

Effects Of Cell-Free Conditioned Media on Healing of Rat Achilles Tendon

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ABSTRACT

Tendon injuries are common musculoskeletal conditions that impose significant challenges to medical professionals, pain and debilitation of patients, and financial burdens to the healthcare system. Due to limited natural healing capacity, tendons typically undergo scar-mediated repair that compromises biomechanical integrity and increases the risk of reinjury. Despite a variety of therapeutic strategies, functional tendon healing remains a major clinical challenge. Mesenchymal stem cells (MSCs) represent an attractive strategy to improve tendon healing, largely due to their paracrine activity via the release of the secretome, a set of bioactive molecules that are known to mimic the immunomodulatory and regenerative properties of their parental cells. More recently, acellular approaches using MSC-secretome derivatives, including conditioned media (CM) and exosomes, have been largely explored for tendon healing. This work begins with a review of the literature surrounding the therapeutic potential of MSC secretome to improve tendon healing, highlighting advantages of MSC secretome over MSC-based therapies, proposed mechanisms of action, manufacturing considerations, and the current state of research. Building on this foundation, it then evaluates the effects of CM derived from TGF β -3-primed tendon stem cells (TSCs) in an acute rat Achilles tendon injury model. *In vitro*, CM from TGF- β 3-primed and unprimed TSCs was profiled by proteomics and functionally characterized using cell proliferation/viability and immunofluorescence assays. *In vivo*, the effects of CM on Achilles tendon healing were assessed via intralesional administration of CM, blended with fibrin gel for controlled delivery, in a rat Achilles tendon injury model. Overall, TGF β -3 priming of TSCs produced a matrix-organizing secretome that

accelerated tenogenic marker expression *in vitro*, provided cytoprotection under nutrient stress conditions, and improved gross tendon repair quality *in vivo*.

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CHAPTER I: INTRODUCTION

INTRODUCTION

Tendons are well-organized connective tissues attaching muscles to the skeleton. They play an important role in transmitting high contraction forces generated by muscle to bone to maintain posture and produce joint movement¹⁻⁶. Tendons consist primarily of densely packed collagen fibers and tendon-specific cell types, tenocytes and tenoblasts, embedded in an extracellular matrix (ECM^{2,4,7}). The ECM is the non-cellular component, composed of collagen fibers, primarily collagen type I, and other non-collagenous proteins, predominantly proteoglycans, glycoproteins and glycosaminoglycans^{2,8-11}. Those components provide physical support to the collagen fibers, essential to ensuring the mechanical function of the tendon, and maintain a microenvironment crucial for tissue homeostasis, differentiation, and morphogenesis^{8,9,12}. Collagen, synthesized by tenocytes, forms the fundamental force-transmitting unit of the tendon. They are aligned with the muscle-bone axis to form a collagen structure with unique biomechanical properties that can resist tensile and compressive forces^{2,4,13}. However, overuse or mechanical loading above the biomechanical limit of the tissue commonly results in injuries, such as rupture, tear, or degeneration of the tendon^{2,14-16}.

Tendon injuries are debilitating musculoskeletal disorders that commonly affect human and veterinary patients^{15,17,18}. These injuries are characterized by localized pain, swelling of the tendon, loss of function, and impaired performance^{16,17,19,20}. They also represent significant financial hardship and medical challenges^{7,21}. In the United States alone, musculoskeletal injuries account for an estimated \$30 billion annually, with 45% to 55% involving tendons and ligaments^{2,22,23}.

Natural tendon healing is a lengthy process and often yields suboptimal structural, mechanical and functional outcomes⁷. Limited and inefficient intrinsic healing capacity, owing to relative hypocellularity and hypovascularity of the tissue, impairs restoration of an organized ECM^{24,25}. Despite intensive remodeling after injury, the naturally healed tendon appears scar-like and does not completely recover its biomechanical properties²⁶⁻²⁹, resulting in a healed tendon with reduced tensile strength and elevated risk of reinjury^{28,30}.

Current treatment options for tendon injuries depend on the severity of the lesion and are generally limited to either conservative management or surgical intervention¹³. Mild to moderate injuries that do not significantly impair functions are typically treated conservatively. However, conservative approaches often show limited success in severe cases, such as complete tendon ruptures, making surgery necessary. Surgical management is also recommended when conservative treatment fails¹³. Despite their widespread use, both conservative and surgical treatments are associated with suboptimal long-term outcomes, including prolonged healing times, excessive scar tissue formation, and high reinjury rates²⁵.

To improve outcomes, several strategies have been explored, including stem cells/stromal cells therapy, platelet-rich plasma (PrP), and biomaterials, alone or seeded with stem cells^{7,31}. PrP is an autologous, blood-derived therapy that contains a variable mixture of growth factors and other bioactive substances, including chemokines, cytokines, proteinases and their inhibitors, clotting factors and their inhibitors, and adhesive proteins³². Reported benefits of PRP for tendon healing are controversial, likely due to heterogeneity and poor characterization of its composition, a key factor that determines its

biological activity⁷. Despite the development of multiple therapeutic strategies, functional tendon healing continues to be a major clinical challenge^{7,33,34}. To date, no therapies can fully restore the functional properties of injured tendons^{35,36}. Recently, the therapeutic potential of mesenchymal stem cell (MSC)-secretome derivatives, such as conditioned media (CM) and extracellular vesicles (EVs), have gained attention as acellular therapies with the potential to modulate inflammation, promote matrix remodeling, and enhance tenogenic repair. While promising, more studies are needed to clarify their safety, mechanisms of actions and efficacy.

Study aims and hypotheses. This study evaluates the effects of CM derived from TGF β -3-primed tendon stem cells (TSCs) on tendon healing. We combine (i) *in vitro* functional assays and proteomic profiling of CM with (ii) *in vivo* intralesional administration of CM in a rat Achilles tendon injury model. We hypothesized that TGF β -3 priming would induce a pro-regenerative, tenogenic secretome in TSCs and improve Achilles tendon healing compared with unprimed CM and control.

CHAPTER II: LITERATURE REVIEW

MESENCHYMAL STEM CELL-SECRETOME DERIVATIVES FOR TENDON

HEALING: THERAPEUTIC POTENTIAL, CURRENT CHALLENGES,

MECHANISMS OF ACTION, AND CURRENT STATE

1. MESENCHYMAL STEM CELLS IN TENDON REGENERATION

Among stem cell types that have been explored for regenerative medicine application, mesenchymal stem cells are of particular interest¹⁸. MSCs are multipotent progenitor cells that are able to differentiate into multiple lineages and self-renew^{18,37}. In addition to their differentiation properties, MSCs have other features that make them attractive for clinical application in regenerative medicine, such as their paracrine activity, a type of biological communication between cells via their secreted substances, collectively referred to as the secretome³⁸. The secretome comprises a set of soluble bioactive molecules, including growth factors, cytokines, and chemokines, as well as extracellular vesicles (EVs) that are collectively secreted to the extracellular space, and can be found in the cell culture media in which MSCs are cultured, referred to as conditioned media (CM)^{38,39}.

Mesenchymal stem cell therapy represents an attractive approach for the treatment of tendon injury in human and veterinary sports medicine, given their potential to modulate inflammation and promote tissue repair⁴⁰. In animals, the intralesional implantation of MSCs to treat tendonitis in horses was reported for the first time in 2003⁴¹. Since then, MSC therapy has been widely used in equine sports medicine for the treatment of musculoskeletal injuries, particularly tendon and ligament injuries⁴², achieving tissue regeneration without the production of excessive scar tissue that commonly occurs following tendon injuries⁴³. Studies of sport horses with superficial digital flexor tendon (SDFT) tendonitis reported improved tendon healing and reduced re-injury occurrence following intralesional application of MSCs^{44–46}. The SDFT is analogous to the human Achilles tendon in terms of function and injury susceptibility. This is of particular interest

for studying the pathogenesis of tendon injuries in humans, as the horse represents the only available animal model in which spontaneous, naturally occurring large tendon injury occurs^{47,48}. The therapeutic potential of MSC therapy for tendon injuries in humans has been demonstrated in a number of studies^{49,50}. However, the translation of MSC-based therapies into human clinical trials and clinical use remains largely disappointing^{35,51}. This is particularly related to MSCs' heterogeneity, regulatory challenges, difficulties in standardizing isolation and expansion protocols, and ongoing safety concerns, issues that will be further discussed in this review. To date, surgery and conservative treatment combined with physical therapy remain the only widely accepted treatment options for tendon injuries in humans⁵².

Bone marrow-derived MSCs (BM-MSCs) are the best studied and well-characterized MSC type, making them the most commonly explored for tendon regeneration strategies to date^{40,53}. Adipose tissue-derived MSCs (ASCs) also have drawn attention due to the ease of accessibility, minimally invasive harvesting procedures, and less patient morbidity⁵⁴. More recently, tendon stem cells (TSCs) have been characterized⁵⁵ and increasingly explored as a promising cell source for tendon healing therapies^{54,56-58}. These cells represent 1-4% of the nucleated cells within tendon tissue and share key MSC properties, including self-renewal, trilineage differentiation (bone, cartilage, and fat), and desirable surface marker expression^{55,59}. Importantly, TSCs can differentiate into tenocytes and form tendon-like tissue both *in vitro* and *in vivo*^{60,60-62}. In a comparative study using a rat Achilles tendon rupture model, TSCs showed superior regenerative potential over BMSCs, including enhanced tendon healing, improved tissue organization, and greater

collagen fiber alignment⁶³. These findings suggest that TSCs may represent a better stem cell source for tendon healing.

2. MSC-DERIVED SECRETOME AS A NEW REGENERATIVE APPROACH

The direct replacement of necrotic or apoptotic cells, through local engraftment and differentiation into tissue-specific lineage of cells, was initially proposed as the primary therapeutic mechanism of action of MSCs^{64–67}. Indeed, studies using labeled MSCs showed that MSCs were able to migrate to the site of injury after *in vivo* administration with some evidence of differentiation^{68–70}. The potential of MSCs to repair tissues through differentiation was continuously supported by *in vitro* cell culture studies reporting MSC differentiation into non-mesenchymal cells, including neural cells, cardiomyocytes, hepatocytes, and endothelial cells under appropriate experimental conditions^{71–74}. However, several *in vivo* studies showed evidence that MSCs were able to promote functional improvements with little evidence of engraftment or differentiation of the cells, suggesting that MSCs have poor grafting potential and survive only for a short period of time in damaged tissue after transplantation. For example, a study using a mouse model of myocardial infarction⁷⁵ reported improved cardiac function after systemic administration of human MSCs (hMSCs). However, despite cardiac improvement, there was no evidence of cell engraftment in the hearts after 3 weeks. In another study⁷⁶, children with osteogenesis imperfecta administered with MSCs showed significant improvement in clinical outcomes but less than 1% of donor MSCs were detected in the bone or other tissues of the recipient children collected four to six weeks after infusion of MSCs.

Over time, the understanding of the MSCs fate after *in vivo* transplantation and the mechanisms through which MSCs exert their therapeutic effects has evolved. Given the

evidence supporting the poor engraftment and survival of MSCs after *in vivo* transplantation, despite improved functional outcomes, it has become more evident that the therapeutic benefits of stem cells are primarily mediated via their paracrine activity through the release of the secretome rather than their long-term presence in the tissue or stemness potential^{38,77–80}. In fact, the term “mesenchymal stem cells” has been reconsidered as “medicinal signaling cells” to more accurately reflect the mechanisms through which those cells exert their immunomodulatory and regenerative effects⁸¹. This idea has been supported by studies demonstrating that cell culture media conditioned by MSCs contain large amounts of bioactive proteins and EVs that mirror the immunomodulatory and regenerative capabilities of their parental cells, thus presenting with the potential for promoting *in vitro* and *in vivo* therapeutic effects similar to those promoted by the transplantation of cells.

2.1 MSC-DERIVED CONDITIONED MEDIA (CM)

Conditioned media (CM) is the media in which MSCs have been cultured. It contains the complete regenerative milieu of MSC-sourced secretome, including soluble factors and EVs^{38,82}. Both MSC-CM and the EV fraction have been demonstrated to be sufficient to trigger tissue regeneration in a variety of diseases, including skin wounds, lung injury, liver injury, uveitis, myocardial infarction, renal disease, and soft tissue injuries such as tendon and ligament injuries^{56,79,83–92}. In a recent study, TSC-CM improved Achilles tendon healing in rats by modulating ECM composition, promoting angiogenesis, improving tendon fiber alignment, suppressing inflammation, and strengthening biomechanical properties of tendons⁵⁶. Similarly, ASC-EVs promoted the healing of injured Achilles tendon in rabbits. ASC-EVs were found to promote ECM remodeling,

improve the mechanical strength of the injured tendon, reduce inflammation, and promote more organized and aligned collagen fibers⁹³.

As the need grows for therapies that enhance tendon healing and restore function, both MSC-CM and EVs have emerged as promising cell-free alternatives. Their therapeutic potential is largely attributed to their immunomodulatory and regenerative properties, which will be explored in detail later in this review.

2.2 EXTRACELLULAR VESICLES (EVs)

Extracellular vesicles (EVs) are heterogeneous, nano-sized, membrane-bound particles that are actively released by nearly all cell types into the extracellular environment via the exocytosis process. EVs are composed of a phospholipid bilayer, originated from the parent cell, which encloses a variety of bioactive cargos, including proteins, lipids, and nucleic acids^{94,95}. EVs are categorized based on their size, origin, and biogenesis pathway, including exosomes (40–160 nm), microvesicles (100–1000 nm), and apoptotic bodies (> 1000 nm). Among these, exosomes and microvesicles are the most studied and are commonly referred to collectively as EVs. They can be distinguished by their size, molecular content, biological function, and mechanism of formation^{38,94,96,97}.

Recently, the therapeutic potential of secreted exosomes has received extensive attention. Among other inherent features of exosomes, their ability to transfer bioactive molecules from their parent cells is of great interest for their potential value in clinical treatments^{94,98,99}. Given the transfer of macromolecules, exosomes mediate intercellular communication, improve cell adhesion and migration, and facilitate the exchange of genetic information between cells^{94,96–98,100}. They have been isolated from cell culture supernatant (i.e., CM) and body fluids, including blood, serum, saliva, breast milk,

amniotic fluid, bronchoalveolar lavage fluid, synovial fluid, and urine^{101–107}. Depending on the type of releasing cell, exosomes may contain a variety of proteins, metabolites, nucleic acids, RNA, DNA, receptors, transcription factors, enzymes, lipids, and ECM proteins from their parent cells^{94,96,108,109}. Given the presence of specific cell binding sites on the surface of exosomes, they serve as signaling molecules that transport these macromolecules cargo into target cells to exert biological functions¹¹⁰. The phospholipid bilayer of exosomes provides them with desirable stability and permeability, allowing them to enter target cells to regulate their function and signalling¹¹¹.

While all cells are known to release exosomes, there is evidence that MSCs release greater quantities of exosomes¹¹⁰. It has been shown that MSC-exosomes have unique biological functions due to the presence of a specific protein subclass cargo⁹⁴. Their ability to promote tissue repair, regeneration, immunomodulation, and angiogenesis¹¹⁰ has rendered them a potential novel therapy for a variety of diseases.

As signaling molecules, the lower immunogenicity¹¹⁰ of MSC-exosomes and their ability to promote the same effects as MSCs, while also having a more stable membrane and better immune tolerance, make EVs attractive as an alternative to cell therapy. Recent studies on tendon injury reported that MSC-exosomes promoted beneficial effects on tendon healing, including reducing the early inflammatory response, inhibiting fibrosis, and regulating ECM remodeling^{57,58,112,113}. Therefore, there is growing interest in exosomes as novel therapeutic agents for clinical applications in tendon injuries.

2.3 BENEFITS OF CELL-FREE THERAPY OVER STEM-CELL BASED APPLICATIONS

There is increasing interest in the therapeutic properties of MSC-secretome derivatives, such as CM and EVs, because of safety concerns and limitations associated

with the transplantation of living, proliferative cells¹¹⁴. Stem cell-based therapies carry risks including immune rejection, poor engraftment and survival, transmission of infections, tumorigenic potential, and undesired spontaneous differentiation^{38,56,80,115,116}. For example, Liu et al. demonstrated that TSCs underwent erroneous differentiation toward chondrogenic and osteogenic lineages when cultured, *in vitro*, on degenerative decellularized tendon slices; this was likely influenced by the damaged matrix in the degenerative microenvironment¹¹⁷.

Stem cell therapies also raise logistical and biological challenges. These include variability in donor cell quality, lack of standardized production protocols, and the need for quality-control that ensures the quality of MSCs and their therapeutic effectiveness after transplantation^{51,118}. Autologous stem cell therapies require invasive cell harvesting, are time-consuming, and require expensive cell culture processes. Additionally, MSC quality and efficacy can be affected by patient factors, such as age and overall health, and exposure to inflammatory environments^{119–123}. A study investigating the effects of an inflammatory environment on the tenogenic properties of equine ASCs found that their tenogenic potential was diminished in inflammatory conditions and in the presence of leukocytes, suggesting that tenogenic differentiation of administered MSCs resulting in cell replacement is unlikely when applied directly into areas of acute tendon disease⁴⁷. These limitations highlight the need to optimize the clinical use of MSCs by exploring the immunomodulatory and regenerative properties of MSC-secretome rather than relying on live cells.

The use of MSC-secretome-based therapies offers key advantages over cell-based approaches. They mitigate many of the risks associated with cell transplantation, including

immunogenicity, tumorigenesis, infection, and undesirable spontaneous differentiation^{38,124–126}, representing a potentially safer alternative compared to cell-based therapies. Moreover, their safety, potency, and dosage can be evaluated in a manner similar to conventional pharmaceutical drugs³⁸. Another key advantage is that MSC-CM can be harvested in large quantities, lyophilized and stored long-term without losing bioactivity¹²⁷, avoiding the need for cryopreservatives present in cell freezing media such as DMSO¹²⁸. In one study, Bermudez et al. demonstrated the use of lyophilized CM from human uterine cervical stem cells to promote corneal healing in rats. The lyophilized CM was stored at -80°C and reconstituted in distilled water prior to administration, maintaining its therapeutic effect¹²⁷.

MSC-secretome derivatives are more practical for clinical applications as they avoid the need for invasive cell collection and complex culture protocols, reducing both treatment costs and time. Further, the absence of cells reduces safety concerns with MSCs handling and implantation^{38,126}. In a study involving 13 horses with spontaneous tendon or ligament injuries, intralesional administration of MSC-CM was well-tolerated, with no adverse reactions, inflammation, or tumor formation observed, demonstrating its safety for intralesional administration¹²⁹.

Importantly, MSC-secretome derivatives can be produced on a large scale using tailor-made standardized cell lines under controlled conditions. This ensures reproducibility and consistent bioactive composition, crucial for clinical application³⁸. Moreover, the low metabolic activity and acellularity of MSC-secretome preparations further simplify quality control and regulatory compliance, presenting a promising prospect to be produced similar to pharmaceuticals^{130,131}. MSC-secretome can be

manufactured, freeze-dried, packaged, stored, and transported more easily than cells¹³¹, making off-the shelf therapies for tendon injuries a feasible option^{38,126,130,132}. These cell-free products can be derived from healthy and vibrant allogeneic donors, enabling cost-effective, readily available treatment with reduced safety concerns^{38,119,133}.

2.4 MSC-SECRETOME MANUFACTURING AND ITS LIMITATIONS

Although the regenerative potential of MSC-secretome and its derivatives has been demonstrated in several studies, advancing towards clinical translation in both veterinary and human practice requires standardized and reproducible manufacturing protocols. Establishing such standards is essential to optimize the manufacturing process, and minimizing batch-to-batch variability in MSC secretion profiles, and ensuring quality control, safety, and therapeutic consistency.

Preparation of MSC-CM typically involves culturing cells for a defined period of time, followed by centrifugation and filtration to remove cell debris and collect the supernatant containing the secretome^{134,135}. However, several variables in this process remain without a standardized method. There is controversy in the literature regarding optimal cell culture conditions, including the choice of basal media, cell confluency at the time of collection, and the period of time for which the cells are cultured before collecting and processing the media, all of which can significantly affect the composition of CM^{136,137}. Most often, cells are cultured in serum-free media and collected during the first 72 hours of culture when cells are 70-90% confluent¹³⁰. Use of fetal bovine serum (FBS) in the culture media for CM production poses risks of immunologic reaction due to contamination from animal proteins and raises concerns regarding the presence of contaminants such as endotoxins and viruses^{138,139}. Moreover, when FBS is added to the media, FBS-derived

growth factors and exosomes may undesirably contaminate the secretome components released by MSCs, compromising purity and confounding experimental outcomes¹³⁰. As a result, serum-free media formulations are the current gold standard for preparing MSC-secretome derivatives¹³⁹. Understanding how cell culture conditions affect the composition, concentration, and types of bioactive molecules secreted by MSCs is key to establishing good manufacturing practices (GMP) that will allow for optimization and large-scale production of MSC-secretome products¹³². This includes defining parameters such as cell type, culture media, culture condition, conditioning time, passage number, number of cells, and processing of CM¹³⁰.

The EV fraction of MSC-secretome, including exosomes, can be isolated from the CM of cultured cells¹⁴⁰. However, manufacturing exosomes for therapeutic use involves several additional challenges, including standardization of isolation, quantification, and characterization methods, purity, and scalability^{124,141,142}. The quantity and quality of exosomes released in culture may vary depending on a variety of factors, including the source of MSC, culture conditions, and the methods used for exosomes isolation^{140,143–145}. The yield of exosomes in the CM is inherently low, with MSCs typically releasing 1–4 µg of exosomes per 10⁶ cells over a 24 hour culture period¹⁴⁴. This limits the use of exosomes in clinical trials, which require quantities that often range between 40 and 50 mg per dose¹⁴⁶.

Traditional methods of exosome isolation include differential centrifugation, ultracentrifugation, size-exclusion chromatography (SEC), immuno-based capturing (IBC), and precipitation methods¹⁴⁷. However, most of these methods are time-consuming, require specialized equipment, and do not guarantee high yield or purity of exosomes¹⁴⁸.

For example, while ultracentrifugation is the gold standard method for exosome isolation in academic research, it has major limitations for clinical use. It is time-consuming and often results in incomplete sedimentation, aggregation of exosomes and contamination with cellular debris, proteins, and other EVs of different sizes^{148–150}. Moreover, these traditional methods cannot be scaled up, hindering their applicability in clinical trials¹⁵¹. In response to these limitations, emerging technologies such as microfluidic-based methods have been explored to enable rapid, effective, and clinically compliant exosomes isolation^{96,152}.

Another major challenge in the manufacturing and clinical translation of exosome-based therapies is their heterogeneity. The structure and cargo of exosomes depend heavily on the cell source, physiological state, and microenvironmental conditions, and even exosomes produced by the same cell type may exhibit significant variability under different cell culture conditions^{94,153,154}. This heterogeneity results in batch-to-batch inconsistencies and limits the standardization of manufacturing processes and the therapeutic reproducibility of EV-based products¹⁵⁵.

2.5 LARGE-SCALE PRODUCTION OF MSC-SECRETOME

Advancing the clinical translation of MSC-secretome requires the development of scalable, cost-effective, and standardized production methods. One of the major limitations to large-scale manufacturing of MSC-secretome lies in the finite proliferative capacity of MSCs, which undergo limited divisions before they reach senescence (the Hayflick limit)^{38,156,157}. This results in the need for continuous derivation and expansion of new MSC batches to replenish the cell source of secretome and EVs, leading to batch-to-batch variability and increased production costs^{38,157}. To overcome this challenge, the use of

immortalized MSC lines has been explored as a promising strategy. For instance, Chen et al. immortalized human embryonic stem cells (hESCs) through *MYC* oncogene-transformation. The exosomes isolated from these *MYC*-transformed MSCs reduced relative infarct size in a mouse myocardial ischemia/reperfusion injury model, suggesting preserved therapeutic activity of exosomes. This study demonstrates the feasibility of oncogene-mediated immortalization as a practical approach to ensure an effectively limitless, uniform MSC supply for scalable secretome production¹⁵⁷.

The implementation of bioreactor systems also has been proposed as an effective approach for large-scale, reproducible MSC-secretome manufacturing. Compared to conventional two-dimensional (2D) cell culture systems, bioreactors offer several advantages, including the elimination of continuous cell passaging, standardized expansion of large numbers of cells under controlled conditions, and improved standardization and reproducibility of MSC-secretome production^{38,158,159}. Combining immortalized cell lines with bioreactor-based culture systems represents a promising integrated approach for scaling up MSC-secretome manufacturing¹⁶⁰.

2.6 PRIMING STRATEGIES TO IMPROVE THE THERAPEUTIC POTENTIAL OF MSC-SECRETOME

The MSC secretome is not a constant mixture of bioactive factors. In fact, the composition of secretome is known to be influenced by different stimuli present in the microenvironment surrounding the MSCs¹⁶¹. For this reason, *in vitro* pre-conditioning or priming strategies have been used to modulate MSC secretome composition and potentiate MSC's therapeutic potential for a given application^{38,51,161,162}. A variety of MSC priming

strategies have been investigated, including: a) cell culture under hypoxic conditions, b) cytokines, growth factors, and hormones, and c) tri-dimensional (3D) cell culture.

Hypoxic preconditioning is known to increase primarily angiogenic cytokine secretion and other factors involved in tissue regeneration⁵¹. Hypoxia upregulates the expression of the hypoxia inducible factor (HIF-1 α), which in turn, increases the expression of angiogenic factors, such as the vascular endothelial-derived growth factor (VEGF)¹⁶³. For example, Lee and Joe¹⁶⁴ found that VEGF and angiogenin were markedly increased in CM derived from MSCs primed with hypoxia. In addition, CM promoted increased migration and tube formation of human umbilical vein endothelial cells (HUVECs). Similarly, exosomes derived from hypoxia-stimulated BMSCs were found to increase the proliferation, migration, and tube formation of HUVECs *in vitro*, compared to PBS (control) or exosomes derived from unprimed BMSCs¹⁶⁵. Hypoxic preconditioning also has been shown to stimulate the release of factors related to ECM remodeling, such as matrix metalloproteinase 2 (MMP-2) and MMP-9^{166,167}. Chen et al. also found that MSCs cultured under hypoxic conditions secreted significantly greater amounts of interleukin 6 (IL-6) and interleukin 8 (IL-8), key mediators of the inflammatory response¹⁶⁸.

Preconditioning of MSCs with inflammatory cytokines enhances their immunomodulatory and regenerative properties by promoting increased secretion of factors involved in immune response regulation^{38,161}. Bulati et al.¹⁶⁹ investigated the immunosuppressive properties of human amnion-derived MSCs (hAMSCs) and found that hAMSCs, alone, produced an insufficient amount of immunomodulatory factors. Further, Sivanathan et al. showed that priming MSCs with interferon-gamma (IFN- γ) resulted in increased expression of genes involved in immune response, humoral response, antigen

processing and presentation, and complement system activation¹⁷⁰. It has been shown that preconditioning with inflammatory cytokines can also enhance the immunomodulatory properties of MSC-derived exosomes. For example, Li et al.¹⁷¹ found that MSCs stimulated with tumor necrosis factor- α (TNF- α) showed increased anti-inflammatory properties and were able to reduce endometrial fibrosis in a murine intrauterine adhesion model. Later, they found that primed MSCs promoted their therapeutic effect mainly through macrophage polarization to M2 phenotype via exosomes.

Stimulating MSCs with other bioactive factors, such as growth factors and hormones, seems to enhance the regenerative capacity of MSCs by favoring the secretion of factors involved in tissue repair, angiogenesis and fibrosis inhibition¹⁶¹. Studies have shown that priming of MSCs with growth factors or hormones can also favor the immunomodulatory profile of MSC secretomes. For example, priming MSCs with prostaglandin E2 (PGE2) has been shown to improve LPS-induced acute lung injury in mice by reducing proinflammatory cytokines while increasing anti-inflammatory cytokines and modulating macrophage polarization¹⁷². Similarly, Zhang et al.⁵⁶ showed that CM derived from TSCs primed with hepatocyte growth factor (HGF) was able to suppress inflammation and improve biomechanical properties in injured Achilles tendon in rats. In rats treated with CM, the expression of IL-6 reduced while the expression of IL-10, an anti-inflammatory cytokine, increased. Further, CM caused increased expression of MMP-2, MMP-9, and VEGF in fibroblasts treated *in vitro*, suggesting that CM from primed MSCs is able to promote angiogenesis and ECM remodeling.

Compared with traditional 2D monolayer cultures, culturing MSCs in a 3D environment aims to more closely mimic the natural microenvironment into which MSCs

would be implanted. Following *in vivo* implantation, these conditions would stimulate the secretion of soluble factors that contribute to cell survival and proliferation^{38,161}. 3D culture of MSCs, such as spheroids, has been demonstrated to increase the expression of CXCR4, a gene that provides instructions for the adhesion to endothelial cells. This, in turn, contributes to the survival and homing of MSCs after transplantation¹⁷³. Moreover, 3D spheroid cultures have been shown to enhance the immunomodulatory and angiogenic properties of MSCs^{173,174}. The secretome profile of CM derived from hBM-MSCs cultured as 3D spheroids showed markedly increased levels of IL-6, VEGF, IL-8, and chemokine ligand 1 (CXCL1) compared with CM from MSCs in 2D culture¹⁷⁵.

3. POTENTIAL MECHANISMS OF MSC-SECRETOME ON TENDON HEALING

Elucidating the mechanisms behind the effects of MSC-secretome in tendon healing lays the groundwork for future clinical studies. The therapeutic effects of MSC-secretome in tendon healing are mediated by a combination of soluble factors, such as cytokines, chemokines, and growth factors, as well as vesicle components, including exosomes. As previously described in this review of the literature, the CM consists of the complete regenerative milieu of secretome bioactive elements, including soluble components and EVs. While the mechanisms of CM and EVs are often discussed separately in literature, all components of MSC-secretome contribute to the paracrine actions of MSCs that modulate inflammation and promote tissue regeneration¹⁷⁶.

3.1 IMMUNOMODULATION AND ATTENUATION OF THE INFLAMMATORY RESPONSE

MSC-secretome has been shown to benefit the injured tissue via modulation of the immune response and control of the early inflammatory response by inducing an attenuated pro-inflammatory cytokine response and an enhanced expression of anti-inflammatory

cytokines such as IL-10¹¹². Zhang et al. found that the secretion of pro-inflammatory cytokines, including IL-1b and IL-6, was significantly reduced in torn human rotator cuff tendons treated with ASC-exosomes¹⁷⁷. Similarly, Lee et al. demonstrated that MSC-CM suppressed the gene expression of pro-inflammatory cytokines, including IL-6 and TNF- α , in an IL-1 β -induced *in vitro* tendinopathic model¹⁷⁸.

The inflammatory process following tendon injury, largely driven by macrophages that migrate to the injured site, is critical in the formation of adhesions during tendon healing^{179,180}, which ultimately affects the prognosis. Exosomes have been shown to regulate macrophage polarization, playing an important role in modulating the early inflammatory process and improving the healing of injured tendons^{177,181–183}. While M1 macrophages participate in the early inflammatory phase of injury, releasing pro-inflammatory cytokines essential to the body's response to injury, M2 macrophages display anti-inflammatory and pro-healing functions, playing a role in tissue regeneration^{184,185}. Wang et al. found that ASC-exosomes inhibited M1 polarization and enhanced M2 polarization, contributing to amelioration of chronic rotator cuff tendinopathy in a mouse model. Tendons in the exosome group showed less infiltration of inflammatory cells, disorganization of collagen fibers, and ground substance deposition¹⁸¹. Shen et al. demonstrated that MSC-EVs target macrophages, blocking macrophage nuclear factor- κ B (NF- κ B) activity and attenuating the early tendon inflammatory response after injury¹⁸².

MSC-exosomes were found to inhibit M1 macrophage polarization as well as the release of proinflammatory cytokines after rotator cuff reconstruction in a rats model¹⁸⁶. Similarly, BMSC-exosomes promoted tendon-bone healing by increasing M2 macrophage polarization, improving biomechanical properties after tendon-bone reconstruction in

mice¹⁸⁷. In another study⁵⁷, the expression of CCR7, a marker for type M1 pro-inflammatory macrophages, was significantly reduced in rats with Achilles tendon injury one week after TSC-exosomes treatment. On the other hand, the levels of CD163, a marker of type M2 anti-inflammatory macrophages, were significantly increased. The same study also observed that the expression of IL-10, an M2 macrophage stimulating factor, was greater in the TSC-exosomes group compared to the control groups, while IL-6, an M1 macrophage stimulating factor, was significantly decreased⁵⁷.

There is evidence that MSC-exosomes prevent the fibrotic healing response that typically develops after tendon injury via different mechanisms⁸³. Zhang et al. found that TSC-exosomes were able to reduce fibrosis and improve tendon healing by significantly decreasing the expression of COX-2 after Achilles tendon injury⁵⁷. In another study, BMSC-exosomes were found to significantly inhibit the fibrogenic process both *in vitro* and *in vivo* via the let-7a/Tgfb1 axis¹¹³.

3.2 REGULATION OF ANGIOGENESIS

Angiogenesis plays an important role in the early stages of tendon healing. It results in neovascularization, allowing the delivery of fibroblasts, inflammatory cells, oxygen, nutrients and soluble factors to the site of injury, while removing waste products^{188–190}. Increase in gene expression for a variety of pro-angiogenic soluble factors have been identified in the MSC-secretome, including VEGF, macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β , IL-8, monokine induced by IFN- γ (MIG), and stromal cell-derived factor-1 (SDF-1)^{56,191,192}. CM derived from hepatocyte growth factor (HGF)-primed TSCs was found to promote angiogenesis through upregulation of VEGF in rats with Achilles tendon injury⁵⁶.

MSC-exosomes play a role in stimulating the migration of endothelial cells to promote angiogenesis. In recent studies, BMSC-exosomes were found to promote proliferation, migration, and angiogenic tube formation of human umbilical vein endothelial cells (HUVECs)^{165,186}. BMSC-exosomes also were able to induce peritendinous angiogenesis at the rotator cuff endpoint after reconstruction in rats¹⁸⁶.

3.3 ATTENUATION OF APOPTOSIS

MSC-secretome is known to have anti-apoptotic effects that may prevent excessive cell loss after injury. MSC-EVs were found to promote tendon healing through the suppression of inflammation and apoptotic cell accumulation after local administration in patellar tendons of rats¹⁹³. Similarly, MSC-exosomes were found to attenuate cell apoptosis after a tendon-bone reconstruction in mice¹⁸⁷. Zhang et al. observed that the expression of caspase 3, a marker of apoptotic cells, was significantly decreased in rats with Achilles tendon injury treated with TSC-exosomes, compared to that in the control groups⁵⁷. Interestingly, MSC-CM was reported to exert a different effect on normal cells and cancer cells. While MSC-CM showed anti-apoptotic activity in normal cells¹²⁷, it was found to induce apoptosis in cancer cells *in vitro* and *in vivo*¹⁹⁴.

3.4 REPOPULATION OF DAMAGED TISSUE THROUGH RECRUITMENT, PROLIFERATION AND TENOGENIC DIFFERENTIATION OF ENDOGENOUS CELL

Tenocytes and TSCs play a crucial role in tendon homeostasis, function, ECM remodeling, and repair^{18,195}. Exosomes derived from TSCs were found to increase the proliferation and migration of TSCs *in vitro* through activation of the TGF β Smad2/3 and ERK1/2 signaling pathways in TSCs¹⁹⁶. Similarly, BMSC-exosomes were found to promote tendon healing by facilitating the proliferation, migration, and tenogenic

differentiation of TSCs *in vitro* and the proliferation of local TSCs *in vivo*¹⁹⁷. Moreover, TSC-exosomes promoted early tendon healing in rats through miR-144-3p-regulated tenocyte proliferation and migration¹⁹⁸ and were shown to be taken up by tenocytes and promote their proliferation and migration in a process that may be dependent on the activation of the PI3K/AKT and MAPK/ERK1/2 signaling pathways⁵⁷. CM derived from TSCs also was found to promote the viability, proliferation and migration of tenocytes^{56,199}.

3.5 BALANCE OF TENDON EXTRACELLULAR MATRIX

ECM degradation typically occurs as a result of the inflammatory response that develops after tendon injury, delaying the repair process²⁰⁰. In a recent study⁵⁸, TSC-exosomes were shown to balance the synthesis and degradation of ECM by decreasing MMP-3 expression and increasing tissue inhibitor of metalloproteinase-3 (TIMP-3), Col-1a1 and tenomodulin (TNMD) expression *in vitro* and *in vivo* in rats with Achilles tendon tendinopathy. In the same study, exosomes also improved the alignment of collagen fibers and increased the biomechanical properties of the injured tendons. Similarly, Zhang et. al.⁵⁷ demonstrated that TSC-exosomes promoted high-quality tendon healing and regulated ECM remodeling in rats with Achilles tendon injury. Treatment upregulated the expression of COL1a1, COL3a1, and tissue inhibitor of matrix metalloproteinase (TIMP-1) while downregulating matrix metalloproteinase-9 (MMP-9) at week 2. By week 8, COL1a1 and TIMP-1 levels remained significantly increased while MMP-9 levels decreased. In parallel, the authors observed a significant downward trend of COL3a1, resulting in a favorable COL1a1/COL3a1 ratio, indicative of improved tendon ECM composition. Similar results were found by Zhang et al. using CM from HGF-primed TSCs to treat Achilles tendon injury in a rat model. TSC-CM altered ECM composition, promoted the orderly

arrangement of tendon fibers, and improved the biomechanical properties of injured Achilles tendons⁵⁶.

4. CURRENT STATUS AND FUTURE PERSPECTIVES IN THE REPAIR OF TENDON INJURIES USING MSC-SECRETOME

MSC-secretome has emerged as a promising area of research for tendon repair in regenerative medicine for both animals and people. As summarized in Table 1, a variety of preclinical animal studies have shown favorable effects of MSC-derived CM and EVs on tendon healing. However, significant challenges remain, particularly the substantial variability across studies in MSC-secretome sources, dosage, frequency of administration, and mode of delivery for tendon repair¹³⁰. The lack of standardization in these parameters limits the comparability of results and represents a major barrier to clinical translation.

As shown in Table 1, most studies rely on small animal tendon injury models, particularly rodent models, which have a faster rate of tendon healing than humans. Among large animals, the horse represents the only available animal model of naturally occurring tendon injuries^{47,48}. Nonetheless, only a single preclinical study has reported intralesional administration of MSC-CM in horses affected by spontaneous tendon or ligament injuries¹²⁹, and one case report has described the use of MSC-EVs in a 6-year-old Dutch Warmblood gelding diagnosed with suspensory ligament injury²⁰¹. Additionally, a pilot study investigating the therapeutic potential of MSC-EVs for acute rotator cuff injury in sheep has been published²⁰². While all three studies reported favorable clinical outcomes and improved tendon tissue quality as assessed by histology or diagnostic imaging, none included objective functional assessments of the tendons.

Most preclinical studies on MSC-secretome for tendon healing lack objective functional assessments such as gait analysis. A recent study reported the use of a video-based gait analysis to assess tendon functional healing after treatment with ASC-EVs in mice with Achilles tendon injury. Gait analysis demonstrated that EVs reduced functional deficits after Achilles tendon healing²⁰³. Functional assessments are key to determine if MSC-secretome treatment yields improvements in tendon function and should be considered in further animal studies.

The relative efficacy of the complete CM versus the EV fraction alone remains poorly understood. Some evidence suggests that the immunomodulatory and regenerative effects of MSC-secretome result from the synergistic action of EV cargo and soluble proteins, implying that complete CM may have greater therapeutic potential than the EV fraction alone²⁰⁴⁻²⁰⁶. Supporting this, a recent study showed that MSC-CM was more effective than EVs alone in modulating inflammation in tenocytes³⁵. However, further research is needed to clarify these differences and identify components that are most critical to therapeutic efficacy.

To date, only one active clinical trial is evaluating MSC-secretome for a tendon disorder: a phase 1/2 study at Shanghai Sixth People's Hospital (NCT07111325) testing the safety and preliminary efficacy of human induced pluripotent stem cell-derived extracellular vesicle (iEV) for lateral epicondylitis of the humerus. There are currently no other active tendon-specific clinical trials testing MSC-secretome derivatives in people or animals. More robust preclinical evidence, generated from large animal models, is a necessary first step toward clinical trials designed to rigorously assess the safety and efficacy of MSC-secretome derivatives for tendon healing.

5. CONCLUSION

Growing evidence supports the therapeutic potential of MSC-secretome for tendon healing. However, further research is needed to fully establish its safety, efficacy, and clinical viability. In particular, addressing the challenges in the manufacturing of MSC-secretome, including standardization of all processes and steps, is critical to minimize variability in secretome components and ensure consistent quality and therapeutic reliability. Moreover, robust preclinical studies focusing on large animal models and well-designed clinical trials are essential to bridge the gap between experimental findings and clinical translation of MSC-secretome for tendon healing. While many challenges remain unresolved, the promise of MSC-secretome as a cell-free therapy for tendon healing warrants continued investigation and development.

Table 1. Summary of preclinical studies evaluating the use of MSC-secretome for tendon healing.

Treatment used (source/ dosage/ frequency)	Animal model	Number of Study Subjects	Outcomes	Reference number
Horse amniotic membrane-derived MSCs CM (AMC-CM) (volume/dose not specified, single local injection)	Horse (spontaneous tendon/ligament injury)	n = 13	Lower re-injury rate (15.38 %), improved clinical/US outcomes	129
ADSC-derived EVs (single injection, dose not specified)	Rabbit (Achilles tendon injury)	n = 36	The mechanical strength, histology, and protein expression in the injured tendons significantly improved 4 weeks after ADSC-EV treatment	207
BMSC-derived EVs (2×10^{10} particles in 50 μ L PBS, single injection)	Rat (patellar tendon injury)	n = 16	Reduced inflammation and apoptotic cell accumulation, improved collagen fibers organization and alignment, increased the expression of genes related to tendon ECM formation and tenogenic differentiation with improved collagen I/III ratio, and increased TSC (CD146 ⁺) presence	208
TSC-exosomes (50 μ g in 20 μ L PBS, single injection).	Rat (Achilles tendon injury)	n = 18	Balanced ECM synthesis/degradation, reducing fibrosis and improving tendon strength.	209
BMSC-derived EVs (low dose: 10 μ g, high dose: 30 μ g, single injection)	Rat (Achilles tendon injury)	n = 16	High-dose EVs significantly improved tendon repair, with better collagen organization and mechanical strength compared to low-dose or control groups	210
MSC-derived EVs (10^{10} particles in 50 μ L PBS, single injection)	Mice (Achilles tendon injury)	n = 32	Attenuated early inflammatory response, reduced the rate of post-repair tendon gap formation and rupture, and facilitated collagen formation	211
Tendon stem cell-derived exosomes (TSC-Exos) embedded in a GelMA hydrogel (10 μ g in 20 μ L PBS, single injection)	Rat (Achilles tendon injury)	n = 54	Reduced inflammation, improved collagen organization, enhanced mechanical strength, and reduced scarring	212

Table 1: Continued

BMSC-derived exosomes (100 µg in 50 µL PBS, single injection)	Rat (patellar tendon injury)	n = 52	Stimulated TSCs proliferation <i>in vivo</i> , promoted ECM remodeling, and enhanced the mechanical properties and histological scores of injured tendons.	213
CM from HGF-treated TSCs (100 µL, injected twice weekly for 2 weeks)	Rat (Achilles tendon injury)	n = 63	CM promoted ECM remodeling, angiogenesis, and collagen fibers alignment, reduced inflammation and reduced scar formation, and enhanced the biomechanical properties of injured tendons.	56
Human umbilical cord MSC-derived exosomes with/without miR-29a-3p overexpression (100 µg in 50 µL fibrin glue, single injection)	Rat (Achilles tendon injury)	n = 90	Exosomes enhanced collagen deposition, tendon-specific matrix components, and biomechanical properties. Engineered exosomes (overexpressing miR-29a-3p) showed superior healing effects compared to natural exosomes.	214
ADSC-derived exosomes embedded in GelMA (200 µg, single injection)	Rat (patellar tendon injury)	n = 63	Activated both SMAD1/5/9 and SMAD2/3 pathways, promoting tenocyte proliferation and ECM synthesis	215
TSCs-derived exosomes in a scaffold of photopolymerizable hyaluronic acid (200 µg in 50 µL PBS, single injection)	Rat (patellar tendon injury)	n = 72	Exosomes promoted early healing of injured tendons, with better fiber arrangement, higher histological scores, and improved biomechanical properties.	198
Large EVs from iPSC-derived MSCs (single injection, dose not specified)	Rat (quadriceps tendon injury)	n = 30	EVs reduced inflammation by regulating macrophage polarization (M1→M2) and promoted tendon regeneration.	216
Human umbilical cord MSC-derived exosomes (dose not specified)	Rat (Achilles tendon injury)	n = 18	Reduced tendon injury via miR-27b-3p/ARHGAP5/RhoA signaling pathway.	217

Table 1: Continued

EVs from TNF- α -primed ADSCs on a collagen sheet (1×10^9 or 5×10^9 EVs, single injection)	Mice (Achilles tendon injury)	n = 32	Both doses of primed EVs reduced inflammation and enhanced intrinsic healing, improving collagen fiber organization and mechanical properties. miR-147-3p was identified as one of active components mediating the anti-inflammatory function of EVs from primed MSCs.	218
Collagen-targeted EVs from TSCs delivered via decellularized tendon scaffolds	Rat (Achilles tendon injury)	n = 72	Scaffold-EVs promoted tendon regeneration with better collagen deposition and biomechanical recovery than scaffold alone	219
TSC-EVs combined or not with GelMA (500 μ g of EVs in 50 μ g of PBS or 500 μ g of EVs in 50 μ g of GelMA, single injection)	Rat (Achilles tendon injury)	n = 96	The healing of injured Achilles tendons was optimized by loading GelMA with TSC-sEVs. The combination of EVs with GelMA resulted in a local slow-release therapeutic effect of EVs for more than 7 days. EVs promoted tendon repair by regulating miR-145-3p	220
TSC-secretome and ASC-secretome, alone or combined (single injection)	Rat (Achilles tendon injury)	n = 14	Combined TDSC + ASC secretome increased Type I collagen synthesis (PINP levels) but showed no significant improvement in collagen alignment or angiogenesis	221

CHAPTER III: MATERIALS AND METHODS

1. ETHICS

All animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee (IACUC Protocol #3020-0224), and performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals²²².

2. ISOLATION, CULTURE, AND CHARACTERIZATION OF TENDON-DERIVED STEM CELLS (TSCs)

Tendon stem cells (TSCs) used in this study were previously isolated from the Achilles tendon of New England rabbits and characterized by Ozmen et al.^{223,224}. Briefly, harvested tendon samples were minced into small pieces, enzymatically digested in a solution of collagenase I (3 mg/mL) and dispase II (4 mg/mL), and incubated with horizontal shaking for 1.5 h at 37°C. Following digestion, undigested tissue fragments were transferred to T25 culture flasks containing Dulbecco's Modified Eagle Medium (DMEM-F12, HyClone™, Wilmington, DE, USA) supplemented with 20% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin–streptomycin–neomycin (Thermo Fisher Scientific, Waltham, MA, USA), and 0.1% amphotericin B (Thermo Fisher Scientific, Waltham, MA, USA). The remaining digest was centrifuged at 1500 x g for 15 minutes at 4°C and the resulting supernatant was collected and transferred to T25 culture flasks containing complete growth media. The residual cell pellet was resuspended in fresh media and plated separately. All cultures were maintained under standard incubation conditions (37°C, humidified atmosphere with 5% CO₂). When cultures reached 80–90% confluency, cells were harvested with 0.25% trypsin for cryopreservation or cell characterization.

Cells were characterized by immunofluorescence detection assays for the expression of CD90, CD44, and CD 29. CD34 was used as a negative control. Tenogenic differentiation of cells was also assessed by immunofluorescence detection assays for the expression of collagen I, tenascin C (TenC) and tenomodulin (TNMD). In addition, proliferation rates of cells were assessed at 1, 3, and 5 days using the CellTiter 96 AQueous One Solution Cell Proliferation (MTS) assay (Promega, Madison, WI, USA).

3. PREPARATION OF CONDITIONED MEDIA

TSCs were thawed and cultured in complete growth media consisting of Dulbecco's Modified Eagle Medium (DMEM-F12, HyClone™, Wilmington, DE USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin–streptomycin–neomycin (Thermo Fisher Scientific, Waltham, MA, USA), and 0.1% amphotericin B (Thermo Fisher Scientific, Waltham, MA, USA), until 80-90% confluency. The cells were harvested with 0.25% trypsin, washed twice with PBS to remove residual FBS, and seeded at a density of 1×10^6 cells per T175 flask, using serum-free media (SFM, DMEM-F12, 1% penicillin–streptomycin–neomycin, and 0.1% amphotericin B). All cells used were passage 3. Two different TSC-CM were prepared: CM1, containing SFM with 10 ng/ml of TGF β -3 (Peprotech, Rocky Hill, NJ, USA), as previously described²²⁵, and CM2, containing SFM only. After 48 h of conditioning, the supernatant from CM1 and CM2 was collected and centrifuged at 3000 x g for 20 minutes at 4°C. The new supernatant was filtered through a 0.22- μ m sterile filter to remove cell debris or other particulate material, and stored at -80°C for use in *in vitro* and *in vivo* experiments. Protein concentration of CM1 and CM2 was determined using the NanoDrop

2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA) in triplicates. SFM was used as a blank.

4. FUNCTIONAL CHARACTERIZATION OF CONDITIONED MEDIA

4.1 PROTEOMIC ANALYSIS

Proteomic analysis of CM from TGF β -3-primed TSCs (CM1) and unprimed TSCs (CM2) was performed by the IDeA National Resource for Quantitative Proteomics (University of Arkansas for Medical Sciences, Little Rock, AR, USA) (Proteome ID: UP000001811 for *Oryctolagus cuniculus*). CM samples from each group (CM1 and CM2) were collected, filtered to remove cell debris and other particulate material, and frozen at -80°C prior to shipment for analysis in triplicates.

FASP Methods – Orbitrap Exploris DIA

Protein samples were reduced, alkylated, and digested on-bead using filter-aided sample preparation²²⁶ with sequencing grade modified porcine trypsin (Promega). Tryptic peptides were then separated by reverse phase XSelect CSH C18 2.5 μm resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 60 min gradient from 98:2 to 65:35 buffer A:B ratio. Eluted peptides were ionized by electrospray (2.4 kV) through a heated capillary (275°C) followed by data collection on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific).

Precursor spectra were acquired with a scan from 385-1015 Th at a resolution set to 60,000 with 100% AGC, max time of 50 msec, and an RF parameter at 40%. DIA was configured on the Orbitrap 480 to acquire 40 x 15 Th isolation windows, normalized AGC target 500%, maximum injection time 40 ms. A second DIA was acquired in a staggered

window (15 Th) pattern with optimized window placements from 390-990 Th. Buffer A = 0.1% formic acid, 0.5% acetonitrile. Buffer B = 0.1% formic acid, 99.9% acetonitrile.

Data Analysis: Spectronaut - VSN

Following data acquisition, data were searched using Spectronaut (Biognosys version 19.9) against the UniProt *Oryctolagus cuniculus* database (2nd version of 2025) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to maxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Protein MS2 intensity values were assessed for quality using ProteiNorm²²⁷. The data was normalized using VSN²²⁸ and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (limma) with empirical Bayes (eBayes) smoothing to the standard errors^{229,230}. Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant (upregulated). Proteins were further classified as enriched if $1.0 < FC < 2.0$, adj. p < 0.05, not differentially secreted (similar in CM1 and CM2) if p > 0.05, and downregulated if $FC \leq 0.5$, adj. p < 0.05.

4.2 CELL PROLIFERATION ASSAYS

To evaluate the effect of CM on the proliferation of TSCs, we assessed the proliferation of rabbit TSCs cultured in CM1 and CM2 at days 1, 3, and 5 using the CellTiter 96 AQueous One Solution Cell Proliferation (MTS) assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. Complete media served as a positive control to verify assay performance, while SFM served as the true control for comparison with CM1 and CM2. Briefly, rabbit TSCs (passage 3) were harvested from

T175 flasks with 0.25% trypsin and seeded into 24-well plates at 2.0×10^4 cells per well in 500 μ L of CM1, CM2, complete media, or SFM. All cells were initially seeded in complete growth media and incubated overnight at 37°C with 5% CO₂ to allow for cell attachment. The wells were then washed twice with PBS to ensure complete removal of residual FBS before adding the treatments. Complete media and SFM were used as blanks. Absorbance was read at 490 nm on a microplate reader (BioTek, Winooski, VT, USA). An additional well was utilized at each timepoint for quantitative viability using Calcein AM dye (Thermo Fisher, Waltham, MA, USA) per the manufacturer's instruction. Stained cells were imaged on a fluorescent microscope (Leica Microsystems, Wetzlar, Germany).

4.3 IMMUNOFLUORESCENCE ASSAYS

To assess the tenogenic differentiation potential of CM, expression of three tendon-associated ECM proteins, collagen I, tenomodulin (TNMD) and tenascin C (TenC), was assessed at days 1 and 3 by immunofluorescence, as previously described^{225,231,232}. Briefly, rabbit TSCs (passage 3) were harvested from T175 flasks with 0.25% trypsin, and transferred to 6-well plates at 5.0×10^4 cells per well in 2 mL of CM1, CM2, or complete media (control). All cells were initially plated in complete media and incubated overnight at 37°C with 5% CO₂ to allow for cell attachment. Wells were then washed twice with PBS to remove residual FBS before adding treatments. On days 1 and 3, cells from each treatment group were fixed with 4% paraformaldehyde for 10 min at room temperature, rinsed twice with HBSS (Hanks' Balanced Salt Solution), and permeabilized with 0.1% Triton X-100. Cells were then blocked with Universal Blocking Reagent (BioGenex, Fremont, CA, USA) for 30 min at room temperature and incubated at 4°C for 24 hours with primary antibodies to collagen I (Bio-Rad, Hercules, CA, USA), TNMD (Biorbyt,

Cambridge, UK), and TenC (Invitrogen, Waltham, MA, USA) at 1–2 μ L per well. Following two HBSS washes, appropriate Alexa Fluor–conjugated anti-mouse or anti-rabbit secondary antibodies were applied for 30 min at room temperature in the dark. Nuclei were counterstained and slides mounted with ProLong Gold antifade reagent containing DAPI (Invitrogen, Waltham, MA, USA). Fluorescent images were acquired on a Leica microscope (Leica Microsystems, Wetzlar, Germany). Images were analyzed in Fiji/ImageJ (Bethesda, MD, USA) to quantify DAPI-labeled nuclei and the numbers of collagen I–positive, TenC–positive, and TNMD–positive cells, following established methods^{225,233,234}. Quantitative outcomes were expressed as the ratio of marker–positive cells to total DAPI–stained nuclei as described²²⁵ and evaluated statistically by one-way ANOVA with Tukey’s post hoc test.

5. FIBRIN GEL

To enable controlled delivery, CM1, CM2, or SFM (control) was incorporated into a fibrin gel fabricated in our laboratory from fibrinogen and thrombin (MilliporeSigma, Burlington, MA, USA) at a ratio of 0.2 U thrombin per 1 mg fibrinogen, as previously described²²⁵. Each gel received 100 μ L of CM or SFM. To verify gel cytocompatibility, viability of rabbit TSCs cultured with the fibrin formulations was assessed on days 1, 3, and 5 using the CellTiter 96 AQueous One Solution (MTS) assay (Promega, Madison, WI, USA) and Calcein AM dye (Thermo Fisher, Waltham, MA, USA). Briefly, fibrin gels containing CM1, CM2, or SFM were prepared in 24-well plates, allowed to gel for approximately 15 minutes, and then incubated for approximately 30 minutes until liquid state of gel was achieved. Cells were seeded onto the gels at 2.0×10^4 cells per well. 50 μ L of complete growth media was added to each well, and the cells were subsequently

incubated for 15 minutes before adding more complete growth media to the wells. All assays and data analysis were performed as described for the prior cell viability experiments.

6. SURGICAL PROCEDURE

Eighteen female Lewis rats (10–11 weeks old; 150–225 g) were acquired and housed according to the NIH Guide for the Care and Use of Laboratory Animals²²². An Achilles tendon injury was created in the right hindlimb of each animal, as previously described^{225,235,236}. Rats were randomized (n = 6/group) to receive intralesional fibrin gel containing SFM (control), CM1, or CM2. Preoperatively, buprenorphine (0.05 mg/kg, SQ) was administered, and anesthesia was induced with isoflurane. The Achilles tendon was exposed through a 1 cm longitudinal incision over the posterior right hindlimb, and the central third was isolated and entirely removed. The assigned treatment was then delivered intralesionally by placing the fibrin gel into the defect with forceps. Simple interrupted sutures were used to close the skin in a single layer. Following the surgical procedure, animals were individually housed and allowed free cage activity. Analgesia (buprenorphine 0.05 mg/kg, SQ) was provided every 12 hours for 3 days and all animals were monitored routinely for overall health status, incision healing, body weight/body score condition, urination/defecation, and lameness. At 30 days, all rats were euthanized, and the gross appearance of the Achilles tendons was evaluated. The tendons were then harvested for further analyses.

8. HISTOLOGICAL ANALYSIS

Tendons specimens were fixed in 10% formalin for 48h and embedded in paraffin. Tissue blocks were cut parallel to the long axis of the tendons and mounted on glass slides.

Slides were stained with hematoxylin and eosin (H&E) and Masson's Trichrome (MT) to evaluate tendon morphology and healing. Subsequently, all slides were imaged using a digital microscope (Keyence, IL, USA). Histological grading was performed by one reviewer that was blinded to treatment groups. For each tendon, the entire slide, comprising a defect from the myotendinous junction to the insertion at the calcaneus, was scored using a semi-quantitative grading score (0 = best score, 3 = worst score, with a total possible score of 18), as previously described^{225,237} (Table 2). The parameters evaluated included fiber structure and arrangement, cell density, cell appearance, inflammation, neovascularization, and fatty deposits.

10. STATISTICAL ANALYSIS

Data handling and cleaning were performed in Microsoft Excel; all statistical analyses were conducted in R (R Foundation for Statistical Computing). Data are reported as mean $\pm$ SD, with statistical significance set at $\alpha = 0.05$. The effects of CM over time for MTS and immunofluorescence (IF) assays were evaluated using two-way ANOVA, with Tukey's test for pairwise comparisons. Between-group differences at days 1, 3 and 5 (MTS) or 1 and 3 (IF) were tested by one-way ANOVA, with significant interactions followed by simple effects with Tukey's adjustment. Ordinal histological scores (0-3) were compared across groups using the Kruskal–Wallis test and summarized as counts and percentages. Finally, a power analysis of *in vivo* histological total score (0-18) results was performed to test sample size power using one-way ANOVA, post-hoc power check (i.e., Kruskal-Wallis test), and Pairwise Hedges' g.

Table 2. Semi-quantitative histological grading score as described by de Girolamo et al. (2019).

Score	Fiber Structure and Arrangement	Cell Density	Cell Appearance	Inflammation	Neovascularization	Fatty Deposits
0	Normal; compact, parallel, and long fibers	Normal	Long, spindle-shaped cells with elongated nuclei	<10%	Normal appearance and number of vascular bundles	Normal; no lipid deposits
1	Slight fragmentation, mildly wavy, disorganized fibers	Slightly increased	Slightly rounded cells	10-20%	Slight increase in vascularity	Slight increase in lipid deposits
2	Moderate fragmentation and disorganization; crossed, wavy, and short fibers	Moderately increased	Moderately rounded cells	20-30%	Moderate increase in vascularity	Moderate increase in lipid deposits
3	Severe disorganization with no appreciable pattern; short and fragmented fibers	Markedly increased	Markedly rounded cells with distinctly round nuclei	>30%	Marked increase in vascularity	Marked increase in lipid deposits

CHAPTER IV: RESULTS

1. ISOLATION, CULTURE, AND CHARACTERIZATION OF TENDON-DERIVED STEM CELLS (TSCs)

The lineage of TSCs was successfully isolated and characterized by Ozmen et al.^{223,224}. Briefly, immunofluorescence assays for the expression of surface markers showed

that isolated TSCs expressed CD90, CD44, and CD29, and they did not express CD34. Tenogenic differentiation of isolated TSCs was confirmed by the expression of collagen I, TenC, and TNMD by immunofluorescence. Cell numbers increased linearly over a period of five days as assessed by MTS assays.

2. PREPARATION OF CONDITIONED MEDIA

Approximately 45 mL of CM1 and CM2 was obtained for use in *in vitro* and *in vivo* experiments. The protein concentration of CM1 and CM2 was determined using the NanoDrop 2000 spectrophotometer. A mean protein concentration of 0.644 $\mu\text{g}/\mu\text{l}$ (± 0.04) was detected in CM1 and a mean protein concentration of 0.203 $\mu\text{g}/\mu\text{l}$ (± 0.03) was detected in CM2.

3. FUNCTIONAL CHARACTERIZATION OF CONDITIONED MEDIA

3.1 PROTEOMIC ANALYSIS

We performed proteomic analyses of CM derived from TGF β -3-primed TSCs (CM1) and unprimed cells (CM2) to investigate changes in the secretome induced by TGF β -3 priming. One of the three CM1 replicates displayed a secretion profile that was markedly distinct from the other samples, as shown by both the normalized heatmap and principal component analysis (PCA) (Figure 1). Because this sample did not cluster with its biological group, it was considered an outlier and excluded from downstream analyses. A total of 537 different proteins were identified in CM1 and 535 proteins in CM2. The normalized heatmap revealed a distinct proteomic profile between CM1 and CM2, which was confirmed by PCA (Figure 2). Differential protein secretion between groups was assessed using volcano plot analysis, with a significance threshold of fold change >2 and adjusted p-value <0.05 (Figure 3). Based on this analysis, 41 proteins were differentially

secreted between CM1 and CM2. Of these, 38 proteins were significantly upregulated and 4 were downregulated in CM1 (Table 3, Appendix). Proteomics showed that TGF β -3 had been depleted from the culture media of CM1 by 48h of culture.

Several proteins that are known to play an important role in tendon healing were identified among the upregulated, enriched, and similar in CM1 and CM2 groups.

Upregulated proteins (FC > 2, p < 0.05) in CM1: fibrillar and non-fibrillar collagens (COL1a1, COL1a2, COL5a2, COL11a1, COL14a1, COL15a1, COL8a1/2), fibronectin (FN1), fibrillin 1 (FBN1), fibromodulin (FMOD), secreted protein acidic and rich in cysteine (SPARC), thrombospondins (TSP1/2), latent transforming growth factor beta binding proteins (LTBP1/2), CCN family member 2 (CCN2), plasminogen activator inhibitor 1 (SERPINE1), biglycan (BGN), periostin (POSTN), Follistatin like 1 (FSTL1), Lysyl oxidase (LOX2/3), and milk fat globule-EGF factor 8 protein (MFGE8). **Enriched in CM1 (1.0 < FC < 2.0, p < 0.05):** tenascin C (TenC), procollagen C-endopeptidase enhancer (PCOLCE), collagen type III alpha 1 chain (COL3a1), collagen type VI alpha 3 chain (COL6a3), metalloproteinase inhibitor 1 (TIMP-1) and peroxidasin (PXDN). **Similar in CM1 and CM2 (p > 0.05):** galectin-3 (LGALS3), nuclear factor interleukin-3-regulated protein (NFIL3), metalloproteinase inhibitor 2 (MMP-2), and metalloproteinase inhibitor 2 (TIMP-2). Figure 4 illustrates key secreted factors of interest for tendon healing detected by proteomics, alongside their potential functional effects on tendon healing.

3.2 CELL VIABILITY ASSAYS

Proliferation of rabbit TSCs was quantified by MTS assay, at 490 nm, on days 1, 3, and 5, and qualitative viability evaluated by Calcein AM staining (Figure 5). On day 1, no statistically significant differences in cell proliferation were observed among groups (p

> 0.05), with all groups presenting similar absorbance values. On day 3, MTS values increased significantly relative to day 1 in the CM1 and CM2 groups ($p < 0.05$), but CM1, CM2, and SFM were not significantly different from each other ($p > 0.05$). On day 5, CM2 showed higher proliferation than SFM ($p < 0.05$), whereas CM1 was not significantly different from SFM or CM2. From day 3 to day 5, all three groups exhibited a decrease in cell proliferation. However, this was not statistically significant for the CM1 group ($p > 0.05$) (Figure 6). Live cell imaging using Calcein AM dye was consistent with MTS results (Figure 5A).

3.3 IMMUNOFLUORESCENCE ASSAYS

The tenogenic differentiation potential of CM was assessed by immunofluorescence for collagen I (Figure 7), TNMD (Figure 8) and TenC (Figure 9) on days 1 and 3. On day 1, no significant differences in collagen I expression were detected among treatment groups ($p > 0.05$). In contrast, TenC expression varied significantly between groups ($p < 0.05$), with CM1-treated cells showing higher expression (0.94 ± 0.02) compared to both CM2 (0.79 ± 0.03) and control (0.79 ± 0.05) groups (Tukey, $p < 0.05$). TNMD expression was also significantly affected by treatment ($p < 0.05$), with CM1 showing markedly higher cell ratios (0.92 ± 0.06) compared to CM2 (0.28 ± 0.02) and control (0.36 ± 0.11).

On day 3, Collagen I expression showed no statistically significant differences between treatments ($p < 0.05$), although CM1 and CM2 displayed numerically higher ratios (0.93 ± 0.02 and 0.87 ± 0.04 , respectively) compared to control (0.76 ± 0.11). TenC expression did not differ significantly between groups ($p > 0.05$). TNMD expression remained significantly different across treatments ($p < 0.05$), with CM1-treated cells (0.91

± 0.02) exhibiting higher expression compared to CM2 (0.28 ± 0.06) and control (0.56 ± 0.08), with all pairwise comparisons significant in Tukey's test ($p < 0.05$).

No significant changes were observed in the expression of TNMD between day 1 and day 3 for any group ($p > 0.05$), while changes in the expression of Collagen I and TenC over time were significant ($p < 0.05$) and are shown in Figure 10. In the CM1 group, collagen I expression was significantly higher on day 3 ($p < 0.05$) compared to day 1, while there was a significant decrease in TenC expression on day 3 ($p < 0.05$). CM1 consistently promoted higher TNMD expression at both time points, but no significant changes in expression were observed on day 3 ($p > 0.05$). In the CM2 group, no significant changes were observed in the expression of collagen I and TNMD between day 1 and day 3 ($p > 0.05$), while a significant increase in TenC on day 3 was observed ($p < 0.05$). In the control group, no significant changes were observed in the expression of any of the markers between day 1 and day 3 ($p > 0.05$).

4. FIBRIN GEL CYTOCOMPATIBILITY

Fibrin gels incorporating CM1, CM2, or SFM (control) were fabricated in our laboratory. To evaluate the cytocompatibility of the gel, TSC proliferation was assessed over a period of five days. No statistically significant differences were found in the proliferation rate of cells between groups (ANOVA, $p > 0.05$). Live cell imaging using Calcein AM dye was consistent with MTS results (Figure 11).

5. INTRALESIONAL ADMINISTRATION OF CONDITIONED MEDIA IN ACHILLES TENDON INJURY

5.1 GROSS MORPHOLOGY OF HARVESTED RAT ACHILLES TENDON

At 30 days post-surgery, the gross appearance of the repaired Achilles tendons varied among the three groups (Figure 12). In all groups, the defect created by central third resection had been bridged by newly formed tissue, but the quality, thickness, and surface characteristics differed. Tendons in the **control group** appeared irregular and hypertrophic at the defect site. The repair tissue was thickened compared to the adjacent native tendon and was opaque and heterogeneous in color. The transition between the newly formed tissue and native tendon was abrupt, with evident surface irregularities. Tendons treated with **CM2** showed improved gross tissue organization relative to the control group. The repair region was more tapered and better aligned with the native tendon, with reduced thickening of the neo-tissue. The tissue color was more uniform, presenting as a glossy, whitish-pink band with fewer surface irregularities. The integration with adjacent native tendon appeared smoother, though mild thickening at the repair site was still present. Grossly, the **CM1** tendons most closely resembled the native tendon. The defect region was bridged by slender, well-aligned tissue with a spindle-shaped morphology that followed the longitudinal axis of the tendon. The repair tissue had a smooth, native-like appearance and was more homogeneous in color compared to control and CM2 tendons. The junctions between the repair site and native tendon were smoother, with minimal evidence of thickening. Adhesions to the skin or surrounding soft tissues were not observed in any of the groups.

5.2 HISTOLOGICAL ANALYSIS

Semi-quantitative grading of H&E- and MT-stained sections, assessing fiber structure and arrangement, cell density, cell appearance, inflammation, neovascularization, and fatty deposits, revealed no significant differences among groups ($p > 0.05$) (Figure 13). Quantitatively, however, tendons treated with CM1 and CM2 appeared more linearly organized than controls. All groups exhibited defect bridging with hypercellular repair tissue and immature collagen, and inflammatory infiltrates were not observed in any of the groups (Figure 14). Complete histologic evaluation was not possible for two CM1 specimens, one CM2 specimen, and one control specimen due to tissue-processing artifacts.

Power analysis and sample size. A post-hoc power check showed low omnibus power (~ 0.37 , $\alpha = 0.05$) and a non-significant rank-based test, indicating a substantial risk of Type II error. Pairwise contrasts had low achieved power (~ 0.10 – 0.46), and, if the observed effects are true, would require 13–91 animals per group, depending on the comparisons, to reach 80% power.

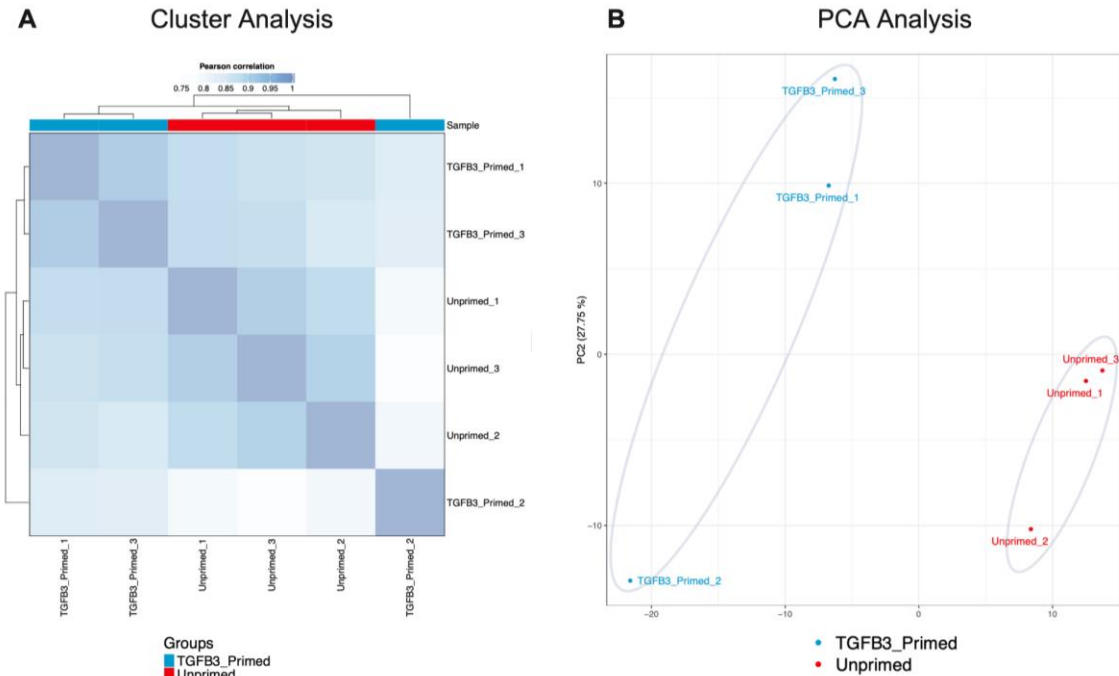


Figure 1. Cluster analysis (A) and PCA analysis (B) showing markedly distinct secretion profile of one CM1 (TGFB3_Primered) replicate (TGFB3_Primered_2) from the other CM1 samples.

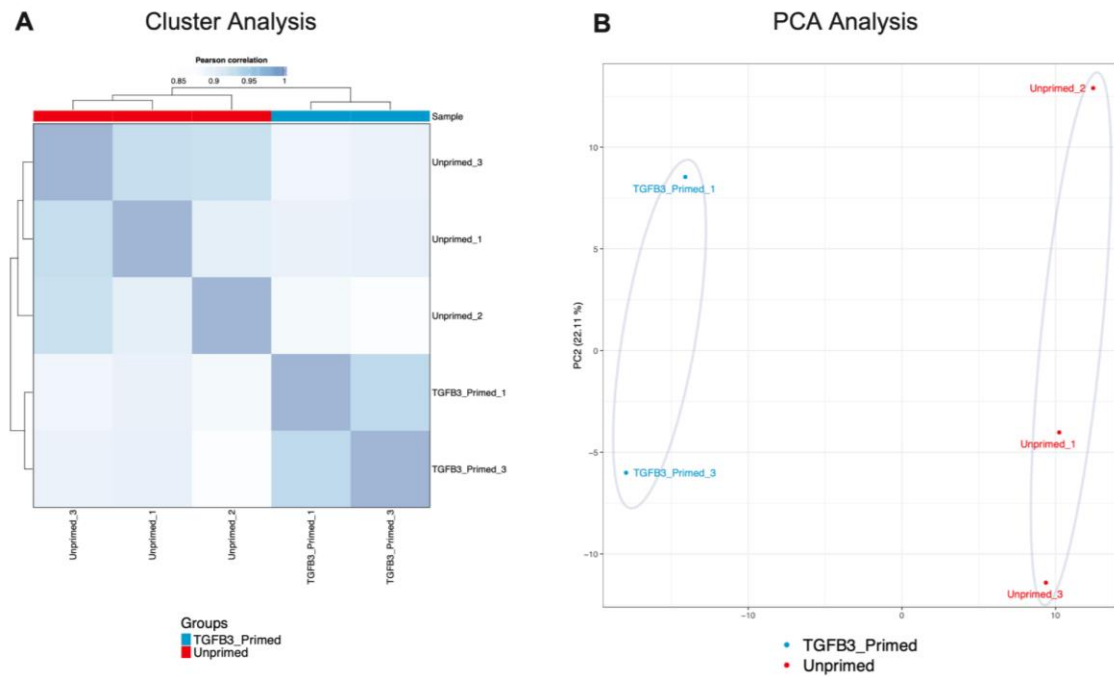


Figure 2. Cluster analysis (A) and PCA analysis (B) showing a different secretion profile between CM1 (TGFB3_Primered) and CM2 (Unprimed) samples.

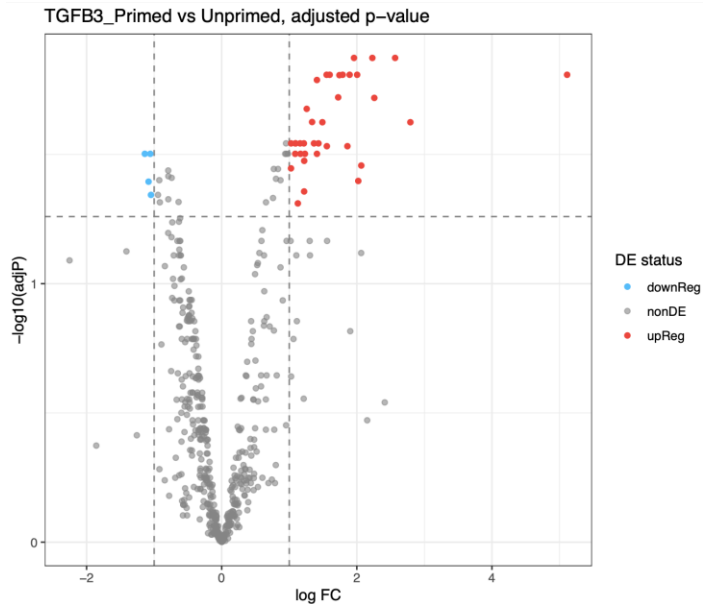


Figure 3. Volcano plot analysis used to assess differential protein secretion between CM1 (TGFB3_Primed) and CM2 (Unprimed). Significance threshold of fold change >1 and adjusted p-value <0.05 were used.

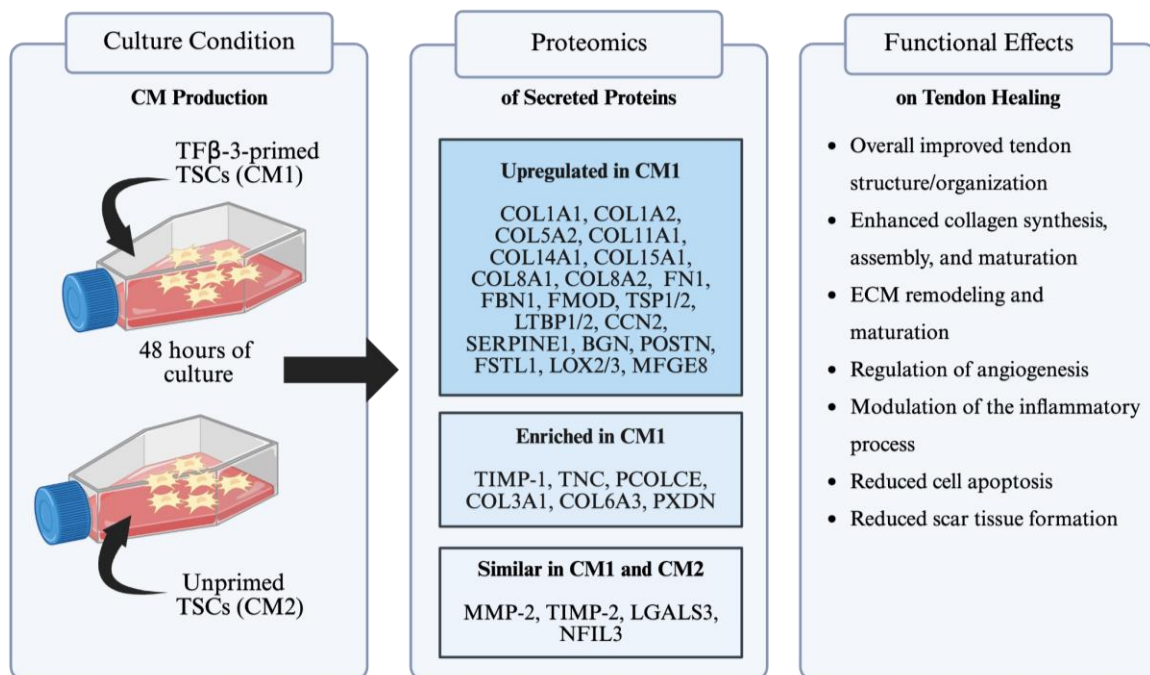


Figure 4. Schematic representation of the effects of TGF-β3 priming on the TSC secretome and putative functional outcomes for tendon healing. Rabbit TSCs were cultured for 48 h either with TGF-β3 (CM1) or without priming (CM2) to generate CM. Proteomics of secreted factors is summarized by three bins: Upregulated in CM1 ($FC > 2$, $p < 0.05$), Enriched in CM1 ($1.0 < FC < 2.0$, $p < 0.05$) and similar in CM1 and CM2 ($p > 0.05$). The right panel lists inferred functional effects on tendon repair.

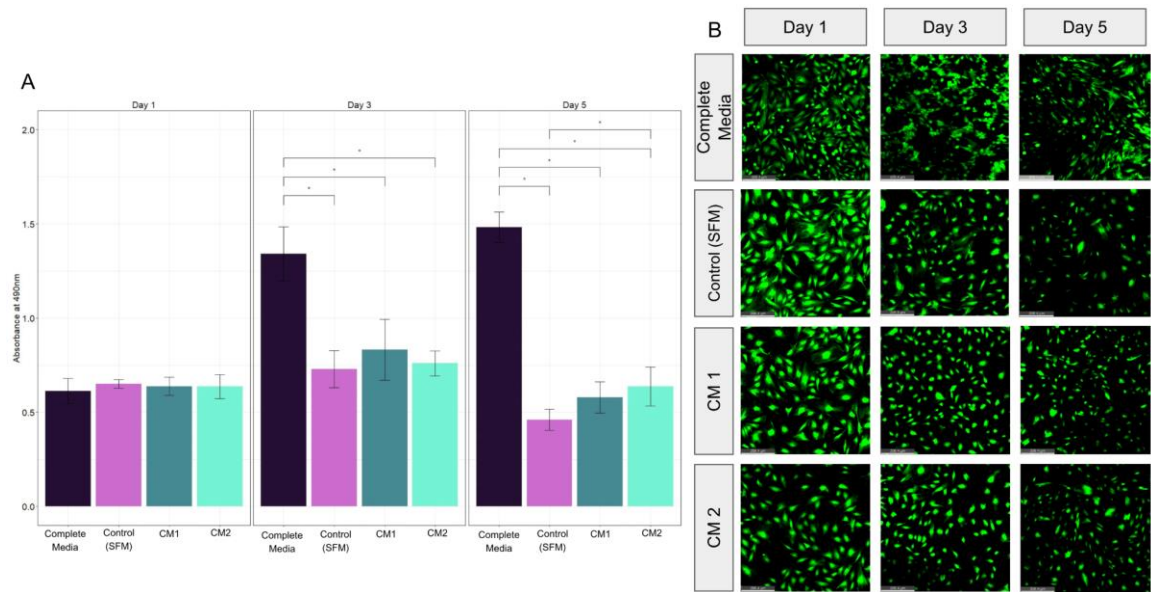


Figure 5. Proliferation and viability assays of rabbit TSCs. (A) Cell metabolic activity measured by MTS (absorbance at 490 nm) on day 1, day 3, and day 5 for complete media, SFM (control), CM1 (TGF- β 3-primed TSC CM), and CM2 (unprimed TSC CM). Bars show mean $\pm$ SD (n=3 per timepoint). Horizontal brackets indicate statistically significant pairwise differences by one-way ANOVA with Tukey's post hoc test ($p < 0.05$). (B) Representative Calcein AM live-cell images (green) acquired at the same timepoints for each condition, illustrating qualitative viability consistent with MTS results. Scale bars as indicated.

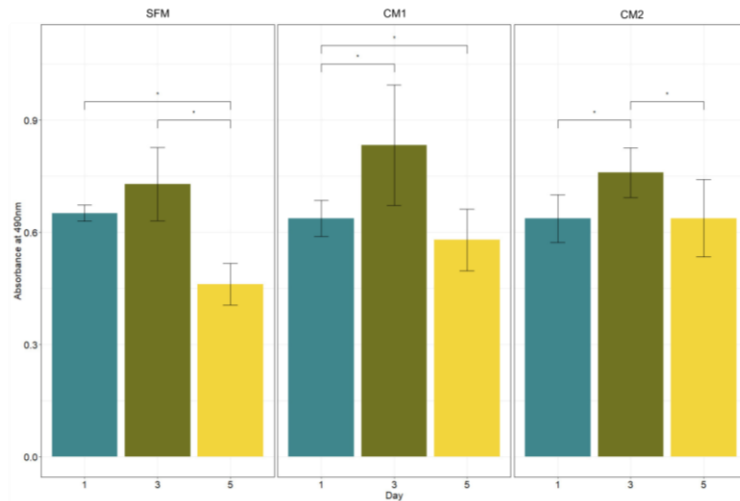


Figure 6. Proliferation of rabbit TSCs across days in SFM (control), CM1, and CM2. Cell metabolic activity was quantified by MTS (A490 nm) at day 1, day 3, and day 5 in SFM, CM1 (TGF- β 3-primed TSC conditioned media), and CM2 (unprimed TSC conditioned media). Bars show mean $\pm$ SD (n = 3). Horizontal brackets indicate significant between-day differences within each condition (one-way ANOVA with Tukey post hoc, $p < 0.05$).

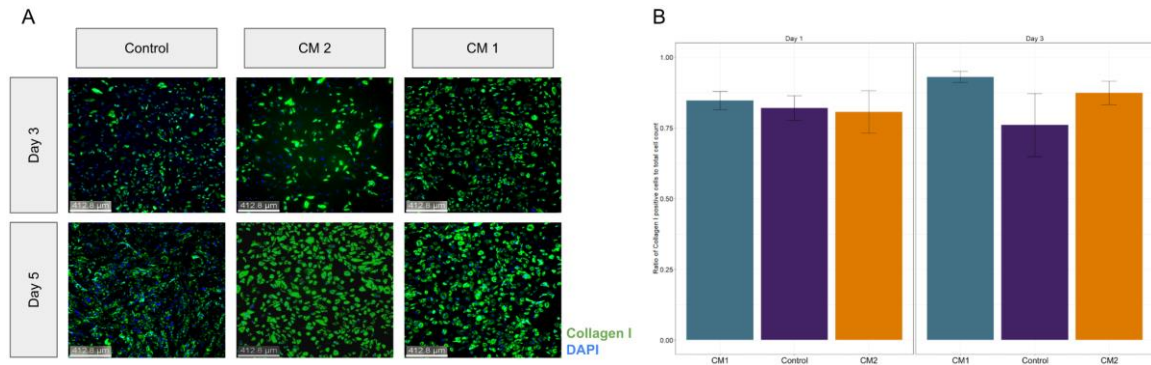


Figure 7. Collagen I immunofluorescence of rabbit TSCs treated with CM1, CM2, or control medium. (A) Representative images showing Collagen I (green) with DAPI nuclear counterstain (blue) at the indicated days (left labels). Scale bar: 412.8 μ m. (B) Quantification of the fraction of Collagen I-positive cells (Collagen I⁺/total DAPI⁺ nuclei) for each condition at the indicated days. Bars show mean $\pm$ SD; statistics by one-way ANOVA with Tukey's post hoc.

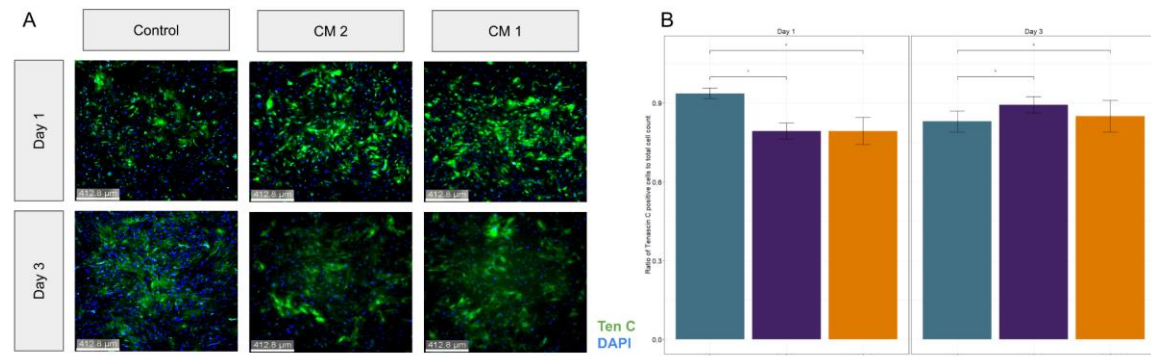


Figure 8. Tenascin C (TenC) immunofluorescence of rabbit TSCs treated with CM1, CM2, or control medium. (A) Representative images showing TenC (green) with DAPI nuclear counterstain (blue) at the indicated days (left labels). Scale bar: 412.8 μ m. (B) Quantification of the fraction of TenC-positive cells (TenC⁺/total DAPI⁺ nuclei) for each condition at the indicated days. Bars show mean $\pm$ SD; statistics by one-way ANOVA with Tukey's post hoc.

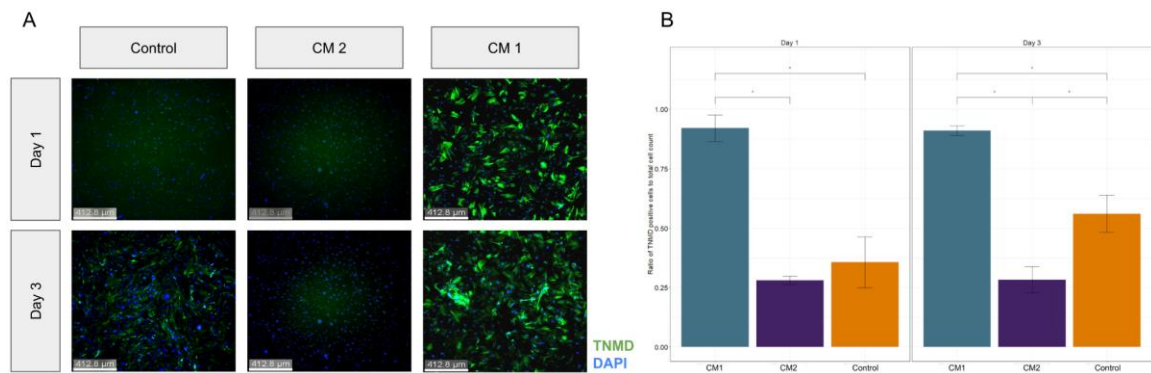


Figure 9. Tenomodulin (TNMD) immunofluorescence of rabbit TSCs treated with CM1, CM2, or control medium. (A) Representative images showing TNMD (green) with DAPI nuclear counterstain (blue) at the indicated days (left labels). Scale bar: 412.8 μ m. (B) Quantification of the fraction of TNMD-positive cells (TNMD⁺/total DAPI⁺ nuclei) for each condition at the indicated days. Bars show mean $\pm$ SD; statistics by one-way ANOVA with Tukey's post hoc.

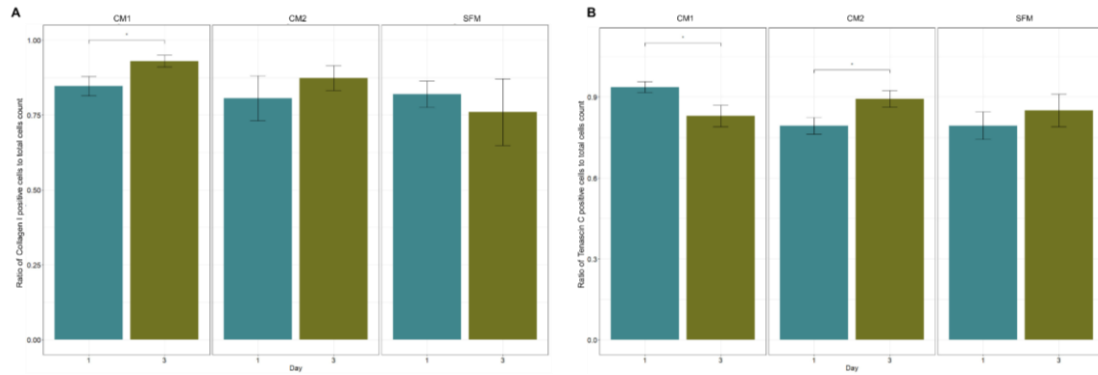


Figure 10. Collagen I (A) and Tenascin-C (B) expression across days. Quantification of immunofluorescence showing the fraction of marker-positive cells (marker⁺/DAPI⁺) on day 1 and day 3 for each condition. Bars represent mean $\pm$ SD (n = 3 fields/replicates per condition, as described in Methods). Horizontal brackets denote significant within-condition differences between days (one-way ANOVA with Tukey's post hoc, $p < 0.05$).

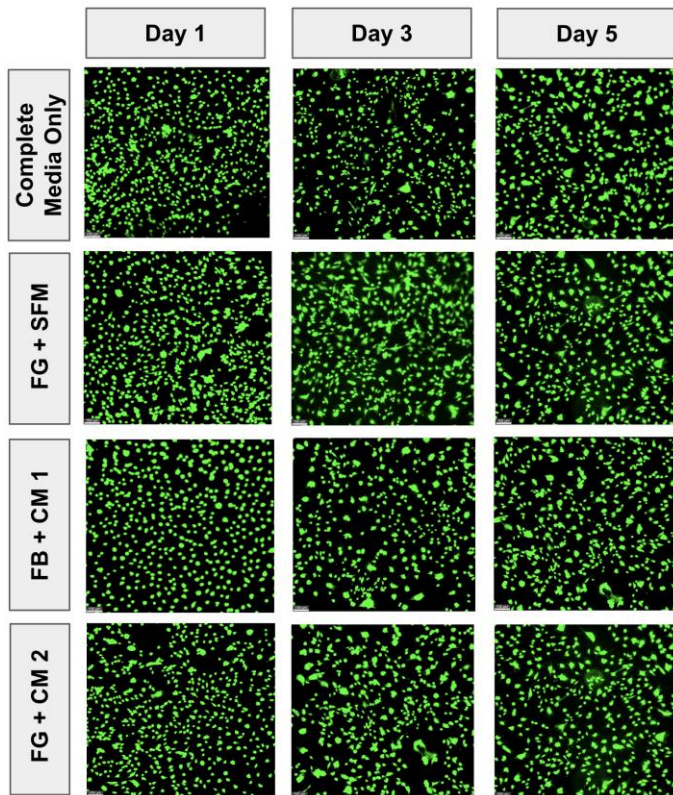


Figure 11. Representative Calcein AM live-cell images (green) acquired at the same timepoints for each condition, illustrating qualitative viability consistent with MTS results. Scale bar: 100 μ m.

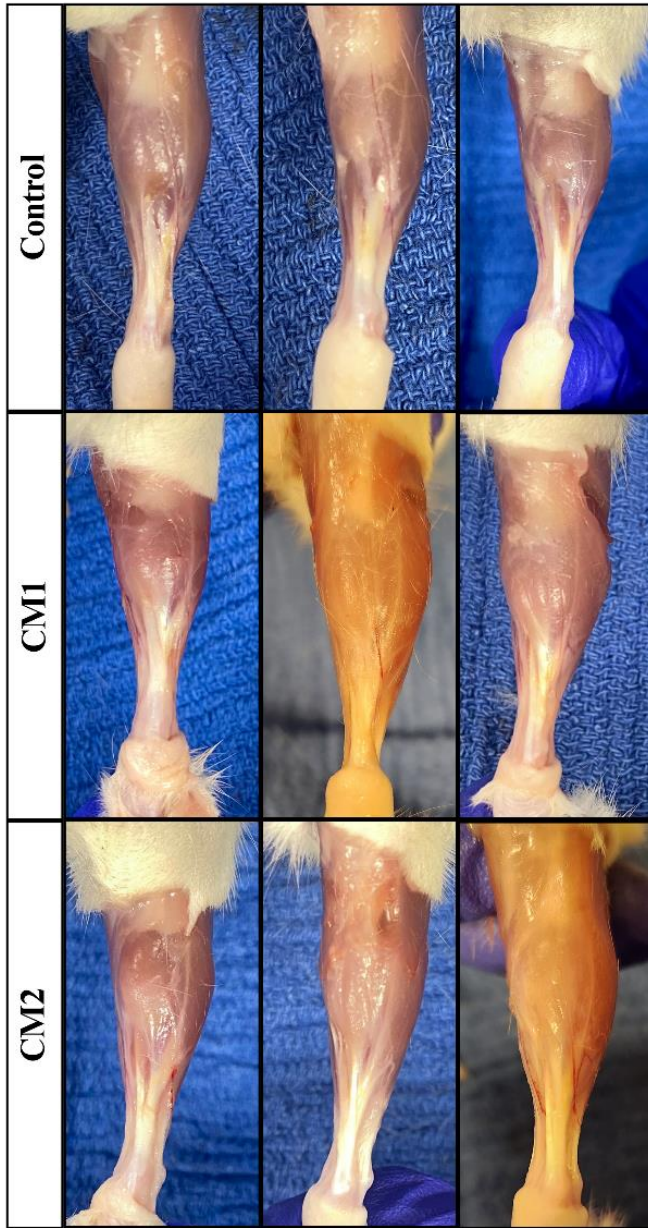


Figure 12. Gross appearance of repaired rat Achilles tendons at 30 days post-surgery. Representative macroscopic images for each treatment group: Control (SFM), CM1 (TGF- β 3-primed TSC conditioned medium), and CM2 (unprimed TSC conditioned medium).

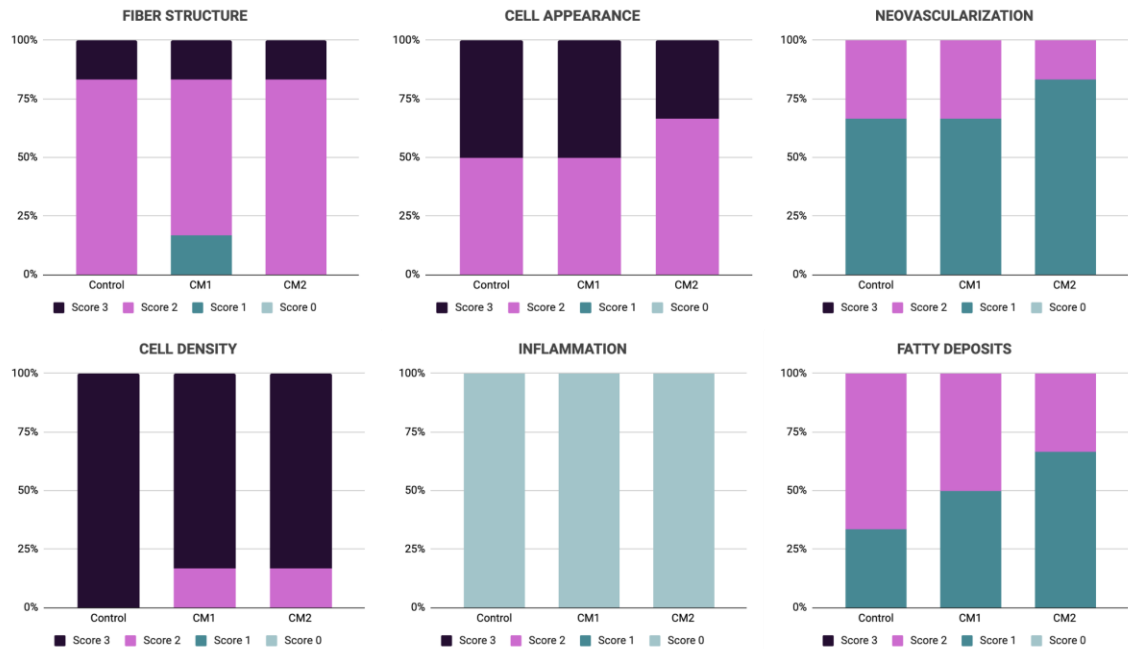


Figure 13. Semi-quantitative histology scores (0–3) at 30 days post-injury. Stacked bars show the proportion of specimens per group (Control, CM1 = TGF- β 3-primed CM, CM2 = unprimed CM) achieving each categorical score (color legend: 0–3) for: Fiber structure, Cell appearance, Neovascularization, Cell density, Inflammation, and Fatty deposits. Categorical scores were summarized as counts and percentages; group comparisons used a Kruskal–Wallis test (no significant differences among groups for any domain, $p > 0.05$).

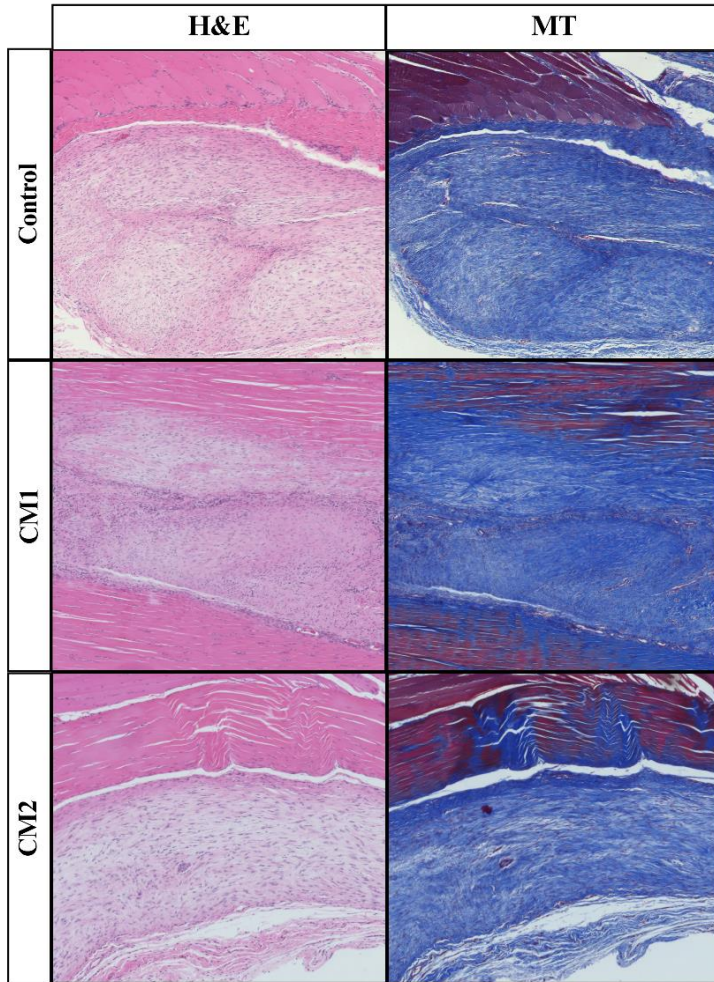


Figure 14. Representative histology of repaired rat Achilles tendons 30 days post-injury. Rows show treatment groups (Control, CM1 = TGF- β 3-primed TSC conditioned media, CM2 = unprimed TSC conditioned media). Columns show H&E (left; cellularity and general architecture) and Masson's trichrome (MT) (right; collagen = blue, muscle = red). All groups demonstrate defect bridging with hypercellular repair tissue and immature collagen. Semi-quantitative grading (fiber structure/arrangement, cell density/appearance, inflammation, neovascularization, fatty deposits) detected no significant between-group differences ($p > 0.05$).

CHAPTER V: DISCUSSION

This study aimed to evaluate the effects of CM derived from TGF β -3-primed TSCs on healing of rat Achilles tendon. Therapeutic potential of CM was assessed through *in vitro* functional characterization and proteomics of CM and intralesional administration of CM, blended with fibrin gel for controlled delivery, in a rat Achilles tendon injury model. TGF β -3 priming of TSCs produced a matrix-organizing secretome that accelerated tenogenic marker expression *in vitro*, conferred cytoprotection under nutrient stress conditions, and improved gross tendon repair quality *in vivo*. Overall, our findings suggest that CM derived from TGF β -3-primed TSCs may be a promising acellular alternative to stem cell-based therapies for tendon healing.

TSCs have been increasingly explored as a promising cell source for tendon healing therapies^{54,56–58} due to their ability to differentiate into tenocytes and form tendon-like tissue both *in vitro* and *in vivo*^{60,60–62}. Building on this rationale, we utilized rabbit TSCs and evaluated whether their secretome alone could improve tendon healing.

The composition and bioactivity of MSC secretome are known to be shaped by different stimuli present in the microenvironment surrounding MSCs¹⁶¹. *In vitro* priming strategies have been used to modulate the composition of MSC secretome and potentiate its therapeutic potential for a given application^{38,51,161,162}. A common priming strategy involves adding inflammatory cytokines, growth factors or hormones to cell culture¹⁶¹. TGF β -3, used to prime TSCs in this study, is notable for promoting tenogenic features, including characteristic changes in cell morphology, tissue alignment/orientation, improved cell viability, and upregulation of tendon-associated glycoproteins²²⁵. Consistent with this biology, our proteomic analyses showed that 48-hour TGF β -3-priming produced a distinct CM profile relative to unprimed CM.

To investigate the effects of TGF β -3 priming on the composition of CM, we performed proteomic analysis of CM derived from primed (CM1) and unprimed (CM2) TSCs. One of the three CM1 replicates was excluded from downstream analyses due to not clustering with its biological group. It is possible that protein degradation occurred in this replicate during sample collection, storage, or transport. As expected, after 48 h of priming, CM1 displayed a different secretion profile compared with CM 2, which shifts in both number and abundance of bioactive components. Notably, proteomic analysis showed that TGF β -3 priming significantly upregulated a diverse array of ECM-related proteins and matricellular regulators in the secretome of TSCs, supporting the induction of a tenogenic secretory phenotype.

Upregulated proteins included multiple fibrillar and non-fibrillar collagens such as type I, II, V, VIII, XI, XIV, and XV. The process of tendon healing depends on fibrillar collagens to restore mechanical strength and to mediate signaling through binding to cell receptors and other ECM components, while non-fibrillar collagens orchestrate ECM remodeling and the precise assembly of fibrillar collagens, alongside other non-collagenous ECM proteins, ensuring tendon-specific structure and organization²³⁸⁻²⁴⁰.

We also observed increased non-collagenous ECM proteins such as fibronectin, fibromodulin, thrombospondins (TSP1 and TSP2), and periostin, consistent with active ECM assembly and remodeling, as well as tissue homeostasis. Fibronectin participates in ECM assembly by binding to cell surface receptors and has been shown to be an important component of the early tendon repair process^{241,242}. Similarly, fibromodulin regulates collagen fibrillogenesis, including cross-linking, packing, and fibril diameter^{243,244}. Periostin plays a role in tendon healing by maintaining ECM homeostasis through

interaction with other proteins and facilitating type I collagen maturation and crosslinking²⁴⁵. Periostin also was shown to improve tissue repair by promoting cell proliferation and macrophage M2 polarization²⁴⁶. Thrombospondins, as matricellular regulators, interact with cells, other ECM components, and soluble molecules to regulate inflammation, angiogenesis, cell adhesion, migration, and proliferation^{247,248}.

TGF β -3 priming also upregulated other matricellular regulators that favor ECM assembly, tissue homeostasis, and controlled remodeling, including biglycan, SPARC, and SERPINE1. Small leucine-rich proteoglycans (SLRPs), such as biglycan, are key ECM components that play an important role in ECM assembly and turnover, cell-ECM interactions, and collagen fibril growth and organization²⁴⁹. Biglycan interacts with other ECM proteins, such as collagen I, and its deficiency has been linked to abnormal collagen fibrillogenesis²⁵⁰. Notably, biglycan helps organize the TSC niche and induce TSC differentiation by modulating bone morphogenetic protein (BMP) signaling, a mechanism that may inhibit ectopic tissue formation during tendon healing⁵⁵. SPARC, a collagen-binding matricellular protein, supports load-induced tendon maturation and homeostasis by regulating the assembly and maturation of basal lamina and collagen fibril^{251,252}. It also optimizes the regenerative environment by reducing inflammation and supporting metabolic balance^{253,254}. Because ECM integrity reflects the balance between synthesis and degradation of ECM components²⁵⁵, SERPINE1's role is context-dependent: although associated with the formation of fibrosis and adhesions in flexor tendons²⁵⁶, it helps maintain tissue homeostasis and wound healing by suppressing MMP activity²⁵⁵, a mechanism that can preserve provisional matrix and nascent collagen during early repair. Notably, CM1 contained ECM-organizing factors, such as SPARC, biglycan, and

fibromodulin, ECM structural components, and cross-linking enzymes such as lysyl oxidase (LOX). Taken together, we interpret SERPINE1 upregulation in combination with these ECM-forming cues as a controlled remodeling state, rather than an inherently fibrotic program.

Proteomics revealed that exogenous TGF β -3 was depleted from the media by 48 hours of culture. The detection of latent TGF- β binding proteins (LTBP1 and LTBP2) and TGFBI (β ig-h3) in CM1 suggests cellular uptake of TGF β -3, upregulation of TGF- β signaling, and formation of large protein structures (i.e., Large Latent Complexes) that hold TGF- β to the ECM to facilitate its storage in a latent form²⁵⁷. In injured tendons, this ensures TGF- β is released as needed to recruit tenocytes and initiate repair processes²⁵⁸. Latent TGF- β binding proteins also control TGF- β release to avoid tenocyte apoptosis and scar tissue formation that may result from excessive TGF- β signaling^{258,259}. The presence of LTBPs and TGFBI also suggests that endogenous TGF- β (1/2/3) may have been secondarily released in response to exogenous TGF β -3 and stored as latent complexes in the ECM, thus could not be detected in the soluble CM fraction.

The upregulation of enzymatic regulators, such as LOX, suggests maturation of the newly formed matrix. Collagen cross-linking is an enzyme-mediated process in which the LOXs play an important role²⁶⁰. Experimental LOX inhibition resulted in significant reduction of tendon elastic modulus without altering collagen morphology or bulk composition, glycosaminoglycan, and collagen content, implicating LOX activity as a key determinant of tendon functional properties²⁶¹. Other upregulated proteins include fibrillin-1, ANGPTL7, and MFGE8. Fibrillins form microfibrils in the ECM that provide structural scaffolding for several ECM proteins and regulate TGF- β bioavailability²⁶². MFGE8 has

been shown to modulate the immune response and promote tendon repair via efferocytosis and macrophage M2 polarization^{263,264}. In a rat patellar tendon injury model, MFGE8 significantly increased the number of M2 macrophages and anti-inflammatory cytokines, reduced tendon cell apoptosis, and enhanced TenC and COL1A1 expression²⁶⁴. Normal tendons are generally scarcely vascularized with few cells embedded in an ECM predominantly composed of collagen²⁶⁵. Although a slight increase in vascularization is needed for the early healing process of injured tendons, neovascularization following tendon injury has been linked to degeneration and poor functional properties at the injury site^{265,266}. ANGPTL7 has been proposed to help maintain the poorly vascular nature of tendons and contribute to ECM organization²⁶⁷. Collectively, the upregulated secretome components support the hypothesis that TGF β -3 primes TSCs to secrete a pro-regeneration, tenogenic matrix enriched in fibrillar collagens, ECM maturation regulators, and immunoresponse modulators, potentially enhancing the therapeutic efficacy of TSC secretome for tendon repair.

Several proteins were significantly enriched in CM1, including TenC, procollagen C-endopeptidase enhancer (PCOLCE), TIMP-1, and collagen III. TenC, an early marker of tendon development, has been shown to be a key factor in tendon healing, playing an important role in cell proliferation and migration, as well as providing elasticity to the tissue^{268,269}. PCOLCE is essential for collagen maturation, including collagen fibril synthesis and assembly²⁷⁰. TIMP-1 is a key regulator of ECM turnover, playing an important role in ECM remodeling by controlling the activity of MMPs²⁷¹. Collagen III plays a role in the early phase of tendon repair. This collagen is produced following injury, serving as a scaffold for the deposition and organization of collagen type I²⁷². The

enrichment of these factors in CM1 reinforces the hypothesis that TGF β -3 priming induces a secretion profile aligned with early tenogenic cues, ECM assembly, and tissue regeneration.

Other proteins were found at similar concentrations in CM1 and CM2, including MMP-2 and TIMP-2. These proteins play central roles in ECM remodeling and turnover²⁷³. MMP-2 also has been shown to play a role in the release of TGF- β embedded within the ECM²⁷⁴. High levels of TIMP-1 and TIMP-2 in CM derived from human uterine cervical stem cells have been linked to enhanced tissue regeneration via increased cell proliferation and inhibiting apoptosis²⁷⁵. Additional factors detected in both CMs include galectin-3 and NFIL3, which are associated with modulation of the inflammatory process and cell survival^{276,277}. The presence of these proteins in both CM1 and CM2 suggests that TSCs inherently secrete factors that support ECM turnover, immune regulation, and cell survival, which may be further tuned by TGF β -3 priming.

Overall, the secretome of TGF β -3-primed TSCs identified in this study was primarily enriched in structural ECM proteins, matricellular proteins, and pro-regenerative factors. While a few proteins with immunomodulatory roles were detected, canonical immunomodulatory factors commonly associated with MSC-based immunomodulation, such as IL-10, were not detected. This is consistent with existing literature showing that MSCs, alone, are not able to produce an adequate amount of soluble immunomodulatory factors and priming with pro-inflammatory cytokines, such as IFN- γ and TNF- α , more robustly enhances the immunomodulatory profile of MSC secretomes^{38,161,170}.

A few proteins were downregulated in CM1, including clusterin and acetyl-CoA acetyltransferase 2 (ACAT2). Clusterin is a multifunctional glycoprotein associated with

cellular response to stress and inflammation, acting as a chaperone that clears misfolded proteins and cellular debris^{278,279}. Its downregulation in CM1 may suggest a lower degree of stress-protective signaling under TGF β -3 priming in serum-free conditions. ACAT2 is a cytosolic enzyme that plays a role in lipid metabolism and biosynthesis²⁸⁰. Its downregulation may reflect a metabolic reprogramming of TSCs under TGF β -3 priming that prioritizes ECM synthesis over lipid biosynthesis or proliferation.

We assessed proliferation of rabbit TSCs cultured in CM1, CM2, and SFM (control) at days 1, 3, and 5. As expected under serum-free conditions, TSCs exhibited limited proliferation, with all three groups showing a decline in proliferation rates from day 3 to day 5. As supported by Calcein AM imaging, this decline suggests cell loss and/or reduced metabolic activity late in culture. Notably, this late decline was not significant for the CM1 group ($p > 0.05$), suggesting that CM1 conferred mild cytoprotection, attenuating the late decline in cell viability under serum-free stress. This is supported by proteomics results showing CM1 contained multiple pro-survival factors that are known to confer cytoprotection, including FN1, POSTN, CCN2, MFGE8, TIMPs, and FSTL1. For example, POSTN has been shown to provide cytoprotection by reducing cell apoptosis and promoting a pro-regenerative M2 macrophage phenotype²⁴⁶. FN1 activates survival signaling pathways such as the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway by binding to integrins²⁸¹. Similarly, CCN2 has been shown to promote cytoprotection by activation of the PI3-kinase/Akt/GSK-3 β signaling pathway²⁸². MFGE8 is also known to activate pro-survival signaling pathways, reduce apoptosis, and attenuate the inflammatory process by directly inhibiting the release of pro-inflammatory cytokines from immune cells²⁸³. Moreover, TIMPs have cytokine functions that are

independent of their function as MMP inhibitors. They promote cytokine effects that include reducing cell apoptosis (TIMP-1) and suppressing inflammation (TIMP-2)^{284–286}. Similarly, FSTL1 has been shown to reduce cell apoptosis and inhibit the release of pro-inflammatory cytokines such as IL-1 β and IL-6^{287–289}.

To assess tenogenic differentiation potential of CM, we assessed the expression of collagen I, tenomodulin (TNMD) and tenascin C (TenC) at days 1 and 3 by immunofluorescence. Prior work shows that TGF β -3 priming of MSCs significantly upregulates tendon-related markers, such as TNMD, TenC, and collagen I, supporting tenogenesis^{225,290–293}. This is in line with our immunofluorescence results showing upregulation of these markers in CM1-treated TSCs. TenC was transiently upregulated in the present study, with protein expression significantly higher on day 1, followed by a decline on day 3. Other studies on tenogenic differentiation trials using TGF β -3 have reported similar TenC expression patterns³². MSC secretome has been shown to promote tendon healing by facilitating the tenogenic differentiation of TSCs¹⁹⁷. Our results suggest that CM1 promoted an earlier and more sustained tenogenic program than CM2 *in vitro*. This is supported by the proteomics signature of CM1, characterized by the upregulation of ECM/matricellular and TGF- β modulatory proteins, suggesting that TGF β -3 priming favors the secretome toward tenogenic differentiation/matrix instruction.

To confirm cytocompatibility of the fibrin gel, we assessed TSC proliferation over a period of five days. MTS and Calcein AM assays showed no significant differences across groups, demonstrating that fibrin gel was cytocompatible and did not impede TSC proliferation.

The effects of CM1 on healing of Achilles tendon were evaluated in a rat Achilles tendon injury model. At 30 days postoperatively, gross assessments showed that CM1 produced the most native-like repair: tendons appeared slender and tapered, with longitudinal fiber alignment and smoother transitions into adjacent native tissue, consistent with a matrix-organizing/tenogenic profile observed for CM1. However, routine histology did not reveal significant differences among groups in fiber structure and arrangement, cellularity, cell morphology, inflammation, neovascularization, or fatty deposits. Notably, no inflammatory responses were detected in any group, indicating that neither the fibrin gel nor the CM elicited adverse tissue reactions.

Despite favorable *in vitro* functional results and proteomic profiling for CM1, these gains did not clearly translate to the histology. A key uncertainty is the release kinetics of the fibrin gel: we were not able to assess how long bioactive factors from CM stayed in the tissues after intralesional administration. It is possible that factors were released immediately postoperatively, or within the first hours, such that additional dosing would be required to elicit a sustained tissue response. For example, in a previous study of CM from HGF-primed TSCs in rat Achilles tendon healing, CM was injected locally three times per week for 14 days⁵⁶. Further studies are needed to determine optimal delivery vehicle, dosing frequency, and total dose. Further, histological analysis was constrained by histologic processing issues, preventing complete evaluation for four rats. In addition, our tissue assessment relied primarily on routine H&E and MT, which may lack sensitivity to subtle architectural or matrix-level changes at 30 days. The gross improvements observed with CM1 could therefore reflect modest healing gains that fall below the detection threshold of these stains. Further, the pilot nature of the study (n = 18; 6 rats/group) limited

statistical power and may have obscured differences between groups. Finally, we did not perform mechanical testing, preventing direct linkage between gross/histologic findings and functional tendon strength.

CHAPTER VI: CONCLUSIONS AND FUTURE DIRECTIONS

Overall, this work demonstrates that TGF β -3 priming of TSCs yields a matrix-organizing, tenogenic secretome. Proteomic profiling of CM1 revealed upregulation or enrichment of fibrillar and non-fibrillar collagens, non-collagenous ECM structural proteins, matricellular regulators, regulators of collagen cross-linking/assembly, immune response modulators, and TGF- β pathway components, alongside downregulation of stress/metabolic cargo. Functionally, CM1 accelerated tenogenic marker expression *in vitro*, conferred cytoprotection under nutrient stress, and improved gross rat Achilles tendon healing quality *in vivo*. Together, these data support TGF- β 3 priming as a strategy to generate a pro-regenerative, tenogenic secretome for tendon repair and motivate follow-up studies to define optimal dosing and delivery, expand sample size, incorporate deeper histologic analyses, and include mechanical testing to assess the functionality of the newly formed tendon.

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APPENDIX

Table 3. List of upregulated and downregulated proteins in CM1.

Upregulated Proteins	Downregulated Proteins
Plasminogen activator inhibitor 1 (SERPINE1)	Ubiquitin conjugating enzyme E2 N
Biglycan	Clusterin
Secreted Protein Acidic and Rich in Cysteine (SPARC)	Acetyl-CoA acetyltransferase 2
Collagen type XIV alpha 1 chain	Proteasome subunit beta
Collagen type XI alpha 1 chain	
Thrombospondin 1	
Fibromodulin	
Plasminogen activator inhibitor 1	
Collagen type II alpha 1 chain	
Collagen type I alpha 1 chain	
Latent transforming growth factor beta binding protein 2	
Fibronectin	
Collagen type I alpha 2 chain	
Collagen type V alpha 2 chain	
CCN family member 2	
Latent transforming growth factor beta binding protein 1	
Thrombospondin 2	
Collagen type XIV alpha 1 chain	
Collagen type XI alpha 1 chain	
Transthyretin	
Desmin	
Collagen type XV alpha 1 chain	
Periostin	

Table 3: Continued

Collagen type VIII alpha 2 chain
Fibronectin type III domain containing 1
Fibrillar Collagen NC1 domain-containing protein
Table 3: Continued
Eukaryotic translation initiation factor 3 subunit J
Phosphopyruvate hydratase
Lysyl oxidase homolog
Dickkopf WNT signaling pathway inhibitor 3
Calsyntenin 1
Serine/arginine-rich splicing factor 1
Collagen type VIII alpha 1 chain
Angiopoietin like 7
Midasin
Legumain
Fibrillin 1
Follistatin like 1
Milk fat globule EGF and Factor V/VIII domain containing

VITA

Tatiana Dias is a veterinarian who earned her degree from the Federal Rural University of Pernambuco, Brazil (2015–2021). She conducted research in the Equine Genetics and Genomics Laboratory at the University of Minnesota (2022–2023), investigating arrhythmias associated with sudden death in racehorses, and in 2024 joined Dr. David Anderson's Tissue Regeneration Laboratory at the University of Tennessee as a master's student. With a profound interest in tendon injuries and regenerative therapies, her work focused primarily on enhancing tendon healing using mesenchymal stem cell (MSC)-derived secretome in a rat Achilles tendon model. Tatiana was also highly involved in other regenerative medicine research focused on bone and tendon regeneration, using different animal models such as goats and rabbits. During graduate studies, she gained extensive experience in tissue engineering, regenerative medicine, and biomechanics, including stem cells, bone and tendon scaffold, and objective gait analysis. Tatiana is incredibly grateful for this experience and the opportunity to work with such an enthusiastic and supporting group. Clinically, her work focuses on equine sports medicine, with an emphasis on physiotherapy and rehabilitation. Tatiana holds certifications as a Certified Equine Rehabilitation Practitioner (CERP, University of Tennessee) and a Certified Veterinary Acupuncturist (CVA, Chi University). She looks forward to applying this combined research and clinical experience throughout her career in equine veterinary medicine.