MEANS OF ACCELERATING TENDON GRAFT HEALING AFTER ACL RECONSTRUCTION

CORINA-ADRIANA GHEBEŞ

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Means of accelerating tendon graft healing after ACL reconstruction

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The artwork is adopted here to represent the goal of this dissertation: to achieve a natural integration of the tendon graft into the bone after ACL reconstruction.

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MEANS OF ACCELERATING TENDON GRAFT HEALING AFTER ACL RECONSTRUCTION

DISSERTATION

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born on June 20th, 1986

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SUMMARY

Rupture of the anterior cruciate ligament (ACL) is one of the most common sport-related injuries. As the ACL is unable to repair itself, reconstructive surgery remains the number one option for the restoration of joint function and the achievement of nearly-normal joint kinematics and kinetics. The current practice of surgical ACL reconstruction uses autografts, such as tendon grafts, to replace the native ligament and ensure a good clinical outcome. Successful ACL reconstruction with a tendon graft requires a strong bond between the soft tissue (graft) and hard tissue (bone) in the femoral and tibial tunnels, followed by functional adaptation and remodelling to resemble the structural properties of the native ACL. After surgery, this process takes approximately 12 months. This relatively long recovery period hinders patients from returning to normal daily activities. The longer they are incapable of normal ambulation and more strenuous activities, the more their muscle and motor skills decline, subsequently causing further delay. This vicious cycle must be broken. The demand for solutions capable of shortening graft healing has prompted scientists to explore new ideas and strategies to improve clinical practice in ACL reconstruction surgery. This thesis has explored three of these approaches to advance ACL healing: harvesting and priming bone marrow cells, utilising the potential of muscle-derived signals and identifying small molecules that can modulate important signalling pathways.

This research has first evaluated the tissue-regenerative properties of the tendon graft compared to the native ligament through an in vitro analysis of the phenotype of cells derived from each tissue. Next, it has identified that the cells lack regenerative properties. The consequent task has been to find cells that do have these properties, as well as osteoinductive properties, that can be used in the ACL reconstruction practice. These properties are found in bone marrow cells that are commonly derived from the ilium. For ACL reconstruction, sourcing these cells proximal to the knee junction would be optimal. However, our comparative study of different bone marrow cell sources has shown that cells derived from the ilium offer the strongest regenerative properties. To enhance these properties further, this research has also investigated different priming methods, such as acoustic stimulation and selection of cells through different isolation methods. Optimisation of the cell source and priming conditions forms a basis for applying these cells in ACL reconstruction research.

This research has used an indirect co-culture system for an in vitro simulation of the crosstalk between different cell types that contribute to the development of tendons and ligaments during embryogenesis. This has revealed that myoblast-derived signals are capable of upregulating classical tendon/ligament gene expression markers on tendon-derived cells, which can contribute to ACL graft healing. Whole transcriptome analysis has shown that co-culturing tendon-derived cells with myoblasts leads to an upregulation of extracellular matrix (ECM) genes and results in enhanced ECM deposition. Using a rat model of ACL reconstruction, we demonstrated *in vivo* that conditioned media derived from muscle tissue accelerates femoral tunnel closure, a key

step for autograft integration. Collectively, these results indicate that muscle-derived signals can be employed to improve ACL graft healing in a clinical setting, where muscle remnants are often discarded.

Modulation of bone morphogenetic protein 2 (BMP-2) and transforming growth factor-β1 (TGF-β1) signalling pathways is essential during tendon/ligament healing. Unfortunately, growth factor delivery in situ is far from trivial and, in many cases, the necessary growth factors are not approved for clinical use. This research has used a BMP-2 and a TGF-β1 reporter cell line to screen a library of 1,280 small molecules approved by the Food and Drug Administration, which has led to the identification of modulators of both signalling pathways. This report presents four relevant compounds and a description of their effects on proliferation and differentiation of tendon-derived cells.

Undoubtedly, this research does not stand alone, but is part of a more extensive field of tendon/ligament research of the musculoskeletal system. While solutions are highly specialised, advancements in the field may benefit from a holistic perspective of the field itself. This research concludes by mapping the field, both visually and conceptually. This visualisation of the world of musculoskeletal tendon and ligament research aims to provide a quick overview of the main contributors to the field and the areas of interest.

SAMENVATTING

Letsel aan de voorste kruisband (anterior cruciate ligament (ACL) in het Engels) is een van de meest voorkomende sportgerelateerde blessures. Omdat de ACL zichzelf niet kan herstellen, blijft reconstructieve chirurgie de beste optie om de gewrichtsfunctie snel te herstellen en voor een nagenoeg normale gewrichtskinematica en -kinetiek. In de huidige praktijk van chirurgische ACL-reconstructie wordt gebruikgemaakt van autotransplantaties zoals peestransplantaties, om het natuurlijke ligament te vervangen en te zorgen voor een klinisch goed resultaat. Een succesvolle ACL-reconstructie met een peestransplantaat vereist een sterke band tussen het zachte weefsel (transplantaat) en het harde weefsel (bot) in de femorale en tibiale tunnels, gevolgd door een functionele aanpassing en reorganisatie om de structuureigenschappen van de oorspronkelijke ACL na te bootsen. Na de operatie duurt dit proces ongeveer twaalf maanden. Deze relatief lange herstelperiode belemmert patiënten de normale dagelijkse activiteiten op te pakken. Hoe langer ze niet in staat zijn om normaal te lopen en inspannendere activiteiten te ondernemen, hoe meer hun spieren en motorische vaardigheden afnemen. Dit zorgt voor verdere vertraging van het herstel. Daarom moet deze vicieuze cirkel worden doorbroken. De vraag naar oplossingen om de duur van het herstel na een transplantatie te verkorten, heeft wetenschappers aangespoord nieuwe ideeën en strategieën te onderzoeken die de klinische praktijk van de ACL-reconstructiechirurgie kunnen verbeteren. Deze dissertatie heeft drie van deze benaderingen om ACL-revalidatie te bevorderen onderzocht: afname en priming van beenmergcellen, gebruikmaken van het potentieel van spier-afgeleide signalen en identificeren van kleine moleculen die belangrijke signaalpaden kunnen moduleren.

Dit onderzoek heeft eerst de weefsel-regeneratieve eigenschappen van het peestransplantaat vergeleken met het oorspronkelijke ligament door een in-vitroanalyse van het fenotype van cellen die afgeleid zijn van elk weefsel. Vervolgens is vastgesteld dat de cellen geen regeneratieve eigenschappen bezitten. Daaruit is voortgekomen dat er een taak ligt om cellen te vinden die deze eigenschappen wel hebben en osteo-inductieve eigenschappen te achterhalen die kunnen worden gebruikt in de reconstructie van ACL. Deze eigenschappen zijn aangetroffen in de beenmergcellen die gewoonlijk zijn afgeleid van het darmbeen. Voor een ACL-reconstructie zou sourcing van de cellen in de buurt van het kniegewricht het best zijn. Ons vergelijkingsonderzoek naar beenmergcellenbronnen heeft echter aangetoond dat cellen die afgeleid zijn van het darmbeen de sterkste regeneratieve eigenschappen hebben. Om deze eigenschappen verder te verbeteren, heeft dit onderzoek ook verschillende priming-methoden onderzocht, zoals akoestische stimulatie en selectie van cellen door verschillende isolatiemethoden. Een optimalisatie van de celbron en de priming-voorwaarden vormt een basis voor de toepassing van deze cellen in een ACL-reconstructieonderzoek.

Dit onderzoek heeft een indirect co-kweeksysteem gebruikt voor een in-vitrosimulatie van de crosstalk tussen verschillende celtypen die tijdens de embryogenese aan de ontwikkeling van pezen en ligamenten bijdragen. Hieruit is gebleken dat signalen van

de myoblast opwaarts klassieke pees- en ligamentgenexpressiemarkers op de pees-afgeleide cellen kunnen reguleren, die aan de ACL-transplantaatrevalidatie kunnen bijdragen. Een volledige transcriptoomanalyse heeft aangetoond dat het co-kweken van pees-afgeleide cellen met myoblasten leidt tot een opwaartse regulatie van extracellulaire matrix (ECM-)genen en resulteert in een verbeterde ECM-depositie. Middels het gebruik van een ratmodel voor ACL-reconstructie hebben we *in vivo* aangetoond dat geconditioneerde media die afgeleid zijn van spierweefsel een femorale tunnelsluiting versnellen. Dit is een belangrijke stap voor de integratie van autotransplantatie. Alle resultaten wijzen erop dat spier-afgeleide signalen kunnen worden ingezet ter verbetering van de ACL-transplantatierevalidatie in een klinische setting, waarbij de spierresten vaak worden verwijderd.

Tijdens de pees- of ligamentrevalidatie is het essentieel dat het bot-morfogenetische eiwit 2 (BMP-2) en de signaalpaden van de transformerende groeifactor-β1 (TGF-β1) moduleren. Groeifactorlevering ter plaatse is helaas verre van triviaal en in veel gevallen zijn de benodigde groeifactoren niet goedgekeurd voor klinisch gebruik. In dit onderzoek zijn een BMP-2 en de TGF-β1-reportercellijnen gehanteerd om een reeks van 1,280 kleine moleculen te onderzoeken, die zijn goedgekeurd door de FDA (Food and Drug Administration). Dit onderzoek heeft geleid tot de identificatie van modulatoren van beide signaalpaden. Dit verslag presenteert vier relevante verbindingen en een beschrijving van de gevolgen daarvan voor de proliferatie en differentiatie van de pees-afgeleide cellen.

Dit onderzoek staat niet op zichzelf, maar is onderdeel van een uitgebreider onderzoeksveld dat gericht is op de pezen en ligamenten van het bewegingsapparaat. Terwijl de oplossingen zeer gespecialiseerd zijn, kunnen de ontwikkelingen op dit gebied profiteren van een holistisch perspectief op het onderzoeksgebied zelf. Ten slotte is het onderzoeksveld zowel visueel als conceptueel in kaart gebracht. Deze visualisatie van de wereld van pees- en ligamentonderzoek heeft als doel een overzicht te bieden van de belangrijkste bijdragen en interessegebieden binnen het veld.

Chapter 1

General introduction and thesis outline

INTRODUCTION

Rupture of the anterior cruciate ligament (ACL) is one of the most common sport-related injuries. Unable to self-repair, reconstructive surgery remains the number one option to restore joint function aiming for rapid recovery and attempting to achieve close to normal join kinematics and kinetics. Current surgical ACL reconstruction practice uses autografts, such as tendon grafts, to replace the native ligament and ensure a good clinical outcome. Successful ACL reconstruction with a tendon graft requires, a strong bond between the soft (graft) and the hard tissue (bone) in the femoral and tibial tunnels, followed by functional adaptation and remodelling to resemble the structural properties of the native ACL. This process is currently only sufficiently healed approximately 12 months after surgery [1], which hinders patients return to normal daily activities and affects their quality of life. The longer they are incapable of normal ambulation and more strenuous activities the more muscle and motor skills are lost. This in turn causes delay in return to normal life and sports. This vicious circle needs to be broken to allow patients more rapid and better return to their desired activities after ACL reconstruction. Solutions capable of shortening graft healing are much needed, prompting scientists to explore new ideas/strategies to improve clinical practice in ACL reconstruction surgery.

Studies report an incidence rate of ACL rupture between 36.9 and 60.9 per 100,000 people per year [2, 3]. ACL rupture often results from twisting or bending the knee [4] and causes significant joint instability that, if left untreated, can cause meniscus tears, cartilage defects and generalised osteoarthritis [5, 6]. Unfortunately, the ACL´s low cellularity, poor vascularization, and a surrounding hostile intra-articular environment [7, 8] do not provide an adequate healing response that can bridge the gap between the ruptured ends of the ACL [9, 10].

ACL RECONSTRUCTION

Today, ACL reconstruction is the standard care procedure to restore function [11]. Several reconstructive procedures have been proposed in the past few decades, differing mainly in terms of graft selection and surgical technique. The autologous graft remains the most popular method for ACL reconstruction, however, and is considered the ‘gold standard’ because of the high rate of success (85-90%) regarding long-term clinical outcomes [12]. One such graft is the hamstring tendon, which, given its mechanical and structural similarities to the native ACL [13] and low morbidity at the harvest site [14], has made it a logical choice for ACL reconstruction [15, 16]. Using the most common ACL reconstruction technique, bone tunnels are drilled into the tibia and femur and the hamstring tendon graft is inserted onto the footprints of the original ACL. The remnant muscle tissue is removed from the graft and the graft is pulled through the bone tunnels and fixed in place [17] (Figure 1). Because the hamstring tendon graft does not have bone plugs, tendon-to-bone healing is largely dependent on the osteointegration of the grafted tendon into the bone tunnels. It may take up to 12 months before a functional

tissue, closely resembling the structural properties of the native ACL, is established and capable of guaranteeing a safe return to similar pre-injury levels of activity [18]. This long recovery after surgery affects patient expectations, especially young individuals and athletes who aim to return soon to high-level sporting activities. Because an earlier return to sports may increase the risk of reincidence, patients are obliged to wait until the transplanted graft is fully remodelled. A firm attachment of the tendon graft to the bone is a crucial factor in facilitating an early aggressive rehabilitation and a rapid return to sports and full activity.

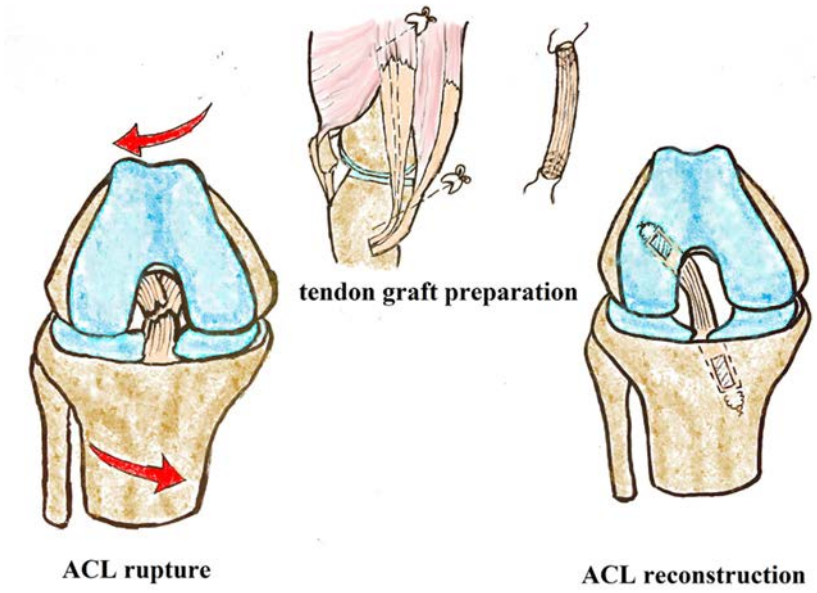

Figure 1. Surgical reconstruction procedure following ACL rupture*. The hamstring tendon graft is harvested; remnant muscle tissue is removed from the graft; and the graft is pulled through the bone tunnels and fixed in place.*

The native ACL insertion into bone is a highly-specialized tissue, comprising a complex transition zone from the ligament to the bone. This transition zone consists of the ligament proper, non-mineralised fibrocartilage, mineralised fibrocartilage, and bone [19] and plays a crucial role in the biomechanics of the knee joint. To restore this interface, progressive mineralisation of the tendon-bone interface must occur. An incorporation of the tendon graft into the surrounding bone, followed by bone ingrowth into the grafts, contributes to the regeneration of the tendon-to-bone junction. To date, there is no proof for an absolute regeneration of this complex transition zone following ligament reconstruction [20] and, perhaps as a consequence, the properties of the grafted tissue remain inferior to the native tissue.

Prior attempts to improve tendon-graft healing include both intraoperative and extracorporeal intervention. A summary and description of these strategies are presented below, and there is a schematic representation in Figure 2.

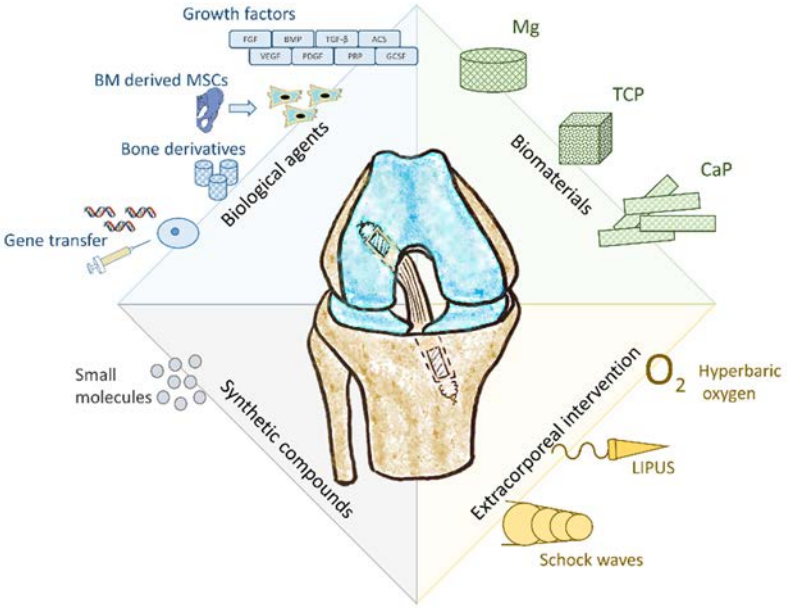

Figure 2. Various strategies employed to stimulate tendon-graft healing.

INTRAOPERATIVE INTERVENTION

Intraoperative interventions include the use of agents and materials, such as biological agents, biomaterials, and small molecules, administered during ACL reconstruction, to provide appropriate molecular signals able to induce tissue specific cell proliferation, differentiation, deposition of extracellular matrix, neovascularisation, or neuro-regeneration

Biological agents

Various biological-based strategies have been proposed involving techniques that can improve the biomechanical, biochemical, and/or biological properties of the tendon graft. Such strategies involve the use of a variety of cells, growth factors, platelet-rich plasma (PRP), periosteum, and gene transfer.

Cell-based therapy

Mesenchymal stromal cells (MSCs), harvested from bone marrow, synovium, or umbilical cord blood [21-23], have been proposed to augment ACL reconstruction. Flourishing preclinical literature suggests that MSC administration can stimulate tissue maturation, improve histological appearance, and favour bone-to bone integration [24]. Using a rabbit model for ACL reconstruction, it has been shown that MSCs embedded within fibrin glue and implanted into the bone tunnel contributed to the formation of a

fibrocartilage interface at the junction that more closely resembles that of a normal ACL [23]. Further studies using similar approaches have shown comparable positive results at the tendon graft to bone integration [22, 25-27]. It seems that MSCs improve the insertion of the graft into the bone, recapitulating some of the features of the natural tissue. The exact mechanism of action of the transplanted MSCs on tendon-bone healing is still largely unknown, however.

In clinical studies, the application of MSCs have been introduced, especially in the field of cartilage regeneration/osteoarthritis; with 18 completed clinical studies found in PubMed and 50 clinical trials listed on clinicaltrials.gov, this carries promise of more breakthrough discoveries [28, 29]. Regarding ACL healing following reconstruction, only one clinical study was identified that used MSCs by the application of bone marrow concentrate harvest from ilium. This study was unable to provide a clear understanding of the actual contribution of the MSCs from bone marrow concentrate to tendon-graft bone tunnel healing [30]. Unfortunately, no characterisation of the aspirate, viability, or numeration of the mononuclear cells (MNCs) was completed on any sample. Since it is possible to obtain MSCs from different sources, and MNC concentration might play a significant role in the augmentation of ACL reconstruction, it would be interesting to determine which source can play a major role in achieving a positive clinical outcome.

The periosteum represents an additional cell-based therapeutic strategy proposed to enhance tendon-graft bone tunnel healing. Consisting of multipotent mesodermal cells that have the capacity to form cartilage and bone, it is used as a wrap around the tendon and inserted into the bone tunnel. Results obtained from preclinical studies showed the formation of fibrocartilage and bone regeneration around the tendon graft [31-34]. Few clinical studies report satisfactory outcomes in patients undergoing ACL reconstruction with periosteum-enveloping tendon-graft technique, however, with regard to bone tunnel enlargement [35, 36].

A new cell-based therapeutic strategy has shown admirable performance on tendon-bone healing in preclinical studies. ACL-derived CD34+ cells have been shown to enhance tendon-bone healing in ACL reconstruction animal models, mostly by enhancing angiogenesis and osteogenesis [37, 38]. Matsumoto et al. revealed the presence of abundant vascular stem cells with a characteristic expression of CD34+ in the injured ACL tissues, which displayed higher expansion and multiple lineage differentiation potential than CD34- cells [39]. In a further study, ACL-derived CD34+ cells were injected into nude mice articular cavities after ACL reconstruction, where more fibrocartilage cells, as well as enhanced angiogenesis and osteogenesis, were suggested in a CD34+ sorted group [38]. Additionally, CD34+ cells were previously isolated from peripheral blood or bone marrow and recognised as a rich population of haematopoietic/endothelial progenitor cells, which have been already used in clinical settings for the repair of various damaged tissues [40]. This can ease their path to clinical application in ACL reconstruction. Isolated from the site of ACL rupture, these cells can provide an important contribution to tendon-bone healing and regeneration.

Growth factors

Platelet-rich plasma (PRP) is an autologous blood-derived product obtained by centrifugation or filtration of peripheral blood to concentrate the platelets. It represents a reservoir of several growth factors and bioactive molecules involved in tissue homeostasis and anabolism [41]. Preclinical studies have shown that application of PRP can promote better and faster ligamentisation of the graft, reduce the proinflammatory factors released immediately after the surgery and contribute, to a less extent, to a better tendon-graft bone integration [42, 43]. In clinical studies, however, the benefits of PRP application in providing a faster and better functional outcome are inconclusive. The addition of platelet concentrate to ACL reconstruction shows a 20 to 30% improvement on graft maturation (progression of cellularity, vessel density, and histologic signs of graft maturity) but with substantial variability, while no significant difference in graft-bone interface healing was observed [44-46].

Targeting specific growth factors can have more promising results than generic PRP injections. Overdosing with PRP can overstimulate the cells, leading to poor differentiation and chaotic scar formation, or might precipitate adverse events such as suppression of osteoclast generation [47].

Bone morphogenetic proteins (BMPs) have been widely acknowledged for their role in cellular differentiation and bone formation. Numerous preclinical studies have reported positive effects of BMP-2 and BMP-7 on healing after ACL reconstruction, mostly by improving the integration between tendon and bone [25, 48-51]. Transforming growth factor beta (TGF-β) has been shown to stimulate matrix protein deposition by generating perpendicular collagen fibres connecting the tendon graft and bone and ultimately increasing the maximum load in preclinical studies. It also modulates tissue healing and remodelling through chemotaxis of neutrophils and monocytes to the wound site [52, 53]. Fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been shown to contribute to fibrous integration between the tendon and bone via vascularisation [25, 54]. Furthermore, granulocyte colony-stimulating factor has been suggested to enhance the ultimate strength and accelerate bone development of the graft in experimental ACL reconstruction animal models [55], while hepatocyte growth factor has been suggested to promote the adhesive healing process at the tendon-bone junction [56]. Finally, platelet-derived growth factor-BB (PDGF-BB) has been shown to increase vascularity and collagen deposition. The application of PDGF-BB on graft incorporation using an ACL model in sheep resulted in higher biomechanical strength, higher vascular density, and higher levels of collagen fibril [57]. Despite their positive role in preclinical studies, only PDGF and autologous conditioned serum (ACS) were introduced in clinical settings to accelerate the healing process following ACL reconstruction. PDGF proved to have limited effect on clinical score, inflammatory markers, or graft appearance on MRI [58, 59], while ACS showed decreased bone tunnel widening [60]. None of this has found its way into clinical practice.

Gene therapy

Gene transfer of therapeutic factors has been developed to accelerate tissue healing, which overcomes the limitations of the direct use of growth factors [61]. Sustained delivery of BMP-2 via gene transfer has been shown to enhance the behaviour of the cells in a manner that improves bone healing response at the tendon-bone interface [50, 62]. Reconstructed tendon-bone interface with PDGF-B transfected bone marrow derived MSCSs enhanced vascularity and collagen deposition [63]. Additionally, viral vector mediated gene transfer of bFGF (both in vitro and in experimentally injured human ACLs) significantly enhanced collagen production and neovascularisation [64, 65].

Bone derivative

Application of the recombined bone xenograft within the bone tunnels following ACL replacement, such as demineralised bone matrix or enamel matrix derivatives, represent a further source of BMPs that can enhance tendon-bone healing and tendon-bone fixation strength by inducing an increase in the growth of fibrocartilage and mineralised fibrocartilage at the tendon-bone interface [66, 67]. As yet, the role of bone derivatives in clinical setting of ACL reconstruction and graft healing has not been assessed.

Biomaterials

Osteoconductive agents such as calcium phosphate (CaP), hydroxyapatite, tricalcium phosphate (TCP), brushite CaP cement, and magnesium adhesive have been used to fix the tendon graft into the bone tunnel and improve its healing via enriched bone ingrowth [68-71]. Some of this technology is used in fixation devices. Currently, one ongoing clinical study is evaluating the application and performance of these materials in revision ACL reconstruction [72].

Small molecules

Simvastatin has been reported to improve endothelial function, have an anti-inflammatory, and to stimulate angiogenesis and bone formation by activating the promoter of the BMP-2 gene [73-76]. Using a rabbit model, a previous study showed that the local administration of low-dose simvastatin-conjugated gelatin hydrogel promoted tendon-to-bone healing during the early phase following ACL reconstruction by enhancing angiogenesis and osteogenesis [77]. Another small molecule, alendronate, has been reported to reduce bone resorption. Using a rat model, the administration of alendronate has been shown to reduce bone resorption and increase mineralised tissue formation inside the bone tunnel [78, 79]. The use of these therapeutic small molecules represents an important alternative to growth factors. Unlikely to induce immune response in the host because they are too small to do so [80], and with no risk of cross-species contamination, as in the case of recombinant protein-based applications [81], small molecules represent the next generation of therapeutic approach.

Extracorporeal intervention

Extracorporeal interventions, such as low-intensity pulsed ultrasound (LIPUS), hyperbaric oxygen, or shock waves are noninvasive therapies that have been used immediately following ACL reconstruction to enhance tendon-to-bone healing. These interventions use mechanotransduction as a mechanism to induce osteogenesis and neoangiogenesis and thus improve local tissue regeneration and remodelling [82-84].

The abovementioned strategies demonstrate the challenge of achieving a secure fix between the tendon graft and the bone tunnel. A current dichotomy between the flourishing preclinical literature and the limited and inconclusive data coming from clinical studies highlights not only the insufficient understanding of the mechanism of action of these powerful agents but also the need for new approaches with better impact on clinical outcomes.

AIM OF THE THESIS

This work aims to find new strategies capable of accelerating tendon-graft healing and improving the future practice of ACL reconstruction in a practical and clinically applicable manner.

OUTLINE OF THE THESIS

The current chapter provides a description of the challenge and a summary of ongoing approaches that aim to address these challenges (Chapter 1).

Given the insufficient regenerative properties of ACL tissue to heal following a severe injury, and the poor healing properties of the tendon graft following ACL reconstruction, it is important to determine the differences in regenerative properties between the ‘to be replaced and the new tissue graft’. This can influence the direction of investigation and the development of strategies to accelerate tendon-graft healing. An evaluation of tissue regenerative properties was achieved by analysing in vitro the phenotype of cells derived from each tissue (Chapter 2).

Based on the information in the previous chapter, that tendon-graft derived cells seem to lack regenerative properties. Aiming for an integration of the tendon graft into the bone, we searched for appropriate cells with regenerative and osteoinductive properties. As an additional project, this chapter provides information regarding possible bone marrow cell sources or cell-priming methods to enhance bone regeneration and implicit enhance tendon-bone tunnel integration (Chapter 3).

Next we provide important evidence (at both in vitro and in vivo levels) that the actual remnant muscle tissue, discarded during ACL reconstruction, can have a beneficial contribution to accelerating tendon-graft healing (Chapter 4). Using a mix of cell types (derived from different tissues), we investigated the influence of intercellular

communication on the expression of genes that direct cell differentiation and extracellular matrix formation. Further analysis, using an in vivo ACL reconstruction rat model, provides proof of concept evidence that remnant muscle tissue releases factors that can accelerate tendon-graft healing.

Our next approach identifies small molecules that can modulate two essential signalling pathways in tendon-graft healing (Chapter 5). Using BMP-2 and TGF-β1 reporter cell lines, we screened a library of small molecules approved by the FDA (Food and Drug Administration) and identified four compounds able to modulate both signalling pathways. Subsequent assays used primary tendon cells to investigate the effect of the selected molecules in tendon cell metabolism and differentiation potential.

Eager to understand the reason why, after decades of research, healing of the ACL (as well as of other ligaments and tendons (L/T)) still represents a persistent clinical challenge, we approached the current research field of L/T from a perspective other than the usual reviews (Chapter 6). A world map summarising the main contributors in the field of L/T and a graphic representation of their main interest can help provide answers to the question and guide researchers towards quickly advancing research in L/T healing.

Ultimately, in the final chapter, we discuss the important findings of this thesis, and reflect upon the relevance these findings have in immediate translation into clinical applications.

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Chapter 2

Anterior cruciate ligament- and hamstring tendon-derived cells: *in vitro* differential properties of cells involved in ACL reconstruction

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ABSTRACT

Anterior Cruciate Ligament (ACL) reconstruction involves the replacement of the torn ligament with a new graft, often a hamstring tendon (HT). Described as similar, the ACL and HT have intrinsic differences related to their distinct anatomical locations. From a cellular perspective, identifying these differences represents a step forward in the search for new cues that enhance recovery after the reconstruction. The purpose of this study was to characterise the phenotype and multilineage potential of ACL- and HT-derived cells. ACL- and HT-derived cells were isolated from tissue harvest from patients undergoing total knee arthroplasty (TKA) or ACL reconstruction. In total, three ACL and three HT donors were investigated. Cell morphology, self-renewal potential, surface marker profiling, expression of tendon/ligament-related gene markers and multilineage potential were analysed for both cell types; both had fibroblast-like morphology and low self-renewal potential. No differences in the expression of tendon/ligament related genes or a selected set of surface markers were observed between the two cell types. However, differences in their multilineage potential were observed: while ACL-derived cells showed a high potential to differentiate into chondrocytes and adipocytes, but not osteoblasts, HT-derived cells showed poor potential to form adipocytes, chondrocytes and osteoblasts. Our results demonstrated that HT-derived cells have low multilineage potential compared to ACL-derived cells, further highlighting the need for extrinsic signals to fully restore the function of the ACL upon reconstruction.

INTRODUCTION

The ACL represents one of the major ligaments in the human knee. Located between the femur and the tibia, its function is to limit the anterior translation and rotation of the tibia with respect to the femur [1, 2], thereby contributing to the stability of the knee joint. Annually, more than 200,000 people report ACL injuries in the USA alone [3]. Due to poor cellularity, limited vascularization, and the intra-articular environment of the ACL [4-6], healing is frequently insufficient to restore functionality and thus surgical reconstruction is performed in 40% of patients [3]. The goal of surgical reconstruction is to restore the mechanical function of the knee and to prevent the knee joint from prolonged exposure to a pro-inflammatory environment, which would otherwise hamper joint homeostasis and, if not treated, could lead to the early development of osteoarthritis [7].

Over the last two decades, reconstruction of the ACL under arthroscopic observation has become a routine surgical procedure [8] and involves the use of autologous or allogeneic grafts and their fixation into femoral and tibial bone tunnels. In 80% of clinical applications autologous grafts are used as they do not pose the risks of disease transmission and immune rejection, associated with allogeneic grafts [9, 10]. HT represents one of the most often used autologous grafts [11, 12], with another option being the patellar tendon graft. The HT graft can restore the function of the ACL while minimizing morbidity at the harvest site, especially when compared to the patellar tendon graft. Its main disadvantage is the greater prevalence of knee instability [12]. The new graft is inserted into previously drilled femoral and tibial tunnels and fixed in place by intramedullar screws or cortical fixation systems (e.g. Endo-button®), therefore restoring the function of the knee joint. Neither of the graft fixation methods showed significant differences in clinical outcomes [13].

Although the anatomical location and function of the HT differs from that of the ACL – the HT is located on the back of the knee joint, attaching the hamstring muscle group to the tibia and thereby transmitting force from the muscle to the bone – its choice for the reconstruction of the ACL is based on its similarity in structure, biology and mechanical properties to the native ACL [14]. Moreover, harvesting the HT is not only easier but also has a low impact on functional disability after use. Studies that characterise the structure and morphology of these tissues have been previously reported and they described closely packed collagen fibres and low cellular content, with cells aligned with the fibres [14, 15]. Efforts to characterise the phenotype and functionality of these cells have been made. Both ACL and HT tissue are composed of fibroblasts/fibrocytes (ligament) and tenoblasts/tenocytes (tendon), with fibroblasts/tenoblasts being immature cells that become fibrocytes/tenocytes upon maturation. The remaining cells consists of chondrocytes at the bone attachment or insertion site (enthesis), synovial cells and vascular cells, including endothelial cells and smooth muscle cells of arterioles [16]. Recently, multipotent mesenchymal stromal cells (MSCs) have also been found to reside within the two tissues [17-20]. However, their presence does not seem to

contribute to the repair of the tissues upon injury, as in the case of bone repair, where bone marrow-derived progenitor cells help to accelerate healing and regeneration [21, 22]. Therefore, further research is needed to better understand the behaviour of the cells derived from these two tissues, in order to harness their regenerative potential. In addition, as the original function of the HT graft changes after an ACL reconstruction, the cells residing in this tissue have to adapt to the new environment and initiate a remodelling process, ultimately restoring the interface between the grafted tissue and the bone. A thorough analysis of the cellular potential of the transplanted HT cells with respect to the original ACL cells will contribute to improve rehabilitation protocols thus reducing failure rates (currently 6-25% of patients undergo revision surgery [23]) and accelerating the return to normal daily activities.

This study was based on the premise that differences in regenerative response may exist between the replaced and the new tissue after an ACL reconstruction, and therefore a characterisation of the two cell populations was performed in order to understand whether new cues are needed to improve the performance of the transplanted cells upon reconstruction. We hypothesised that, based on their anatomical location and mechanical function, ACL- and HT-derived cells display phenotypic and functional differences. Human ACL- and HT-derived cells were isolated from patients undergoing TKA or ACL reconstruction, respectively. Their self-renewal and multilineage potentials as well as surface marker expression profile were assessed. Additionally, the expression of a panel of genes known to be involved in tendon/ligament regeneration was analysed. A scheme of the experimental design is represented in Figure 1.

MATERIALS AND METHODS

Isolation of ACL- and HT-derived cells

Human ACL and HT samples were harvested from patients undergoing TKA or ACL reconstruction. In total, three ACLs and three HTs were harvested; patient information can be found in Table 1. The harvested tissue was washed with phosphate-buffered saline (PBS) and residual tissue was removed prior to dissection of the fascicles in 3 mm^3 pieces (Figure 2A). Only the core portion of the tissue was dissected. For the isolation of cells from the tissue using the outgrowth procedure, the fragments were placed in culture flasks and grown for 8-10 days at 37°C in growth medium (GM), consisting of Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Austria) containing 10% fetal bovine serum (FBS; Lonza), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco) and 0.2 mM ascorbic acid (Sigma). During this period, the cells could migrate from the tissue into the bottom of the flask (Figure 2B). For the isolation of cells from the tissue using the collagenase digestion procedure, dissected tissue fascicles were digested overnight in 0.15% collagenase type II solution (Worthington) at 37°C. The next day, the cells were washed with PBS and placed in culture flasks.

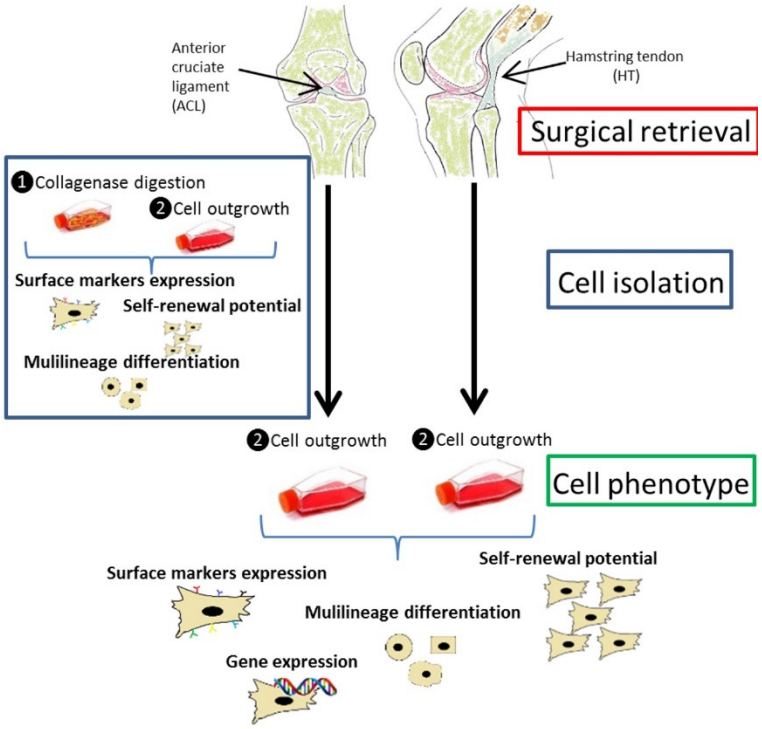


Figure 1. Schematic representation of the experimental design. Surgical retrieval of ACL and HT tissue, identifying the right cell isolation method and characterising the phenotype of the cells derived from ACL and HT.

At semi-confluence the cells were trypsinised and immediately used for the experiments and/or stored in liquid nitrogen for future use. For each experimental condition, cells between passages 2 and 3 were used, after being expanded at a density of 1,000 cells/cm^2 in GM.

Tissue	Gender	Age	Surgery Type
ACL 1	Female	61	TKA
ACL 2	Female	70	TKA
ACL 3	Female	69	TKA
HT 1	Female	24	ACL reconstruction (healthy HT)
HT 2	Female	21	ACL reconstruction (healthy HT)
HT 3	Female	24	ACL reconstruction (healthy HT)

Table 1.Donor information of the ACL and HT samples

The collection and anonymous use of the tissue was performed according to the medical ethical regulations and the guideline ‘good use of redundant tissue for research‘ of the Dutch Federation of Medical Research Societies.

Self-renewal potential

One hundred ACL/HT-derived cells were seeded in a T25 flask and grown in GM for 14 days at 37°C and 5% CO2. The cultures were washed with PBS and fixed with 10% formalin for 15 minutes at ambient temperature, after which freshly filtered 0.5% Crystal violet solution (Sigma) was added for 5 minutes to the monolayers. Samples were rinsed with demineralized water and images were captured using an Epson Perfection V750 PRO scanner. The size of the colonies was measured using Fiji software. In addition, the functionality of the assay was routinely tested on human MSCs [24].

Mineralisation

ACL/HT-derived cells were seeded at 50,000 cells/well in 6-well plates and grown in control medium consisting of GM containing 0.01 M β-glycerophosphate (BGP), in mineralisation medium consisting of GM containing BGP (Sigma) and 10^{-8} M dexamethasone (Dex), or mineralisation medium consisting of GM containing BGP and 100 ng/ml bone morphogenetic protein 2 (BMP2; Shanghai Rebone Biomaterials). Within 21-28 days, the cells were fixed in 10% formalin for 15 minutes at ambient temperature and stained with 2% Alizarin red (Sigma) for 2 minutes, then extensively rinsed with water. Images were captured using a Nikon brightfield microscope. In addition, the functionality of the assay was routinely tested on human MSCs.

Chondrogenesis

ACL/HT-derived cells were seeded at 250,000 cells/well (in triplicate) in a round-bottomed 96-well plate and centrifuged at 500 rcf for 5 minutes in GM. After 24 hours the medium was changed to chondrogenesis control medium (CCM), consisting of GM (without serum), 50 µg/ml insulin transferrin selenium (Gibco) and 40 µg/ml proline (Sigma), and to differentiation medium consisting of CCM containing 10 ng/ml transforming growth-factor-β3 (TGF-β3; R&D Systems) and 10^{-7} M Dex. After 28 days, the cells were fixed in 10% formalin for 15 minutes at ambient temperature and images of the pellets were taken. The size of the pellet was quantified using Fiji software. Subsequently the pellets were dehydrated in an ethanol series and embedded in paraffin. Sections (5 µm) were cut and stained for sulphated glycosaminoglycans (GAGs) with Alcian blue and the nuclei were counterstained with nuclear fast red. Samples were mounted in mounting medium and images were captured with a Nikon Eclipse E600. In addition, the functionality of the assay was routinely tested on human MSCs.

As a result of positive GAG staining in ACL, but not in HT-derived cells, we decided to sequentially expand ACL-derived cells until passage 10 to further investigate their

potential over time. For each passage, the cells were seeded at 1,250 cells/cm^2 and cultured until they reached 90% confluence, upon which they were trypsinized and either used for chondrogenic differentiation or further expanded. Images from the GAG staining of the cell pellets at passage 2, 4, 7 and 10, cultured under chondrogenic differentiation conditions, are shown (Supplementary Figure 4).

Adipogenesis

ACL/HT-derived cells were seeded at 25,000 cells/well in 24-well plates and grown in control medium, consisting of GM, or adipogenic medium, consisting of GM containing 0.2 mM indomethacin, 0.5 mM isobutylmetylxanthine, 10^{-6} M Dex and 10 µg/ml human insulin (all from Sigma). After three weeks, the medium was discarded and the cells were washed with PBS and fixed with 10% formalin for 15 minutes at ambient temperature, following which freshly filtered Oil Red O solution (3 mg/ml in 60% isopropanol) was added to the monolayers. After 5 minutes, the samples were extensively rinsed with demineralized water and images were captured using a Nikon brightfield microscope. The area of fat droplets (µm^2) was quantified using Fiji software. In addition, the functionality of the assay was routinely tested on human MSCs.

Flow cytometry

ACL/HT-derived cells were expanded in a T175 or a T300 flask until they reached confluence. The cells were trypsinised and incubated for 30 minutes in blocking buffer, consisting of 17% bovine serum albumin (BSA; Sigma) in PBS, followed by incubation with conjugated mouse anti-human antibodies for 30 minutes at 4°C in the dark. The samples were then washed three times with a washing buffer, consisting of 3% BSA in PBS. Expression levels of the antibodies were analysed using a FACSAria flow cytometer (BD Bioscience). For phenotypic characterisation, the following antibodies were used: CD34, HLA-DR, CD11b, CD79a, CD14, CD19, CD45, CD105, CD90, CD73, CD44, CD29, CD200, CD166, CD146, CD271, and IgG2a and IgG1 as isotype controls (all from BD Pharmingen). In addition, the functionality of antibodies was routinely tested in our laboratory on human MSCs and haematopoietic cells.

RNA isolation and gene expression profile

ACL/HT-derived cells were seeded at 10,000 cells/well in 6-well plates and grown in GM for seven days. Total RNA was isolated using the NucleoSpin RNA II isolation kit (Macherey-Nagel), per the manufacturer's instructions. RNA was collected in RNase-free water and quantitative analysis was performed using spectrophotometry (Nanodrop). First-strand cDNA was synthesised from 0.5 µg total RNA/sample, using iScript (Biorad) per the manufacturer's instructions. PCR was performed on a real-time PCR detection system (Biorad), using iQ SYBR green supermix (Biorad) for the genes β2-microglobulin (B2M), collagen type I α 1 (COL1A1), collagen type III α 1 (COL3A1), cartilage oligomeric matrix protein (COMP), tenascin C (TNC), alkaline

Target Genes	Primer Sequence
COL IA1	5'-GTCACCCACCGACCAAGAAACC
	5'-AAGTCCAGGCTGTCCAGGGATG
COL IIIA1	5'-GCCAACGTCCACACCAAATT
	5'-AACACGCAAGGCTGTGAGACT
TNC	5'-TGGGCAGATTTCACGGCTG
	5'-TGCTCTGAGCCCGAATGTC
COMP	5'-GTCCGCTGTATCAACACCAG
	5'-GGAGTTGGGGACGCAGTTA
ALP	5'-ACAAGCACTCCCACTTCATC
	5'-TTCAGCTCGTACTGCATGTC
BGLAP	5'-GGCAGCGAGGTAGTGAAGAG
	5'-GATGTGGTCAGCCAACTCGT

Table 2. Primers used for Real-Time Polymerase Chain Reaction Analysis

phosphatase (ALP) and bone γ-carboxyglutamic acid-containing protein (BGLAP) or TaqMan Universal MasterMix for scleraxis (SCX). Primer sequences are described in Table 2. Gene expression was normalised to the reference gene B2M and fold induction calculated using the $\Delta\Delta C_T$ method.

Image processing

Images showing the capacity of ACL/HT-derived cells to form colonies were acquired using an Epson Perfection V750 PRO scanner at a resolution of 800 × 800 dpi. The acquired images were then processed using Fiji software. The images were zoomed 100 times, followed by the selection of a rectangular region of interest representing 3.5 × 3.5 cm of the total surface area of a T25 flask. The images were then transformed to monochrome (8 − bit) and the brightness and contrast were adjusted in a way that allowed the visualisation of the small colonies.

Images showing the presence of fat droplets in differentiated cells were processed using Fiji software. The images were transformed to monochrome. Brightness and contrast were adjusted, similarly in test and control samples, and the fat droplets were selected and their area measured.

Images showing the size of the cell pellets cultured under chondrogenic conditions were quantified using Fiji software. The images were transformed to monochrome, the scale was set from pixel to μm. The threshold was adjusted so that only the pellet area was selected and measured.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software. Unpaired Student's *t*-test was used to compare the data when two groups were analysed. $p \leq 0.05$ indicates a statistically significant difference. Results are shown as mean ± standard deviation (SD).

RESULTS

Cell isolation procedure: outgrowth vs. collagenase tissue dissociation

ACL-derived cells were isolated either by cell outgrowth or by collagenase tissue dissociation, and their cell phenotype was compared based on self-renewal, multilineage differentiation potential and surface marker expression. In total, three donors were used. We found that both cell isolation methods showed similar self-renewal potential, with colonies similar in size and shape (Supplementary Figure 1A,B) and similar multilineage potential (Supplementary Figure 1C-P). We found no mineralisation potential in the presence of Dex (Supplementary Figure 1C,F) but positive GAG staining in the chondrogenic pellets (Supplementary Figure 1I,K) and similar amounts of fat droplets formation (Supplementary Figure 1M,N).

Positive cell surface markers, such as CD105 and CD44, were slightly but not significantly higher in the cells isolated by collagenase tissue dissociation compared to cell migration (Supplementary Figure 2).

Based on these findings, we decided to further isolate the cells using the cell migration method, as no significant differences were observed between the two cell isolation methods and as it is less invasive for the cells because no chemical additives are used.

Isolation and characterisation of ACL- and HT-derived cells

Upon tissue dissection, migration of ACL- and HT-derived cells occurred within 7-10 days. During this period, the medium was changed once in order to minimise the disturbance and potential loss of the adherent cells. We found that cells migrated from the tissue threads and attached to the bottom of the tissue culture flask (Figure 2B). Moreover, we saw that cell colonies were larger underneath the tissue threads than in other isolated spots in the flask (Figure 2B,C).

The morphology of the obtained cells was mostly spindle-shaped and fibroblast-like. Nevertheless, some elongated or large flattened and star-shaped cells were also present, indicating a heterogeneous cell population (Figure 2C). Throughout the culture period, the afore-mentioned heterogeneous population of ACL- and HT-derived cells evolved to a more homogenous fibroblast-like population, suggesting an enrichment of certain cell populations to the detriment of others (Figure 2C-E).

Self-renewal potential (CFU-F assay)

ACL- and HT-derived cells were seeded at low density in order to analyse their self-renewal potential *in vitro*, as measured by the capacity to form individual colonies. After 14 days in culture, small colonies were observed in both cell types, with mean colony-forming unit (CFU) diameters of 0.14 ± 0.03 and 0.04 ± 0.01 cm for ACL-

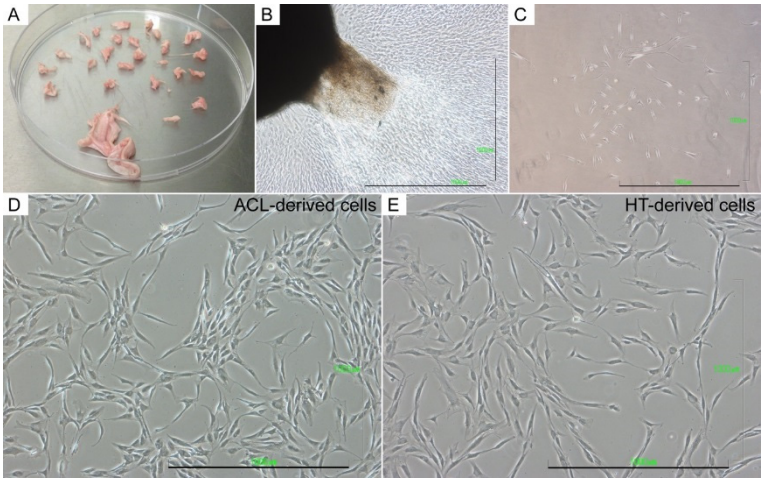


Figure 2. Isolation and morphological characterisation of ACL- and HT-derived cells*. Harvested HT tissue was dissected in small fragments (A) and placed in culture flasks. HT-derived cell migration from the tissue (black mass) to the bottom of the flask (B) and the obtained heterogeneous cell population (C) at passage P0. ACL-derived (D) and HT-derived cells (E) at P2 and P3, respectively; in both cases the cells show a fibroblastic-like structure. Scale bar indicates 1,000 µm.*

derived and HT-derived cells, respectively (Figure 3). Moreover, in the case of HT-derived cells, the colonies obtained showed irregular boundaries, rather than the typical round boundaries characteristic of bone marrow derived MSCs [25]. In contrast, ACL-derived cells formed colonies with well-defined borders, similar to the ones previously reported for MSCs. Our statement was based on visual observations.

Osteogenesis

ACL- and HT-derived cells cultured in osteogenic differentiation medium did not stain positive for calcium deposits, as measured by Alizarin red staining, with the exception of cells from one HT-derived donor (Figure 4D). To further confirm the reproducibility in calcium deposition for this donor, we cultured the cells derived from the same donor in osteogenic induction medium and observed the same phenomena (Supplementary Figure 5A). In addition to this, for the same donor, we examined a panel of osteogenic-related markers and their expression after 14 days of culture in osteogenic induction medium. We found no statistically significant differences in collagen IA1, ALP and BGLAP expression in the osteogenically induced group compared to the control (Supplementary Figure 5B), although a trend towards higher expression for ALP and BGLAP was noticeable. The addition of BMP-2 to the medium did not induce calcium deposition in either ACL- or in HT-derived cells (Figure 4B, E).

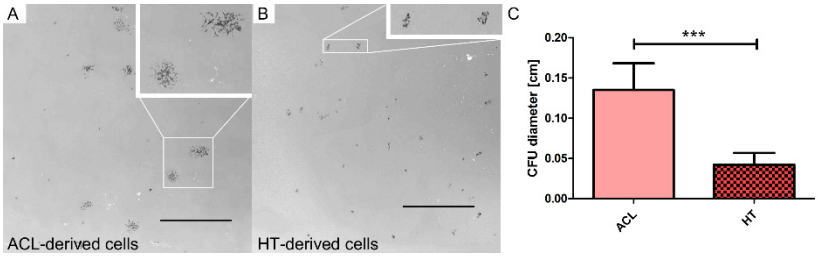


Figure 3. Crystal violet staining of ACL- (A) and HT-derived cells (B). *To investigate their self-renewal potential, cells were seeded at clonal density (100 cells/25 cm2). (C) Quantification of colony size by measuring the diameter (cm); unpaired Student's t-test of the quantified colony size was performed; *** P < 0.001. Scale bar represents 1 cm.*

Chondrogenesis

Pellet cultures were established for ACL- and HT-derived cells and grown for 28 days in control or chondrogenic induction medium. After 28 days of culture, ACL-derived cell pellets cultured under chondrogenic conditions were greater in size than HT-derived cell pellets (Supplementary Figure 3), probably resulting from an increase in extracellular matrix (ECM) deposition. The increase in ECM production was confirmed by Alcian blue staining, which revealed a uniform staining of GAGs in ACL-derived cells (Figure 4G), in contrast to HT-derived cells, where GAG staining was absent (Figure 4I). Despite the positive staining, it was noteworthy that the morphology of the obtained cartilage was more consistent with fibroblast-like cartilage than with the typical round morphology of a chondron. Interestingly, extensive *in vitro* culture did not hamper the chondrogenic potential of ACL-derived cells, as demonstrated by the presence of Alcian Blue-positive cells up to passage 10 (Supplementary Figure 4). Cells cultured under control conditions stained negative in both cases (Figure 4 H,J).

Adipogenesis

The capacity of ACL- and HT-derived cells to form adipocytes upon exposure to induction medium was analysed, and we found a statistically significant difference between ACL- and HT-derived cells (Figure 4K, L). The formation of fat droplets was five times higher in ACL- than in HT-derived cells (Figure 4N). Moreover, the results were not a result of contamination of the sample with adipocytes, since the control samples stained negative for adipogenesis (Figure 4M).

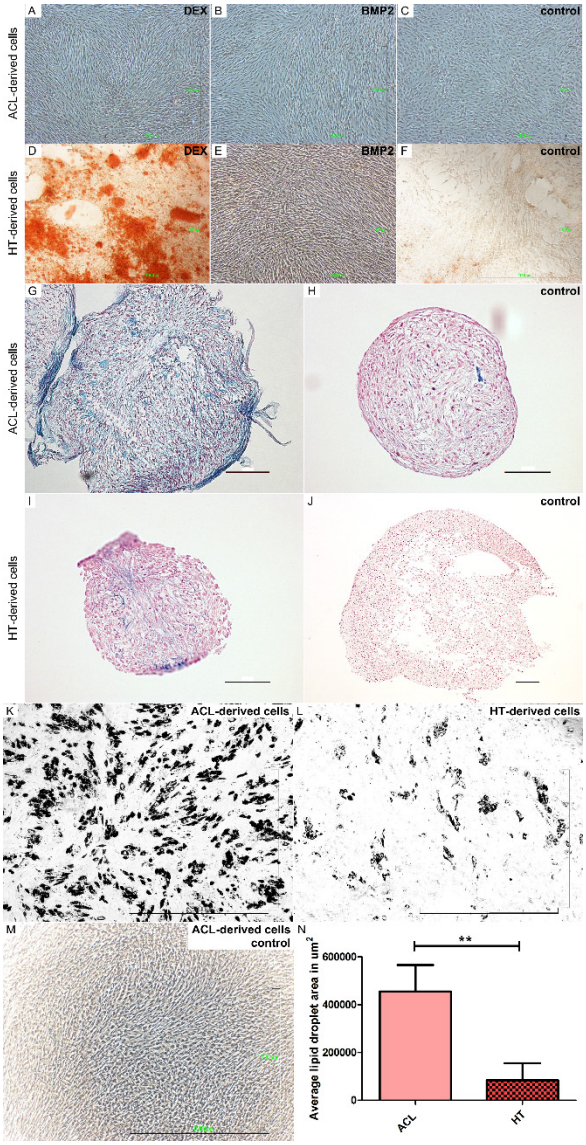


Figure 4. Multilineage potential of ACL- and HT-derived cells. *Alizarin red staining of calcium nodules after osteogenic induction of ACL-derived (A-C) and HT-derived cells (D-F); from left to the right, cells were exposed either to 10^{-8} M Dex, 100 ng/ml BMP2 or 0.01 M BGP alone. Osteogenic differentiation of ACL/HT-derived cells occurred in only one HT donor, as shown in (D); scale bar represents 1,000 µm. Alcian blue staining of GAG in ACL-derived (G, H) and HT-derived cell pellets (I, J) after chondrogenic induction (G, I) and in the control (H, J); scale bar represents 100 µm. Oil Red O staining of fat droplets in ACL-derived (K) and HT-derived cells (L) after adipogenic induction. (M) ACL-derived cells cultured in growth media (control) show no fat droplets formation after staining with Oil Red O; the image is representative also for HT-derived cells. (N) Quantification of fat droplets by measuring their area (μm^2); unpaired Student's t-test of the quantified fat droplets was performed; ** $P < 0.01$. Scale bar represents 1,000 µm.*

Gene expression profile (RT-PCR)

A panel of tendon/ligament-related genes was analysed in order to compare the potential of ACL- and HT-derived cells to differentiate into tendon/ligaments. To do so, we measured the expression of COL1A1, COL3A1, Scleraxis, TNC and COMP (Figure 5A). We found no statistically significant differences between the expression of these markers in the two cell types (for each marker the average over each cell population was calculated and compared) (Figure 5B).

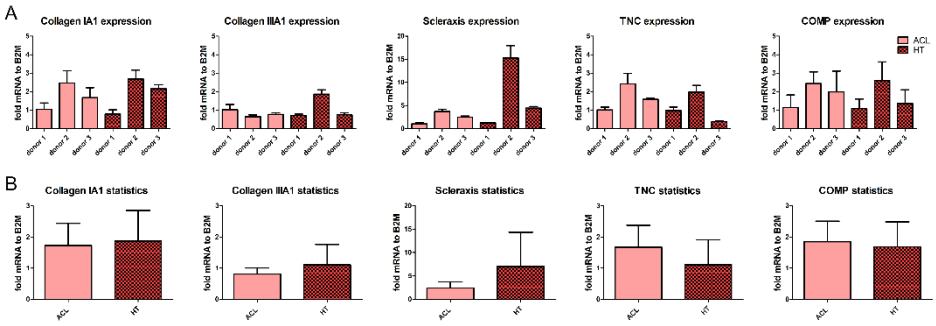


Figure 5. mRNA expression of ligament/tendon-related markers in ACL- and HT-derived cells *(A); ACL donor 1 represents the base line. (B) No statistically significant differences between the ACL- and HT-derived cells were observed; unpaired Student's t-test per gene and on the averaged expression per cell type was performed.*

Expression of surface markers

The analyses of surface markers in ACL- and HT-derived cells showed that, in both cases, a substantial portion of cells expressed CD90 (60 − 70%) marker, whereas a small number expressed CD44 (20%) and CD73 (7 − 9%) and almost no cells expressed CD105 (2 − 3%); see Figure 6. On average, a higher expression of these markers, identified in a large variety of stromal cells belonging to different tissues, was found in HT- than in ACL- derived cells. The remaining markers for hematopoietic stem cells (CD34, CD45, CD11b), pericytes (CD146), B or T cells (CD79a, HLA-DR, CD166, CD200, CD19) and others (CD14, CD29, CD271) were not expressed in either of the two cell types.

DISCUSSION

A key component in the successful regeneration of a reconstructed ACL is the understanding of the differences and similarities between the replaced and the new tissue. Fibroblasts/tenoblasts isolated from ligaments/tendons have been studied separately for musculoskeletal tissue regeneration [13, 26]. Their poor healing capacity increased the interest of using human MSCs isolated from various tissues to improve the healing of these tissues. Although some studies showed that cells isolated from

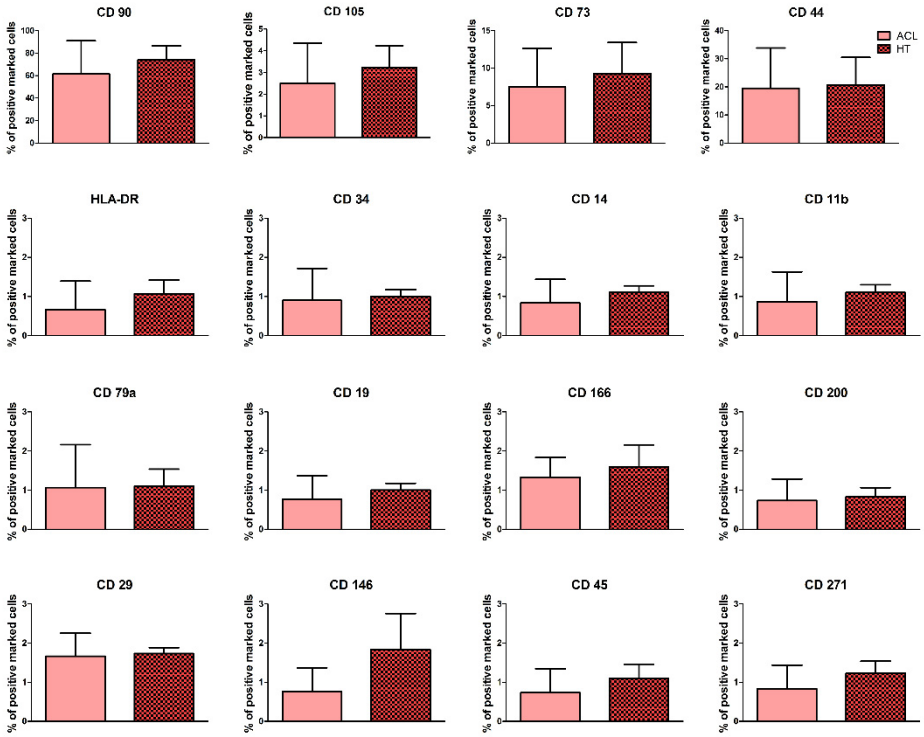


Figure 6. Surface marker expression (in percentage) of ACL- and HT-derived cells, represented as a bar-plot*; each bar stands for percentage of surface marker found on the cells of the different donors corresponding to the individual cell population. Selected sets of cell surface markers expressed positive on multipotent MSCs (A-D), expressed negative on multipotent MSCs (E-J) and other surface markers (L-Q); no statistically significant differences were found between the two cell populations (unpaired Student's t-test was performed).*

ligaments/tendons share similar properties with hMSCs [20, 27], their low number is insufficient for tissue regeneration without prior *in vitro* expansion.

In this study we analysed the phenotype of ACL- and HT-derived cells. Due to the fact that ACL and HT could not be harvested from the same patient, we performed the experiments with tissues isolated from different donors, and hence donor variation should be accounted for when critically analysing the present results. To obtain intact ACL tissue, we performed the collection during TKA, whereas the HT tissue was collected during ACL reconstruction (the ACL was totally torn, and therefore could not be retrieved for analysis). The age of the patients corresponded with the surgical procedure, with TKA performed predominantly in older patients [28] (in this study the average age was 70 years), whereas ACL reconstruction was performed in young and active patients (average age 23 years). Differences in age between the donors may influence the phenotypic outcome for the different cell types, as it has been shown that there is a decline in tissue regeneration potential with age [29]. Our results showed that

ACL- and HT-derived cells have a similar morphology, consistent with previous reports indicating the presence of spindle and fibroblast-like cells [30, 31]. In contrast, the self-renewal potential and the multilineage potential differed between the two cell types. Self-renewal potential, as usually defined for bone marrow MSCs, is absent in both cell types, as no colonies > 2.5 mm were formed [25]. Nevertheless, the size of colonies formed by the HT-derived cells was significantly lower than in the case of ACL-derived cells. These findings differ from other studies, where ACL- and HT-derived cells showed a 20 − 40% potential to form colonies [17, 20], defined by the presence of colonies > 2.5 mm. The differences in performance can be ascribed to differences in tissue collection or *in vitro* culture conditions.

Regarding multilineage potential, our results showed that, although ACL- and HT-derived cells had a low potential to differentiate into the osteogenic lineage, they clearly differed in their adipogenic and chondrogenic potential, with HT-derived cells showing limited potential to differentiate into both lineages. In addition, we showed that the chondrogenic potential of the ACL-derived cells was maintained over time, suggesting that, upon injury, a prochondrogenic remodelling process may occur, leading to functional differences between the remodelled and the original tissue. We believe that ACL-derived cells show chondrogenic and adipogenic potential due to their location in the diarthrodial joint capsule, where other tissues, such as cartilage, synovial membrane, meniscus and intrapatellar fat pad, are bathed in the synovial fluid [32]. Potentially, during development and maturation, the intercellular communication between the ACL-derived cells and the cells residing in the aforementioned tissues might explain the propensity of ACL-derived cells to differentiate towards chondrocytes and/or adipocytes when the right stimuli are applied. Contrariwise, HT-derived cells do not share the same anatomical neighbours as ACL-derived cells, hence they may lack the inherent capacity to respond to chondrogenic or adipogenic stimuli. Despite the differential properties of ACL-derived cells, this does not explain their poor healing capacity. Perhaps, the hostile intra-articular environment toward healing of the injured ACL [33, 34], such as alterations in the post-injury inflammatory response and cell metabolism, intrinsic cell deficiencies, different vascular environments and load-bearing characteristics, might explain this conflict. We believe that these absent cues, among other biological and mechanical factors [35], might also have an important contribution to the delay in formation of a properly functioning enthesis. To better mimic the transition zone upon reconstruction of the native ACL by the HT, we believe that external help is needed. Co-delivery of growth factors such as TGFβ-1 [36] or rhBMP-2 [37]; chemical and biological agents such as matrix metalloproteinases (MMPs) inhibitors [38]; multipotent autologous cells [39] or biomaterials functionalised with extra cues, such as hydroxyapatite [40], have already been shown to have a beneficial effect in accelerating healing between the tendon graft and the bone.

Here, we showed that the cells derived from one HT sample had the potential to differentiate towards the osteogenic lineage and, by doing so, will potentially facilitate the integration of the soft tissue into the bone. As mentioned before, a larger number of donors should be tested and experimental designs, including functional analysis *in vitro*

and clinical outcomes *in vivo*, should be considered in future studies. The similar gene expression profile and the similar expression of a panel of cell surface markers showed that both cell types share common biological properties. The fact that we did not observe differences in the expression of genes involved in matrix production (COL1A1 and COL3A1) [41], cell fate (SCX) [42], cell growth, division and cartilage turnover (COMP) or cell migration and collagen assembly (TNC) [43], and furthermore the similarities in surface markers, also confirm the gene expression data, suggesting that the HT is a good candidate to replace the native ACL. Interestingly, we also observed that, although the cells derived from the two tissues showed similar expression of a panel of surface markers, their differentiation potential is different. Studies have shown that the expression of certain surface markers correlates with the potential to differentiate towards a certain lineage, suggesting that surface expression profiles can be used to preselect cells with a specific lineage potential [44, 45]. Herein, given the similar expression of the surface markers tested, the differentiation potential should have been similar. Notwithstanding, we believe that the differences are likely related to intrinsic differences in the tissues used (ACL vs. hamstring), the respective milieux and the heterogeneous nature of the obtained cell populations. The fact that HT-derived cells show limited multilineage potential suggests that accessory cell types or biological cues might be needed to maintain the transplanted tissue upon reconstruction. A solution for this could be the co-delivery of hMSCs during the ACL reconstruction, or their recruitment from the tunnels drilled during the reconstruction procedure.

Based on these observations, we propose that the intrinsic properties of ACL-derived cells can be, to a certain extent, replaced by HT-derived cells. The search for new molecules capable of modulating and reducing differences between the two cell types can help improve clinical outcome and accelerate rehabilitation.

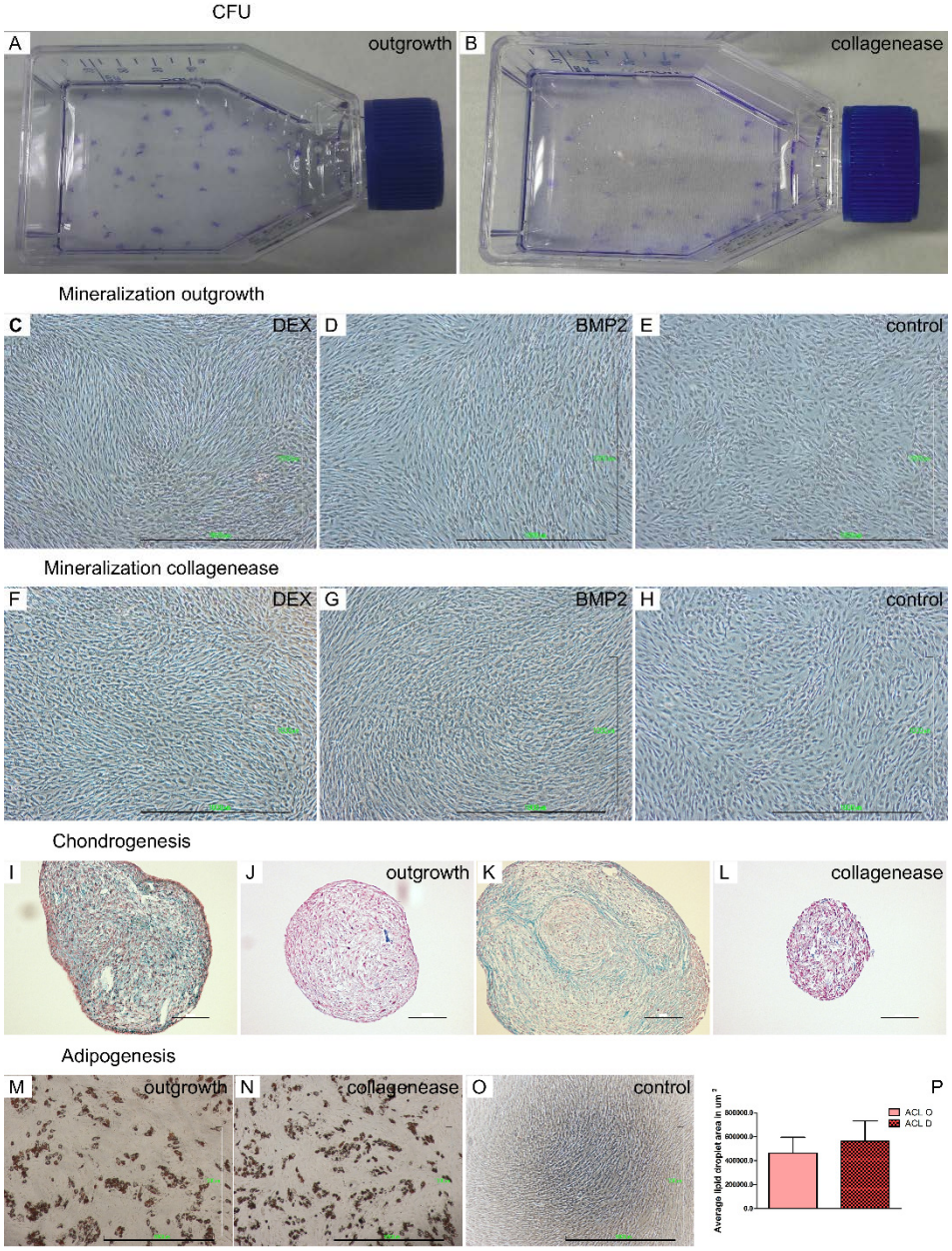
Finally, we would like to emphasise that the findings obtained in this study should be used with caution, due to donor/age variation, the small number of donors tested, differences in cell response between the two-dimensional (2D) and the three-dimensional (3D) environment and absence of mechanical loading present *in situ*. We showed that ACL-derived cells from older donors have chondrogenic and adipogenic properties, while HT-derived cells from young donors did not. With that in mind, we assume that younger donors in the age range comparable to the HT donors would potentially show much better performance than what we have shown in this study with older ACL donors, as it is well known that the regenerative capacities of multiple cell types diminish with age [29]. Regarding donor variation, we believe that the high reproducibility of the properties (with exception of mineralisation potential) in all donors with respect to their cell type accentuates the credibility that what we found is tissue specific rather than donor specific. As regarding donor number, three samples are rather insufficient to perform a robust statistical analysis but are sufficient to offer indicative information. The choice of using the 2D in detriment to the 3D system, and additionally ruling out the biomechanical role, was based on our purpose to understand the basic biological mechanism that underlie the differences in cellular functions

between the two tissue types. This 2D model might not faithfully capture the physiological behaviour of cells *in vivo* but is suitable to address our research question.

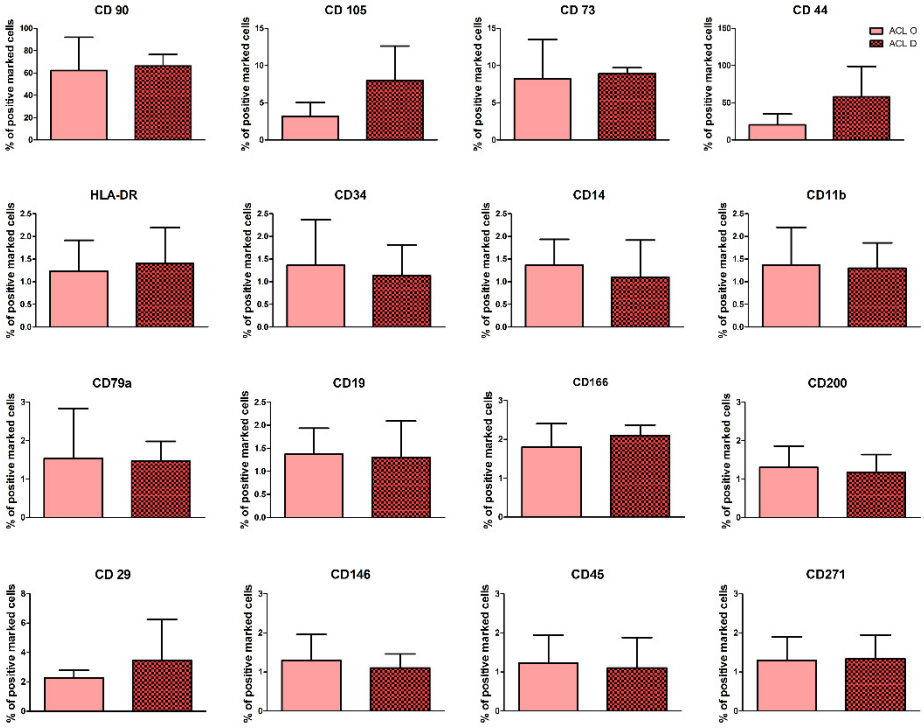
ABBREVIATIONS

Anterior Cruciate Ligament (ACL); hamstring tendon (HT); total knee arthroplasty (TKA); colony-forming unit (CFU); mesenchymal stromal cells (MSCs); phosphate-buffered saline (PBS); growth medium (GM); β-glycerophosphate (BGP); dexamethasone (Dex); bone morphogenetic protein 2 (BMP2); chondrogenesis control medium (CCM); transforming growth-factor-β3 (TGF-β3); glycosaminoglycans (GAGs); bovine serum albumin (BSA); β2-microglobulin (B2M); collagen type I α 1 (COL1A1); collagen type III α 1 (COL3A1); cartilage oligomeric matrix protein (COMP); tenascin C (TNC); alkaline phosphatase (ALP); bone γ-carboxyglutamic acid-containing protein (BGLAP); scleraxis (SCX); standard deviation (SD).

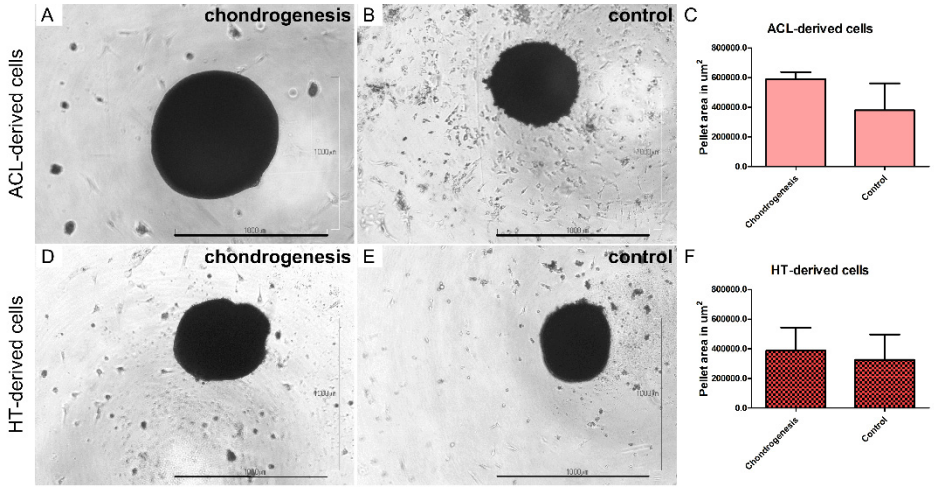
SUPPLEMENTARY FIGURES



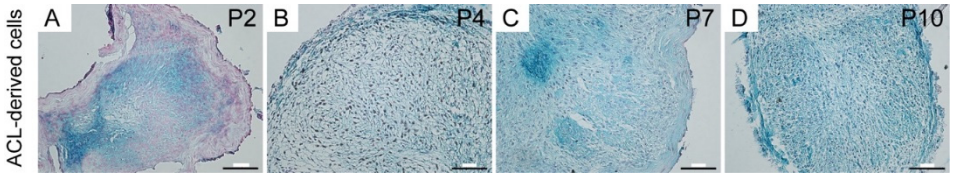
← ***Supplementary Figure 1. Phenotype characterisation of ACL-derived cells isolated by either tissue collagenase digestion or cell outgrowth.*** *Crystal violet staining showing colony forming unit potential of cells isolated by cell outgrowth (A) or tissue collagenase digestion (B). Alizarin red staining of outgrowth isolated cells after osteogenic induction by Dex (C) or BMP2 (D) and control (E); and tissue collagenase digested isolated cells after osteogenic induction by Dex (F) or BMP2 (G) and control (H). Scale bar represents 1,000 µm. Alcian blue staining of the chondrocenic induced cell pellet and control of outgrowth cells (I, J) and cells isolated by tissue collagenase digestion (K, L). Scale bar represents 100 µm. Oil Red O staining of fat droplets in outgrowth cells (M) and cells isolated by tissue collagenase digestions (N) after adipogenic induction. (O) Cells isolated by tissue collagenase digestions in control media, which is representative also for cell outgrowth. (P) Quantification of lipid droplets area. Scale bar represents 1,000 µm.*



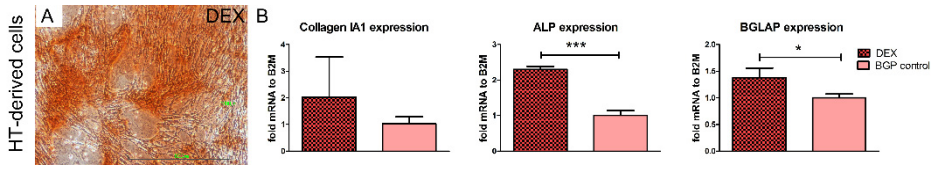
Supplementary Figure 2. Surface marker expression (in percentage) of ACL-derived cells isolated by either cell outgrowth or tissue collagenase digestion, represented as a bar-plot. *No statistically significant differences were found between the two cell populations (Unpaired Student t-test was performed).*



Supplementary Figure 3. Chondrogenic pellets of ACL-derived cells *cultured under chondrogenic induction media (A) or basic media (B) and HT-derived cells cultured under chondrogenic induction media (D) or basic media (E). Quantification of the size of the cell pellet from ACL-derived cells (C) and from HT-derived cells (F).Scale bar represents 1,000 µm.*



Supplementary Figure 4. Alcian blue staining of GAG in ACL-derived cell pellets. *The cells were expanded in vitro and cultured in the presence of chondrogenic induction media at different passages as follow: (A) passage 2, (B) passage 4, (C) passage 7 and (D) passage 10. Scale bar represents 100 µm.*



Supplementary Figure 5. Alizarin red staining of HT-derived cells showing the reproducibility of this donor to stain positive for calcium deposits (A). *Scale bar represents 1,000 µm. Expression of osteogenic related gene markers after exposing the cells for 14 days to osteogenic induction media (DEX) or basic media (BGP).*

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Chapter 3

Means of enhancing bone fracture healing: optimal cell source, isolation methods, and acoustic stimulation

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ABSTRACT

The human body has an extensive capacity to regenerate bone tissue following trauma. Large defects, however, such as long bone fractures of the lower limbs cannot be restored without intervention and often lead to nonunion. Therefore, the aim of the present study is to assess the pool and biological functions of human mesenchymal stromal cells (hMSCs) isolated from different bone marrow locations of the lower limbs, and to identify novel strategies to prime the cells prior to their use in bone fracture healing. Bone marrow from the ilium, proximal femur, distal femur, and proximal tibia was aspirated and the hMSCs isolated. Bone marrow type, volume, number of mononuclear cells / hMSCs and their self-renewal, multilineage potential, extracellular matrix (ECM) production, and surface marker profiling were analysed. Additionally, the cells were primed to accelerate bone fracture healing either by using acoustic stimulation or varying the initial hMSCs isolation conditions. We found that the more proximal the bone marrow aspiration location, the larger the bone marrow volume was, the higher the content of mononuclear cells / hMSCs, and the higher the self-renewal and osteogenic differentiation potential of the isolated hMSCs were. Acoustic stimulation of bone marrow, as well as the isolation of hMSCs in the absence of fetal bovine serum, increased respectively the osteogenic and ECM production potential of the cells. Our results show that bone marrow properties change with the aspiration location, potentially explaining the differences in bone fracture healing between the tibia and the femur. Furthermore, we show two new priming methods capable of enhancing bone fracture healing.

INTRODUCTION

Musculoskeletal disorders that affect the body's muscles, bones, joints, tendons, ligaments, and nerves and are the leading cause of chronic disabilities in adults [1]. Significant research efforts have been undertaken during the past few decades to ease this disability and improve patient mobility and quality of life. Bone fracture repairs have been intensively investigated at both the clinical and fundamental level and yet still 5-10% of fractures resulted in either delayed repair (delayed union) or no repair (nonunion) [2]. At present, there are two primary treatment strategies: (1) surgical intervention that implies the use of bone autograft/allografts, demineralised bone matrix, or synthetic materials; and (2) noninvasive treatments, such as the application of acoustic energy that has been shown to be beneficial in fracture healing [3, 4]. Nevertheless, these strategies rely on the patient's own cells – either stem and/or committed – to induce bone regeneration, posing a challenge in situations where those cells are missing and/or less active. In these cases, cell-based alternatives, such as the use of human mesenchymal stromal cells (hMSCs) were proposed [5].

In this study, we explored the yield, proliferation, multilineage differentiation, and extracellular matrix (ECM) production potential of hMSCs isolated from bone marrow (BM) aspirated from the lower limbs, such as the ilium, proximal femur, distal femur, and proximal tibia. Besides that, we examined the inter- and intra-donor variation between the BM-derived hMSCs from the different locations. Currently, the nonunion rate in bone fracture healing (BFH) differs regarding location, with fractures at tibia diaphysis healing slower (nonunion rate of 18.5% [6]) than fractures in the femoral shaft (nonunion rate of 1.7% [7]). Accordingly, we hypothesise that BM located at the fracture site might play an important role in fracture healing rate, due to differences in cell number, self-renewal-, proliferative-, ECM production- and multilineage-differentiation potential.

Additionally, as cell-based therapies are already used in musculoskeletal pathologies, such as bone fracture, pseudarthrosis, and osteochondral defects [8, 9], we explored the potential of priming BM-derived hMSCs towards a better osteogenic differentiation potential in order to accelerate tissue regeneration upon reimplantation. We explored two distinct priming strategies: (1) the use of acoustic energy applied on BM; and (2) varying the initial culture conditions of the isolated hMSCs.

Ultrasound has been shown to have beneficial effects on BFH, showing an increase in bone formation [10, 11], though not consistently [12-14]. Moreover, the 42% acceleration rate in fracture healing in patients exposed to a 20 minute daily ultrasound treatment is still not excellent [11]. Therefore, we believe that the use of acoustically stimulated BM injected at the fracture site might have a greater impact on BFH than standard ultrasound treatment. Mechanical stimulation has been shown to pre-commit hMSCs towards the osteogenic lineage [15], so we hypothesise that acoustic energy applied directly on BM might induce the commitment of hMSCs towards osteogenesis. It is clinically feasible and simple to apply a short period of acoustic sound cell

manipulation on a BM aspirate during fracture surgery, after which the BM can be administrated to the fracture site either in initial surgery during high risk cases or as an adjunct to revision surgery in case of pseudarthrosis.

Secondly, cell-based therapies often involve the in vitro expansion of cells, where the isolation procedure plays an important role in the selection of desired cell population [16-18]. The isolation of hMSCs from BM is mainly achieved by plastic adherence and it is recognised that both the number of mononuclear cells (MNCs) plated, and the culture media, have a strong influence on the selection of certain hMSCs populations [19]. Accordingly, we hypothesise that low MNC seeding density might select hMSCs prone to higher self-renewal potential, while the use of serum free (SF) media might select an hMSCs subpopulation with enhanced potency. The phenotype of the isolated hMSCs under the aforementioned procedures were compared to a previously described isolation procedure [20].

With this study we aimed to find the optimal ratio between aspirated BM volume and MNC concentration to explain the difference in cell phenotype between the different BM locations of lower limb extremities, and to propose new methods that could accelerate BFH. A schematic overview of the experimental design is presented in Figure 1.

METHODS

Aspiration of bone marrow

Bone marrow aspirates were obtained from patients undergoing total hip arthroplasty (THA) or total knee arthroplasty (TKA). An 8G Jamshidi BM needle fitted with a 50-mL Luer lock syringe containing 1 mL of 1,000 U heparin per 10 mL BM was used to aspirate the BM. Subsequently, the BM was transferred to blood collection tubes (BD-367526) for transport from the operating theatre to the laboratory. The BM was kept at ambient room temperature until being processed the same day.

Bone marrow was aspirated from four different locations: the supra acetabular sulcus (ilium) in 12 donors; the medullary cavity or lateral diaphysis of the femur (proximal femur) in seven donors; and the epiphysis or medullary cavity from the distal femur or proximal tibia in seven more donors.

The collection and anonymous use of the BM aspirate was approved and performed in compliance with the relevant laws and institutional guidelines of the Medisch Spectrum Twente, Twente Ethische Toetsings Commissie (Ref. no.: K13-46). Patients provided written informed consent after being provided with a verbal explanation and an opportunity for questioning.

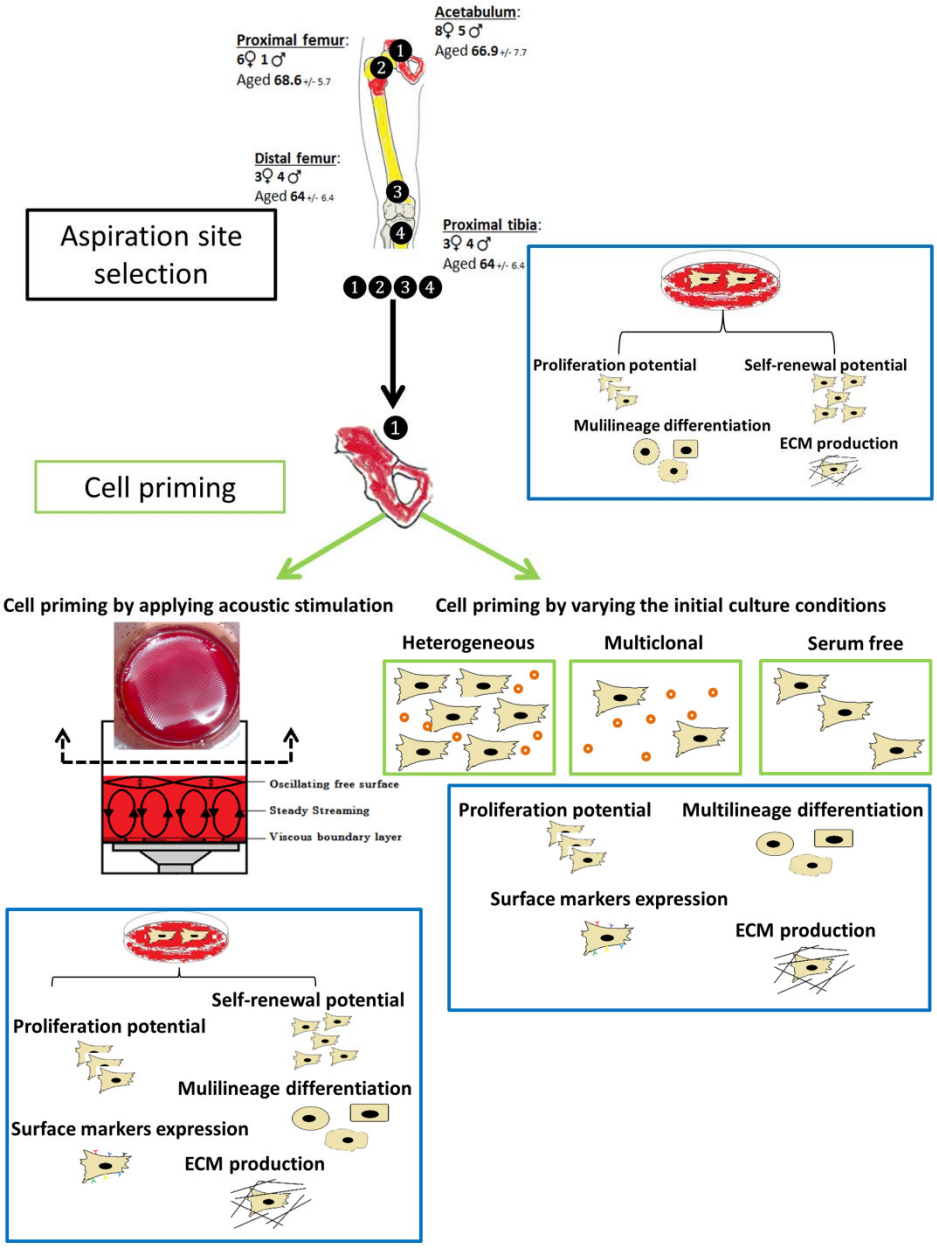

Figure 1. Schematic representation of the experimental design. *Aspiration of BM from different locations of lower limb extremities and selection of the optimal cell source, based on hMSC number and phenotype. In vitro priming of hMSC by use of acoustic stimulation or varying the initial culture conditions with the final aim to enhance in vivo BFH.*

Isolation and culture of hMSCs

Bone marrow aspirate was passed through a 70 µm pore-size cell strainer, to remove the presence of tissue pieces, after which MNC concentration from the ilium and proximal femur was analysed using the Beckman coulter ACT diff 2. The number of MNCs for samples collected from the distal femur and proximal tibia was not analysed due to technical limitations.

Based on the isolation method used, different concentrations of MNCs/cm^2 were plated. We defined three isolation/culture conditions: heterogeneous (classical MNC seeding density, previously described and standardised hMSCs isolation protocol within our laboratory [21]); multiclonal (low MNC seeding density, permissive for single cell clonal expansion); and SF (high MNC seeding density in the absence of fetal bovine serum proteins during the initial phase).

For the heterogeneous isolation condition, BM aspirate was plated at a density of 5x10^5 MNCs/cm^2 and cultured in growth media (GM) consisting of α-minimal essential media (*α*MEM, Life Technologies – Cat. No.: 22571-020); 10% Fetal Bovine Serum (FBS, Gibco – Cat. No.: 10270106); 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (Sigma – Cat. No.: A8960); 2 mM L-glutamine (Gibco – Cat. No.: BE17-605E); 100 units/mL penicillin; and 100 mg/mL streptomycin (Gibco – Cat. No.: 15140-122).

For the multiclonal isolation condition, BM aspirate was plated at a clonal density of 5x10^4 MNCs/cm^2 and cultured in GM.

For the SF condition, BM aspirate was plated at a cell density of 1.5x10^6 MNC/cm^2 in α-MEM containing no additives for the first three days.

On the fourth day, the non-adherent cell fraction was removed and the media were changed to GM for all three conditions. Hereafter, the media were refreshed twice a week. At semi-confluence, the cells were trypsinised and used for sub-culturing or stored in liquid nitrogen for future use.

In total, BM was aspirated from 19 donors and subsequently divided between the different experiments. BM from 14 donors was used to evaluate the most convenient aspiration site location. BM was plated under heterogeneous conditions, with the exception of the distal femur and proximal tibia where 2ml BM was plated each time, as the initial amounts of MNCs were unknown. BM from 11 donors was used to evaluate the effects of acoustic energy. The BM was plated under heterogeneous conditions. And BM from six donors was used to evaluate the effects of varying the initial isolation conditions of the hMSCs.

Donor number, BM aspiration location, BM volume, and the concentration of MNCs/ml can be found in Table 1. BM was cultured at 37°C and 5% CO_2.

Donor number	*BM aspiration location*	*BM colour*	*BM aspirated volume (ml)*	*MNC/ml (*E+06)*
Donor 1	Ilium	Red	20	31.6
Donor 2	Ilium	Red	35	12.3
Donor 3	Ilium	Red	15	17.9
Donor 4	Ilium	Red	25	20.2
Donor 5	Ilium	Red	30	13.5
Donor 6	Ilium	Red	28	6.8
	Proximal Femur	Red	11.5	16.3
Donor 7	Ilium	Red	20	16.1
	Proximal Femur	Red	1	15.1
Donor 8	Ilium	Red	13	34.7
	Proximal Femur	Red	6	16.2
Donor 9	Ilium	Red	20	7.3
	Proximal Femur	Red	6.5	7.9
Donor 10	Ilium	Red	22.5	14.5
	Proximal Femur	Red	9	22.4
Donor 11	Ilium	Red	22.5	7.4
	Proximal Femur	Red	5	6.4
Donor 12	Ilium	Red	7.5	26.6
	Proximal Femur	Red	5	37
Donor 13	Distal Femur	Yellow	3.5	-
	Proximal Tibia	Yellow	2.5	-
Donor 14	Distal Femur	Yellow	5	-
	Proximal Tibia	Yellow	7.5	-
Donor 15	Distal Femur	Yellow	9	-
	Proximal Tibia	Yellow	3	-
Donor 16	Distal Femur	Yellow	2	-
	Proximal Tibia	Yellow	1.5	-
Donor 17	Distal Femur	Yellow	6	-
	Proximal Tibia	Yellow	7	-
Donor 18	Distal Femur	Yellow	8	-
	Proximal Tibia	Yellow	4.5	-
Donor 19	Distal Femur	Yellow	2	-
	Proximal Tibia	Yellow	2	-

Table 1. BM information. *From left to right: donor number, BM aspiration location, type, aspiration location and concentration of MNCs/ml.*

hMSCs population doubling

To assess hMSC proliferation, cells from passage 1 (P1) were seeded in GM at 5,000 cells/cm^2 in T175 tissue culture flasks. At semi-confluence, the cells were trypsinised and counted. Population doubling (PD) was calculated per the formula PD = $\log_2(N_E/N_i)$, where N_E and N_I are the number of hMSCs obtained at passage 2 (P2) and P1, respectively.

Colony forming unit and colony forming unit-osteoblast potential (mineralisation)

The colony forming unit (CFU) assay was used as an indicator of self-renewal potential of the hMSCs, and the CFU-osteoblast (CFU-Ob) assay was used as an indicator of osteoprogenitor presence in the formed CFUs. Two million MNCs were seeded in duplicate in T25 culture flasks and grown in GM for the first seven days, followed by transition to mineralisation media for a further seven days. The mineralisation media consisted of GM containing 0.01 M β-glycerophosphate (BGP, Sigma – Cat. No.: G9422) and 10^{-8} M Dexamethasone (Dex, Sigma – Cat. No.: D8893). At day 14, the cultures were fixed with 10% formalin for 15 min at ambient temperature, after which alkaline phosphatase (ALP) positive colonies were stained using the Leukocyte Alkaline Phosphatase Kit -ALP (Sigma – Cat. No.: 85L2) following the manufacturer´s instructions. Subsequently, the total number of colonies formed were stained using 0.5% Coomassie Briljant Blue staining (Fluka – Cat. No.: 27815) solution added to the monolayer for 10 minutes. Images of the stained colonies were acquired using an Epson Perfection V750 PRO scanner. The total number of CFUs and ALP positive colonies was quantified using ImageJ 1.45s software and the percentage of ALP positive CFUs calculated.

Extracellular matrix production

hMSCs at P2 or P3 were seeded in quadruplicate of 100,000 cells/well in a 384-well plate in GM (without serum) consisting of 50 µg/mL insulin transferrin selenium-premix (Sigma – Cat. No.: I3146) and 40 µg/mL proline (Sigma – Cat. No.: P5607) and incubated for 24h to allow cell adhesion. The following day, the medium was refreshed and 10 ng/mL transforming growth factor beta 3 (R&D Systems – Cat. No.: 243-B3) and 10^{-7} M Dex were added to the wells. After seven days, the formed nodules were fixed in 10% formalin for 15 min at ambient temperature and images were captured using a Nikon bright field microscope. The nodule area and the number of nodules formed were quantified using ImageJ 1.45s software. The early cell condensation phenotype and the increase in nodule size were associated with ECM production.

Alizarin red staining (mineralisation)

hMSCs at P2 or P3 were seeded in triplicate of 50,000 cells/well in T25 and grown in control media consisting of GM containing 0.01 M BGP and in a mineralisation medium consisting of GM containing BGP and 10^{-8} M Dex. The media were refreshed twice a week. After 28 days, the cells were fixed in 10% formalin for 15 minutes at ambient temperature and stained with 2% Alizarin red solution (Sigma – Cat. No.: A5533) for five minutes. Images were captured using a Nikon bright field microscope.

Oil Red O staining (Adipogenesis)

hMSCs at P2 or P3 were seeded in triplicate of 25,000 cells/well in 24-well plates and grown in a control medium consisting of GM or adipogenic medium consisting of GM containing 0.2 mM indomethacin (Cat. No.: I7378); 0.5 mM isobutylmethylxanthine (Cat. No.: I5879); 10^{-6} M Dex; and 10 µg/mL human insulin (Cat. No.: I9278), all from Sigma. The media were refreshed twice a week. After three weeks, the cells were fixed with 10% formalin for 15 minutes at ambient temperature, after which the cell monolayer was incubated for five minutes in 60% isopropanol, and subsequently stained with Oil Red O solution (3mg/mL in 60% isopropanol, Sigma – Cat. No.: O0625). After five minutes, the samples were rinsed with demineralised water and images were captured using a Nikon bright field microscope. After the imaging, Oil Red O staining was extracted from the cells in 4% Nonidet P40 (Fluka, Cat. No.: 74385) in isopropanol and absorbance was measured at 540 nm (Lambda 40; Perkin Elmer). One hundred percent Oil Red O was included in the calibration curve measurements, from which the percentage of Oil Red O staining was calculated.

Flow cytometry

hMSCs at P3 or P4 were expanded in T175 until they reached confluence. The cells were trypsinised and incubated for 30 minutes in blocking buffer consisting of 17% bovine serum albumin (Sigma – Cat. No.: F7524) in phosphate buffered saline (PBS). This was followed by incubation with FITC- or PE-conjugated mouse anti-human antibodies for 30 minutes at 4°C in the dark. The samples were then washed three times with a washing buffer consisting of 3% bovine serum albumin in PBS. The expression levels were analysed using a FACSAria flow cytometer (BD Bioscience). For phenotypic characterisation, the following antibodies were used: CD90, CD73, CD146, CD105, CD271, CD34, CD14, CD79a, HLA-DR, CD45, and IgG1 and Ig G2a as isotype controls (all from BD pharming).

Acoustic stimulation of bone marrow

Acoustic stimulation of BM was achieved using the bone marrow aspirate concentration device, previously described by Ridgway et al. [22]. BM was placed into the processing chamber of the device and acoustic vibration was applied using a voice-coil that produced a geometric standing waveform pattern on the BM fluid surface. Different frequencies were tested by manual adjustment using an Oscilloscope (Agilent Technologies, InfiniVision, MSO-X-3014A Mixed Signal Oscilloscope) and two frequencies, 300 Hz (48 mW/cm^2) and 500 Hz (73 mW/cm^2), were selected for further experimental research. The BM was processed one time for five and 10 minutes for both selected frequencies. The baseline was defined as unstimulated BM.

Part of the BM was plated to assess the self-renewal and proliferation potential as previously described, while the rest of the BM was plated under the heterogeneous hMSCs isolation condition in order to assess the multilineage differentiation potential,

ECM production, and surface markers expression of the hMSCs at a later passage, as previously mentioned.

Bone marrow viscosity

Bone marrow viscosity from six donors (three donors for ilium and proximal femur and three donors for distal femur and proximal tibia) was measured using the Rheometer Physica MCR-301. A total of 30 different points, with an increasing shear rate from 0 to 250 1/s and periodic pause of 10 seconds between each point, were measured. The volume of BM used for the measurements was 350 µl per measuring cycle. All samples were measured in duplicates at ambient room temperature.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software. An unpaired Student's *t*-test and Mann-Whitney post-test were performed to compare the data when two groups were analysed. One-way or two-way analysis of variance (ANOVA) and a Tukey or Bonferroni post-test were used to compare the data when more than two groups were analysed. The uniform distribution of data, to test inter-donor variation, was assessed using a Chi-squared test. A $P \leq 0.05$ indicates a statistical significant difference. The results are shown as mean ± standard deviation.

RESULTS

Inter-donor variability in bone marrow aspirate

The volume of BM aspirated from the different locations varied significantly, with larger BM volumes obtained from the ilium (22 ± 7.6 ml) than the proximal femur (6 ± 3.3 ml), distal femur (5 ± 2.9 ml), or proximal tibia (4 ± 2.6 ml). BM volumes from the ilium yielded a higher concentration of MNCs for volumes close to 10 ml (2.6×10^7 MNC/ml), while volumes close to and larger than 20 ml yielded a lower concentration of MNCs (1.4×10^7 MNC/ml). This, however, was not statistically significant ($p = 0.15$). Similarly, BM aspirated from the proximal femur showed higher MNC yield for volumes lower than 5 mL, 2×10^7 MNC/mL versus 1.6×10^7 MNC/mL. Again, however, this was not statistically significant ($p = 0.7$) (Figure 2A and Table 1).

The concentration of hMSCs obtained at the end of the expansion phase (14 days after MNC seeding) was, on average, 566,494 hMSC/ml for the ilium; 245,549 hMSC/ml for the proximal femur; 76,250 hMSC/ml for the distal femur; and 122,321 hMSC/ml for the proximal tibia samples (Figure 2B). No statistically significant differences were found between the groups; however, lower p values were obtained from hMSCs isolated from proximal versus distal locations (Supplementary Table 1).

Macroscopically, the BM aspirated from the ilium and proximal femur was red, while BM aspirated from distal femur and proximal tibia was yellow, consistent with a higher

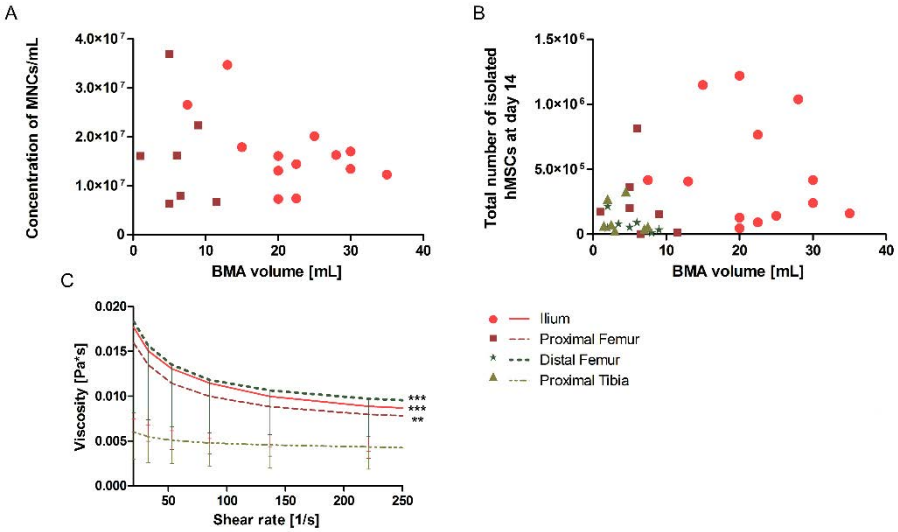

Figure 2. Characterisation of BM aspirated (BMA) from different locations. *(A) Correlation between aspirated BM volume and MNC concentration, for the ilium (circle) and proximal femur (square). (B) Correlation between the plated BM volumes and the number of isolated hMSCs, heterogeneous isolation condition only. (C) BM viscosity curves from different aspiration locations, represented as correlation between the shear rate and the viscosity. The values represented the mean ± standard deviation of three BM donors (n = 3). Statistically significant differences were found with ***p < 0.001 and **p < 0.01.*

presence of lipid droplets in the latter (Supplementary Figure 1). Compared with the other aspiration locations, we observed a significant decrease in BM viscosity for BM aspirated from the proximal tibia (Figure 2C). The morphological appearance of expanded hMSCs did not show any visible differences between the different BM aspiration locations (data not shown).

Effect of aspiration location on biological characteristics of hMSCs

Proliferation, self-renewal ECM production, and multilineage potential (osteo and adipogenic) were assessed for hMSCs isolated from the different locations (Figure 3). Proliferation capacity and ECM production of hMSC showed a uniform distribution across all donors, for each BM aspiration location independently (Figure 3A, E). An average across all donors showed a statistically significant increase in proliferation of hMSCs isolated from the distal femur (0.64 ±0.07) and proximal tibia (0.71 ±0.08) when compared with the ilium (0.47 ±0.09) and proximal femur (0.48 ±0.13) (Figure 3B). No statistical significant differences were seen in ECM production, however (Figure 3F).

In contrast, the CFU capacity of hMSCs showed a non-uniform distribution across all donors, independent of the BM location (Figure 3C). The average across all donors showed a trend towards a higher CFU capacity of hMSCs isolated from the proximal

femur 54 ± 42 CFU compared with the ilium 31 ± 22 CFU, distal femur 14 ± 24 CFU, and proximal tibia 19 ±5 CFU (Figure 3D). The obtained p values can be visualised in Supplementary Table 2.

Similarly, the mineralisation capacity showed a similar trend with a higher CFU-Ob potential in hMSCs isolated from the ilium 12 ± 11 CFU-Ob (36% ± 18 CFU-Ob/CFU) and proximal femur 11 ± 10 CFU-Ob (26% ± 16 CFU-Ob/CFU) compared with the distal femur 1 ± 1 CFU-Ob (13% ± 20 CFU-Ob/CFU) and proximal tibia 5 ±11 CFU-Ob (17% ±22 CFU-Ob/CFU) (Figure 3H). The obtained p values can be visualised in Supplementary Table 2. The high standard deviation is attributed to the non-uniform distribution across the donors (Figure 3G).

The adipogenic potential of hMSCs showed a uniform distribution across all donors for BM aspirated from the ilium and proximal femur, but not from the distal femur and proximal tibia (Figure 3I). The average across all donors showed a significant increase in stained fat droplets in the proximal tibia 21% ±6.85 when compared with the ilium 9% ±2.5. No statistically significant differences were observed between the other groups (Figure 3J).

Effect of acoustic stimulation on hMSCs

Self-renewal, proliferation, ECM production, and multilineage potential (osteo and adipogenic) were assessed from the acoustic stimulated hMSCs.

The different BM volumes harvested from the different donors – 11.5 ml (donor 3), 10 ml (donor 5), 8 ml (donor 4), 6 ml (donor 1), and 5 ml (donor 2) (Supplementary Figure 2A) – were stimulated at a frequency of 300 Hz for five and 10 minutes (Supplementary Figure 2B-F). Upon acoustic stimulation, a significant increase in CFU, mineralisation, and adipogenesis was observed for hMSC isolated from small BM volumes (5 and 6 mL) rather than larger volumes (8, 10, or 11.5 ml). No statistically significant differences were observed in proliferation or ECM production between the conditions. Based on the abovementioned results, subsequent experiments were performed using small BM volumes (4 ml). An illustration of the device, while 4 ml of BM is acoustically stimulated at 300Hz, is presented in Figure 4.

Acoustic stimulation of BM at 300 and 500 Hz for five and 10 minutes showed no statistically significant difference in hMSC proliferation between the conditions (Figure 5A, B). In contrast, an increase (not significant) in CFU, ECM production, and mineralisation but not in adipogenic potential was observed upon acoustic stimulation (Figure 5C – J).

Surface marker expression on hMSCs isolated from acoustically stimulated BM (300Hz for five minutes) showed a decrease, though not statistically significant, in expression of positive surface markers such as CD105 (22 ± 3 % versus 32 ± 17 %); CD90 (21 ± 5 % versus 23 ± 7 %); CD146 (3 ± 1 % versus 4 ± 1 %); and CD73 (20 ± 8 % versus 23 ± 17 %) when compared with the baseline (Supplementary Figure 3).

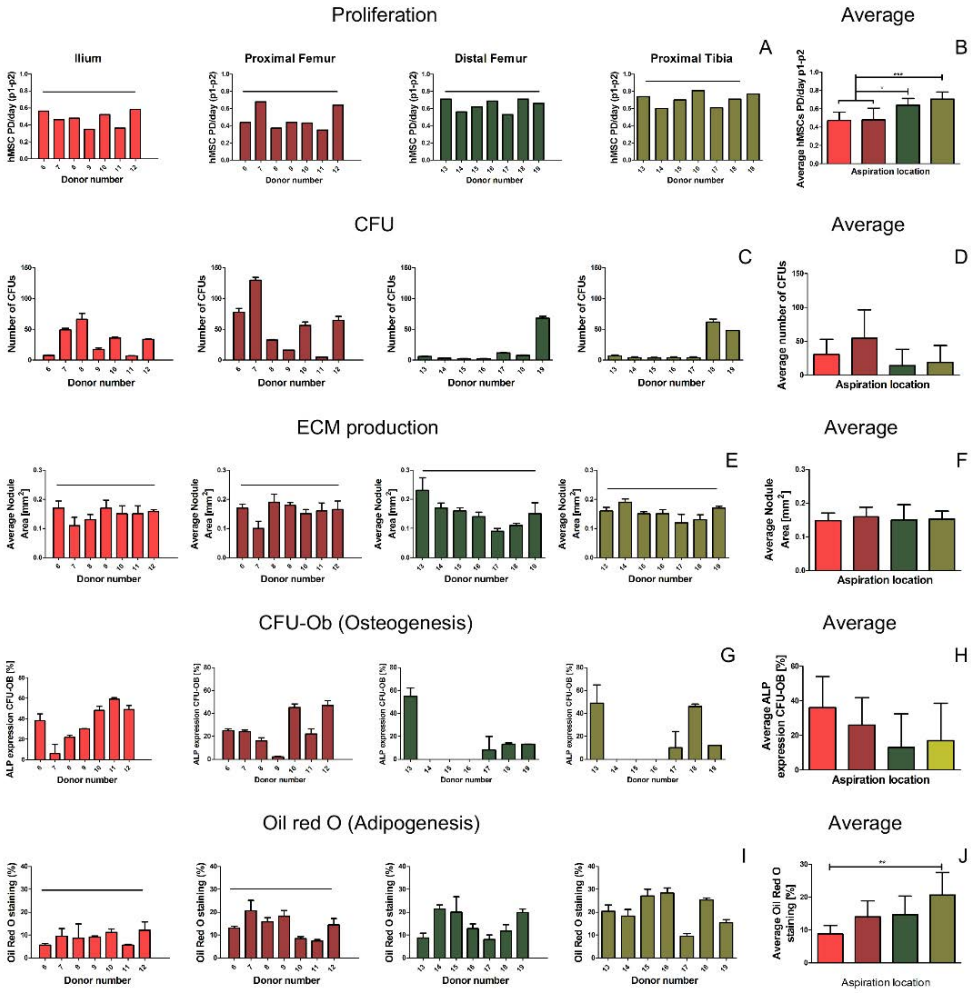

Figure 3. Biological characterisation of hMSCs isolated from different BM locations. *(A) Proliferation of hMSCs calculated as PD/day from P1 to P2, donor and location dependent. (B) Proliferation average across the donors. (C) CFU potential of hMSCs, donor and location dependent. (D) CFU average across the donors. (E) ECM production, quantification of nodule size area in mm² after cell condensation, donor and location dependent. (F) ECM production average. (G) Osteogenic potential calculated as percentage of ALP positive colonies within the CFUs, donor and location dependent. (H) Osteogenesis average. (I) Adipogenic potential, quantification of Oil Red O staining relative to 100% Oil Red O staining solution, donor and location dependent. (J) Adipogenesis average. The uniform distribution of data, to test inter-donor variation, was assessed using Chi-squared test and presented as a line above all donors. Values are represented as the mean ± standard deviation of at least three independent experiments ($n \geq 3$). Statistically significant differences were found with $***p < 0.001$, $**p < 0.01$ and $*p < 0.05$.*

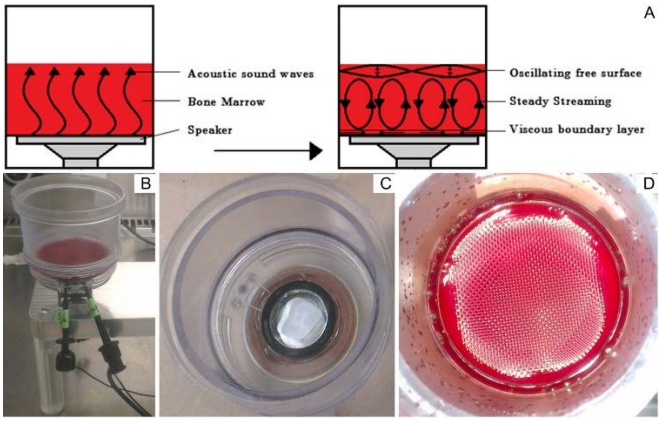

Figure 4. Acoustic stimulating device. *(A) Sketch of the fluid flow within the processing chamber and the formation of a standing wave. (B) Processing chamber. (C) Speaker, in white, located on the bottom of the processing chamber. (D) Standing wave pattern formed in BM at 300Hz.*

Effect of varying the initial culture condition on hMSCs

The isolation of hMSCs from the BM was assessed by varying the initial culture conditions and their proliferation, ECM production, multilineage differentiation potential, and cell surface marker expression. No difference in proliferation (Figure 6A, B, and Supplementary Table 3) and osteogenesis (Supplementary Figure 4) was observed between the different isolation conditions. In contrast, the isolation of hMSCs under SF conditions showed a trend in increased ECM production, with four out of six donors showing a statistically significant increase (Figure 6C). When averaged across all donors, p values of 0.11 and 0.18 were obtained when compared with heterogeneous and multiclonal conditions (Figure 6D and Supplementary Table 3). Additionally, SF conditions showed a trend in decreased adipogenesis, with five out of six donors showing a statistically significant decrease (Figure 6E). When averaged across all donors, p values of 0.48 and 0.13 were obtained when compared with heterogeneous and multiclonal conditions (Figure 6F and Supplementary Table 3); while isolation of hMSCs under multiclonal condition showed a trend in increased adipogenesis (Figure 6E). A statistically significant increase in adipogenesis was observed in five out of six donors in multiclonal when compared with SF isolated hMSCs (Figure 6E and F, Supplementary Table 3).

The expression of CD271, CD34, CD14, CD79a, CD45, and HLA-DR was absent in all conditions, regardless of the isolation procedure; while no significant differences were observed in the expression of CD90 (46 ±31 % heterogeneous versus 36 ± 26 % multiclonal and 43 ± 36 % serum free condition); CD105 (11 ±6 % heterogeneous versus 23 ± 14 % multiclonal and 34 ± 31 % serum free condition); CD73 (27 ±7 % heterogeneous versus 33 ± 18 % multiclonal and 43 ± 33 % serum free condition); and CD146 (5 ± 4 % heterogeneous versus 5 ± 4 % multiclonal and 11 ± 16 % serum free

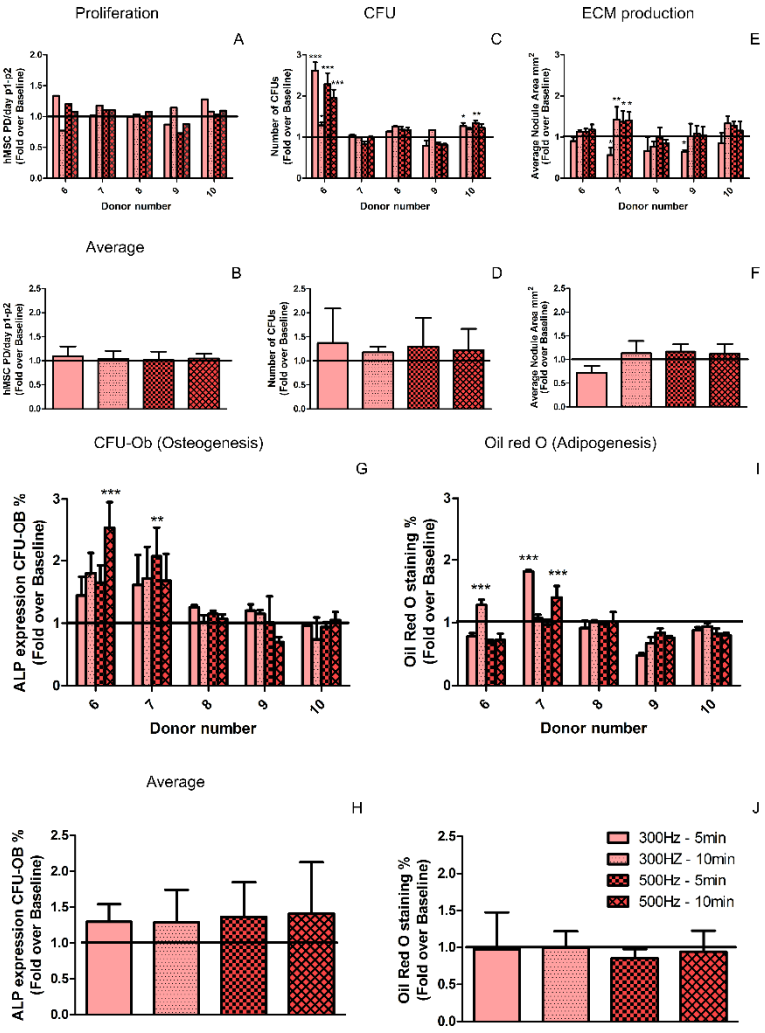

Figure 5. Biological characterisation of isolated hMSCs from acoustically stimulated BM at 300 and 500Hz for five and 10 minutes. *The results are presented as the fold change over the non-stimulated BM (baseline). (A) Proliferation of hMSCs calculated as PD/day from P1 to P2, donor and stimulation dependent. (B) Proliferation average. (C) CFU potential of hMSCs, donor and stimulation dependent. (D) CFU average. (E) ECM production, quantification of nodule size area in mm², donor and stimulation dependent. (F) ECM production average. (G) Osteogenic potential calculated as percentage of ALP positive colonies within the CFUs, donor and stimulation dependent. (H) Osteogenesis average. (I) Adipogenic potential, quantification of Oil Red O staining relative to 100% Oil Red O staining solution, donor and stimulation dependent. (J) Adipogenesis average. Values are represented as the mean ± standard deviation of at least three independent experiments ($n \geq 3$). Statistically significant differences were found with ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.*

condition) between the isolation conditions. A trend in higher expression of CD105 ($p = 0.28$), CD73 ($p = 0.57$), and CD146 ($p = 0.66$) was observed, however, in the hMSCs isolated in SF media when compared with the heterogeneous condition (Supplementary Figure 5). The high standard deviation is the result of inter-donor variation.

DISCUSSION

The human body has an extensive capacity to regenerate bone tissue following trauma. Large defects cannot be restored without intervention, however, and often lead to nonunion. Long bone fracture repair has been extensively studied at both clinical as well as fundamental research level; however, little is known about the differences in fracture repair between the femur and the tibia [6, 7]. Therefore, the aim of the present study was to assess the pool and biological functions of BM-derived hMSCs in the lower limbs, such as the ilium, proximal femur, distal femur, and proximal tibia. Additionally, we broadened our research interest towards methods to prime BM-derived hMSCs for later reimplantation at the fracture site. This should facilitate their homing and commitment towards quicker bone regeneration; it has already been shown that a reduced pool of proliferative and multipotent hMSCs are present at low healing fractures [23].

We showed that the pool of BM-derived hMSCs differ with respect to BM aspiration location. We found that after 14 days, the number of hMSCs isolated from the ilium and proximal femur was higher (Figure 2B), and they showed higher self-renewal and osteogenic differentiation potential (Figure 3D, F) in comparison with hMSCs isolated from the distal femur and proximal tibia, with the latter showing higher adipogenic potential. These findings correspond to the macroscopic appearance of the BM, as described by Malkiewicz et al. [24], with red BM found in the ilium and proximal femur, suggesting an active participation to hematopoiesis; and yellow BM found in the distal femur and proximal tibia, containing a considerable admixture of fat. During ageing, red marrow is replaced by yellow marrow, and this change in the marrow compartment might differ in fracture repair rates [24]. In this context, we strongly believe that the differences in BFH rate between the femur and tibia are the result of insufficient amounts of hMSCs present at the fracture site, as well as their poor self-renewal and osteogenic ability. Additionally, previous studies demonstrated the use of BM aspirate and its efficacy in the treatment of fracture nonunion or high nonunion rate repair [25, 26]. Therefore, we propose that the isolation of BM from the ilium, and its delivery in tibial fractures to enhance bone healing, could improve the current clinical treatment strategy.

In the process of quantifying the concentration of MNCs regarding the aspirated BM volume, we found that 10 ml of BM yields the highest MNC concentration. Higher BM volumes yielded low concentrations of MNCs, due to dilution with peripheral blood during aspiration; while lower BM volumes also yielded lower concentration of MNCs, as described by Fennema EM et al. [27]. Interestingly, in both studies, the same average

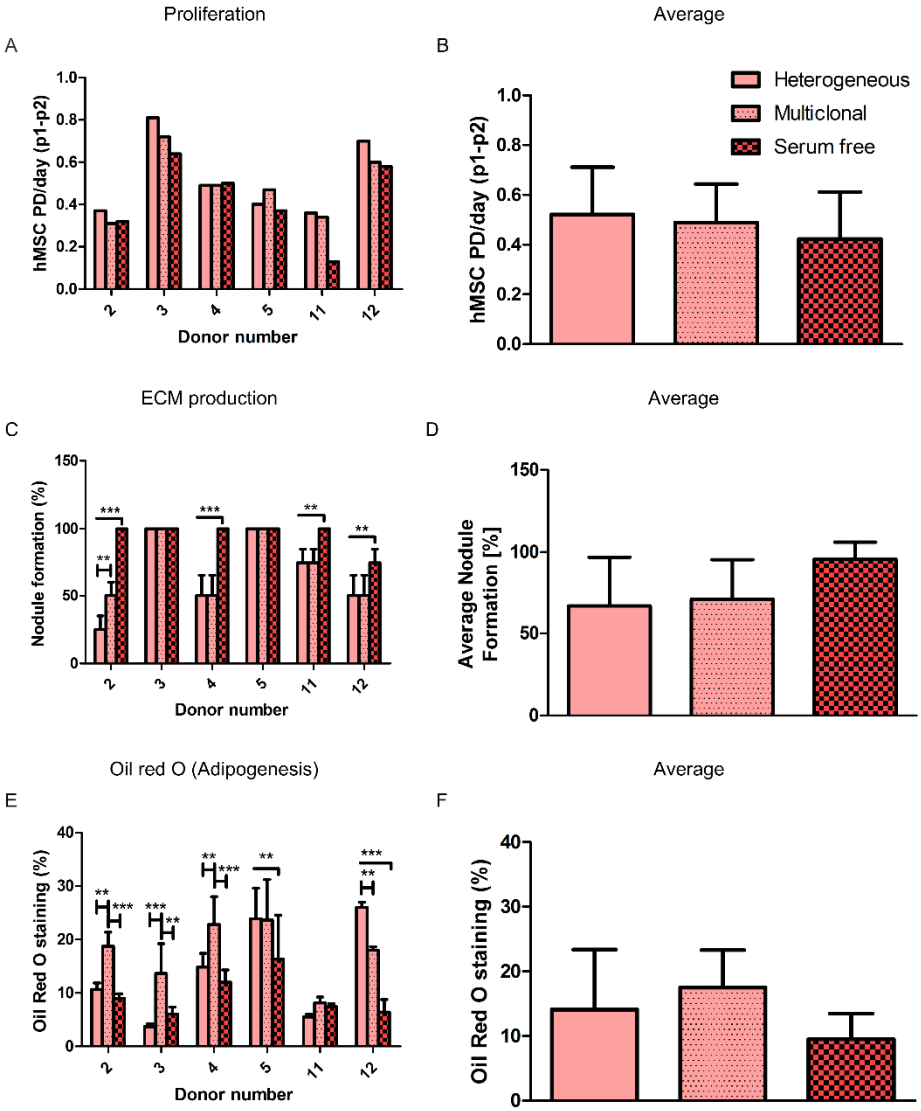

Figure 6. Biological characterisation of hMSCs isolated from BM under different isolation procedures *(A) Proliferation of hMSCs calculated as PD/day from P1 to P2, donor and isolation procedure dependent. (B) Proliferation average. (C) ECM production, percentage of formed nodules, donor and isolation procedure dependent. (D) ECM production average. (E) Adipogenic potential, quantification of Oil Red O staining relative to 100% Oil Red O staining solution, donor and isolation procedure dependent. (F) Adipogenesis average. Values are represented as the mean ± standard deviation of at least three independent experiments (n ≥ 3). Statistically significant differences were found with ***p < 0.001 and **p < 0.01.*

concentration of MNCs ($2.6*10^7$ MNCs/ml) was found at 10 ml aspirated BM, henceforth encouraging the surgeons to limit the aspirated BM volume from the ilium to 10 ml.

To increase the contribution of cells to bone repair, a new dynamic vision emerged in tissue regeneration, focusing on rhythms and oscillatory patterns capable of orchestrating cell fate decision. The use of physical energy, such as ultrasound vibration, has shown to affect cell fate and increase the rate of bone repair [7, 28, 29]; however, at insufficient cell numbers the therapy has been rather inefficient (tibia) [6]. Therefore, we propose a different approach: the delivery of acoustically stimulated BM from the ilium (rich in hMSCs) at the fracture site. Based on a previous study by Ridgway, J. et al., where acoustic vibration was used to separate cells from BM suspension, by trapping the cells in the pressure node planes of the standing wave and reducing the volume, an increase in CFU-Ob potential was observed in the processed BM [22]. We believe that this increase was not only resulted in BM volume reduction, but also a change in cell fate. To test this, we selected two different frequencies in the range of acoustic vibration, 300 and 500 Hz, and two time points: five and 10 minutes. The results showed a trend towards an increased self-renewal, ECM production, and a shift towards osteogenic but not adipogenic differentiation in acoustically stimulated BM, suggesting that hMSCs may sense the acoustic vibratory frequencies. The long expansion period necessary to obtain sufficient cell numbers to perform the assays eventually led to a decrease in the multilineage potential, however, because cell potential is known to diminish with increased in vitro culture time [30, 31]. In addition, we speculate that the decrease in positive hMSCs surface markers in acoustically stimulated BM is the result of integrin reorganisation (cellular mechanoreceptor on the cell surface), followed by surface marker reorganisation [32] and a change in cell fate. To our knowledge, this is the first study where acoustic energy was applied directly to BM and not on cultured cells, paving the way to its implementation into a one-step surgical procedure for bone repair. The harvested BM can be first exposed to acoustic stimulation during surgery in the sterile field. After this short period of cell manipulation, the suspension of BM components can be simply administrated to the location of interest, either in the initial treatment of high risk cases or in revision surgery for pseudarthrosis.

While acoustic sound vibration focuses on changing the phenotype of the cells, variation of the initial hMSCs isolation conditions focuses on the selection of a defined cell pool. We found that isolation of hMSCs in SF media selects a pro-ECM cell population, which could be of great help in accelerating the rebuilding process of a native ECM following a bone fracture. Additionally, we found that isolation of hMSCs at a reduced MNC plating number selects a pro-adipogenic cell population. These findings underline the importance of carefully selecting the right isolation procedure for the right application.

CONCLUSION

Overall, our results suggest that novel approaches to BFH can be developed based on our improved understanding of BM cell biology. Based on our results, we hypothesise that poor BFH in the tibia might be the result of insufficient cell numbers, as well as their poor osteogenic potential. Based on this, we suggest the aspiration of BM from the ilium and its delivery into the tibia to accelerate fracture healing. Moreover, we proposed two new possible therapeutic approaches for BFH: acoustic stimulation of BM and use of preselected pro-ECM hMSCs pool for delivery at the fracture site.

ACKNOWLEDGEMENTS

We would like to acknowledge the orthopaedic department at Medisch Centrum Twente for the collection of BM; Smith & Nephew for the financial support to perform this research; as well as its collaborators, A Au, C Wan, T Kapur, and JN Ridgway for their support and design of the acoustic stimulation device.

ABBREVIATIONS

human mesenchymal stromal cells (hMSCs); bone marrow (BM); bone fracture healing (BFH); mononuclear cells (MNCs); serum free (SM); total hip arthroplasty (THA); total knee arthroplasty (TKA); fetal bovine serum (FBS), growth media (GM); α-minimal essential media (α-MEM); population doubling (PD); passage 1 (P1); passage 2 (P2); colony forming unit (CFU); colony forming unit osteoblast (CFU-Ob); β-glycerophosphate (BGP); dexamethasone (Dex); alkaline phosphatase (ALP), extracellular matrix (ECM).

SUPPLEMENTARY FIGURES

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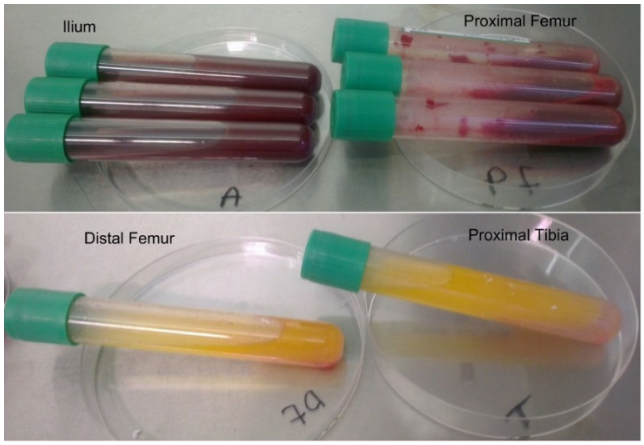
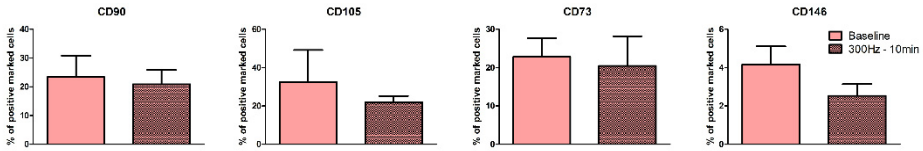
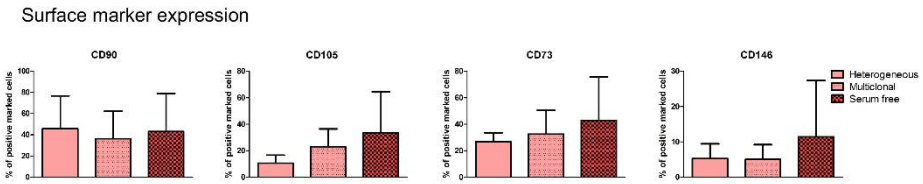


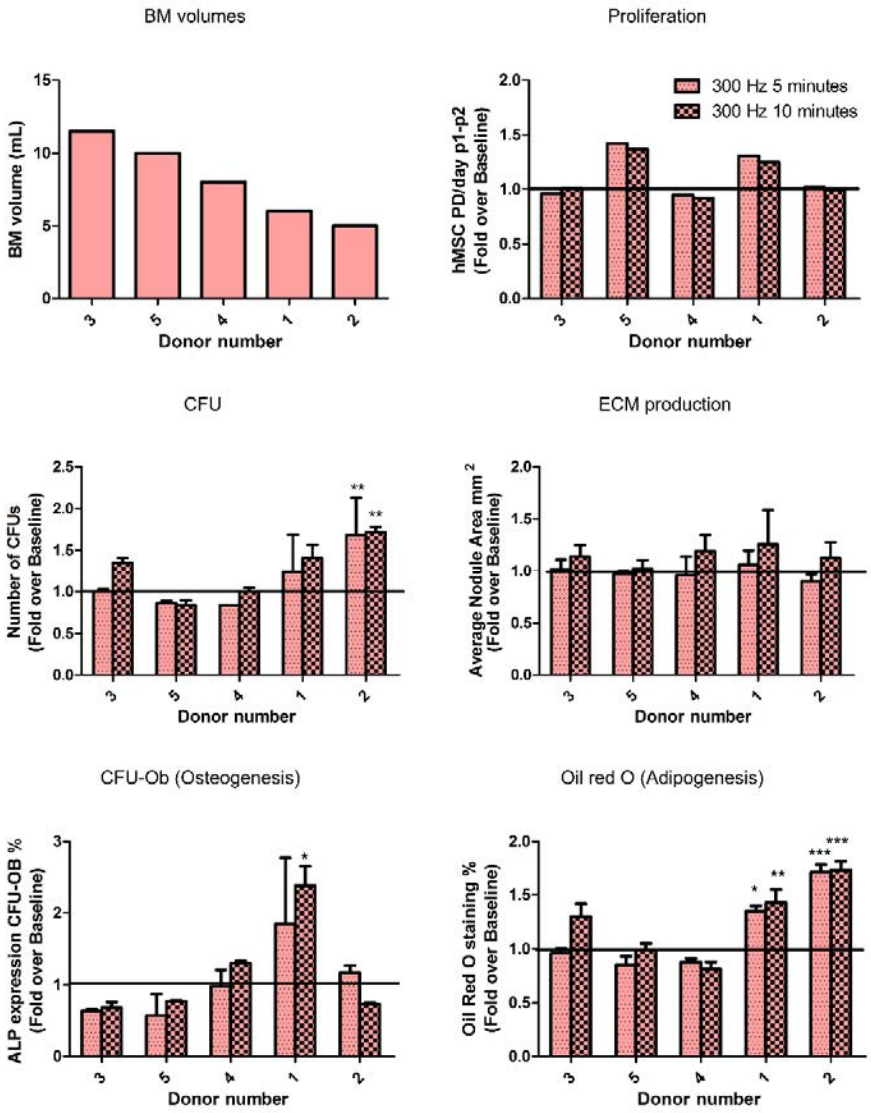
Supplementary Figure 1. Example of bone marrow aspirated from different locations*: ilium, proximal femur, distal femur, and proximal tibia.*



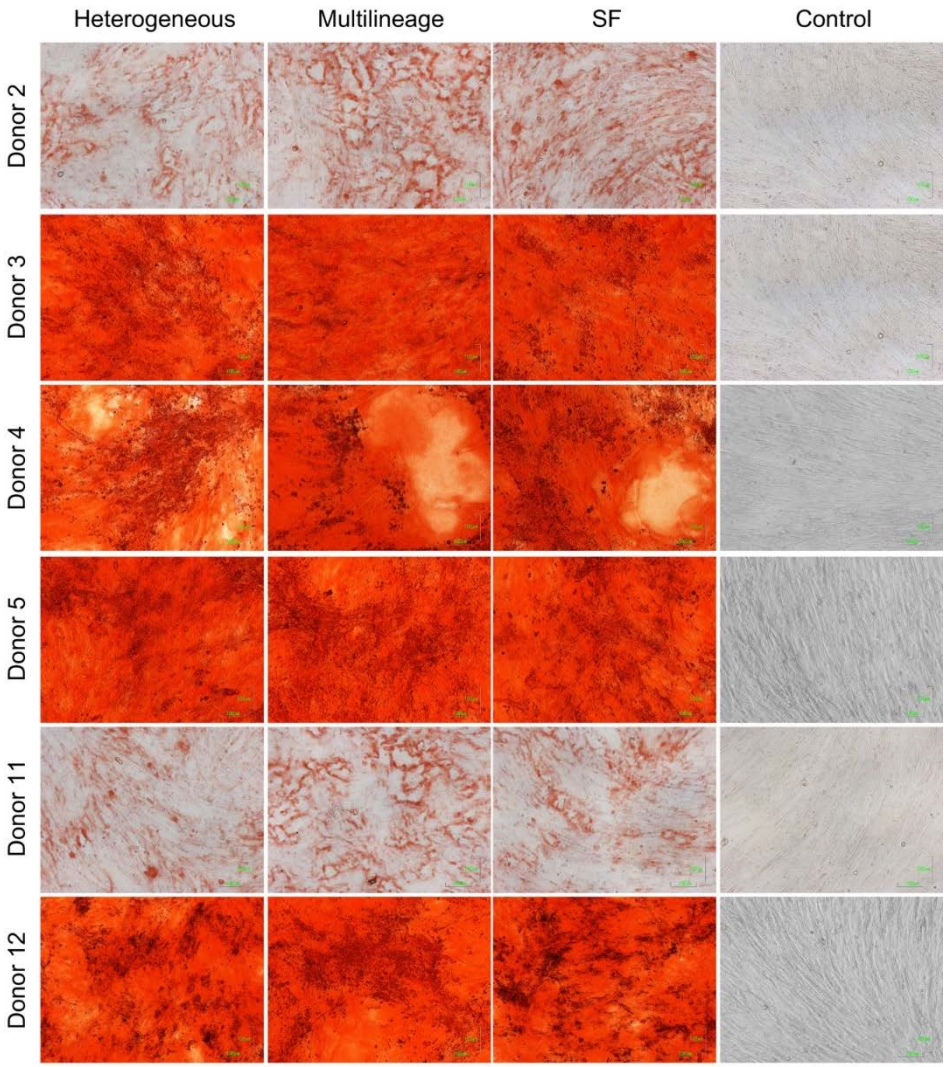
Supplementary Figure 3. Surface marker expression (in percentage) of the acoustically stimulated cells represented as a bar plot*; each bar stands for the average across the percentage of surface markers obtained from three donors. The selected sets of cell surface markers expressed positive on hMSC. All the other investigated sets expressed negative for both conditions, and are therefore not shown. Not statistically significant differences were found between the two conditions.*



Supplementary Figure 5. Surface marker expression (in percentage) of the varying culture conditions represented as a bar plot*; each bar stands for the average over the percentage of surface markers obtained from three donors. The selected sets of cell surface markers expressed positive on hMSC. All the other investigated sets expressed negative for both conditions, and are therefore not shown. No statistically significant differences were found between the three conditions.*



Supplementary Figure 2. Biological characterisation of isolated hMSCs from acoustically stimulated BM at 300 Hz for five minutes at different volumes, 11.5, 10, 8, 6, and 5ml. *The results are presented as the fold change over the non-stimulated BM (baseline). (A) Graphic representation of the BM volumes, donor dependent. (B) Proliferation of hMSCs calculated as PD/day from P1 to P2, donor and volume dependent. (C) CFU potential of hMSCs, donor and volume dependent. (D) ECM production, quantification of nodule size area in mm^2, donor and volume dependent. (E) Osteogenic potential calculated as percentage of ALP positive colonies within the CFUs, donor and volume dependent. (F) Adipogenic potential, quantification of Oil Red O staining relative to 100% Oil Red O staining solution, donor and volume dependent. Values are represented as the mean ± standard deviation of at least three independent experiments ($n \geq 3$). Statistically significant differences were found with ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.*



Supplementary Figure 4. Alizarin red staining of calcium nodules after osteogenic induction of hMSC isolated under varying culture condition from different donors. *No differences were observed between the culture conditions, though differences between the donors were identified. Donor 2 and 11 showed less calcium nodule formation than the rest of the donors. All the controls stained negative for calcium nodules formation. Values are represented as the mean ± standard deviation of at least three independent experiments (n = 3).*

SUPPLEMENTARY TABLES

BM location comparison	P value
Ilium vs. proximal femur	0.36
Ilium vs. distal femur	0.07
Ilium vs. proximal tibia	0.12
Proximal femur vs. distal femur	0.86
Proximal femur vs. proximal tibia	0.94
Distal femur vs. proximal tibia	0.99

Supplementary Table 1. Testing the significance of difference in number of hMSCs isolated from different BM aspiration locations. *The identified p value is presented after performing a one-way ANOVA and Tukey's multiple comparison test.*

Experiment	BM location comparison	P value
CFU	Ilium vs. proximal femur	0.45
	Ilium vs. distal femur	0.72
	Ilium vs. proximal tibia	0.87
	Proximal femur vs. distal femur	0.07
	Proximal femur vs. proximal tibia	0.13
	Distal femur vs. proximal tibia	0.99
CFU-Ob	Ilium vs. proximal femur	0.76
	Ilium vs. distal femur	0.1
	Ilium vs. proximal tibia	0.21
	Proximal femur vs. distal femur	0.5
	Proximal femur vs. proximal tibia	0.73
	Distal femur vs. proximal tibia	0.98

Supplementary Table 2. Testing the significance of difference in CFUs and CFU-Ob from different BM aspiration locations. *The identified p value is presented after performing a one-way ANOVA and Tukey's multiple comparison test.*

Experiment	Cell culture condition	P value
Proliferation	Heterogenous vs. multiclonal	0.94
	Heterogenous vs. serum free	0.62
	Multiclonal vs. serum free	0.81
ECM production	Heterogenous vs. multiclonal	0.95
	Heterogenous vs. serum free	0.11
	Multiclonal vs. serum free	0.18
Oil Red O (Adipogenesis)	Heterogenous vs. multiclonal	0.66
	Heterogenous vs. serum free	0.48
	Multiclonal vs. serum free	0.13

Supplementary Table 3. Testing the significance in proliferation, ECM production, and adipogenesis of different hMSCs isolation and cell culture conditions. *The identified p value is presented after performing a one-way ANOVA and Tukey's multiple comparison test.*

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Chapter 4

Human muscle-derived factors accelerate ACL graft healing: an *in vitro* and *in vivo* analysis

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ABSTRACT

One of the ligaments most often damaged during sports – the anterior cruciate ligament (ACL) – has poor healing capacity. Upon damage, reconstructive surgery is performed to restore the mechanical stability of the knee and to reduce the inflammatory milieu otherwise present in the joint. A return to normal activities, however, takes between 9 and 12 months. Thus, strategies capable of accelerating/improving ACL graft healing are needed.

Embryonic development of tendon and ligament (T/L) is regulated by a crosstalk between different cell types. Using an indirect co-culture system, we discovered that myoblast-derived signals – but not osteoblasts, chondrocytes, or stromal-derived signals – are capable of upregulating classical T/L markers such as Scleraxis and Tenomodulin on human hamstring tendon-derived cells (hTC), which contribute to ACL graft healing. Whole transcriptome analysis showed that co-culturing hTC with myoblasts led to an upregulation of extracellular matrix (ECM) genes and resulted in enhanced ECM deposition. In vivo, using a rat model of ACL reconstruction, showed that conditioned media derived from human muscle tissue accelerated femoral tunnel closure, a key step for autograft integration. Collectively, these results indicate that muscle-derived signals can be employed to improve ACL graft healing in a clinical setting where muscle remnants are often discarded.

INTRODUCTION

The anterior cruciate ligament (ACL) is one of the most often damaged ligaments in the knee and, unfortunately, has very limited healing capacity, leaving surgical reconstruction as the only therapeutic option. More than 200,000 ACL reconstructions are performed annually in the United States alone [1]. A commonly used graft to replace the original ACL and restore its function is the hamstring tendon, which, in comparison with the bone-patellar tendon-bone graft, gained popularity due to its accessibility, reduced pain, and lower morbidity rates [2].

Following an ACL reconstruction, the mechanical stability of the knee joint is readily restored by means of fixation of the graft into the bone, while the necessary biological properties for long-term maintenance lag behind. In particular, vascularisation, extracellular matrix (ECM) deposition, and integration of the soft tissue into the bone are key limiting steps in the regenerative process. Thus, improving at least one of these processes will contribute to ameliorating the recovery period and improving the patient´s quality of life.

In a recent review, Schweitzer et al. discuss the findings that highlight the musculoskeletal assembly in the vertebrate embryo, findings that emphasise the reciprocal interactions between the forming tendons, muscle, and cartilage tissue. Accordingly, the formation of complex tissues during embryonic development requires a continuous bidirectional communication between cells of different origins, such as muscle and tendon, or cartilage and tendon, to allow precise tissue assembly [3].

Based on the abovementioned, we hypothesised that co-culturing human hamstring tendon-derived cells – the cells present in the most commonly used autograft for ACL reconstruction – with myoblast-, osteoblast-, chondrocyte-, or stromal-derived cells could enhance the expression of tendon and ligament (T/L) markers and contribute to accelerating the healing process.

To test this, we used an *in vitro* indirect co-culture system using transwell chambers where we cultured human tendon-derived cells (hTC) in the presence of the different cell types and investigated the expression of several T/L markers firstly by qPCR and later using whole transcriptome and secretome analysis. Our results clearly indicate the co-culturing hTC with myoblast or human muscle tissue (hMT)-derived condition media (CM) significantly increases the expression of T/L markers, ultimately leading to an increase in ECM deposition *in vitro*. Based on these findings we used an *in vivo* rat model of an ACL injury and, as expected, demonstrated that human muscle-CM accelerated femoral tunnel closure. A schematic representation of the experimental design is presented in Figure 1.

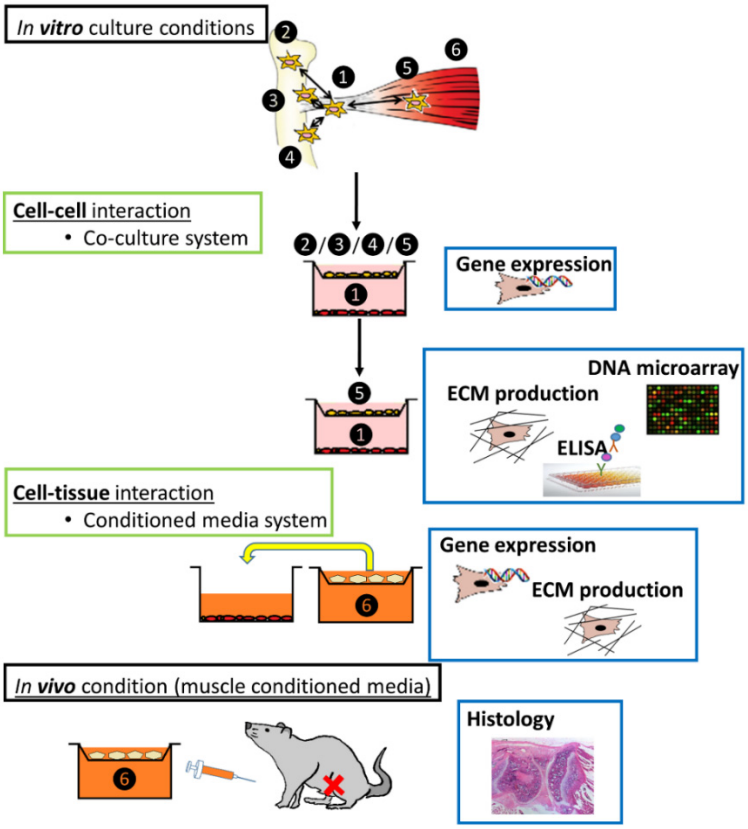


Figure 1. Schematic overview of the experimental design. *The number represents different type of cells or tissue:* ❶ *hTC,* ❷ *BM-hMSC,* ❸ *chondrocytes,* ❹ *osteoblasts,* ❺ *myoblasts,* ❻ *muscle tissue.*

MATERIAL AND METHODS

Cell culture

Samples of human hamstring tendon, cartilage harvested from tibial/femoral ends, and bone marrow (BM) harvested from ilium were obtained from patients undergoing ACL reconstruction, knee replacement, or total hip arthroplasty, respectively. Cell isolation was carried out within 24 hours of procurement, as previously described [4-6]. The collection and anonymous use of the tissues and BM was approved and performed according to the medical ethical regulations and the guideline ‘good use of redundant tissue for research’ of the Dutch Federation of Medical Research Societies (Ref. no.: K13-46). Patients provided informed consent after being provided with a verbal explanation and an opportunity for questioning.

C2C12 (myoblast), MC3T3 (osteoblast), and ATDC5 (chondrocyte) mouse cell lines were obtained from American Type Culture Collection and cultured according to standard mammalian tissue culture protocols and sterile technique [7-9].

Cells were expanded in basic media consisting of α-minimal essential media (αMEM, Life Technologies); 10% fetal bovine serum (FBS, Lonza); 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAP, Sigma); 100 units/mL penicillin (Gibco); and 100 mg/ml streptomycin (Gibco). Primary cells were never used beyond passage 4.

Indirect cell co-culture system

An *in vitro* commercially available biocompartment culture system was used (1 μm pore size; Greiner Bio-One) to allow for an indirect exchange of humoral factors between the different cell types. In brief, hTCs were seeded onto the 6-well outer tissue culture plate, while bone marrow-derived human mesenchymal stromal cells (BM-hMSC) or mouse myoblasts, chondrocytes, and osteoblasts were seeded on the inner culture insert, at 100,000 cells per well/insert at a ratio of 1:1 and in triplicate. The cells were cultured for one day in basic medium to allow an efficient cell attachment, after which the media was changed to serum reduced media (SRM) consisting of basic media with 1% FBS, rather than the abovementioned 10%. Media were refreshed every 3-4 days until the end of the experiment.

Conditioned media collection and use

hMT harvested from patients undergoing ACL reconstruction (remnant hamstring muscle) was washed in PBS, minced into small pieces, and plated on non-tissue culture treated 6-well plate at approximately 2 g tissue/plate in SRM. Condition medium (CM) was collected at day 3 and 7 and passed through a 100 μm filter. One day prior to the collection of hMT CM, hTCs were plated at 100,000 cells/well on tissue culture 6-well plates in triplicate. The next day, half of the media were replaced with hMT CM and SRM and media were refreshed every three days until the end of the experiment.

RNA isolation and qRT-PCR assessment

Total RNA was isolated from hTCs using the NucleoSpin RNA II isolation kit (Macherey-Nagel), per the manufacturer's instructions. RNA was collected in RNase-free water and a quantitative analysis was performed using spectrophotometry (Nanodrop). First-strand cDNA was synthesised from 800 ng total RNA/sample using iScript (BioRad) per the manufacturer's instructions. qPCR was performed on a real-time PCR detection system (Biorad) using iQ Syber green supermix (BioRad) for a panel of primers, described in Supplementary Table 1, or using TaqMan Universal MasterMix for beta-2-microglobulin (B2M) and scleraxis (SCX). Gene expression was normalised to the reference gene B2M and fold induction calculated using the ΔΔCt method.

Microarray expression profiling

Gene expression profiling of two hTC donors cultured in triplicate in the absence or presence of mouse myoblasts was carried out using the Illumina microarray platform. cRNA was synthesised from 750 ng of total RNA using the Illumina TotalPrep RNA amplification kit (Ambion) per the manufacturer's instruction, and cRNA quality was assessed using Bioanalyser 2100 (Agilent). Next, cRNA was hybridised on Illumina HT-12 v4 expression beadchips overnight, after which the array was washed and blocked. Then, by adding streptavidin Cy-3, a fluorescent signal was developed and the arrays were scanned on an Illumina Beadarray reader and raw intensity values were background corrected in BeadStudio (Illumina). Further data processing and statistical testing were performed using R and Bioconductor statistical software [10]. The probe-level raw intensity values were quantile normalised and transformed using variance stabilisation (VSN). A linear modelling approach with empirical Bayesian methods, as implemented in the Limma package [11], was applied for differential expression analysis of the resulting probe-level expression values. P values were corrected for multiple testing using the Benjamini and Hochberg method [12]. Genes were considered differentially expressed between hTC cultured in the presence of myoblasts and hTC cultured alone, when a corrected p value below 0.05 was reached with an absolute log-fold change above 0.5. A venn diagram (using Venny 2.1.0 BioinfoGP online tool) was used to represent the overlapping genes between the different conditions and donors. The differentially expressed genes (combining both up- and down-regulated genes) overlapping between both donors were subjected to Gene Ontology (GO) analysis using DAVID bioinformatics resources [13, 14]. The GO terms were considered significantly overrepresented when a Benjamini-Hochberg corrected p value of 0.05 was reached.

Collagen content assessment

hTCs were co-cultured with myoblasts or hMT CM for 29 days and media were refreshed every three days, as previously described. At day 29, the cells were fixed in 10% (v/v) buffered formalin for 15 minutes at room temperature and stained for collagen deposition using Picrosirius red staining kit (Polyscience Inc.), following the manufacturer´s instructions. Images of the stained cells were acquired using a Nikon bright field microscope and quantification was performed using FIJI open source software. Images were converted to 8-bit and background subtracted using rolling ball radius function. The image was then converted into binary, the area of Picrosirius red staining was selected and measured in total number of pixels.

ELISA

The supernatant of co-cultured hTCs and myoblasts cultured on SRM for three days was collected and immediately processed to assess the secretion of a panel of 23 cytokines and growth factors using ELISA plate array I for profiling 23 mouse cytokine proteins (BioCat), following the manufacturer´s instructions.

ACL reconstruction in rat

The animal experiments were approved and performed in strict accordance with the recommendations and guidelines of the Animal Experimentation Ethics Committee in The Chinese University of Hong Kong (Ref. no.: 15-166-MIS). ACL reconstruction was performed in 15 male Sprague Dawley rats (12 weeks old) on the right knee using ipsilateral flexor digitorum longus tendon, as previously described [15]. Under general anaesthesia with ketamine and xylazine (75 and 25 mg/kg body weight, respectively), the flexor digitorum longus tendon (25 mm in length and 1 mm in diameter) was harvested by longitudinal medial incision of the right heel and the muscle tissue was removed from the graft. The knee joint capsule was opened, native ACL was excised and the successful transection confirmed by a positive Lachman test. Tibia and femoral tunnels of 1.1 mm diameter, 9 mm, and 6 mm in length were created from the footprints of the native ACL to the medial side of the tibia and the lateral femoral condyle. The tendon graft was inserted through both bone tunnels and the graft was fixed to the tibial periosteum first. Through a pulley system, a freely suspended weight was used to provide a constant tensioning force of 4N to the graft during its fixation to the neighbouring femoral periosteum. Finally, the fascia and other soft tissues were closed in layers. All animals were given 0.03 mg/kg body weight buprenorphine hydrochloride as analgesics and were allowed free cage activity after operation.

Post-operative intra-articular injection

hMT and tendon tissue harvested from the same patient undergoing ACL reconstruction (hTC 7) were washed in PBS, minced into small pieces, and plated in non-tissue culture treated plates separately at approx. 2 g per plate (Supplementary Figure 1). Tissue samples were cultured in SRM for three days, after which CM was collected, strained through 100 μm filters, aliquoted, and stored at -80°C until use. SRM alone was collected and used as control. Five animals per condition were used. Intra-articular injection was performed on day 7, 14, 21, 28, and 35 post-operatively. The different CM were individually loaded into a 50 μl syringe with a 26G removable needle (Hamilton Company, NV, USA). Under general anaesthesia with a mixture of isofluorane/O_2, with the knee flexed, the needle was inserted perpendicularly through the patellar tendon to access the joint space, and 50 μl of treatment solution was slowly injected. A total of five weekly injections were given per animal during the experiment.

Histological analysis

At six weeks post-operation, the rats were euthanised with an overdose of pentobarbital. The harvested knee joints were fixed in 10% (v/v) buffered formalin, decalcified in 9% formic acid for five weeks and embedded in paraffin. Five-micrometre-thick paraffin sections along the sagittal plane of the knee were collected in groups of 10-20 sections at 500 μm intervals. From each animal, 2 or 3 sections from each group of sections were chosen for histological examination of femoral tunnels, intra-articular graft mid-

substance, and tibial tunnels [16]. Sections from the epiphyseal region were chosen for comparison of graft healing inside tunnels. H&E stained sections were examined under bright field light (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software. Two-way ANOVA and a Tukey post hoc test or Unpaired Student's t-test were used to compare the different conditions. A $P \leq 0.05$ indicates a statistically significant difference. Results are shown as the mean ± standard deviation.

RESULTS

Upregulation of tendon/ligament markers in hTC co-cultured with myoblasts

Indirect co-culture of hTC with BM-hMSCs, myoblasts, osteoblasts, and chondrocytes resulted in differential expression of lineage specific markers compared with the untreated control. Although co-culture of hTC with BM-hMSC and osteoblasts did not change the expression of T/L genes – neither genes related with chondrogenesis and osteogenesis – it was interesting to observe the effect of co-culturing hTC with myoblasts and chondrocytes. Co-culturing hTC with chondrocytes significantly increased the expression of chondrogenic (28.6 ± 28.5 and 3.7 ± 3.6-fold increase vs. control for cartilage oligomeric matrix protein (COMP) and aggrecan (ACAN), respectively) and osteogenic marker (12.6 ± 18.77-fold increase vs. control for ALP). It is notable that co-culturing hTC with myoblasts led to a statistically significant increase in the expression of SCX (5.5 ± 4.4-fold vs. control) – the master transcription factor involved in T/L specification. Moreover, tenomodulin (TNMD) – a well-recognised T/L lineage marker – was also upregulated solely on hTC exposed to myoblasts (8.1 ± 1.7-fold) (Figure 2 and Supplementary Figure 2). Furthermore, T/L-related genes such collagen IA1 (COL1A1) (2.8 ± 2-fold), tenascin (TNC) (2.4 ± 1.6-fold), and COMP (8.6 ± 6-fold) were also upregulated in hTC exposed to myoblasts but did not achieve a statistically significant difference.

Co-culture of hTC with chondrocytes does not increase the expression of T/L markers

Based on the abovementioned results, we decided to validate our findings on human-derived cells. To that end, we exposed primary isolated human hTC to primary isolated human chondrocytes and investigated the expression of a set of T/L- and cartilage-related genes. With the exception of TNMD, which was down-regulated, co-culturing human primary hTC with human primary chondrocytes did not change the expression of T/L markers compared with the control (Supplementary Figure 3). Based on these findings, we decided to focus on the role of myoblasts on hTC.

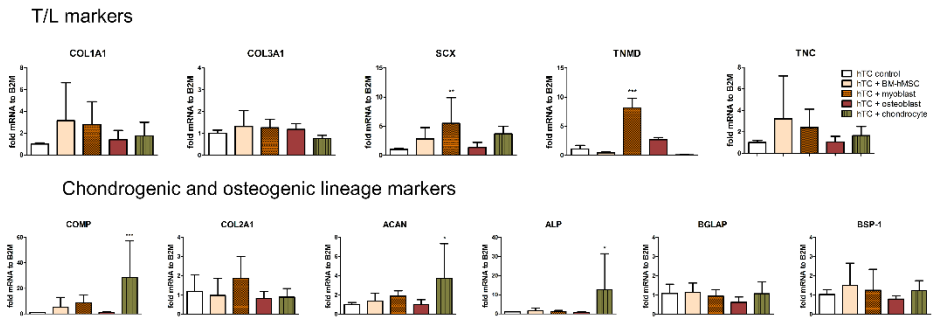


Figure 2. Gene expression of tendon-/cartilage- and bone-related markers in hTC when cultured in the presence of BM-hMSC, myoblasts (C2C12), osteoblasts (MC3T3), or chondrocytes (ATDC5) for seven days in 1% FBS media. *The graphs represent the average in gene expression over 3 TC donors. Statistically significant differences were found with ***p < 0.001, **p < 0.01 and *p < 0.05.*

Genome-wide analysis of gene expression in hTC exposed to myoblasts

Based on the abovementioned results highlighting the positive role of co-culturing hTC with myoblasts, and in order to unravel the molecular players governing this interaction, we performed a genome-wide gene expression analysis to identify which genes were differentially regulated on hTC (from two different donors) exposed to myoblasts compared with the control. Transcriptional profiling revealed a total of 60 genes up-regulated and 90 genes down-regulated in hTC cultured in the presence of myoblasts vs. control (Figure 3A). The top 20 up- and down-regulated genes and respective fold change are presented in Figure 3B; while the complete list comprising gene name, fold change, and location of gene function and gene function can be found in Supplementary Table 2. A brief analysis of the top 20 genes reveals an enrichment in genes involved in ECM modulation, such as COMP; tissue inhibitor of metalloproteinases 3 (TIMP3); Collagen type XI alpha 1 (COL11A1); retinoic acid receptor responder 2 (RARRES2); polypeptide N-acetylgalactosaminyltransferase 1 (GALNT1); stimulator of chondrogenesis 1 (SCRG1); fibronectin type III domain containing 1 (FNDC1); chitinase 3 like 1 (CHI3L1); C-X-C motif chemokine ligand 12 (CXCL12); platelet derived growth factor receptors like (PDGFRL) and complement C1r subcomponent (C1R) (Figure 3B, pattern-containing bars). Additionally, using the identified up- and down-regulated genes (60 + 90) and GO enrichment analysis, we found significantly enriched GO categories in biological adhesion (GO:0022610, fold enrichment 2.9, P value = 0.05); response to wounding (GO: 0009611, fold enrichment 3.6, P value = 0.03); proteinaceous extracellular matrix (GO: 0005578, fold enrichment 4.4, P value = 0.004); extracellular region part (GO: 0044421, fold enrichment 2.7, P value = 0.003); extracellular matrix (GO: 0031012, fold enrichment 4.8, P = value 0.0006); and extracellular region (GO: 0005576, fold enrichment 2.4, P value = 0.000008) (Figure 3C). This analysis clearly demonstrates the influence myoblasts exert on the modulation of ECM molecules in hTC. Additionally, we found that within these ECM modulators,

several genes were involved in the formation and remodelling of cartilage ECM, such as COMP, COL 11A1, TIMP3, SCRG1, or CHI3L1 (Figure 3D, orange circles). Furthermore, GO enrichment analysis revealed that, although not significant, a large number of genes were involved in the regulation of cell migration, motion, and locomotion, such as laminin subunit alpha 4 (LAMA4); podoplanin (PDPN); ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2); platelet derived growth factor receptor alpha (PDGFRA); thrombospondin 1 (THBS1); adenosine A1 receptor (ADORA1); tropomyosin 1 (TPM1); CXCL12; LIM zinc finger domain containing 2 (LIMS2); annexin A2 (ANXA2); tensin 3 (TNS3); RARRES2; actin; and alpha 2 smooth muscle aorta (ACTA2) (Figure 3D, dark green circles). Finally, we analysed the location of the identified gene functions and found that the majority of the gene functions were located within the cytoplasma (34%), followed by plasma membrane (23%), extracellular space (18%), and nucleus (10%) (Figure 3E). Gene function analysis showed an enrichment in genes involved in enzyme (17%), while a small percentage of genes was involved in kinase, transmembrane receptor, peptidase, and transcription regulator (each 5%), or phosphatase (3%) and cytokine and G-protein coupled receptor (2%). The remaining genes showed enrichment in other classes of genes (52%) (Figure 3F).

Analysis of the secretome of myoblast co-cultured with hTC

To identify which factors secreted by myoblasts may be responsible for the effects observed on hTC, we performed a protein array study in which we analysed a panel of cytokines and growth factors released in the culture media during indirect co-culture of myoblasts with hTC. Our results showed that, compared with the control (hTC alone), co-culture with myoblasts upregulated the expression of monocyte chemioattractant protein-1(MCP-1) 7.4-fold, vascular endothelial growth factor (VEGF) 1.4-fold, interleukin 6 (IL-6) 1.8-fold, and Rantes 1.7-fold (Figure 4).

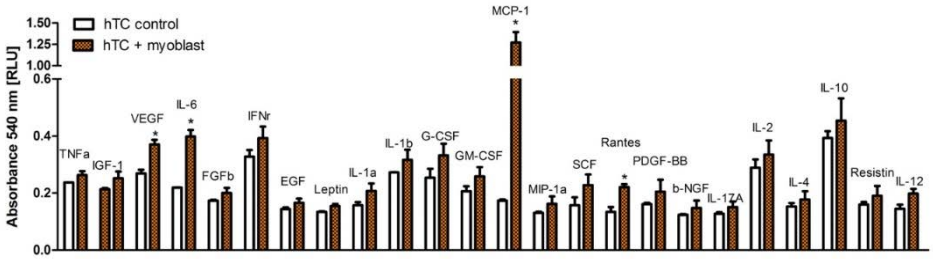


Figure 4. Protein array study showing cytokines and growth factors produced by mouse myoblast cell line when co-cultured with hTC1 and hTC2. *The values are shown as the average over the two hTC donors and represented as a relative light unit. Mouse myoblasts have been shown to produce VEGF, IL-6, MCP-1, and Rantes. Statistically significant differences were found with *p < 0.05*

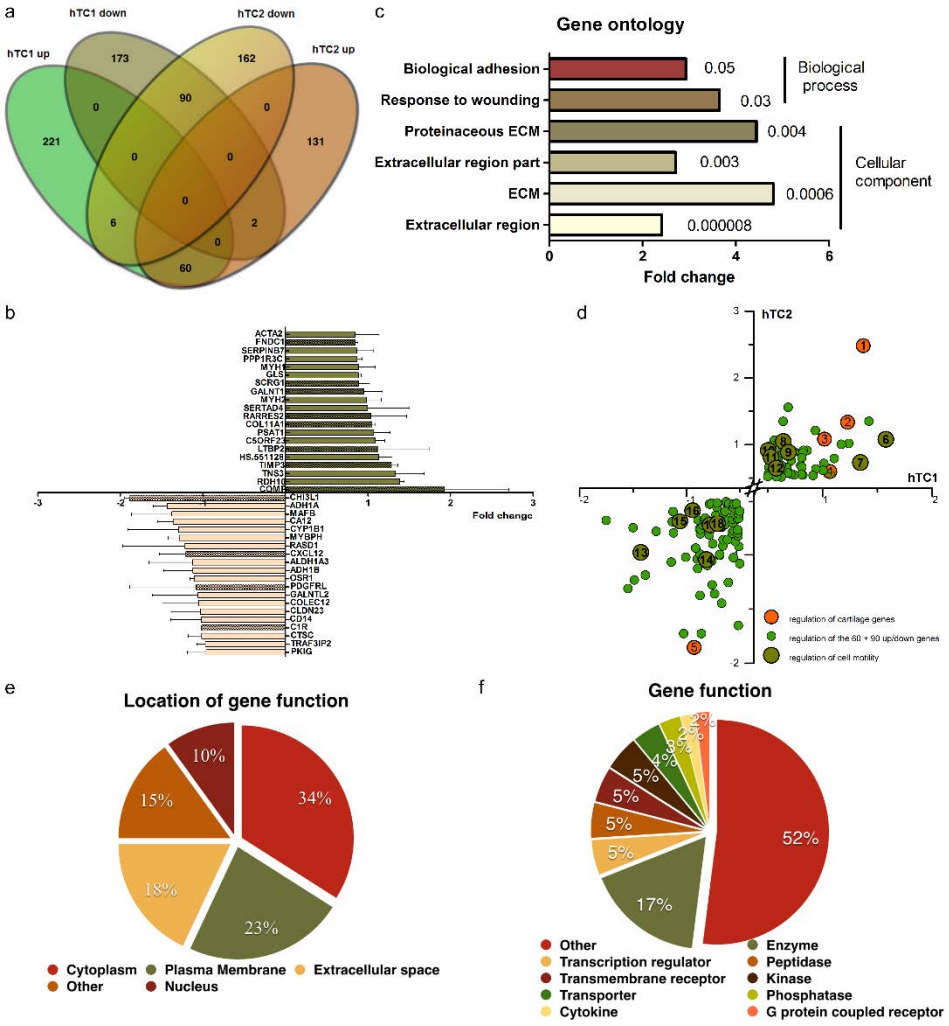


Figure 3. Genome-wide microarray and GO analysis of differentially expressed probe sets. *(A) Venn diagram of differentially expressed genes. The number of significant up- and down-regulated genes in hTC1 and hTC2 and their intersection, indicating the number of common and individually regulated genes. Differentially expressed genes were identified by pairwise comparison analysis of hTC + myoblasts and hTC alone. (B) Map of the top 20 up- and down-regulated genes. Pattern graphs represent genes involved in ECM remodelling. (C) GO analysis of all common differentially expressed genes and the identified significant enriched biological process and cellular component GO clusters. (D) hTC1 hTC2 plot of fold change of the differentially expressed genes. Positive fold change defines positive expression of genes in hTC indirect co-cultured with myoblasts and reverse for negative fold change. Orange circles represent genes that regulate cartilage ((1) COMP, (2) TIMP3, (3) COL11A1, (4) CHRDL2, (5) CHI3L1), dark green circles represent genes that regulate cell motility ((6) TNS3, (7) RARRES2, (8) ACTA2, (9) ANXA2, (10) TPM1 (11), LIMS2, (12) THBS1, (13) CXCL12, (14) ENPP2, (15) ADORA1, (16) LAMA4, (17) PDPN, (18) PDGFRA), while small green circles represent the rest of the 60 + 90 commonly expressed genes. (E) Pie diagram representing the location of gene function. (E) Pie diagram representing gene function.*

hTC exposed to hMT CM upregulated T/L genes

To translate our findings into a potential therapy for T/L healing we decided to analyse whether hTC responded similarly when exposed to soluble factors obtained from hMT. To do so, we exposed hTC to hMT CM from three different donors for seven days. In line with the results obtained for myoblasts, our results showed a statistically significant upregulation for TNMD (40 ± 40-fold), TNC (1.5 ± 0.3-fold), and COMP (2 ± 0.7-fold) between hMT CM treated cells vs. control (Figure 5 and Supplementary Figure 4).

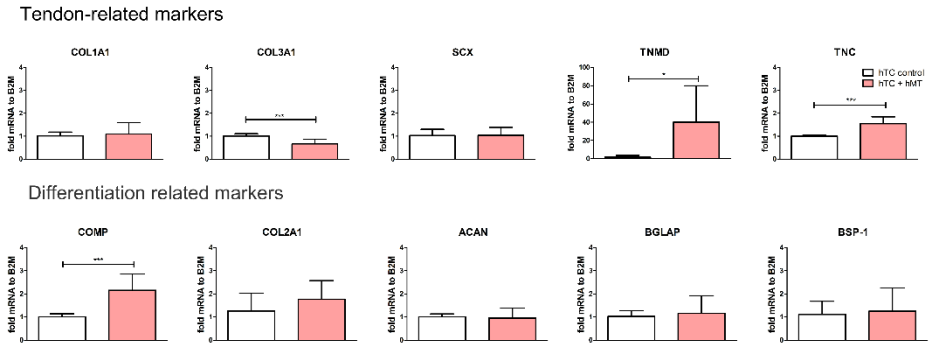


Figure 5. Gene expression of tendon, cartilage, and osteogenic related markers in hTCs when cultured in the presence or absence of muscle conditioned media. *The graphs represent the average in gene expression over three hTC donors. Statistically significant differences were found with ***p < 0.001.*

Myoblast indirect co-culture and hMT CM enhance collagen production

Given the effects observed in the expression of ECM-related genes upon indirect co-culture of hTC with myoblasts and hMT CM, we proceeded to quantify the amount of collagen produced by hTC exposed to the respective condition for 29 days. We found that, compared with the control, hTC exposed to myoblasts significantly increased the production of collagen (1.7-fold for hTC1 and 2.2-fold for hTC2 (Figure 6 A-B, D-E, G-H)), while the addition of hMT CM resulted in a 3.3-fold increase for hTC6 (Figure 6 C, F, I). Based on these results, we decided to test the effect of hMT CM in a rat model of ACL reconstruction.

Treatment of hMT CM improved healing after ACL reconstruction

A rat ACL reconstruction model was used to test the effects of hMT CM. Tendon tissue CM was used as a control for the *in vivo* study. At six weeks post-operation, healing responses were evident in all ACL reconstructed rat knees, with respect to bone tunnel healing, bone-to-graft incorporation, and tendon-graft remodelling (Figure 7). The presence of Sharpey's fibre and/or fibrocartilage interface (Figure 1D-I) was observed

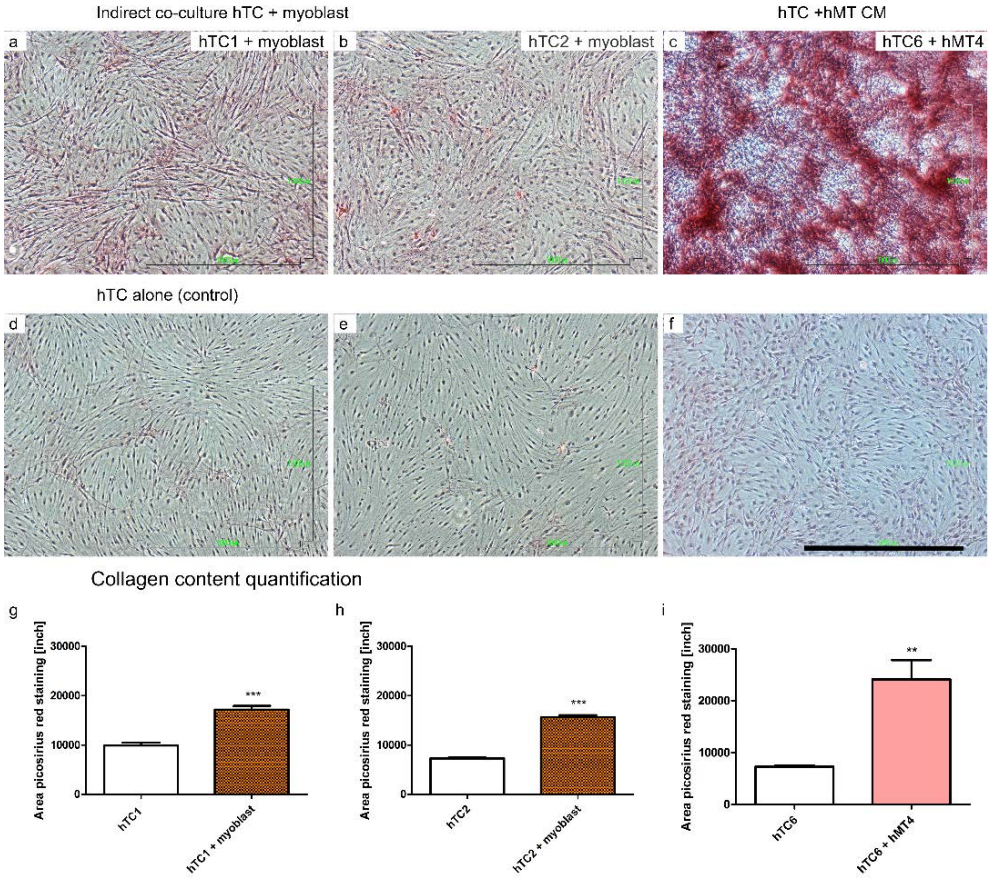


Figure 6. Picrosirius red staining of hTCs indirect co-cultured with myoblasts or exposed to muscle tissue CM. *(A) hTC1 indirect co-cultured with myoblasts. (B) hTC2 indirect co-cultured with myoblasts. (C) hTC6 exposed to muscle tissue CM. (D, E, F) hTC1/hTC2/hTC6 cultured alone (negative control). (G, H, I) Quantification of Picrosirius red staining using FIJI software. Statistically significant differences were found with ***p < 0.001 and **p < 0.01. Scale bar represents 1mm.*

at the graft incorporation to surrounding bone in both femoral and tibial tunnels and in all groups. Additionally, the cellularity in the tendon graft at the intra-articular portion was high in all groups; whereas the degree of cell infiltration in bone tunnels varied between different groups (Figure 7 J-L). Bone tunnel closure did not show any significant differences in tibial bone tunnel closure between groups; however, significant femoral bone tunnel closure was observed in hMT CM group (four out of five samples) (Figure 7C and F) as compared with the basic media group (one out of five samples) (Figure 7A and D) and tendon CM group (one out of five samples) (Figure 7B and E).

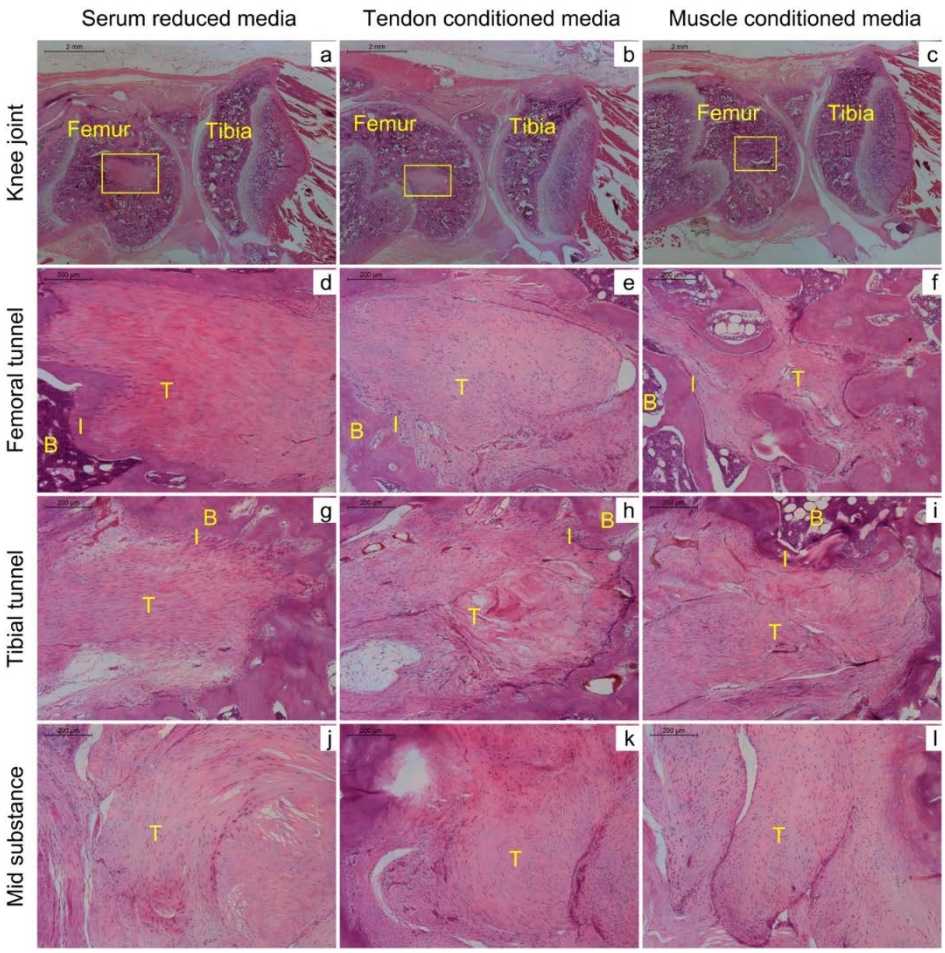


Figure 7 Histological images showing sagittal views of ACL reconstructed rat knees at six weeks post-operation, treated with either basic media, tendon conditioned media, or muscle conditioned media. *D-F are magnified images of the tendon graft, marked in squares of A-C respectively. Significant bone tunnel closure is observed in the femoral tunnels of muscle conditioned media group (C, F), but not in the other two groups. Sharpey's fibre and fibrocartilage zone is present at bone-to-tunnel interface in all groups, as indicated by 'I' in D-I. (H&E staining. Optical magnification: 12.5x in A-C, 100x in D-L). B = bone, I = Bone-tendon interface, T = tendon graft.*

DISCUSSION

Intra-articular healing and remodelling of tendon grafts, following an ACL reconstruction, has been described in the literature as a complex and long-term process, which delays the time for patients to return to work and sport activities [17, 18]. To accelerate the healing process and the integration of the tendon graft into the new environmental conditions, we approached the remodelling process by engaging the use

of cellular crosstalk known to trigger signalling cascades beneficial in tissue remodelling. Inspired by the embryonic development of T/L, where tendon cells experience behavioural changes as result of interaction with surrounding tissue cells, we explored, in a simplified 2D in vitro model, the effects of surrounding mature tissue cells, such as myoblasts, chondrocytes, osteoblasts, or BM-hMSC on the expression of a panel of ECM, and differentiation genes in tendon cells. We found that myoblasts and chondrocytes modulated tendon cellular responses, resulting in an increased expression of tendon and cartilage related markers. The expression of SCX in hTC exposed to myoblasts accompanied the expression of TNMD and a trend towards increased expression in COLIA1, TNC, and COMP. This regulatory effect exerted by skeletal muscle cells on tendon cells by exchange of paracrine factors has been previously reported to occur during tendon development where the expression of SCX and TNMD were described to be dependent on muscle signals [19, 20]. To better understand the role that myoblasts play in modulating tendon cell commitment, we performed a genome-wide analysis on tendon cells, using the same experimental and donor setting. We found that a large majority of differentially expressed genes were involved in ECM modulation. Since it is known that ECM remodelling plays a key role in restoring the biology and function of T/L during the healing process, and since myoblasts play a key role in tissue contraction and organ fibrosis during wound healing [21, 22], our findings suggest that in a simplified 2D environment, with no tensile forces, the chemokines released by myoblasts were enough to affect matrix synthesis and turnover. Further analysis identified that some of the differentially expressed ECM genes – COMP, COL11A1, TIMP3, SCRG1, CHI3L1, CHRDL2 – are known to be involved in cartilage matrix remodelling [23-28], suggesting an important role in guiding tendon-bone interface regeneration. Other regulated genes were found to be involved in cell migration and motion, such as TNS3, RARRES2, ACTA2, ANXA2, TPM1, LIMS2, THBS1, CXCL12, ENPP2, ADORA1, LAMA4, PDPN, and PDGFRA [29-41], suggesting an important role for cell migration on the repopulation of the tendon graft, while influencing revascularisation and tendon-bone closure. To elucidate the molecular signals exchanged between myoblasts and hTC, we analysed a panel of cytokines and identified MCP-1, VEGF, Rantes, and IL-6 as being secreted by myoblasts during indirect co-culture with both hTC donors. We propose two explanations that relate the expression of the identified cytokines to the regulation of genes. One explanation is the fact that MCP-1, VEGF, and Rantes are known chemotactic cytokines [42-44], which may explain the regulation of a large number of genes involved in cell migration and motion. Secondly, Rantes and IL-6 are also known as inflammatory cytokines, which may explain the regulation of genes involved in GO category response to wounding.

In our attempt to change to a more clinically relevant approach, we examined the influence hMT may have on hTC. Consequently, the remnant hMT removed during preparation of the tendon graft and harvest from a patient undergoing ACL reconstruction [45] offered us the chance to investigate its role in tendon cell commitment and its potential beneficial use in accelerating tendon graft remodelling. We found that CM collected from hMT induces, similarly to myoblasts co-culture, an

increase in expression of T/L and cartilage related markers, which in turn are involved in synthesis and turnover of the ECM. These findings in gene expression were further confirmed by increased collagen production in hTC exposed to myoblasts or hMT CM. While Murray and Spector have identified the presence of myofibroblasts in the ruptured human ACL and hypothesised that the contractile role of myofibroblasts in the ACL could be responsible for the wrinkling of the ECM and the formation of crimp [46], Lei Sun et al. have shown in a rabbit model that hMT left on tendon graft promotes the intra-articular healing and remodelling of the graft [47]. Here we speculate that collection and later stage delivery of hMT CM, at the end of the inflammatory stage, would influence tendon remodelling by increasing the number of cells, blood vessels, and remodelling of the ECM for a better ligamentisation. We used an ACL reconstruction rat model and injected hMT CM, tendon tissue CM, or SRM control into the operated joints of the animals. We showed that femoral tunnel closure was promoted by hMT CM, but tibial tunnel closure was not affected. This difference might be attributed to the different mechanical environments inside femoral and tibial tunnels owing to the relative positions of graft and the tunnels. Moreover, the distribution of bioactive factors in the knee joints upon injection of hMT CM may also affect treatment outcomes. A controlled delivery system will be necessary to direct the bioactive factor to the healing site along the tendon graft.

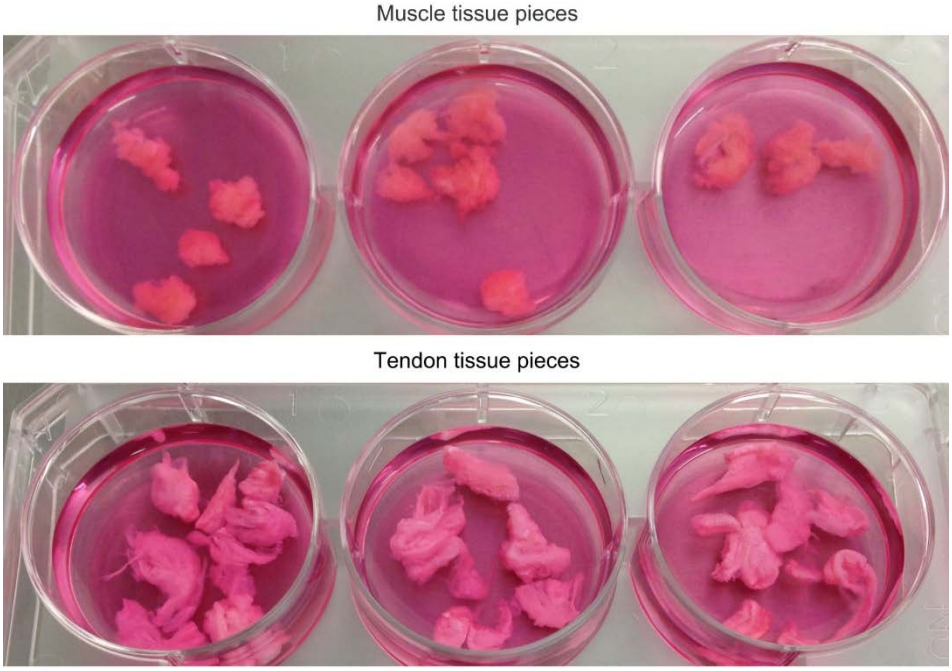
Overall, we showed that myoblast-derived molecules influence hTC commitment and ECM remodelling and are capable of accelerating intra-articular healing in an ACL reconstruction rat model. Consequently, these findings provide preliminary proof that remnant hMT may become a useful tool in accelerating ACL reconstruction healing.

ABBREVIATIONS

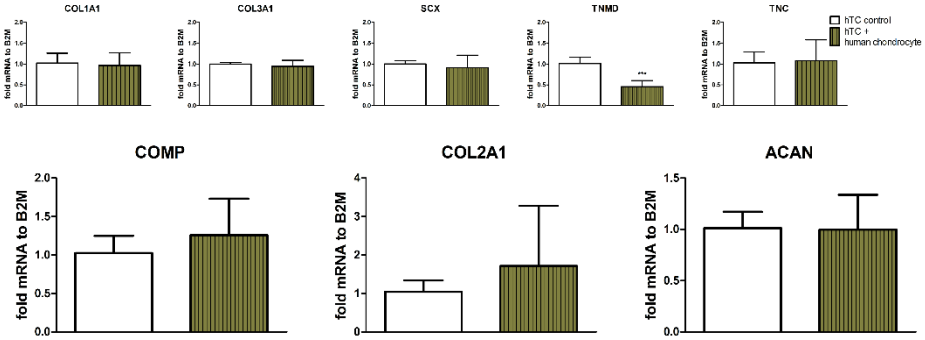
anterior cruciate ligament (ACL); tendon and ligament (T/L); human hamstring tendon-derived cells (hTC); extracellular matrix (ECM); human muscle tissue (hMT); condition media (CM); basic media (BM); L-ascorbic acid 2-phosphate magnesium salt (ASAP); fetal bovine serum (FBS); serum reduced media (SRM); beta-2-microglobulin (B2M); scleraxis (SCX); Gene Ontology (GO); bone marrow-derived human mesenchymal stromal cells (BM-hMSC); cartilage oligomeric matrix protein (COMP); aggrecan (ACAN); tenomodulin (TNMD); Collagen IA1 (COL1A1); tenascin (TNC); tissue inhibitor of metalloproteinases 3 (TIMP3); Collagen type XI alpha 1 (COL11A1); retinoic acid receptor responder 2 (RARRES2); polypeptide N-acetylgalactosaminyltransferase 1 (GALNT1); stimulator of chondrogenesis 1 (SCRG1); fibronectin type III domain containing 1 (FNDC1); chitinase 3 like 1 (CHI3L1); C-X-C motif chemokine ligand 12 (CXCL12); platelet derived growth factor receptors like (PDGFRL) and complement C1r subcomponent (C1R); laminin subunit alpha 4 (LAMA4); podoplanin (PDPN); ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2); platelet derived growth factor receptor alpha (PDGFRA); thrombospondin 1 (THBS1); adenosine A1 receptor (ADORA1); tropomyosin 1 (TPM1); LIM zinc finger domain containing 2 (LIMS2); annexin A2 (ANXA2); tensin 3 (TNS3); alpha 2 smooth muscle aorta (ACTA2); monocyte chemioattractant protein-1(MCP-1); vascular endothelial growth factor (VEGF); interleukin 6 (IL-6).

SUPPLEMENTARY FIGURES

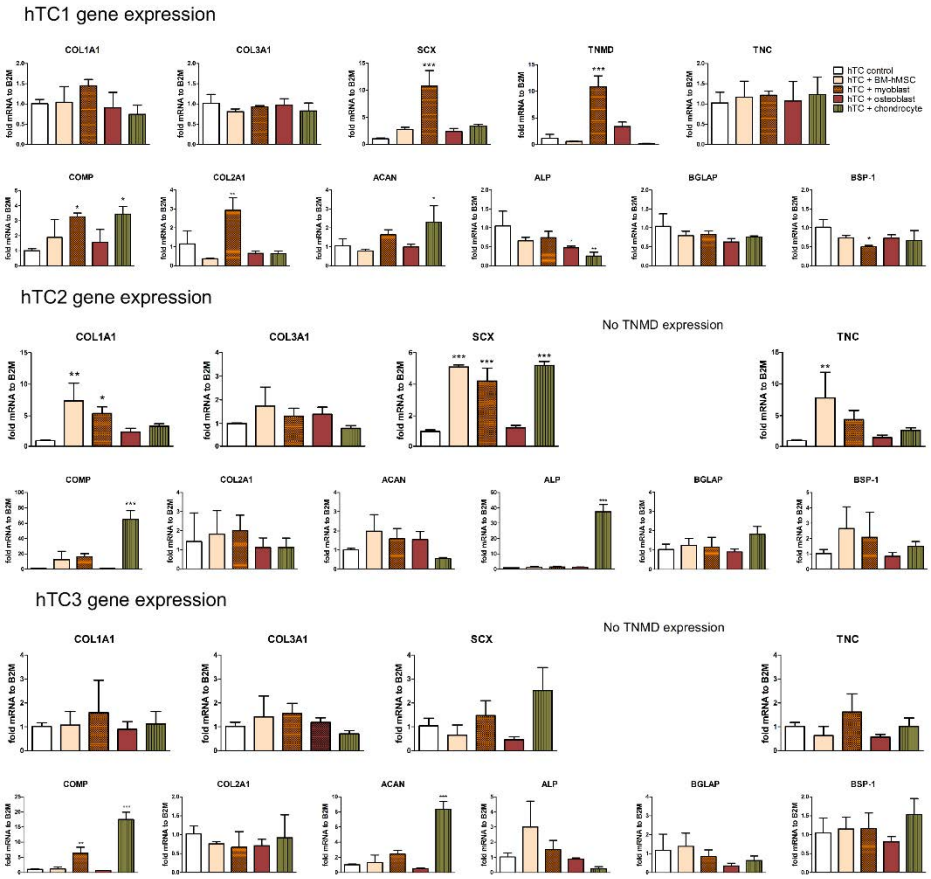
Please note: cluster of figures (1 and 3)



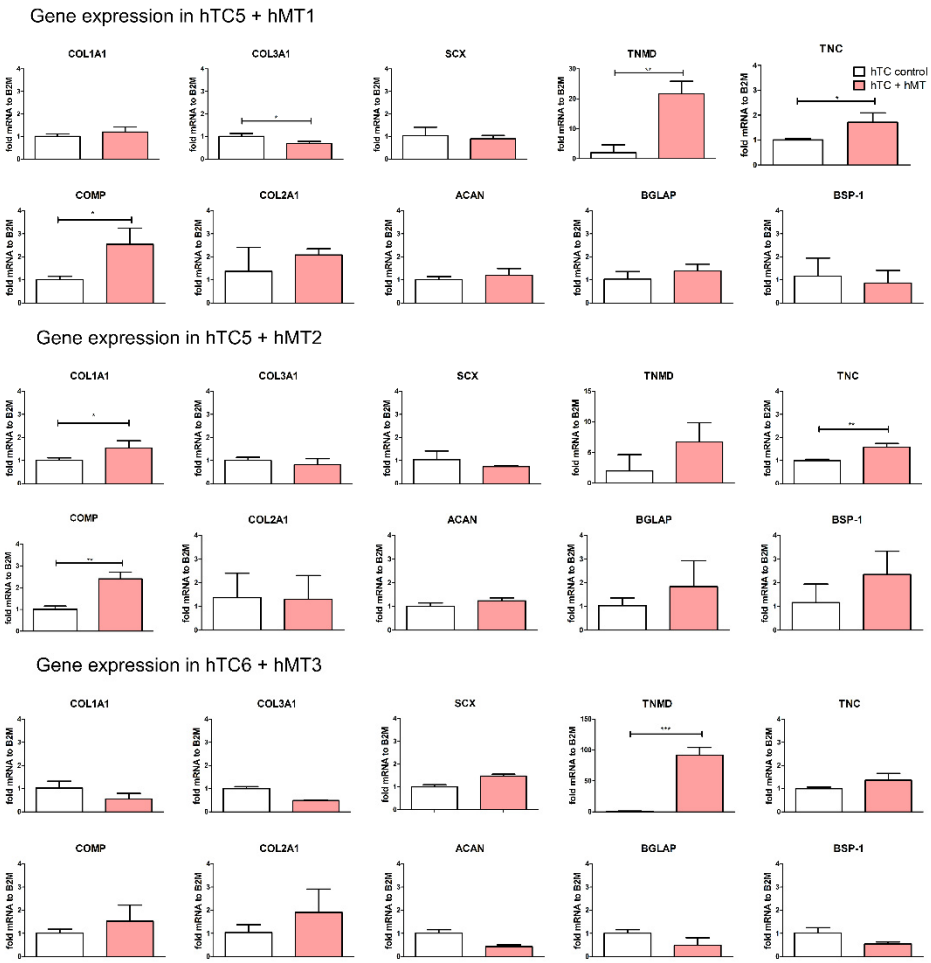
***Supplementary Figure 1. Collection of CM** from (A) muscle tissue and (B) tendon tissue. Tissue was plated at 2g per 6-well plates.*



***Supplementary Figure 3. Donor dependent gene expression of tendon and cartilage related markers in hTC indirectly co-cultured with chondrocytes**. Statistically significant differences were found with **p < 0.01 and *p < 0.05.*



Supplementary Figure 2. Donor dependent gene expression of tendon/cartilage and bone-related markers in hTC indirectly co-cultured with BM-hMSC, myoblasts, osteoblasts, or chondrocytes. *Statistically significant differences were found with ***p < 0.001, **p < 0.01 and *p < 0.05.*



Supplementary Figure 4. Gene expression of tendon, cartilage, and osteogenic related markers in hTC cultured in the presence or absence of hMT CM. *Statistically significant differences were found with ***$p < 0.001$.*

SUPPLEMENTARY TABLES

Gene	Symbol	Primer Sequence
β2-microglobulin	B2M	5'-ACAAAGTCACATGGTTCACA
		5'-GACTTGTCTTTCAGCAAGGA
collagen type I alpha 1	COL1A1	5'-GTCACCCACCGACCAAGAAACC
		5'-AAGTCCAGGCTGTCCAGGGATG
collagen type III alpha 1	COL3A1	5'-GCCAACGTCCACACCAAATT
		5'-AACACGCAAGGCTGTGAGACT
cartilage oligomeric matrix protein	COMP	5'-GTCCGCTGTATCAACACCAG
		5'-GGAGTTGGGGACGCAGTTA
tenascin C	TNC	5'-TGGGCAGATTTCACGGCTG
		5'-TGCTCTGAGCCCGAATGTC
tenomodulin	TNMD	5'-TGTATTGGATCAATCCCACTCTAAT
		5'-TTTTTCGTTGGCAGGAAAGT
collagen type II alpha 1	COL2A1	5'-CCAGATGACCTTCCTACGCC
		5'-TTCAGGGCAGTGTACGTGAAC
aggrecan	ACAN	5'-GGCCTCTCCAGTCTCATTCTC
		5'-AGGCAGCGTGATCCTTACC
bone sialoprotein I	BSP-I	5'-CCCCACCTTTTGGGAAAACCA
		5'-TCCCCGTTCTCACTTTCATAGAT
Bone gamma-carboxyglutamic acid-containing protein	BGLAP	5'-GGCAGCGAGGTAGTGAAGAG
		5'-GATGTGGTCAGCCAACTCGT
Alkaline phosphatase	ALP	5'-ACAAGCACTCCCACTTCATC
		5'-TTCAGCTCGTACTGCATGTC

Supplementary Table 1. Primers used for qRT-PCR analysis

Gene symbol	Gene description	LogFC *	Location	Function
COMP	Homo sapiens cartilage oligomeric matrix protein	1.93	Extracellular space	other
RDH10	Homo sapiens retinol dehydrogenase 10 (all-trans)	1.39	Nucleus	enzyme
TNS3	Homo sapiens tensin 3	1.33	Plasma Membrane	phosphatase
TIMP3	Homo sapiens TIMP metallopeptidase inhibitor 3	1.28	Extracellular Space	other
HS.551128	Homo sapiens MSTP131	1.13		
LTBP2	Homo sapiens latent transforming growth factor beta binding protein 2	1.12	Extracellular Space	other
C5ORF23	Homo sapiens chromosome 5 open reading frame 23	1.09	-	-
PSAT1	Homo sapiens phosphoserine aminotransferase 1	1.07	Cytoplasm	enzyme
COL11A1	Homo sapiens collagen, type XI, alpha 1, transcript variant A	1.05	Extracellular Space	other
RARRES2	Homo sapiens retinoic acid receptor responder (tazarotene induced) 2	1.04	Plasma Membrane	transmembrane receptor
SERTAD4	Homo sapiens SERTA domain containing 4	1	Other	other
MYH2	Homo sapiens myosin, heavy chain 2, skeletal muscle	0.98	Cytoplasm	enzyme
GALNT1	Homo sapiens UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1	0.95	Cytoplasm	enzyme
SCRG1	Homo sapiens scrapie responsive protein 1	0.89	Extracellular Space	other
GLS	Homo sapiens glutaminase	0.89	Cytoplasm	enzyme
MYH1	Homo sapiens myosin, heavy polypeptide 1, skeletal muscle, adult	0.88	Plasma Membrane	enzyme
PPP1R3C	Homo sapiens protein phosphatase 1, regulatory (inhibitor) subunit 3C	0.87	Cytoplasm	phosphatase
SERPINB7	Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 7	0.87	Cytoplasm	other
FNDC1	Homo sapiens fibronectin type III domain containing 1	0.85	Plasma Membrane	other

ACTA2	Homo sapiens actin, alpha 2, smooth muscle, aorta	0.84	Cytoplasm	other
CHRDL2	Homo sapiens chordin-like 2	0.83	Cytoplasm	other
SCARF2	Homo sapiens scavenger receptor class F, member 2	0.83	Plasma Membrane	transmembrane receptor
COL8A2	Homo sapiens collagen, type VIII, alpha 2	0.82	Extracellular Space	other
C1ORF133	Homo sapiens chromosome 1 open reading frame 133	0.82	-	-
BHLHB2	Homo sapiens basic helix-loop-helix domain containing, class B, 2	0.82	Nucleus	transcription regulator
DDIT4	Homo sapiens DNA-damage-inducible transcript 4	0.81	Cytoplasm	other
TRIL	Homo sapiens TLR4 interactor with leucine rich repeats	0.81	Other	other
VLDLR	Homo sapiens very low density lipoprotein receptor	0.81	Plasma Membrane	transporter
HAPLN1	Homo sapiens hyaluronan and proteoglycan link protein 1	0.79	Extracellular Space	other
ANXA2	Homo sapiens annexin A2 (ANXA2), transcript variant 2	0.79	Plasma Membrane	other
MYLK	Homo sapiens myosin light chain kinase	0.78	Cytoplasm	kinase
NUAK1	Homo sapiens NUAK family, SNF1-like kinase, 1	0.78	Nucleus	kinase
NPR3	Homo sapiens natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	0.77	Plasma Membrane	G-protein coupled receptor
PAWR	Homo sapiens PRKC, apoptosis, WT1, regulator	0.75	Nucleus	transcription regulator
ANXA2P1	Homo sapiens annexin A2 pseudogene 1 on chromosome 4	0.74	Other	other
ITGA11	Homo sapiens integrin, alpha 11	0.74	Plasma Membrane	other
CSRP1	Homo sapiens cysteine and glycine-rich protein 1	0.74	Nucleus	other
CAP2	Homo sapiens CAP, adenylate cyclase-associated protein, 2 (yeast)	0.73	Plasma Membrane	other
TPM1	Homo sapiens tropomyosin 1 (alpha), transcript variant 3	0.71	Cytoplasm	other
HS.557431	RC3-BN0425-011200-022-c08 BN0425 Homo sapiens cDNA	0.7	-	-

COL8A1	Homo sapiens collagen, type VIII, alpha 1, transcript variant 2	0.69	Extracellular Space	other
TLE2	Homo sapiens transducin-like enhancer of split 2	0.68	Nucleus	transcription regulator
HPS5	Homo sapiens Hermansky-Pudlak syndrome 5	0.68	Cytoplasm	other
PLOD2	Homo sapiens procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	0.68	Cytoplasm	enzyme
LIMS2	Homo sapiens LIM and senescent cell antigen-like domains 2	0.67	Cytoplasm	other
SEPT11	Homo sapiens septin 11	0.67	Nucleus	other
TPD52L1	Homo sapiens tumor protein D52-like 1	0.67	Cytoplasm	other
PDLIM7	Homo sapiens PDZ and LIM domain 7 (enigma)	0.66	Cytoplasm	other
ALDH1L2	PREDICTED: Homo sapiens aldehyde dehydrogenase 1 family, member L2	0.65	Cytoplasm	enzyme
PID1	Homo sapiens phosphotyrosine interaction domain containing 1	0.64	Cytoplasm	other
RUSC2	Homo sapiens RUN and SH3 domain containing 2	0.64	Cytoplasm	other
ZNF365	Homo sapiens zinc finger protein 365	0.63	Cytoplasm	other
PCK2	Homo sapiens phosphoenolpyruvate carboxykinase 2 (mitochondrial)	0.62	Cytoplasm	kinase
THBS1	Homo sapiens thrombospondin 1	0.61	Extracellular Space	other
CRABP2	Homo sapiens cellular retinoic acid binding protein 2	0.59	Cytoplasm	transporter
IL32	Homo sapiens interleukin 32	0.57	Extracellular Space	cytokine
C1ORF198	Homo sapiens chromosome 1 open reading frame 198	0.57	-	-
HS3ST3A1	Homo sapiens heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1	0.55	Cytoplasm	enzyme
HOXC8	Homo sapiens homeobox C8	0.54	Nucleus	transcription regulator
SERAC1	Homo sapiens serine active site containing 1.	0.51	Extracellular Space	other

CHI3L1	Homo sapiens chitinase 3-like 1	-1.89	Extracellular Space	enzyme
ADH1A	Homo sapiens alcohol dehydrogenase 1A (class I), alpha polypeptide	-1.43	Cytoplasm	enzyme
MAFB	Homo sapiens v-maf musculoaponeurotic fibrosarcoma oncogene homolog B	-1.38	Nucleus	transcription regulator
CA12	Homo sapiens carbonic anhydrase XII	-1.36	Plasma Membrane	enzyme
CYP1B1	Homo sapiens cytochrome P450, family 1, subfamily B, polypeptide 1	-1.29	Cytoplasm	enzyme
MYBPH	Homo sapiens myosin binding protein H	-1.28	Cytoplasm	other
RASD1	Homo sapiens RAS, dexamethasone-induced 1	-1.22	Cytoplasm	enzyme
CXCL12	Homo sapiens chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	-1.20	Extracellular Space	cytokine
ALDH1A3	Homo sapiens aldehyde dehydrogenase 1 family, member A3	-1.12	Cytoplasm	enzyme
ADH1B	Homo sapiens alcohol dehydrogenase IB (class I), beta polypeptide	-1.12	Cytoplasm	enzyme
OSR1	Homo sapiens odd-skipped related 1	-1.10	Nucleus	other
PDGFRL	Homo sapiens platelet-derived growth factor receptor-like	-1.08	Plasma Membrane	kinase
GALNTL2	Homo sapiens UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 2	-1.06	Cytoplasm	enzyme
COLEC12	Homo sapiens collectin sub-family member 12	-1.05	Plasma Membrane	transmembrane receptor
CLDN23	Homo sapiens claudin 23	-1.03	Plasma Membrane	other
CD14	Homo sapiens CD14 molecule	-1.02	Plasma Membrane	transmembrane receptor
C1R	Homo sapiens complement component 1, r subcomponent	-1.01	Extracellular Space	peptidase
CTSC	Homo sapiens cathepsin C	-1.01	Cytoplasm	peptidase
TRAF3IP2	Homo sapiens TRAF3 interacting protein 2	-0.97	Cytoplasm	other

PKIG	Homo sapiens protein kinase (cAMP-dependent, catalytic) inhibitor gamma	-0.96	Other	other
FAM20A	Homo sapiens family with sequence similarity 20, member A	-0.95	Extracellular Space	other
SRPX	Homo sapiens sushi-repeat-containing protein, X-linked	-0.95	Cytoplasm	other
SLC9A9	Homo sapiens solute carrier family 9 (sodium/hydrogen exchanger), member 9	-0.95	Cytoplasm	transporter
AKR1C4	Homo sapiens aldo-keto reductase family 1, member C4 (chlordecone reductase; 3-alpha hydroxysteroid dehydrogenase, type I; dihydrodiol dehydrogenase 4)	-0.93	Cytoplasm	enzyme
SCARA5	Homo sapiens scavenger receptor class A, member 5 (putative)	-0.93	Cytoplasm	other
ENPP2	Homo sapiens ectonucleotide pyrophosphatase/phosphodiesterase 2	-0.93	Plasma Membrane	enzyme
ANGPTL2	Homo sapiens angiopoietin-like 2	-0.93	Extracellular Space	other
AKR1B10	Homo sapiens aldo-keto reductase family 1, member B10 (aldose reductase)	-0.91	Cytoplasm	enzyme
ALDH1A1	Homo sapiens aldehyde dehydrogenase 1 family, member A1	-0.91	Cytoplasm	enzyme
ADORA1	Homo sapiens adenosine A1 receptor	-0.87	Plasma Membrane	G-protein coupled receptor
VWCE	Homo sapiens von Willebrand factor C and EGF domains	-0.86	Other	other
PCOLCE2	Homo sapiens procollagen C-endopeptidase enhancer 2	-0.85	Extracellular Space	other
LDLR	Homo sapiens low density lipoprotein receptor (familial hypercholesterolemia)	-0.84	Plasma Membrane	transporter
CLDN11	Homo sapiens claudin 11 (oligodendrocyte transmembrane protein)	-0.84	Plasma Membrane	other
RCAN2	Homo sapiens regulator of calcineurin 2	-0.83	Other	other
CTSB	Homo sapiens cathepsin B	-0.82	Cytoplasm	peptidase

C10ORF10	Homo sapiens chromosome 10 open reading frame 10	-0.82	-	-
KIAA1324L	Homo sapiens KIAA1324-like	-0.80	Other	other
PPAP2B	Homo sapiens phosphatidic acid phosphatase type 2B	-0.79	Plasma Membrane	phosphatase
AKR1C2	Homo sapiens aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	-0.79	Cytoplasm	enzyme
PENK	Homo sapiens proenkephalin	-0.79	Extracellular Space	other
CFD	Homo sapiens complement factor D (adipsin)	-0.79	Extracellular Space	peptidase
DDIT4L	Homo sapiens DNA-damage-inducible transcript 4-like	-0.78	Cytoplasm	other
SNTB1	Homo sapiens syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)	-0.77	Plasma Membrane	other
C13ORF33	Homo sapiens chromosome 13 open reading frame 33	-0.76	-	-
LAMA4	Homo sapiens laminin, alpha 4	-0.76	Extracellular Space	enzyme
RNASE4	Homo sapiens ribonuclease, RNase A family, 4	-0.76	Extracellular Space	enzyme
ALDH3A2	Homo sapiens aldehyde dehydrogenase 3 family, member A2	-0.76	Cytoplasm	enzyme
PDPN	Homo sapiens podoplanin	-0.75	Plasma Membrane	other
RARRES3	Homo sapiens retinoic acid receptor responder (tazarotene induced) 3	-0.75	Cytoplasm	enzyme
LRRN4CL	Homo sapiens LRRN4 C-terminal like	-0.74	Other	other
IFI16	Homo sapiens interferon, gamma-inducible protein 16	-0.74	Nucleus	transcription regulator
ABCA8	Homo sapiens ATP-binding cassette, sub-family A (ABC1), member 8	-0.74	Plasma Membrane	transporter
PPL	Homo sapiens periplakin	-0.73	Cytoplasm	other
DCN	Homo sapiens decorin	-0.71	Extracellular Space	other

PDGFRA	Homo sapiens platelet-derived growth factor receptor, alpha polypeptide	-0.71	Plasma Membrane	kinase
CCBE1	Homo sapiens collagen and calcium binding EGF domains 1	-0.71	Extracellular Space	other
IL1R1	Homo sapiens interleukin 1 receptor, type I	-0.70	Plasma Membrane	transmembrane receptor
MAFF	Homo sapiens v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	-0.70	Nucleus	transcription regulator
FAM65C	Homo sapiens family with sequence similarity 65, member C	-0.70	Other	other
CTSL1	Homo sapiens cathepsin L1	-0.69	Cytoplasm	peptidase
BDKRB2	Homo sapiens bradykinin receptor B2	-0.69	Plasma Membrane	G-protein coupled receptor
CPXM1	Homo sapiens carboxypeptidase X (M14 family), member 1	-0.68	Extracellular Space	peptidase
HLA-B	Homo sapiens major histocompatibility complex, class I, B	-0.68	Plasma Membrane	transmembrane receptor
UNC84A	Homo sapiens unc-84 homolog A (C. elegans)	-0.68	Nucleus	other
MARCKSL1	Homo sapiens MARCKS-like 1	-0.67	Cytoplasm	other
PROCR	Homo sapiens protein C receptor, endothelial (EPCR)	-0.67	Plasma Membrane	other
MAP3K8	Homo sapiens mitogen-activated protein kinase kinase kinase 8	-0.67	Cytoplasm	kinase
RNF144	Homo sapiens ring finger protein 144	-0.66	Nucleus	other
DUSP5	Homo sapiens dual specificity phosphatase 5	-0.65	Nucleus	phosphatase
IFFO1	Homo sapiens intermediate filament family orphan 1	-0.65	Other	other
ARHGEF3	Homo sapiens Rho guanine nucleotide exchange factor (GEF) 3	-0.64	Cytoplasm	other
LOC100134304	PREDICTED: Homo sapiens similar to hCG1983233	-0.64	-	-
C16ORF45	Homo sapiens chromosome 16 open reading frame 45	-0.64	-	-

SIPA1L2	Homo sapiens signal-induced proliferation-associated 1 like 2	-0.63	Other	other
TNFRSF21	Homo sapiens tumor necrosis factor receptor superfamily, member 21	-0.63	Plasma Membrane	transmembrane receptor
HLA-F	Homo sapiens major histocompatibility complex, class I, F	-0.62	Plasma Membrane	transmembrane receptor
FYN	Homo sapiens FYN oncogene related to SRC, FGR, YES	-0.61	Plasma Membrane	kinase
CD59	Homo sapiens CD59 molecule, complement regulatory protein	-0.61	Plasma Membrane	other
CDKN2C	Homo sapiens cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	-0.60	Nucleus	transcription regulator
C1ORF21	Homo sapiens chromosome 1 open reading frame 21	-0.59	-	-
STEAP1	Homo sapiens six transmembrane epithelial antigen of the prostate 1	-0.59	Plasma Membrane	transporter
PELI2	Homo sapiens pellino homolog 2 (Drosophila)	-0.57	Cytoplasm	other
PHKG1	Homo sapiens phosphorylase kinase, gamma 1 (muscle)	-0.57	Cytoplasm	kinase
HLA-H	Homo sapiens major histocompatibility complex, class I, H (pseudogene)	-0.56	Plasma Membrane	other
NTNG1	Homo sapiens netrin G1	-0.56	Extracellular Space	other
C1S	Homo sapiens complement component 1, s subcomponent	-0.56	Extracellular Space	peptidase
ZCCHC14	Homo sapiens zinc finger, CCHC domain containing 14	-0.54	Other	other
PSMB8	Homo sapiens proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	-0.54	Cytoplasm	peptidase
SASH1	Homo sapiens SAM and SH3 domain containing 1	-0.53	Extracellular Space	other

Supplementary Table 2. Two donor gene co-regulation in tendon-derived cells co-cultured with myoblasts for 7 days.

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Chapter 5

High-Throughput Screening Assay Identifies Small Molecules Capable of Modulating the BMP-2 and TGF-β1 Signalling Pathway

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ABSTRACT

Modulating the bone morphogenetic protein 2 (BMP-2) and transforming growth factor-β1 (TGF-β1) signalling pathways is essential during tendon/ligament (T/L) healing. Unfortunately, growth factor delivery in situ is far from trivial and, in many cases, the necessary growth factors are not approved for clinical use. Here we used a BMP-2 and a TGF-β1 reporter cell line to screen a library of 1280 Food and Drug Administration-approved small molecules and identify modulators of both signalling pathways. We identified four compounds capable of modulating BMP and TGF signalling on primary human tendon-derived cells (huTCs) and describe their effects on proliferation and differentiation of these cells.

INTRODUCTION

Despite significant advances in unravelling the mechanisms governing tendon/ligament (T/L) healing and improving the clinical management of these injuries, the outcome is far from ideal. Animal and cellular models developed to identify the molecular drivers of T/L healing and repair lend evidence to the importance of specific growth factors in the process. Secreted growth factors (GFs) such as bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) have been identified as key players in the process of T/L specification during embryonic development as well as on the healing and repair upon injury [1, 2]. Several reports demonstrated that GFs promoted cell differentiation, upregulated cartilage/bone markers known to be involved in enthesis formation and induce cytoskeletal organization, thus contributing to the restoration of tissue strength and functionality [3-5]. Nonetheless, the concentration and spatial distribution of these endogenous GFs during T/L healing are not sufficient to shorten the repair period that may take from several months to a year [6]. In an attempt to overcome these limitations, several groups suggested that a combination of scaffolds and recombinant human GFs, such as rhTGF-β1 and rhBMP-2, could accelerate T/L healing [7, 8]. However, even though recombinant human material has shown great potential in tissue regeneration, their biological nature poses several challenges for their production regarding batch variability, stability, biological activity, and production costs [9], emphasizing the need for an alternative such as the identification and/or development of small molecules with the desired biological properties.

Consequently, we screened a library of 1,280 Food and Drug Administration (FDA)-approved small molecules using a BMP-2 and a TGF-β1 reporter cell line and identified small molecules capable of modulating both pathways. Subsequent assays used human tendon-derived cells (huTCs) to investigate the effect of the selected molecules in huTC metabolism as well as differentiation potential. A schematic overview of the experiment design is represented in Figure 1.

MATERIALS AND METHODS

Cell types

Mink lung epithelial cells (MLEC) previously transfected with a reporter plasmid consisting of TGF-β-responsive elements from the PAI-1 promoter fused to a luciferase reporter gene [10], and C2C12 transfected with a reporter plasmid consisting of BMP-responsive elements from the Id1 promoter fused to a luciferase reporter gene [11] were a generous gift from DB Rifkin.

huTCs were isolated from human hamstring tendon tissue using 0.15 % (w/v) collagenase type II solution (Worthington) digestion in basic media (see below) as previously described [12]. The collection and anonymous use of the tissue was performed

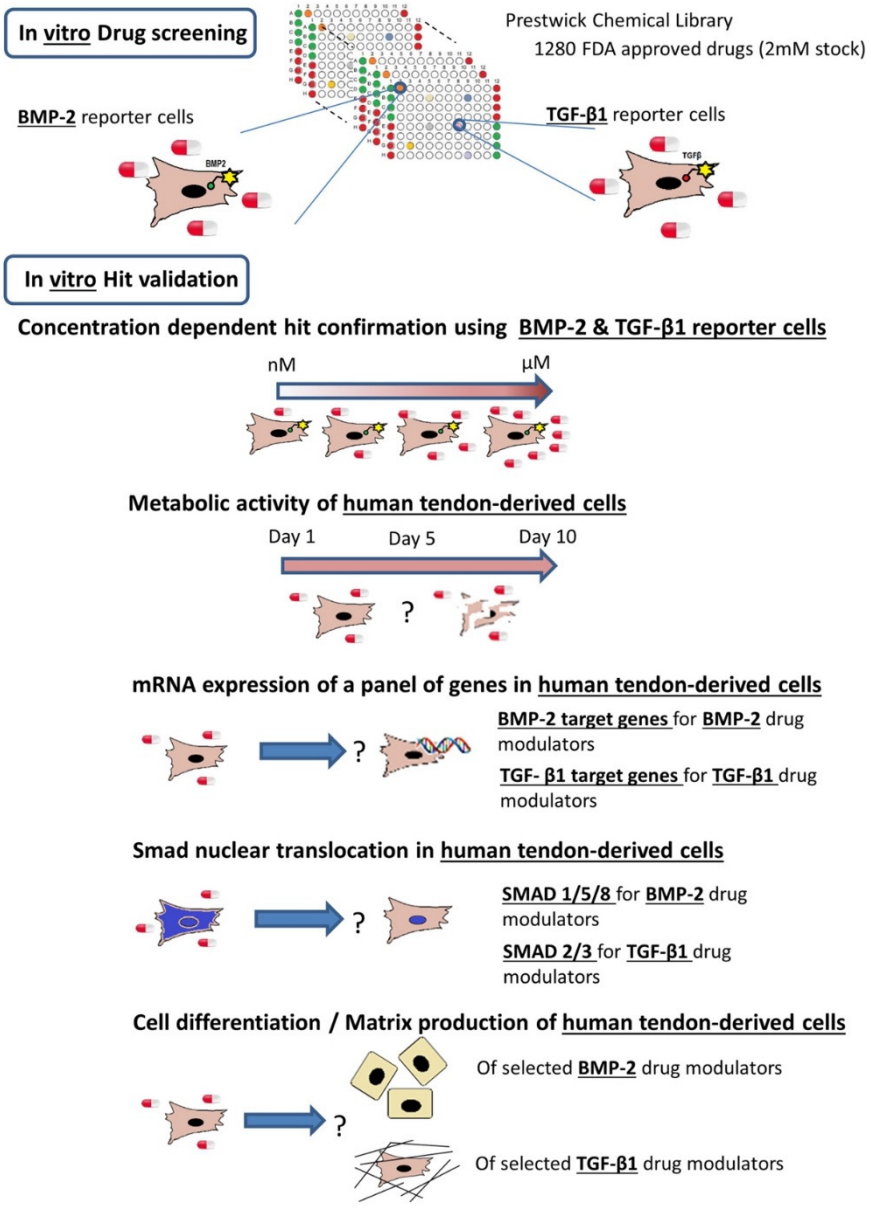


Figure 1. Schematic overview of the experimental design.

according to the medical ethical regulations and the guideline 'good use of redundant tissue for research of the Dutch Federation of Medical Research Societies'.

All cells were cultured in basic media (BM) consisting of DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) and 0.2 mM ascorbic acid (Sigma).

Small-molecule high throughput screening

The Prestwick Chemical Library consisting of 1,280 FDA-approved small molecules (Prestwick Chemical, Inc.) was dissolved in DMSO to a final concentration of 2 mM.

BMP-2 reporter cells were seeded onto 96-well cell culture plates at 10,000 cells/well in BM and allowed to attach for 24 hours at 37°C, 5% CO_2 and 95% humidity. Small molecules were then added to the wells at a final concentration of 5 µM and DMSO treated cells were used as negative control, while cells treated with 2.5 nM rhBMP2 were used as a positive control. Cells were further cultured for 24 hours upon which media containing the small molecules was removed, cells were washed one time with PBS and Luciferase assay (Promega) was used per the manufacturer´s protocol.

Similarly, TGF-β1 reporter cells were seeded onto 96-well cell culture plates at 20,000 cells/well and allowed to attach for 24 hours at 37°C, 5% CO_2 and 95% humidity. Small molecules were then added to a final concentration of 5 µM and DMSO treated cells were used as the negative control, while cells treated with 0.8 nM rhTGF-β1 were used as positive control. Cells were further cultured for 24 hours upon which media containing small molecules was removed, cells were washed one time with PBS and Luciferase assay (Promega) was used per the manufacturer´s protocol.

Luminescence was read at 590 nm on a Victor plate reads (Perkin Elmer, Wellesley, MA) and reported as relative light units (RLU). A hit was identified if, upon exposure to a small molecule, the resulting luciferase activity was at least 2 times higher than the negative control. Small molecules that fulfilled this threshold were purchased from Prestwick Chemical, Inc. and retested in a dose-dependent manner (from nM to µM) under the same seeding and culture conditions. The compounds showing an increased luciferase activity compared to the negative control were selected for further investigation using huTCs.

Metabolic activity

huTCs were seeded on 96-well cell culture plates at 4,000 cells/well in BM and allowed to attach for 24 hours. The next day, cells were treated with different concentrations of the selected compounds in a dose-dependent manner (from nM to µM). After 1, 5, and 10 days metabolic activity was measured after incubation for 1 hour with 10% (v/v) Presto Blue Cell Viability Reagent (Invitrogen) in BM and absorbance was measured at 590 nm (n=3).

Gene expression analysis

huTCs were seeded on 6-well cell culture plates at 50,000 cells/well in BM and cultured until confluent. BM was then replaced with starvation media (SM) consisting of BM without FBS and further cultured for 24 hours upon which the selected compounds were added in BM for further 24 hours.

Total RNA was isolated using the NucleoSpin RNA II isolation kit (Macherey-Nagel), per the manufacturer's instructions. RNA was collected in RNase-free water and a quantitative analysis was performed using spectrophotometry (Nanodrop). First-strand cDNA was synthesized from 0.6 µg total RNA/sample, using iScript (BioRad) per the manufacturer's instructions. PCR was performed on a real-time PCR detection system (Biorad), using iQ Syber green supermix (BioRad) for different genes, as presented in Table 1, or TaqMan Universal MasterMix for B2M and scleraxis (SCX). Gene expression was normalized to the reference gene B2M and fold induction calculated using the ΔΔCt method, presented on a Log2 scale.

Target Genes	Primer Sequence
B2M	5' -GACTTGTCTTTCAGCAAGGA 5' -ACAAAGTCACATGGTTCACA
COL IA1	5'-GTCACCCACCGACCAAGAAACC 5'-AAGTCCAGGCTGTCCAGGGATG
COL IIIA1	5'-GCCAACGTCCACACCAAATT 5'-AACACGCAAGGCTGTGAGACT
TNC	5'-TGGGCAGATTTCACGGCTG 5'-TGCTCTGAGCCCGAATGTC
TIMP1	5' -CTTCTGCAATTCCGACCTCGT 5' -ACGCTGGTATAAGGTGGTCTG
TIMP3	5' -CCAGGACGCCTTCTGCAAC 5' -CCTCCTTTACCAGCTTCTTCCC
MMP1	5' -GGGAGATCATCGGGACAACTC 5' -GGGCCTGGTTGAAAAGCAT
MMP3	5' -TGGCATTCAGTCCCTCTATGG 5' -AGGACAAAGCAGGATCACAGTT
BMP-2	5' -GCTAGACCTGTATCGCAGGC 5' -TTTTCCCACTCGTTTCTGGT
BMP-4	5' -AGCGTAGCCCTAAGCATCAC 5' -AGTCATTCCAGCCCACATCG
ALPL	5' -ACTGGTACTCAGACAACGAGAT 5' -ACGTCAATGTCCCTGATGTTATG
RUNX2	5' -GGAGTGGACGAGGCAAGAGTTT 5' -AGCTTCTGTCTGTGCCTTCTGG

Table 1. Primers used for Real-Time Polymerase Chain Reaction Analysis

SMAD2/3 and SMAD 1/5/8 nuclear translocation

huTCs were seeded on 96-well cell culture plates at 5,000 cells/well in BM and allowed to attach for 24 hours. The next day, BM was replaced with SM for 5 hours followed by 30 minutes or 2 hours incubation in BM in the presence of quinacrine (5 μM), niclosamide (100, 500 nM or 1 μM), vorinostat (5, 10 μM) or hycanthone (5, 10 μM). Cells exposed to 2.5 nM rhBMP-2 or 0.2 nM rhTGF-β1 were considered as positive control. Following, the cells were washed with PBS and fixed with 10% formalin solution neutral buffered (Sigma) for 15 minutes, after which they were stained either for SMAD2/3 (for TGF-β mediated translocation) or SMAD1/5/8 (for BMP mediated translocation). Fixed cells were permeabilised with 0.2% (v/v) Triton X-98 in PBS for 15 minutes and then blocked in 0.2% (v/v) bovine serum albumin/0.2% Triton X-98/PBS for 1 hour. The primary antibody against SMAD2/3 (mouse anti-smad2/3, BD Bioscience) or SMAD1/5/8 (rabbit anti-smad1/5/8, Santa Cruz Biotechnology) were used at a dilution of 1:200 in 0.2% bovine serum albumin/PBS and incubated for 3 hours. The secondary antibody Alexa Fluor 546 goat anti-mouse (Life Technologies) was used against SMAD2/3 and Alexa Fluor 549 goat anti-rabbit (Invitrogen) was used against SMAD1/5/8 at a dilution of 1: 200 in 0.2% bovine serum albumin/PBS for 1 hour in the dark. Afterwards, cells were counterstained with 2.8 mM DAPI (Invitrogen) in 0.2% bovine serum albumin/PBS for 15 minutes, upon which cells were washed with PBS. All incubation steps were performed under orbital shaking and at ambient room temperature.

Fluorescence images were taken using BD Pathway 435 after adjusting the staining intensity to the isotype (secondary antibody alone) and quantification of SMAD nuclear translocation was assessed using CellProfiler open source software [13]. Following, the integrated intensity measured both for the cytoplasmic and the nuclear SMAD was used to calculate the ratio nucleus:cytoplasm SMAD.

Alkaline phosphatase activity

huTCs were seeded on 12-well cell culture plates at 50,000 cells/well in BM. The next day, BM was replaced with fresh media and 100 nM niclosamide or 4 nM rhBMP-2 was added to the wells (n=3). Cells were cultured for 7 days with one media change at day 3. At day 7, cells were fixed in 10% formalin solution neutral buffered (Sigma) for 15 minutes and stained using the Leukocyte Alkaline Phosphatase Kit -ALP (Sigma, 85L2-1KT) following the manufacturer´s instructions. Images of the stained wells were acquired using an Epson Perfection V750 PRO scanner and quantification was assessed using FIJI open source software [14]. Equal sized squares were cropped from the wells, converted to 8-bit and background subtracted using the rolling ball radius function. The image was then converted into binary, the area of ALP staining was selected and the total number of pixels measured (Supplementary Figure 1).

Collagen content analysis

huTCs were seeded on 6-well cell culture plates at 50,000 cells/well in BM and cultured until confluent, upon which media was refreshed and vorinostat (5 and 10 μM) or 0.2 nM rhTGF-β1 was added to the wells (n=3). Vorinostat or rhTGF-β1 were freshly added every three days when media was refreshed and at day 10, cells were fixed in 10% formalin for 15 minutes at room temperature and stained for collagen deposition using the Picrosirius red stain kit (Polyscience Inc.) following the manufacturer´s instructions. Images of the stained wells were acquired and quantified using FIJI open source software as described above.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6 software. Two-way ANOVA and a Tukey post hoc test were used to compare the different conditions. A value of $p \leq 0.05$ indicates a statistical significant difference. Results are shown as mean ± standard deviation.

RESULTS

Identification and validation of small molecules capable of modulating TGF-β1 and BMP-2 reporter cell lines

A library of 1,280 FDA-approved small molecules was screened to identify modulators of BMP-2 and TGF-β1 signaling. BMP-2 and TGF-β reporter cell lines were exposed for 24 hours to the small molecules (5 μM) and hits were selected if a compounds´ luciferase activity was at least two times higher than the negative controls. Seven BMP-2 and three TGF-β1 small molecules were identified as primary hits. Pirlindole mesylate, pramipexole, nylidrin, butylscopolammonium (n-) bromide, quinacrine dihydrochloride dehydrate (quinacrine), chlorhexidine and niclosamide were identified as modulators of the BMP-2 reporter cell line, whereas hycanthone, etoposide and vorinostat were identified as modulators of the TGF-β1 reporter cell line (Figure 2A, C and Supplementary Figure 2).

Subsequently, dose-response curves (1 nM to 100 μM, n=3) were generated for the selected primary hits. Quinacrine and niclosamide were validated as potential BMP-2 modulators, while hycanthone and vorinostat were validated as potential TGF-β1 modulators (Figure 2B, D).

Effect of the validated hits on metabolic activity of huTCs

Given that our final goal is to use the identified hits for T/L regeneration, we tested the identified hits on huTCs previously isolated and described [12]. To that end, a time dependent dose-response curve was performed and metabolic activity was measured at

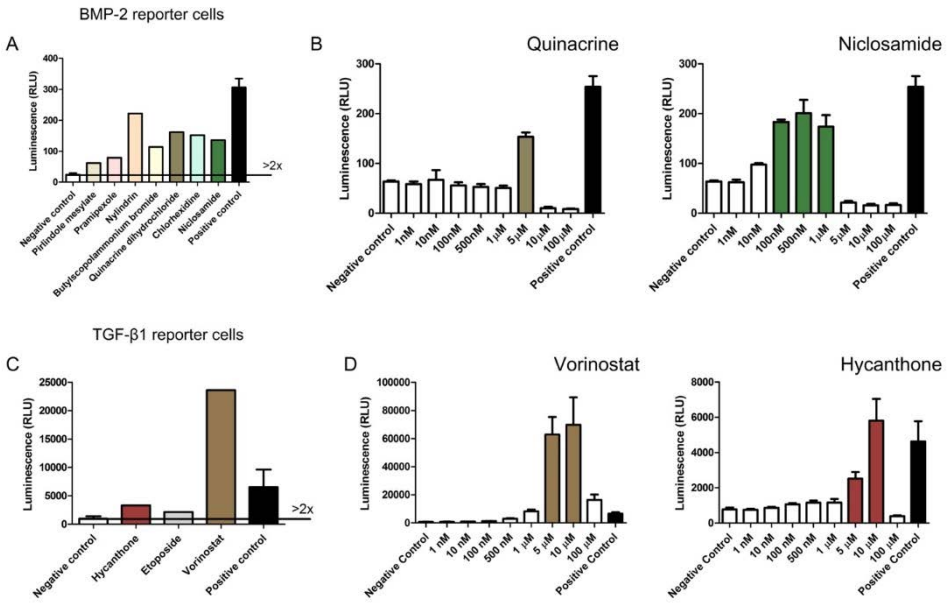


Figure 2. Small-molecule library screening for BMP-2 and TGF-β1 modulators. *BMP-2 and TGF-β1 reporter cell lines were independently plated on to 96-well cell culture plates and compounds were added for 24 hours at a concentration of 5 μM, after which cells where lysed and luciferase activity measured. Positive control are cells cultured in the presence of 2.5 nM rhBMP-2 or 0.8 nM rhTGF-β1, while negative control are cells cultured in basic media. A hit was identified if, upon exposure to a small molecule, the resulting luciferase activity was at least 2 times higher than the negative control. (A) BMP-2 hits identified in the screening assay. (B) Compounds were retested in a dose-dependent manner (from nM to μM) under the same seeding and culture conditions. We identified quinacrine and niclosamide as BMP-2 modulators. (C) TGF-β1 hits found in the screening assay. (D) Compounds were retested in a dose-dependent manner (from nM to μM) under the same seeding and culture conditions. We identified vorinostat and hycanthone as TGF-β1 modulators. RLU, relative light units.*

day 1, 5 and 10. For BMP-2 modulators our results showed a statistical significant increase in metabolic activity for quinacrine (250 nM) and niclosamide (100 nM) when compared to rhBMP-2 (Figure 3A). Moreover, for the time and concentrations tested herein, an increase in metabolic activity during time was observed for all concentrations, with exception of quinacrine (5 μM) and niclosamide (1 μM) which inhibited metabolic activity both at day 5 and 10. Noteworthy, all the groups treated with rhBMP-2 or the BMP-2 modulators showed lower metabolic activity relative to BM suggesting that both BMP-2 and BMP-2 modulators reduce the metabolic activity of huTCs cultures, possibly by reducing cell proliferation in favour of cell differentiation.

For TGF-β1 modulators, all concentrations showed an increase in metabolic activity during the first 5 days. However, when compared to rhTGF-β1 and BM control, the metabolic activity for all the concentrations tested was statistically significant reduced with exception of 100 nM hycanthone. At day 10, metabolic activity either reached a

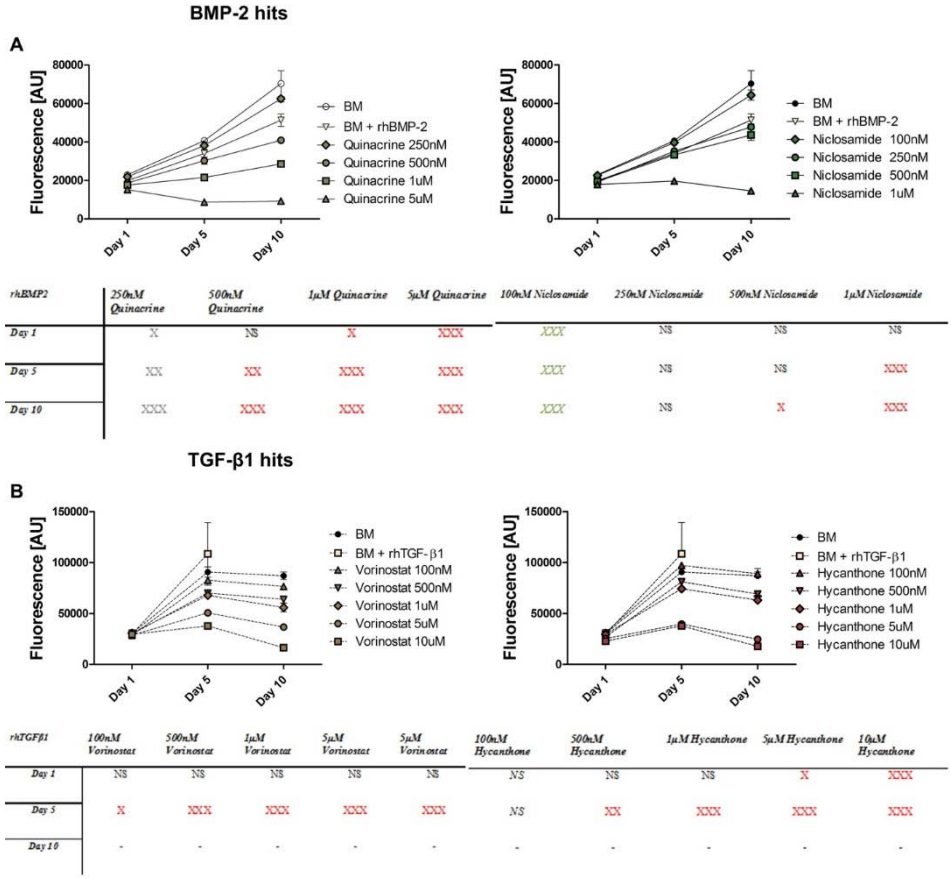


Figure 3. Metabolic activity of huTCs exposed to (A) BMP-2 and (B) TGF-β1 modulators in a dose- and time-dependent manner. *Positive control are cells cultured in the presence of 2.5 nM rhBMP-2 or 0.8 nM rhTGF-β1, while negative control are cells cultured in basic media. Note: rhTGF-β1 positive control was lost at day 10 due to confluent cell layer and pellet cell formation. Statistically significant difference is represented in the table, where green stands for a statistical significant increase and red for decrease in metabolic activity compared to rhBMP-2 or rhTGF-β1 control, with XXX $p < 0.001$, XX $p < 0.01$ and X $p < 0.05$. NS, not significant.*

plateau or became partially inhibited, possible due to high number of cells which, in the case of TGF-β1 control, led to cell detachment and pellet formation by day 10 (Figure 3B).

Following, as the concentrations found to induce BMP-2 and TGF-β1 activation in the reporter cells inhibited the metabolic activity of huTCs, we decided to test whether acute exposure (24h) rather than chronic exposure (10 days) could modulate both signalling pathways in huTCs.

Identified hits modulate BMP-2 and TGF-β1 target gene expression

To examine whether the identified hits modulate the expression of genes involved in BMP-2 or TGF-β1 pathway signalling, we analysed the expression of a panel of genes after acute exposure (24h) of huTCs to the to the selected hits.

Our results showed that 100 nM niclosamide significantly increased the expression of Collagen IA1 (COL1A1) by 1.3-fold ± 0.2, followed by a trend in increased expression in runt related transcription factor 2 (RUNX2) by 0.7-fold ± 0.3, alkaline phosphatase, liver/bone/kidney (ALPL) by 0.6-fold ± 0.3, BMP2 by 0.4-fold ± 0.3 and BMP4 by 0.4-fold ± 0.2, when compared to both BM and rhBMP-2 control. Next, we found that 5 μM quinacrine significantly increases the expression of BMP2 and BMP4 by 2.8 ±0.4 but not the expression of other osteogenic target genes, which were significantly downregulated when compared to both BM and rhBMP-2 control (Figure 4A).

For the TGF-β1 hits, our results showed that exposing huTCs to vorinostat partly induced expression of tendon and extracellular matrix (ECM) genes. Compared to BM, both 5 and 10 μM vorinostat significantly increased the expression of SCX by 0.6 ± 0.1 and 1.5-fold ± 0.1, TIMP metallopeptidase inhibitor 1 (TIMP1) by 0.8-fold ± 0.04 and 1-fold ± 0.2 and TIMP3 by 0.6-fold ± 0.03 and 1.1-fold ± 0.2. Additionally, further increase, though not statistically significant, was observed for TNC by 0.3-fold ± 0.03 and 0.2-fold ± 0.2, and matrix metallopeptidase 1 (MMP1) by 0.7 ± 0.4 and 0.5 ± 0.4. COL 1A1, COL 3A1 and MMP3 remained at comparable levels to BM. In contrast to vorinostat, 5 and 10 μM hycanthone showed an inhibition of all genes with exception of MMP1 and MMP3 where a statistically significant increase was observed for the highest concentration tested (Figure 4B).

Similar results were obtained with a second donor (Supplementary Figure 3).

BMP-2 and TGF-β1 hits modulate SMAD nuclear translocation

To disclose the mechanism of action of the identified BMP-2 and TGF-β1 hits, we investigated how they modulate SMAD nuclear translocation in huTCs. To do so, cells were exposed to the selected hits for 30 min or 2 hours and SMAD1/5/8 (BMP-2 signalling pathway) or SMAD2/3 (TGF-β1 signalling pathway) nuclear translocation was analysed using immunocytochemistry.

Exposure of huTCs to BMP-2 hits for 30 minutes or 2 hours in BM or SM did not affect SMAD1/5/8 nuclear translocation. Although not statistically significant, after 30 minutes a trend towards increased SMAD1/5/8 nuclear translocation was observed for 5 μM quinacrine and 1 μM niclosamide and after 2 hours for 100 and 500 nM niclosamide.

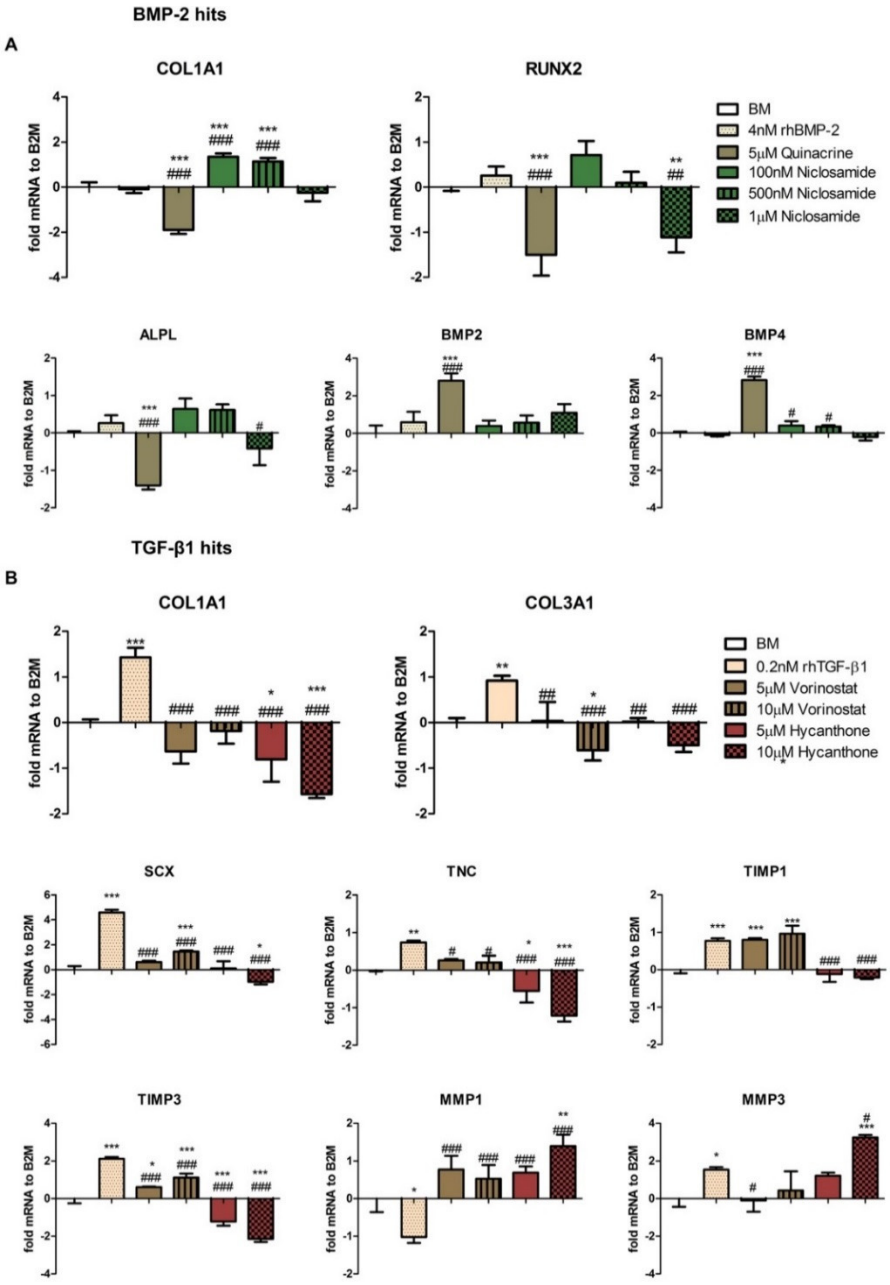


Figure 4. Gene expression analysis of huTCs exposed for 24 hours *to (A) BMP-2 and (B) TGF-β1 modulators. Statistically significant differences were analysed between BM and BMP-2/TGF-β1 modulators (*) or between rhBMP-2/rhTGF-β1 and BMP-2/TGF-β1 modulators (#), with ***/###p < 0.001, **/##p < 0.01 and */#p < 0.05.*

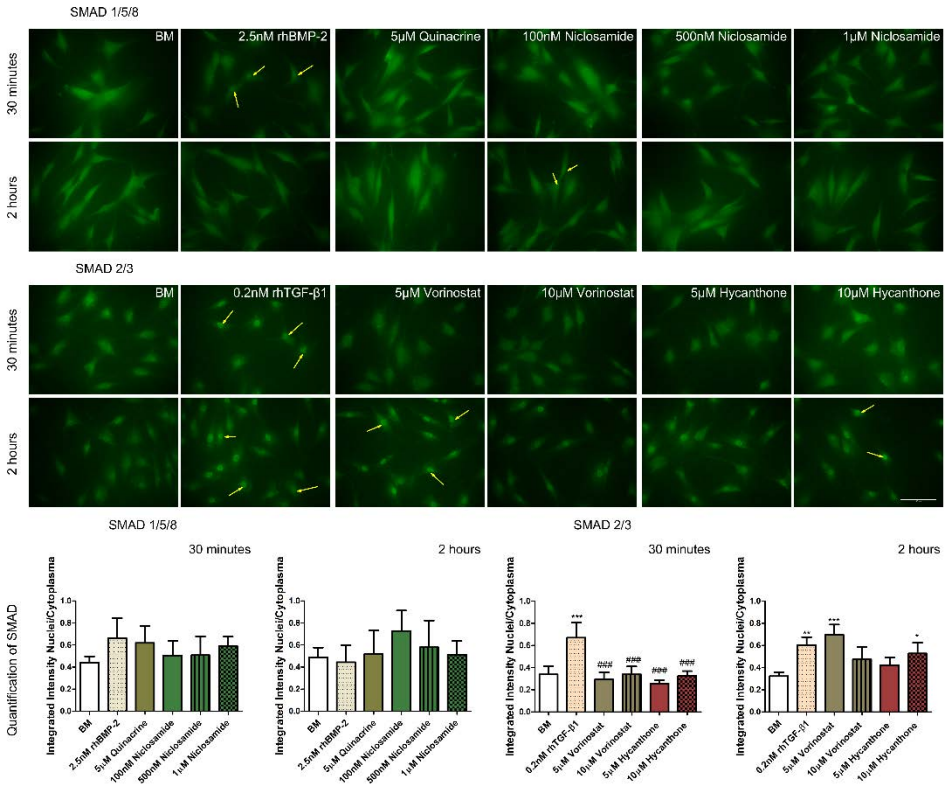


Figure 5. Immunofluorescence staining of SMAD1/5/8 and SMAD2/3 on huTCs exposed to BMP-2 or TGF-β1 modulators for 30 minutes or 2 hours. *Yellow arrows indicate SMAD nuclear translocation. Quantification of SMAD1/5/8 or SMAD2/3 nuclear translocation is presented as a ratio between integrated staining intensity between nuclei and cytoplasm. Values are represented as mean ± standard deviation of at least three independent pictures from different wells (n ≥ 3). Statistically significant differences were found only when comparing BM with TGF-β1 modulators (*) or rhTGF-β1 with TGF-β1 modulators (#). Scale bar corresponds to 100 μm.*

For the identified TGF-β1 hits, exposure of huTCs to the selected hits for 30 minutes did not increase SMAD2/3 nuclear translocation when compared to BM. However, after 2 hours exposure to 5 μM vorinostat and 10 μM hycanthone a statistically significant increase in SMAD2/3 nuclear translocation was observed compared to BM. Interestingly, this increase was similar to the one observed with rhTGF-β1 control (Figure 5).

BMP-2 hits modulate ALP activity

Following the increase in metabolic activity and in the expression of osteogenic genes upon exposure of huTCs to 100 nM niclosamide we decided to analyse its effect on ALP activity, an early marker for *in vitro* osteogenesis. During a 7 days exposure of huTCs to 100 nM niclosamide the ALP activity did not increase neither interfered with

BMP2-mediated increase in ALP activity (Figure 6A). Additionally, exposure of huTCs to 5 µM quinacrine during 7 days led to cell death and consequently no ALP activity could be analysed.

TGF-β1 hits modulate collagen production

To investigate whether vorinostat influences the production of ECM we exposed huTCs to 5 and 10 µM vorinostat, intermittently for 2 hours a day, for 10 days and analysed collagen production using a picrosirius red staining. Our results demonstrate that, as expected, exposure of huTCs to rhTGF-β1 led to an increase in collagen production whereas exposure to vorinostat, regardless of the concentration used, did not increase collagen production (Figure 6B).

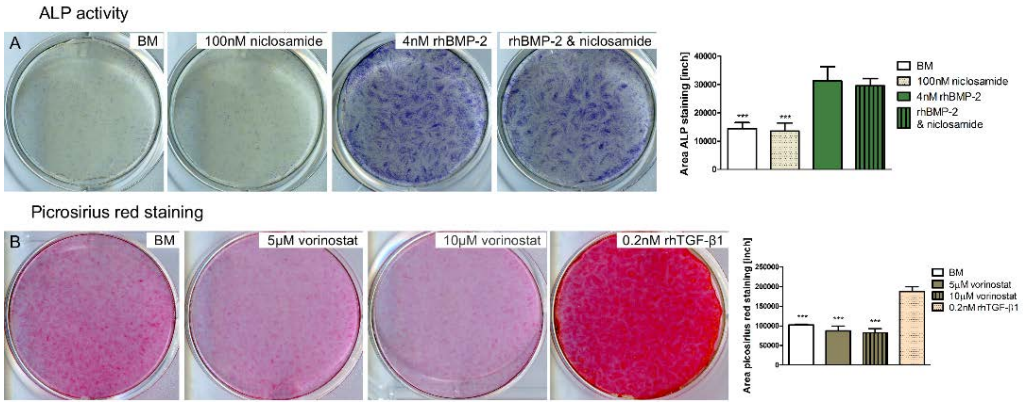


Figure 6. (A) ALP activity staining of huTCs exposed to niclosamide and rhBMP-2 for 7 days. (B) Picrosirius red staining of huTCs exposed to vorinostat intermittently for 2 hours each day, during a period of 10 days. *The intensity of ALP and picrosirius red staining was quantified using Fiji open-source software and statistically significant differences were identified between rhBMP-2/rhTGF-β1 and the other conditions, with ***p < 0.001 and **p < 0.01.*

DISCUSSION

In this study we aimed to identify FDA-approved compounds capable of modulating BMP and TGF-β signalling. The identification of such molecules could pave the way to new therapies capable of inducing bone and matrix formation, key actors in tendon/ligament enthesis formation [15] and middle core repair [16, 17]. Currently, the only approved drug that stimulates bone formation is parathyroid hormone (PTH), which was lately reported to stimulate bone resorption while increasing bone mineral density [18]. Furthermore, rhBMP2 was approved as a bone graft substitute to achieve fracture repair in clinical use [19]. However, the need to use supra-physiological doses and the high production costs creates a barrier for routine clinical application and emphasizes the need to discover new bone anabolic agents that either replace or reduce the basal concentration of rhBMP-2 used in patients. rhTGF-β has not been approved

for clinical application. Other than the previously described compounds, there are no other FDA-approved compounds that modulate the TGF-β pathway.

We screened a commercially available library of FDA-approved compounds using genetically modified reporter cell lines for BMP-2 and TGF-β1, and identified several small molecules that modulate BMP and TGF-β signaling pathways. Dose-response curve analysis led to the identification of two BMP-2 (quinacrine dihydrochloride dehydrate and niclosmaide) and two TGF-β1 (hycanthone and vorinostat) candidates. All four compounds have been previously described in literature to have antineoplastic potential, showing different mechanism of action. Quinacrine has been described to intercalate with DNA, inhibit some flavoprotein enzymes and uncouple oxidation and phosphorylation [20]. Niclosamide has been described to inhibit glucose uptake, oxidative phosphorylation and anaerobic metabolism [21, 22]. Hycanthone has been described to intercalate into DNA and inhibit RNA synthesis *in vitro* [23] and vorinostat is a powerful reported histone deacetylases inhibitor [24].

According to their antineoplastic properties, we were concerned that the identified compounds may dominate our screen with antiproliferative properties, which we identified by a decrease in metabolic activity for quinacrine, vorinostat, hycanthone but not for niclosamide. Following, we decided to test whether acute exposure (24h) rather than chronic exposure (10 days) could modulate both signalling pathways in TCs and investigated transcriptional activation of BMP and TGF-β responsive genes. Niclosamide (100 nM) was found to induce expression of BMP signalling genes, in line with a recent study that screened for osteogenic compounds using a SP7:luciferase transgenic zebrafish model and where niclosamide was identified as a potential lead osteogenic compound [25]. In an attempt to better understand its function we screened the literature and found that niclosamide treatment does not inhibit the Wnt/β-catenin signalling pathway [26], which can act downstream of BMP-2 signalling to promote osteoblast cell fate decision in multipotent progenitors [27]. We also showed that quinacrine induces expression of BMP signalling genes (BMP2 and BMP4) in both donors and reached up to 4 times higher levels than the addition of rhBMP-2 alone. This finding corroborates with a previous study reporting that quinacrine stimulates endogenous BMP-2 upregulation *in vitro* [28]. According to literature and based on our findings, we believe that quinacrine specificity to intercalate with the DNA [29] is not random but rather selective, as we and others have shown that it promotes BMP transcription and where future expertise is needed.

From the TGF-β1 modulators we have shown that only vorinostat, but not hycanthone, induces the expression of TGF-β responsive genes, like SCX, TNC, TIMPs and MMPs. As a HDAC inhibitor, vorinostat upregulates the cellular acetylation level, and therefore accelerates the natural epigenetic process [30] that we believe to potentially be of great help during tendon remodeling.

So far, we have shown that our identified compounds modulate the expression of BMP-2 and TGF-β1 target genes. However, we could not observe an increase in ALP activity for niclosamide and quinacrine and neither did we observed an increase on ECM

production in huTCs exposed to vorinostat. Our concern that the antiproliferative or cytotoxic effects of the compounds might hinder downstream assays to study the functionality of the cells turned out to be true for quinacrine but not for niclosamide or vorinostat. Even though we did not observe an increase in ALP activity in the presence of niclosamide or collagen production in the presence of vorinostat, the protein expression levels remained at comparable levels with basic media with no observable changes on cell morphology. By investigating whether the identified hits modulate BMP or TGF-β signalling by modulating SMAD nuclear translocation, we found that even if vorinostat - but not niclosamide - affected SMAD nucleus translocation, their mechanism of action differ from the native TGF-β1 and BMP-2 suggesting that other mechanisms may be involved.

Additionally, the reason why niclosamide and vorinostat, in contrast with rhBMP-2 and rhTGF-β1, did not show an effect on the selected functional assays – ALP and ECM production, respectively - suggests that an increase in mRNA production does not necessary translate to an increase in protein levels. These results warrant further studies to unravel the relation between transcription and translation of the target proteins in huTCs with special emphasis on the molecular regulators of ALP activity and ECM production and turnover.

Moreover, with the advent of gene editing technologies such as the CRISPR/Cas9, one could accelerate the discovery of compounds capable of modulating the endogenous promoters in the cellular context of interest rather than using minimal promoter(s) cloned on reporter plasmids. This is highly relevant for future work since it is well-recognized that the genetic and epigenetic landscape of a given promoter is a key modulators of its activity [31].

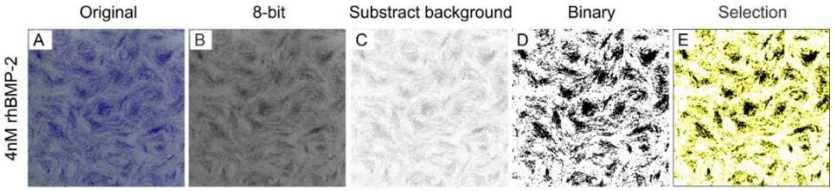
ABBREVIATIONS

Bone morphogenetic protein 2 (BMP-2); recombinant human bone morphogenetic protein 2 (rhBMP-2); transforming growth factor-β1 (TGF-β1); recombinant human transforming growth factor-β1 (rhTGF-β1); tendon/ligament (T/L); human tendon-derived cells (huTCs); growth factors (GFs); Food and Drug Administration (FDA); mink lung epithelial cells (MLEC); basic media (BM); starvation media (SM); fetal bovine serum (FBS); relative light units (RLU); scleraxis (SCX); alkaline phosphatase (ALP); extracellular matrix (ECM); collagen IA1 (COL1A1); runt related transcription factor 2 (RUNX2); alkaline phosphatase; liver/bone/kidney (ALPL); TIMP metallopeptidase inhibitor 1 (TIMP1); matrix metallopeptidase 1 (MMP1).

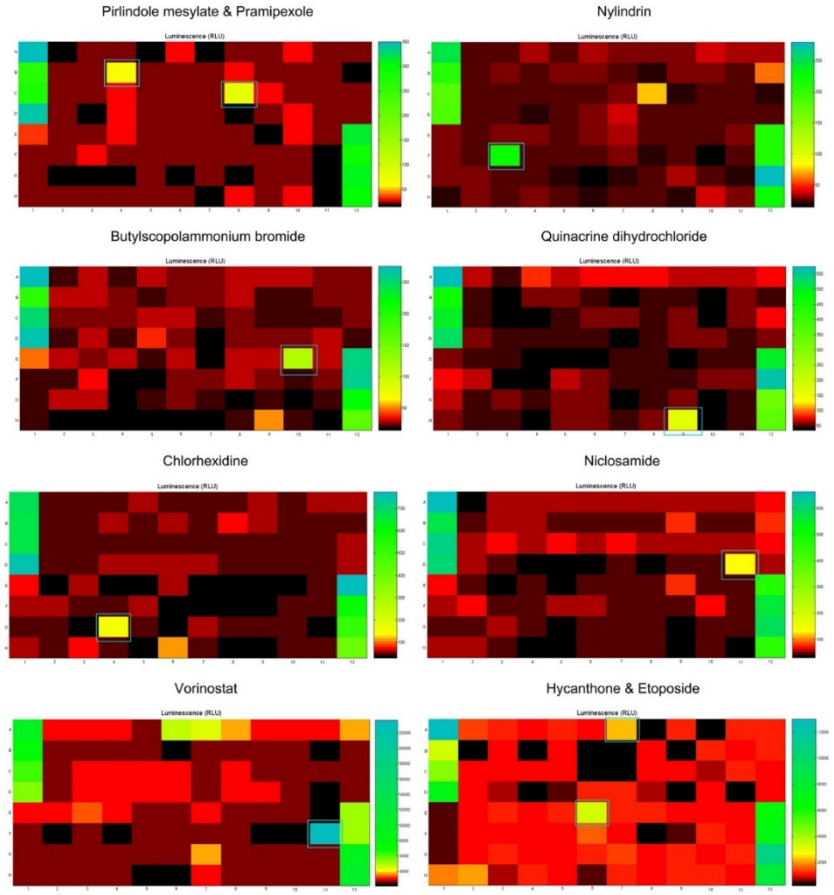
ACKNOWLEDGEMENTS

This work was supported by the University of Twente.

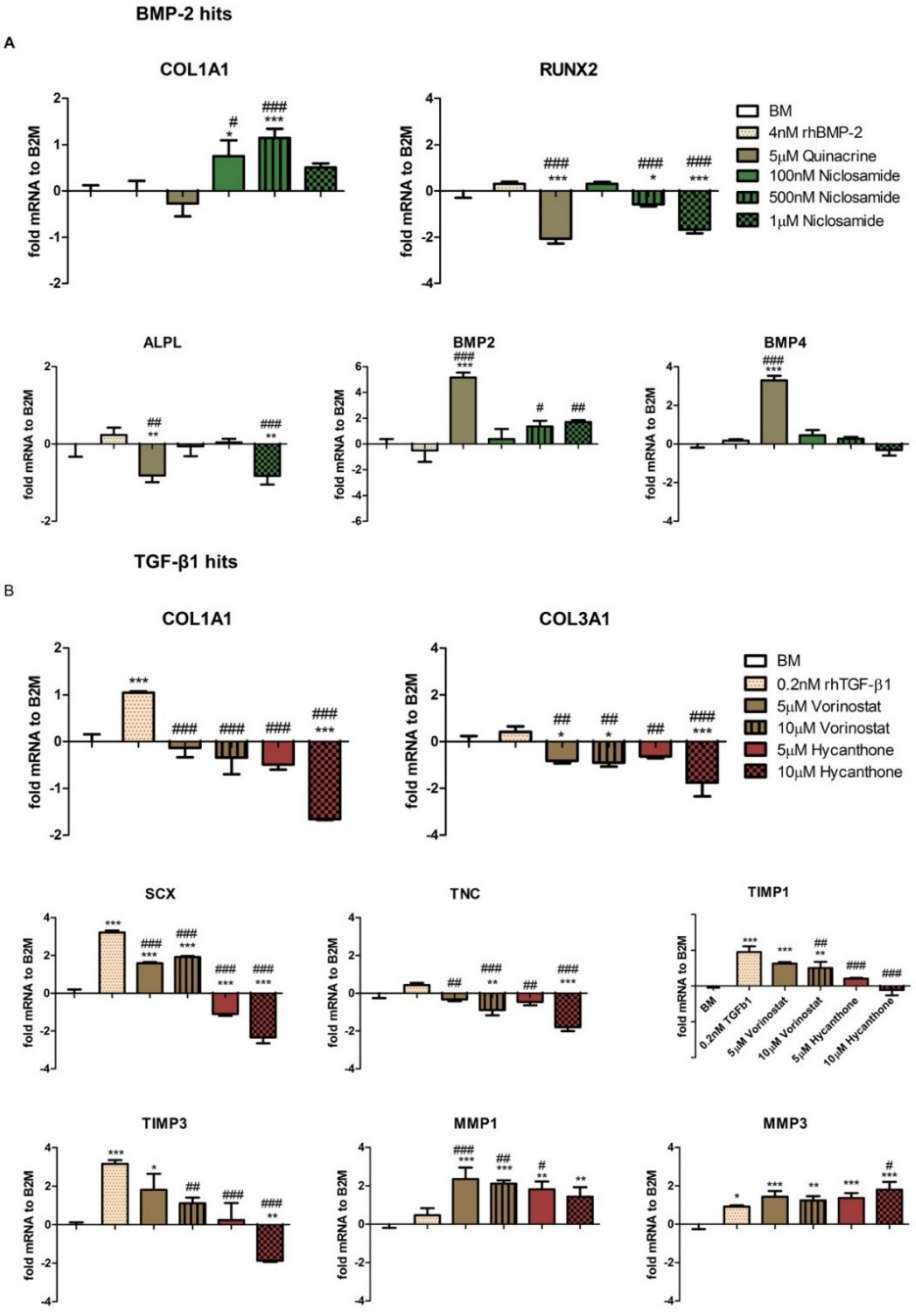
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Image based quantification of ALP staining on huTC using FIJI open source software. *(A) Equal size squares were selected from the original image. (B) The squares were converted into 8-bit image. (C) The background was subtracted using rolling ball function. (D) The image was converted into a binary image. (E) The region of interest were selected and the area measured.*



Supplementary Figure 2. Heat map of the identified hits in the 96 cell culture well plate. *BMP-2 and TGF-β1 reporter cells were exposed to the library of small molecules (2-11 A-H) or to rhTGF-β1 or rhBMP-2 positive control (1A-D and 12 E-H) and BM negative control (1E-H and 12A-D) and luciferase was measured.*



Supplementary Figure 3. Gene expression analysis of huTCs (second donor) exposed for 24 hours *to (A) BMP-2 and (B) TGF-β1 modulators. Statistical significant differences were analysed between BM and BMP-2/TGF-β1 modulators (*) or between rhBMP-2/ rhTGF-β1 and BMP-2/TGF-β1 modulators (#), with ***/###p < 0.001, **/##p < 0.01 and */#p < 0.05.*

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Chapter 6

Infographic

A View on the World of Musculoskeletal Tendon and Ligament Research

Ghebeş C.A., van Eemeren T., Fernandes H.A.M. and Saris D.B.F.

THE CONTEXT OF DOING TENDON AND LIGAMENT RESEARCH:

Increased participation in sport and ageing populations have raised the rates of musculoskeletal tendon/ligament (T/L) injuries. In the U.S. alone, this type of injury accounts for 50% of the 33 million injuries in the musculoskeletal system every year [1]. Decades of research on this problem has yielded extensive knowledge on the biological, structural, and biomechanical properties of these connective tissues. It has also advanced tissue engineering to new treatment strategies for T/L healing. On the world map, an overview of the past 14 years of research is presented. Even though this research has been translated into surgical advances that can prevent major disability, T/L injuries remain a persistent clinical challenge. The current treatment strategies continue to fail to restore the properties of repaired tendons and ligaments to the level of native tissue.

A GEOGRAPHICAL MAP PRESENTING THE RESEARCH FIELD OF T/L (PUBLICATIONS 2000-2014)

We identified the main geographical regions that have contributed to advancements in the field of T/L research between 2000-2014 by gathering and analysing a large body of literature. The geographical map presents a selection of scientists who contributed to the field, the number of articles they published, and how often these articles were cited. Publication and citation rates are used as a measure of academic performance by many global ranking schemes and academic institutions [2]. Nevertheless, these numbers should always be used with caution. Citations are a measure of the number of times a paper is cited by others as a reference but do not necessarily indicate the quality of the research. When there is a high citation rate, we assume that the work has influenced other scientists, but it does not necessarily mean that the paper has generated additional impact on society. Such caution must be taken into account also, given the fact that we have chosen to make use of these indicators to map the field of T/L research.

In this map, we highlighted countries or states that participate in T/L research by colouring them green. The research of single scientists mentioned in publications of the past few years is identified by means of circles positioned on top or proximate to their location. The size of the circle is an indication of the number of publications, and the number within the circle refers to the scientist listed in the legend. There can also be found the name, location, number of publications (indicated with a *P*), and sum of citations for these publications (indicated with a *C*).

We identified that:

- T/L research is found to be localised in North America, West Europe, East & South Asia, and Australia.
- The density and size of circles on North America's east coast indicate it to be the hotspot of T/L research.

- The ratio between the number of publications and citations (not presented on this map) varies greatly. The map shows a distribution per publication achievements. This does not, however, necessitate high citation indices as well. We found scientists with a high number of citations relative to their limited number of publications (Tabin CJ; Schweitzer R; Lu HH; Altman GH & Kaplan DL; Duprez D; and Shukunami C & Hiraki Y). Likewise, we found scientists with a high number of publications relative to their lower citation indices.
- The type of research also has an influence on the number of citations. Published papers from basic science (that study nature of function, injury, and repair) have, in general, higher citation numbers than those from analytical science (which are concerned with tissue engineering strategies or biomechanical studies).
- The highest number of citations per publication is found in research attempting to understand the mechanisms underlining T/L embryonic development (Tabin CJ; Schweitzer R; Birk DE; Shukunami C & Hiraki Y; and Duprez D).

INTERESTS OF THE FIELD

Generally, researchers tend to find solutions to more isolated problems, and not look at the field of T/L research as a whole. In publications concerned with these problems, scientists have identified various strategies, trends, and interests. We found six different categories to be most prevailing in these publications (found below), and they provided a proper overview of the research field. Though we did not find publications mentioning all six of these categories, to our knowledge, no other categories have been mentioned in a significant manner. We have classified the body of publications of this study in these six categories, placing each paper in one or more of these categories, and presented the results in the bar graph.

The six categories:

Biology

These studies include a wide range of research that focuses on describing the morphology, structure, and molecular biology of healthy T/L tissues, and how these are affected upon injury and repair. Additionally, it includes research describing the biological assembly of tissue engineered tendons and ligaments and the biological integration of engineered scaffolds.

Scaffold & Biomaterials

In this area, a wide range of biological materials, biodegradable polymers and composite materials are developed. These are used to develop scaffolds, in various designs, that mimic the tissue structure and biomechanical properties native to tendons

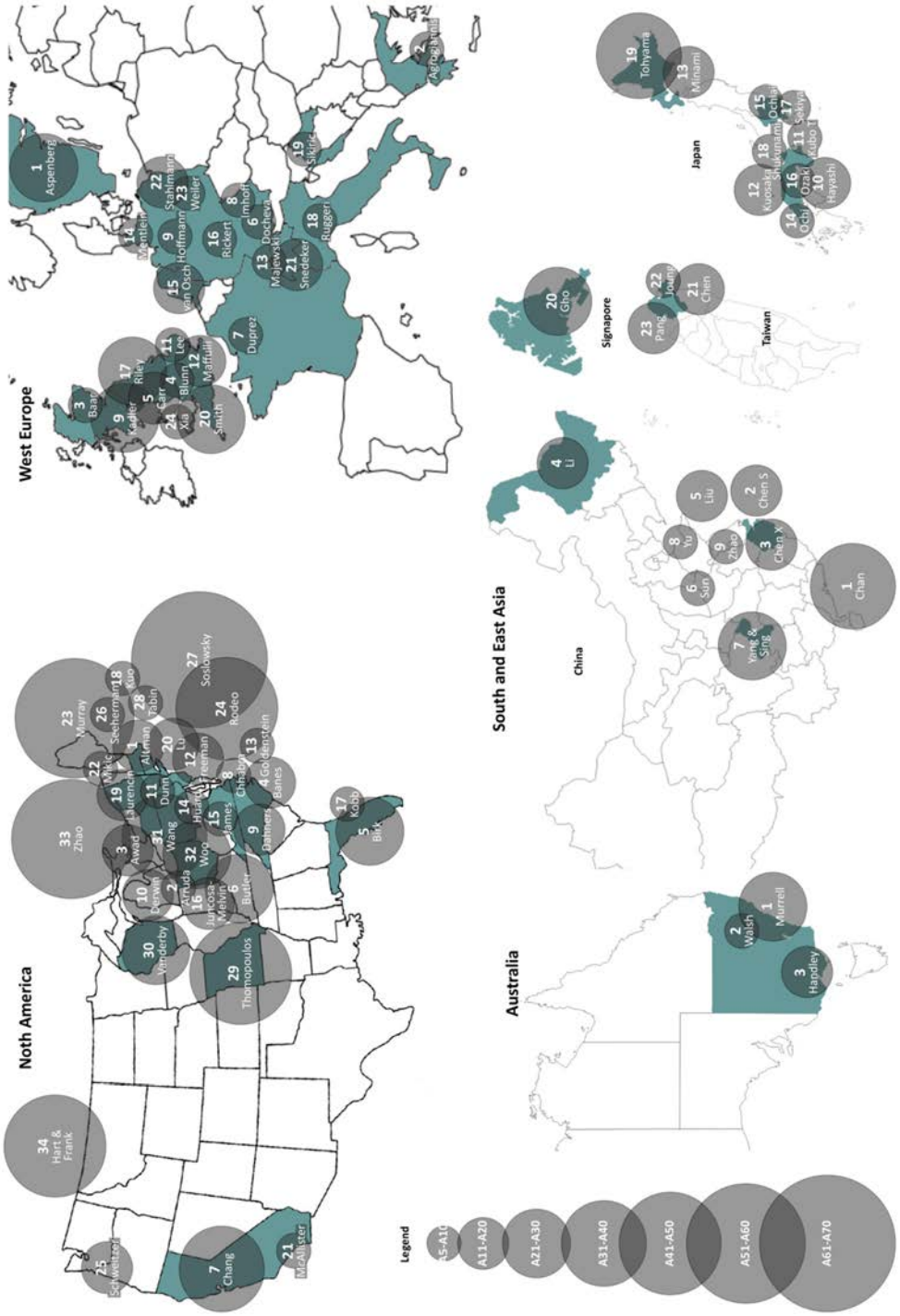

Figure 1. A geographical map presenting the field of musculoskeletal tendon and ligament research (publications 2000-2014).

→ Legend per country: name; location; number of publications (P); number of citations (C).

North America

USA

1	**Altman GH & Kaplan DL**	Boston	P13/C265
2	**Arruda EM**	Ann Arbor	P8/C100
3	**Awad HA**	Rochester	P12/C153
4	**Banes AJ**	Chapel Hill	P17/229
5	**Birk DE**	Tampa	P23/C432
6	**Butler DL**	Cincinnati	P29/C512
7	**Chang J**	San Francisco	P32/C197
8	**Chhabra AB**	Charlottesville	P6/C79
9	**Dahners LE**	Durham	P12/C48
10	**Derwin KA & Iannotti JP**	Cleveland	P17/C148
11	**Dunn MG**	New Jersey	P8/C87
12	**Freeman JW**	New York	P13/C43
13	**Goldstein AS**	Blacksburg	P6/C79
14	**Huard J**	Pittsburg	P8/C81
15	**James R**	Charlottesville	P6/C33
16	**Juncosa-Melvin N**	Cincinnati	P18/311
17	**Kobb TJ**	Tampa	P7/C26
18	**Kuo CK**	Boston	P6/C97
19	**Laurencin CT**	Connecticut	P11/C156
20	**Lu HH**	New York	P18/C393
21	**McAllister DR**	Los Angeles	P5/C22
22	**Mikic B**	Northampton	P8/C86
23	**Murray MM**	Boston	P54/C765
24	**Rodeo SA & Hannafin JA**	New York	P47/C529
25	**Schweitzer R**	Portland	P12/C544
26	**Seeherman H**	Boston	P6/C72
27	**Soslowsky LJ**	Philadelphia	P68/C1123
28	**Tabin CJ**	Boston	P9/C490
29	**Thomopoulos S**	St. Louis	P47/C902
30	**Vanderby R jr**	Madison	P23/C177
31	**Wang JH & Zhang J**	Pittsburg	P37/C534
32	**Woo SL**	Pittsburg	P26/255
33	**Zhao C**	Rochester	P52/C349

Canada

34	**Hart DA & Frank CB**	Calgary	P48/C266

South and East Asia

China

1	**Chan KM**	Hong Kong	P40/C345
2	**Chen S**	Shanghai	P12/C18
3	**Chen X**	Hanzhou	P12/C147
4	**Li Z**	Habin	P17/C93
5	**Liu W**	Shanghai	P14/C98
6	**Sun L**	Tai'an	P5/C37
7	**Yang L & Sing KL**	Chongqing	P23/C74
8	**Yu C**	Beijing	P6/C37
9	**Zhao J**	Shanghai	P8/C29

Japan

10	**Havashi K**	Osaka	P15/C52
11	**Kubo T**	Kyoto	P9/C71
12	**Kurosaka M & Kuroda R**	Kobe	P20/C92
13	**Minami A & Nishimura S**	Sapporo	P15/C88
14	**Ochi M**	Hiroshima	P10/C87
15	**Ochiai N**	Tsukuba	P9/C41
16	**Ozaki T**	Okayama	P6/C35
17	**Sekiva I**	Tokyo	P5/C75
18	**Shukunami C & Hiraki Y**	Kyoto	P9/C71
19	**Tohyama H & Yasuda K**	Hokkaido	P33/C113

Singapore

20	**Gho JC**	Singapore	P27/C295

Taiwan

21	**Chen CH**	Taoyuan	P16/C80
22	**Joung TH**	Taipei	P6/C46
23	**Pang JH**	Taoyuan	P15/C76

Australia

1	**Murrell GA**	Sydney	P26/C221
2	**Walsh WR**	Sydney	P7/C12
3	**Handley CJ**	Melbourne	P15/C89

West Europe

1	**Aspenberg P**	Linkoping	P27/C211
2	**Agrogiannis G**	Athens	P8/C60
3	**Baar K**	Dundee	P10/C106
4	**Blunn GW**	London	P6/C44
5	**Carr AJ & Chaudhury S**	Oxford	P20/C39
6	**Docheva D**	München	P7/C113
7	**Duprez D**	Paris	P9/C152
8	**Imhoff AB**	München	P6/C32
9	**Hoffmann A**	Hannover	P6/C88
9	**Kadler KE**	Manchester	P21/C297
10	**Lee DA**	London	P8/C76
11	**Maffulli N**	London	P16/C99
12	**Maiewski M**	Basel	P5/C37
13	**Mentlein R**	Kiel	P7/C48
14	**Van Osch GJ & van Schie HT**	Rotterdam	P14/C136
15	**Rickert M**	Heidelberg	P5/C65
16	**Riley GP**	Norwich	P24/C258
17	**Ruggeri A**	Bologna	P7/C40
18	**Sikiric P**	Zagreb	P5/C10
19	**Smith RK**	Hatfield	P23/C188
20	**Snedeker JG**	Zurich	P20/C131
21	**Stahlmann R & Shakibaei M**	Berlin	P13/C95
22	**Weiler A**	Berlin	P10/C94
23	**Xia Z**	Swansea	P6/C7

and ligaments. Ideally, the scaffold should support cell adhesion and must be biodegradable to enable tissue ingrowth, which is crucial for the new T/L to form.

Biomechanics

The biomechanical properties of native T/L tissue, subjected to a wide variety of loading conditions including tension, compression, and shear, as well as those of newly engineered scaffolds and T/L tissues, are the subject of this category.

Bioactive molecules

Papers fitting this category describe the influence of intrinsic or extrinsic delivered bioactive molecules, such as growth factors, and cytokines on T/L healing. They cover also the influence of oxygen tension and the delivery of pharmaceutical small molecules.

Cell study

This area describes the phenotypic properties of cells derived from T/L tissues; exploring new potential cell sources, as well as the use of genetically modified cells to enhance T/L healing.

Developmental study

This advances the understanding of T/L development, which provides a basis of knowledge for improving T/L tissue repair, regeneration, and healing. The research of T/L embryonic development has resulted in the discovery of important markers of tendon cell identity, matrix molecules, and signalling regulators.

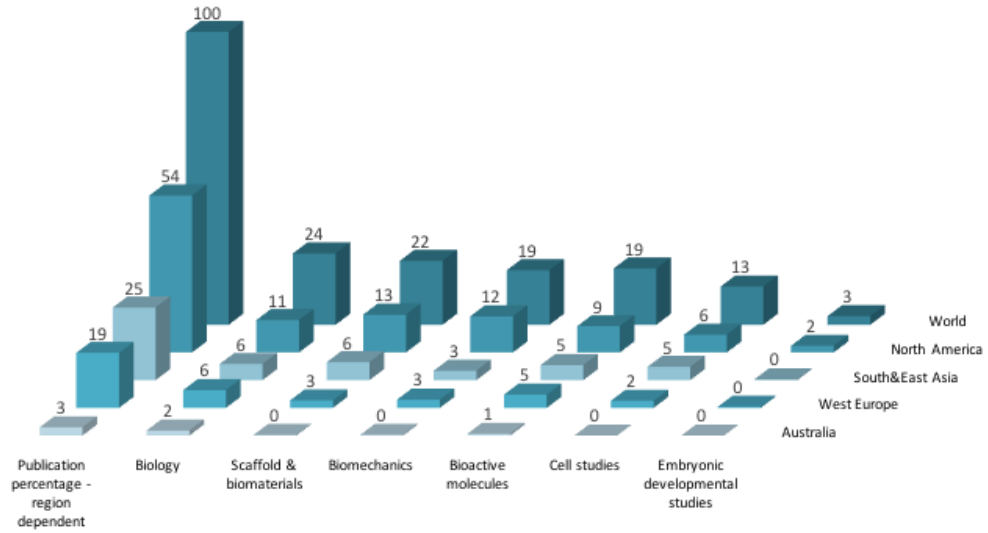

Figure 2. Tendon and ligament research interests

In the bar graph, along the horizontal axis, the research is distributed across the different categories and, along the vertical axis, across the different world regions. Each bar represents a percentage of the total research (e.g. research on biomechanics in America makes up 12% of the total research). This graphic provides an overview of the type of research performed in different regions around the world during 14 years of research. North America, also in this graph, is shown to be the most significant contributor to the field. Asia and Europe together show a similar contribution to the field, with Australia taking up a significantly smaller percentage. Furthermore, in this graph, it becomes clear that even though the category of 'cell studies' shows a slightly lower percentage worldwide, the category of 'embryonic development' shows an especially significant lower percentage than the others. The latter coincides with issues previously addressed in various reviews that argue for further understanding to advance the field of T/L research [3-5].

In conclusion, our approach of taking a step back and looking at the whole field, rather than at isolated problems, makes two points visible: that there are six main categories into which the field of T/L research can be classified, and that the category of embryonic developmental studies receives significantly less attention than others.

CRITERIA OF SELECTION

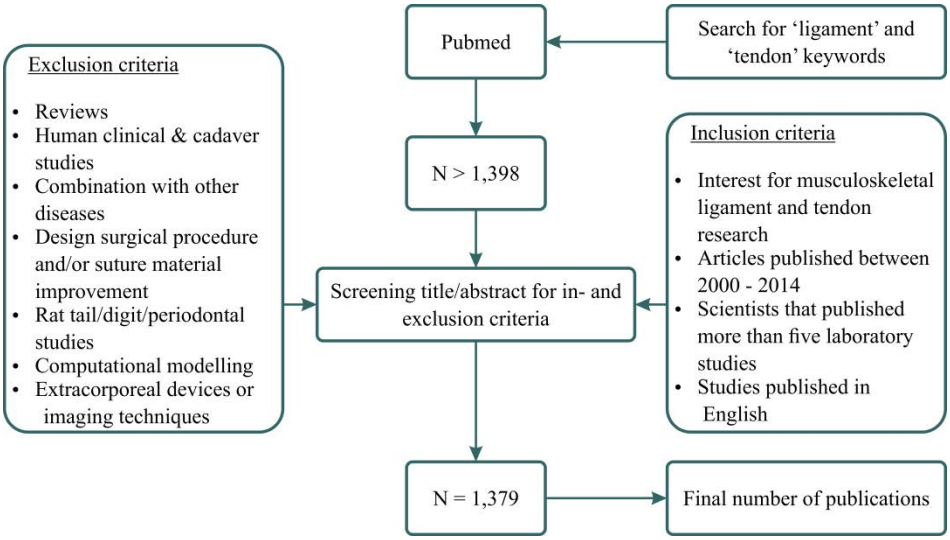

Figure 3. Search strategy and selection criteria

A PERSPECTIVE FOR INTERESTED RESEARCHERS AND OTHERS

As a young researcher, it takes a lot of time and effort to get to know the field. Traditional means to achieve this are, among others, visiting conferences and keeping up to date on scientific literature in the field. The visual view of the world of musculoskeletal tendon and ligament research provided here aims to be an additional means to this end. It provides a quick overview of the main contributors to the field and the areas of interest.

Besides those interested from within the field, exterior parties that looking for collaborations, or interested in another way, could find in this map a coherent overview of possible business partners.

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Chapter 7

General Discussion

INTRODUCTION

Curious about how we can accelerate and improve tendon-graft remodelling and about finding a new approach to biological augmentation of ACL reconstruction in the near future, we set out on this adventure.

With a rise in participation in organised sports, which has brought not only health benefits but also an increase in knee trauma and ACL ruptures, the desire of athletes to return to sports and others to resume their normal life in a the shortest time as possible calls for action. While the surgical procedure of ACL reconstruction is well established, the adaptation of the tendon graft to the new environment remains critical and seems to be the time limiting factor.

Multiple disciplines, including biology, engineering, material science, and chemistry, have converged to advance tendon-graft healing. We identified the emerging approaches that address this challenge, these include the administration of cells, that are able to differentiate into tissue specific cells or secrete bioactive factors important for tissue regeneration; growth factors, which provide important signals for tissue formation and differentiation; gene therapy techniques, which ensure the prolonged presence of molecules important for the healing process; bone derivatives, which provide fixation strength and bone-tendon ingrowth; biomaterials, which provide adhesion and fixation strength; and small molecules capable of modulating molecular signals. Thus, while these graft-enhancing methods have improved the structure and composition of the graft tissue, a major challenge remains in accelerating the healing process for early rehabilitation and a rapid return to full activity.

The native ACL attaches to the surface of bone through a direct insertion, which contains four distinct zones: ligament, uncalcified fibrocartilage, calcified fibrocartilage, and bone. In the human body, however, there are no sites where a tendon enters a bone tunnel, and therefore no native situation analogous to tunnel-tendon graft exists that can be resembled [1]. Tendon-graft healing appears to begin with the formation of fibrovascular tissue at the tendon-to-bone (T-B) interface, ingrowth of monocytes, macrophages, and bone marrow-derived cells; and progresses with a non-uniform pattern of cartilaginous interface and bone trabeculae, which reach a steady maturation after two years post-reconstruction [2]. The functional and structural recapitulation of the native counterpart, however, the enthesis, is never accomplished as result of the generation of a fibrovascular scar rather than regeneration of a graded fibrocartilaginous transition. The advantage of tissue regeneration over repair lies in the potential to mimic embryonic development, which results in the nearly complete reconstitution of the original structure, function, and mechanical properties of the tissue [3].

Consequently, tendon-graft healing following ACL reconstruction should ideally be accelerated and bear regenerative properties. In this thesis, we evaluated the tissue regenerative properties of the tendon graft versus the native ligament by analysing in vitro the phenotype of cells derived from each tissue. Similarly, we evaluated the tissue

regenerative properties of BM cells harvested from different locations and their potential role in tendon-graft regeneration. Furthermore, inspired by embryonic development, where intercellular communication plays an important role in tissue formation, we addressed the potential influence of mature cells, derived from the same progenitor pool as tendon cells, on tendon-graft healing. Finally, we searched for therapeutic factors that can direct cell fate and influence tissue regeneration.

DISCUSSION

To progress towards a successful healing of the reconstructed ACL, it is important to understand the differences and similarities between the native ACL and the replacement graft. In current surgical practice, the majority of procedures are performed using an autologous tendon graft due to its availability and similarity in structure, physiology, and mechanical properties to the native ACL [4, 5]. One such graft is the hamstring tendon. Given the insufficient regenerative properties of the ACL tissue to heal following severe injury and the poor healing properties of the tendon graft, an understanding of the behaviour of the cells, derived from these two tissues, is important in order to harness their regenerative potential.

The regenerative potential of cells involved in ACL reconstruction

In *Chapter 2* we analysed in vitro the phenotype of heterogeneous isolated cells from the ACL and the hamstring tendon graft. We found that ligament- and tendon-derived cells share similar biological properties in terms of morphology, gene expression profile, and cell surface markers, though not in terms of regenerative potential. Both cell types showed limited potential for self-renewal and trilineage differentiation, compared with the in vitro regenerative properties of MSCs described in the literature [6], and where tendon-derived cells showed even less regenerative properties than ligament-derived cells. Conversely, regenerative stem/progenitor cells were previously identified to reside in the ECM of tendons [7], albeit in reduced numbers. When inserted into a window-defect in a rat patellar tendon, after being expanded in vitro, they significantly promoted healing with increased collagen production and alignment, cell alignment, and overall increase in ultimate stress [8]. Nevertheless, this limited cell population is insufficient to contribute to natural tissue regeneration, unless it is expanded in vitro. Our findings suggest that the poor in vitro regenerative properties of the derived cells can explain the poor in vivo regenerative properties of the two tissues. Additionally, the inferior regenerative properties of the tendon graft compared to the replaced ACL tissue emphasises the major challenge remaining, i.e. to find means to accelerate tendon-graft regeneration and consequently ACL graft healing.

We addressed two distinctive methods/solutions, that may initiate tendon-graft regeneration. First, the regenerative properties of the graft tissue may be activated by the addition/implantation of an external cell source known to hold regenerative

properties. Second, external factors that have the potential to instruct the biological system of the tendon graft and potentiate tissue regeneration.

Many of the therapeutic benefits seen in the augmentation of current surgical procedures are mediated by the activation of transplantation of MSCs [9]. Their effectiveness upon transplantation is mediated by their ability to differentiate into cells that participate in tissue regeneration, and due to the release of paracrine factors, such as growth factors and cytokines. From in vivo animal models, it is hypothesised that the presence of bone marrow-derived MSCs may improve tendon-graft regeneration and accelerate the repair process by laying down extracellular matrix, releasing growth factors, and triggering the necessary immune responses [10-12]. Nevertheless, to date, only one study has reported the use of BM concentrate harvest from the ilium to enhance ACL reconstruction healing [13]. The poor characterisation of the BM aspirate, however, like MNC concentration or cell viability, made it difficult to draw a conclusion regarding its contribution to tendon-graft healing. Thus, the challenge lies in optimising the treatment. We believe that an optimal treatment strategy to accelerate tendon-graft healing using BM aspirate would be to harvest BM proximal to the knee joint, to concentrate and/or activate it, and to place it back at the graft-bone insertion site. Collecting BM close to the ACL reconstruction site – either distal femur and/or proximal tibia – would not require an additional surgical step (as with BM harvested from the ilium) making it an appealing source to accelerate the healing process.

In *Chapter 3*, we evaluated means to enhance BM function for regenerative musculoskeletal applications. We determined the in vitro regenerative properties of MSCs isolated from different BM locations, including the ilium, proximal femur, distal femur, and proximal tibia. We found that the regenerative properties of the isolated MSCs differ in relation to the BM aspiration location, with larger amounts, higher self-renewal, and osteogenic differentiation potential for MSCs isolated from the ilium rather than the distal femur and proximal tibia. Caution is needed regarding the results obtained, however, since the age of the BM donors is 50+ years and the patients we address, i.e. undergoing ACL reconstruction, is 20+ years. Previous studies have shown that an increase in donor age is associated with decreased proliferation rates and differentiation potential of the MSCs [14, 15], which illustrates that age is an important factor when analysing the regenerative potential of BM. For ACL reconstruction, the attractive regenerative properties of BM harvested from the ilium makes it to a valuable source of regenerative cells. It remains to be clarified, however, whether in young patients the regenerative properties of BM cells harvested from the distal femur and proximal tibia are comparable with those from ilium.

We evaluated also the potential use of acoustic energy to activate BM aspirate. Current in vivo animal studies have shown encouraging results using low-intensity pulsed ultrasound (LIPUS) as an extracorporeal method able to promote healing at the T-B interface following ACL reconstruction [16, 17]. Known to prime osteogenic differentiation of MSCs and enhance proliferation and differentiation of osteoblasts, we hypothesised that the direct application of acoustic energy on BM aspirate would

activate MSCs prior to their reimplantation. We found that the application of acoustic stimulation on BM can increase self-renewal and the osteogenic differentiation potential of the MSCs, though not in all BM donors. This result represents the first attempt in exploring the use of acoustic stimulation as a system to prime BM cells. While further in vitro and in vivo investigations are needed, the use of acoustic stimulation as a tool to activate BM during its administration in ACL reconstruction surgery represents a feasible clinical approach.

Therapeutic factors capable of modulating ACL graft healing

Remnant muscle tissue can accelerate ACL graft healing

Cell-to-cell interactions are of crucial importance in the formation of connective tissues [18]. Upon maturation, however, these interactions are lost due to the abundant extracellular matrix surrounding the cells, which results in impaired tissue regeneration. Aiming to regenerate the original structure, function, and mechanical properties of the native ACL using a tendon graft, in *Chapter 4* we analysed the influence of intercellular communication between tendon cells and cells involved in the development of tendon tissue. Using an *in vitro* co-culture system and evaluating the expression of genes that direct cell differentiation and extracellular matrix formation, we found that muscle-tendon cell interaction can play an important role on extracellular matrix remodelling. Considering such potential, the possibility of applying muscle tissue to enhance ACL reconstructive surgery appears attractive, because residual muscle is removed during preparation of the tendon graft. Using an in vivo ACL reconstruction rat model, we injected muscle tissue factors (muscle condition media) into the operated joints of the animals five times over a period of one month. Histological examination showed that femoral tunnel closure was promoted via muscle tissue factors. These positive in vitro and in vivo preliminary results emphasise that muscle tissue factors have an influence on tendon-graft matrix remodelling and can accelerate T-B junction integration. Nevertheless, our approach is, for now, insufficient to conclude whether muscle tissue factors have an influence on tendon-graft regeneration resembling the structure, function, and mechanical properties of native ACL. A more in detail evaluation of the muscle tissue modulatory factors and T-B junction remodelling/integration would make it possible to make that statement.

In this thesis, we described the potential use of muscle tissue factors; however, the preservation of muscle tissue itself on tendon grafts, as an envelope, could represent another feasible method to accelerate T-B junction integration. This method was previously described by Sun et al., who found that retention of muscle remnants on tendon grafts accelerated histological intra-articular healing of ACL reconstruction in a rabbit model [19]. Consequently, the use of remnant muscle represents a promising option to facilitate an early stage post-surgical rehabilitation and improve clinical outcomes for ACL reconstruction.

Small molecules capable of indirect modulation of ACL graft healing

Small molecule-mediated tissue regeneration is emerging as a promising strategy for the regeneration of various tissues. Having unique advantages over other growth factors, such as no risk of cross-species contamination as with the use of recombinant protein-based growth factors [20], and being more stable, highly soluble, and cost-effective [20], makes small molecules an important alternative to growth factors.

In this study, we searched for small molecules that can modulate two essential signalling pathways involved in tendon development and healing: TGF-β and BMP (*Chapter 5*) [18, 21]. TGF-β1 and BMP-2 reporter cell lines were used to screen a library of FDA-approved small molecules. The identified small molecules were further confirmed to mediate the expression of BMP-2 and TGF-β1 target genes in primary tendon-derived cells. The translation to target proteins was not yet successful, however, when analysing functional assays, such as ALP activity and ECM production. The identified small molecules require much further preclinical in vitro and in vivo testing to confirm their beneficial role in tissue regeneration and their safety in future clinical applications.

CONCLUSION

Searching for methods to accelerate and improve ACL reconstruction, we identified differences in regenerative properties between the cells residing in the tendon graft and the injured ligament tissue. Unable to regenerate the tissue by themselves, we searched for an alternative cell sources with regenerative properties; and we illustrated three systems that have the potential to instruct the biological system of the tendon graft to regenerate and restore the biology and function of native tissue. At the preclinical incipient phase, these systems have the potential for future successful clinical applications.

- Exposing BM to acoustic energy prior to delivery of the activated cells at the tendon to bone junction is a new system with the potential to enhance tendon to bone junction integration via activation of MSCs pre-commitment towards osteogenesis.
- Co-delivery of small molecules (using a carrier system) that can provide different biological cues, such as ECM production and bone integration.
- Integration of muscle tissue factors at the tendon-graft site represents a new system that can promote intra-articular healing by graft remodelling and tendon to bone junction integration via ECM deposition. The ‘tomorrow’ strategy to accelerate ACL reconstruction healing, muscle tissue factors represents a good system that can be easily implemented into the clinical practice.

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List of publications

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Anterior cruciate ligament- and hamstring tendon-derived cells: In vitro differential properties of cells involved in ACL reconstruction.

Ghebeş CA, Kelder C, Schot T, Renard AJ, Pakvis DFM, Fernandes HA, Saris DB. J. Tissue. Eng. Regen. Med. 2015 Mar; 11. Doi:10.1002/term.2009.

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Human muscle-derived factors accelerate ACL graft healing: an in vitro and in vivo analysis

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Characterisation of hamstring tendon-derived cells and their crosstalk with neighbouring cell types involved in embryonic development of tendons.

CA Ghebeş, T Schot, C Kelder, E Ekwueme, AJ Renard, HAM Fernandes, DBF Saris. NBTE, 2013, The Netherlands.

Bio-instructive approach to accelerate ligament/tendon healing.

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Ways to enhance musculoskeletal repair.

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Cell source for anterior cruciate ligament reconstruction.

HAM Fernandes, **CA Ghebeş**, C Kelder, C van Blitterswijk, DBF Saris. TERMIS, 2012, Austria.

Hamstring tendon-derived cells, a cell source for anterior cruciate ligament reconstruction.

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PATENT

Bone fracture healing with primed distal bone marrow mesenchymal stromal cells (WO/2016/029148)

Wan C, Hsiong S, Au A, **Ghebeş CA**. Smith & Nephew Inc. and University of Twente.

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Biography

Corina-Adriana Ghebes was born on June 20th 1986 in Sibiu, Romania. In 2009 she received her Bachelor of Science diploma in Mechanical Engineering - Management from the Politehnica University of Bucharest (Romania). In 2011 she graduated as Master of Science in Biomedical Engineering with main focus in biomaterials and biomechanics from Vienna University of Technology (Austria).

Later, in 2012 she started her PhD training under the supervision of Prof. Dr. D.B.F. Saris and Dr. H.A.M. Fernandes. Her doctoral research focused on means to accelerate tendon graft healing after ACL reconstruction. This thesis describes the findings of her research work.

Cover
- Henrique Oliveira's *Desnatureza* -
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Galerie Vallois, 2011

Design by:
Tjebbe van Eemeren

The artwork is adopted here to represent the goal of this dissertation: to achieve a natural integration of the tendon graft into the bone after ACL reconstruction.

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