a 3-0 Ethicon suture (Johnson & Johnson, Somervile, New Jersey, USA). Subsequently the tendon was sharply detached from the proximal humerus with a scalpel blade, and any residual fibrocartilage at the footprint was removed by gently decorticating the greater tuberosity to expose the bone marrow with a 1.0 mm crosscut burr (Stryker, Kalamazoo, Michigan, USA). A bone tunnel was made using the burr at the anterior margins of the insertion two millimetres from the articular surface. Both ends of the suture from the Mason-Allen stitch were passed through this tunnel and tied over a bony bridge on the lateral aspect of the proximal humerus.

Prior to securing the tendon repair, the rat was randomized to one of the groups to receive either the fibrin sealant carrying endothelial progenitor cells or fibrin sealant only. The experimental group received $10^6$ endothelial progenitor cells suspended in 25 microlitres of the fibrin sealant
carrier and the fibrin group received only 25 microlitres of the fibrin sealant carrier. The cells were first suspended in 12.5 microlitres of thrombin, and then injected at the central hole simultaneously with 12.5 microlitres of fibrinogen. The clot formed at the bony trough made by decorticating the fibrocartilaginous footprint. The supraspinatus tendon was approximated to the exposed marrow by pulling the suture and tying it over the humeral metaphyseal cortex. The quality of the repair was evaluated by applying slight tension to the tendon. The deltoid muscle and the skin were closed separately using a standard suturing technique with 5-0 Ethicon suture (Johnson & Johnson, Somervile, New Jersey, USA).

The rat was then transferred to the recovery chamber until it recovered from the anaesthesia, and was then housed individually. Two doses of buprenorphine (0.05 mg/kg) were administrated to each rat during the post-operative period for two days. All rats were allowed normal weight bearing and cage activities, standard diet and water ad libitum.
2.4 End Points, Sacrifices, and Dissections

There were three end points for biomechanical testing (two, four, and six weeks after rotator cuff repair), and two for histological analysis (four and six weeks after rotator cuff repair). End points for cell tracking were three days, one week, two weeks, and four weeks after rotator cuff repair. From the experimental and the fibrin control groups, ten rats were dedicated for biomechanics and the other four for histology at each end point.

All dissections were performed post mortem. The rats were sacrificed via anaesthesia with isoflurane (5 %) in combination with oxygen (2 L/min) in the induction chamber followed by cardiac injection of a dose of diluted euthanizing agent, T61. Each rat received 0.5 millilitres of T61 diluted with 0.5 millilitres of saline. The shoulder regions were then shaved to fully expose the skin.

For biomechanics, the rat shoulders were dissected of irrelevant tissues; each specimen contained only the humerus, the supraspinatus tendon, and the supraspinatus muscle. Each specimen was immediately wrapped in gauze which was soaked in phosphate buffered saline, and kept in -80°C freezer until use.

For histology, the rat shoulders were harvested en bloc; each specimen included the humerus, the scapula, and the adjacent soft tissues. Each specimen was immediately immersed in 10 % neutral buffered formalin and kept at room temperature for at least five days before any subsequent tissue processing.
2.5 Biomechanical Testing

The rotator cuff specimens, consisting of the supraspinatus tendon attached to the humerus, were stored in the -80°C freezer. The suture was removed and was not included in the testing. Before the biomechanical testing, the specimens were thawed overnight in the 4°C refrigerator. They were warmed to room temperature prior to testing. The area of the tendon-bone interface (enthesis) was measured using a digital caliper. The area was estimated to be rectangular and length and width were recorded for each specimen. The greater tuberosity of the humerus was used as the main landmark for tendon/scar length measurement. Additionally, the central tunnel for transosseous sutures was present in all repaired shoulders, and was used to approximate the width of the healing tendon/scar.

i. Specimen mounting

The tendon end was stripped of muscle tissue, gripped with sandpaper and ethyl cyanoacrylate adhesive (crazy glue), and clamped with custom screw grip. The tendon was aligned to the humerus in a linear fashion (see Figure 2-2). The tendon footprint area was measured using a digital caliper.
ii. Tendon loading

The testing protocol included a preload to secure the tendon, a session of cyclic preconditioning to obtain a consistent tendon loading history followed by a ramp to failure to determine the ultimate load.

In detail, the tendons were preloaded to 0.10 N to eliminate the slack. Ten preconditioning cycles from 0.10 N to 0.50 N at a rate of 1.0 mm/min were given to each of the tendon to reach a steady state of mechanical equilibrium. The ramp to failure was performed at a rate of 1.0 mm/min. Two sites of failure were anticipated: at the enthesis and at the tendon. An enthesis failure can be a disruption at the tendon-bone interface with or without bony fragments attached. Disruption at the tendon body can be considered tendon rupture, and the load to failure was not included in the final analysis.
iii. Parameters

The measured parameters included the area of the footprint, tendon displacement, load, and ultimate tensile load. The calculated parameters included the stiffness and stress.

(1) Tendon stress: tendon force relative to the tendon cross sectional area

\[
\text{Stress} = \frac{F_{\text{tendon}}}{CSA}
\]
(2) Tendon stiffness: change in tendon length in relation to the force applied to the tendon

\[
Stiffness = \frac{\Delta F_{\text{tendon}}}{\Delta L}
\]

These parameters were subjected to statistical analysis, comparing the experimental and the fibrin control groups using the t-test for four and six weeks animals (n = 10 in each group), and non-parametric test for two weeks animals (n = 4 in each group). Analysis of variance (ANOVA) and multiple comparison test (Bonferroni’s test) were performed to compare the effect of healing over time.
2.6 Histological Analysis

The specimens for histology consisted of the scapula, the humerus, the distal portion of the clavicle, and all the attached musculature. The specimens were kept in 10 % neutral buffered formalin and stored at room temperature for a minimum of five days. The shoulders were processed in groups for consistency. After fixation, the specimens were trimmed of irrelevant tissues and decalcified in Immunocal (Decal, Congers, New York, USA) for one week minimum.

The dehydration process was executed using Leica TP1020, an automated tissue processing machine. The specimens were secured in a plastic cassette and went through 12 stations of various solvents or solutions. See table below for details in each station.

Table 2-2. Tissue dehydration process by Leica TP1020

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After the specimens completed the dehydration process, they were embedded in paraffin in a geometry which the supraspinatus was approximately perpendicular to the shaft of the humerus. The specimens were positioned for sectioning in the coronal plane. The positioning of the tissue sample was achieved with fast solidification of the paraffin wax on ice using Leica EG 1160. The embedded samples were then stored at room temperature.

The sectioning was performed using a low profile blade on Leica RM 2235. Each section was five microns thick in the coronal plane, cutting through the head of humerus, the tendon-bone junction, and the supraspinatus tendon. The sections were mounted on positively charged microscope slides.
2.6.1 Hematoxylin and Eosin Staining

The hematoxylin and eosin staining was performed using Leica Autostainer XL. Slides were then examined for proper section orientation and depth as well as infection.

Table 2-3. Hematoxylin and eosin staining protocol using Lecia Autostainer XL

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</tbody>
</table>
2.6.2 Safranin O/ Fast Green FCF Staining

The staining for cartilage involved three parts: (1) deparaffinization/rehydration, (2) cartilage staining, and (3) dehydration. The deparaffinization/rehydration steps were a replication of haematoxylin staining using Leica Autostainer XL.

Table 2-4. Deparaffinization and rehydration of slides using Leica Autostainer XL

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</tr>
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<tr>
<td>22</td>
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</tbody>
</table>

After the slides were retrieved, the cartilaginous tissues were stained with Safranin O and fast green FCF. First the slides were submerged in 0.1 % aqueous fast green FCF stain (OmniPur, EMD Chemicals, Darmstadt, Germany) for 3 minutes followed by an acetic acid (1 %) wash for 30 seconds. Then they were submerged in 0.1% aqueous Safranin O (Harleco, EMD Chemicals, Darmstadt, Germany) for 5 minutes.

For the dehydration process, the slides were transferred to the Leica Autostainer XL.

Table 2-5. Dehydration of slides using Leica Autostainer XL
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<td>EXIT</td>
<td>-</td>
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</tr>
</tbody>
</table>

The proteoglycans in the cartilage/fibrocartilage stained red, the nuclei stained black, and the cytoplasm stained green.

Safranin O binds to proteoglycans in the fibrocartilage and presents a contrasting red colour against the green background. The fibrocartilage was outlined on three sequential slides for each rotator cuff with a software program and the area for each slide was calculated. The mean area and the standard deviation for each group were determined and compared.

2.6.3 Collagen Birefringence

The collagen parallelism was examined with polarized light microscopy. When a beam of light passes through a polarizing filter, the light will exist with vibration in one direction. When it encounters another polarizing filter with a main axis perpendicular to that of the first filter, the light will not pass through, resulting in the dark field effect. Collagen has the ability to rotate the axis of light passing through called birefringence. Once the vibration direction of the light has
been rotated, the light will pass through the second filter, and collagen appears bright against the dark background.

The parallelism of the collagen fibres close to the insertion can be evaluated with the parallelism index. The index is calculated based on the amount of light signal fluctuation. Higher fluctuation indicates more organized fibre directions as light intensity is greatest when collagen fibres are parallel (anisotropy). Likewise, unorganized fibres will generate smaller fluctuation of light intensity as multiple fibre orientations are represented.

The slides were first deparaffinized/rehydrated using Leica Autostainer XL, then stained in picrosirius red (Sirius red, Sigma-Aldrich, St Louis, Missouri, USA) for one hour and washed twice with an acetic acid (1 %) wash for 30 seconds each. The slides were dehydrated using the Leica Autostainer XL.

Picrosirius red staining was used to enhance the birefringent property of collagen. The light intensity, the rotation of the filter, and the focus of the camera were standardized for all the measurements. The specimens were orientated so that the tendons were parallel to the edge of the stage. The field of view was imaged and the mean light intensity was recorded for each specimen using an image analysis program based on language IDL 6.3 (ITT Visual Information Solutions, Boulder, Colorado). The mean and the standard deviation for each group was determined and compared.
2.6.4 Vessel Staining

The tissues were stained for CD 31 (PECAM-1). The slides were deparaffinized/rehydrated in the same manner as before using the Leica AutoStainer XL. The antigen retrieval step involved incubating the slides with 10 mM of sodium citrate at 98°C for 20 minutes. The slides were then treated with 3% hydrogen peroxide for 30 minutes to quench the endogenous peroxide. They were washed thoroughly and followed by incubation with Dako protein block (Dako, Glostrup, Denmark) for 30 minutes. The serum was wiped off and the rabbit anti-CD 31 antibody (Abcam, Cambridge, England, United Kingdom) was applied to the slides. After incubation overnight at 4°C, the slides were washed and the secondary antibody (Goat anti-rabbit antibody: Biotinylated, Dako, Glostrup, Denmark) was added at 1:200 dilution and incubated at room temperature for 30 minutes. The VECTORSTAIN Elite ABC System (Vector Labs, Burlingame, CA, USA) was used for detection. Solutions A and B were both diluted 1:50 for avidin-biotin/horse raddish peroxidase binding. The DAB reagent was then applied for colour development, incubated at room temperature for 10 minutes. The slides were rinsed, dehydrated, and mounted for analysis.

Images of the new tendon insertion (the area of interest where the tendon bordered the bone and the adjacent tendinous tissues) were captured and selected, and the vascular objects were counted as vascular density (vessel/mm²) using an image analysis program, Visiopharm Integrator System (Visiopharm, Hoersholm, Denmark). The mean and the standard deviation for each group were determined and compared.
2.7 Cell Tracking

Green fluorescent protein (GFP) was used to track transplanted endothelial progenitor cells. The endothelial progenitor cells were isolated from the bone marrow of transgenic GFP Fischer 344 rats [F344-Tg(UBC-EGFP)F455Rrrc, RRRC#: 307; Rat Resource and Research Center, Columbia, MO, USA] using the same isolation techniques and procedure as previously described. Additionally, GFP cell pellets were collected for positive control staining. The GFP positive endothelial progenitor cells were cultured in the same manner, and delivered to the rotator cuff tendon insertion with fibrin sealant. The rats were allowed to heal for three days, one week, two weeks, and four weeks. Two rats were allocated per time point. Regular non-GFP fluorescent endothelial progenitor cells were used for control.

Once the shoulders were dissected, they were processed in the same manner as previously described for histology. Again, the specimens consisted of the scapula, the humerus, the distal portion of the clavicle, and all the attached musculature. They were then fixed, decalcified, and embedded. Five micron sections were cut in preparation to stain for GFP protein using immunohistochemistry.

After the slides were cut, they were deparaffinized and rehydrated. The antigen retrieval step involved incubating the slides with 10 mM of sodium citrate at 98°C for 20 minutes. Then the endogenous hydrogen peroxidases were blocked with 3 % hydrogen peroxide solution for 30 minutes. Dako protein block (Dako, Glostrup, Denmark) was applied and incubated for 30 minutes at room temperature. The primary antibody (Chicken IgY anti-GFP antibody,
Invitrogen, Carlsbad, California, USA) was added at 1:500 dilution and incubated at 4°C overnight. The secondary antibody (Goat anti-chicken antibody: Biotinylated, Vector Labs, Burlingame, CA, USA) was added at 1:200 dilution and incubated at room temperature for 30 minutes. The VECTORSTAIN Elite ABC System (Vector Labs, Burlingame, CA, USA) was used for detection. Solutions A and B were both diluted 1:50 for avidin-biotin/horse radish peroxidase binding. The DAB reagent was then applied for colour development, incubated at room temperature for 10 minutes. The slides were rinsed, dehydrated, and mounted for analysis.

The transplanted endothelial progenitor cells (GFP-expressing) were identified by the presence of the brown stain under a microscope.
3 Results

3.1 Endothelial Progenitor Cells

3.1.1 Flow cytometry

Cells were selected out of all detected events in the flow cytometry. Then single cells, CD 34 positive, and CD 133 and Flk-1 positives, and lastly, CD 31 positive cells were gated. The analysis found 98.3 % of single cells positive for CD 34. Of the CD 34 positive cells, 96.8 % were positive for both CD 133 and Flk-1. These antigens indicated progenitor and endothelial characteristics. The “stemness” of the progenitor cells was illustrated by the high expression of CD 34 and CD 133, reflective of the hematopoietic lineage. The presence of Flk-1, also known as vascular endothelial growth factor receptor-2 (VEGFR-2), indicated the response to angiogenic signals. With respect to the total single cell population, 98 % of the cells were positive for all three surface antigens.

Lastly, CD 31 was gated to determine endothelial commitment. CD 31, platelet endothelial cell adhesion molecule 1 (PECAM-1), is a mature endothelium marker. This set of surface antigen profile in the EPC population indicated its origin from the hematopoietic lineage and commitment to the endothelial lineage. The level of CD 31 expression, 60 % positivity, was not as high as the other markers because the endothelial progenitor cells have yet to lose their
stem/progenitor cell phenotype. The cells possessed more of the characteristics of stem cells than the terminally differentiated endothelial cells.

Please refer to Appendix A for the full cytometric analysis.

Figure 3-1. Flow cytometry analysis: 98.3% of the cultured cells expressed CD 34 on the surface (left); of these CD 34 expressing cells, 96.8% of the cells expressed CD 133 and Flk-1 (right). In summary, 95% of the cultured cells expressed all three surface antigens.
3.1.2 Functional Assays

i. AcLDL and lectin

The isolated cell population was identified by DAPI, staining the nucleus blue. The cells showed high positivity for both Dil-AcLDL and Alexa Fluor 488 conjugated GS-II, suggesting the uptake of AcLDL and the binding to lectin molecules. AcLDL uptake and lectin binding are characteristics of the endothelial cell lineage. The results indicate that the isolation population possesses endothelial phenotypes.
Figure 3-2. AcLDL and lectin stains: DiI-AcLDL (red fluorescence) staining and DAPI (blue fluorescence) staining (top); GS-II lectin DAPI (green fluorescence) staining and DAPI DAPI (blue fluorescence) staining (bottom). Bar = 50 μm.
ii. **Tube formation**

The angiogenic potential of the progenitor cells was assessed by their ability to form tube-like structures on the basement membrane matrices, Matrigel. The mesenchymal stem cell control did not have tube-like structure 24 hours after seeding.

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![Figure 3-3](image-url)

Figure 3-3. Tube formation assay 24 hours after seeding: endothelial progenitor cells (after ten days of culturing, left) and mesenchymal stem cells (right). The endothelial progenitor cells formed tube-like structures while the mesenchymal stem cells were in isolated colonies.
3.1.3 Cell survival in fibrin sealant

The cells survived the clot formation from adding thrombin to fibrinogen. The general cell shape remained intact at 24 hours after mixing the components to produce fibrin. After the growth media was supplemented, the endothelial progenitor cells were able to proliferate at the edges of the fibrin clot. They were able to find a surface to adhere to and assumed the cell adherent morphology. The cells remained active after 48 hours in the fibrin clot.

When delivered in vitro, the fibrin clot is expected to be disintegrated by the host within a day. The cells will then be exposed to the host tissue environment which will provide the essential nutrients and oxygen for metabolism. From this result, the endothelial progenitor cells could be expected to survive the delivery and remain active in the host.

Figure 3-4. Cell survival: cells suspended in fibrin for 24 hours (left) and cell proliferation at 48 hours (right). After the cells were placed into a flask after being in the fibrin clot for 24 hours, they were able to adhere, resume monolayer morphology, and proliferate.
3.2 Biomechanical Testing

The average weight of the Fibrin group at the time of repair was 283 ± 21 g. The weight of the Fibrin group at the three respective endpoints, two, four, and six weeks, was 297 ± 16 g, 319 ± 20 g, and 331 ± 14 g. The average weight of the EPC group at the time of repair was 281 ± 13 g. The weight of the EPC group at the three respective endpoints, two, four, and six weeks, was 293 ± 16 g, 315 ± 10 g, and 332 ± 18 g. There were no differences between the Fibrin and the EPC groups at the time of the repair or at the endpoints.

Figure 3-5. The weight of rats. The weights of the rats in EPC groups and Fibrin groups were not significantly different at any of the time points. Error bars represent standard deviations.
3.2.1 Ultimate Load

For the healthy contra-lateral shoulders, the failure sites were either at the physis or the tendinous tissue close to the interface. Only the ones that failed at the tendon-bone interface were included. However, the failure modes of the biomechanical tests for the operated tendons (with sutures removed) were consistently at the tendon-bone interface. The fibrous scar at the attachment was significantly weaker than the tendinous tissue.

The healthy shoulders tended to have a clean slope to failure with one distinctive peak and a characteristic drop in force, indicating a complete rupture. The operated shoulders, however, displayed discontinuous ramps, multiple peaks, and a gradual decrease in force.

The healthy insertions had an average ultimate force of $26.39 \pm 5.27$ N with a sample size of 10 specimens. For the Fibrin group, the ultimate force at two, four, and six weeks was determined to be $9.33 \pm 3.86$ N, $10.67 \pm 3.29$ N, and $19.09 \pm 3.48$ N. The ultimate load for the EPC group at two, four, and six weeks was $9.14 \pm 3.00$ N, $11.58 \pm 2.74$ N, and $24.64 \pm 3.58$ N*. However, at six weeks, there was an appreciable difference between the Fibrin and the EPC groups ($p=0.0025$). ANOVA and Bonferroni’s multiple comparison test indicated no differences in ultimate force between at two and four weeks, but significant increase in ultimate force from four to six weeks in both groups. Refer to Appendix B for details of Bonferroni’s multiple comparison test.
Table 3-1. Ultimate Load (N)

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Fibrin Group</th>
<th>EPC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 2</td>
<td>9.33 ± 3.86 N</td>
<td>9.14 ± 3.00 N</td>
</tr>
<tr>
<td>Week 4</td>
<td>10.67 ± 3.29 N</td>
<td>11.58 ± 2.74 N</td>
</tr>
<tr>
<td>Week 6</td>
<td>19.09 ± 3.48 N*</td>
<td>24.64 ± 3.58 N*</td>
</tr>
</tbody>
</table>

Significance: * p = 0.0025

Figure 3-6. The ultimate load at the insertions. The ultimate load to failure is significantly higher in the EPC treated repairs at 6 weeks (t-test, p = 0.0025). Error bars represent standard deviations. The dotted line represents the ultimate load for healthy tendon, 26.39 N.
3.2.2 Interface Area and Ultimate Stress

The area of the tendon-bone interface was significantly smaller in the healthy insertions compared to the operated groups. These healthy insertions were better defined and their boundaries were clear. On the other hand, the operated insertions in the Fibrin and the EPC groups were filled with scar tissues. For the Fibrin group, the interface area at two, four, and six weeks was determined to be $7.69 \pm 0.49 \text{ mm}^2$, $6.08 \pm 0.76 \text{ mm}^2$, and $6.71 \pm 0.49 \text{ mm}^2$. The interface area for the EPC group at two, four, and six weeks was $7.58 \pm 0.51 \text{ mm}^2$, $6.24 \pm 1.22 \text{ mm}^2$, and $6.43 \pm 0.54 \text{ mm}^2$. There was no significant difference in the area between the groups. The interface area remained larger than that of the healthy tendon insertion at all the time points. ANOVA and Bonferroni’s multiple comparison test indicated a decrease in the cross sectional area in the Fibrin group from two to four weeks. The difference was not significant in the EPC group due to large standard deviation. However, there were no significant differences between the EPC and the Fibrin groups in either time point. Refer to Appendix B for details of Bonferroni’s multiple comparison test.
Table 3-2. Interface Cross-Sectional Area (mm²)

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Fibrin Group</th>
<th>EPC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 2</td>
<td>7.69 ± 0.49 mm²</td>
<td>7.58 ± 0.51 mm²</td>
</tr>
<tr>
<td>Week 4</td>
<td>6.08 ± 0.76 mm²</td>
<td>6.24 ± 1.22 mm²</td>
</tr>
<tr>
<td>Week 6</td>
<td>6.71 ± 0.49 mm²</td>
<td>6.43 ± 0.54 mm²</td>
</tr>
</tbody>
</table>

Figure 3-7. Area of tendon-bone attachments. No differences in the insertion area were found at any time point. Error bars represent standard deviations. The dotted line represents the area of tendon-bone attachment for healthy insertions, 2.71 mm².
The ultimate stress was determined by dividing the ultimate load with the corresponding tendon-bone interface area. This normalized the attachment strength with respect to the size of the insertion. For the Fibrin group, the ultimate stress at two, four, and six weeks was determined to be 1.21 ± 0.52 MPa, 1.79 ± 0.66 MPa, and 2.83 ± 0.35 MPa. The ultimate stress for the EPC group at two, four, and six weeks was 1.20 ± 0.33 MPa, 1.89 ± 0.44 MPa, and 3.83 ± 0.43 MPa*. Significant difference was detected at six weeks, with the EPC group demonstrating significantly higher ultimate stress (p<0.0001). ANOVA and Bonferroni’s multiple comparison test indicated gradual improvements from two weeks to six weeks in the EPC group over time. The Fibrin group did not show significant differences between two and four week due to large standard deviation. However, differences between the Fibrin and EPC groups were not seen until six weeks. Refer to Appendix B for details of Bonferroni’s multiple comparison test.
Table 3-3. Ultimate Stress of Tendon-Bone Interface (N/mm$^2$ or MPa)

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Fibrin Group</th>
<th>EPC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 2</td>
<td>1.21 ± 0.52 MPa</td>
<td>1.20 ± 0.33 MPa</td>
</tr>
<tr>
<td>Week 4</td>
<td>1.79 ± 0.66 MPa</td>
<td>1.89 ± 0.44 MPa</td>
</tr>
<tr>
<td>Week 6</td>
<td>2.83 ± 0.35 MPa</td>
<td>3.83 ± 0.43 MPa*</td>
</tr>
</tbody>
</table>

Significance: * p < 0.0001

Figure 3-8. Ultimate stress at the insertion. The stress significantly higher in the EPC group 6 weeks after the repair (p < 0.0001). Error bars represent standard deviations. The stress for healthy insertions is 9.84 MPa (not shown on the graph).
3.2.3 Stiffness

The stiffness of the tendon and scar tissues were determined by the largest value of slope in the linear region of the ramp to failure. For the Fibrin group, the stiffness at two, four, and six weeks was determined to be $6.77 \pm 2.23$ N/mm, $8.17 \pm 3.11$ N/mm, and $13.17 \pm 3.93$ N/mm. The stiffness for the EPC group at two, four, and six weeks was $8.11 \pm 1.75$ N/mm, $8.56 \pm 2.62$ N/mm, and $12.78 \pm 2.68$ N/mm. There was no significant difference between the Fibrin and the EPC group. ANOVA and Bonferroni’s multiple comparison test indicated no differences in stiffness between at two and four weeks, but significant increase in stiffness from four to six weeks in both groups. Refer to Appendix B for details of Bonferroni’s multiple comparison test.
### Table 3-4. Stiffness of Tendons (N/mm)

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Fibrin Group</th>
<th>EPC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 2</td>
<td>6.77 ± 2.23 N/mm</td>
<td>8.11 ± 1.75 N/mm</td>
</tr>
<tr>
<td>Week 4</td>
<td>8.17 ± 3.11 N/mm</td>
<td>8.56 ± 2.62 N/mm</td>
</tr>
<tr>
<td>Week 6</td>
<td>13.17 ± 3.93 N/mm</td>
<td>12.78 ± 2.68 N/mm</td>
</tr>
</tbody>
</table>

**Figure 3-9.** The stiffness of the tendons. No differences in stiffness were found at any time point.

Error bars represent standard deviations. The dotted line represents the stiffness for healthy insertions, 23.17 N/mm.
Table 3-5. Summary of biomechanical values for 2, 4, and 6 Week Groups

<table>
<thead>
<tr>
<th></th>
<th>Fibrin (n=4)</th>
<th>EPC (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 Week Groups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultimate Load (N)</td>
<td>9.33 ± 3.86</td>
<td>9.14 ± 3.00</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>6.77 ± 2.23</td>
<td>8.11 ± 1.75</td>
</tr>
<tr>
<td>Insertion Area (mm$^2$)</td>
<td>7.69 ± 0.49</td>
<td>7.58 ± 0.51</td>
</tr>
<tr>
<td>Stress (N/mm$^2$)</td>
<td>1.21 ± 0.52</td>
<td>1.20 ± 0.33</td>
</tr>
<tr>
<td><strong>4 Week Groups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultimate Load (N)</td>
<td>10.67 ± 3.29</td>
<td>11.58 ± 2.74</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>8.17 ± 3.11</td>
<td>8.56 ± 2.62</td>
</tr>
<tr>
<td>Insertion Area (mm$^2$)</td>
<td>6.08 ± 0.76</td>
<td>6.24 ± 1.22</td>
</tr>
<tr>
<td>Stress (N/mm$^2$)</td>
<td>1.79 ± 0.66</td>
<td>1.89 ± 0.44</td>
</tr>
<tr>
<td><strong>6 Week Groups</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultimate Load (N)</td>
<td>19.09 ± 3.48</td>
<td>24.64 ± 3.58*</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>13.17 ± 3.93</td>
<td>12.78 ± 2.68</td>
</tr>
<tr>
<td>Insertion Area (mm$^2$)</td>
<td>6.71 ± 0.49</td>
<td>6.43 ± 0.54</td>
</tr>
<tr>
<td>Stress (N/mm$^2$)</td>
<td>2.83 ± 0.35</td>
<td>3.83 ± 0.43**</td>
</tr>
</tbody>
</table>

Significance: * p = 0.0025; ** p < 0.0001
3.3 Histological Analyses

All stains were performed on the operated shoulders as well as the unoperated, healthy contralateral shoulders.

Figure 3-10. Hematoxylin and eosin, Safranin O, picrosirius red, and CD 31 antigen staining (from left to right) of healthy rotator cuff tendons. Bar = 2 mm.

3.3.1 Hematoxylin and Eosin Staining

Qualitative analysis of the shoulder indicated that the operated tendons were damaged, and that scar tissue was apparent. The central hole in the humeral head at the native tendon insertion was obvious in the operated samples. The sections made were of the correct orientation and at similar depth.
Figure 3-11. Hematoxylin and eosin. The orientation and the depth of the shoulder sections were appropriate. Bar = 2 mm.

### 3.3.2 Safranin O staining

No fibrocartilage was observed at the insertion site at four or six weeks after the repair in both the Fibrin and the EPC groups.
Figure 3-12. Safranin O and fast green staining. No cartilage formation was detected at the tendon-bone interface. Bar = 2 mm.

### 3.3.3 Picrosirius Red Staining

The polarization birefringence property was enhanced using picrosirius red staining. The measured brightness intensity demonstrated that there was no difference between the Fibrin and the EPC groups, indicating that the degree of collagen immaturity and disorganization were similar.
Figure 3-13. Picrosirius red staining. The organization of collagen fibers was assessed under polarized light. There was no difference in collagen organization between the Fibrin and the EPC groups. Bar = 2mm.
Table 3-6. Collagen birefringence measured in mean optical density

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Fibrin Group</th>
<th>EPC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>89.82 ± 20.22</td>
<td>96.69 ± 12.30</td>
</tr>
<tr>
<td>Week 6</td>
<td>108.20 ± 9.52</td>
<td>91.23 ± 7.35</td>
</tr>
</tbody>
</table>

Figure 3-14. Polarization of tendinous insertion. No significant differences were found between the groups at any time point. Error bars represent standard deviations.
3.3.4 Vessel Staining

There was no significant difference between the Fibrin and the EPC groups in the number of vessels. Although the values did not reach statistical significance, the vascular density was higher in the EPC group and both time points. In addition, an increase of vascularity was observed in the EPC group between four and six weeks, while the Fibrin group exhibited the opposite trend.

Figure 3-15. CD 31 staining. The insertion region was selected for analysis. The EPC group showed an increase in vascularity from Week 4 to Week 6. Bar = 2 mm.
Table 3-7. Vascular density (vessels/mm²)

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Fibrin Group</th>
<th>EPC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>279 ± 141 (n = 4)</td>
<td>376 ± 126 (n = 3)</td>
</tr>
<tr>
<td>Week 6</td>
<td>188 ± 78 (n = 4)</td>
<td>457 ± 146 (n = 3)</td>
</tr>
</tbody>
</table>

Figure 3-16. Number of blood vessels per millimeter squared (vascular density) at the insertion site as identified by CD 31 immunohistochemistry staining. Although the differences were not statistically significant, the density of vessels was increased in the EPC group at both time points and the EPC group showed a trend towards increasing vessel density from 4 to 6 weeks, whereas the Fibrin group showed a decrease in vessel density from 4 to 6 weeks. Error bars represent standard deviations.
3.4 Cell Tracking

Green fluorescent protein (GFP) positive endothelial progenitor cells were observed at the rotator cuff repair site at three days, one week, two weeks, and four weeks. The presence of the cells indicates survival and retention at the site of injury.

Most GFP positive cells were surrounded by tendinous scar tissues. Only a few were incorporated into the endothelium.
Figure 3-17. The brown HRP-DAB stain indicated GFP-expressing endothelial progenitor cells (transplanted cells) at Week 2 in the scar tissue at the injury site. Few of the GFP positive cells incorporated into the endothelium as indicated by the red arrows.
4 Discussion

Rotator cuff retear is a common complication after surgical repair. Causes for retear have been proposed and strategies to augment tendon healing have been investigated. Scaffolds, biological factors, surgical techniques, cells, and even rehabilitation have been indicated in variable studies to improve integration of the tendinous and bony tissues at the insertion. However, there is currently a lack of clinically effective means to augment rotator cuff repair biologically. Recently, there has been significant interest in the investigation of adult pluripotent stem cells to aid rotator cuff tendon healing. Cell-based therapies are of a particular interest among other therapeutic strategies because of their ability to contribute to healing through self-renewal, paracrine effects, and/or differentiation into tissue-specific cells that contribute to the healing process.

Our study was built on previous findings of restricted blood supply to the supraspinatus tendon insertion and the therapeutic angiogenic effect of endothelial progenitor cells. Vascular ingrowth is critical to facilitate the delivery of nutrients and cells that respond to the requirements of the stages of healing. Since the successful identification of endothelial progenitor cells by Asahara et al.⁶, there has been a wealth of evidence that these cells induce angiogenic responses in various in vivo models. With endothelial progenitor cells and their known ability for neovascularization, we hoped to develop a novel cell therapy for rotator cuff repair to augment healing at the poorly vascularized tendon-bone interface.
The rat rotator cuff repair model is a well-established surgical model for the investigation of tendon-bone healing and therapeutic interventions. The fibrin sealant delivery method and the survival of transplanted cells were assessed in previous studies. The use of autologous progenitor cells minimized immunogenic responses in the host. No adverse immunogenic response was observed in the animals.

The tracking of the transplanted green fluorescent protein-expressing endothelial progenitor cells using immunohistochemistry at three days indicated the survival of the transplanted cells. However, the survival percentage of transplanted cells is not quantified. Determining the survival percentage should give light to the effectiveness of cell delivery through fibrin sealant.
4.1 Endothelial Progenitor Cells

The Ficoll density separation limited the cells collected from the bone marrow to be strictly mononuclear, which contains the progenitor cells including endothelial progenitor cells as well as mesenchymal stem cells. The fibronectin coating and the growth media supplementation favoured the adherence and proliferation of endothelial progenitor cells in vitro culturing.

The high expression of CD 34 and CD 133 suggested that the isolated endothelial progenitor cells possessed the characteristics of stem cell characteristics, especially that of the hematopoietic lineages. As importantly, the presence of vascular endothelial growth factor receptor-2 (VEGFR-2) indicated response to angiogenic signals. The same population of endothelial progenitor cells also demonstrated endothelial capacity: expression of CD 31 (PECAM-1), uptake of AcLDL and binding of lectin, and in vitro tube formation. Collectively these assays confirmed that the population of cells isolated from the bone marrow was indeed functional endothelial progenitor cells.

The cells were only cultured for ten days prior to surgery, and thus were considered early endothelial progenitor cells. Cells that were cultured for more than two weeks would be considered late endothelial progenitor cells. Early endothelial cells are believed to predominantly provide paracrine effects, and the presence of these cytokine secreting early endothelial progenitor cells was important for in vivo angiogenic mechanisms.\textsuperscript{208} The result in this study supports the paracrine effects of transplanted early endothelial progenitor cells (see Chapter 4.4).
4.2 Biomechanical Testing

The ultimate force for this rat rotator cuff repair model is considered the most appropriate biomechanical outcome as it is the most repeatable measurement with the least source of error. The injury no doubt weakened the strength at the tendon-bone interface. All operated specimens ruptured at the tendon-bone interface (repair site, sutures removed). Even at six weeks after the repair, the attachment (Fibrin group) remained weaker than the healthy, uninjured insertions. In this study, the percentage of strength recovered at each time point are 35 %, 43 %, and 72 % of the normal insertion strength, versus the reported value at 22 % and 52 % at two and four weeks. Additionally, Gulotta et al found no benefits of applying fibrin sealant during rat rotator cuff repair.

We found no significant differences between the Fibrin and the EPC groups at two or four weeks in the ultimate force, interface cross-sectional area, stiffness, and ultimate stress. Similar results were reported by Gulotta and colleagues when they used mesenchymal stem cells alone in the same rotator cuff repair model. From gross observation, it is evident that fibrous tissues filled the interface and attached over the greater tuberosity of the humeral head in a disorganized fashion. The coverage area was much larger than the original insertion, likely a reparative mechanism to compensate for the weaker scar fibers, providing stability during the initial stages of healing. Results of rotator cuff repair using cell therapy by Gulotta et al also indicated large amount of scar at the attachment.

Significant improvements in the tendon ultimate load and stress were detected after six weeks of healing with the addition of endothelial progenitor cells. The ultimate load of the rotator cuff
insertion in the EPC group (24.64 ± 3.58 N) was comparable to that in the healthy insertions (26.34 ± 5.27 N). Manning and co-workers have reported that the attachment strength in repaired tendon insertions (TGF-treated and control groups) approaches the normal/uninjured strength at 28.8 N eight weeks after repair. The large cross-sectional area of these fibrous tissues at the tendon-bone interface offered strength to the attachment more so than the quality of the tendon tissue. Extensive scar formation was observed in studies using the same rotator cuff model. Consequently, the ultimate stress at six weeks (3.83 ± 0.43 N/mm²) remained inferior to that of the healthy insertions (9.84 ± 2.15 N/mm²).

The trend showed a slow healing progress in the first four weeks followed by a rapid increase in strength. This is likely from the effect of the remodeling phase in which the weaker type III collagen fibrils were replaced by the stronger, more organized type I collagen fibrils. Galatz and colleagues reported an increased expression of mRNA and protein contents in both types I and III collagen at ten days post-surgery. Between two to four weeks, there was a drop in type I collagen content and synthesis and that of the type III collagen plateaued. After four weeks, types I and III collagen synthesis exhibited opposing trends, with increasing type I and decreasing type III, suggesting structural remodeling. The biomechanical effect in our study was consistent with the trend observed in the previous literature.
4.3 Histology

The histology results indicated that there was no significant difference between the Fibrin group and the EPC group. At four weeks, the literature reported high type III collagen content and synthesis. Disorganized fibers were present in abundance even at six weeks in our study. Collagen birefringence showed less dense and less mature collagen fibers at the insertion of repaired shoulders compared to the healthy, non-injured tendons. Scarring has been associated with transforming growth factor beta (TGF-β) signaling. TGF-β1 level peaked at day ten after rotator cuff repair, corresponding to the peak cell proliferation and extracellular matrix synthesis. The administration of TGF-β3 during tendon-bone healing led to increased formation of fibrovascular scar tissue. It seems that scarring is an inevitable process in adult rotator cuff healing, and provides stability and strength to the injured tissue prior to structural remodeling as suggested in the previous TGF-β3 study. Whether or not having abundant scar tissue at this stage of healing is therapeutic would require longer endpoints fully assess.

We did not observe fibrocartilage at the repair site in either treatment group. Ide et al did not find safranin O-stained fibrocartilage in the FGF-untreated shoulders at two or six weeks. Using unmodified mesenchymal stem cells alone did not result in increased fibrocartilage. In the cell therapy studies where increased fibrocartilage was detected, molecular signaling (transduction of MT1-MMP gene or Scleraxis gene into mesenchymal stem cells) was incorporated to induce tissue regeneration. Collectively, this evidence suggests that early regeneration of this fibrocartilaginous transition zone between the tendon and the bone requires more than just the host’s intrinsic anabolic cells and the transplanted adult stem cells. The repair
site likely lacks the critical biological factors/molecules and the appropriate reparative cells to recreate a fibrocartilaginous insertion. It is, however, uncertain if angiogenic therapy will promote the recreation of the native fibrocartilaginous insertion due to the nature of the hypovascular transition zone.

The attempt to increase the blood supply to this region did not improve the healing response or encourage early remodeling at four weeks. While the vascularity data did not reach statistical significance in either time point, it is apparent that the EPC group had increasing vascularity at the injury site between four to six weeks after the repair. At the same time the Fibrin group showed decreasing vascularity. The endothelial progenitor cells were capable of sustaining vascular supply, and possibly enhancing it, weeks after the surgery when the tendon naturally progresses into its “hypovascular state”. The presence of the avascular critical zone in healthy insertions is believed to have negative impact on the subsequent rotator cuff tendon-bone healing. The questionable blood supply to the repair site, especially after massive rotator cuff repairs, has been implicated in repair failure in clinical studies. Other clinical studies have found increased vascular distribution to the repair site compared to the uninjured states; however, the studies showed either delayed or a lower magnitude of response than the surrounding tissues at the repair site. In our study, the observed increase in vascularity correlated with the mechanical advantages of the attachment site seen in the biomechanical testing. It is plausible that prolonged supply of nutrient and cellular access to the attachment was responsible for the superior strength. The alteration of the vascular environment perhaps allowed reparative cells to engage in a higher anabolic level, for a longer period of time, than those without. The finding here confirms that the lack of sustained vascular supply during insertion healing could have detrimental consequences. However, given the histological findings in the tissue quality at the
repair site, the specific mechanism by which vascular supply improves attachment strength remains unclear.

The conventional histological methods were unable to detect differences between the Fibrin and the EPC groups. This suggests that the increase in strength is independent of the presence of fibrocartilage or better collagen fibre organization. It seems that more sensitive and targeted histological techniques are required to detect changes in the healing rotator cuff tendons. Immunohistochemical characterization of collagen types (eg. types I, II, III, and X) and tendinous ground substances (eg. aggrecan, decorin, and biglycan) at the insertion may shed light on small changes in repair tissue and the distribution of these extracellular matrix molecules. Changes in tissue levels of matrix metalloproteinases, modulators of tendon remodeling, may help uncover the remodeling processes that led to stronger attachments. Moreover, the transition zone includes the tendon and the bone, and endothelial progenitor cells have been shown to promote bone healing. While we did not specifically assess bone healing at the insertion histologically, such healing may have contributed to the increase in attachment strength observed.
4.4 Cell Tracking

The ubiquitous green fluorescent protein (GFP) expression allows the identification of the transplanted endothelial progenitor cells in regular non-GFP expressing Fischer 344 rats. The GFP positive endothelial progenitor cells were seen at all cell tracking time points, suggesting survival in the host environment without adverse immunogenic responses. Endothelial incorporation was not observed on day 3, but was seen at one, two, and four weeks. This is consistent with reports in the literature that peak capillary proliferation was seen at seven days post-surgery \(^{54}\). However, most of the GFP positive endothelial progenitor cells were stray cells in the scar tissue rather than incorporating into endothelium, suggesting that their activity may be limited to paracrine effects, such as secreting molecular factors beneficial to the repair process. More studies are needed to delineate the fate of transplanted endothelial progenitor cells in rotator cuff tendon healing.

Gulotta et al used the metabolism of β-galactosidase to determine the presence of transplanted mesenchymal stem cells (MSCs) at the rotator cuff tendon repair site \(^{187}\). While the metabolism of β-galactosidase indicated that the cells were alive and metabolically active at the site of injury, neither the cells’ location nor morphology could be determined using such method. Our detection of transplanted endothelial progenitor cells via immunohistochemistry allowed us to identify and locate the cells in the shoulder sections. It also revealed cell fate (incorporation into the endothelium) after transplantation.
4.5 Limitations

The study has several limitations that require consideration. As previously mentioned, several potential sources of error are inherent to the biomechanical testing. While the testing was performed in a consistent manner, variability between specimens was inevitable. This is especially the case when mounting the specimens (gauge length) and measuring the attachment area. The distribution glue between the sand paper and the tendons, the time needed for the glue to dry, and the penetration of glue into the tissues are examples of potential variability. The graphs of the biomechanical test results were not “clean” load to failure curves, making stiffness calculations problematic. This likely contributed to the variability in stiffness and ultimate stress, although the variability observed in this study was actually quite low. It is also important to note that a single load-to-failure test construct used in this study (and others in the literature) does not represent what the clinical population experiences on a daily basis. Continuous cyclic loading may be a better testing regime for replicating tendon overuse.

While the shoulder sections were made by experienced technicians and the sample orientation and depth were examined by the pathologist during the cut to the best of their abilities, these variables could have affected our histological data. The histology serves as supplementary results to support our biomechanical findings. Thus, the histology by itself cannot provide conclusive results of rotator cuff healing. In spite of our biomechanical finding, our histology methods may not have been sensitive enough to detect differences in the healing tissues.

In this study, a single dose of one million endothelial progenitor cells was used for transplantation. This number was chosen in accordance to previous studies of cell therapy for
rotator cuff repairs 63, 64, 65, 66. Varying numbers of cells were not tested. It is likely that more than one million cells are required to result in detectable histological differences. However, it is expected that at some point the aggregation of transplanted cells will become a burden to healing. A dose-response curve will be crucial to determine the optimal amount of cells before becoming detrimental to tendon healing.

Another potential limitation of this study is that the model used healthy young rats. This may not be representative of the older population who often experiences rotator cuff tears. Compromised healing rat models should be investigated in future studies. These may include diabetic models, steroid or collagenase injections to simulate tendon degeneration, and induced overuse via activity. Aged rats are expected to show slower healing due to their diminished cellular activities. The augmentation of blood supply in these conditions will perhaps have a greater effect in healing and result in further statistical significance.
4.6 Clinical Relevance

We found the transplantation of autologous endothelial progenitor cells during rotator cuff repair to result in improved biomechanical strength without adverse effects or immunogenic responses. The difference in the ultimate load to failure between the EPC group (24.64 N) and the Fibrin group (19.09 N) is 5.55 N, a 29% increase from the Fibrin group. This improvement in insertion strength is considered clinically significant as we previously defined clinical significance to be 20% or more, in agreement with the literature15, 16, 34, 62, 63, 73. Although it is unfortunate that we cannot directly link the difference in insertion strength to retear rates in a clinical setting, we can associate stronger attachment with better tendon-bone integration, and a higher threshold for rupture. While the regeneration of the fibrocartilaginous transitional zone has been regarded as the hallmark for the regeneration of native tendon-bone insertion34, 62, 64, 79, 80, we found attachment strength increase to be independent of fibrocartilage formation at the insertion site in this study.
5  Conclusion and Future Directions

5.1  Conclusion

The delivery of endothelial progenitor cells during rotator cuff repair improves the attachment strength at the supraspinatus insertion six weeks after the surgery without detrimental effects to the tendinous tissue. Further studies are required to gain a better understanding of the multiple facets of tendon-bone healing and to ultimately lead to effective biological therapies for rotator cuff repair.

5.2  Future Directions

Attributing a single factor or cause to rotator cuff injury is difficult due to the large number of pathogenic factors that may come into play. Targeting each pathogenic condition is necessary for individualized medical treatment. Similarly, while rotator cuff tears can be treated surgically or managed non-operatively, the post-operation treatments will need to address the various facets of the condition for the rotator cuff healing to be optimized.
In this experiment, a cellular strategy for vascular enhancement was investigated. Endothelial progenitor cells have been shown this study, and many others, to have the ability to induce and enhance localized vascular growth in injured tissues. The cells are appropriate candidates for therapeutic angiogenesis.

### 5.2.1 Identifying the Angiogenic Mechanisms

The current study sought to address the functional outcome via biomechanical testing. The load to failure represents the strength that the healing tendinous structure can sustain before it detaches completely. Biomechanical testing is the most clinically representative outcome, as functional strength is a well-defined clinical assessment for rotator cuff injuries. In this study, the histomorphometric analyses provide only supplementary confirmation of tissue healing. Histology generates pictures for the healing phases, and histological grading can be related to biomechanical functions.

The study draws connections between the transplanted cells and the outcomes. The cellular mechanism by which vessel growth is promoted by the endothelial progenitor cell population is still yet to be fully delineated. Possible mechanisms include secretion of angiogenic growth factors such as vascular endothelial growth factors, angiopoietin-1, and platelet derived growth factor, and the recently proposed mechanism of secreting vesicles containing miRNA, and differentiation into endothelial cells to aid the expansion of the local vascular network.
The factors responsible for the angiogenic mechanisms could be confirmed by genetically modifying the endothelial progenitor cells to enhance the synthesis of angiogenic molecules, such as vascular endothelial growth factors, angiopoietin-1, and platelet derived growth factor. Pharmaceutical agents, alternatively, could be employed to block the activity or production of angiogenic molecules to more precisely define the downstream effect of the factors at play.

5.2.2 The Mesenchymal Lineage

The transition zone helps transfer mechanical forces from the tendon to the bone by minimizing stress concentration at the interface. Accordingly, for optimal healing at the tendon-bone interface, the regeneration of this specialized tendon-bone transition zone must occur. In addition to meeting the metabolic and vascular demands, this healing process requires the proliferation and hypertrophy of chondrocytes at the interface, proper mineral deposition in the extra-cellular matrices, and remodeling of collagenous structures.

The current study focused on the augmentation of vascular supply in hopes that this would set stage for the following healing and remodeling processes. Indeed, meeting the local metabolic demands of the tissue is crucial for optimizing tendon-bone healing. However, fibrocartilage chondrogenesis and structural remodeling are other facets of the healing that could be targeted.

In the future, a mixture of endothelial progenitor cells and mesenchymal stem cells could be investigated. Both cell types are found in the bone marrow mononuclear cell population. The functions and effects of the endothelial progenitor cells are described previously. The
mesenchymal stem cells can differentiate into the various stromal cell lineages that aid the repair of musculoskeletal tissues; osteoblasts, chondrocytes, and tenocytes/fibroblasts that are essential for the production of extracellular matrix of the tendon-bone junction. This is where blood supply plays a crucial role in meeting the metabolic demands of these active cells. As previously mentioned, the fact that autologous transplantation of mesenchymal stem cells alone did not yield superior healing \(^{63}\) is possibly due to the discrepancy between nutrient supply and metabolic demand at the repair site. With the presence of endothelial progenitor cells, the vascularized tendon-bone interface would possibly support the high metabolic requirement of active cells at the repair site.

While the mononuclear cell population has already been employed in clinical studies, endothelial progenitor cells constitute only a small fraction of the population \(^{46}\). Given the results of this study, it would reasonable to investigate an endothelial progenitor cell-enriched as well as mesenchymal stem cell-enriched mononuclear population in rotator cuff tendon healing after surgery.

### 5.2.3 Clinical Application

Moving forward, a dose-response relationship between the quantity of endothelial progenitor cells and the healing/angiogenic response needs to be established. The study was done in an animal model in which the rats received one million endothelial progenitor cells for their treatment. Whether the positive effect can be seen in a much larger scale in that of a human rotator cuff is still unknown. A recent study of mesenchymal stem cell therapy in human rotator
cuff repair has shown successful healing with an average of 51 000 cells given to each patient compared to the one million mesenchymal stem cells given in previous studies. Various amounts of endothelial progenitor cells need to be transplanted to determine a curve that will indicate the appropriate dose of endothelial progenitor cells to administer to the patients.

The cells are rare and their phenotype alters during in vitro expansion, and thus finding the optimal number cells for transplantation requires ensuring the quality of the cells as well as the quantity. Accordingly, it is crucial to standardize and optimize methods to rapidly and efficiently acquire sufficient endothelial progenitor cells from the bone marrow with or without in vitro culturing for use during the surgery. Maintaining early endothelial progenitor cell phenotypes may be key to higher levels of angiogenic secretions. In addition, investigations of multiple deliveries over the span of the recovery period could further determine the best treatment regime. While the first dose of endothelial progenitor cells could be given during the surgery, subsequent doses would have to be administered via local injection or intravenous delivery. Accordingly, the method of cell delivery is also a topic that requires further investigation.

Most importantly, not all rotator cuff injuries should be treated the same. Identifying the patients who would be most likely to benefit from endothelial progenitor cell transplantation during rotator cuff repair is paramount. Older populations suffering from tendon degeneration are more likely to benefit from the addition of endothelial progenitor cells than the young, athletic population with traumatic rotator cuff injury. Comparisons of healing potential between aged rat, diabetic rats, and young mature rats will confirm the possible different responses to endothelial progenitor cell therapy.

The use of endothelial progenitor cells in surgical settings to aid the healing of rotator cuff tendon is feasible as the cell population can be extracted from autologous sources: the bone
marrow and the blood. Employing adult stem cell-like progenitor cells for rotator cuff surgeries bypasses the many ethical issues and technical issues that surround the use of embryonic stem cells and induced pluripotent stem cells. Nonetheless, the cost-efficiency of such procedure would need to be addressed.
References


Appendix A

Flow cytometry analysis for CD 31, CD 34, CD 133, and Flk-1

The first gate selected cells from the total events registered by the flow cytometer (top left). The second gate limited the analysis to single cells only (top right). Using unstained cells as well as fluorescence minus one (FMO) samples, we determined the threshold for CD 34 positivity to be at $10^3$ (bottom left). Cells with a fluorescence of $10^3$ or higher were considered CD 34 positive. The analysis indicated 98.3 % of the single cells express CD 34. Then we gated for both CD 133 and Flk-1 at the same time (bottom right). Again, using unstained cells as well as FMO samples, we determined the threshold for CD 34 positivity to about $10^3$. The analysis indicated 96.8 % of the CD 34 expressing cells express both CD 133 and Flk-1. In other words, approximately 95 % of the single cells express all three markers.
CD 31 is a mature endothelial marker. In this study, CD 31 was used to evaluate endothelial commitment of the endothelial progenitor cells.

The unstained endothelial progenitor cell population exhibited some level of auto-fluorescence (top picture). The gating showed that approximately 60% (after accounting for auto-fluorescence) of the endothelial progenitor cells were positive for CD 31 (middle picture). While the shift in fluorescence was small, the detection of CD 31 with the antibody was sensitive as indicated by overlapping the unstained cell data with the stained (bottom picture).

Flow cytometry showed that most cells possessed the endothelial progenitor cell markers CD 34, CD 133, and Flk-1, but only some expressed the endothelial marker C 31. This indicates that the cell population exhibited stronger stem/progenitor cell characteristics than the terminally differentiated endothelial cells.
### Appendix B

**Bonferroni’s Multiple Comparison Test**

#### Ultimate Force

<table>
<thead>
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<th>Significant?</th>
<th>Adjusted P Value</th>
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<tr>
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#### Stiffness

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### Cross Sectional Area

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### Ultimate Stress

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<td>Week 2 vs. Week 6</td>
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