There remains a critical need to more thoroughly elucidate the mesenchymal stem cell (MSC) mechanism of action to improve their utilization as a cellular therapy. This is apparent in recent research, which has steered a paradigm shift toward support of the primary therapeutic benefit of MSCs being due to paracrine secretion and efferocytosis and less from local engraftment and cell differentiation.\textsuperscript{1,2} To this end, considerable effort is being expended in techniques to manipulate MSCs prior to transplantation to enhance these functions. Many of these efforts focus on direct bioactive factor or cell-to-cell stimulation to enhance the MSC secretion of select proteins that could directly improve treatment of specific diseases.\textsuperscript{3–6} This stimulation or priming, referred to as MSC licensing, shows promise.

Equine superficial digital flexor tendon (SDFT) injuries are a naturally occurring injury in a veterinary

**OBJECTIVE**
The study objectives were to 1) determine the mesenchymal stem cell (MSC) surface expression of major histocompatibility complex (MHC) class I and transcriptome-wide gene expression changes following IL-1β + TGF-β2 dual licensing and 2) evaluate if IL-1β + TGF-β2 dual-licensed MSCs had a greater ability to positively modulate tenocyte function compared to naive MSCs.

**SAMPLE**
Equine bone marrow–derived MSCs from 6 donors and equine superficial digital flexor tenocytes from 3 donors.

**METHODS**
Experiments were performed in vitro. Flow cytometry and bulk RNA sequencing were utilized to determine naive and dual-licensed MSC phenotype and transcriptome-wide changes in gene expression. Conditioned media were generated from MSCs and utilized in tenocyte cell culture assays as a method to determine the effect of MSC paracrine factors on tenocyte function.

**RESULTS**
Dual-licensed MSCs have a reduced expression of MHC class I and exhibit enrichment in functional pathways associated with the extracellular matrix, cell signaling, and tissue development. Additionally, dual-licensed MSC-conditioned media significantly improved in vitro tenocyte migration and metabolism to a greater degree than naive MSC-conditioned media. In tenocytes exposed to IL-1β, dual-licensed conditioned media also positively modulated tenocyte gene expression.

**CLINICAL RELEVANCE**
Our data indicate that conditioned media containing paracrine factors secreted from dual-licensed MSCs significantly modulates in vitro tenocyte function, which may confer benefits in vivo to healing tendons following injury. Additionally, due to reduced MHC class I expression in dual-licensed MSCs, this technique may also provide an avenue to provide an effective “off-the-shelf” allogenic source of MSCs.

**Keywords:** mesenchymal stem cell, tendon, MHC class I, IL-1β, TGF-β2

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population that has benefited from local administration of naïve autologous MSCs. Specifically, naïve cells injected within the core of midsubstance SDFT lesions has led to improved tissue architecture and reduced clinical reinjury rates in both experimental and clinical studies.1,2-10 However, MSC therapy has not completely abolished reinjury rate nor have consistent improvements in biochemical and biomechanical strength been demonstrated. Additionally, effective translation of MSC therapy from this representative large-animal model to human tendon injury has been ineffective, and current human guidelines do not advise MSC therapy for tendon injuries.11 Enhancing the function of MSCs in a large-animal model could therefore improve outcomes for veterinary patients and may justify their translation into human clinical trials.

Successful therapy with autologous MSCs is limited by MSC quality and timely preparation due to cell isolation and expansion techniques. An “off-the-shelf” MSC therapy with allogenic cells with a known functional profile could enhance therapy; however, it is understood that major histocompatibility complex (MHC)-mismatched cells are targeted for death by anti-MHC antibodies.12,13 Recent work indicates that TGF-β2 can be used to reduce the MSC cell surface expression of MHC without altering their immunomodulatory properties.14,15 Additionally, both IL-1β and TGF-β2 licensing of equine bone marrow-derived MSCs modulates their transcriptional profile and protein expression in a way that could drive the tendon microenvironment toward tissue healing.16,17 Taken together, it is possible that the dual licensing of equine bone marrow–derived MSCs with both IL-1β and TGF-β2 could provide a source of MSCs with an enhanced functional profile with a reduced MHC expression less likely to elicit an immune response in vivo and, therefore, improved tendon tissue healing.

Therefore, the objectives of this study were to 1) determine the MSC cell surface expression of MHC class I and transcriptome-wide gene expression changes following IL-1β + TGF-β2 dual licensing and 2) determine if IL-1β + TGF-β2 dual-licensed MSCs had a greater ability to positively modulate equine SDFT tenocyte function in vitro compared to naïve MSCs.

Methods

Bone marrow–derived MSCs

Bone marrow aspirates from live animals were collected with the approval of the Institutional Animal Care and Use Committee of North Carolina State University under protocol #23-399. Bone marrow aspirates from postmortem animals were collected from client-owned horses following pentobarbital euthanasia for reasons unrelated to this study whose owners selected and signed off on the following statement from the Consent for Patient Disposition Form as approved by the North Carolina State University Veterinary Hospital: “I authorize the use of any needed organs and tissues for medical research and education.” Aspirates were plated in tissue culture–treated flasks containing naïve MSC isolation media containing 1 g/dL glucose DMEM (Corning), 20% fetal bovine serum (FBS; Cytiva Life Sciences), 1M HEPES buffer, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin, 1 ng/mL recombinant human basic fibroblast growth factor (Corning), and 250 μg/mL amphotericin B. After 24 hours, half of the flask volume of bone marrow aspirate in naïve MSC media was split into a second flask (50:50 split). Media were exchanged 72 hours later and then every 48 hours thereafter. Fibroblast colonies were allowed to proliferate until passage at 10 to 14 days following initial plating, lifted at 80% subconfluency by using Accutase cell dissociation solution (Innovative Cell Technologies Inc), and frozen in naïve MSC media containing an additional 10% FBS and 10% DMSO for future use. For all experiments, frozen MSCs were thawed, counted, and seeded in naïve MSC media (media as above but with 10% FBS and without amphotericin B) and grown until approximately 80% confluent. Cells were lifted with Accutase, counted, and seeded at 2,850 cells per cm² into new plates in quadruplicate. Following 24 hours of attachment, media were exchanged based on treatment group to naïve MSC media or licensing media. Cytokine dose was determined utilizing a previous publication16 that determined peak concentration of IL-1β in surgically induced equine SDFT injury and by previous in vitro work15,17 evaluating the effect of TGF-β2 on MSC immunogenicity and changes in the MSC transcriptome. Licensing media consisted of naïve media with IL-1β (2 ng/mL), TGF-β2 (1 ng/mL), or both IL-1β and TGF-β2 (dual licensed). Cells were treated for 72 hours, when licensing media was removed and exchanged with fresh licensing media at 48 hours. All MSCs utilized for the experiments were within the fourth passage.

Tenocytes

Tendons were collected postmortem from client-owned horses following pentobarbital euthanasia for reasons unrelated to this study whose owners selected and signed off on the following statement from the Consent for Patient Disposition Form as approved by the North Carolina State University Veterinary Hospital: “I authorize the use of any needed organs and tissues for medical research and education.” An approximately 6-cm length of the forelimb SDFT was aseptically harvested and transported to a tissue culture hood in sterile PBS containing 100 U/mL penicillin and streptomycin. The paratenon was removed, and the tendon proper was diced into 1 cm² pieces with 2-mm tendon explants and added to tenocyte isolation media containing 1 g/dL glucose DMEM (Corning), 10% FBS (Cytiva Life Sciences), 100 U/mL penicillin and streptomycin, 2 mM HEPES buffer, 2 mM L-glutamine, 20 μg/mL α-ketoglutaric acid, and 50 μg/mL ascorbic acid with the addition of 0.3% collagenase type I (Gibco, Thermo Fisher Scientific Inc). The tendon was digested for 6 to 8 hours, digested tendon media was filtered through a 100 μm Nylon cell strainer, and cells were pelleted by centrifugation at 800 x g. Viability and cell count were obtained, and cell numbers were expanded by
culture on 100-mm tissue culture plates at 8,800 cells per cm² with standard tenocyte media as listed above but without collagenase. Tenocytes were passaged with trypsin-EDTA (0.25%) when plates became 80% to 90% confluent and were frozen in tenocyte media containing an additional 10% FBS and 10% DMSO. For tenocyte experiments, FBS was reduced to 5% to lessen negative effects on tenocyte phenotype. All tenocytes utilized in the experiments were within the fourth passage.

**Immunophenotyping of MSCs**

Cells were lifted with Accutase, washed with PBS, pelleted, and immunophenotyped with an LSRII flow cytometer (BD Biosciences) equipped with FACS Diva analysis software (BD Biosciences). Antibodies for these markers were previously validated for the horse: LFA-1 (cz3.2, Antczak Lab), CD29 (TDM29, Millipore Sigma), CD44 (CVS18, BioRad), CD45RB (DH16, Washington State University), CD90 (DH24A, Washington State University), MHC I (cz3, Antczak Lab), and MHC II (cz11, Antczak Lab). Dilutions of 1:200 (CD29, CD90), 1:100 (CD44), or 1:10 (MHC class I, MHC class II, LFA-1, CD45RB) were used according to the manufacturer’s directions for commercial antibodies and according to previous experience. Phenotype of isolated MSCs was confirmed by evaluation of naive bone marrow-derived MSC cell surface expression of CD29, CD44, CD45, CD90, LFA-1, and MHC class I and II. For licensed MSCs, only MHC class I and II expression were analyzed and compared to naive MSCs as previous publications have not shown alteration to other cell surface expression following exposure to inflammatory cytokines or TGF-ß2.

Cells were pelleted in aliquots containing approximately 1 X 10^6 cells on 96-well V-bottom plates and treated with a 20-minute blocking step by using 10% normal goat serum in PBS. The cells were pelleted and resuspended in unconjugated primary antibody and incubated for 45 minutes at 4°C. MSCs were then washed and resuspended in a secondary allophycocyanin-conjugated goat anti-mouse immunoglobulin antibody (BD Biosciences) and incubated for an additional 30 minutes at 4°C. MSCs stained with the secondary antibody alone were used as negative controls. Cells were gated as previously described, and data were collected on a minimum of 1 X 10^4 cells for each sample. The relative geometric mean fluorescent intensity was calculated after normalization to naive MSC expression for MHC class I, and the percentage of positive cells was determined for MHC class II expression due to overall low expression.

**MSC RNA sequencing**

Previous publications have reported transcriptome-wide changes in equine bone marrow-derived MSCs following licensing with the same concentrations of IL-1ß or TGF-ß2 but not following concurrent dual licensing with both cytokines. Therefore, the same approach was used to examine differential gene regulation in IL-1ß + TGF-ß2 dual-licensed MSCs compared to their naive counterparts. Total RNA was extracted from paired naive and dual-licensed MSCs using the RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. RNA amount and quality were assessed by spectrophotometry (NanoDrop, Thermo Fisher Scientific Inc). All samples had an absorbance 260/280 ratio of greater than 1.8. Libraries were generated, and poly(A) was enriched using 1 µg of RNA as input. Indexed samples were sequenced using a 150-bp paired-end protocol and a sequencing depth of approximately 30 million reads per sample on a HiSeq 2500 (Illumina) according to the manufacturer’s protocol. Sequence reads were trimmed to remove possible adaptor sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the Equus caballus reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. Unique gene hit counts were calculated using featureCounts from the Subread package v.1.5.2. Using DESeq2, a comparison of gene expression between naive and dual licensed bone marrow-derived MSCs was performed. The Wald test was used to generate P values and fold changes. Genes with an adjusted P value < .05 and log2 fold change > 1 were determined to be differentially expressed genes (DEGs) for each comparison. The quantification and poly(A) selection of mRNA, library preparation, sequencing, and bioinformatics were outsourced to GENEWIZ (Azentra Life Sciences). The raw sequence data and normalized cells counts were deposited in a publicly available database (Gene Expression Omnibus accession number GSE256139). Functional enrichment analysis utilizing DEGs was determined using a web-based server (g:Profiler).

**Generation of tendon and MSC-conditioned media**

To standardize conditioned media (CM) between cell types, CM was generated by preparing an assay medium that had the overlapping components of both MSC media and tenocyte media. This ensured that CM generated from both tenocytes and MSCs was different only in the cellular component and not due to media composition. This assay medium consisted of 1 g/dL glucose DMEM (Corning), 5% FBS (Cytiva Life Sciences), 100 U/mL penicillin and streptomycin, 1 M HEPES buffer, and 2 mM L-glutamine. For experiments determining CM effects on tenocytes, control wells consisted of tenocyte CM (TENO CM) generated from tenocytes grown for 72 hours in standard tendon media with a final 24 hours of growth in the assay medium. Tenocyte donors were exposed to their own CM in triplicate as TENO CM control wells. MSC CM (NAIVE CM or DUAL CM) were generated from MSCs grown for 72 hours in either naive or dual-licensed MSC media with a final 24 hours in the assay medium. The CM supernatant was collected from each treatment group, centrifuged at 1,000 X g for 10 minutes to remove cell debris, and immediately used in tenocyte migration, metabolism, and gene expression experiments.
Tenocyte migration (scratch wound) assay

For migration analysis, tenocytes were seeded at 57,125 cells per cm² in 12-well plates to ensure confluency after 24 hours. Following 48 hours of attachment, cells were serum deprived for 24 hours in standard tenocyte medium without FBS. The following day, a single vertical scratch wound was created in each well with a 200-µL pipette tip, wells were washed twice with PBS, and the appropriate CM was added to each well. Images were obtained at 4X magnification with an inverted microscope as previously described.17 Three images per treatment group were captured at the same location every 12 hours for a 36-hour duration without medium exchange. A free open-source image software program (ImageJ, National Institutes of Health) with scratch wound assay plugin was utilized to determine the pixel area remaining. The percentage of wound closure of each scratch wound was calculated as (pixel area baseline – pixel area time point at X h/pixel area baseline) X 100.

Tenocyte metabolic activity

Tenocyte metabolic activity was determined by the EZMTT Cell Proliferation Assay (Sigma-Aldrich), a colorimetric assay utilizing the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazolium salt. Cells with a higher absorbance correlate to a higher metabolic rate. Tenocytes were plated in 12-well tissue culture–treated plates at 2,850 cells per cm² in standard tenocyte media and allowed to grow for 48 hours. Cells were washed with PBS, and media was replaced with serum-free tenocyte media (negative control) or TENO CM, NAIVE CM, or DUAL CM. Cells were grown for 24 hours before the addition of MTT as per the manufacturer’s recommendation. The absorbance at 450 nm was measured 48 hours after the addition of MTT (72 hours after the addition of CM).

Tenocyte gene expression

For gene expression analysis, tenocytes were seeded at 8,500 cells per cm² in 12-well tissue culture–treated plates. Following 24 hours of attachment in standard tenocyte media, cells were inflammatory stimulated with IL-1β (10 ng/mL) for 24 hours. Twelve hours into stimulation, half of the well volume was removed, and the appropriate CM was applied, where control tenocyte CM with inflammatory stimulation (IL-1β) was compared to inflammatory stimulation with MSC treatments as IL-1β + NAIVE CM or IL-1β + DUAL CM. Additional exogenous IL-1β was added with the CM to maintain stimulation at 10 ng/mL for the final 12 hours. After 24 hours, cells were washed twice with PBS, and total RNA was extracted from tenocytes using the RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. The RNA amount and quality were assessed by spectrophotometry (NanoDrop, Thermo Fisher Scientific Inc).

Changes in gene expression were assessed using the nCounter MAX system (NanoString Technologies), which utilizes sequence-specific capture and reporter probes for genes of interest in an amplification-free system where expression is measured by counting the amount of mRNA in each sample using a digital analyzer. A custom codeset was designed for our laboratory and included genes for tendon and osteoarthritis-specific processes (Supplementary Table S1). Three housekeeping genes, actin beta (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hypoxanthine phosphoribosyltransferase 1 (HPRT1), were included with 47 other genes of interest. Approximately 200 ng of RNA was hybridized for 18 to 20 hours before samples were prepared and analyzed on the nCounter MAX system. Data quality control, thresholding, and normalization were performed using nSolver Analysis Software (NanoString Technologies) based on the manufacturer’s recommendation and similar to a previous report.21 Normalized mRNA counts were then utilized for statistical comparisons for each gene between treatment groups.

Statistical analyses

The functional enrichment analysis of RNA-sequencing data was performed using g:Profiler (version e111_eg58_p18_30541362) with g:SCS multiple testing correction method applying a significance threshold of 0.05. For all other experiments, data were reported as the mean and SD. Continuous data were examined for normality using the Shapiro-Wilk test with parametric data analyzed by a one-way ANOVA with Tukey’s test for multiple comparisons. Statistical significance was considered P < 0.05. All analyses were performed in GraphPad Prism version 10.1.0 (GraphPad Software Inc).

Results

IL-1β + TGF-β2 dual licensing of MSCs reduces the expression of MHC class I

MSCs utilized in the experiments were harvested from 3 mares and 3 geldings (n = 6, horses 1 through 6) with a mean age of 9 years (SD, 4.6 years). Naïve MSCs from all 6 horses grew with a fibroblast morphology and were adherent to tissue culture–treated cell culture dishes. These cells were positive for CD29, CD44, CD90, and MHC class I and negative for CD45 and LFA-1 (Supplementary Figure S1). Five of the 6 horses were also negative for MHC class II. Compared to naïve MSCs and as previously reported,14 MSCs licensed with TGF-β2 remained MHC class I positive but had significantly reduced MHC expression amongst the entire cell population (P < 0.001). Similarly, MSCs dual licensed with IL-1β + TGF-β2 remained MHC class I positive but had significantly reduced MHC class I expression compared to naïve MSCs (P < 0.01, Figure 1). MSC licensing with IL-1β did not significantly alter MHC class I expression. In the 5 horses without MHC class II expression, licensing did not alter expression. In the single horse expressing MHC class II, expression was maintained throughout regardless of licensing (Supplementary Figure S2).
Equine SDFT tenocytes express tendon-associated markers

Tenocytes were isolated from the SDFT of 2 geldings and 1 mare (n = 3, horses 7 through 9) with a mean age of 16 years (SD, 1 year). These cells had a fibroblast morphology, were adherent to tissue culture–treated cell culture dishes, and expressed a variety of tendon-specific genes, including scleraxis.
IL-1β + TGF-β2 dual-licensed MSCs undergo changes in gene expression linked to extracellular matrix production and cell signaling

The results of RNA sequencing (RNAseq) revealed that IL-1β + TGF-β2 dual licensing led to 1,341 DEGs, with downregulation of 671 genes and upregulation of 670 genes (Figure 2). The top 10 enriched pathways each for molecular functions, biologic processes, and cellular components are presented (Figure 2). Differential gene expression in dual-licensed MSCs was linked to multiple pathways associated with extracellular matrix (ECM) production, cell signaling, development, and integrin binding.

The most significantly upregulated genes (Figure 2) included inflammatory mediators (IL11, CXCL6) and integrins (ITGAE, ITGB3). The most significantly downregulated genes (Figure 2) include those associated with fatty acid oxidation (ACAD10), the tumor necrosis factor (TNF) ligand family (TNFSF13), and a serine protease inhibitor (SERPINB6). Additionally, genes associated with immunomodulation and tissue repair, vascular development, and ECM production and remodeling were also differentially regulated (Supplementary Figure S4). Based on RNAseq data, 3 horses (horses 2, 3, and 5) with representative expression of a variety of genes of interest following licensing were selected to be used to generate DUAL CM for migration and metabolism experiments with all tenocyte donors (horses 7 through 9), and a single MSC donor (horse 2) was used with all tenocyte donors to determine the effect of CM on tenocyte gene expression.
DUAL CM enhances tenocyte migration

Following scratch wounding of confluent tenocytes, DUAL CM was able to significantly enhance tenocyte migration over TENO CM at 24 hours (P = .027) and 36 hours (P = .0033) (Figure 3). NAIVE CM did not significantly enhance migration over control wells at any time point.

DUAL CM enhances tenocyte metabolism

As expected, tenocytes grown in CM from all control and MSC groups had significantly higher (P < .001) absorbance, indicative of a higher metabolic rate, compared to tenocytes grown in serum-free media (Figure 4). Additionally, tenocytes grown in DUAL CM had significantly greater absorbance than both TENO CM (P < .001) and naive CM (P < .001). NAIVE CM was not able to significantly enhance tenocyte metabolism over TENO CM.

DUAL CM positively alters IL-1β–induced tenocyte gene expression

All samples passed quality control parameters as determined by nSolver. Of the normalized mRNA counts of 47 genes within the custom codeset, the baseline tenocyte expression prior to IL-1β stimulation in most samples for C-C motif chemokine receptor 2 (CCR2), growth differentiation factor 7/bone morphogenetic protein 12 (GDF7/BMP12), hyaluronan synthase 1 (HAS1), HAS3, IL1β, IL4, IL6, IL10, IL17, matrix metalloproteinase 13 (MMP13), platelet factor 4/C-X-C motif chemokine ligand 4 (PF4/CXCL4), pro-platelet basic protein/C-X-C motif chemokine ligand 7 (PPBP/CXCL7), proteoglycan 4 (PRG4), tenomodulin (TNMD), and transient receptor potential cation channel subfamily V member 4 (TRPV4) was below 20 mRNA counts, and therefore, a threshold of 20 was established. However, following IL-1β stimulation, CCR2, elastin (ELN), and HAS2 were the only genes that remained below measurable expression levels regardless of treatment and were not included in analysis. IL-1β significantly stimulated proinflammatory and matrix remodeling genes, like ADAM metalloepitidase with thrombospondin type 1 motif 5 (ADAMTS5); PPBP/CXCL7; C-X-C motif chemokine ligand 6 (CXCL6); IL17; C-C motif chemokine ligand 2/monocyte chemotactic protein (MCP1/CCL2); C-C motif chemokine ligand 5/regulated upon activation, normal T cell expressed and presumably secreted (CCL5/RANTES); and TNFa (Supplementary Table S1). While MMP1, MMP13, and MMP3 expression was dramatically increased, wide variations in expression between tenocyte donors resulted in non-significant differences. Extracellular matrix proteins were significantly affected, with increased expression of BGN, COL2A1, COL3A1, cartilage oligomeric matrix protein (COMP), DCN, lumican (LUM), PRG4, and TNC and decreased expression in aggrecan (ACAN), COL1A1, COL1A2, and fibromodulin (FMOD).

DUAL CM significantly reduced the tenocyte expression of CCL2/MCP1, COL2A1, DCN, LUM, MMP1, and MMP13 and significantly enhanced the tenocyte expression of IL6 and VEGF compared to IL-1β–stimulated tenocytes (Table 1; Supplementary Table S2). NAIVE CM resulted in similar significant differences for the genes CCL2/MCP1 (to a greater degree than DUAL CM), DCN, MMP13, and VEGF (to a lesser degree than DUAL CM), with the addition of significantly reduced expression of ADAMTS5, PPBP/CXCL7, IL17, FGF2, and SCX that was not significant with DUAL CM. Significant differences identified between NAIVE CM and DUAL CM indicated that DUAL CM resulted in tenocytes with greater expression of the genes IL6 and VEGF and lesser expression of DCN and MMP3.
Table 1—Dual-licensed conditioned media (DUAL CM) significantly alters IL-1β–induced tenocyte inflammatory and extracellular matrix (ECM) remodeling gene expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>TENO CM mean fold change</th>
<th>NAIVE CM mean ± SD fold change</th>
<th>DUAL CM mean ± SD fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACAN</td>
<td>1.0a</td>
<td>0.878 ± 0.106a</td>
<td>0.879 ± 0.204a</td>
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<td>ACTA2</td>
<td>1.0a</td>
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<td>0.985 ± 0.013a</td>
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<td>CCL2/MCP1</td>
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<td>0.894 ± 0.018b</td>
<td>0.947 ± 0.010b</td>
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<td>CCL5/RANTES</td>
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<td>1.092 ± 0.073a</td>
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<td>CCR2</td>
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<td>–</td>
<td>–</td>
</tr>
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<td>0.793 ± 0.136ab</td>
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<td>0.771 ± 0.043a</td>
<td>0.914 ± 0.165ab</td>
</tr>
<tr>
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<td>1.221 ± 0.104a</td>
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<tr>
<td>VEGF</td>
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<td>1.182 ± 0.172a</td>
<td>1.538 ± 0.133b</td>
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</table>

Tenocytes were stimulated with IL-1β for 24 hours with tenocyte control CM (TENO CM) or mesenchymal stem cell (MSC) CM (NAIVE CM or DUAL CM) added 12 hours into stimulation. NAIVE and DUAL CM were generated from a single MSC donor and applied to tenocytes. Total mRNA counts of all 3 groups were normalized to TENO CM, where gene fold change for TENO CM is 1.0. Normalized counts were then analyzed by 1-way ANOVA with Tukey multiple comparisons (n = 3). Groups with different letters (a, b) written in bold superscript indicate a significant difference (P < .05) between groups while groups with the same letter written in superscript indicates no significant difference. Data presented as mean (SD) fold change.

Discussion

Dual cytokine licensing of equine bone marrow-derived MSCs with IL-1β and TGF-β2 altered the MSC transcriptome and enriched pathways associated with the ECM, cell signaling, and tissue development. This strategy also suppressed MSC cell surface expression of MHC class I, which may be favorable to reduce in vivo immune recognition of allogenic MSCs. Additionally, CM from dual-licensed MSCs also significantly improved in vitro tenocyte metabolism, migration, and response to IL-1β-induced inflammation, which could result in enhanced in vivo tendon healing following injury. These findings suggest that this cytokine licensing technique may provide an avenue to enhance MSC therapy for tendon injuries by providing an allogenic source of MSCs that exhibits improved secretion of reparative paracrine factors, warranting future exploration.

Allogeneic MSCs may be better suited for broad therapeutic applications rather than autologous MSCs for a variety of reasons. Foremost, it is understood that MSC efficacy is affected by donor age, health status, and comorbidities. Additionally, the implementation of timely therapy is hindered by the time associated with isolation and in vitro culture and expansion of autologous MSCs. Thus, a source of allogenic MSCs with a known functional profile as an “off-the-shelf” option is attractive for more efficacious and expedited therapy. Yet, a key obstacle remains. Allogenic equine bone marrow-derived MSCs are known to elicit an immune response in MHC-mismatched donors and are targeted for death by anti-MHC antibodies, which likely limit their in vivo efficacy. However, recent in vitro evidence indicates that MHC class I expression can be downregulated in a SMAD3-dependent manner following treatment with TGF-β2. Additionally, TGF-β2 also enhances in vitro expression of equine bone marrow-derived MSC paracrine factors with known associations to tendon healing. Our data confirmed this MHC class I downregulation with TGF-β2 licensing. Additionally, MHC class I was also significantly downregulated with IL-1β + TGF-β2 dual licensing but to a lesser degree. Interestingly, IL-1β alone did not significantly increase MHC class I expression as previously reported for the other proinflammatory cytokines IFN-γ, IL-17, and TNF-α. Therefore, the dual licensing of MSCs with both IL-1β and TGF-β2 might not only impart beneficial immunomodulatory and reparative effects but also result in an allogenic MSC product that is less likely to elicit an immune response in MHC-mismatched recipients. In vitro and in vivo immune assays are needed for confirmation.

Previous work has shown that exosomes isolated from murine tendon-derived stem cells enhance murine tenocyte migration in a VEGFA-dependent manner. In equine-specific tissues, Harman et al. showed that peripheral blood-derived MSCs enhanced dermal fibroblast migration through a plasminogen activator inhibitor 1 (PAI-1)- and tenasin-C-mediated process. And while previous work has examined the effect that equine bone marrow-derived MSCs licensed with TGF-β2 have on equine tenocyte migration, neither the previous study nor our current work has investigated the mechanism involved in the modulation of migration. However, the dual licensing of MSCs did significantly enhance the gene expression of VEGFA and TNC, which could be at least partially responsible for the enhanced migratory effect on equine tenocytes in vitro. If this enhanced migration translates to the in vivo environment, it may confer benefits to the healing tendon, especially in the early stages of injury following the loss of endogenous stromal cell populations.

Following injury, tendon tissue undergoes stages of healing similar to wounds, where increased tenocyte metabolism and proliferation is key to early healing. Pechanec et al. utilized the MTT assay to assess cell proliferation based on metabolic function and noted that naive adipose-derived MSCs were able to overall enhance tenocyte proliferation of peritenon-based tenocytes over deeper isolated tendon proper tenocytes but saw no difference between specific time points. Similarly, for tenocytes isolated from the tendon proper, NAIVE CM did not enhance metabolism but instead required CM from cytokine-licensed MSCs. It should be noted that assay outcomes may vary due to differences in tenocyte donors, MSC source, and experimental strategy. Similarly, tenocytes utilized in our experiments were isolated from the tendon proper, and peritenon-specific tenocyte proliferation may be a more relevant requirement for tendon healing. As SDFT core lesions occur within the anatomic center, further studies elucidating the mechanisms of location-specific tenocyte proliferation and their association with healing are warranted to distinguish the benefit of dual-licensed MSCs more accurately on in vitro tenocytes assays.

Stimulation of equine tenocytes with IL-1β induced gene expression changes as previously reported, including the expression of proinflammatory mediators, matrix remodeling enzymes, and alterations to ECM-related genes. We also observed tenocyte expression of a variety of chemokines. The addition of DUAL CM, but not NAIVE CM, significantly enhanced the tenocyte expression of IL6, which is known to enhance tenocyte proliferation, viability, migration, and angiogenesis and stimulate collagen synthesis. VEGF, which also enhances proliferation and can positively augment tenogenic gene expression, was significantly upregulated in the RNAseq of dual-licensed MSCs as well as tenocytes exposed to both DUAL and NAIVE CM but to a greater degree in DUAL CM. The presence of both MSC and tenocyte-specific VEGF may therefore have blunted the IL-1β-induced alterations in the tenocyte expression of COL1A1, COL1A2, and TNC, although the changes were not statistically significant. These improvements of tenocyte gene expression, especially in that of VEGF, might confer benefits to the healing equine tendon, particularly if present early in the course of injury. Finally, CCL2/MCP1 is thought to contribute to the development of fibrosis in a variety of organs, while

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increased monocyte-specific levels are recognized in tendinopathy. Reductions in tenocyte expression by CM from both MSC groups could therefore provide fibrosis-modifying benefits to the healing tendon. Gene expression data taken with tenocyte function following exposure to DUAL CM indicates that the licensing of MSCs with both IL-1β and TGF-β2 deserves further investigation in an in vivo setting.

Despite data supporting the efficacy of licensed MSCs in a variety of murine disease models, only a few studies have examined licensed MSCs for tendon injury. Kim et al. utilized a hypoglycemic agent, pioglitazone, for MSC licensing, which imparted beneficial effects to rat Achilles tenocyte migration and collagen secretion. Aktas et al. implemented TNF-α-licensed MSCs in a murine Achilles segmental defect model and reported increased type I procollagen along with enhanced failure stress of treated tendons. We are aware of a single study in horses examining licensed MSC therapy for tendon injuries. Recently, the efficacy of “tenogenic primed” allogeneic peripheral blood MSCs was reported, which improved tissue architecture on ultrasound and tissue biochemical composition on histopathology in a surgically induced model of equine tendon injury. However, due to the proprietary nature, the licensing strategy was not reported. Finally, providing exogenous cytokines in vitro to initiate MSC licensing appears the most appropriate for the treatment of the equine SDFT as previous work utilizing ultrafiltration probes within surgically induced tendon lesions has demonstrated that cytokine levels of IL-1β and TGF-β2 within the tendon microenvironment do not persist past the first 2 weeks. Further work implementing licensed MSCs for tendon injury is warranted.

The limitations of this study include the lack of protein expression data for IL-1β + TGF-β2 dual-licensed MSCs. However, previous work has shown that in differentially regulated genes from RNAseq following single cytokine licensing, downstream protein production is significantly altered. Utilizing conditioned media ignores the contribution of direct cell interactions that occur between MSCs and tenocytes in vivo; however, CM might be the most relevant paracrine fraction to examine MSC therapeutic effect. The tenocyte outcomes presented do not reflect the likely contribution of the tendon niche, including the tendon ECM, local and infiltrating immune cell populations, and vasculature, and thus must be interpreted with caution. Additionally, because donor age is known to affect the tenocyte transcriptome, assay outcomes from cells utilized in this study may not be representative of horses of different ages. Finally, the reported changes in tenocyte gene expression are biased by the small set of genes examined and by the use of a single cytokine to simulate inflammation. More robust changes in tenocyte gene expression in response to CM might have also occurred with longer stimulation and treatment times or by including more than 3 biologic replicates.

Dual cytokine licensing of MSCs with IL-1β and TGF-β2 reduced the expression of MHC class I and significantly altered the transcriptome in a positive tendon-relevant manner. Further, when dual-licensed DUAL CM was applied to tenocytes in vitro, tenocyte migration and metabolism was enhanced, and gene expression was positively modulated during IL-1β stimulation. These data indicate that dual cytokine licensing may be a strategy to enhance the secreted paracrine factors of MSCs, which could enhance in vivo healing. Additionally, due to reduced MHC class I expression, this technique may also provide an avenue to provide an effective “off-the-shelf” allogeneic source of MSCs and support the testing of dual-licensed MSCs in a preclinical equine model of tendon injury.

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The RNAseq dataset has been made publicly available through the Gene Expression Omnibus repository (accession No. GSE256139).

Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

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**Supplementary Materials**

Supplementary materials are posted online at the journal website: avmajournals.avma.org.

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