ON ASPECTS OF INTRA-ARTICULAR LIGAMENT RECONSTRUCTION

PhD Thesis

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"There are two objects of medical education:

to heal the sick and to advance the science."

Charles Horace Mayo (1865-1939)

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List of abbreviations

ACL: anterior cruciate ligament
ACLR: anterior cruciate ligament reconstruction
ANOVA: analysis of variance
BMD: bone mineral density
BMP: bone morphogenic protein
BPTB: bone-patella-tendon-bone
CI: confidence interval
COX: cyclo-oxygenase
μ-CT: micro-computed tomography
DXA: dual energy x-ray absorptiometry
EZH2: enhancer of zeste homolog 2
FDR: false discovery rate
HMWPE: high molecular weight polyethylene
H&E: hematoxylin and eosin
HR: hazard ratio
HT: hamstrings tendon
IACUC: Institutional Animal Care and Use Committee
IL: interleukin
IQR: inter-quartile range
KOOS: Knee Injury and osteoarthritis outcome score
MSC: mesenchymal stem cell
N: Newton
NAR: National Arthroplasty Registry
NIH: National Institute of Health
NKLR: Norwegian Knee Ligament Registry
NSAID: non-steroidal anti-inflammatory drug
OA: osteoarthritis
OR: odds ratio
OPG: osteoprotegerin
PG: prostaglandin
PROM: patient-reported outcome measure
qRT-PCR: quantitative reverse-transcription polymerase chain reaction
RANK: receptor activator of nuclear factor kappa-B
RANKL: receptor activator of nuclear factor kappa-B ligand
RCT: randomized controlled trial
RUNX2: Runt-related transcription factor 2
SD: standard deviation
TGF: transforming growth factor

List of publications

Paper I:

Fibrin glue mediated delivery of bone anabolic reagents to enhance healing of tendon to bone.

Soreide E, Denbeigh JM, Lewallen EA, Samsonraj RM, Berglund LJ, Dudakovic A, Cool SM, Nordsletten L, Kakar S, van Wijnen AJ.

J Cell Biochem. 2018 Feb 1. doi: 10.1002/jcb.26755.

Paper II:

In vivo assessment of high-molecular-weight polyethylene core suture tape in intra-articular ligament reconstruction**.**

Soreide E, Denbeigh JM, Lewallen EA, Thaler R, Xu W, Berglund L, Yao JJ, Martinez A, Nordsletten L, van Wijnen AJ, Kakar S.

Bone Joint J 2019;101-B:1238–1247. Published online: 30 Sept, 2019

Paper III:

The effect of limited perioperative nonsteroidal anti-inflammatory drugs on patients undergoing anterior cruciate ligament reconstruction.

Soreide E, Granan LP, Hjorthaug GA, Espehaug B, Dimmen S, Nordsletten L.

Am J Sports Med. 2016 Dec; 44(12):3111-3118.

Introduction and background

Joint stability is a well-orchestrated collaboration between proprioception and both static and dynamic stabilizers. Typically, static stability is determined by the bony congruity of a joint combined with the ligaments, while neuromuscular control, via feedback from mechanoreceptors, is essential for dynamic stabilization. In a hip joint, which is characterized by a high grade of bony congruity between the femoral head and the acetabulum, the ligaments may be of less importance to the overall static stability. By contrast, a low grade of bony congruity increases the demands of the ligaments to maintain a stable joint, as evident in the knee joint, the gleno-humeral joint, the elbow and the wrist. Furthermore, muscular activation, based on proprioceptive feedback via highly sensible mechanoreceptors, provides dynamic stability, which is typically needed to maintain stability in a loaded joint to protect the static stabilizers.

Extra-articular ligaments and connective tissue have significant healing capacity. By contrast, intra-articular ligaments have limited intrinsic healing potential. Despite increased research attention, the precise mechanisms are not yet fully understood. Several factors are considered to be responsible for this difference in healing capacity. The micro-environmental localization in the synovial fluid is unfavorable for tissue healing as the initial fibrin clot formation needed to provide a tissue scaffold to facilitate tissue healing and ingrowth is degraded by enzymes in the synovial fluid. In addition, other factors have been reported to be involved, including limited vascularization, biomechanical aspects and intrinsic cell deficiencies.[1]

A ligament serves as a tissue-connection between two adjacent bones and may be injured if exposed to sudden extensive loads. A ligament injury can be graded on a severity scale ranging from a sprain via partial tear to complete rupture of the tissue.

While a sprain or partial tear is a severe stretch of the ligament causing elongation or rupture of part of the ligament tissue, the continuity of the tissue remains and usually sufficient joint stability persists. On the other hand, a complete rupture causes discontinuity of the tissue and thus loss of joint stability.

Using a canine model, Murray et al. showed that in the healing process of a specific extra-articular ligament, the medial collateral ligament (MCL), fibrin clot formation within the wound site provided a scaffold which supported tissue ingrowth and remodeling.[2] In contrast, in an intra-articular setting, the upregulated production of enzymes transforming plasminogen to plasmin leads to fibrin degradation, thus preventing the clot formation needed for tissue ingrowth and remodeling.[3] Furthermore, the lack of a stable scaffold reduces levels of inflammatory signaling cytokines, which may reduce the healing potential of a ruptured intra-articular ligament. Consequently, the clinical outcome of an intra-articular ligament rupture remains unpredictable, and high failure rates have been reported.[4,5]

Severe ligament injuries may lead to short-term functional impairment in sports and daily activities, as well as long-term disability due to the early onset of painful osteoarthritis.[6] Given this, ligament reconstruction, using autograft and allograft tendon grafts, is commonly performed to restore joint stability and regain function. A successful intra-articular ligament reconstruction depends on solid incorporation of the tendon graft to the adjacent bones, as well as sufficient remodeling of the intra-articular part of the tendon graft in order to adopt the biomechanical properties of a native ligament.

Incidence

Anterior cruciate ligament (ACL) injuries are common, with an estimated incidence of 68.9 per 100,000.[7] The annual incidence of ACL reconstructions (ACLRs) in Norway is estimated to be 34 per 100,000, but with considerably higher numbers in the population at risk, the younger population.[8,9] In 2017, more than 1800 primary ACL reconstructions were conducted in Norway, including >450 primary reconstructions in patients aged 15–19 years.[10] Similar numbers have been reported from other Scandinavian countries.[11] Worldwide, more than 400,000 ACL reconstructions are conducted annually.[12]

Intra-articular ligament injuries, such as ACL injuries, occur mainly in the younger and more active portion of the population.[9,13] A previous study also reported a significantly higher risk of ACL ruptures in female athletes than in males.[14] According to the Norwegian Knee Ligament Registry (NKLR), the main contributing sports are soccer, European team handball and alpine skiing.[10]

Unfortunately, despite evolving surgical techniques, including improved graft fixation devices, permanently restoring joint stability with a normal range of motion remains a significant challenge.[6,15,16] Consequently, there is a very real need to improve our current surgical options in order to create more predictable and better long-term outcomes.

Management of ligament ruptures: a historical perspective

Anatomy and biomechanics

Claudius Galen (131–201 BC) is often considered to have provided the first description of the cruciate ligaments in the knee joint.[17] In 1836, the Weber brothers described the importance of the ACL in the maintenance of normal knee kinematics, as they

demonstrated increased anterior-posterior translation of the tibia following sectioning of the ACL.[18] Over subsequent years, a range of new studies contributed to our increasing knowledge of the functional properties of the ACL as well as anatomical aspects of the ACL,[19] surrounding knee structures,[20] knee kinematics,[21,22] injury patterns/mechanisms of the ACL,[23-25] and the clinical presentation of ACL ruptures.[26] Robert Adams described the first clinical case of an ACL rupture in 1837.[27] This was based on history taking and clinical examination, which continue to be extremely valuable tools for the assessment of ACL injury. ACL injuries were initially treated conservatively by immobilization in plaster of Paris, or various types of braces or walking apparatus, often for long periods of time. However, these commonly resulted in a poor functional outcome for the patients involved.[28]

Direct repair

The surgical treatment of ACL ruptures emerged at the end of the 19th century. This was largely the consequence of the limited functional outcome reported in ACL ruptures that had been treated conservatively, combined with a reduction in surgery-associated morbidity and mortality. Sir Arthur Mayo-Robson performed the first ACL repair in 1895 (Figure 1), although details of the first successful direct ACL repair were published in 1900.[29] Sir Mayo-Robson conducted a suture repair in a 41-year-old miner who had sustained an ACL rupture 3 years earlier. Except for somewhat limited knee flexion, good functional results were reported when followed-

Figure 1: Sir Arthur Mayo-Robson (1853–1933), British surgeon, recognized for performing the first ACL suture repair in 1895.

up 6 years later.[30] In 1913, Goetjes published an extensive review of 23 patients who had undergone direct ACL suture repair and concluded that all acute and chronic cases of ACL ruptures with inferior knee function should be recommended for suture repair.[31] Despite these promising early results, the outcome following direct repair remained unpredictable, possibly explaining why some surgeons continued to prefer non-operative treatment for ACL injuries.

In 1938, Palmer published his observations on the limited healing capability of ACL ruptures, and put forward his argument for the importance of an early repair.[32] Later, O´Donoghue published good results following a repair consisting of a suture weave through the tibia stump that was passed through a bone tunnel in the femur to reattach the torn ACL to the femur.[33] This contributed substantially to the increased popularity of ACL repairs in the USA during the 1950s. O´Donoghue emphasized, as Palmer previously had, that an early repair was essential to achieve good outcome. ACL repair remained a relatively popular procedure until 1980, and was supported by convincing clinical results.[34,35] Yet, the popularity for the repair procedure started to decrease after Feagin and co-workers published a 5-year follow up on ACL repairs in 1976.[4] These authors reported poor clinical outcomes as most of the 35 patients they studied presented with some degree of painful instability, in addition to a high rate of re-ruptures. These findings were further confirmed by Engebretsen et al., who presented devastating long-term results of primary ACL suture repair in 1990.[36]

ACL reconstruction

Ernest William Hey Groves is recognized for performing the first ACL reconstruction using an autologous tissue graft in 1917 (Figure 2). He was well aware of the

importance of an oblique graft placement, similar to the native ACL, to restore appropriate joint stability.[37,38] Unfortunately, his understanding of the ACL's contribution to knee biomechanics was not fully appreciated during his lifetime and it took almost a century to confirm his observations in a biomechanical study that was later verified in a clinical setting.[39,40] Subsequently, Hey Groves modified his own technique and, together with Alwyn Smith, laid down the basic principle for anatomic ACL reconstruction surgery; these principles are still valid today.[38,41]

Figure 2: Ernest William Hey Groves (1872–1944), British Professor of surgery and a pioneer orthopedic surgeon, attributed for performing the first ACL reconstruction in 1917.

Despite new surgical techniques with promising results, the debate continued as to whether surgical repair or reconstruction was the optimal treatment for ACL injuries. Physicians promoting the non-operative management of these injuries noted that reconstruction "could not give any benefit other than that derived from the period of immobilization following the surgical procedure."[42] After examining several ACL-reconstructed knees, Sir Robert Jones concluded that there was "no perfect result, but several have been much improved."[43] Over the next few decades, physicians continued to debate ACL reconstruction from a variety of different perspectives, including the type of graft tissue, graft/tunnel placement, fixation methods, the timing of surgery and indications for surgery.

Extra-articular ACL reconstruction was initially introduced with the aim of functionally replacing the ruptured ACL without opening the joint. Numerous procedures were

proposed, which used free tendon grafts to stabilize the medial side and thus restore antero-medial rotatory stability.[44-46] Subsequently, similar stabilizing procedures evolved that aimed to reduce antero-lateral instability on the lateral side.[47-50] Because ACL assessment up to 1970s was predominantly based upon the anterior drawer test on a 90° flexed knee, which is usually only positive in situations with concomitant injuries to the menisci or capsuloligamentous structures, it seems logical that the medial or lateral side was prioritized in order to reduce anterior translation of the tibia in relation to the femur. Around this time, MacIntosh's non-anatomic ACL reconstruction, using a fascia lata graft with an intact tibial insertion, began to gain popularity.[51] In this particular technique, the graft was passed under the lateral collateral ligament and attached to the intermuscular septum to prevent anterior translation of the tibia.

The introduction of the Lachman test, and the pivot shift test, in the 1970s led to improvements in the clinician's ability to assess isolated ACL ruptures. ACL reconstruction had been considered a formidable surgical procedure for decades due to substantial surgical exposure, prolonged rehabilitation and the considerable risk of adverse events. Therefore, the introduction of arthroscopic surgery was welcomed by surgeons and rapidly increased in popularity during the 1970s as new and better instruments were developed. This advancement enabled more sophisticated surgical procedures to be conducted. Dandy performed the first arthroscopically assisted ACL reconstruction in 1980.[52]

Over the years, various types of grafts have been used to replace the ACL. For example, Hey Groves introduced the use of fascia lata, with preserved proximal muscle belly; in 1927, Ekenbary used this tissue as a free graft in a modification of Hey Groves' original procedure.[53] Subsequent work by John Insall, using a band of fascia lata as

graft, made a valuable contribution to intra-articular ACL reconstruction.[54] Fascia lata continued to be a common graft until the 1990s; it was around this timepoint when other types of graft began to gain popularity. For a short period, some surgeons considered the menisci, a fibrocartilage structure already present in the intra articular environment, to be a suitable graft. However, the popularity of using the menisci as a graft dropped rapidly at the end of 1980s; this was due to our expanding knowledge relating to the menisci's important contribution to normal load transmission and knee kinematics and increasing concerns relating to the deleterious consequences of its removal.[55-58] The use of patellar tendon as graft was first published in 1928.[59] Initially, the distal tibia insertion was kept intact, and it was not until 1982 that Clancy used the bone patella tendon bone (BPTB) as a free tendon graft in an intra-articular ACL reconstruction.[60]

Galeazzi first described the use of hamstring tendon (HT) as a graft for ACL reconstruction in 1934.[61] However, it was not until surgeons began to pay more attention to issues associated with patellar tendon graft harvesting, such as anterior knee pain, patella fractures and knee flexion contracture, that the use of hamstring tendon as a graft for ACL replacement became more popular.[62] Furthermore, the evolution of knee arthroscopy during the 1970–80s, the development of better surgical instruments and, perhaps most importantly, innovative graft fixation devices were all important events for the revival of HT as a graft.[62] In addition, a study published in 1982 reported only a minor reduction in knee flexion strength using both the gracilis and semitendinosus tendons, thus providing valuable support for the use of HT grafts in ACL replacement surgery.[63]

The quadriceps tendon graft was introduced in 1984, and aimed to reduce donor site morbidity while providing a mechanically robust graft.[64] This form of graft was shown

to exhibit comparable capability to restore and maintain knee joint stability as BPTB and HT grafts, but was associated with less donor site morbidity compared to the BPTB graft and superior knee flexion strength compared to the HT graft.[65-67] Despite not receiving the same popularity as HT or BPTB, the quadriceps tendon graft remains an appropriate alternative for selected patients, and is particularly useful for revision ACL reconstruction (ACLR).

The xenograft was first introduced in 1929, and involved the use of a kangaroo tendon for ACL replacement; however, this technique never gained popularity outside of Australia.[68] Human allografts for ACL replacement were introduced in 1986, following biomechanical assessments showing that these allografts had similar properties to autografts.[69,70] However, the risk of transmitting infectious diseases was a concern and high-dosage gamma irradiation and ethylene oxidation were considered the only available methods that could limit this risk. Unfortunately, these sterilization methods have been shown to cause detrimental effects to collagen fibers, thus reducing the biomechanical strength of the graft and leading to high failure rates. Modern methods of sterilization, using low-dosage radiation, are now considered to be more graft friendly and have reduced the risk of disease transmission; however, these techniques still weaken the tensile properties of a ligament.[71] Recent meta-analyses report that low-dose irradiation reduces graft strength, while non-irradiated grafts yield the same clinical outcome as autografts.[72,73] As a consequence, the risk of transmitting an infectious disease can be limited using effective screening protocols, serological screening and by eliminating high-risk populations. Nevertheless, allografts have never fully regained popularity, but are a valuable alternative in selected patients and for revision surgery, despite the cost involved.

In 1903, the German surgeon Fritz Lange was the first to introduce the use of a synthetic graft, a braided silk construct, for ACL replacement.[74] Since then, a variety of materials have been used, including carbon fiber, Gore-Tex, polyester and modified silk scaffolds. Despite encouraging results over the short term, such synthetic grafts have been associated with only limited functional long-term outcomes and a high rate of graft failure over time.[36,75-77] A recently published 25-year follow-up of ACL reconstruction using BPTB grafts, compared to BPTB augmented with synthetic grafts, did not report any significant difference in functional outcomes, re-ruptures or the presence of osteoarthritis.[78]

Double bundle anatomic reconstruction was introduced because it was thought that this form of reconstruction would better resemble the properties of the native ACL better than the conventional single bundle technique.[79] However, prospective comparative studies have since taught us that the number of bundles does not appear to affect either the functional outcome or the rate of osteoarthritis, as long as the bone tunnels are adequately placed.[66,80,81]

Over the years, ACL reconstruction has evolved from an extra-articular, non-anatomical reconstruction using open surgical approaches to intra-articular anatomical arthroscopic-assisted procedures. The current standard of practice to restore joint stability is to use a free autologous tendon graft (hamstring tendon, bone-patella-tendon-bone) or allograft placed through bone tunnels aligned along the oblique course of the native ACL.

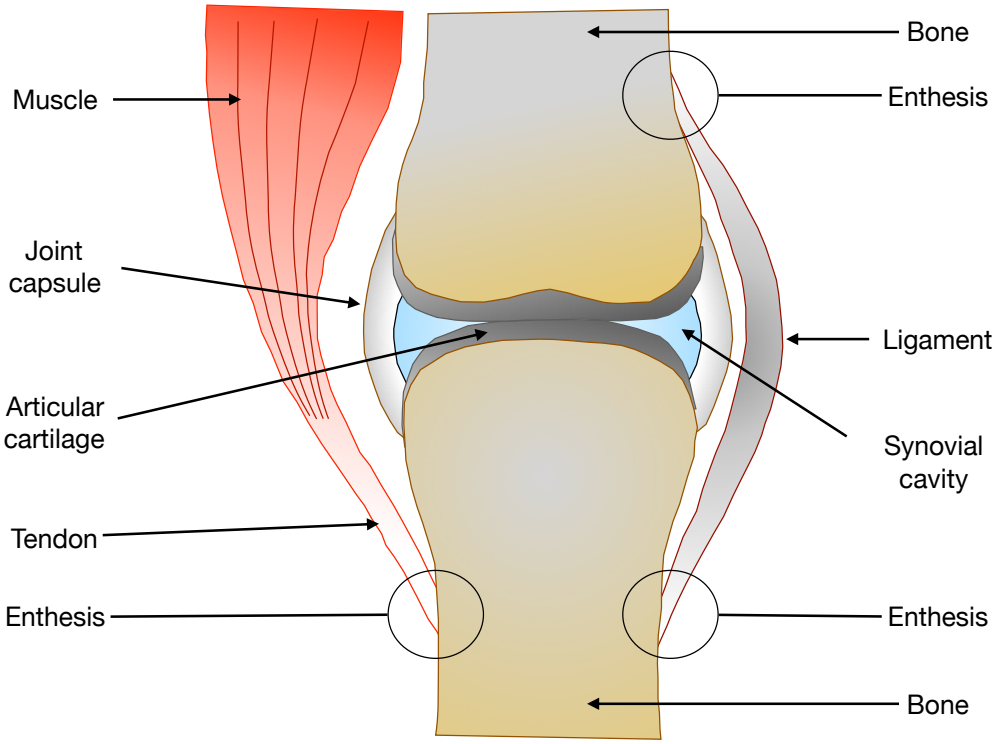

Figure 3. A simplified drawing of a joint, demonstrating how ligaments attach to the adjacent bone to provide static stability, while tendons attach muscle to bone to generate motion and provide dynamic stability.

Tendon to bone tunnel healing

The primary function of tendons is the efficient transformation of tensile loads from muscles to bone. Together with the ligaments, the tendons provide stability and motion in the musculoskeletal system. While tendons connect muscles to bone, transforming muscle contraction to motion and dynamic joint stability, ligaments contribute to static joint stability as they link the adjacent bones and guide the joint motion (Figure 3). Consequently, joint stability and kinematics depend on a well-balanced and dynamic interaction between tendons and ligaments.

Tendons and ligaments attach to bone through a highly specialized fibrous connective tissue zone that is complex in terms of its architectural structure and cellular composition.[82-84] This transitional zone, recognized as the enthesis, consists of four distinct tissue zones that gradually transform tendon/ligament to bone via non-

mineralized fibrocartilage and mineralized fibrocartilage (Figure 4).[85-87] These tissue types differ in both cellular composition, mineralization and extracellular architecture, thus defining their specific structural properties.[88] However, the gradual transition from soft tissue to bone tissue leads to a reduction in strain at the junction of these tissues; this is a biomechanical requirement that permits the distribution of tensile loads over this integrated tissue junction with varying elastic modulus.[89,90]

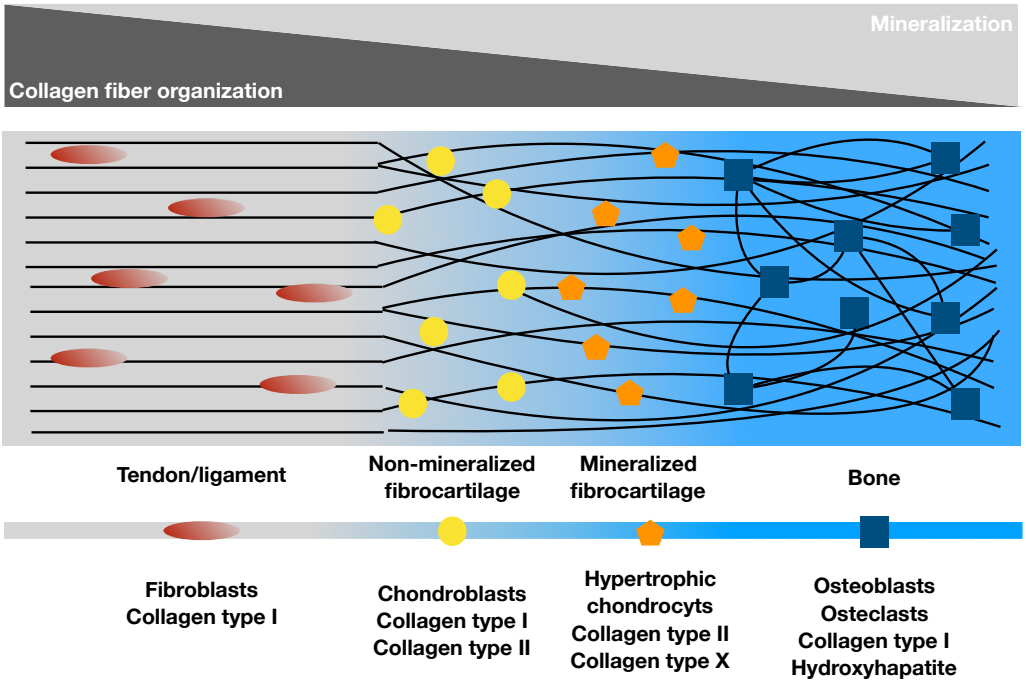

Figure 4. An overview of the enthesis, the transition zone between tendon/ligament and bone, demonstrating its gradual transition in cellular composition and structural architecture needed for distribution of tensile forces over the junction site of two tissues with vast discrepancy in biomechanical properties. From Tellado et al. (2015).

The use of tendon grafts for ligament reconstruction depends on a number of biomechanical aspects to be successful. First, it requires the appropriate selection of a graft with sufficient strength to replace the ruptured tissue. Correct placement of bone tunnels is essential in the restoration of joint stability. Incorporation of a tendon graft into the bone tunnels, which is considered to be the weakest link in the initial healing phase, is crucial for long-term outcome and survival of the reconstruction.[91] Typically, to prevent early failure or recurrent instability, early post-operative mobilization and

rehabilitation is limited. Second, a prolonged remodeling process needs to occur in the graft, characterized by an early hypocellular healing phase that exposes the tendon graft for elongation until sufficient repopulation and the synthesis of extra-cellular matrix has taken place.

The native enthesis is not regenerated following injury or surgical repair.[88] Thus, the healing process for a tendon to bone reattachment occurs through the progressive mineralization of the initially fibrovascular scar tissue established at the interface between the tendon graft and the bone.[92] Tendon to bone healing can be divided into four stages: 1) the inflammatory phase; 2) the proliferative phase; 3) matrix synthesis; and 4) matrix remodeling.

The initial phase is characterized by the increased infiltration of inflammatory cells and the recruitment of mesenchymal stromal cells (MSCs). In addition, angiogenesis is induced as a response to the cellular release of cytokines, hypoxia and growth factors; this promotes the ingrowth of blood vessels and further cellular migration and repopulation of the area. Chemotactic factors induce cellular differentiation and the proliferation of mesenchymal stromal cells to fibroblasts and chondroblasts. This subsequently leads to an increase in the rate of matrix synthesis, which forms an initial scaffold between the graft and the bone. During the matrix-remodeling phase, mature osteoblasts contribute to the ingrowth of new bone in the bone tunnel, combined with the progressive maturation and mineralization of hypertrophic chondrocytes within the interface tissue. In addition, the formation of primarily type III collagen fibers, resembling Sharpey's fibers, cross the interface to re-establish the tendon to bone attachment and graft incorporation.[93-95]

The interface between the tendon and the bone is considered to represent the weakest link during tendon to bone reattachment. Studies have shown a direct correlation

between the formation of new bone and biomechanical strength, suggesting that osteogenesis is critical for the tendon to bone healing.[96,97] The formation of new bone within the bone tunnel is considered to occur through endochondral bone formation. Furthermore, the formation of new bone is regulated partly by micro-environmental factors, including hypoxia, mechanical stress and inflammation.[98] These complex regulatory pathways, which are not yet fully understood, control the differentiation, proliferation and activity of osteoblasts, and represent key factors in achieving a secure anchorage of a tendon graft in the bone tunnel.[99-104]

The initial healing phase is slow, followed by a more accelerated healing period characterized by the increased maturity of the interface tissue and ingrowth of newly formed bone, thus reflecting improved biomechanical properties.[96] Using a canine model, Rodeo and collaborators demonstrated the continuity of collagen fibers between the tendon and surrounding bone at 26 weeks, indicative of complete healing.[92] However, another longitudinal study, which investigated morphological changes for up to 104 weeks in sheep, found that tendon to bone reattachment did not result in the re-establishment of the anatomic enthesis, but resembled a functional repair with a hypercellular fibrocartilage tissue.[94] The temporal aspects of tendon-to-bone tunnel healing remain uncertain and exhibit inter-species differences.

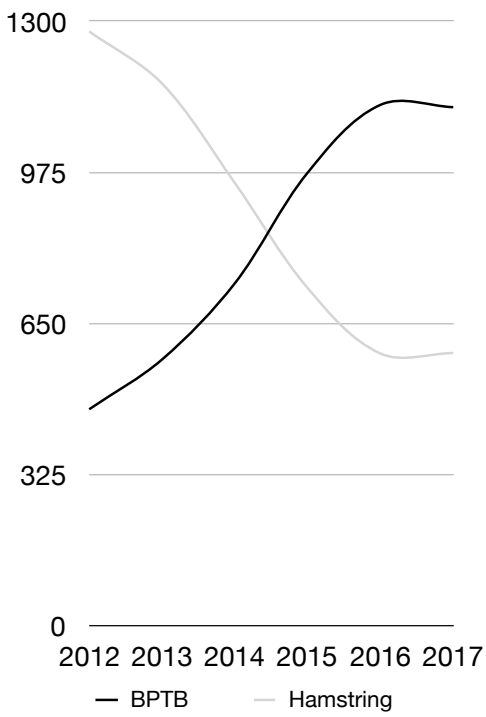

Figure 5. Graft types used for ACLR in Norway in recent years demonstrating an obvious change from hamstring tendon grafts to bone-patella-tendon-bone grafts.

In Norway, auto-tendon grafts are most commonly used for ACLRs, particularly BPTB and HT grafts.[105] Both of these grafts are dependent on bone ingrowth, along with the remodeling/mineralization of the initially formed fibrovascular tissue at the interface between the bone and graft to ensure solid anchorage to the adjacent bone. However, there is an important difference between the two grafts, as the BPTB graft involves healing between bone and bone. In contrast, HT grafts depend upon the healing of two heterogenous tissue types with vast differences in morphological composition and biomechanical properties.[92] It is also worth noting the change in graft use observed by the Norwegian Knee Ligament registry over the last few years, where BPTB has steadily increased in popularity at the cost of HT grafts (Figure 5). This change is partly attributed to Persson's study, which demonstrated a significantly increased risk for revision in patients where ACL is replaced with HT grafts compared to BPTB grafts,[106] a finding which was also confirmed by the Kaiser Permanente ACLR registry.[107]

As mentioned above, tendon-to-bone tunnel healing is a fragile process, influenced by both biological and mechanical factors (Figure 6).[108] Improper healing at the tendon-

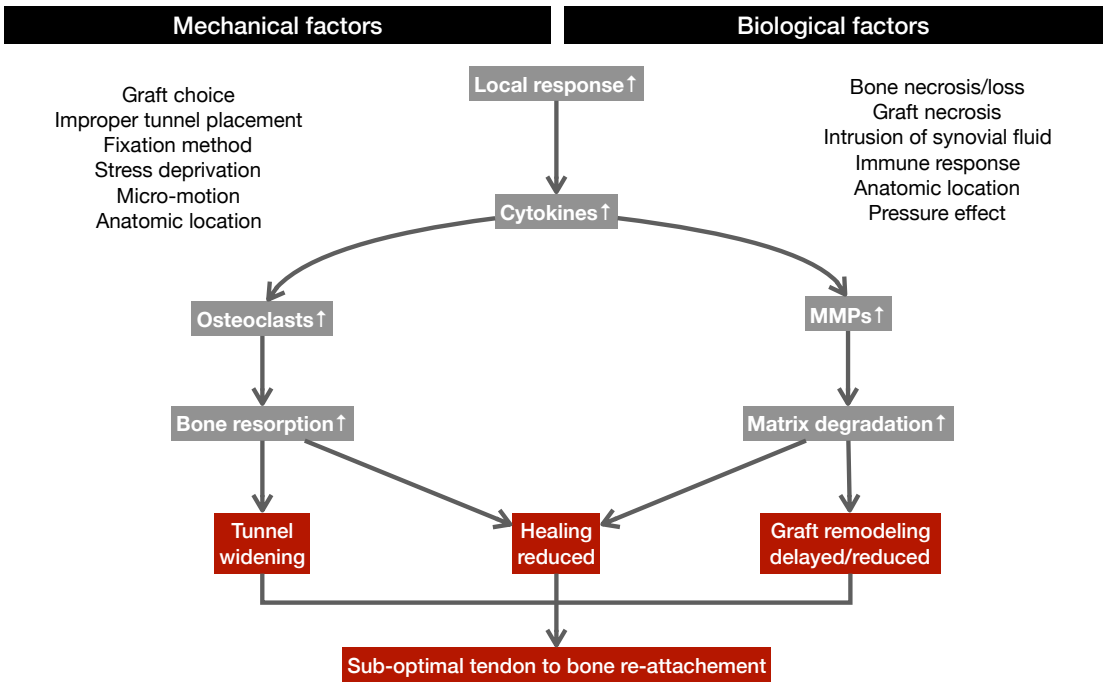

Figure 6. Both biological and mechanical factors may contribute to sub-optimal tendon to bone reattachment.

bone-interface site may lead to poor graft fixation, while insufficient graft remodeling could possibly increase the risk of elongation and creep. Should either of these issues occur, there may be consequential impediment on joint stability and joint function.

Roles of BMP-2 and GSK126 in bone formation

Bone is in a constant state of remodeling, and the balance between formation and resorption remain stable under physiological conditions. Osteoblasts and osteoclasts are the two main cell types involved in this remodeling process, which is regulated by osteocytes, different cytokines, hormones and signaling pathways. Cellular communication between osteoblasts and osteoclasts is important for inducing the initial resorption phase and progression to the termination phase; this occurs via the transition phase (Figure 7).[102] Osteogenesis is a cascade involving the migration and mitosis of mesenchymal stromal cells (MSCs), successive stages of differentiation via osteoblast progenitors and pre-osteoblasts into mature osteoblasts that deposit osteoid (Figure 8).[109]

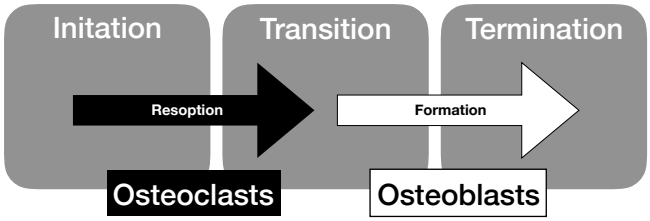

Figure 7. Three-phase model of bone remodeling. Osteoblast differentiation is induced by osteoblast lineage cells expressing signaling molecules such as RANKL, activating osteoclasts to resorb bone. Transition from resorption to the formation of bone is regulated via coupling factors such as OPG, and BMP2 from the bone matrix. The termination phase ensures that osteoblasts flatten to form a layer of lining cells over the newly formed bone.

Osteoclasts are derived from hematopoietic stem cells via mononuclear cells and committed to osteoclast differentiation via the activation of the surface receptor RANK. Osteoblasts and bone marrow stromal cells express RANK ligand (RANKL), which activates osteoclastogenesis and bone resorption by binding to RANK. Osteoprotegerin (OPG) competes with RANKL in its binding to RANK and can inhibit

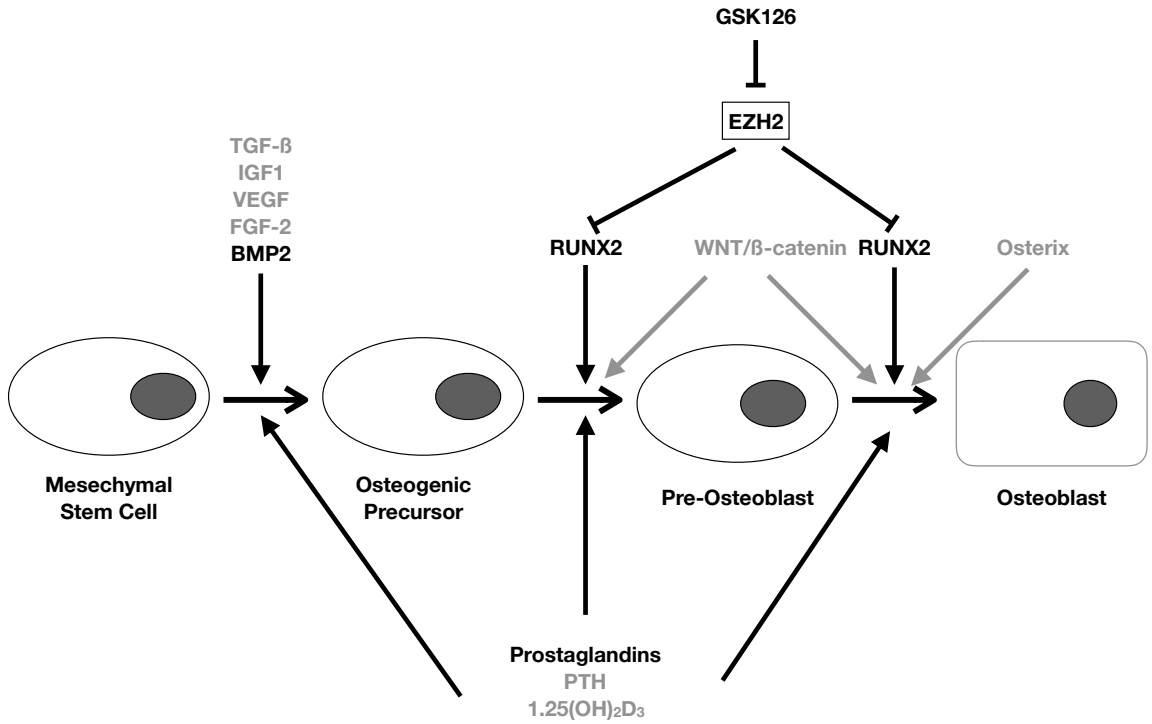

Figure 8. Overview of osteoblast differentiation, demonstrating the involvement of regulatory pathways and factors, in addition to the fundamental role of the transcription factor RUNX2. From Dalle Carbonare et al. (2012).

osteoclastogenesis, thus illustrating the importance of balancing between RANKL and OPG for osteoclastogenesis and bone remodeling.[98]

The initial fixation of the tendon graft is highly dependent on new bone formation in the bone tunnel. As previously mentioned, the biomechanical properties of the tendon to bone reattachment correlates to the mineralization and new bone formation during the initial phase.[92,96] Therefore, increased new bone formation may enhance the biomechanical and morphological outcomes during tendon to bone healing. Anabolic bone factors capable of inducing osteoblast lineage differentiation and proliferation, such as bone morphogenic protein (BMP), may therefore enhance tendon to bone tunnel healing by increasing the formation of new bone. BMPs, first discovered in 1965, are a group of extracellular multifunctional cytokines belonging to the large superfamily of transforming growth factor beta (TGF-β).[98,100,104] BMPs are a group of growth factors shown to play a key role in regulating tissue architecture and development throughout

the body. Some of these, and BMP-2 in particular, have been shown to have osteo-inductive properties, as demonstrated in both *in vitro* cell culture and *in vivo* animal models.[110] Osteo-inductive BMPs have been shown to represent a useful clinical adjunct for spine fusions and open tibial fractures.[111-113]

Exogenous BMPs act by phosphorylating BMP receptors on the cellular membrane. Intracellular signal transduction is then transduced via downstream SMADs (group of cytoplasmic proteins), thus upregulating the transcription of target genes and the translation of proteins. This causes the induction of osteoblast differentiation, and hence increased rates of new bone formation[104]. Increased new bone formation in a bone tunnel may enhance the anchoring of a tendon graft. BMP-2 may also have a direct enhancing effect upon osteoclastogenesis via the RANK pathway, as activated osteoblasts express the osteoclast-activating signal RANKL; this is important for the initial resorption phase in bone remodeling.[113-116]

Runt-related transcription factor 2 (RUNX2) is an essential transcription factor for regulating the differentiation of osteoblastogenic precursor cells, and therefore new bone formation.[117,118] BMP/SMAD signaling interacts with RUNX2 in order to co-regulate target genes controlling the osteoblastic differentiation of MSCs. Enhancer of zeste homolog 2 (EZH2) is a subunit of the polycomb repressive complex 2 (PRC2) involved in the regulation of RUNX2-dependent osteoblast differentiation at the epigenetic level.[99,119] EZH2 catalyzes the trimethylation of histone 3 lysine 27 (H3K27), thus promoting the formation of heterochromatin. Consequently, EZH2 silences the RUNX2 gene by making it less available for gene transcription (Figure 9).

GSK126 is a selective inhibitor of EZH2 and helps to unpack chromatin, thus increasing the accessibility of RUNX2 and therefore upregulating the transcription of specific genes to increase the levels of osteoblast differentiation and new bone formation[117,120-

[122]. Furthermore, GSK126 has been reported to be capable of inhibiting osteoclastogenesis.[123] Silencing the communication between osteoclasts and osteoblasts may enhance the effect of new bone formation as a reduced level of osteoclast activity may contribute to less post-traumatic bone loss in the tunnel.[124,125]

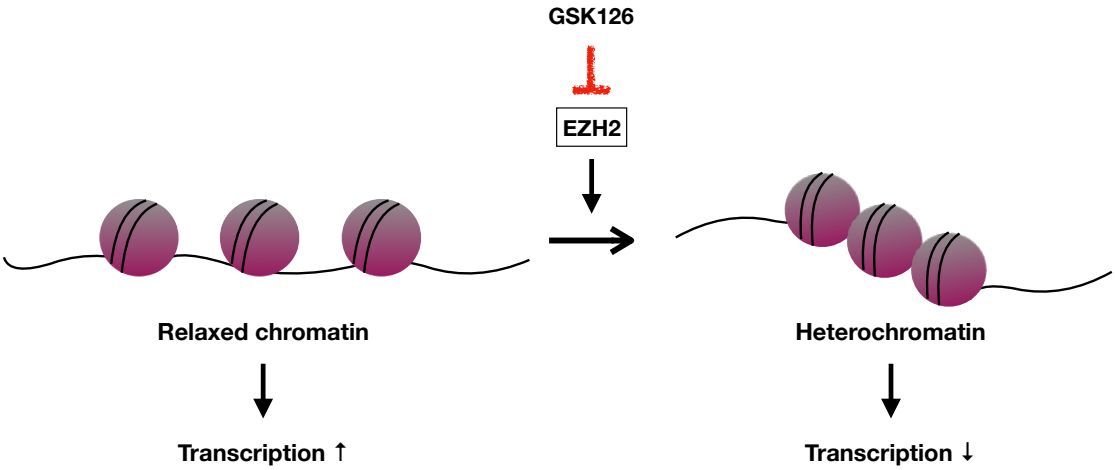

Figure 9. EZH2 promotes heterochromatin formation, silencing transcription of RUNX2, shown to be an essential regulator for bone development and osteoblast differentiation. However, GSK126 inhibits EZH2, thus increases the DNA accessibility for transcription by unpacking of the chromatin.

Tendon graft remodeling and augmentation

Primary ACL suture repair aims to promote direct tissue healing by maintaining reduction and fixation of the ruptured ends and thus support the healing process. However, this has shown limited and unpredictable outcomes in the past due to unacceptably high failure rates.[36] During the healing process in an extra-articular ligament, a fibrin clot first forms at the wound site, thus creating a temporary scaffold that permits tissue ingrowth and healing. By contrast, the upregulation of enzymes within the intra-articular environment can lead to degradation of the fibrin clot serving as a scaffold, thus inhibiting the healing process. Consequently, ligament reconstructions, where the ruptured ligament is replaced by a graft, are commonly performed and aim to stabilize the joint, both in the short and long term. The choice of

graft is determined by the physiological demands placed on the graft during normal activity and, in order to optimize outcome, should preferably restore joint stability and allow early active rehabilitation immediately following surgery.

Tendons are commonly used for the reconstruction of ligaments. Both tendons and ligaments are composed of dense connective tissue-containing cells, proteoglycans and collagen. However, their exact composition and structural architecture can undergo dynamic variations to meet their functional demands. Compared to tendons, ligaments are more metabolically active and have a slightly different composition and organization of collagen fibers, but less total collagen, more proteoglycans, a different degree of cross linking and a different distribution of collagen fibril diameters.[126] Collectively, the cellular composition and structural hierarchy of ligaments defines their biomechanical capabilities to withstand tensile forces and maintain joint homeostasis.[127]

"Ligamentization," a phenomenon attributed to Amiel et al., refers to the time-dependent morphological and biochemical changes occurring in a patellar tendon used for ACL reconstruction.[128] At 30 weeks, these authors found increased levels of collagen type III, increased levels of proteoglycans, and changes in cross-linking patterns; collectively, these processes redefined the structural architecture of the tissue to resemble native ACL. Other studies have attempted to further characterize this continuous remodeling process, which can be distinguished into three phases: early phase, proliferative phase and maturation phase.[129-131] In the early phase, the tissue is characterized by hypocellularity, reduced vascularity and alterations in the ECM organization; collectively, these processes influence the biomechanical properties of the tissue. During the proliferative phase, the graft is increasingly repopulated to become hypercellular with a high cellular activity level and revascularization of the graft. ECM synthesis is enhanced during this phase, particularly collagen type III. In the final

maturation phase, the cellularity and vascularity reduce to levels observed in native ligament tissue. ECM synthesis begins to diminish, and the tissue gains maturity as collagen fibers regain their organization into fascicles and bundles become more densely packed with a parallel orientation along the longitudinal axis of mechanical tension.

Although Marumo et al. described collagen content and the number of crosslinks within the ECM of HT grafts to resemble native ACL tissue within a year of ACL replacement,[132] others have characterized graft remodeling at this point in time as being immature.[133] There is currently no clear consensus for the overall duration of the time taken for the transition of structural and morphological parameters from tendinous to ligamentous tissue in terms of appearance and tensile qualities.

Compared to animal studies, the remodeling process in humans is considered to progress at a slower and less intense rate.[134] Animal studies have revealed cellular necrosis in the early phase; this process has not been confirmed human studies. The revascularization appears to occur in a peri-ligamentous manner, rather than by central ingrowth. Despite this, there is strong evidence that “ligamentization” is a continuous process in which tendon grafts gain biomechanical properties that are similar to native ligaments, and that this occurs via a remodeling process involving the cellular composition and morphological structure, causing a change to ligament-like tissue. The persistent small diameter of the collagen fibrils, combined with increased levels of collagen type III, may explain the difficulties involved with the restoration of the structural properties in native ligaments. Reinnervation of the graft is also important for proprioception. When, and if, this occurs in humans remains unclear. For example, Aune et al. described reinnervation in rat ACL grafts, but were not able to confirm this finding in human grafts after 5–37 months of observation.[135]

One way of improving the biomechanical properties of a ligament reconstruction is the biological augmentation of the auto- or allograft.[136] This could potentially improve the capabilities to withstand creep and elongation of the tendon graft during the early revascularization and remodeling phases.[137,138] It is important to enhance the biomechanical properties of a ligament reconstruction during the early phase to facilitate early and perhaps more aggressive rehabilitation and optimized functional outcome.

Recent advances in tissue engineering techniques have yielded important knowledge for the signaling pathways and epigenetic regulation involved in healing, remodeling and the regeneration of musculoskeletal tissue.[1,139] A range of cell signaling processes are involved in regulating the synthesis, composition and architecture of extracellular matrix proteins, and therefore defining the biomechanical capabilities of the tissue itself. However, the translation and implementation of this knowledge from bench to bed-side has been limited, thus far.

Tissue engineering strategies can be utilized to fabricate a tissue matrix with a cellular composition and biomechanical properties on a scaffold, which is sufficient to meet the initial tensile demands within the joint. An ideal scaffold should mimic the mechanical properties of the native tissue it replaces. This scaffold should degrade at a predictable rate so that it provides appropriate support for tissue formation and the creation of a viable structure. However, the balance between tissue formation and scaffold degradation is essential, as the biomechanical capabilities of the newly formed tissue should increase at least at the same rate as the scaffold degrades; this would ensure the structure retains sufficient tensile properties. An unpredictable rate of degradation

could lead to a sudden failure of the scaffold but without sufficient biomechanical strength in the regenerated ligament tissue to provide sufficient joint stability.

Several biological materials, biodegradable polymers and composite materials have been, or are currently being, evaluated for the construction of ligaments.[140,141] Yet, an ideal ligament scaffold must be capable of withstanding mechanical demands within the joint, while also facilitating tissue regeneration and degrading at a predictable rate to transfer functional demands to the regenerated tissue as its capabilities improves. No such ligament scaffold or replacement exists at the present time.

Effects of non-steroidal anti-inflammatory drugs on bone metabolism

Maintaining a high rate of outpatient reconstruction surgery is a key requirement in providing a modern and cost-effective management service for ACL injuries. Over recent years, the incidence of outpatient surgery has been continuously increasing, and currently accounts for over 70% of the annual ACL reconstructions performed in Norway (Figure 10).[10] Effective post-operative pain management is essential in ensuring early and safe discharge, and is a key factor in maintaining a perioperative complication rate of less than 3%. Appropriate post-operative management is important for many aspects of ACL reconstruction, including wound healing, infection rate, the

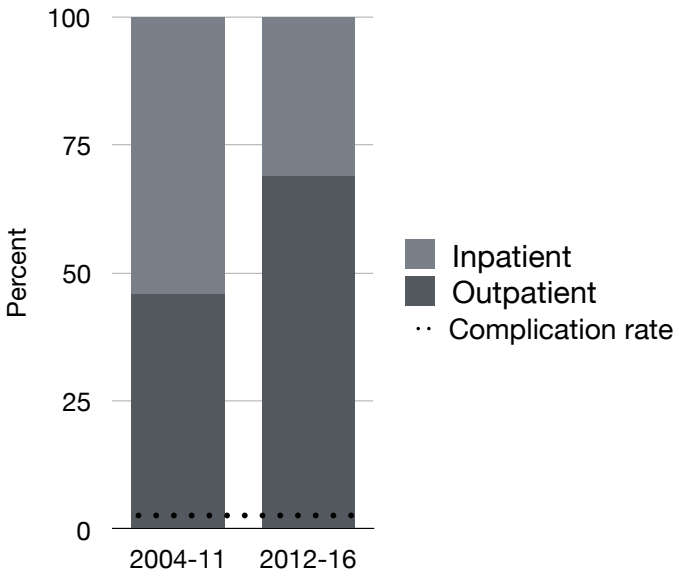

Figure 10. The outpatient ACL surgery rate has increased over the last decade in Norway, while overall perioperative complication rate remains low, and for the latest period < 3%. (NKLR Annual Report 2018).

development of chronic pain and the prevention of cardiopulmonary complications. In addition, pain management is needed to ensure that the patient can perform early range of motion exercises and undergo active rehabilitation; these practices are critical in optimizing functional outcome following ACL reconstruction. Therefore, procedure-specific pain management regimens have evolved over the years. These regimens consists of different analgesics, which aim to provide sufficient pain management, but limit the use of opioids.[142] Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to provide good pain relief in patients with moderate to severe pain, and are considered to represent an important factor in the resolution of post-operative pain following orthopedic surgery, such as ligament reconstructions.[143-145]

NSAIDs inhibit cyclooxygenase (COX), which plays a key role in regulating the formation of cell signaling prostaglandins and interleukins from arachidonic acids (Figure 11).[101] Depending upon the physiological demands of the bone, prostaglandins (PGs) can either promote bone resorption by the direct stimulation of osteoclast activity or enhance bone production via the induction of osteoblast differentiation. Two sub-classes of the COX enzyme, COX-1 and 2, are directly involved in the regulation of PG expression, and are therefore involved in regulation of musculoskeletal tissue healing. COX-1

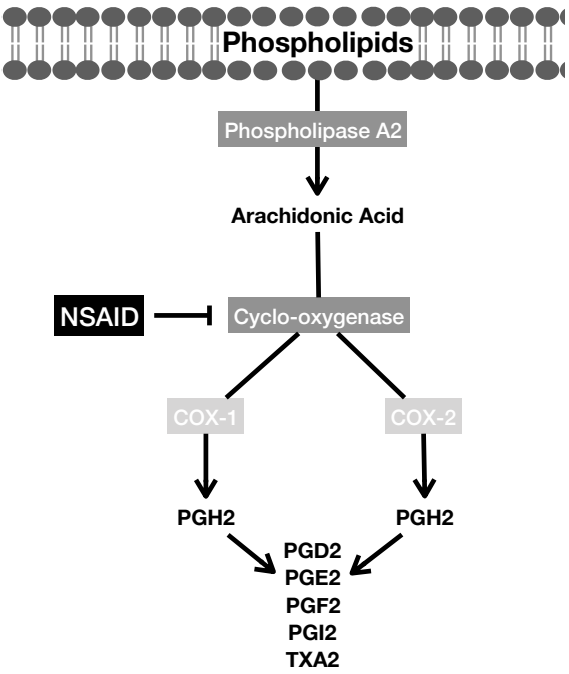

Figure 11. Overview of the arachidonic acid pathway, demonstrating how the enzymatic activity of COX plays a key role in regulating the production of cell signaling molecules such as prostaglandins. From Su and O'Connor (2013).

is continuously expressed and primarily involved in the regulation of homeostasis. On the other hand, COX-2 is rapidly inducible as a stress reaction gene and is directly involved in the production of PGs and early response to changes in the cellular environment, such as acute inflammation.[146-148] Following a fracture, or in a newly created bone tunnel in a ligament reconstruction procedure, there is a very clear increase in the release of PGs.[149] As PGs promote both osteoclast activity and induce osteoblast differentiation for increased bone formation, they represent important regulators of balanced bone remodeling (Figure 12). COX-2 is essential for the acute stress reaction in bone remodeling, and is also essential in the healing process for endochondral fractures.[150]

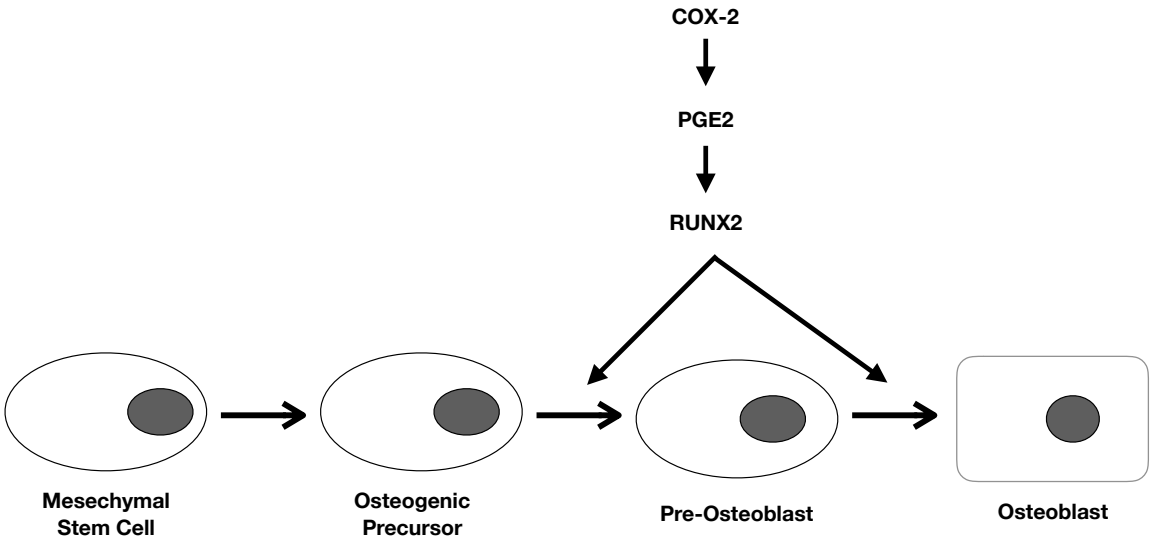

Figure 12. COX-2 contributes to upregulated osteoblast differentiation via RUNX2.

The inhibition of COX-2 is involved in various aspects of endochondral bone formation and is important for tendon graft to bone tunnel healing, and in the healing process of fractures. Normal COX-2 function is important for the differentiation of MSCs into osteoblasts and also for maintaining osteoblast function.[151] PGs are also promoters of the angiogenic processes that take place during the initial phase of endochondral healing.[152,153] In addition, COX-2 is involved in the terminal differentiation of

chondrocytes in a fracture callus, and is therefore critical in the healing process of fractures (Figure 13).[154]

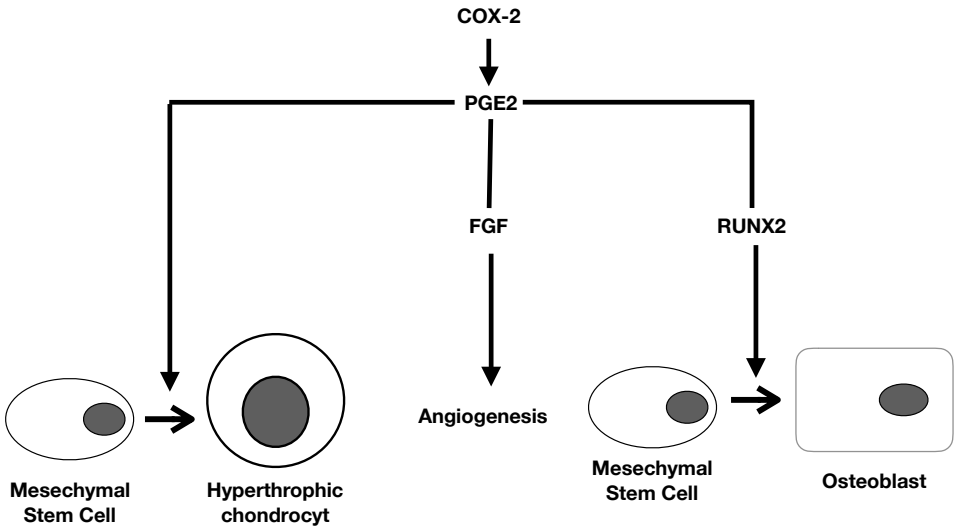

Figure 13. COX-2 is an important contributor to endochondral bone formation as it induces chondrogenesis, angiogenesis and osteoblastogenesis.

Concerns have been raised about the potential negative impact of NSAIDs upon bone metabolism.[101,155-157] Previous experimental studies on non-selective and selective COX-inhibitors have reported negative effects on musculoskeletal healing, including fracture healing, tendon to bone tunnel healing and tendon repair.[158-165] While animal studies and *in vitro* studies have advanced our understanding of the critical signaling pathways and regulatory mechanisms involved in normal musculoskeletal repair, the overall effect of NSAIDs on bone metabolism, tendon to bone healing and fracture healing have still not been fully elucidated. A range of factors should be considered when translating knowledge from preclinical studies to clinical practice, including interspecies differences, the type of NSAID, bioactivity, timing and duration of administration and total exposure.[162,166-168] A recent systematic review investigated the methodological quality of clinical studies on the effects of NSAIDs on bone healing and highlighted that studies not recommending NSAIDs for fracture healing were of poorer quality than studies which did recommend the use of NSAIDs.[166] The authors of this

review also found that a significantly higher number of clinical studies were cited in review articles that did recommend the use of NSAIDs. The authors concluded that further studies are needed to reach a firm conclusion on the safety aspects of using NSAIDs for fracture healing, and emphasized that readers should take a critical approach when drawing conclusions upon the existing literature, especially given the wide variation in quality and methodological limitations. Because of the potential drawbacks of NSAIDs on bone metabolism and musculoskeletal healing, and because of a lack of supportive evidence in the existing literature, the use of NSAIDs for pain management following orthopedic surgery has remained controversial.

Norwegian Knee Ligament Registry

The national Norwegian Knee Ligament Registry (NKLR) was established in 2004 and is owned and co-managed by the Norwegian Association of Orthopedic Surgery and the Norwegian Arthroplasty Registry in Bergen. The NKLR is an approved National Medical Quality Registry and funded by Norwegian National Health Authorities.

The registry prospectively collects surgeon-reported data from patients undergoing ACL reconstruction;[8] 1400–1800 patients are enrolled into the registry each year. All patients included in the registry provide informed written consent and can withdraw from the registry at any timepoint. Previously, this registry has been associated with high rate of reported complete datasets. However, some concerns have been raised over recent years because the rate of reported complete datasets has declined to approximately 84%.[105,169] The registry aims to have a rate of completeness above 90%, and it is hoped that the transition to electronic registration forms, combined with continued effort from surgeons, will help us to reach this goal and therefore ensure that future data is of high quality.

The NKLR aims to monitor important aspects of ACL injury management, such as revision rates, patient-reported outcomes over time and patient demographic data, along with injury- and surgery-related data. This strategy will help to improve our understanding of the incidence of ACL injury, injury mechanisms and patterns over time, as well as changes in management practice. It is vital to consider these aspects if we are to optimize management strategies and improve outcomes for patients with ACL ruptures. Furthermore, a key role for the NKLR, and other registries, is to identify inadequate procedures and surgical devices, and to combine this information with data relating to prognostic factors.[9] The NKLR records both hard and soft endpoints, which includes patient-reported outcome scores at 2, 5 and 10 years following ACL reconstruction. All Norwegian citizens receive a unique 11-digit identification number at birth, enabling the NKLR to link the primary ACL reconstruction to any subsequent knee-related surgical procedure. Confidentiality, for both patients and surgeons, is strictly ensured and approved by the Norwegian Data Protective Authority.

Knee Injury and Osteoarthritis Outcome Score (KOOS)

Patient-reported outcome measures (PROMs) are used to survey the course of disease and evaluate treatment outcomes. PROMs are based on a patient's self-evaluation of their perceived health status, and not on an interpretation by an observer, thus limiting reporting bias. In general, a PROM should be designed with a content that is relevant for the construct of interest and target population ('validity'). Furthermore, a PROM should measure intended dimensions, be consistent for repeated measures ('reliability') and should be able to detect changes in perceived status over time ('responsive'). To ensure a high rate of completeness, and to limit the amount of missing data, a PROM must also be self-explanatory and user-friendly ('feasibility').

The KOOS score was developed in 1998 as a self-administrated PROM with which to assess knee function outcome following knee injury and treatment.[170] The KOOS score is a validated tool with which to evaluate the course of knee injuries and treatment outcome, including ACL reconstructions.[170,171] In addition, the KOOS score is considered to be user friendly; the KOOS questionnaire form includes 42 items categorized into five separate subscales: symptoms, pain, function in activity of daily living, function in sports and recreation, and knee-related quality of life (QOL) (Appendix 1). A KOOS subscale score of 0 corresponds to a patient enduring extreme problems, while a score of 100 corresponds to a patient being free of problems.

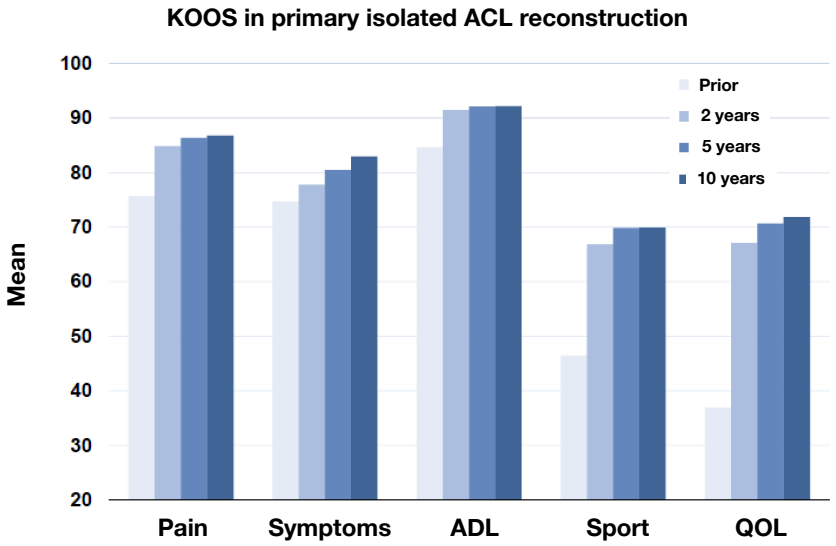

Figure 14. Changes in KOOS score for patients undergoing isolated primary ACL reconstruction. Sport and QOL are the most responsive sub-scores. Data extracted from the NKLR Annual Report 2018.

Of the five subscales, 'QOL' and 'Sport & Recreation' are considered to be the two most reliable and responsive subscales, and assess both short- and long-term outcomes following ACL reconstruction (Figure 14).[172] Frobell et al. proposed the use of KOOS as a tool to identify patients with inferior knee function and stated that a KOOS subscale quality of life score <44 was the equivalent of "clinical failure."[173] Granan et al. confirmed this cut-off value, reporting increased risk for future revisions

in patients with a KOOS QOL score <44 at 2-year follow-up.[154] In a subsequent cross-sectional study, all patients reporting unacceptable symptoms and self-perceived treatment failure had a KOOS QOL score <44. Of note, a KOOS QOL score <44 was also reported by a small portion of patients reporting unacceptable symptoms, but answering "no" for treatment failure, categorized as "undecided intermediate".[174] As indications for revision surgery may vary over time, and between different patients, the KOOS QOL score is considered to represent a very useful tool with which to survey the course of perceived knee function, and to detect failures and inferior results, regardless of a patient's decision to actually undertake revision surgery and the necessary rehabilitation program.

Clinical challenges facing ACL reconstruction

Long-term clinical follow-up has shown equivalent results for joint stability, knee function and the incidence of osteoarthritis(OA) irrespective of whether HT or BPTB grafts are used for ACL reconstruction.[175,176] Even so, acute and chronic joint instability following ligament injuries remains challenging to manage. Few patients with ACL injury are able to return to a pre-injury level of activity without appropriate treatment; surgical ACL reconstruction can substantially increase the number of patients reaching such levels of activity, although surgery is not always successful.[177] Furthermore, a higher risk of secondary knee injuries has been reported for patients who have previously undergone ACLR.[178] According to the NKLR, the survival rate of ACL reconstructions at 8 years is 94%.[10] Moreover, inferior functional outcome and increased revision rates have been reported following revision ACLR.[179] The prevalence of post-operative OA following ACL reconstruction increases with time after surgery.[180-184]

The relatively high incidence of ACL injuries in the younger population, combined with long-term concerns for osteochondral lesions, meniscal lesions and a high rate of early onset OA, has led to uncertainty in selecting the optimal form of management for these injuries. Despite our increased knowledge of fundamental biomechanics, advances in surgical techniques and improved rehabilitation techniques, these patients remain at risk for early onset knee OA, potentially requiring joint replacements at a relatively young age.[6,185] The currently available evidence for joint replacements is primarily based on an older population, with a lower demand and with different expectations to return to recreational and sport activities than a younger patient population. Therefore, the survival rates of the currently available joint replacement implants for young patients remain uncertain and it is difficult to predict joint function over the long term. Collectively, these concerns indicate that we have so far failed to meet our aim of permanently restoring normal knee joint homeostasis and kinematics following the surgical management of ACL ruptures.

Aims of the thesis

Overall aim

Ligament injuries can be a serious condition causing painful instability that may limit function in sport, recreational activities and even the activities of daily living. As these injuries commonly occur in the younger, more active members of the population, it is critical to be able to reliably restore joint stability and regain function. In this thesis, I aimed to investigate different aspects of intra-articular ligament reconstructions, focusing particularly upon factors affecting incorporation of the tendon graft in the bone tunnel and the enhancement of ligament reconstruction by augmenting a tendon graft.

Specific aims

Paper I: To investigate the local delivery of bone-stimulating growth factors in a validated *in vivo* model of tendon to bone tunnel healing.

Paper II: To evaluate *in vivo* the use of ultra-high molecular weight polyethylene suture tape to enhance the biomechanical properties of a ligament reconstruction and support ligament regeneration needed to regain joint function.

Paper III: To assess the effects of NSAID administration upon patients undergoing ACLR.

Materials and methods

Paper I: *Fibrin glue mediated delivery of bone anabolic reagents to enhance healing of tendon to bone.*

Design: This was an experimental laboratory study.

Materials: 45 skeletally mature, female, Wistar rats.

Methods: This experiment used an established model of tendon to bone tunnel healing to evaluate the effect of administering BMP-2 (250 μg), GSK126 (30 μg) or saline on tendon to bone tunnel healing during the early healing phase. Fibrin sealant was used as a delivery vehicle. The animals had free access to food and water, and there were no restrictions placed upon ambulation at any time point. All animals were euthanized at 4 weeks.

Outcome parameters: The primary outcome for this experiment was biomechanical assessment, the quantification of ultimate load to failure, stiffness, elongation and energy absorption of the construct. Secondary outcomes included quantitative micro-computed tomography (μ-CT) for the evaluation of new bone formation and descriptive histology.

Statistical analysis: Continuous data are presented as medians with inter-quartile range (IQR). Comparison analysis was performed using the Kruskal-Wallis test.

Ethical considerations: The Mayo Clinic Institutional Animal Care and Use Committee (IACUC #A1182, Mayo Clinic, Rochester, MN) reviewed and approved the study protocol. Animal handling was in strict accordance with the National Institutes of Health guidelines. There are no in vitro models available that could allow us to assess the healing of a tendon graft in a bone tunnel, and a clinical trial would neither be feasible nor ethically justified. Thus, an experimental study design was chosen, using a previously verified animal model for tendon to bone tunnel healing.[96,186]

Paper II: *In vivo assessment of high-molecular-weight-polyethylene core suture tape in intra-articular ligament reconstruction.*

Design: This was an experimental laboratory study.

Material: 18 skeletally mature, female, New Zealand rabbits

Methods: This experiment used an established rabbit model of ACL reconstruction. All animals were randomly allocated to undergo bilateral ACL reconstruction using autograft, FiberTape or FiberTape augmented with autograft only. Tenodesis screws were used as the fixation method for all types of grafts. Free access to food and free ambulation was allowed throughout the study period of 8 weeks.

Outcome parameters: The primary outcome of this experiment was biomechanical testing to failure for quantifying ultimate load, stiffness, elongation and energy absorption of the reconstruction. Secondary outcomes included μ-CT for quantitative evaluation of new bone formation, descriptive histological evaluation and quantitative assessment of specific gene expression in tissues using quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

Statistical analysis: Biomechanical data are presented as medians and distribution as IQR. The Kruskal-Wallis test was used to evaluate overall significance, and subsequent pairwise comparative analysis was conducted using Mann-Whitney U tests. Bone mineral density (BMD) is presented as mean and standard deviation (SD), and groups were compared by one-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc tests. Gene expression levels were analyzed using ANOVA with false discovery rate (FDR) adjustments for multiple comparisons. All tests were two-sided, and significance level (alpha) was set to 0.05.

Ethical considerations: This study was approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC #A34511, Mayo Clinic, Rochester, MN). Animal handling was in strict accordance to the National Institutes of Health guidelines. We used an in vivo experimental design because there are no in vitro tools available with which to evaluate augmentation of a ligament reconstruction; a clinical trial would not be feasible and would be unethical.

Paper III: *The effect of limited perioperative nonsteroidal anti-inflammatory drugs on patients undergoing anterior cruciate ligament reconstruction.*

Design: This was a cohort study featuring prospectively collected data from the NKLR.

Patients: This study included 7822 patients, aged > 15 years, who had undergone isolated primary ACL reconstruction and were registered between 2008 and 2013.

Methods: Evaluate the effect of perioperative NSAIDs administration on graft survival (risk for revision) and knee function (risk for KOOS QOL < 44) to patients undergoing primary isolated ACLR.[99]

Outcome parameters: Primary outcome was the evaluation of risk for revisions, and secondary outcome was the assessment of inferior knee function, as evaluated by KOOS score QoL < 44. Statistical analysis: Kaplan-Meyer survival analysis was used to assess graft survival, and groups were compared using the Log Rank test. Adjusted Cox regression analysis was used to assess relative differences in the risk for revision, hazard ratio (HR). Logistic regression was used to assess the risk of inferior knee function, for example, odds ratio (OR). Potential confounders were evaluated and included in the final multivariate analysis if $p < 0.2$, for both the risk for revision and inferior knee function analysis.

Ethical considerations: The NKLR is approved by the national health authorities in Norway and the Norwegian Data Protective Authority. All participants provided written informed consent prior to inclusion and are allowed to withdraw at any time point. Data from the registry is non-identifiable, to ensure confidentiality for both patients and surgeons, which is important for general data protection in addition to facilitating a high inclusion rate and completeness, thus limiting reporting bias.

Summary of results

Paper I: *Fibrin glue mediated delivery of bone anabolic reagents to enhance healing of tendon to bone.*

This study evaluated the effects of locally administering bone-stimulating factors to a validated model of tendon to bone tunnel healing. Biomechanical testing yielded encouraging, but not statistically significant, trends for BMP-2- and GSK126-treated animals with regards to energy absorption ($p = 0.116$) and elongation ($p = 0.054$); load to failure ($p = 0.691$) and stiffness ($p = 0.404$) did not show any discernible differences. Cross-sectional areas of the tunnel were reduced in animals treated with BMP-2, but neither BMP-2 nor GSK126 administration led to any significant change in BMD ($p = 0.492$) or bone volume ($p = 0.258$). Histological analysis demonstrated immature fibrovascular tissue at the interface between bone and the tendon as expected at this early time point. The analysis failed to identify any significant differences in new bone formation between the groups. Interestingly, there was no increased accumulation of inflammatory cells in treated animals compared to controls. Collectively, these data indicate that the fibrin glue delivery of BMP-2 and GSK126 appears to be safe and may have the potential to enhance tendon to bone tunnel healing in ligament reconstructions, although the results from this study did not reflect significantly enhanced biomechanical outcomes.

Paper II: In vivo assessment of high-molecular-weight-polyethylene core suture tape in intra-articular ligament reconstruction.

This study aimed to evaluate the capability of a collagen-coated high-molecular-weight-polyethylene (HMWPE) suture tape to withstand tensile forces after ligament

reconstruction and thereby enhance ligament regeneration at an early time point during tendon bone healing.

At 8 weeks, FiberTape alone, or as an autograft augmentation, demonstrated significantly increased biomechanical strength compared to an autograft alone in terms of ultimate load ($p = 0.035$), elongation ($p = 0.006$) and energy absorption ($p = 0.022$). FiberTape grafted samples also demonstrated significantly increased bone formation in the bone tunnel, as measured by BMD ($p = 0.039$). All grafts appeared to be integrated into the bone tunnels, as evidenced by the formation of fibrovascular tissue at the graft bone interface and by the ingrowth of new bone from the perimeter. Overall, limited and comparable signs of inflammation between the groups were observed. No prolonged inflammation was detected by the quantification of inflammatory markers. We did not observe the regeneration of ligament-like tissue along the suture tape within the time frames of this study. Except for one autograft failure, there were no adverse events observed during the entire study period or when evaluating the study samples post-operatively. FiberTape therefore appears to be safe for intra-articular use in ligament reconstructions, as it did not exert a negative impact upon new bone formation in the bone tunnels or induce prolonged inflammation within the time frames of this study.

Paper III: *The effect of limited perioperative nonsteroidal anti-inflammatory drugs on patients undergoing anterior cruciate ligament reconstruction.*

In total, 7822 patients were included in our analysis of graft survival and our assessment of risk for revision; 4144 patients were administrated with NSAIDs post-operatively. The mean duration of follow-up was 2.8 years (range: 0–5.9 years). Overall, the administration of NSAIDs did not affect graft survival. Adjusted Cox

regression analyses confirmed this result for revision risk, with a HR of 1.0 (95% confidence interval [CI]: 0.8–1.3). In patients undergoing reconstruction with a BPTB autograft there was a reduced risk for revision in patients administered with NSAIDs, with an HR of 0.3 (95% CI: 0.1–0.8). Using logistic regression analysis to assess the risk for inferior knee function at 2 years, defined as a KOOS subscale QOL-score < 44, we found a reduced OR in patients administrated with NSAIDs, with an OR of 0.8 (95% CI: 0.6–0.9).

Our study therefore showed that limited peri-operative administration of NSAIDs to patients undergoing ACL reconstruction does not reduce graft survival or increase the risk for either revision or inferior knee function, as evaluated by KOOS subscale QOL < 44 at 2 years of follow-up.

Discussion

The success of a ligament reconstruction depends on several elements such as patient specific factors, surgery related factors, mechanical factors, and post-operative rehabilitation. In addition, biological healing of musculoskeletal tissue, such as tendon to bone healing and the remodeling process of the tendon graft known as “ligamentization”, are crucial to restore and maintain joint stability following a ligament reconstruction.

This project aimed to investigate tendon to bone healing and tendon graft augmentation in ligament reconstruction. We combined clinicals registry data and animal studies in order to be able to evaluate all timepoints in the healing and recovery process. Therefore, this thesis consists of 2 experimental animal studies and one clinical, registry-based study.

Paper I and Paper III assessed factors potentially affecting tendon to bone healing, important for incorporation of the tendon graft to the adjacent bones. Paper II investigated the use of synthetic suture tape as an augmentation of a tendon graft, or on its own, to improve the biomechanical capabilities of the reconstruction. As such, this thesis consists of studies on improving the outcome of future ligament reconstructions.

In Paper I bone anabolic reagents were investigated to evaluate their potentially enhancing effect of the healing process. Paper II investigated the early phase of a ligament reconstruction using a synthetic suture tape as an augmentation of a tendon graft, or on its own, in an intra-articular ligament reconstruction. As neither of these studies would not have been feasible using a clinical study design, *in vivo* animal models were used to answer these questions. Biomechanical assessment was chosen as main outcome for both studies.

Pre-clinical studies have demonstrated a negative effect of NSAIDs on musculoskeletal tissue healing, such as tendon to bone healing. Therefore, NSAIDs have been used with precaution in orthopedic surgery, despite limited clinical data available confirming the negative results from preclinical studies. As cohort studies are capable to detect low incidence events, such as ACLR failure, data from the NKLR were analyzed to assess the effect of NSAIDs on an intra-articular ligament reconstruction, as presented in Paper III.

Experimental animal studies

Etichal considerations

No *in vitro* models are currently available to permit the assessment of the healing process of a tendon in a bone tunnel or the synthetic augmentation of a tendon graft into an intra-articular ligament reconstruction at an early time point. Studying these processes clinically would hardly be feasible, although there have been histological studies of the graft itself after re-arthroscopy in ACLR knees.[135] Therefore, we used an experimental study design in a verified small animal model, as presented in Paper I.[96,186] The experimental animal study presented in Paper II, used an established rabbit model of ACL reconstruction, which has previously been shown to be reliable for intra-articular ligament reconstruction.[187,188]

The study protocols for the experimental studies presented in Paper I and Paper II were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic. The studies were conducted in accordance to guidelines provided by the National Institutes of Health (NIH) to ensure the highest level of animal welfare. Animals were evaluated daily throughout the study periods in order to identify potential adverse events. The accredited animal facilities featured a controlled environment to ensure animal welfare, including temperature, humidity and light/dark

cycles. The staff at the facility are highly trained to provide optimal care for the animals. A veterinary doctor was available at all times for consultation, clinical evaluation and the initiation of treatment, if required.

Tendon to bone healing model

Female Wistar rats were used for the study presented in Paper 1. Rodents, including rats, are commonly used in experimental orthopedic studies as the anatomy and physiology of these animals have been documented extensively. Furthermore, the bone metabolism in rats is considered to be comparable to humans.[189] Tendon to bone healing processes are assumed to be comparable to those in humans, but appear to be faster in rats.[190] We used only female rats for this study to limit potential inter-gender differences in terms of healing and drug metabolism.

The tendon to bone tunnel healing model used in this study has previously been used within our department to evaluate the effect of NSAIDs and zoledronic acid on tendon to bone healing.[186,191] In addition, a longitudinal evaluation of the time-dependent changes in the tendon to bone tunnel healing process has also been documented.[96] As described by Hjorthaug, healing of a tendon to bone tunnel is slow during the first 4 weeks followed by an accelerated period of healing. Furthermore, a correlation between increased biomechanical properties and increased mineralization of the tendon bone interface was demonstrated in that study.[96]

In contrast to some previous studies, a 1.5 mm diameter drill bit was used to create the bone tunnel in the distal tibia. This was to limit adverse events related to fractures and cut out. This practice also provided a better fit between the graft and bone tunnel and thus improved the potential for healing. Furthermore, the scar tissue surrounding the Achilles tendon at the dorsal aperture of the bone tunnel was not removed during dissection as this was considered part of the healing tissue.

The local administration of the growth factors, BMP-2 and GSK126, was chosen to limit potential systemic effects and adverse events, and to improve the clinical applicability of the study. Growth factor dose (250 µg GSK126 or 15 µg BMP-2) was based upon previous studies in the laboratory and a search of the literature.[192,193]

Fibrin glue is approved for clinical use as a hemostat, sealant and adhesive.[194] This was chosen as a delivery vehicle to allow drug administration locally at the dorsal aperture of the bone tunnel without diffusion of the drug into the surrounding tissue. The intention of using a fibrin sealant as a delivery vehicle for growth factors was to ensure the sustained release of a relevant dose to archive therapeutic effect over days, rather than short-term supra-physiological growth factor concentrations.[194-196] Encapsulation of the growth factors into fibrin sealant prevented the rapid degradation of the drug and provided the sustained delivery of a bioactive drug, as the release rate is dependent upon the degradation rate of the fibrin sealant.[195,197,198] As well as permitting sustained drug release, fibrin sealant is also completely absorbed *in vivo*.[199]

Although the prolonged release of growth factors should be beneficial for new bone formation, the degradation rate may have been insufficient to release the necessary concentrations of growth factors required to promote new bone formation at this stage of tendon to bone healing. By contrast, the rapid degradation of fibrin sealant may have released a high dose of BMP-2, which is known to induce osteoclast differentiation with increased initial bone resorption.[116] In our case, we do not know how the drugs diffused into the tunnel. Consequently, the anticipated increased new bone formation following the local administration of growth factors may have been insufficient to override the increased initial bone resorption at this early phase of healing.

Tendon graft augmentation model

For Paper 2, New Zealand rabbits were used. They are commonly used in experimental orthopedic animal studies. They are the largest of the small animals used in the laboratory, and this particular breed is non-aggressive, making them easy to handle and observe. They also have fewer health problems compared with other breeds of rabbit.[200] A rabbit knee is considered a sufficiently sized knee for a reproducible ACL reconstruction. However, an important difference from the human knee is the natural hyper-flexed resting position in the rabbit knee. We used skeletally mature female animals for this study to limit biological variability in skeletal healing. As with all experimental animal studies, we acknowledge that the translational potential to human biology may be uncertain due to interspecies differences.

All animals were randomly allocated to receive either FiberTape, autograft or FiberTape-augmented autograft in a bilateral ACL reconstruction. All animals received the same graft bilaterally. In all animals, the semitendinosus tendons were removed; this ensured an equal magnitude of surgical trauma in all animals, even though it was not used as graft in the FiberTape-only group. Native ACL footprints were used as a guide for tunnel placement, creating bone tunnels 2.5 mm in diameter in both the femur and the tibia. Grafts were fixed using 3 x 8 mm tenodesis screws to ensure a solid fixation in both the tibia and femur. No restraint on ambulation was applied at any time point to allow “early active rehabilitation” and the cyclic loading of grafts. Post-operative pain management was closely monitored to ensure a rapid return to normal ambulation and a natural resting position.

Biomechanical assessment

The main outcome for Paper I and Paper II was biomechanical assessment. An experienced engineer from the biomechanical laboratory conducted all the tests to limit technical/operator variability.

For Paper I, the load to failure test was performed using a custom-made electro-mechanical testing machine. The distraction force was aligned along the direction of the bone tunnel, pulling the Achilles tendon straight out from the distal tibia. Custom-made clamps were made to (1) ensure rigid assembly to the testing machine and (2) avoid applying pressure to the bone tendon interface, or the tendon itself; either of these factors could have affected our test results (Figure 15). We did not experience any problems during testing with this test set-up. Our sample size was based upon previous studies, but could have been underestimated and therefore not able to detect a true difference between the groups (a type II error).

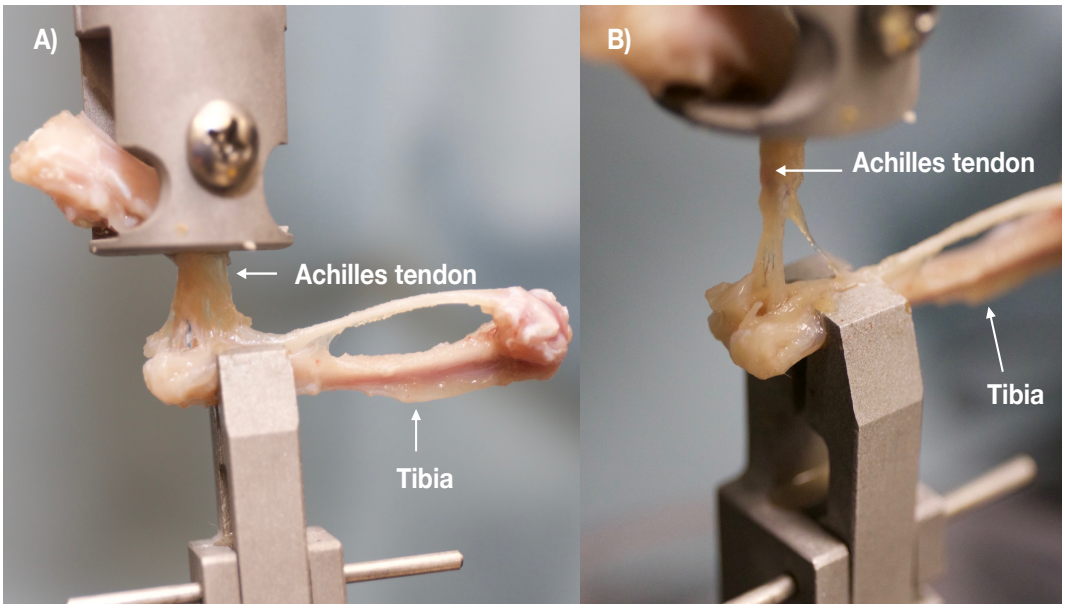

Figure 15. Procedure used for the force to failure test for tendon to bone tunnel healing. (A) Custom-made clamps were used to avoid applying pressure to the tested interface, while ensuring rigid fixation of specimens in the testing jig. (B) Achilles tendon pulled out from bone tunnel in the distal tibia, distraction force aligned parallel to the axis of the bone tunnel in the distal tibia.

For Paper 2 we used a hydraulic material testing machine (MTS, Eden Prairie, MN, USA) for the biomechanical assessment. The tibia and femur shafts of each specimen were potted into polymethylmethacrylate (PMMA) for secure fixation in the custom testing jig. Following accurate and rigid fixation in the testing machine, all of the remaining soft tissue, except for the ACL reconstruction, was carefully transected to permit an isolated assessment of the reconstruction only. The knee joint was flexed 20° degrees during the test, to align the distraction force along the longitudinal axis of the bone tunnels. One could argue that a tibia anterior translation test may have been more clinically relevant; however, our decision to conduct a distraction test was based on an anticipated higher degree of reproducibility in this small animal model.

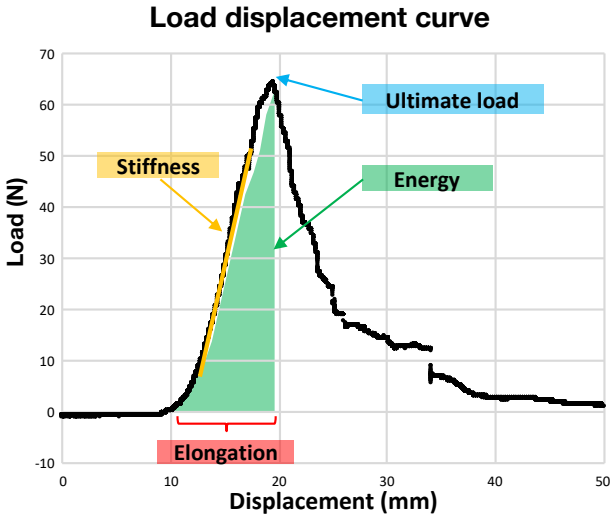

Figure 16. Typical load displacement curve. Ultimate load was defined as the peak of curve. Stiffness was computed as the slope in the linear region of the curve. Energy was computed as the area under the curve until the peak of the curve. Elongation was estimated as the distance between inclination from baseline to the peak of the curve.

For both studies, data relating to load, time and displacement were collected, allowing us to create load displacement curves to determine ultimate load to failure, stiffness, elongation and total energy absorption in the reconstructions (Figure 16).

Quantitative μ-CT assessment

While dual-energy x-ray absorptiometry (DXA) determines BMD in two dimensions, with the result expressed as areal density, μ-CT analysis provides a volumetric three-dimensional analysis for the quantification of BMD. The region of interest was not two-

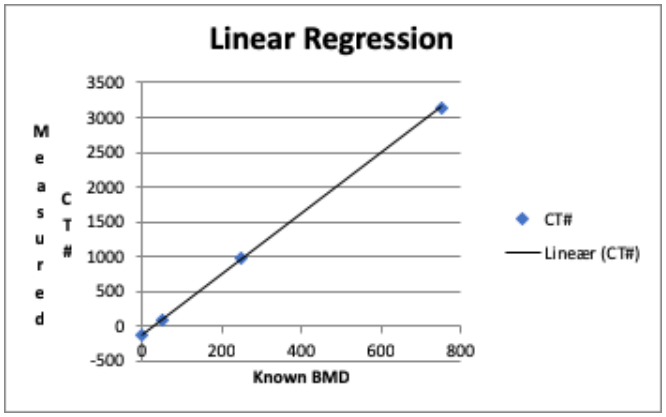

Figure 17. Linear regression was used to determine the relationship between known and measured CT numbers, using BMD = (CT# - Beta CT)/Sigma CT.

dimensional as in DXA scanning, but three-dimensional with increased accuracy for anatomical localization. This yields a more precise result for the BMD values in the region under investigation.

New bone formation was quantified as BMD, and cross-sectional area (mm^2) was measured by μ-CT. An Inveon CT/PET module scanner (Siemens Medical Solutions INC., Knoxville, TX) was used. A calibration phantom, containing samples with known calcium concentrations, was scanned to determine the CT number (density per voxel) for known calcium levels. To determine the relationship between the measured CT numbers and known CT number, we used linear regression (Figure 17).

A three-dimensional region of interest was defined, aligning a cylinder with a diameter equal to the drill bit used (1.5 mm) along the longitudinal axis of the bone tunnel. Two concentric cylinders were placed around this cylinder such that each would have the same volume as the cylinder, thus allowing measurement of mean CT number for each volume and bone range. An experienced biomedical image analyst performed the final analysis to ensure accuracy and reproducibility for both studies.

For Paper I, the evaluation did not yield any differences in terms of bone volume, BMD or cross-sectional area, which might have been because there was no detectable effect in response to the applied growth factors, or because of false negative findings, as the sample size for this outcome was limited. Furthermore, the μ-CT imaging was performed only at the end of the studies. It is not possible to rule out increased early

bone resorption with BMP-2. Additional time points could have provided useful longitudinal data on the healing process, and enabled adjustment for baseline bone volume and density. This would have also provided an evaluation of bone tunnel placement and diameter, and possibly monitoring of tunnel widening, which may affect the initial incorporation of a tendon graft in a bone tunnel.

Histological evaluation

Histological evaluation of the tendon bone interface was performed using hematoxylin and eosin (H&E) stained sections. Sections were aligned normally to the longitudinal axis of the bone tunnel, thus allowing evaluation of the graft incorporation and tissue formation in the bone tunnel. A descriptive evaluation was performed to evaluate tendon graft incorporation into the bone tunnel, as evidenced by new bone formation and fibrocartilage tissue formation at the tendon bone interface. We used a modified scoring system to quantify integration of the graft, as we did not expect mature fibrocartilage formation at this early time point.[201]

For Paper II, interference screws were not removed prior to the fixation and embedding of tissue in methacrylate; this was to preserve the graft–bone interface intact for descriptive review. Non-decalcified tissue was embedded in methyl methacrylate and sectioned using a fine bandsaw with a diamond blade. Sections were created with the aim of providing a cross-sectional presentation of the graft within the bone tunnel. A trained musculoskeletal pathologist performed the descriptive histological evaluation. No quantitative histological assessments were conducted. H&E-stained sections were evaluated to identify potential adverse events, such as fractures and the presence of inflammatory cells, including infection. Evaluation of Sanderson's Rapid Bone Stains aimed to get a better impression of mineralization within the tissue at the interface between the graft and bone tunnel, as

this stain differentiates better between mineralized and non-mineralized tissues. The formation of new bone and fibrocartilage tissue in the bone tunnel was evaluated to assess the maturity of tendon graft incorporation in the bone tunnel.

Unfortunately, we experienced technical issues with the decalcification of tissues, which complicated the sectioning and staining. This meant that some slides were of sub-optimal standard for the final analysis. Furthermore, sections were not perfectly aligned to the bone tunnel, and we experienced difficulties creating sections from a similar anterior-posterior position in the bone tunnel to allow comparison between groups.

Gene expression analysis

qRT-PCR is a highly sensitive and consistent technique that is able to detect the presence or absence of gene expression; it can also quantify the expression of specific genes. Therefore, qRT-PCR is a useful tool with which to quantify alterations in gene expression. Our aim was to assess specific alterations in genes related to the deposition of extra-cellular matrix and inflammatory markers in peri-graft tissue.

Although PCR is a highly sensitive technique, it can produce misleading results due to contamination. The isolation of RNA from *in vivo* tissue increases the risk of contamination compared to *in vitro* cell culture. All PCR primers were tested prior to their use on rabbit tissue to verify their specificity. However, primers can anneal to sequences that are similar but not completely identical to the target gene, thus producing false results. As our study sample was limited by this outcome parameter, our results were exposed to type II error.

Registry based study

In the era of modern evidence-based and value-driven healthcare, the hierarchy of evidence is considered to represent a critical foundation. Physicians are encouraged to seek the highest level of evidence available to guide in everyday clinical decision-making, at both the population and individual level.[202] The development of the Cochrane library, which provides clinical guidelines based on systematic reviews of the management of numerous medical conditions, has made a valuable contribution to improvements in the standards of medical care.

Appropriately designed randomized controlled trials (RCTs) permit systematic blinded comparison between groups to provide sound evidence for cause and effect, and considered to be the gold standard in biomedical research. However, there are limitations to RCTs, especially when investigating rare diseases or events, when considering the surveillance of management with rapid changes in technology or devices, the treatment of complex injury patterns, numerous treatment combinations, and the evaluation of actual standards in medical practice.[203] All of the above mentioned situations appear to be valid considerations for the management of ACL injuries. Registry-based observational cohort studies provide solid information in these circumstances; this was the basis for establishing the Norwegian Arthroplasty Registries (NAR) and subsequently the NKLR. A prospective cohort study provides useful information with which to improve treatment outcomes by providing feedback to hospitals and surgeons. It is also a useful tool with which to monitor the real-world management of selected diseases/injuries in a broader population. Thus, large cohort studies can detect the premature failure of devices and medical implants, identify changes in management over time, reveal adverse events, estimate the risk of inferior

treatment and identify prognostic factors that might affect outcomes, such as the administration of NSAIDs to patients undergoing primary ACLR.

Kaplan-Meyer curves are commonly used to analyze survival data by predicting the length of time to an event, as in our study time from ACL reconstruction to revision.[204] A common feature of survival analyses is that a number of study participants do not reach the main end-point before the end of the study. We used right censoring in the present study, at the time of revision, discontinued follow-up or at the end of the study, whichever occurred first. Survival between groups was compared using the log rank test, which tests the null hypothesis that there is no difference in probability between the groups, for revision in our case.[205] The log-rank test is a significance test that can be used to detect differences between groups, without providing an effect estimate.

Cox regression analysis is used to analyze the relationship between an event, such as ACL revision, and possible explanatory variables, such as the administration of NSAIDs. The effect estimate in Cox regression analysis is the hazard ratio (HR), which is considered to be comparable to relative risk for the outcome to occur at any timepoint. The HR was calculated as the hazard of ACL revision in patients given NSAIDs, divided by the hazard of ACL revision in patients not given NSAIDs. Given this, an HR equal to 1 would indicate that the risk of ACL revision is the same for both groups, whereas an HR exceeding 1 would indicate an increased risk for revision in the exposed group compared to the un-exposed group. The 95% confidence interval (CI) is used to estimate the precision of the HR; a narrow CI indicates a high level of precision, while a wide CI indicates a low level of precision. Furthermore, if the 95% CI does not include unity, i.e. HR=1, then it is considered to be statistically significant.[206-209]

The odds ratio (OR) is a measure of association between an exposure (NSAIDs) and an outcome, inferior knee function as evaluated by KOOS QOL < 44 in the present study.[206] We calculated the OR for inferior knee function using logistic regression analysis, adjusted for selected confounders. Given that the odds for an outcome to occur is the same as for it not to occur, independently of exposure, the OR would be equal to 1. Thus, if an exposure is associated with higher odds of an outcome, then OR > 1; by contrast, if an exposure is associated with lower odds, then OR < 1. For construction of CI, the same assumptions regarding precision and overlap of 1 as described for Cox regression are valid for OR.

Potential confounders were analyzed for both risk of revision and inferior knee function. Variables with a significance level < 0.2 were included in the final multivariate analysis. An experienced statistician, affiliated with the NKLR, supervised and controlled the statistical analysis conducted during this study presented in Paper III.

To ensure a continuously high external validity of the registry, the NKLR aims for the complete inclusion of all primary ACLRs performed in Norway. The reporting completeness rate has shown a decline over recent years, to approximately 84% in 2018.[10,105] This registry can identify patient demographics, describe injury patterns and the complexity of surgical management, and longitudinally track clinical outcomes in a real-world setting. The surgeon conducting the procedure reports the data; this limits the potential for reporting bias. Furthermore, to ensure a high rate of completeness, and to limit information bias, the identity of the surgeon is not recorded. Patient identities remain confidential, as the registry only provides non-identifiable data to researchers. The KOOS was chosen as an outcome measurement because it has been validated for this patient population, and for short- and long-term patient-reported functional outcomes after ACL reconstruction. The KOOS is patient-administered, self-

explanatory and can be completed within reasonable time limits; this ensures that most patients complete the survey. The main outcome estimate in the NKLR is revision, as with the NAR. The unique personal identification number, which all Norwegian citizens receive at birth, can be linked to future surgical procedures, thus enabling the NKLR to identify accurate numbers of knee-related surgeries, including revisions. In addition, the KOOS is included as a secondary endpoint, to prospectively monitor long-term knee functions up to 10 years post-operatively. The KOOS is an important tool with which to track the outcome of ACL surgery, as it identifies patients with inferior knee function and early failures, independent of the decision to undergo revision of the failed ACL reconstruction. The administration of NSAIDs was included in the reporting form for the NKLR in 2007. However, the reporting rate for the first year was somewhat uncertain, and was therefore not included in this particular study. The surgeon reports the type of NSAID and the duration of administration. The timing of administration, in relation to the surgery itself, was not reported. Patient compliance to the surgeon's recommended and prescribed use of NSAIDs was not evaluated. Combined with the fact that there was some missing information for certain patients with regards to administration, the type of NSAIDs used, and the duration of administration, this represents an uncertainty that should be taken into consideration when interpreting the findings of this study.

General discussion

Tendon to bone healing

Ligament reconstructions using tendon grafts depend on the secure attachment of the graft in the bone tunnel. The vast discrepancy in elastic modulus in these two types of tissues makes reattachment of a tendon to bone difficult. Also, the native enthesis is not regenerated in the reattachment of tendon to bone. While this attachment process

is not fully understood, it is considered to be a fragile and prolonged process, and occurs through gradually increased mineralization of fibrovascular scar tissue initially forming at the interface between bone and tendon tissue combined with bony ingrowth from the surrounding native bone. It has previously been shown that the biomechanical strength of tendon to bone healing correlates with the amount of bony ingrowth, mineralization and remodeling of the fibrovascular tissue at the interface.[92,96] The bone–soft tissue interface is considered to represent the weakest link in a reattachment, thus an enhanced bone formation is favorable to improve the biomechanical properties of tendon to bone tunnel healing at an earlier time point.

Both BMP-2 and GSK126 have shown osteo-inductive capabilities, thus increasing new bone formation.[98,104,110,139,210] The study presented in Paper I, revealed a tendency of increased bone formation in animals treated with BMP-2 and GSK126, which was reflected in increased yield load; however, our findings were not statistically robust. A larger study sample would increase the statistical power of the findings. In addition, an increased dosage of the treatment combined with a vehicle delivering a prolonged higher drug concentration could enhance new bone formation to yield statistical differences between the treatment groups. The time frame used for this study period may not be appropriate to show statistical differences between the groups.

Using a canine model, and a collagen sponge as a delivery vehicle for BMP-2, Rodeo et al. previously demonstrated a significant increase in pull-out after just 2 weeks in a tendon to bone tunnel healing model.[211] These authors also noted more extensive bone formation at all time points up to 8 weeks, which was attributed to enhanced bone ingrowth following BMP-2 treatment. In a rabbit model of ACL reconstruction, Pan et al. observed prolonged osteogenic effects of BMP, with stronger fixation of the graft when using different vehicles for BMP-2 delivery.[199] In contrast, Thomopoulos et al. did

not observe enhanced tendon to bone healing in response to local BMP-2 administration in a canine flexor tendon model after 3 weeks.[212] These authors concluded that the anticipated bone anabolic effect at this time point was insufficient to override the post-surgical bone loss. Furthermore, using rabbits, Martinek et al. showed increased incorporation of a tendon graft in the bone tunnel by adenovirus-infected gene transfer, which caused the expression of BMP-2 to be upregulated. Increased osteoblastogenesis, visible by histology, was reflected in enhanced strength up to 8 weeks after surgery.[213] In another study, Takigami et al. demonstrated that BMP-2 injected into the end of the tendon graft prior to implantation in a bone tunnel could induce tendon tissue transformation to bone.[214] Histological findings were also reflected in improved failure load and stiffness, indicating enhanced tendon to bone tunnel healing. This confirms the previously described ability of BMP-2 to induce the differentiation of fibroblasts into osteoblast-like cells.[214,215]

Collectively, these studies demonstrate the potential enhancing effect of BMP-2 on tendon to bone healing. However, transiently increased osteoclast activity due to intercellular signaling between osteoblasts and osteoclasts, BMP-2 induced differentiation of osteoclasts[113] or BMP-2 dose-dependent osteoclast stimulation can cause increased bone resorption during the initial phase.[114,115] Given this, these studies confirm that both anatomical location, species, drug delivery vehicle and drug dosage contribute to the outcome of experimental studies on tendon to bone tunnel healing.

Previous studies have reported bone tunnel enlargement following the HT reconstruction of ACL.[216] Mechanical factors, such as suspensory device fixation or improper graft fixation, may be associated with micro-motion, thus causing a bungee effect, which might also contribute to bone tunnel enlargement. Furthermore, both the

misplacement of bone tunnels, and graft fixation outside of the tunnel, may contribute to “windscreen-wiper effect” at the entrance as the grafts oscillate at the aperture. This has also been reported to be associated with tunnel enlargement. In addition, biological factors, such as increased osteoclast activity, the presence of synovial fluid in the bone tunnel and immature bone–tendon healing during the initial phase, may enhance tunnel enlargement.[217] Thus, in addition to accurate surgical technique, the biological enhancement of the tendon–bone interface may positively affect the outcome of ligament reconstructions.

In the early phase of healing, as determined in our present study, inflammation-driven endochondral bone formation is immature with limited mineralization of the fibrovascular tissue formed at the interface. In addition, induced osteoclast activity during this early stage of healing can contribute to tunnel widening, as indicated by our μ-CT findings of increased cross-sectional area of the bone tunnels in all groups. We did not identify any differences between the groups in terms of BMD in the bone tunnel, which may be consistent with post-traumatic upregulated osteoclast activity and immature bone formation during this early phase of healing. In addition to their bone-enhancing effect by osteoblast induction, both BMP-2 and GSK126 may affect osteoclast differentiation and bone resorption.[113,123] While BMP-2 can increase osteoclastogenesis via RANKL upregulation or autocrine BMP-2 effects, GSK126 has been shown to exert inhibitory effects on osteoclast differentiation via gene silencing mechanisms.[123]

EZH2 is important for the epigenetic regulation of skeletal development and osteoblast differentiation via the essential transcription factor RUNX2.[99,139] GSK126 is reported to stimulate bone formation by inhibiting EZH2; this occurs via the inhibition of chromatin formation.[210] Our study evaluated a new application of GSK126, namely its ability to

enhance tendon to bone tunnel healing. Despite promising trends showing somewhat increased bone volume, reflected in increased median yield load, neither outcome parameter demonstrated statistically significant differences at this early time point with the dosage, model and sample size used herein. In contrast to our study, which investigated the local administration of a single dose, a previous study reported the anabolic effects of GSK126 in bone following oral administration for 6 weeks.[210] The effect of GSK126 has yet to be investigated in a fracture-healing model, as previous studies has focused skeletal development using small animal models. Given this, GSK126 may play a more essential role in development than in the healing of skeletal tissue. Furthermore, our current findings might be related to various factors, including the route of administration, treatment duration and the utilized dosage.

BMP-2 has been shown to possess the ability to induce the differentiation of fibroblasts into bone-producing osteoblast-like cells.[214] While not reported to be an issue in previous tendon to bone healing studies, heterotopic ossification is a potential adverse effect of exogenous treatment with BMP-2, and potentially other bone-promoting factors. We did not observe the formation of heterotopic ossification in any of our animals; this was consistent with previous reports.[199,211,212] Neither did we observe any other adverse events, such as the formation of seroma, an increased rate of infection, wound issues, skin rash or pronounced osteolysis in any of our experimental groups. Thus, the local application of osteo-inductive factors, using fibrin sealant as a delivery vehicle, to promote new bone formation appears to be safe. However, in our model we were unable to identify increased levels of fixation after 4 weeks.

Tendon graft augmentation in ligament reconstruction

Paper 2 demonstrated stronger intra articular ligament reconstructions grafted with FiberTape alone or as an autograft augmentation at an early timepoint of tendon to

bone tunnel healing. While autografted samples failed by gradual elongation of the graft until it eventually ruptured, FiberTape and FiberTape-augmented autografts withstood such elongation and failed by sudden pull-out from the bone tunnel after higher tensile loads were applied. FiberTape, either alone or as autograft augmentation, yielded significantly higher ultimate load and energy absorption than autograft alone. Within the time frame of this study, FiberTape did not negatively affect bone tunnel healing, and we did not observe prolonged increased inflammation in FiberTape samples compared to autograft only.

The early healing phase of a tendon graft is characterized by partial necrosis and hypocellularity, thus causing a weakening of the graft[129,188,218]. This may be reflected in our study where mid-substance mode of failure was more common in autografts. Our present results are similar to previous studies of ACL reconstruction in rabbits, in terms of ultimate load and stiffness.[187,219] This confirms previous considerations for longitudinal graft healing and re-modeling, which is unsufficient to restore native biomechanical properties within 8 weeks. In addition, graft pull-out from the tibial side was more common than on the femoral side, which could be due to microstructural differences in the bone between the proximal tibia and the distal femur.[201] Given this, the protective properties of FiberTape may allow for earlier rehabilitation without compromising the integrity of the remodeling tendon graft.

Post-traumatic bone loss, and tunnel widening, may limit tendon graft to bone tunnel healing in the initial phase.[124,211,220] Numerous factors are considered to contribute to this issue, including inflammation, increased hydrostatic pressure in the extracellular bone matrix due to synovial fluid infiltration, the perioperative administration of non-steroidal anti-inflammatory drugs, micro-movements of the graft and increased osteoclast activity.[125,186,221] A pre-experimental concern was that FiberTape could

induce a foreign-body reaction in the surrounding tissue and joint, potentially exerting deleterious effects upon new bone formation. However, we observed increased BMD in the FiberTape group, compared to autograft & FiberTape, indicating acceptable conditions for osseus graft incorporation in the bone tunnel in FiberTape samples. While the augmented graft filled the bone tunnel more extensively than the autograft or FiberTape alone, one could assume that this created more favorable conditions for new bone formation; however, this was not observed in either region. One possible explanation for this could be that the hybrid graft provides less space for new bone formation in a bone tunnel.

Prior to the experiment, we were concerned about HMWPE suture tape induced foreign-body inflammation. However, our gene expression analysis did not indicate increased levels of inflammation in the grafts, bone tunnels or joint capsule. Histology revealed only sparse signs of inflammation, and there were no differences in histological appearance between the groups. Similar formations of bone and fibrovascular tissue were observed at the bone–tendon graft interface and we did not observe any inflammation-induced adverse events, such as arthrofibrosis or heterotopic ossification in any of our animals. Based on this, the limited extent of inflammation observed in this study could simply be related to surgical trauma within the time frame of this study.

In a previous study, Cook et al. reported promising results for an arthroscopic ACL reconstruction in a canine model, and compared allograft quadriceps tendon augmented with FiberTape to native non-operated ACL.[138] At 6 months, these authors found morphologically solid incorporation of the augmented graft in the bone tunnels, and remodeling of the mid substance graft indicated by neovascularization and changes in the organization of collagen fibers. These morphological findings were

reflected in biomechanical properties when compared to the native ACL. These authors observed no signs of osteoarthritis when evaluated by gross dissection, arthroscopic evaluation or radiographically; consequently, they considered sufficient joint stability to have been re-established following the surgical reconstruction of the ACL at 6 months.

Previous attempts to use a synthetic graft for ligament reconstruction have been associated with concern over cyclic fatigue. Compared to biological tissue, these synthetic grafts lack the ability to self-repair. While a minor tear in a biological ligament would undergo self-repair to restore the original structural composition, repeated small tears of a synthetic graft will add up over time, potentially leading to a sudden failure. Therefore, the biological incorporation of a synthetic graft is crucial when restoring the self-repair of a composite tissue with sufficient capacity to permanently meet the required demands. Cook reported neovascularization and remodeling of the extracellular matrix of a mid-substance graft, in addition to tissue ingrowth into FiberTape, at 6 months.[138] This finding may indicate that FiberTape does not limit the mechano-transduction needed for morphological and structural remodeling of the mid-substance tendon graft to restore the biomechanical properties.

The histological evaluation did not indicate tissue regeneration along the intra-articular part of the FiberTape. An optimal augmentation of a tendon graft should mimic the properties of the replaced native tissue, allow for mechanical stress transfer to induce remodeling, in addition to having a predictable degradation rate to provide protection to the biological tissue until ii can withstand the tensile demands. These characteristics were not proven for FiberTape in this study.

Early patient mobilization after ligament reconstruction is recommended to prevent joint stiffness and need for prolonged rehabilitation. Yet, early mobilization may expose

a tendon graft to elongation associated with increased joint laxity and reduced stability of the joint.[222] Thus, augmenting a tendon graft reconstruction with a biocompatible suture tape to increase the biomechanical capabilities of the reconstruction is encouraging during the initial phase of early mobilization. However, there is a paucity of currently available literature to provide any evidence for the use of this technique in humans.

Does NSAID administration negatively affect ligament reconstructions?

Effective post-operative management following ACL reconstruction is critical for safe recovery, effective rehabilitation, and to enhance patient satisfaction and functional outcome. NSAIDs are well-established and efficacious drugs for modern multi-modal pain therapy after surgery.[142,223,224] Psychological factors, including pain and quality of life, are important predictors for the outcome following ACL reconstruction.[225,226] Sufficient pain control is needed to ensure safe and comfortable discharge from hospital post-operatively, particularly in the out-patient setting where ACLRs are being conducted at an increasing rate.

Graft failure following ACL reconstruction remains a devastating complication for ACL reconstruction and is commonly associated with recurrent joint instability and loss of function; in these cases, revision ACL reconstruction is often indicated. However, functional results following revision ACL reconstruction are worse and yield lower return to sports rates, higher failure rates and lower patient satisfaction, compared to primary ACL reconstructions.[179,227,228] The potential impairing effect of NSAIDs upon musculoskeletal healing, including tendon–bone and bone–bone interfaces,[159,186] could represent a valid reason to be cautious and restrictive when administering NSAIDs in orthopedic surgery.

Despite some clinical studies demonstrating the detrimental effect of NSAIDs on fracture healing,[229,230] the currently available literature referring to the effect of NSAIDs for human ACL reconstruction healing is sparse. In a retrospective study, Mehta and co-workers reported increased laxity at 6 weeks in patients treated with ketorolac following ACL reconstruction.[231] Furthermore, Pullen et al. found a small, but significantly increased risk for revision associated with NSAIDs following ACL reconstruction in active military personnel;[232] the estimated median duration of use was 30 days (IQR: 14–30), which is considerably longer than the mean of 6.7 days in our cohort. From experimental studies, we know that both the timing and duration of NSAIDs administration can affect the healing of musculoskeletal tissue. [160,233]

NSAIDs are effective for post-operative pain management and reduce the consumption of opioids in orthopedic patients.[143,234] However, NSAIDs are associated with an increased risk for cardiovascular and gastrointestinal adverse effects.[235] NSAIDs should also be avoided during pregnancy as they increase the risk of early loss and fetal risks including renal injury and intracranial hemorrhage.[236] Thus, one should be precautious and assess each patient individually prior to administering NSAIDs. We should take special care to limit the dosage and duration of NSAIDs administration to ensure sufficient pain management, while reducing the exposure to any other known and unknown adverse effects.

An interesting finding was that NSAIDs administration was associated with reduced risk for revision in patients reconstructed using BPTB graft. Based on our study there is no obvious explanation for this finding. However, better post-operative pain control, could allow for earlier range of motion rehabilitation, possibly affecting the incorporation and remodeling of the graft. In addition, patients reconstructed with BPTB graft and administrated NSAIDs, more commonly underwent surgery in an outpatient

setting. This could indicate that surgeons experience may play a role as high volume institutions could have a higher rate of outpatient surgery, and administrate NSAIDs more commonly to ensure pain control. Looking the collective study cohort, patients administrated NSAIDs had less acute meniscal injuries, less severe cartilage injuries, less frequent previously surgery to affected knee, and shorter time from injury to surgical reconstruction. This could affect the outcome and revision rate.

An ACL reconstruction aims to restore joint stability, whether using an HT or BPTB graft. Typically, the outcome of a ligament reconstruction depends on a staged healing process to incorporate the tendon graft to the adjacent bones, in addition to remodeling of the intra-articular portion of the graft to transform the biomechanical capabilities to withstand the tensile demands within the joint and thus ensure restored and permanent joint stability. While BPTB depends upon on bone to bone healing for incorporation of the graft, the HT depends on tendon to bone healing. Both types of graft depend on mineralization of the fibrous tissue formed initially at the interface between graft and bone, in addition to bony ingrowth from the surrounding bone. However, due to the extensive difference in elastic modulus and morphological structure between tendons and bone tissue, tendon to bone healing is considered to be more fragile compared to bone to bone healing.[92] Considering these differences, in addition to previous studies showing higher revision rates in HT grafted knees,[15,106] one could assume that NSAIDs could limit the incorporation of a HT graft more extensively than a BPTB graft. However, based on the findings from our present study, this difference cannot be attributed to the administration of NSAIDs. We observed no difference in the risk for revision within the HT group, while NSAID administration in the BPTB group was associated with a small, but significant reduced risk for revision.

Conclusions

This thesis aimed to investigate aspects of intra-articular ligament reconstructions, focusing on tendon to bone healing and tendon graft augmentation for enhancement in ligament reconstruction.

Paper I demonstrated favorable trends in biomechanical properties and increased levels of bone formation following the local administration of BMP-2, and the novel application of GSK126, in a rat model of tendon to bone tunnel healing at 4 weeks. However, with the sample size, study set up and time frame these differences were not statistically significant.

Our experimental study presented in Paper II demonstrated enhanced biomechanical properties of intra articular ligament reconstructions grafted with FiberTape alone, or as an augmentation of an autograft, compared to autograft alone. FiberTape enhanced the capabilities to tolerate the immediate tensile demands within the joint while protecting the tendon graft in the initial healing phase of a tendon grafted intra articular ligament reconstruction at 8 weeks in a rabbit model. We did not observe regeneration of ligament-like tissue along the suture tape within the time frames of this study.

Based on the analysis of prospectively collected data from the NKLR, presented in Paper III, we identified that the short-term perioperative use of NSAIDs following ACLR is safe and does not exert deleterious effects upon graft survival, or increase the risk of graft revision or a poor functional outcome. However, we should take care to ensure that the dosage and duration of NSAIDs administration is low enough, and short enough, respectively, to ensure sufficient pain management, while limiting exposure to any other adverse effects.

Future research perspectives

Tendon to bone healing

To optimize both short- and long-term outcomes, it is important to gain improved knowledge about the gradual healing process in tendon to bone healing. This includes characterizing the factors that control cellular processes, such as signaling pathways and cytokines, in addition to micro-environmental factors, particularly mechano-stimuli, as we know little about these factors. The cellular processes involved lead to cellular proliferation and the differentiation of chondroblasts, fibroblasts and osteoblasts, in addition to the synthesis and remodeling of the extra-cellular matrix.

The strength of tendon to bone healing is dependent upon bone ingrowth, mineralization and the maturation of the healing tissues. Thus, regulatory pathways involved in the early mineralization of the fibrovascular tissue, and remodeling of the collagen fibers to create continuity between the tendon and bone, are potential targets for biological enhancement stimulation.

A better understanding of both enthesis development and soft tissue to bone healing may allow us to develop regenerative strategies for enhanced healing, with bench to bedside potential for improved clinical outcome after ligament reconstruction and tendon to bone reattachment repair. Synergistic effects between various growth factors and bone resorption inhibitors may be one way forward. However, the timing of administration to restore the most native-like enthesis remains uncertain at this moment in time.

GSK126 contributes to increased osteoinduction. Further studies should aim to evaluate the ability of GSK126 to enhance fracture healing and tendon to bone healing, and to determine the optimal duration, dosage and timing of administration. In addition, the potential synergistic combination of GSK126 and BMP-2 should be assessed, as

they both involve RUNX2-dependent osteoblast differentiation. Further pre-clinical studies are now needed to determine the translational potential of GSK126 as a biological enhancement in the setting of orthopedic surgery.

Studies have shown the enhancement of tendon to bone healing in response to biological factors. However, the existing literature demonstrates considerable variation in study designs and methodological aspects, which could complicate the translational potential of pre-clinical experiments. Thus, further studies on tendon to bone healing should preferably utilize pre-verified animal models at standardized timepoints to allow the comparison of dosage-effect and applied biological enhancement, such as cellular therapy or growth factors, between studies.

The development of repair strategies for the improved restoration of the proprioceptive capabilities of ligament reconstructions should also be investigated. Regained collaboration between static and dynamic stabilizers may prevent over-loading of the ligament reconstruction and thus improve joint function.

Despite promising results of adjuvant biological treatment in pre-clinical settings, these findings have yet to be translated to the clinical setting. Clinical trials, proving both safety and efficacy, are now needed before biological adjuvants can be introduced into regular guidelines. Cost-effectiveness should also be considered in a modern healthcare system.

New non-invasive diagnostic tools, such as advanced imaging modalities, are also needed to clinically evaluate the strength of tendon to bone healing. This would be useful both in a research setting to assess the outcome following novel treatment application, and to better guide post-operative rehabilitation and return to recreational and sport activities.

Tendon graft augmentation of ligament reconstructions

New augmentation devices should preferably have a structural composition that supports cellular attachment and ingrowth to replace the scaffold as it degrades at a predictable rate to maintain the strength of the regenerated tissue. Also, self-repair mechanisms are not available in current augmentation devices. A form of augmentation that delivers growth factors for segmental differentiation along the reconstructed ligament could improve both the incorporation and adjust remodeling of the graft for more rapid and enhanced remodeling. Such a factor is not currently available. Our goal should be to restore native or near-native tissue while reducing the risk of treatment failure. New augmentation devices must be assessed in a clinical setting to demonstrate both safety and efficacy over the short and long term.

Currently available augmentation devices for ligament reconstruction, require further investigation for long-term failure rate and functional outcome. Clinical trials and cohort studies are also needed to evaluate to what extent the augmentation of ligament reconstructions affect the time from surgery to a return to high demand activities. In addition, long term outcomes, including adverse events, rate of recurrent joint instability, rate of revision and time from primary reconstruction to failure should be evaluated.

Use of NSAIDs in ligament reconstruction surgery

As we still do not fully understand the effect of NSAIDs upon the various healing phases of musculoskeletal tissue and tendon to bone healing, further pre-clinical studies should aim to more accurately determine safe dosage, duration and the timing of administration to clarify the safety aspect, including the provision of sufficient pain management without limiting the healing process. These findings should be confirmed in a clinical study, preferably a prospective randomized trial. Also, to increase the external validity of our findings of no negative effects associated with the limited

perioperative administration of NSAIDs to patients undergoing ACL reconstruction, our findings should be confirmed by other ACLR registries.

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Appendix

Appendix I-IV: Knee Injury and Osteoarthritis Outcome Score (KOOS) and Paper I-III

HELSE **BERGEN**
Haukeland universitetssjukehus

NASJONALT KORSBÅNDSREGISTER
Nasjonalt Register for Leddproteser
Helse Bergen HF, Ortopedisk klinikk
Haukeland universitetssjukehus
Møllendalsbakken 11
5021 BERGEN
Tlf: 55976454

DATO: _____

FØDSELSNR (11 siffer): _____

NAVN: _____

KOOS – Spørreskjema for knepasienter.

Veiledning: Dette spørreskjemaet inneholder spørsmål om hvordan du opplever kneet ditt nå. Informasjonen vil hjelpe oss til å følge med i hvordan du har det og fungerer i ditt daglige liv. Besvar spørsmålene ved å krysse av for det alternativ du synes stemmer best med deg (kun ett kryss ved hvert spørsmål). Hvis du er usikker, kryss likevel av for det alternativet som føles mest riktig.

KRYSS AV FOR RIKTIG KNE (NB: Ett skjema for hvert kne**):** ☐[1] **VENSTRE** ☐[0] **HØYRE**

Røyker du? ☐[0] Nei ☐[1] Av og til ☐[2] Daglig
Hvis du røyker daglig –
hvor mange sigaretter per dag: _____

Vekt: _____ kg

Høyde :_____ cm

Symptom

Tenk på **symptomene** du har hatt fra kneet ditt den **siste uken** når du besvarer disse spørsmålene.

S1. Har kneet vært hovent?

Aldri	Sjelden	I blant	Ofte	Alltid
☐[0]	☐[1]	☐[2]	☐[3]	☐[4]

S2. Har du følt knirking, hørt klikking eller andre lyder fra kneet?

Aldri	Sjelden	I blant	Ofte	Alltid
☐[0]	☐[1]	☐[2]	☐[3]	☐[4]

S3. Har kneet haket seg opp eller låst seg?

Aldri	Sjelden	I blant	Ofte	Alltid
☐[0]	☐[1]	☐[2]	☐[3]	☐[4]

S4. Har du kunnet rette kneet helt ut?

Alltid	Ofte	I blant	Sjelden	Aldri
☐[0]	☐[1]	☐[2]	☐[3]	☐[4]

S5. Har du kunnet bøye kneet helt?

Alltid	Ofte	I blant	Sjelden	Aldri
☐[0]	☐[1]	☐[2]	☐[3]	☐[4]

Stivhet

De neste spørsmålene handler om **leddstivhet.** Leddstivhet innebærer vanskeligheter med å komme i gang eller økt motstand når du bøyer eller strekker kneet. Marker graden av leddstivhet du har opplevd i kneet ditt den **siste uken.**

S6. Hvor stivt er kneet ditt når du nettopp har våknet om morgenen?

Ikke noe	Litt	Moderat	Betydelig	Ekstremt
☐[0]	☐[1]	☐[2]	☐[3]	☐[4]

S7. Hvor stivt er kneet ditt senere på dagen etter å ha sittet, ligget eller hvilt?

Ikke noe	Litt	Moderat	Betydelig	Ekstremt
☐[0]	☐[1]	☐[2]	☐[3]	☐[4]

Smerte

P1. Hvor ofte har du vondt i kneet?

Aldri	Månedlig	Ukentlig	Daglig	Hele tiden
☐0	☐1	☐2	☐3	☐4

Hvilken grad av smerte har du hatt i kneet ditt **den siste uken** ved følgende aktiviteter?

P2. Snu/vende på belastet kne

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

P3. Rette kneet helt ut

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

P4. Bøye kneet helt

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

P5.Gå på flatt underlag

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

P6. Gå opp eller ned trapper

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

P7. Om natten (smerter som forstyrrer søvnen)

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

P8. Sittende eller liggende

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

P9. Stående

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

Funksjon i hverdagen

De neste spørsmålene handler om din fysiske funksjon. **Angi graden av vanskeligheter du har opplevd den siste uken ved følgende aktiviteter på grunn av dine kneproblemer.**

A1. Gå ned trapper

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A2. Gå opp trapper

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A3. Reise deg fra sittende stilling

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

Angi graden av **vanskeligheter** du har opplevd ved hver aktivitet **den siste uken.**

A4. Stå stille

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A5. Bøye deg, f.eks. for å plukke opp en gjenstand fra gulvet

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A6. Gå på flatt underlag

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A7. Gå inn/ut av bil

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A8. Handle/gjøre innkjøp

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A9. Ta på sokker/strømper

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A10. Stå opp fra sengen

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A11. Ta av sokker/strømper

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A12. Ligge i sengen (snu deg, holde kneet i samme stilling i lengre tid)

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A13. Gå inn/ut av badekar/dusj

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A14. Sitte

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A15. Sette deg og reise deg fra toalettet

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A16. Gjøre tungt husarbeid (måke snø, vaske gulv, støvsuge osv.)

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

A17. Gjør lett husarbeide (lage mat, tørke støv osv.)

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

Funksjon, sport og fritid

De neste spørsmålene handler om din fysiske funksjon. **Angi graden av vanskeligheter du har opplevd den siste uken ved følgende aktiviteter på grunn av dine kneproblemer.**

SP1. Sitte på huk

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

SP2. Løpe

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

SP3. Hoppe

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

SP4. Snu/vende på belastet kne

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

SP5. Stå på kne

Ingen	Lett	Moderat	Betydelig	Svært stor
☐0	☐1	☐2	☐3	☐4

Livskvalitet

Q1. Hvor ofte gjør ditt kneproblem seg bemerket?

Aldri	Månedlig	Ukentlig	Daglig	Alltid
☐0	☐1	☐2	☐3	☐4

Q2. Har du forandret levesett for å unngå å overbelaste kneet?

Ingenting	Noe	Moderat	Betydelig	Fullstendig
☐0	☐1	☐2	☐3	☐4

Q3. I hvor stor grad kan du stole på kneet ditt?

Fullstendig	I stor grad	Moderat	Til en viss grad	Ikke i det hele tatt
☐0	☐1	☐2	☐3	☐4

Q4. Generelt sett, hvor store problemer har du med kneet ditt?

Ingen	Lette	Moderate	Betydelige	Svært store
☐0	☐1	☐2	☐3	☐4

Tilleggsspørsmål

T1. Har du pådratt deg noen ny akutt skade i kneet etter korsbåndsoperasjonen?

☐[0] Nei

☐[1] Ja

T2. Hvis ja, hva slags skade (kryss av for hver skadetype, hvis flere strukturer er skadet):

☐[1] Fremre korsbånd	Dato (mm.åå.):
☐[2] Bakre korsbånd	Dato (mm.åå.):
☐[3] Andre leddbåndsskader	Dato (mm.åå.):
☐[4] Meniskskade	Dato (mm.åå.):
☐[5] Bruskskade	Dato (mm.åå.):
☐[6] Bruddskade	Dato (mm.åå.):

T3. Hvis du har pådratt deg en ny korsbåndsskade, hvordan ble diagnosen stilt:

☐[0] MR-undersøkelse (”magnetrøntgen”)

☐[1] Artroskopisk undersøkelse (”kikkhullsoperasjon”)

☐[2] Undersøkelse av lege

☐[3] Undersøkelse av annet helsepersonell (fysioterapeut, manuell terapeut etc.)

Takk for at du tok deg tid og besvarte samtlige spørsmål!

Bergen Grafisk as - 06.2012

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RESEARCH ARTICLE

WILEY Journal of Cellular Biochemistry

Fibrin glue mediated delivery of bone anabolic reagents to enhance healing of tendon to bone

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Abstract

Tendon graft healing in bone tunnels for the fixation of intra-articular ligament reconstructions may limit clinical outcome by delaying healing. This study assesses the effects of hydrogel-mediated delivery of bone anabolic growth factors in a validated model of tendon-to-bone tunnel healing. Forty-five Wistar rats were randomly allocated into three groups (BMP2-treated, GSK126-treated, and placebo). All animals underwent a tendon-to-bone tunnel reconstruction. Healing was evaluated at 4 weeks by biomechanical assessment, micro-computed tomography (bone mineral density, bone volume, cross sectional area of bone tunnels), and traditional histology. Adverse events associated with the hydrogel-mediated delivery of drugs were not observed. Results of our biomechanical assessment demonstrated favorable trends in animals treated with bone anabolic factors for energy absorption ($P = 0.116$) and elongation ($P = 0.054$), while results for force to failure ($P = 0.691$) and stiffness ($P = 0.404$) did not show discernible differences. Cross sectional areas for BMP2-treated animals were reduced, but neither BMP2 nor GSK126 administration altered bone mineral density ($P = 0.492$) or bone volume in the bone tunnel. These results suggest a novel and positive effect of bone anabolic factors on tendon-to-bone tunnel healing. Histological evaluation confirmed absence of collagen fibers crossing the soft tissue-bone interface indicating immature graft integration as expected at this time point. Our study indicates that hydrogel-mediated delivery of BMP2 and GSK126 appears to be safe and has the potential to enhance tendon-to-bone tunnel healing in ligament reconstructions.

KEYWORDS

BMP2, connective tissue, GSK126, ligament reconstruction, orthopedic, tendon-to-bone healing

1 | INTRODUCTION

Intra-articular ligament injuries are common and challenging to manage. For example, anterior cruciate ligament (ACL) ruptures occur in 35 of 100 000 individuals and cost more than $3 billion to treat in the United States each year.[1] Within the upper extremity, the scapholunate ligament (SL) in the wrist, and rotator cuff in the shoulder are sites of common injury that are difficult to manage.[2,3] Once injured, ligaments lack the intrinsic ability to heal. If left untreated, joints may become unstable leading to pain, stiffness, and post-traumatic arthritis. Although surgical techniques for reconstructing

ligaments are advancing, long-term clinical data demonstrate persistent, and recurrent joint-related symptoms, pain, and degenerative joint disease. Given their limited healing capacity, the current standard of care for ligament reconstruction involves using autograft (self) or allograft (cadaver) tendons. Surgical reconstruction depends on the fixation of two tissues (bone and tendon) with vastly different morphological and biomechanical properties, and depends on new bone formation to anchor the tendon in the bone tunnel during early recovery.

Intra-articular ligament injuries (eg, anterior cruciate ligament ruptures) are most common among young, active people.[4] Severe ligament injury may lead to short-term functional impairment of sport activities and daily living, as well as long-term disability due to early onset osteoarthritis.[5] The healing potential for intra-articular ligament injuries is low, and surgical reconstruction by tendon graft is often required to restore joint stability and function.[6–8] A successful intra-articular ligament reconstruction must withstand multi-directional tensile forces within the joint. In contrast to the native tendon-bone insertion site developed during embryological development, healing of tendon-bone attachment in surgical reconstructions occurs through the formation of fibrovascular scar tissue. The enthesis, a transition zone between native ligaments or tendons and bone, is not regenerated,[9] and therefore the outcome of reconstruction depends on the secure anchorage of grafted tendon-to-bone tunnel.[10–13] Poor outcomes following tendon-to-bone tunnel healing can be caused by circumferential bone loss, potentially limiting the anchorage of the tendon graft. Collagen formation across the tendon-bone interface can further delay the healing process and limit integration of the graft.[13] Additional strategies are urgently needed to enhance the fixation of tendon grafts to bone tunnels during the surgical repair of intra-articular ligaments.

Bone anabolic factors, such as bone morphogenic protein (BMP), may enhance tendon-to-bone tunnel healing by increasing new bone formation and reducing circumferential bone tunnel loss. BMPs are part of the transforming growth factor beta superfamily (TGF-β),[14–16] and BMP2 has osteoinductive properties that are well documented in both cell culture and animal models.[17] Clinically, BMPs are sometimes used to improve bone healing after spine fusions or to repair tibial non-union fractures.[18] Exogenous BMPs act via phosphorylation of BMP receptors, which activates the cell signaling pathway via intracellular SMADs to upregulate transcription of target genes and translation of proteins to induce osteoblastogenesis, and therefore increase new bone formation.[16] New bone formation in the localized region of a bone tunnel can therefore provide a critical mechanism for the anchoring of tendon grafts used to repair ruptured ligaments and restore joint stability.

Furthermore, RUNX2 is a key transcriptional regulator of the Wnt pathway, involved in osteoblast differentiation and bone formation.[19] BMP/SMAD signaling interacts with RUNX2 for co-operative regulation of target genes involved in osteoblastic differentiation of mesenchymal stromal/stem cells (MSCs). The enhancer of zeste homolog 2 (EZH2) is an important epigenetic enzyme that regulates RUNX2 dependent osteoblast differentiation and a principal subunit of the polycomb repressive complex 2 (PRC2).[20] EZH2 catalyzes the trimethylation of histone three lysine 27 (H3K27), promoting heterochromatin formation, and silencing the RUNX2 gene. The therapeutic reagent GSK126, a selective inhibitor of EZH2 can unpack chromatin, and increase accessibility of RUNX2 to bone-specific genes that stimulate bone-specific transcription, osteoblast differentiation and new bone formation.[19,21–23] GSK126 may also suppress osteoclastogenesis, which can further increase new bone formation by silencing cell communication between osteoclasts and osteoblasts.[24] In addition, increased osteoclast activity can contribute to circumferential bone loss in the bone tunnel,[25,26] an undesirable outcome during the tendon graft-based restoration of human joint stability.

Our study aimed to assess the effect of locally administered bone anabolic drugs in a tendon-to-bone tunnel healing model of rat achilles tendon.[27,28] This is a novel and clinically applicable approach in the use and evaluation of these bone anabolic factors. Primary measureable outcomes were biomechanical assessment, (ie, force to failure, stiffness, elongation, and energy absorption), quantitative evaluation of cross sectional bone tunnel area, bone mineral density, and bone volume, as well as a semi-quantitative histological evaluation of the bone-to-soft tissue interface. We hypothesized that tendon-to-bone tunnel healing would be enhanced by applying bone anabolic growth factors. Specifically, we assessed whether local hydrogel-mediated delivery of BMP2 or GSK126 enhances tendon-to-bone tunnel healing. The main findings of our study were that BMP2 and GSK126 treatments do not have adverse effects, but may result in favorable biomechanical trends during ligament repair. Further, bone tunnel diameters were reduced and bone volumes increased in BMP2 treated animals at 4 weeks after surgery.

2 | MATERIALS AND METHODS

2.1 | Animal handling

All animal work performed during this study was approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC #A1182, Mayo Clinic, Rochester, MN). Reduction, replacement and refinement strategies were considered to ensure animal welfare. Animals were maintained according to the guidelines provided by National

Institute of Health. Female Wistar rats ($n = 45$), mean weight 239 g (SD ±10) were kept three animals per cage, in an accredited animal facility with controlled temperature, humidity, and light/dark cycles. Animals were allowed free access to food and water throughout the study period. No restriction to ambulation was applied at any time. The animals were allocated by computerized randomization into three groups (GSK126-treated, BMP2 treated, and saline "placebo"), and study members were blinded to animal group assignment until final analyses were completed (Figure 1). Anesthesia was induced using an intra-peritoneal (IP) administration of ketamine (10 mg/kg), acepromazine (0.3 mg/kg) and xylazine (2 mg/kg) and maintained during the surgical procedure by inhalation of isoflurane (1.0-2.5% and 100% oxygen). Buprenorphine Sustained Release (SR) (0.6 mg/kg) was administered post-operatively to manage pain, and antibiotics were not indicated.

2.2 | Surgical procedure

The surgical procedure was done as previously described.[28] Following sterile preparation, a 10 mm longitudinal skin incision was made on the medial side of the right Achilles tendon. The tendon was released proximally at the tendon calf muscle transition zone, while the distal calcaneal insertion was left intact. A holding suture was applied to the proximal end of the Achilles tendon in a Kessler suture fashion using a monofilament non-resorbable suture (Prolene 5-0, Ethicon, Somerville, NJ) (Figure 2A). A drill hole was made in the distal tibia in a dorsoventral direction 3 mm proximal to the ankle joint using a 1.0 mm drill bit followed by drilling with a 1.5 mm drill bit (Figure 2B). The bone tunnel was irrigated with saline water, before a suture passer was used to guide the sutures through the bone tunnel in a dorsoventral direction and securely sutured to the ventral soft tissue while keeping the ankle joint in a 90° flexed position (Figure 2C).

Selected growth factors were administered locally to the dorsal opening of the bone tunnel, adding 45 µL of fibrin glue containing either drug (BMP2, GSK126) or placebo. Fibrin was prepared as previously described.[29] Clotting of the fibrin glue was confirmed before the skin incision was closed using resorbable sutures (Ethicon, Somerville, NJ). Animals were closely observed until awake, and no restrictions to ambulation were applied at any time. All animals were euthanized at 4 weeks by carbon dioxide asphyxiation.

The animal model used for this study allowed assessment of tendon-to-bone tunnel healing at a single insertion site in metaphyseal bone as the distal insertion of the Achilles tendon to the calcaneal bone is preserved. In addition, free load bearing mimics active rehabilitation, which is important to prevent secondary joint stiffness due to immobilization in a clinical setting. GSK126 (BioVision, Inc., Milpitas, CA) and BMP2 (BioVision, Inc.,) reagents were dissolved in phosphate buffered saline (PBS) to concentrations of

Unilateral
Surgery
Day 0

Sacrifice
4 Weeks

Administration of
treatment in fibrin glue

R Leg Treatment	*n*
Placebo (saline)	16*
BMP2 (15 ug/animal)	14
GSK126 (250 ug/animal)	15
	45

Tissue
Assessment

	n
µCT and Histology	
Placebo	4
BMP2	4
GSK126	4
Biomechanics	
Placebo	10
BMP2	10
GSK126	11

FIGURE 1 Experimental outline of animal study for tendon-to-bone tunnel healing. The rat model of ligament repair was designed to have intra-operative delivery of bone anabolic reagents (BMP2 or GSK126) within fibrin glue at day 0 (45 animals), and animal killing 4 weeks later. Tissues were preserved for analysis by µCT, histology and biomechanical properties (force to failure, stiffness, elongation, and energy absorption). (*2 animals died during surgery due to anesthesia complications)

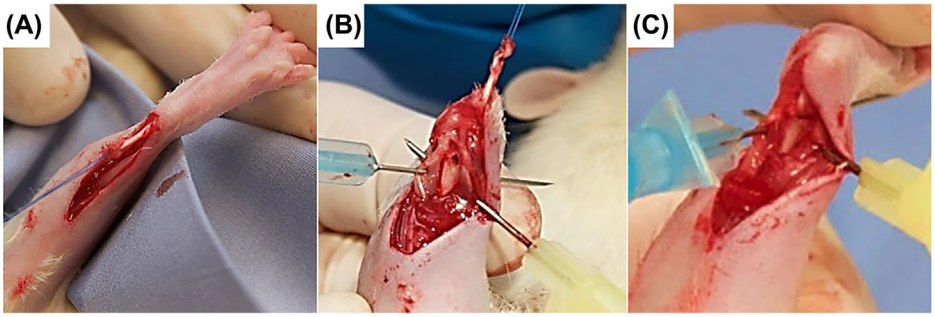


FIGURE 2 The surgical procedure involved (A) achilles tendon excision proximally and addition of a holding suture, (B) distal tibia exposure for bone tunnel drilling (dorsal view), and (C) achilles tendon passage through the bone tunnel

50 μg/μL and 3 μg/μL, respectively. Based on previous studies,[30–32] this drug delivery strategy ensures sustained local administration because 5 μL of this solution was mixed and encapsulated into 45 μL of Tisseel fibrin glue (Baxter Healthcare Corp., Deerfield, IL) immediately prior to delivery, which allows in situ clotting. Dosage was based on previous experiments in our lab and search of the literature.[33,34]

2.3 | Biomechanical assessment

A custom-made electromechanical testing machine was used for assessments of biomechanical properties. Specimens were secured in custom-made clamps, designed to avoid applying pressure to the insertion site on either the tibia or Achilles tendon. Using a 10 lb. ultra-precision mini load cell (Transducer Techniques LLC, Temecula, CA), the displacement rate was set to 0.1 mm/sec and data were collected via linear potentiometer at 100 Hz with customized LabView program. This sampling strategy allowed collection of force, time and displacement data for final analyses in MatLab software (MathWorks Inc., Natick, MA).

2.4 | MicroCT analysis

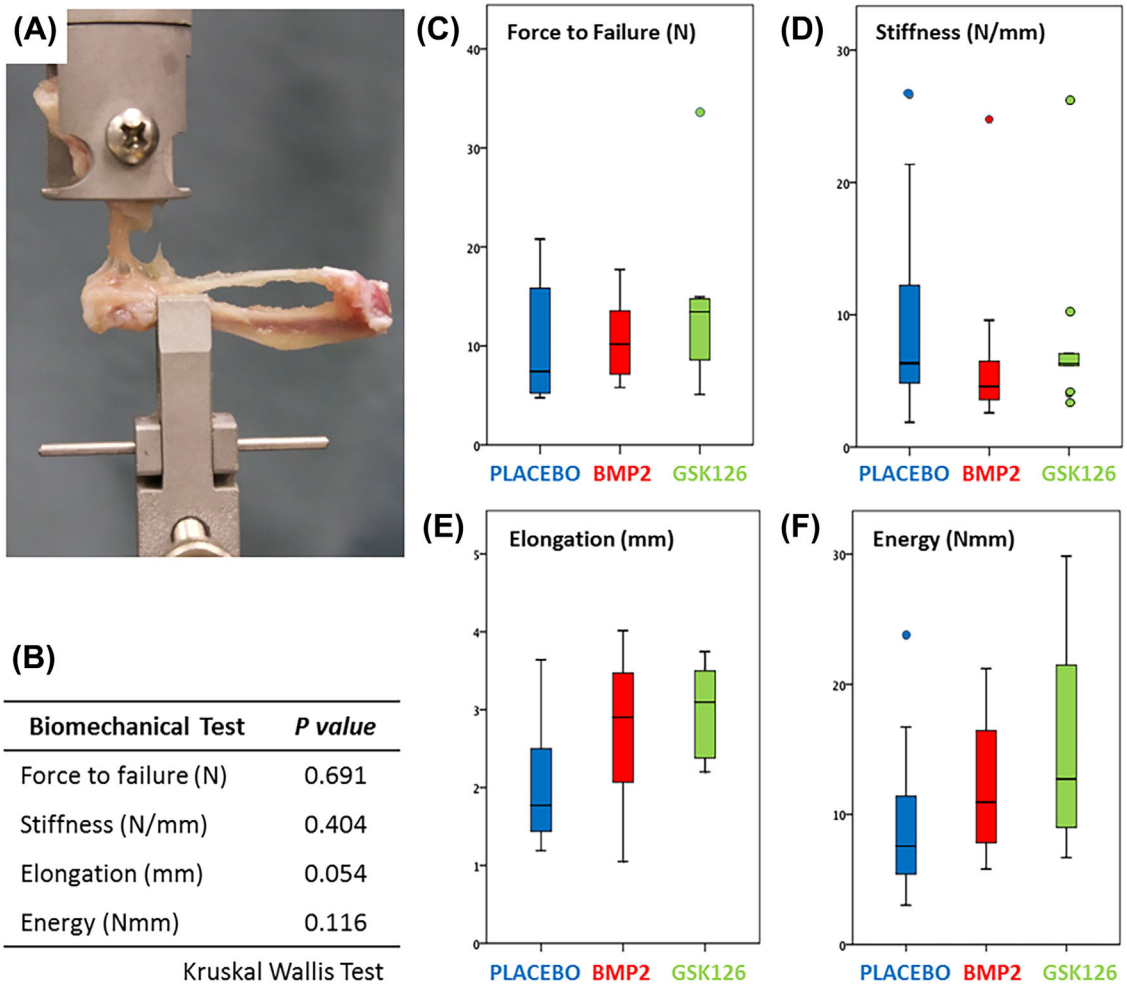
Skin- and muscle-tissues were removed from all specimens following ex-articulation of legs at the knee joint. The forefoot was resected though the proximal aspect of metatarsal bones. Specimens were preserved in 10% neutral buffered formalin (NBF) until scanning. Bone mineral density (BMD) and cross-sectional area (mm^2) of the bone tunnels were measured using microcomputed tomography (μCT). An Inveon CT/PET module scanner (Siemens Medical Solutions, INC., Knoxville, TX) was used, at pixel size 26.93, energy setting of 60 kV and projection time of 1000 ms. Final analyses of the images were conducted by an experienced biomedical imaging analyst using the software Analyze (AnalyzeDirect Inc., Overland Park, KS). One phantom specimen containing samples with known calcium concentrations was scanned to determine the CT number (density per voxel) for known calcium levels. BMD and bone volume was measured by aligning a cylinder with diameter equal to the drill bit used for the surgery (1.5 mm) along the longitudinal axis of the bone tunnel (Figure 4A). Further, two concentric circles were placed around the cylinder such that each would have approximately the same area, and allowing measurement of Mean CT number for each volume and bone range. To determine the relationship between the measured CT numbers and known CT numbers, we used linear regression to determine $\sum$CT and βCT. BMD estimates were then calculated using the following relationship: CT number = BMD × $\sum$CT + βCT.

2.5 | Histology

Following μCT scanning, the specimens were used for histological evaluation. Tissues were fixed in 10% NBF, and decalcified using RDO- Rapid Decalcifier Solution (Apex Engineering Products Corporation, Aurora, IL) until sufficient decalcification was verified by needle testing. Subsequently, tissues were embedded in paraffin and sectioned along the coronal plane to get a cross-sectional view of the tendon inside the bone tunnel. Slides were stained with H&E (Sigma-Aldrich, St Louis, MO) before histological evaluation by a certified veterinary pathologist as well as two additional independent reviewers. Assessments aimed to evaluate bone formation and maturity in the bone tunnel, as well as graft integration on the bone tunnel. More specifically, each sample was assessed for degree of bone tunnel filling (new bone formation) on a scale from 0 to 2, where a score of two indicated complete integration of the graft in the bone tunnel, a score of one indicated partial integration, and a score of 0 indicated poor attachment of tendon graft in the bone tunnel.

2.6 | Statistics

The primary endpoint of biomechanical analysis was pullout strength. Secondary endpoints were stiffness, energy absorption,



(B)

Biomechanical Test	*P value*
Force to failure (N)	0.691
Stiffness (N/mm)	0.404
Elongation (mm)	0.054
Energy (Nmm)	0.116

Kruskal Wallis Test

FIGURE 3 Biomechanical data for tendon-to-bone healing in rats were collected on three groups of animals: placebo (PBS, $n = 10$); GSK126 ($n = 11$); BMP2 ($n = 10$). Measurements were made using (A) custom-made electromechanical testing machine with custom-made clamps, and a 250 g Model 31 load cell, to assess (B) *P*-values derived using the Kruskal-Wallis test on (C) force to failure, (D) stiffness, (E) elongation, and (F) energy adsorption

elongation, bone tunnel cross sectional area, and BMD and bone volume as compared among the groups. Histograms and normal q-q-plots did not demonstrate normal distribution. Hence, results are presented as median and inter quartile range (IQR) distributions. Comparison analysis was done using Kruskal-Wallis tests. The alpha level was set to 0.05 and all statistical analyses were done using IBM SPSS Statistics for Macintosh v. 23.0 (IBM Inc., Chicago, IL).

3 | RESULTS

Successful surgical ligament reconstruction depends on the biomechanical properties of implanted tissue and its osseointegration at origin and insertion sites. Primary reasons for failed ligament reconstruction are graft loosening and incomplete fixation of graft-to-bone. We used rats as a model to assess whether bone-anabolic reagents (*BMP2* and GSK126[17,35]) would improve the fixation of tendon grafts to bone.

3.1 | Animals

Two rats died peri-operatively, most likely due to an anesthesia-related complication. Additionally, two surgical reconstructions failed, which left 41 animals in our final analysis. No post-operative infections or other adverse events were noted. All rats were closely observed throughout the study period to prevent complications and ensure animal welfare, although no additional injections of Buprenorphine were indicated. Overall, animal weights increased significantly ($P = 0.001$) during the study period (final weights = 283.5 ± 17.32 g), and differences in body weight between the study groups were not significant ($P = 0.931$).

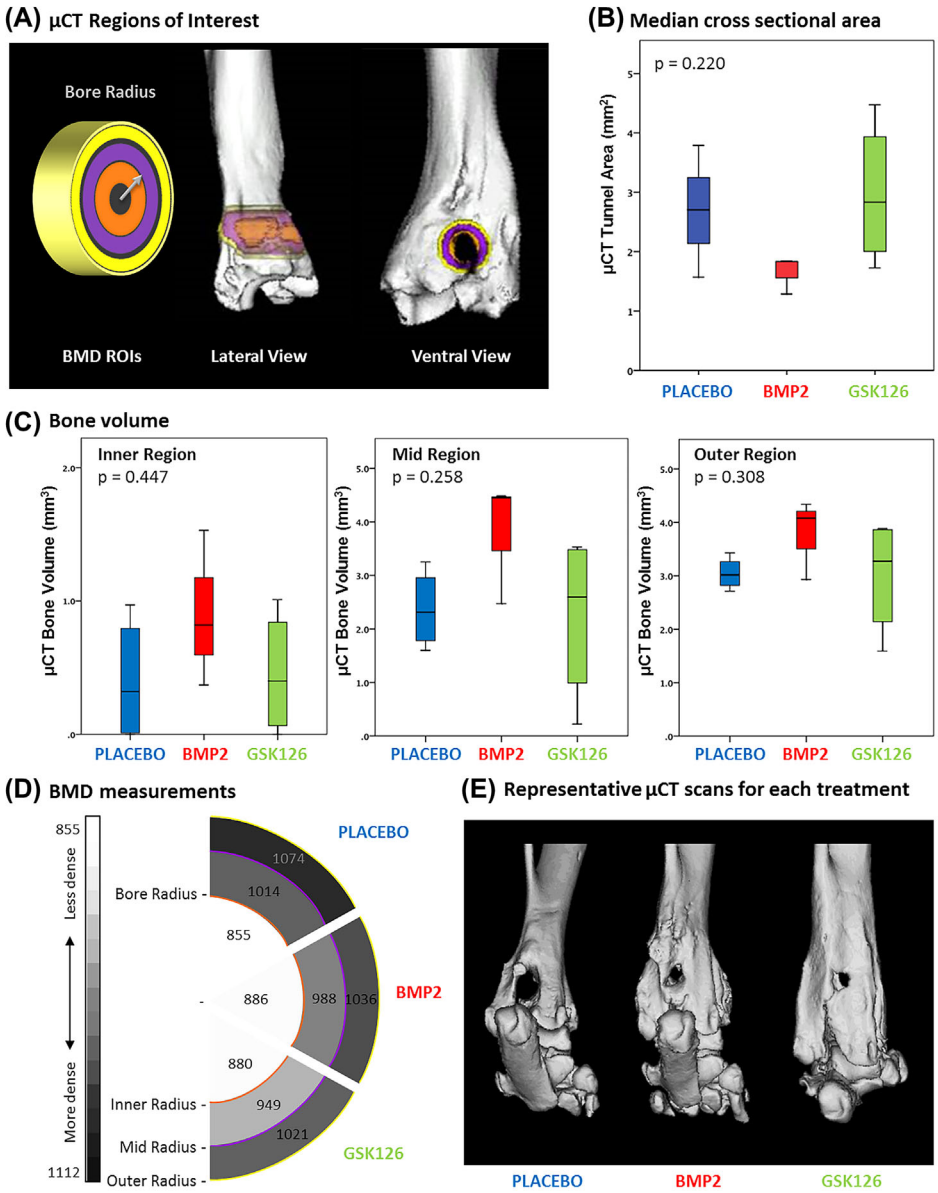


FIGURE 4 MicroCT assessment of rat tendon-to-bone tunnel healing. A, Schematic showing the areas (rings) used to assess bone mineral density (BMD) measurements. The (orange) inner circle has a radius equal to the radius of the bore. Lateral and ventral views of the limb are presented with the measurement areas imposed on top. B, Box and whisker plot of the median µCT bone tunnel area measurements versus treatment. BMP2 presents the smallest bone tunnel area. C, Bone volume quantification demonstrates a trend of increased median bone volume values, particularly in BMP2 treated animals. D, Schematic of the bone mineral density measurements for each treatment group and ring radius, with increasing bone density represented by the darkest intensities. No significant differences in BMD were observed for treatment groups. E, Representative µ-CT 3D reconstructions, supporting the trend of reduced cross sectional area and increased bone volume in the bone tunnel of BMP2 and GSK126 treated animals

The absence of treatment related adverse events indicates that the drug regimen does not cause harmful effects.

3.2 | Biomechanical data

Biomechanical assessments were conducted on 31 specimens: BMP2 ($n = 10$), GSK126 ($n = 11$) and placebo ($n = 10$). All tested reconstructions failed at the tendon-bone interface (Figure 3A). Overall, the median (IQR) force to failure was 10.1N (±8.46). The median (IQR) force to failure was 10.2 (±7.3) N and 13.4 (±5.7) N for BMP2 and GSK126 specimens respectively, compared to 7.4 (±2.2) N for the placebo group ($P = 0.691$) (Figures 3B and 3C). Stiffness measurements resulted in median(IQR) values of 6.32 N/mm (±5.53) for placebo, 4.58 N/mm (±3.83) for BMP2, and 6.32 N/mm (± 3.53) for GSK126-treated animals

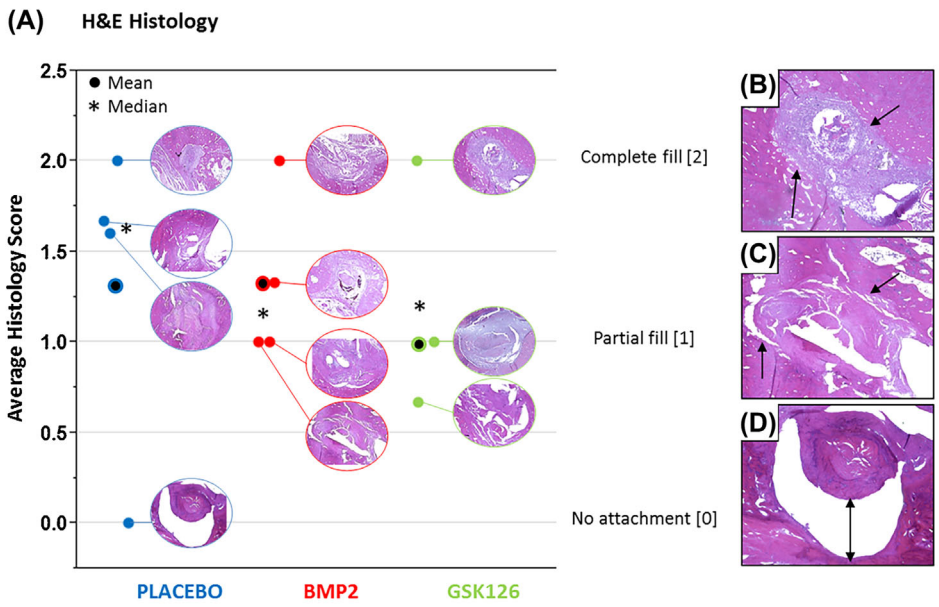


FIGURE 5 Histological Assessment. A, The bone tunnels for all histology samples are shown in the graph with the average and median histology scores ($n = 3$ blinded readers) presented. Representative images of (B) complete fill, score of 2; (C) partial fill, score of 1; (D) no attachment, score of 0. Notably, only the placebo group was observed to have a sample with a score of 0 (no attachment)

($P = 0.404$) (Figures 3B and 3D). Both BMP2 and GSK126 treated animals had increased median elongation values by 61% and 72% respectively, compared to placebo ($P = 0.054$) (Figures 3B and 3E). Energy absorption was higher in BMP2 and GSK126 treated animals, 43% and 67% respectively, however not significant ($P = 0.116$) (Figure 3).

3.3 | MicroCT imagery

MicroCT imaging was used to examine bone tunnel area (Figures 4A and 4B), bone volume (Figure 4C) and bone mineral density near the bone tunnel (Figure 4D). Median (IQR) cross sectional area of the bone tunnel was 1.7 ($\pm$0.69) mm^2 for BMP2, 2.95 ($\pm$2.76) mm^2 for GSK126 and 2.67 ($\pm$1.95) mm^2 for the placebo group (Figure 4B) ($P = 0.21$). Bone volume measurement did not show statistical differences between the groups, however median bone volume was higher in BMP2 and GSK126 in the inner and mid regions compared to placebo (Figure 4C). BMD measurement did not reveal statistically significant differences in comparisons among predefined zones, inner ($P = 0.957$), mid ($P = 0.492$) and outer ($P = 0.741$) (Figure 4D). Representative μ-CT 3D reformations confirmed trend of reduced cross sectional areal and increased bone volume in the bone tunnel in BMP2 and partly GSK126 treated animals (Figure 4E).

3.4 | Histology outcomes

One score of 0 was noted in the placebo group, and may be related to graft decay/failure to integrate. Additionally, our analysis did not reveal clear differences in graft osseointegration, or new bone formation or maturity, or vascularization among the sample groups (Figure 5). In general, tissues from the treated animal groups were not histologically dissimilar than the placebo group, supporting the safety of our strategy. Collagen fibers crossing the tendon-bone interface were not observed in any samples or groups. This result indicates an immature fixation of the tendon graft in the bone tunnel that highly depends on new bone formation to anchor the tendon graft in the bone tunnel at this early time point.

4 | DISCUSSION

Because BMP2 and GSK126 are known to increase osteoblast differentiation and new bone formation, we attempted to improve the healing of tendon-to-bone tunnel in a rat model of ligament repair. Although biomechanical analyses yielded no statistical difference between groups for force to failure or stiffness, energy absorption and elongation measurements were marginally significant among the groups. MicroCT analyses of bone tunnels revealed smaller cross-sectional areas for the BMP2 group ($P < 0.001$), which suggests that BMP2 may improve the short-term integration of tendon-to-bone in a simulated ligament repair model. Tendon-to-bone tunnel healing is a slow process, especially during the early post-operative phase. Thus, early range of motion exercises to prevent joint stiffness limit the initial surgical fixation of the reconstruction. An enhancement of the initial healing phase may therefore improve the early mobilization of the

reconstruction, potentially promoting normalization of joint stability and earlier return to daily activities (including sports) for patients.

Using a canine model similar to the rat model we used in this study, Rodeo et al[36] demonstrated that BMP can accelerate healing of tendon-to-bone tunnel. In particular, they found higher loads to failure in BMP2 treated animals after only 2 weeks. Histologic and radiographic presentation of more excessive bone formation in these animals confirmed their interpretations. Also using a canine model, Thomopoulos et al[37] found that BMP did not improve the biomechanical properties of tendon-to-bone tunnel compared to placebo specimens, and concluded that 3 weeks is insufficient for the bone anabolic effect of BMP to overcome post-surgical bone loss. Collagen sponge delivery, anatomic location, and dosing of bone anabolic reagents can have dramatic effects on the outcome of studies designed to recapitulate endogenous tendon osseointegration. For example, gene transfection by adenovirus-infected autografts was shown to enhance tendon graft integration in a bone tunnel. Up-regulation of BMP2 by gene transfection in semitendinosus autografts improved the biomechanical properties and increased bone formation in a rabbit ACL.[38] These studies demonstrate the potential benefit of locally upregulating the BMP2 signaling pathway in tendon-to-bone tunnels, and highlight the need for further research to optimize delivery vehicles that ensure a localized and sustained delivery of growth factor to promote bone ingrowth and minimize adverse events.

Most ACL models involve the complete resection of the tendon including all native vascular elements. The model used for this study maintains critical vascularization to the tendon and leaves the other interface for experimental assessments of the effects of treatments that affect the tendon-bone interface. Our in vivo model improves data reliability because tendon grafts remain alive throughout the healing process by specifically leaving the native tendon intact at the calcaneal bone insertion site. Furthermore, other modifications of our model, including use of custom-made clamps that enable solid fixation of specimens in the testing device, prevent application of excess forces on the tendon—bone interface that is being evaluated for different treatment modalities. The utility of this model is reflected by the previous demonstration that non-steroidal anti-inflammatory drugs have negative effects on tendon-to-bone healing.[27,28]

Biomechanical analysis of the experimentally manipulated tendons indicated a broad range of values for graft rupture in force to failure experiments that prevent our results from reaching statistical significance. Although the median force to failure was 38% higher in BMP2 specimens and 81% higher in GSK126 specimens compared to placebo specimens, these comparisons were not statistically significant. This broad statistical variation of our biomechanical data may be attributable to soft tissue callus formation at the dorsal aspect of the insertion site of the Achilles tendon in the tibia. The latter was not entirely removed, as it is considered a part of the overall tendon-bone reconstruction. Furthermore, the study presented here only evaluated a single dose and administration time for the two bone anabolic factors we investigated. Time and dosage of drug delivery can both have profound effects on healing and integration of the tendon graft in bone tunnel. The used dosage and timing has previously demonstrated positive effect of the evaluated growth factors in in vivo critical bone defect experiments. We analyzed early stage healing, yet this phase can be influenced by inflammation-driven endochondral bone formation. The first healing phase is also characterized by increased osteoclast activity with bone resorption at the perimeter of the bone tunnel. Our μCT data (median cross sectional area of the bone tunnel), indicated that all groups had increased cross sectional areas compared to the size of the largest drill bit (1.5 mm). Taken together, there are a number of factors that cause experimental variation and it is encouraging that our studies reveal interesting numerical trends that warrant follow-up studies on the use of bone anabolic reagents to enhance tendon integration in bone tunnels.

The bioactivity of a growth factor is sometimes limited by short half-life and loss through diffusion. However, as shown by Yang et al[31,32], sustained delivery with preserved bioactivity is achievable by encapsulating a growth factor into conjugated fibrin. Drug doses used in our study were identified based on literature and other experiments performed by members of our research group. To prevent any bias of inter-gender difference in drug metabolism, only female animals were used for the experiment. Exogenously delivered BMP and other bone anabolic factors can promote heterotopic ossification, although we did not observe heterotopic ossification in any of our samples, as evaluated by gross anatomic dissection, histology, or μCT. No other adverse effects were observed, including seroma, skin healing issues, or osteolysis. Thus, we consider fibrin-glue-mediated local administration delivery of bone anabolic factors to be safe and potentially effective.

Mechanically induced signaling is important for bone remodeling and new bone formation, which increases strength by adding collagen fibers that span the soft tissue interface and increase graft integration. The model used for this experiment allows free weight bearing to apply mechanical loading that mimics clinical rehabilitation. Reorganization of microstructural collagen fibers crossing the bone-soft tissue interface were not observed; possibly related to the early timing of our biomechanical evaluation. Additionally, we did not find any differences in BMD for any group comparison, which is consistent with upregulated osteoclast activity and/or increased bone resorption during the initial healing phase measured in this study. As a future strategy, a bone resorption inhibitor could be used to limit the

early (healing phase) resorption to accentuate the effects of BMP/GSK126 and promote rapid bone formation. However, co-delivery of multiple reagents can be complicated by dynamic release kinetics and time-dependent interactions among reagents that increase the favorability of single-reagent delivery methods.

EZH2 is a crucial contributor to skeletal development and early bone formation. The inhibitory properties of GSK126 on EZH2 promote commitment of precursor cells into differentiation in the osteoblastic lineage, and thus increase bone formation. This study elucidates the novel approach of using GSK126 to enhance tendon-to-bone tunnel healing. Additional biomaterials may permit sustained release of growth factors and mitogens (eg, BMP2). Of particular interest are bivalent biopolymer tapes that contact the tendon grafts via a TGFβ1 side and the bone tunnel via a BMP2 side as a strategy for facilitating osseointegration of the graft. Further testing of other bone-anabolic reagents (eg, PTH) will also have significant translational potential in promoting tendon-to-bone healing because many such compounds are already in clinical use for other indications.

In conclusion, our study presents promising results of fibrin-glue-mediated delivery of bone anabolic drugs, both BMP2, and a novel application of GSK126, in a clinically relevant and validated rodent tendon-to-bone tunnel healing model, with trends of smaller cross sectional areas and increased bone volume in the inner two regions for BMP2 compared to placebo. Further work aimed at characterizing the molecular and cellular differences between tendon and ligament will permit targeted strategies for biologically enhancing tendon tissue in a manner that permits adoption of the mechanical strength characteristic of ligament tissue, and facilitate osseointegration of the tendon graft. Such strategies, including the results presented herein, have potential to improve both short- and long-term clinical outcomes of tendon-based ligament repair by increasing graft fixation and retention.

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