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High-Plex RNA Expression Profiling of Formalin-Fixed Paraffin-Embedded Synovial Membrane Indicates Potential Mechanism of Mesenchymal Stromal Cells in the Mitigation of Posttraumatic Osteoarthritis

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Continued advances in understanding the pathobiology of posttraumatic osteoarthritis (PTOA) and mesenchymal stromal cells (MSCs)-based therapies can be achieved by probing archived samples to ask *a posteriori* questions. Herein, we isolated RNA and performed 42-plex gene analysis on synovial membrane samples that were embedded in paraffin and stored at room temperature for more than a year. Samples were obtained as part of an *in vivo* study where intra-articular administration of integrin α10-selected MSCs was shown to protect cartilage and subchondral bone from traumatic injury in equine talocrural joints.[1] Interestingly, synovial membrane histology scores were significantly higher in treated joints at 6 months due to a mononuclear cellular infiltrate of macrophages and lymphocytes. To gain insight into possible mechanisms driving the chondroprotective effects of integrin α10-selected MSCs, RNA was isolated from the synovial membrane for gene expression analysis using the NanoString nCounter platform (NanoString Technologies Inc., Seattle, WA) and a custom-designed 42-gene equine panel. Six months after initiation of PTOA, there was decreased expression of TIMP2 ($P = 0.028$) and NF-κB ($P = 0.031$) in treated joints, suggesting that integrin α10-selected MSCs diminish long-term pro-inflammatory and catabolic states after injury. Treatment with integrin α10-selected MSCs also resulted in increased expression of CCL5 ($P = 0.049$). Through promoting macrophage reprogramming (M1/M2 transition), CCL5 may be aiding in the reparative response elicited by the integrin α10-selected MSCs.[2] This study demonstrates the ability to use archived synovial membrane samples to gain insight into mechanisms of action, and identification of potential targets to mitigate PTOA.

Methods, Results, and Discussion

Horses ($n = 8$) received bilaterally focal impact injuries to the talar articular surface and were treated intra-articularly with 20×10^6 allogeneic, adipose-derived integrin α10-selected MSCs (treated) or vehicle only (control), as previously described.[1] Synovial biopsies were obtained at the time of surgery (before injury), at 6-week second-look arthroscopy, and postmortem at 6 months. Samples were fixed in 10% neutral-buffered formalin for 24 hours, embedded in paraffin (herein referred to as FFPE samples), and stored at room temperature for 17 to 23 months prior to this study. RNA was extracted from the FFPE samples using High Pure FFPET RNA Isolation Kit (Roche, Indianapolis, IN) and according to NanoString's recommendations, where a minimum tissue input of 48 mm^2 is recommended to obtain about 10 ng/μL of RNA. For this study, ten 10-μm thick sections from FFPE blocks were required to obtain RNA of sufficient quantity and quality to perform gene expression analysis in most samples.

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Table 1. Custom CodeSet Panel: List of Genes Selected for the NanoString Gene Expression Analysis Based on Their Contributions to the Pathogenesis of Posttraumatic Osteoarthritis.

Inflammation	Immunoregulation	Cartilage Matrix Homeostasis	Housekeeping Genes
CCL2	ARG1	MMP-1	GAPDH
COX1	CCL5	MMP-3	HPRT1
COX2	CD163	MMP-13	UBC
CXCL12	CTLA-4	TIMP1	
CXCL8	FOXP3	TIMP2	
IFN-γ	GATA-3		
IL-12	IL-1RA		
IL-17A	IL-4		
IL-23	IL-10		
IL-1β	MRC1		
NF-κB	STAT6		
NOS-2	TGF-β1		
PRG4			
RUNX2			
SOX9			
T-bet			
TNF-α			
IL-2			
IL-6			
IL-13			
IL-22			
PGE2			

Quality control criteria to select samples for NanoString analysis, included (1) minimum RNA concentration (>10 ng/μL) and (2) RNA purity (A260/A280 ratio between 1.7 and 2.3) measured on a NanoDrop (Thermo Scientific, Waltham, MA). For samples that did not meet these criteria, the RNA extraction was repeated on 10 more sections. If criteria were still not met, the sample was excluded from the NanoString analysis. The minimum input of RNA from each sample to be used for the analysis was calculated based on the extent of RNA degradation, as estimated by smear analysis on the basis of the RNA Quality Number (RQN, <7) and amount of fragments sized 50 to 300 nucleotides (nt) (expressed as percentage of total RNA) measured using the AATI Fragment Analyzer (Advanced Analytical Technologies Inc., Ames, IA). Synovial membrane biopsies that were collected at the initial surgery and 6-week second-look arthroscopy were obtained with an arthroscopic biopsy punch, resulting in very small (<8 mm^2) samples, and the RNA extracted from 21 of 28 samples did not meet the inclusion criteria for NanoString analysis. Therefore, only the results obtained from samples harvested during necropsy at 6 months after injury are reported, finally including synovial membrane from control and treated joints of 7 horses ($n = 14$).

A custom NanoString CodeSet was developed to measure expression of 39 genes associated with inflammation, immunoregulation, and cartilage matrix homeostasis in early PTOA (**Table 1**).[3,4] NanoString nSolver 4.0 Analysis Software was used to process raw data. Background threshold was calculated from the raw data as the mean ±2 SD mRNA count across all the negative controls. Data were normalized against 3 housekeeping genes (GAPDH, HPRT1, and UBC). Normalized log transform data of the gene counts were exported for further analysis in JMP Pro 13 (SAS, Cary, NC). The expression of each gene was compared between treated and control joints using a paired *t* test, to account for the dependency of the matched joints within the same horse, with $P < 0.05$ considered significant.

The concentration of the extracted RNA was 272.4 ± 127.5 ng/μL (range 50.9-468 ng/μL). On smear analysis, 30.5% ± 8.7% (range 17%-42.5%) of RNA was represented by 50 to 300 nt length fragments, from which we derived the percentage of RNA with length >300 nt (69.5% ± 8.7%, range 57.5%-83%). Based on the quality control analysis results, a volume was calculated for each sample so that 200 ng of RNA was run against the custom CodeSet on nCounter SPRINT profiler. Gene expression analysis revealed that treatment with integrin α10-selected MSCs, resulted in increase of CCL5 expression ($P = 0.049$) in treated joints 6 months after injury, and additionally revealed decreased expression of TIMP2 ($P = 0.028$) and NF-κB ($P = 0.031$) (**Fig. 1A-C**).

Although CCL5 is classically considered an inflammatory chemokine, recent evidence suggests that CCL5 is

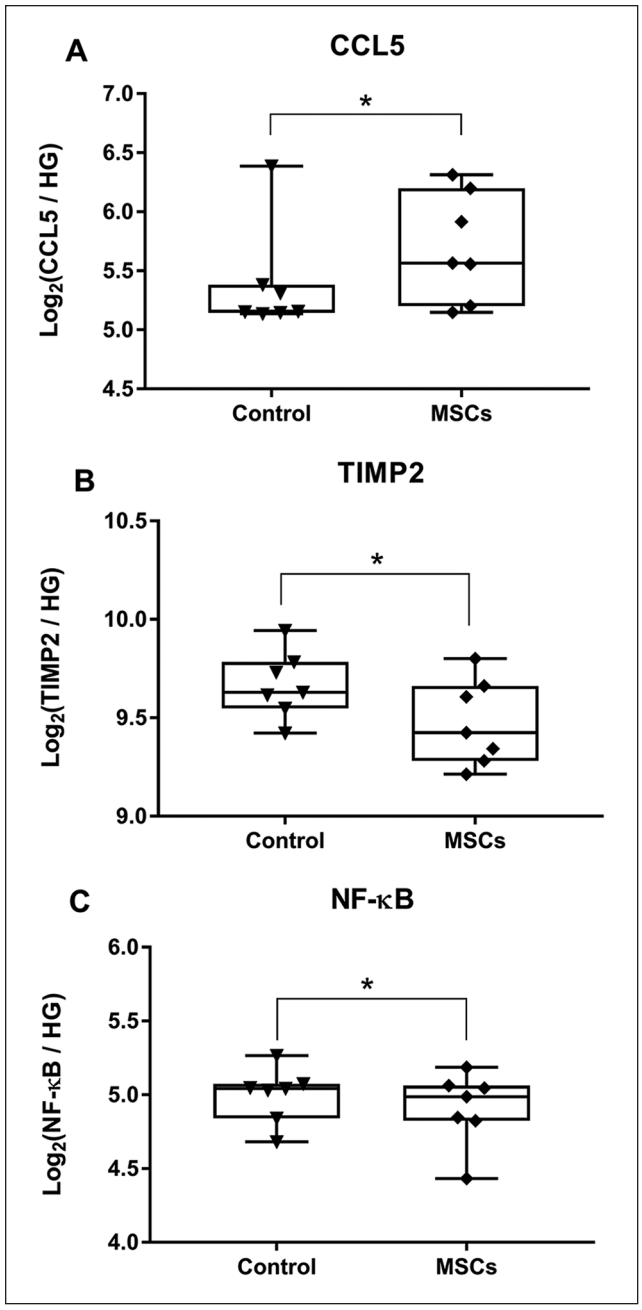

Figure 1. Intra-articular injection of integrin α10-selected mesenchymal stromal cells (MSCs) following acute injury results in long-term changes in gene expression of the synovial membrane including (**A**) increased expression of CCL5, (**B**) decreased expression of TIMP2, and (**C**) decreased expression of NF-κB. Data are expressed as $\log_2$ transformed gene counts relative to 3 housekeeping genes (GAPHD, HPRT1, UBC) detected using NanoString. Data are represented as single datapoints, with box-and-whiskers plot indicating median with interquartile range ($n = 7$ in each group) (*$P < 0.05$, paired t test). HG = housekeeping genes.

critical in the reprogramming of macrophages (M1/M2 transition) during the resolution phase of inflammation, and that CCL5 is actively secreted in high concentrations by resolution phase macrophages.[2,5] A transition from M1 to M2 phenotype is also consistent with the decrease in NF-κB expression, a key driver of M1 phenotype, which is upregulated during activation of pro-inflammatory M1 macrophages.[6] Additionally, monocytes are capable of secreting TIMP2 in response to inflammation.[7] TIMP2 is critical for maintenance of tissue homeostasis through its role as a matrix metalloproteinases antagonist. Although counter intuitive, early suppression of TIMPs seems predictive of a positive therapeutic outcome, as normal physiological extracellular matrix remodeling is restored when catabolic tissue destruction by aberrant matrix metalloproteinase release is resolved.[8] The decreased expression of these two genes in joints treated with integrin α10 selected MSCs may be indicative of earlier recovery from the post-traumatic pro-inflammatory and catabolic state.

In the original study, the synovial membrane 6 months postinjury showed a considerable mononuclear cellular infiltrate in the treated joints compared with the control, an unexpected finding that initiated this *a posteriori* investigation.[1] It has been previously shown that MSCs are detected within injured tissues only during the first 3 to 8 days after administration, thereafter they undergo transdifferentiation or they disappear.[9,10] In mice, following intravenous injection, MSCs are found in the lungs where they recruit macrophages and are subsequently phagocytosed within 72 hours, inducing a regulatory macrophage phenotype.[11] The aforementioned studies have reported that autologous, allogeneic, and xenogeneic MSCs are capable of triggering macrophage recruitment and phagocytosis of MCSs, subsequently inducing macrophage polarization to an immunosuppressive phenotype. These events could account for the findings in our study.

The use of the NanoString nCounter platform allowed us to gain valuable insight into long-term changes in gene expression occurring within the synovial membrane following intra-articular traumatic injury and injection of integrin α10-selected MSCs. While methods such as real-time quantitative polymerase chain reaction (RT-qPCR), microarrays and RNA-seq can also be used to analyze RNA gene expression, they require higher quantity and quality of RNA than NanoString.[12] Archived FFPE tissue samples contain RNA that can be valuable for use in retrospective clinical studies.[13] However, the process of formaldehyde fixation, embedding in warm paraffin, and room temperature storage, collectively result in reduced quality and yield of extracted RNA.[14] The NanoString nCounter platform is sensitive, specific, reproducible, and does not require the same complex manipulation stages as platforms like RNA-seq.[12] Additionally, the nCounter platform requires no amplification, as it utilizes a dual-probe system that uses a combination of target-specific capture probe and color-coded reporter probe to directly quantify RNA sequences.

The probes recognize a 100-base target region, and therefore, RNA degradation does not typically affect quality of the data which is important for studies involving FFPE samples.

In an ideal study design, tissues are *a priori* preserved for RNA isolation using snap freezing in liquid nitrogen.[15] However, for *a posteriori* analysis of musculoskeletal tissues preserved using FFPE, NanoString is a suitable platform to analyze up to 800 genes from clinical samples. NanoString exhibits multiple advantages over other RNA expression methods, including RT-qPCR, microarray, and RNA-seq because of its documented sensitivity, specificity, and robustness. Use of the NanoString nCounter platform can aid in answering mechanistic questions and connect the dots when analyzing histological changes seen in archived clinical samples.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

All procedures performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, federal and state regulations, and were approved by the Cornell University Institutional Animal Care and Use Committee.

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