Mesenchymal stem cell licensing: enhancing MSC function as a translational approach for the treatment of tendon injury

Drew W. Koch, DVM, PhD, DACVS1,2,* and Lauren V. Schnabel, DVM, PhD, DACVS, DACVSMR1,2

1Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC
2Comparative Medicine Institute, North Carolina State University, Raleigh, NC

*Corresponding author: Dr. Koch (dwkoch@ncsu.edu)
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ABSTRACT
Tendon injuries are common in both veterinary and human clinical patients and result in morbidity, pain, and lost athletic performance. Consequently, utilizing naturally occurring injuries in veterinary patients as a comparative model could inform the development of novel therapies and increase translation for the treatment of human tendon injuries. Mesenchymal stem cells (MSCs) have shown considerable efficacy for the treatment of experimental and clinical superficial digital flexor tendon injury in the horse; however, the reinjury rate following treatment can remain high and MSC efficacy in treating other tendons is less well known. Additionally, the translation of MSC therapy to human tendon injury has remained poor. Recent evidence indicates that naïve MSC function can be enhanced through exogenous stimulation or manipulation of their environment. This stimulation or activation, herein termed MSC licensing, markedly alters MSC functions associated with immunomodulation, extracellular matrix remodeling, vascular development, bioactive factor production, and endogenous stromal/progenitor cell support. Additionally, a variety of licensing strategies has proven to influence MSC-secreted factors that have positively influenced outcome parameters in both in vitro and in vivo disease models separate from musculoskeletal tissues. Therefore, identifying the optimal licensing strategy for MSCs could ultimately provide an avenue for reliable and repeatable treatment of a broad range of tendon injuries of both veterinary and human clinical patients. This article details current evidence on the effects of licensed MSCs in both in vitro and in vivo disease models of different species and provides commentary on how those effector functions identified may be translated to the treatment of tendon injuries.

Keywords: mesenchymal stem cell, licensing, activation, tendon, MSC function

The Pathophysiology of Tendon Injury

Naturally occurring tendon injuries in animals provide a translational avenue to study pathophysiology and evaluate novel therapeutics that benefit both veterinary and human clinical patients. Specifically, injuries to the equine superficial digital flexor tendon and canine common calcaneal tendon, as discussed in the companion Currents in One Health by Schnabel and Koch, JAVMA, October 2023, can provide insight into improved treatment strategies that are relevant to human disease. However, despite their high incidence and large clinical and research expenditure for their treatment in both human and veterinary patients, our understanding of tendon pathophysiology is still evolving.1–3 Tendon healing after injury follows a similar path to wound healing: (1) an inflammatory phase characterized by a platelet clot at the site of tissue damage that serves as cytokine secretion to recruit immune cells to infiltrate and remove debris; (2) proliferative phase heralded by vascular development and fibroblast infiltration for collagen and extracellular matrix (ECM) production resulting in a fibrovascular scar; and (3) remodeling with reorganization of newly deposited collagen and ECM with the goal of realignment of tenocytes and collagen fibers along axis of tissue stress along with a reduction in vascularity, cellularity, and water content in the previous scar.4,5 These stages occur in a coordinated interaction between tenocytes (tendon fibroblasts) of the stromal compartment, the endogenous immune sensing compartment of innate immune cells, and the infiltrating compartment composed of cells of both the innate and adaptive

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immune system. It is known that tendon injury can be incited due to acute overloading injuries, chronic repetitive stresses, and other internal and external factors. Additionally, predispositions for injury are also likely due to genetics, tendon location, and health status of the individual. This results in imbalances of the tendon biochemical composition primarily recognized as a movement away from a collagen type I-dense ECM to increasing amounts of collagen type III at the site of injury. Alterations to other ECM proteins likely occur but are less well recognized. Regardless, a loss of biomechanical strength is the result. This “tendinopathy” is characterized by histopathology and immunohistochemistry by disordered collagen fibers, neovascularization, increases in noncollagenous ECM, cell proliferation, and increased proteoglycan and glycosaminoglycan content. Following injury, healing is impeded by chronic inflammation, continued mechanical overload, improper remodeling, or a combination thereof, which prevents a return to native biochemical composition and biochemical strength. It is these factors that current treatments for tendon injury seek to overcome in an effort to repair pathologic changes within the tendon and revert it back to its native composition and strength.

Since the first report of their experimental and clinical use in the early 2000s, intralesional administration of naïve MSCs has become a mainstay for the treatment of equine superficial digital flexor tendon injuries. While shown to decrease the clinical reinjury rate and enhance tissue architecture in horses, the reinjury rate remains high in certain equine disciplines. Additionally, the therapeutic effect of MSCs has been poorly translated to human tendon injuries. Therefore, strategies to improve MSC function could enhance the treatment of both veterinary and human clinical patients and warrant continued research. A more in-depth discussion of the current use of MSCs for the treatment of veterinary tendon injuries is addressed in the companion Currents in One Health by Schnabel and Koch, JAVMA, October 2023.

MSC Licensing Strategies

It is becoming apparent that the secretory function of MSCs is critical to their capacity as a therapeutic. Further, manipulation of MSCs to enhance this function, deemed MSC licensing, may provide a method to enhance their therapeutic potency. The primary methods to achieve licensing have focused on exposing MSCs to inflammatory cytokines, growth factors, or ligands that stimulate specific membrane receptors. However, it is now known that hypoxia, various small molecules, and mechanical and physical stimuli can also be utilized to license MSCs as recently reviewed (Figure 1). These strategies have been successfully implemented to generate enhanced

![Figure 1](https://www.biorender.com)
MSCs to treat a wide variety of inflammatory and autoimmune diseases; however, a lack of studies has focused on their utility for tendons or other musculoskeletal diseases. This article, therefore, is not intended to repeat these exemplary reviews on MSC licensing. Instead, the objective of this review is to examine how data gleaned from the successful implementation of licensed MSCs within in vitro and in vivo studies could be leveraged as a translational approach for the treatment of tendon injuries.

**Effector Function of Licensed MSCs**

**Innate immune cell modulation**

Immune cells of both the innate and adaptive immune systems are emerging as players in the response to tendon injury and the development of pathology. While neutrophils and mast cells likely contribute to tendon healing, we understand to a much greater degree the role of both endogenous and infiltrating monocyte-derived macrophages in tendon homeostasis and response following injury. Generally, it appears the healthy tendon harbors minimal macrophages unless responding to injury where increases in total number are then apparent. Specifically, it appears that on the continuum of macrophage polarization, those skewed toward the classical, inflammatory phenotype (M1) are detrimental to tendon healing while anti-inflammatory (M2) macrophages provide a more reparative function in the tendon. Furthermore, the contribution of macrophages to tendon healing is clear as ablation of macrophages to tendon healing is clear as ablation greatly reduces cell proliferation, matrix deposition, and functional parameters after injury. MSCs secreted paracrine factors and extracellular vesicles can greatly alter macrophage polarization. Prostaglandin E2 (PGE2), IL-1 receptor antagonist, IL-10, indoleamine 2,3-dioxygenase (IDO), and C-C motif ligand 18, secreted factors from MSCs, are specifically known to promote M2 polarization. Further, extracellular vesicles from naive bone marrow-derived MSCs have been shown to alter macrophage function and improved tendon healing following in vivo transplant. With this knowledge, the purposeful introduction of licensed MSCs able to polarize endogenous and infiltrating macrophage populations may exceedingly enhance tendon healing following injury. In a study that evaluated licensed MSCs for tendon healing, TNF-α–licensed MSCs in a seeded scaffold implanted into a murine Achilles tendon defect significantly reduced the number of M1 macrophages at 2 weeks, enhanced levels of M2 macrophages, and contributed to higher levels of type I procollagen and higher tendon stiffness. Proof of licensed MSC modulation of macrophage polarization in other tissues could also be beneficial. Retinoic acid licensing has been shown to enhance PGE2 secretion, which could enhance M2 polarization, and IL-1β licensing has resulted in enhanced M2 macrophages in an induced model of colitis.

**Adaptive immune cell modulation**

Lymphocytes of the adaptive immune system appear to have little effect on tendon development and instead have a more relevant role in tendon healing as infiltrating cells following injury. However, most of our knowledge to date focuses on the presence of specific subpopulations of cells and not their functional role in the tendon following injury. Following injury, total numbers of CD4+ T cells have been noted to peak at 2 weeks in the Achilles tendon with CD8+ having a prolonged infiltration out to 8 weeks. This regulation of T lymphocytes also appears mechanically dependent as regulatory T-cell (Treg) infiltration following injury has been prevented when the limb cannot be loaded. Additionally, the interaction of T lymphocytes and tenocytes appears to have effects on inflammation, proliferation, ECM production, and fibrosis of the latter. Regarding B lymphocytes, other than animal models of injury indicating their presence, their role in healing is unknown.

The adaptive immune cell-altering function of MSCs is well known and relies on their ability to respond to inflammatory molecules and alter the production of immunosuppressive molecules and cell surface receptors. In addition, while naive MSCs are able to provide some immunosuppression at baseline, such as limiting T-cell proliferation, they cannot suppress T-cell effector function without out inflammatory stimulation in the form of licensing. Licensed MSCs then utilize inducible nitric oxide synthase, IDO, and IDO-independent mechanisms to suppress T-cell proliferation and effector function. Additionally, these cells contain the ability to secrete tumor necrosis factor-inducible gene 6, responsible for the promotion of FoxP3+ Treg cells. While rarely the sole focus, a handful of in vivo murine studies have examined how licensed MSCs affect the adaptive immune system during injury and healing. Murphy and colleagues showed tumor necrosis factor-α (TNF-α)/IL-1β dual-licensed MSCs could support corneal allograft survival in rats. They reported that this combination of dual cytokine licensing enhanced corneal allograft survival through Treg enhancement. Following induction of colitis, IL-1β–licensed MSCs abrogated colon pathology through macrophage polarization and shifting proinflammatory Th1 and Th17 cells to anti-inflammatory Th2 and Treg cells. While more data are not available for the in vivo effects of licensed MSCs on the adaptive immune system, these preliminary reports indicate their ability to modulate T-lymphocyte function in clinically relevant disease models. If these successes can be translated to a tendon injury, similar modulation of lymphocytes may also be beneficial.

**Source of bioactive factors**

A variety of cytokines and growth factors have been implicated in tendon pathology and its development, progression, and healing. While human tendon samples are typically not obtained until sufficient pathology or clinical pain has arisen, animal models have better informed our understanding of tendon...
pathophysiology.\textsuperscript{45,46} Both pro- and anti-inflammatory cytokines, growth factors, proresolving lipids, and varying molecules and enzymes appear responsible for these cascades.\textsuperscript{1,47,48} Despite a better understanding of the molecular players associated with tendon injury, targeted single anticytokine therapy has not emerged as a successful technique to modulate tendon healing. Therefore, secretion of bioactive factors from licensed MSCs would likely serve 2 main roles in tendon injury: alteration of local and infiltrating immune cells (as discussed above) and enhancing the reparative ability of the stromal compartment.

The presence of PGE\textsubscript{2} within the tendon is believed to have beneficial effects on healing through modulation of immune cell populations, tenocyte proliferation, and improved tendon structural and material properties when supplied exogenously.\textsuperscript{1,28,31,49} Licensing of MSCs with all-trans retinoic acid improved secretion of PGE\textsubscript{2} and enhanced wound closure in a murine model.\textsuperscript{50} Further, TNF-α/IL-1β dual-licensed MSCs secreted significantly more PGE\textsubscript{2} than their naive counterparts and were responsible for enhanced corneal allograft survival in rats.\textsuperscript{44} In addition, while less is understood about its role in tendon healing, licensed MSC secretion of hepatocyte growth factor-induced via hypoxia exposure, fibroblast growth factor-2, or spheroid culture may also enhance tendon healing through support of endogenous tendon stem progenitor cells (TSPCs).\textsuperscript{50,51} Additionally, tumor necrosis factor-inducible gene 6 secretion by TSPCs has been shown to enhance tendon healing.\textsuperscript{54} Thus, 3-D aggregate culture of MSCs, which enhances its suppression and is protective in induced murine peritonitis, may be yet another benefit of aggregate-culture licensing of MSCs for tendon.\textsuperscript{55}

### Vascular development and extracellular matrix support

In the early stages of tendon healing, neovascularization is important to encourage infiltrating immune cells, platelets, and growth factors to the site of damage.\textsuperscript{1,2} Yet, chronic neovascularization is 1 of the hallmarks of chronic tendon injury. As expected, the utility of vascular development following injury is likely multifactorial and poorly understood; however, data indicate that administration of exogenous factors associated with vascular development, like VEGF, can in fact enhance tendon healing.\textsuperscript{54,57} Therefore, the secretion of factors associated with vascular development by licensed MSCs could encourage tendon healing, especially in the early stages of injury. Groups studying ischemia and cardiac repair have focused on the angiogenic potential of licensed MSCs. Retinoic acid, hydrogel encapsulation, hypoxia, fibroblast growth factor-2, and other molecules have significantly enhanced licensed MSC secretion of angiogenic molecules and improved local tissue vascularization.\textsuperscript{51,52,58-60} Following licensing with 2,4-dinitrophenol, intramyocardial injection of MSCs enhanced vessel numbers within the infarcted mouse myocardium.\textsuperscript{59} Similarly, when a prolyl hydroxylase inhibitor was used to mimic hypoxic preconditioning, licensed MSCs had significant upregulation of angiogenic factors that likely contributed to improved cardiac function and reduced fibrosis following induction of myocardial infarcts.\textsuperscript{58} These studies indicate that the translation of licensed MSCs from cardiac and ischemia research could be highly relevant to tendon healing.

Providing a source of ECM components might also enhance tendon healing. While ECM production from licensed MSCs has not been of direct interest during in vivo studies, cytokine licensing has been shown to enhance in vitro gene expression and protein secretion of collagenous and noncollagenous ECM molecules and growth factors that could augment tendon ECM.\textsuperscript{46,61} Following IL-1β licensing of equine bone marrow-derived MSCs, collagen type V alpha fragments (COL5A1, COL5A2), IL6, and IL11, and various matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases genes were enhanced.\textsuperscript{46} Additionally, TGF-β2 licensing more broadly enhanced both collagenous and noncollagenous gene and protein expression such as tenasin-C (TNC), biglycan (BGN), elastin (ELN), and various relevant collagens.\textsuperscript{61} What does appear beneficial from murine studies, however, is the reduction in ECM fibrosis and the number of alpha-smooth muscle actin (αSMA)-positive myofibroblasts. In induced models of pulmonary disease, where a reduction in fibrosis is critical for treatment, utilizing hypoxia to license MSCs has been shown to significantly reduce fibrosis.\textsuperscript{62,63} Following radiation-induced lung injury, hypoxia-licensed MSCs significantly reduced pulmonary fibrosis as determined by Masson trichrome and antibody staining for αSMA.\textsuperscript{63} A similar effect was noted in a model of bleomycin-induced lung fibrosis where a reduction in Masson trichrome staining and tissue expression of fibrosis-related connective tissue growth factor and collagen type III were reduced.\textsuperscript{62} Additionally, these benefits were attributed to hypoxia-licensed MSC secretion of hepatocyte growth factor.\textsuperscript{52} Where elevated levels of collagen type III and αSMA-positive myofibroblasts likely contribute to tendon fibrosis, hypoxic licensing of MSCs is yet another strategy that could be employed to enhance MSC function and subsequently, tendon healing.\textsuperscript{5,64,65}

### Modulation of endogenous and retention of exogenous stromal cells

Endogenous stromal cells, primarily those of tenocytes and TSPCs are a key cell population of the tendon microenvironment during injury and repair.\textsuperscript{2,5,15,66} Protecting these endogenous cells from apoptosis and enhancing their proliferative capacity, especially in the early stages of tendon injury, may be beneficial. Additionally, exogenous stromal cells in the form of culture-expanded MSCs delivered systemically or locally can benefit the tissue microenvironment through direct support and homing of endogenous stromal cells or alternatively by polarization of immune cells that subsequently affect the endogenous stromal cells.\textsuperscript{67,68} Finally, it is plausible that increasing local retention of exogenously
delivered MSCs may provide them further time to positively modify the microenvironment.

Retention of viable MSCs within the target tissue is highly variable and based on administration technique. While their paracrine function and uptake by macrophages likely lead to benefits long after administration, improving retention may have an additive effect. Through the application of hydrogel scaffolds, hypoxia, lipopolysaccharide, and retinoic acid, MSCs have shown improved retention and survival in vivo. Following hypoxic licensing, MSCs have enhanced survival in ischemic tissues associated with the hypoxia-inducible factor-1α–78-kDa glucose-regulated protein–Akt axis. Additionally, this protected ischemic mouse tissues from stress and apoptosis-related proteins. This licensing strategy also decreased local inflammatory mediator production in radiation-induced lung injury following intravenous injection. Finally, MSC exposure to curcumin not only reduced MSC apoptosis but was noted to reduce myocardial apoptosis in myocardial infarction as determined by terminal deoxynucleotidyl transferase dUTP nick end label staining. Despite increasing knowledge that MSC apoptosis and subsequent macrophage–led efferocytosis likely contribute considerably to MSC therapeutic benefit, enhanced MSC retention with a reduction in apoptosis may conversely be beneficial.

**The Future of MSC-based Therapies for Tendon Injury?**

MSC function can be markedly altered through licensing strategies. Through specific stimulation or alteration of their environment, enhancement of MSC functions associated with immunomodulation, extracellular matrix remodeling, vascular development, bioactive factor production, and endogenous stromal/progenitor cell support can occur (Figure 2). Despite positive data suggesting both in vitro and in vivo benefits of licensed MSCs, their utilization for the treatment of tendon injury has been greatly understudied. As described above, murine studies have provided data that licensing can enhance MSC therapy of an Achilles tendon defect and stimulate tenocyte expression of tendon-relevant genes. Additionally, RNA sequencing of equine MSCs indicates cytokine licensing strategies alter beneficial tendon-relevant gene expression.

**Figure 2**—Pathophysiologic changes in tendon injury and proposed mechanism of effect of licensed MSCs. Created with BioRender.com. CCL18 = C-C motif ligand 18. ECM = Extracellular matrix. FGF-2 = Fibroblast growth factor-2. HGF = Hepatocyte growth factor. IDO = Indoleamine 2,3-dioxygenase. INOS = Inducible nitric oxide synthase. LPS = Lipopolysaccharide. PGE2 = Prostaglandin E2. TNF-α = Tumor necrosis factor-α. Treg = Regulatory T cell. TSG-6 = Tumor necrosis factor-inducible gene 6.

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protein expression and enhance tenocyte migration in vitro. More studies are needed to determine how licensed MSCs might enhance tendon healing by inducing macrophage polarization toward an M2 phenotype, enhancing the generation of Treg lymphocytes, providing a source of growth factors and extracellular matrix molecules, and supporting local stromal and progenitor cell populations. Therefore, MSC licensing may overcome key barriers to more reliable and repeatable treatment of tendon injuries in both veterinary and human clinical patients and warrants continued study.

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