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Low Molecular Weight Fraction of Commercial Human Serum Albumin Induces Morphologic and Transcriptional Changes of Bone Marrow-Derived Mesenchymal Stem Cells

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ABSTRACT

Osteoarthritis (OA) is the most common chronic disease of the joint; however, the therapeutic options for severe OA are limited. The low molecular weight fraction of commercial 5% human serum albumin (LMWF5A) has been shown to have anti-inflammatory properties that are mediated, in part, by a diketopiperazine that is present in the albumin preparation and that was demonstrated to be safe and effective in reducing pain and improving function when administered intra-articularly in a phase III clinical trial. In the present study, bone marrow-derived mesenchymal stem cells (BMMSCs) exposed to LMWF5A exhibited an elongated phenotype with diffuse intracellular F-actin, pronounced migratory leading edges, and filopodia-like projections. In addition, LMWF5A promoted chondrogenic condensation in "micromass" culture, concurrent with the upregulation of collagen 2α1 mRNA. Furthermore, the transcription of the CXCR4-CXCL12 axis was significantly regulated in a manner conducive to migration and homing. Several transcription factors involved in stem cell differentiation were also found to bind oligonucleotide response element probes following exposure to LMWF5A. Finally, a rapid increase in PRAS40 phosphorylation was observed following treatment, potentially resulting in the activation mTORC1. Proteomic analysis of synovial fluid taken from a preliminary set of patients indicated that at 12 weeks following administration of LMWF5A, a microenvironment exists in the knee conducive to stem cell infiltration, self-renewal, and differentiation, in addition to indications of remodeling with a reduction in inflammation. Taken together, these findings imply that LMWF5A treatment may prime stem cells for both mobilization and chondrogenic differentiation, potentially explaining some of the beneficial effects achieved in clinical trials. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:1–11*

SIGNIFICANCE

This study describes the effect of a biologic currently under development for the treatment of osteoarthritis to induce both cytoskeletal and transcriptional changes in bone marrow-derived mesenchymal stem cells. These changes may have implications for the regenerative potential of low molecular weight fraction of commercial 5% human serum albumin and could help explain some of the clinical findings in the clinical trials conducted using this drug.

INTRODUCTION

Osteoarthritis (OA) is the most common chronic disease of the joint, affecting an estimated 40% of the population aged older than 60 years [1, 2]. Traditionally considered a degenerative disease, it is now widely accepted that inflammation is a critical feature of OA [3]. Abnormal mechanical loading and the production of inflammatory mediators drive the production of catabolic proteases that erode cartilage, ultimately leading to the functional failure of the joint [4].

Therapeutic options for those suffering from OA are often limited to pain management with analgesics and nonsteroidal anti-inflammatory drugs [5]. As the disease progresses, more aggressive interventions are warranted, such as intra-articular (IA) steroids or hyaluronic acid injections, which provide some temporary relief but have not met with long-term clinical success. In advanced cases, total knee replacement is still the only viable therapeutic option. As a result of these shortcomings, a critical need exists for the development of effective methods to promote

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articular cartilage formation and healing for those suffering from degenerative joint diseases such as OA.

Experiments previously conducted in our laboratory demonstrated that commercial human serum albumin (HSA) solutions possess anti-inflammatory properties. Cytokine release from stimulated peripheral blood mononuclear cells and T-cell lines was inhibited by commercial human serum albumin solutions in vitro [6]. This activity was lost after dialysis, implicating low molecular weight components, including aspartyl-alanyl diketopiperazine (DADKP) [7].

As a result of these findings, a prepared low molecular weight fraction (<5 kDa) of commercial 5% human serum albumin (LMWF5A) is currently under development as a therapeutic for OA of the knee. In a recently completed clinical trial involving a total of 329 randomized patients, it was found that a single IA injection of LMWF5A resulted in a significant 42.3% reduction in pain observed at 4 weeks after injection that persisted to the completion of the trial, versus saline controls [8]. The afforded relief was most pronounced in patients with severe OA (Kellgren Lawrence grade IV) with almost double the pain reduction exhibited following administration compared with lower grades. Based on these observations, LMWF5A was deemed both safe and effective for the treatment and intervention of moderate to severe OA of the knee.

The purpose of this investigation was to help elucidate some of the biological mechanisms by which LMWF5A alleviated pain and enhanced knee function in the clinical trial participants. Although the anti-inflammatory activity of LMWF5A probably plays a pivotal role in its in vivo activity, the prolonged nature and return of function seen in these patients cannot be easily explained by a singular anti-inflammatory event. We hypothesized that LMWF5A can affect the activity of cells involved in the healing process, in particular, bone marrow-derived mesenchymal stem cells (BMMSCs). Intracellular F-actin organization, gene expression profiling, transcription factor binding, and the phosphorylation of key cell-signaling proteins were assessed following exposure to LMWF5A. We found pivotal changes in BMMSCs following treatment that may have potential implications for self-renewal, transcriptional regulation, and differentiation. It is our hope that these findings will aid understanding of the overall activity of LMWF5A observed in clinical trials.

Materials and Methods

SPRING Study Synovial Fluid Sample Preparation and Proteomic Analysis

The safety and efficacy of LMWF5A was evaluated in 329 patients with moderate to severe OA of the knee in the multicenter, randomized, vehicle-controlled, double-blind, phase III SPRING study (ClinicalTrials.gov identifier NCT01839331) [8]. The protocol for the SPRING study was approved by the institutional review boards from SUNY-Buffalo Health Sciences and Liberty IRB (Deland, FL, https://www.libertyirb.com/) and complies with the code of ethics of the World Medical Association. Written informed consent was obtained from all participants involved in the study. According to the protocol, the first 20 randomized patients were slated to have synovial fluid extracted from the index knee for proteomic analysis, with samples drawn at baseline and week 12 (study end). Samples were then processed and analyzed by SomaLogic (Boulder, CO, http://www.somalogic.com). A proprietary protocol was developed and optimized to address the viscosity of the synovial fluid samples and improve handling. Briefly, frozen samples were thawed, centrifuged, and then disrupted with a Bead Ruptor 24 machine and tubes (Cole-Parmer, Court Vernon Hills, IL, http://www.coleparmer.com). The samples were then subjected to proteomic analysis by the highly multiplexed proteomic SOMAscan biomarker assay (SomaLogic), which uses 1,129 slow off-rate modified aptamers (SOMAmers) to simultaneously measure protein expression levels.

Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, https://www.sigmaaldrich.com) unless otherwise stated. DADKP was synthesized by NN Scientific Ltd. (Cardiff, Wales, U.K.). The ≤ 5-kDa filtrate of commercial 5% HSA was isolated by Sypharma Pty Ltd. (Dandenong, Victoria, Australia, http://sypharma.com.au) using tangential flow filtration and a 5-kDa molecular weight cutoff Hydrosart filter membrane (Sartorius Stedim Biotech GmbH, Germany, http://www.sartorius.com).

Primary Stem Cells and Subculturing

Human BMMSCs were purchased from Cyagen Biosciences (Santa Clara, CA, http://www.cyagen.com) and subcultured in TheraPEAK MSCGM-CD growth medium (Lonza, Walkersville, MD, http://www.lonza.com) at 37°C and 5% CO_2. Passage 5 cells were used for all experiments and released from subculturing flasks using trypsin/EDTA (Lonza).

F-Actin Staining

Round glass cover slips (12 mm, #1.5) were coated with 2 μg/ml fibronectin and placed in 24-well tissue culture plate wells. Next, 1 ml Lonza TheraPEAK medium containing 10,000 BMMSCs was then added and incubated for 24 hours. The medium was then removed and replaced with 1 ml RPMI medium containing 0.5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After 24 hours, the medium was exchanged with saline or LMWF5A diluted 1:4 in serum-reduced medium and incubated for an additional 24 hours. Following treatment, the cells were fixed using 3.6% formaldehyde for 10 minutes at room temperature and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes. Alexa Fluor 568-phalloidin (100 μl, Invitrogen; Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com) diluted 1:50 in PBS was then added for 20 minutes, and representative frames were photographed using a Zyla sCMOS camera (Andor Technology, South Windsor, CT, http://www.andor.com) mounted on a Nikon Eclipse Ti inverted microscope (Nikon, Tokyo, Japan, http://www.nikon.com). Cellular structures were measured using ImageJ software (NIH, Bethesda, MD, http://imagej.nih.gov/ij) [9].

GTPase Activation Assays

BMMSCs were expanded in 75-cm^2 tissue culture flasks using Lonza TheraPEAK medium until 50%–70% confluent and then placed in RPMI containing 0.5% FBS and 1% penicillin/streptomycin for 24 hours. The medium was then replaced with saline or LMWF5A diluted 1:4 in serum-reduced medium and incubated on the cells for 10 minutes or 1 hour. Total protein was isolated, and activation of RhoA, Rac1, and Cdc42 was determined

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using G-LISA Biochem assay kits purchased from Cytoskeleton (Denver, CO, http://www.cytoskeleton.com).

Chondrogenic Differentiation

Mesenchymal stem cell chondrogenic differentiation medium was purchased from Cyagen and formulated without dexamethasone. To induce differentiation, suspensions of 1×10^7 cells per milliliter were prepared in warmed differentiation medium, and 20 μl was spotted to the center of 24-well tissue culture plate wells. The plates were incubated for 1 hour, and then 720 μl of warmed differentiation medium and 250 μl of saline or LMWF5A were layered over the cells. In addition, 250 μl of commercial 5% HSA excipients (3 mM N-acetyl-tryptophan [NAT] and 0.6 mM caprylate prepared in saline), the known component DADKP (400 μM in saline), and the anti-inflammatory dexamethasone (4 μM in saline) were tested. Finally, transforming growth factor β3 (TGF-β3) was added to a final concentration of 10 ng/ml, and the cells were incubated with medium exchanges performed every 3 days. Cultures were photographed using a Nikon D5000 camera mounted on a Motic AE31 inverted microscope (Richmond, BC, Canada, http://www.motic.com).

Hanging Drop Spheroid Three-Dimensional Culture

Hanging drop cultures were used as follows. Briefly, 100,000 BMMSCs were placed in 750 μl of mesenchymal stem cell growth medium (Lonza) and combined with 250 μl of saline, LMWF5A, or 800 μM cobalt chloride (final 200 μM). The resulting suspensions were added drop-wise to the underside of sterile petri dish lids (Greiner Bio One, Monroe NC, http://www.greinerbioone.com) in 25 μl increments and inverted on bottoms containing 20 ml of sterile phosphate-buffered saline. Following incubation, the cells were washed from the lids with 10 ml of warmed saline and centrifuged at 1,000 rotations per minute for 5 minutes.

Quantitative Reverse Transcription Polymerase Chain Reaction and Gene Expression Profiling

Total RNA was isolated from chondrogenic cultures 7 days following induction or after 3 days in hanging drop culture using Qiagen (Valencia, CA, http://www.qiagen.com) RNeasy plus columns. Complementary DNA was then generated from 10 μl of the isolated RNA with Qiagen Omniscript kit reagents. Quantitative polymerase chain reaction (qPCR) was then performed on a Roche 480 LightCycler using SYBR green I master mix (Roche Diagnostics, Indianapolis, IN, http://www.roche.com) with qPCR assay primers or RT^2 Profiler Signal Transduction Pathfinder Pathway PCR arrays purchased from Qiagen, following the manufacturer's protocol. Relative gene expression was calculated using $\Delta\Delta$Ct analysis versus saline controls normalized to housekeeping gene expression. Functional annotation and pathway analysis was performed using Ingenuity pathway analysis (Qiagen) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics 6.7 (National Institute of Allergy and Infectious Diseases, Frederick MD, http://www.niaid.nih.gov) software [10, 11].

Transcription Factor Activation Profiling and Phosphokinase Arrays

BMMSCs were expanded in 175-cm^2 tissue culture flasks in Lonza TheraPEAK medium until 50%–70% confluence was obtained and then starved for 24 hours in RPMI containing 0.5% FBS and 1% penicillin/streptomycin. The cells were then treated for 1 hour with saline or LMWF5A diluted 1:4 in the serum-reduced medium. To determine transcription factor activation profiles, nuclear protein was isolated and transcription factor activation profile plate arrays (Signosis, Santa Clara, CA, http://www.signosisinc.com) were performed adhering to the supplied protocol with luminescence detected using a GloMax 96 microplate luminometer (Promega, Madison WI, https://www.promega.com). To evaluate phosphorylation of signaling proteins, total protein was harvested and proteome profiler human phosphokinase arrays (R&D Systems, Minneapolis, MN, http://www.rndsystems.com) were performed following the manufacturer's recommendations. Photography and densitometry calculations were performed on a Carestream molecular imaging system (Carestream, Woodbridge, CT, http://www.carestream.com).

PRAS40 Cell-Based Enzyme-Linked Immunosorbent Assay

BMMSCs were seeded into black 96-well microplates with clear bottoms at 20,000 cells per well in 200 μl of RPMI containing 0.5% FBS and penicillin/streptomycin. After 24 hours, the medium was aspirated and replaced with 150 μl of RPMI containing 0.1% FBS. Next, 50 μl of saline or LMWF5A was added, and the cells were incubated for the indicated times and then fixed in 100 μl of 10% formalin (Sigma-Aldrich). Cell-based enzyme-linked immunosorbent assays (ELISAs) for the detection of total and phosphorylated PRAS40 (T246) were performed using reagents purchased from R&D Systems, and fluorescence was measured using a Spectramax M2^e plate reader (Molecular Devices, Sunnyvale, CA, http://www.moleculardevices.com).

Statistical Analysis

Distributions were determined, and the paired Student's *t* test was applied to data sets using Microsoft Excel (Microsoft, Redmond, WA, https://www.microsoft.com), with statistical significance set at $\leq$.05. SOMAscan assay relative fluorescence unit measurements from the synovial fluid samples were log-transformed, and differential expression of analytes was evaluated using a repeated measures mixed model that included treatment category (LMWF5A or control), time point (baseline or week 12), and interaction of treatment and time point (treatment $\times$ time point) as fixed effects. For proteins with a significant interaction, pairwise comparisons were also performed. Given the exploratory nature of this study, significant differences in protein expression were determined at a cutoff of α = .05 and were not corrected for multiple testing.

RESULTS

Morphologic, Cytoskeletal, and GTPase Activity Changes Triggered by LMWF5A Treatment of BMMSCs

To determine whether LMWF5A alters cellular morphology and intracellular F-actin organization, BMMSCs were treated with LMWF5A for 24 hours and then stained with fluorescently labeled phalloidin. A higher number of LMWF5A-treated BMMSCs exhibited an elongated phenotype with pronounced lamellipodia-like leading edges when examined microscopically under $\times$10 magnification (Fig. 1A, 1B). In addition, LMWF5A-treated cells contain elevated amounts of diffuse F-actin

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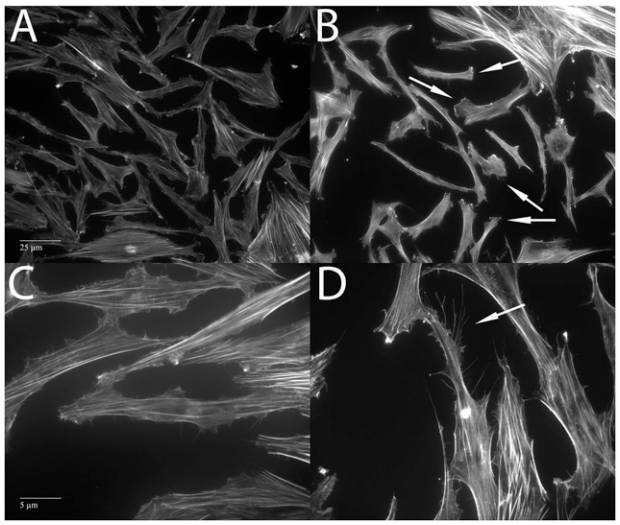

Figure 1. Low molecular weight fraction of commercial 5% human serum albumin (LMWF5A) treatment of bone marrow-derived mesenchymal stem cells (BMMSCs) induces changes in cytoskeletal organization. Serum-starved BMMSCs were stained for intracellular F-actin with fluorescently labeled phalloidin 24 hours after treatment. **(A):** Under ×20 magnification, saline-treated controls exhibited F-actin arranged primarily in stress fibers. **(B):** After treatment with LMWF5A, intracellular F-actin was more diffuse, and the cells exhibited migratory fronts (arrows). **(C):** At higher magnification (×100), filopodia-like structures were observed on saline controls. **(D):** LMWF5A increased both the length and number of filopodia found around the periphery of the cell (arrows). Scale bar = 25 μm **(A, B)** and 5 μm **(C, D)**.

throughout the cytosol. Furthermore, under higher magnification (×100 oil immersion), a marked increase in filopodia-like projections can be observed versus controls (Fig. 1C, 1D).

For affirmation, cellular structures in the ×20 images were measured using ImageJ software (supplemental online Fig. 1). Saline-treated cells possessed a median number of 32 filopodia per cell (first quartile: 27; third quartile: 39; minimum: 17; maximum: 51) in these images, whereas those exposed to LMWF5A exhibited a significant increase of 38 (p = .02), and the distribution skewed toward higher numbers (first quartile: 35; third quartile: 51; minimum: 20; maximum: 56). Furthermore, the overall length of the protrusions significantly increased with treatment. Vehicle-treated control cells had a median filopodia length of 17.4 pixels (first quartile: 12.6; third quartile: 18.8; minimum: 9.4; maximum: 36.5). The median filopodia length of LMWF5A-treated cells was 18.2 pixels, skewed to longer filopodia present (first quartile: 16.7; third quartile: 24.0; minimum: 10.3; maximum: 29.7; p = .05 vs. control). Finally, the overall length of the cells trended to increase in the treatment group. Saline-treated cells exhibited a median length of 576.4 pixels (first quartile: 511.9; third quartile: 748.1; minimum: 298.2; maximum: 983.3), and cells exposed to LMWF5A proved to have a median of 661.3 (first quartile: 551.2; third quartile: 780.9; minimum: 437.0; maximum: 1,069.8; p = .16 vs. control).

GTPase activity was then evaluated to help substantiate these observations using commercially available G-LISA kits (supplemental online Fig. 2). At 10 minutes following treatment with LMWF5A, the detectable amount of active GTP-bound RhoA decreased by 60%. Conversely, the activated amount of Rac1 increased 14%. When examined after 1 hour, both GTPases exhibited reduced activity of 51% and 6% for RhoA and Rac1, respectively. RhoA is a GTPase intimately associated with stress fiber formation of thick-cable F-actin that serves as scaffolding for myosin motors [12]. The observed reduction in RhoA activity could explain both the loss of stress fibers and the increase in diffuse actin seen within the cytosol. In contrast, Rac1 mediates lamellipodia development through Arp 2/3 [12]. A rapid increase in Rac1 may propagate the development of these structures on the leading edge of migratory fronts. Unexpectedly, we also observed a drop in Cdc42 activity at both time points tested (data not shown). Filopodia formation can be driven by Cdc42 activation of the formin protein mDia1 [12]. It is possible that a loss in Cdc42 activity combined with an increase in Rac1 and a disproportionate drop in RhoA may drive filopodia development in these cells.

LMWF5A Enhances BMMSC Condensation in Chondrogenic Culture

In addition to altering the morphology of cells cultured on cover glass, phenotypic changes were also observed in chondrogenic cultures treated with LMWF5A. To induce chondrogenic differentiation, BMMSCs were cultured serum free with TGF-β3 while in "micromass" aggregates. LMWF5A enhanced the ability of BMMSCs to condense into aggregates under these conditions. When observed at 1 week after initiation under ×4 magnification, LMWF5A-treated cultures appeared as tight pellets, whereas saline controls maintained a raised "disk-like" architecture (Fig. 2A, 2B). Several known components of LMWF5A were also tested at concentrations found in the final product: 3 mM NAT, 0.6 mM sodium caprylate, and 100 μM DADKP were all added to chondrogenic cultures, but DADKP was the only compound tested that accelerated condensation (Fig. 2C). Interestingly, dexamethasone, the anti-inflammatory and known enhancer of chondrogenesis, also exhibited an ability to induce condensation (Fig. 2D).

Collagen 2α1 Transcription Is Upregulated by LMWF5A During Chondrogenic Differentiation

To confirm induction and determine the effect of LMWF5A on chondrogenic differentiation, qPCR was performed for several key markers. In our model, mRNA for SOX9, MMP13, collagen 2α1, and aggrecan were upregulated by 2.8-, 2.1-, 43-, and 16.1-fold, respectively (data not shown) compared with confluent monolayers cultured under standard growth conditions after 7 days. When LMWF5A was added to the differentiation medium, collagen 2α1 transcription increased by an additional 4.3 $\pm$ 1.1-fold (Fig. 3). This was the only chondrocyte marker tested that exhibited this effect and did so only in conjunction with TGF-β3 in micromass. When individual components were tested, NAT and DADKP alone did not significantly increase collagen 2α1 transcription (1.4 $\pm$ 0.3- and 2.2 $\pm$ 2.0-fold changes, respectively). Sodium caprylate, however, had a significant effect, but it was not as pronounced as that of the complete LMWF5A product (1.96 $\pm$ 0.4 vs. 4.30 $\pm$ 1.1).

Expression Profiling and Functional Annotation of BMMSCs Treated With LMWF5A During Chondrogenic Differentiation

Qiagen RT^2 Profiler Signal Transduction Pathfinder PCR arrays were used to establish gene expression profiles of chondrogenic cultures following treatment with LMWF5A. Total RNA was

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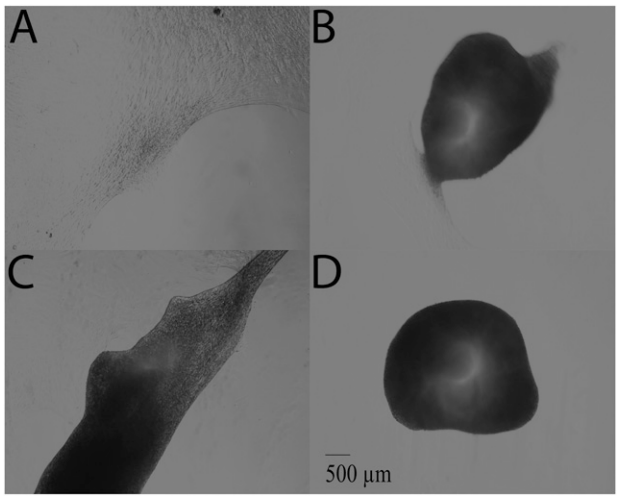

Figure 2. Morphologic alterations observed in chondrogenic cultures treated with low molecular weight fraction of commercial 5% human serum albumin (LMWF5A). Phenotypic changes were observed by microscopy (×4 magnification) 7 days after induction of chondrogenesis in cultures treated with LMWF5A. Saline-treated controls **(A)** remained in a disk-like configuration, whereas LMWF5A-treated cells **(B)** condensed into tight pellets. Known excipients and components found in LMWF5A were also tested. Cultures treated with 100 μM aspartyl-alanyl diketopiperazine **(C)** also exhibited accelerated condensation, but this effect was not as pronounced as the full LMWF5A product. **(D):** 1 μM dexamethasone, a strong anti-inflammatory and known enhancer of chondrogenesis, also exhibited an ability to accelerate condensation. Scale bar = 500 μm.

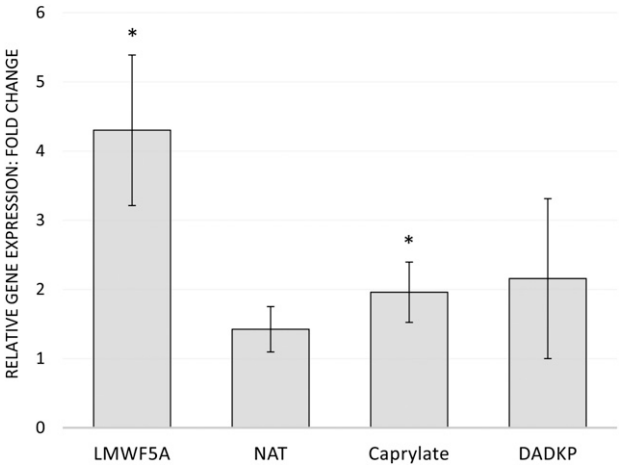

Figure 3. Collagen 2α1 transcription is upregulated by LMWF5A during chondrogenesis. Collagen 2α1 mRNA levels were determined by quantitative polymerase chain reaction 7 days following initiation of chondrogenic differentiation. In the presence of LMWF5A, a 4.3 + 1.1-fold increase in collagen 2α1 mRNA level was observed. Known LMWF5A excipients and components were also tested: 3 mM NAT and 100 μM DADKP alone did not significantly increase collagen 2α1 transcription (1.4 $\pm$ 0.3- and 2.2 $\pm$ 2.0-fold changes, respectively). Although 0.6 mM sodium caprylate had a significant effect, it was not as pronounced as that of the complete LMWF5A product (2.0 $\pm$ 0.4 vs. 4.3 $\pm$ 1.1). Data are presented as mean of three separate experiments $\pm$ SEM. *, $p \leq .05$. Abbreviations: DADKP, aspartyl-alanyl diketopiperazine; LMWF5A, low molecular weight fraction of commercial 5% human serum albumin; NAT, N-acetyl-tryptophan.

isolated when condensation was observed in the LMWF5A-treated samples (approximately day 7), and mRNA levels were compared with saline controls (Table 1). A total of 36 genes included on the array were found to be upregulated $\geq$1.5-fold, whereas 9 were downregulated. The biggest changes in transcription were observed with *ICAM-1* and *Notch-1* following exposure (>20-fold). Among genes exhibiting slight increases were some associated with chondrogenesis, including bone morphogenetic protein-2 (*BMP-2*), *BMP-4*, and *Wnt* (5.2-, 1.6-, and 2.9-fold, respectively). In addition, a protein implicated in stem cell renewal, *MYC*, was upregulated 2-fold.

The differentially expressed genes were functionally annotated with two separate tools. DAVID Bioinformatics analysis identified enrichment in gene ontology terms for regulation of cell proliferation, positive regulators for transcription, and negative regulators of apoptosis. In addition to these pathways, Ingenuity pathway analysis software also identified enrichment in cell cycle, cellular movement connective tissue development, and function pathways. The top regulator effect network identified by the program was AKT1.

CXCR4 and CXCL12 Transcription Is Altered by LMWF5A Treatment of BMMSCs

To determine whether LMWF5A could affect the transcription of key stem cell trafficking molecules, mRNA levels for the chemokine CXCL12 (also known as SDF-1α) and CXCR4 were monitored by qPCR. These investigations were performed in three-dimensional hanging drop culture to preserve the transcription of CXCR4, which is rapidly lost following ex vivo expansion of stem cells [13]. Treatment for 3 days with LMWF5A led to significant changes in both CXCR4 and CXCL12 mRNA (Fig. 4). CXCR4 transcription increased 12.3 $\pm$ 5.1-fold, whereas CXCL12 decreased 11.6 $\pm$ 7.5-fold. Other transcripts, including collagen 2α1 and MMP13, exhibited no significant change (1.5 $\pm$ 0.5 and $-$0.6 $\pm$ 0.9, respectively). Regulation of the cartilage-specific proteoglycan aggrecan trended to be higher (4.7 $\pm$ 5.2) but was not statistically significant. A similar genotypic CXCR4/CXCL12 axis pattern was exhibited by cells cultured under chemically induced hypoxia (200 μM cobalt chloride), a known inducer of CXCR4 (data not shown).

Transcription Factor Array Analysis of BMMSCs Treated With LMWF5A

To identify transcription factors activated by LMWF5A, stimulated BMMSCs were subjected to array analysis. Nuclear protein was isolated 1 hour following treatment and then incubated with DNA oligonucleotides matching consensus binding sequences of known transcription factors. A doubling in signal was deemed significant for this assay, and a representative experiment is presented in Table 2. A number of transcription factors implicated in stem cell differentiation and self-renewal were actively binding oligonucleotide response element probes following LMWF5A treatment, including AP2, COUP-TF, GATA, Gfi-1, Myb, NFAT, PPAR, RXR, SOX2, VDR, and XBP [14–25].

Kinase Array and PRAS40 In-Cell Analysis

To elucidate signaling pathways activated by LMWF5A, kinase arrays were performed. BMMSCs were treated with saline or LMWF5A for 1 hour, and total protein was isolated. The resulting

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Table 1. Differentially expressed genes of LMWF5A treated BMMSC during chondrogenesis

Gene	Gene symbol	Unigene number	Fold regulation
Notch 1	*NOTCH1*	Hs.495473	22.13
Intercellular adhesion molecule 1	*ICAM1*	Hs.643447	21.08
Carbonic anhydrase IX	*CA9*	Hs.63287	14.50
Aryl hydrocarbon receptor nuclear translocator	*ARNT*	Hs.632446	11.22
Chemokine (C-C motif) ligand 5	*CCL5*	Hs.514821	8.99
Adrenomedullin	*ADM*	Hs.441047	5.97
BTG family, member 2	*BTG2*	Hs.519162	5.81
Bone morphogenetic protein 2	*BMP2*	Hs.73853	5.20
BCL2-related protein A1	*BCL2A1*	Hs.227817	5.16
Patched 1	*PTCH1*	Hs.494538	4.72
Heme oxygenase (decycling) 1	*HMOX1*	Hs.517581	4.56
Acyl-CoA synthetase long-chain family member 5	*ACSL5*	Hs.11638	4.40
Cyclin D1	*CCND1*	Hs.523852	3.83
Epithelial membrane protein 1	*EMP1*	Hs.719042	3.70
Wingless-type MMTV integration site family, member 1	*WNT1*	Hs.248164	2.90
Wingless-type MMTV integration site family, member 5A	*WNT5A*	Hs.643085	2.77
FOS-like antigen 1	*FOSL1*	Hs.283565	2.73
Sequestosome 1	*SQSTM1*	Hs.724025	2.62
Epidermal growth factor receptor	*EGFR*	Hs.605083	2.22
Vascular endothelial growth factor A	*VEGFA*	Hs.73793	2.22
Lactate dehydrogenase A	*LDHA*	Hs.2795	2.13
V-myc myelocytomatosis viral oncogene homolog (avain)	*MYC*	Hs.202453	2.03
Axin 2	*AXIN2*	Hs.156527	1.98
Fc fragment of IgE, low affinity II, receptor for (CD23)	*FCER2*	Hs.465778	1.98
Interferon, γ	*IFNG*	Hs.856	1.98
Growth arrest and DNA-damage-inducible, α	*GADD45A*	Hs.80409	1.94
Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	*ID1*	Hs.504609	1.90
Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	*SERPINE1*	Hs.713079	1.86
Peroxisome proliferator-activated receptor delta	*PPARD*	Hs.696032	1.83
Glutathione reductase	*GSR*	Hs.271510	1.81
Glutamate-cysteine ligase, catalytic subunit	*GCLC*	Hs.654465	1.79
Thioredoxin reductase 1	*TXNRD1*	Hs.654922	1.70
BCL2-like 1	*BCL2L1*	Hs.516966	1.67
Wingless-type MMTV integration site family, member 6	*WNT6*	Hs.29764	1.62
Bone morphogenetic protein 4	*BMP4*	Hs.68879	1.57
Interferon regulatory factor 1	*IRF1*	Hs.436061	−1.51
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	*CDKN1B*	Hs.238990	−1.54
Fas (TNF receptor superfamily, member 6)	*FAS*	Hs.244139	−1.76
Glutamate-cysteine ligase, modifier subunit	*GCLM*	Hs.315562	−1.84
Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	*HERPUD1*	Hs.146393	−1.95
Growth arrest and DNA-damage-inducible, β	*GADD45B*	Hs.110571	−1.96
Proliferating cell nuclear antigen	*PCNA*	Hs.744934	−2.45
Disabled homolog 2, mitogen-responsive phosphoprotein (*Drosophila*)	*DAB2*	Hs.696631	−2.75

BMMSCs were cultured in aggregate, under serum free conditions, in the presence of 10 ng/ml TGFβ3 to induce chondrogenesis. Total RNA was harvested at the time of condensation and Qiagen RT^2 Profiler Signal Transduction Pathfinder polymerase chain reaction arrays were utilized to determine differential transcription in cultures treated with LMWF5A versus saline controls. A total of 36 genes present on the array exhibited ≥1.5-fold increase in regulation after normalization as compared with saline-treated controls, while 9 were downregulated.
Abbreviations: BMMSC, bone marrow-derived mesenchymal stem cell; LMWF5A, low molecular weight fraction of commercial 5% human serum albumin.

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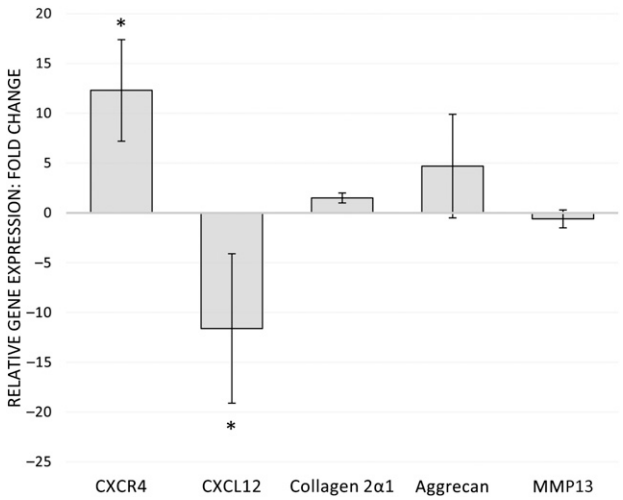

Figure 4. Transcriptional changes in CXCR4 and CXCL12 following treatment with low molecular weight fraction of commercial 5% human serum albumin (LMWF5A). Bone marrow-derived mesenchymal stem cells in hanging drop culture were exposed to LMWF5A for 3 days, and mRNA levels were evaluated by quantitative polymerase chain reaction. Stimulation with LMWF5A resulted in an increase in CXCR4 (12.3 $\pm$ 5.1-fold) concurrent with a decrease in CXCL12 (11.6 $\pm$ 7.5-fold). The transcription of other genes such as collagen 2α1, aggrecan, and MMP13 were unaffected by treatment. Data are presented as mean of three separate experiments $\pm$ SEM. *, $p \leq .05$. Abbreviation: MMP13, matrix metalloproteinase 13.

extracts were incubated with nitrocellulose membranes spotted with antibodies against phosphoproteins associated with cellular signaling pathways. A 2-fold increase in PRAS40 phosphorylation at residue threonine 246 was observed at the time point chosen (Fig. 5A, 5B). The phosphorylation of PRAS40 at threonine 246 was primarily the result of protein kinase B/Akt activity, suggesting that this pathway is triggered by LMWF5A [26].

To confirm this finding, in-cell ELISAs for the detection of both total and phospho-T246 PRAS40 were performed on fixed cells. Elevated phosphorylation was detected as early as 5 minutes following exposure and persisted for at least 1 hour (Fig. 5C). The peak LMWF5A-induced PRAS40 T246 phosphorylation was found at 15 minutes of treatment (23.4% $\pm$ 1.6% change vs. control).

Differential Synovial Fluid Protein Expression Associated With LMWF5A Treatment

A preliminary proteomic analysis was performed on synovial fluid collected from patients enrolled in the SPRING study. Of the first 20 patients randomized, 11 patients did not have sufficient synovial fluid in their intra-articular space to sample; these patients were considered "dry taps." Of the remaining nine patients, who had enough synovial fluid to sample, six received LMWF5A and three received saline.

A total of 35 proteins were identified that exhibited significant differential expression in the LMWF5A–treated synovial fluid samples compared with the saline-treated controls using the highly multiplexed SOMAscan proteomic assay. The expression of 23 proteins (supplemental online Table 1) was significantly elevated after LMWF5A treatment at week 12. The lowest interaction *p* value was observed with clusterin, the expression of which was highly stimulated in samples from all six patients treated with LMWF5A. Expression of the mitochondrial complement C1q subcomponent-binding protein (C1QBP, also called hyaluronic acid-binding protein 1) was also significantly increased in all six patients treated with LMWF5A. Other proteins of interest with increased expression included monocyte chemoattractant protein 1, interleukin-11, matrix metalloproteinase 3 (MMP3), basic fibroblast growth factor (bFGF), and tissue inhibitor of metalloproteinase 1 (TIMP). Conversely, the expression of 12 proteins (supplemental online Table 2) significantly decreased in LMWF5A-treated synovial fluid at week 12 relative to saline-treated fluid. Several of these proteins were kinases, including mitogen-activated protein kinase-activated protein kinase 2 and 3, β-adrenergic receptor kinase 1, and c-Src kinase. In addition, we also saw decreases in aggrecanase 1 and Noggin.

Discussion

For the first time, we demonstrated that LMWF5A—in addition to its immune-modulating properties—shows an ability to alter the morphology and transcriptional activity of BMMSCs. Our findings suggest that LMWF5A treatment could prime these cells in ways that could aid recruitment and collagen deposition. The inherent low cellularity and avascularity of articular cartilage presents serious challenges for the natural regeneration of synovial joints. To counteract these limitations, a number of cell-based interventions have been under investigation, including the transplantation of expanded autologous chondrocytes or BMMSCs [27]. Although the preliminary findings have been encouraging, these techniques necessitate invasive procedures for harvesting viable tissue and cells. A drug that potentiates BMMSC mobilization and chondrogenic differentiation could be beneficial for healing and cartilage regeneration clinically without the need for invasive harvesting and expansion of autologous cell types.

One of our initial observations was that treatment of BMMSCs with LMWF5A changes actin nucleation in the cytosol and at the periphery of the cell. We also detected a rapid switch in GTPase activity patterns within minutes of application. These findings could explain the observed morphologic changes and have broader implications clinically. It has long been appreciated that F-actin organization is integral to stem cell differentiation. Mesenchymal stem cells entering into chondrogenesis exhibit decreased RhoA activity with a consequent change in shape [28]. Conversely, overexpression of RhoA decreases chondrogenesis, marked by a loss of SOX9 expression, a transcription factor that is intimately associated with chondrocyte commitment [28]. Furthermore, disrupting actin with cytochalasin-D, an inhibitor of actin polymerization, encourages chondrogenesis in both animal models and in culture [28]. Taken together, manipulation of the cytoskeleton could directly affect the differentiation potential of these cells.

LMWF5A also appears to exert an ability to alter BMMSC transcription. Gene profiling of chondrogenic cultures showed that several genes linked to stem cell differentiation and chondrogenesis were upregulated following treatment, including BMP-2 and Wnt [29, 30]. Other genes that are implicated in stem cell expansion or self-renewal also increase after exposure to LMWF5A. Notch-1 signaling, for example, plays a role in the expansion of hematopoietic stem cells from progenitor cells [31]. In addition, the transcription factor MYC induces proliferation when inserted

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Table 2. Transcription factor activation profile of BMMSCs following stimulation with LMWF5A

Transcription factor	Gene description	Fold change
AP2	Activator protein 2	82.06
HEN	Helix-loop-helix protein	17.90
ER	Estrogen receptor	13.91
HOX4C	Hox4c homobox	13.59
XBP	X-box binding protein 1	10.32
Gfi-1	Growth factor independent 1 transcription	8.39
ROR	Retinoic acid receptor-related orphan	7.98
Stat-3	Signal transducer and activator of transcription 3	7.47
COUP-TF	Nuclear receptor subfamily 2, group F	6.18
AR	Androgen receptor	5.74
YY1	YY1 transcription factor	5.55
Stat-6	Signal transducer and activator of transcription 6	5.26
SOX2	SOX protein 2	4.74
CBF	CCAAT/enhancer binding protein (C/EBP), ζ	4.73
Stat-5	Signal transducer and activator of transcription 5	4.56
GATA	GATA transcription factor	4.40
FREAC2	Forkhead-related activator 2	4.33
CAR	Nuclear receptor subfamily 1, group 1, member 3	4.26
Nkx3-2	Homeobox protein Nkx-3.2	4.14
RXR	Retinoid X receptor	4.03
PPAR	Peroxisome proliferator-activated receptor	3.88
AP4	Activator protein 4	3.85
SMAD	SMAD family	3.81
Myb	v-myb myeloblastosis viral oncogene homolog	3.65
FoxC1	Homeobox C1	3.62
VDR	Vitamin D (1,25-dihydroxyvitamin D3)	3.55
PXR	Pregnane X receptor	3.51
Pit	Pituitary-specific transcription factor 1	3.31
Brn-3	POU domain, class 4, transcription factor 1	3.25
E2F-1	E2F transcription factor 1	3.21
MZF	Zinc finger type transcription factor MZF	3.14
C/EBP	CCAAT/enhancer binding protein (C/EBP), α	3.13
TR	Thyroid hormone receptor	2.89
USF-1	Upstream transcription factor	2.86
NF-1	Nuclear factor 1	2.74
Prox1	Prospero homeobox protein 1	2.72
ATF2	Activating transcription factor 2	2.71
Snail	Snail 1 zinc finger protein	2.68
Nkx2-5	Homeobox protein Nkx-2.5	2.66
CREB	cAMP responsive element binding protein 1	2.63
FOXG1	FOXbox G1	2.50
IRF	Interferon regulatory factor	2.47
TFIID	TATA box binding protein	2.46
NFAT	Nuclear factor of activated T cells	2.32
AP3	Activator protein 3	2.27
Sp1	SP1 transcription factor	2.21
Ets	v-ets erythoblastosis virus E26 oncogene homolog 1	2.17
Stat-4	Signal transducer and activator of transcription 4	2.16
PIT1	POU class 1 homeobox 1	2.14
Pax2	Pair box-2 protein	2.11
HNF-1	Hepatocyte nuclear factor 1	2.07
FOXD3	Forkhead box D3	1.96

Serum-starved BBMSCs were analyzed for transcription factor DNA binding after exposure to LMWF5A using Signosis TF profiling arrays. Nuclear protein extracts were harvested 1 hour following stimulation with LMWF5A and then incubated with biotin-labeled consensus sequence oligonucleotide probes. A marked increase in response element oligonucleotide binding was observed for 52 different transcription factors. A twofold increase in binding was deemed significant activation.

Abbreviations: BMMSC, bone marrow-derived mesenchymal stem cell; LMWF5A, low molecular weight fraction of commercial 5% human serum albumin.

into stem cells using a retroviral vector [32]. Intriguingly, one of the most overexpressed genes detected was the adhesion molecule *ICAM-1*. This finding is of interest because *ICAM-1* is upregulated in BMMSCs undergoing chemotaxis [33]. Although limited by the small number of genes present on the array used, the expression profiles of LMWF5A-stimulated BMMSCs suggested that these cells experience increases in growth, survival, motility, and transcription.

Because of the incomplete coverage of the arrays used, we chose to investigate the effect of LMWF5A on genes associated with chondrogenesis and stem cell trafficking. Collagen 2α1 was the only chondrogenic marker tested that exhibited enhanced transcription following LMWF5A-treatment. Interestingly, this increase was only achieved jointly with TGF-β3, suggesting an interaction with this signaling pathway. CXCR4 and CXCL12 mRNA levels were also evaluated after treatment in three-dimensional culture. CXCL12, also known as SDF-1, and its receptor, CXCR4, are the primary axis of stem cell trafficking and have been shown to promote BMMSC migration to the site of tissue injury, aiding and accelerating repair [34]. We found that the transcription of CXCR4 was upregulated by LMWF5A with a corresponding drop in CXCL12. The observed increase in CXCR4 transcription could potentially prime cells to respond to chemical gradients of CXCL12, whereas a reduction in locally derived ligand may enhance the ability of the cell to escape the autocrine effect of surrounding cells. Taken together, LMWF5A could help prime BMMSCs for infiltration and collagen deposition.

Activation of the phosphoinositide-3 kinase (PI3K) and AKT pathway could partially explain the findings of this investigation. PRAS40 is an inhibitory component of the mammalian target of rapamycin complex 1 (mTORC1), and phosphorylation at threonine 246 by PI3K/AKT leads to the dissociation of PRAS40 from the complex, relieving its inhibitory constraint [26]. Once free, mTORC1 activates the ribosomal protein S6 kinase, leading to elevated transcription and subsequent cellular proliferation [26]. It has also been shown that mTOR plays a role in the lineage commitment of stem cells by upregulating critical transcription factors at the appropriate time [35]. In addition to reducing proliferation, rapamycin treatment of the chondrogenic line ATDC5, when delivered within the first 2 days of the

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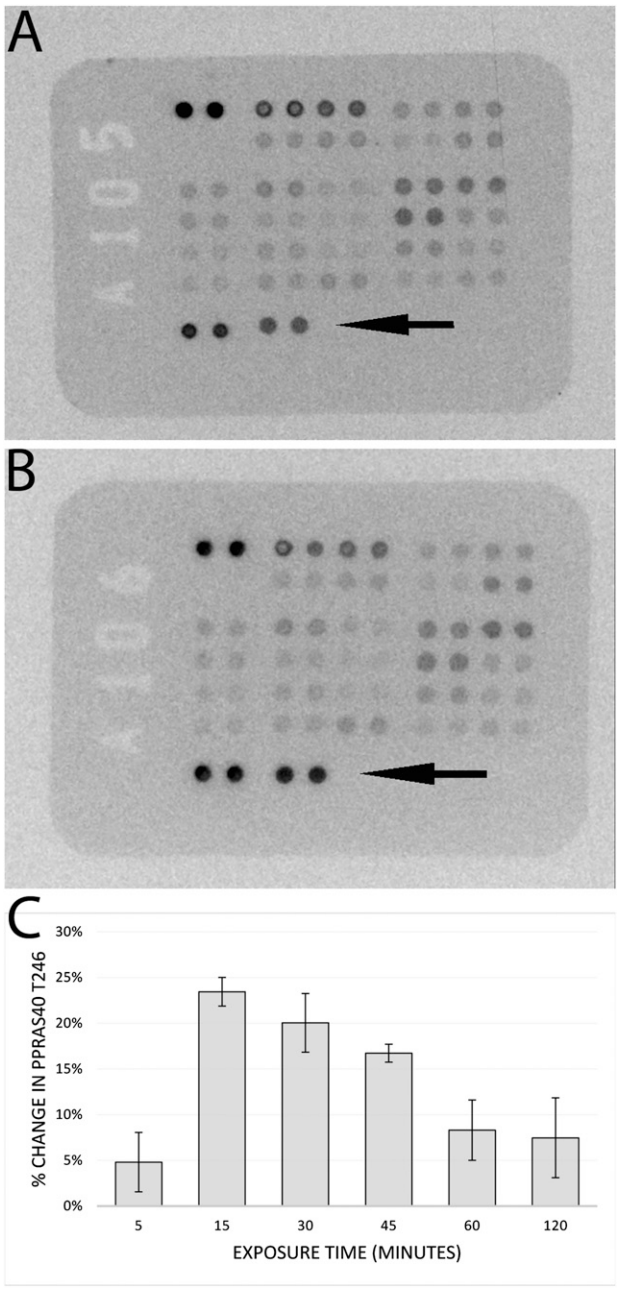

Figure 5. PRAS40 phosphorylation identified by kinase and in-cell enzyme-linked immunosorbent assay (ELISA) following treatment with low molecular weight fraction of commercial 5% human serum albumin (LMWF5A). **(A):** R&D Systems proteome profiler human phosphokinase array was performed on saline-treated bone marrow-derived mesenchymal stem cell (BMMSC) controls. **(B):** One hour after the addition of LMWF5A, a 2-fold increase in phospho-T246 PRAS40 was observed. **(C):** This was confirmed using an in-cell ELISA for the detection of both total and phosphor-T246 PRAS40. BMMSCs were treated with LMWF5A and fixed at the indicated time points. Phosphorylation of PRAS40 at T246 was elevated above background in as little as 5 minutes, with a peak at 15 minutes following addition of LMWF5A. This elevated phosphorylation persisted for at least 120 minutes.

differentiation process, greatly reduces chondrogenesis [36]. The cytoskeletal changes observed also could have a root in this pathway. The knockdown of PRAS40 by small interfering RNA not only reduces apoptosis but also drastically changes cell shape [37]. One of the classic routes of Rac1 activation is through PI3K, and AKT has been found to colocalize with actin bundles at the leading edge of migratory cells [38, 39]. Some research has even suggested that actin can be directly phosphorylated by AKT and that the cortical actin remodeling seen during filopodia formation is dependent on AKT-activated proteins [38]. Similarly, prenylated proteins identified for their involvement with filopodia formation in cancer cells have been implicated in migration and participate in the PI3K/AKT pathway [40]. Although we feel that the activity of LMWF5A is not limited to the activation of Akt, evidence suggests that some of the effects of this biologic on BMMSCs can be explained by stimulation of this pathway.

No direct evidence of stem cell markers was observed in the synovial fluid of patients receiving treatment with LMWF5A. This is to be expected because synovial fluid is a cocktail of proteins derived from multiple cell types to which stem cells will contribute little. Instead, we saw a microenvironment conducive to stem cell infiltration, maintenance, and differentiation, with evidence of a reduction in inflammation and active remodeling. The most significantly stimulated proteins in synovial fluid from the knee of OA patients treated with LMWF5A, clusterin and complement C1q, both promote stem cell migration through CXCR4-mediated pathways [41, 42]. In addition, monocyte chemoattractant protein, another proven chemokine for stem cells, increases after treatment compared with control [43]. The self-renewal or proliferation of stem cells would also be enhanced by the presence of mitogenic growth factors such as bFGF [44]. Conversely, a strong antagonist of differentiation, Noggin, decreases in the presence of LMWF5A [45]. Differentiation and proliferation has also been shown to be enhanced by clusterin [46]. In addition, we observed evidence of remodeling because MMP3 and TIMP increased, whereas aggrecanase 1 decreased. Elevated expression levels of clusterin in synovial fluid and tibial bone have been observed during disease progression in OA and could indicate that tissue repair processes have been activated by surrounding cells [47]. Clusterin is highly expressed in human cartilage that is undergoing active repair, and the distribution of clusterin in repaired tissue differs from that in normal cartilage [48]. We speculate that all of these processes could modulate tissue repair in response to LMWF5A.

CONCLUSION

Because of limitations with the experimental use of LMWF5A (contains 0.01% human serum albumin, which could lead to a xenogenic immune response), we chose to investigate human cell culture models designed to mimic different phases of BMMSC function in vitro. We think that the findings presented in this investigation, however, demonstrate that LMWF5A can alter stem cells function and that the proteomic changes in the synovial fluid of patients receiving treatment indicate that these observations translate, in part, in vivo. Consequently, LMWF5A could prove to be a viable alternative to the existing strategies for the treatment of OA of the knee or possibly enhance the efficacy of existing protocols by locally affecting extracellular matrix deposition, enhancing mesenchymal stem cell differentiation and/or mobilizing of mesenchymal stem cells into the compartment.

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Author Contributions

D.B.-O. and G.W.T.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; L.T.R. and E.D.G.: conception and design, data analysis and interpretation, manuscript writing; P.R.: data analysis and interpretation, final approval of manuscript; E.B.: conception and design, data analysis and interpretation, final approval of manuscript.

Disclosures of Potential Conflicts of Interest

D.B.-O. has compensated employment and uncompensated intellectual property rights, stock, and stock options with Ampio Pharmaceuticals. G.W.T. is a compensated employee of Ampio Pharmaceuticals, is on patents regarding LMWF5A, and has compensated stock options in Ampio Pharmaceuticals. L.T.R. is a compensated employee of Ampio Pharmaceuticals and Trauma Research and is a compensated shareholder of Ampio Pharmaceuticals. E.D.G. is a compensated employee of Ampio Pharmaceuticals and has compensated stock options from Ampio Pharmaceuticals. P.R. is a compensated consultant on the advisory board of Ampio Pharmaceuticals. E.B. is a compensated advisor to Ampio Pharmaceuticals and has uncompensated stock options in Ampio Pharmaceuticals.

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