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AAO Foundation Final Report Form
(a/o 2/9/2021)

Type of Award: Biomedical Research Award (BRA)

Name of Principal Investigator: Kyungsup Shin, D.M.D., Ph.D.

Institution: University of Iowa

Title of Project: Extracellular Vesicles for Endogenous Progenitor Cell Homing to Regenerate Condylar Fibrocartilage in Temporomandibular Joint

Period of AAOF Support: 07-01-2020 to 6-30-2021

Amount of Funding: $30,000.00

Summary/Abstract:

The overall objective for this project is to elucidate the therapeutic potential of characterized exosomes (extracellular vesicles) as a chemoattractant to stimulate fibrocartilage stem cell (FCSC) migration and fibrocartilage regeneration.

We have successfully completed the following Specific Aims;

Aim 1. Characterize bone marrow stromal cell-derived exosomes (BMSC-Exos) and identify Exo contents. *Working hypothesis: Highly-purified BMSC-Exos will have specific microRNA (miRNA) families regulating TMJ fibrocartilage regeneration.* Highly-purified BMSC-Exos will be characterized for size distribution, morphology, and protein markers. For Exo contents, Next-Generation Sequencing will be performed for miRNA profiling.

Aim 2. Determine the effects of BMSC-Exos on *in vitro* FCSCs migration and fibrocartilage regeneration. *Working hypothesis: BMSC-Exos will play an important role in cell-to-cell communication for FCSC migration and TMJ fibrocartilage differentiation.* The efficiency of BMSC-Exo on FCSC migration and fibrocartilage regeneration will be evaluated using *in vitro* cell invasion assay and 3-dimensional pellet culture, respectively.

1. Characterization of BMSCs and FCSCs (Aim 1)

BMSCs and FCSCs showed a fibroblast-like morphology (Figure 1A and 2A) and highly expressed stem cell positive markers (CD29, CD45, CD105, and CD44) compared to negative markers (MHC-2 and CD34) in both batches (Figure 1E and 2E). In addition, both bovine BMSCs and FCSCs were capable of adipogenic, osteogenic, and chondrogenic differentiation (Figure 1B-D and 2B-D).

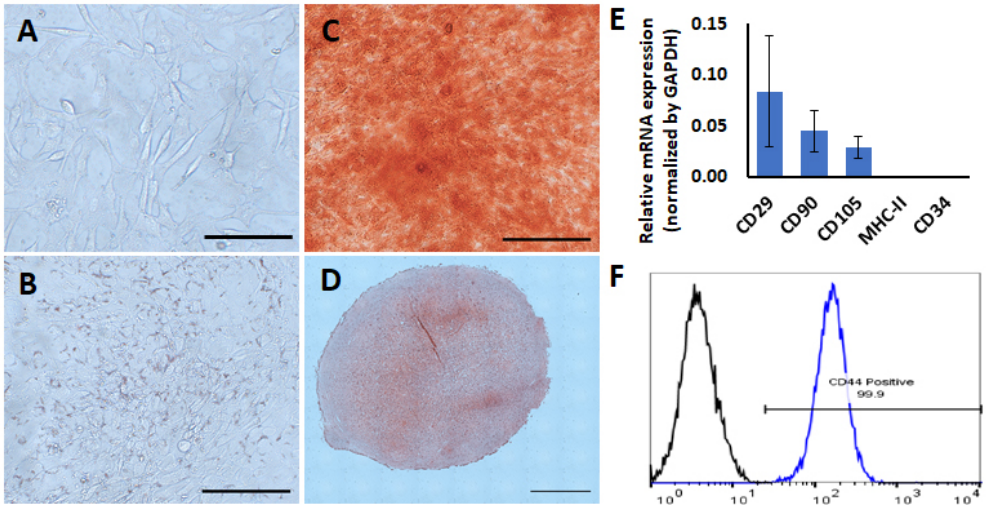

Figure 1. Identification and characterization of bovine bone marrow stromal cells (BMSCs). (A) Morphology of BMSCs (scale bar = 100 µm). (B) Oil Red O for adipogenesis (scale bar = 200 µm). (C) Alizarin red for osteogenesis (scale bar = 200 µm). (D) Safranin-O for chondrogenesis (scale bar = 500 µm). (E) TaqMan™ PCR with putative stem cell positive markers (CD29, CD90, and CD105) and negative markers (MHC-2 and CD34) (n = 3). (F) Flow cytometry analysis with stem cell positive marker (CD44; blue) and negative control (black) (n = 3).

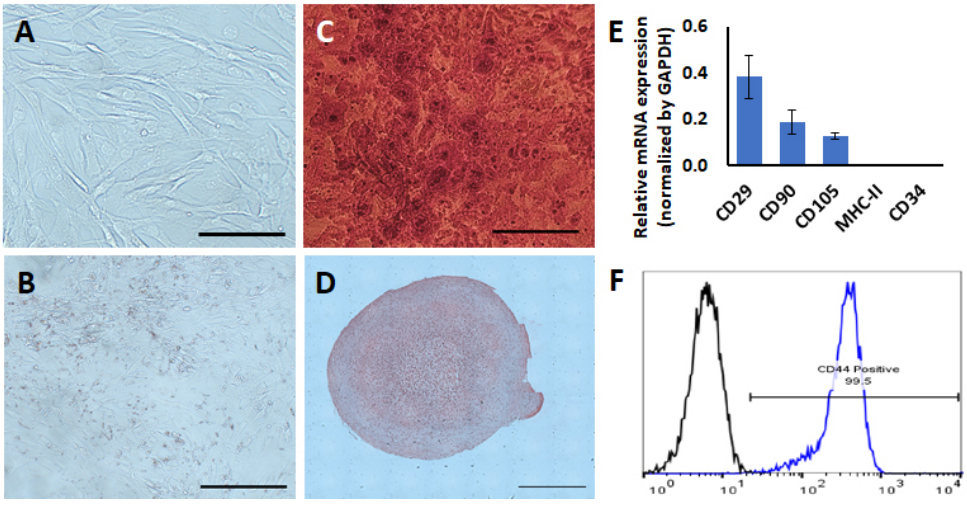

Figure 2. Identification and characterization of bovine fibrocartilage stem cells (FCSCs). (A) Morphology of FCSCs (scale bar = 100 µm). (B) Oil Red O for adipogenesis (scale bar = 200 µm). (C) Alizarin red for osteogenesis (scale bar = 200 µm). (D) Safranin-O for chondrogenesis (scale bar = 500 µm). (E) TaqMan™ PCR with putative stem cell positive markers (CD29, CD90, and CD105) and negative markers (MHC-2 and CD34) (n = 2). (F) Flow cytometry analysis with stem cell positive marker (CD44; blue) and negative control (black) (n = 3).

2. Isolation and Characterization of BMSC-Exos (Aim 1)

Ultracentrifugation (UC) is one of the most common methods and considered as a gold standard for exosome isolation, but the low exosome yield and the co-sedimentation of the exosome with various contamination including non-vesicular proteins and unknown aggregates are major issues that may need to be improved (PMID: 31373715 and 26044649). In this regard, we compared SEC and UC, and as shown in Figure 3, SEC provided a higher yield and purity of BMSC-Exos. These results indicate that SEC would be beneficial to obtain a sufficient amount and target concentration of BMSC-Exos for the downstream applications.

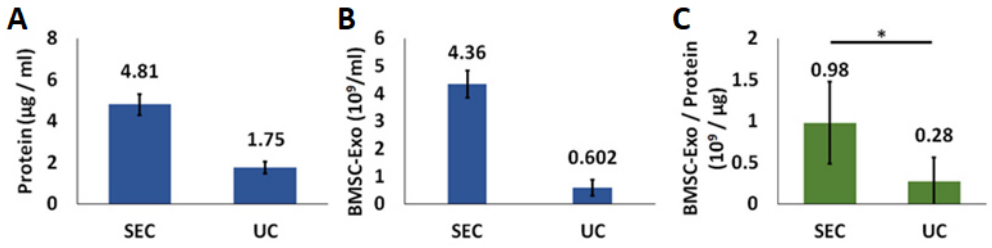

Figure 3. Comparison of the yield and purity of BMSC-Exo isolated by ultracentrifugation (UC) and size exclusion chromatography (SEC). (A) Total protein measured by BCA protein assay. (B) Numbers of exosome vesicles measured by nCS1. (C) The purity of BMSC-Exos obtained by total number of exosomes per total amount of proteins (n = 3, *p < 0.05).

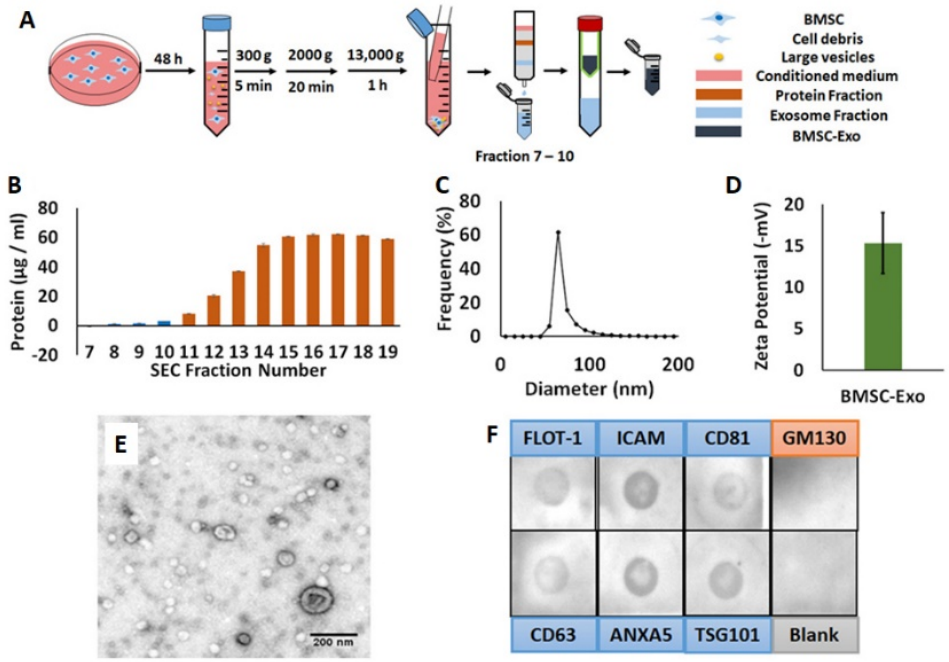

Figure 4. Isolation and characterization of BMSC-Exos. (A) Schematics of BMSC-Exo isolation using size exclusion chromatography (SEC). (B) The amount of proteins in SEC fractions (n=3). (C) Representative size distribution of BMSC-Exos evaluated by nCS1. (D) Membrane potential (surface charge) of BMSC-Exos measured by Zetasizer (n=3). (E) Morphology of BMSC-Exos visualized by TEM (scale bar = 200 nm). (F) Immunoblotting assay with purported exosomal positive markers (FLOT-1, ICAM, CD81, ANXA5, and TSG101) and a negative marker (GM130).

Following the 6 eluting fractions (void volume) from the SEC column, four exosome fractions (7^{th}-10^{th}) were collected to maximize the BMSC-Exo yield while minimizing the protein contamination (Figure 4A and 4B). In nCS1 and Zetasizer analyses, the distribution of bovine BMSC-Exos was ranged from 50 nm to 100 nm with -15 mV of zeta potential (Figure 4C and 4D). TEM images showed the similar size and cup-shape morphology of exosomes satisfying the criteria of the exosomes (Figure 4E). In the immunoblotting assay, BMSC-Exos were positive in exosome protein markers, flotillin-1 (FLOT-1), intercellular adhesion molecule (ICAM), CD81, CD63, annexin A5 (ANXA5), and tumor susceptibility gene 101 (TSG101), but negative in cis-Golgi matrix protein (GM130), which is a marker of cellular contamination (Figure 4F).

3. miRNA profiling using Next-Generation Sequencing (Aim 1)

A

Group	Sample	RNA Concentration (ng/ul)	RNA Quality (260/280)
1	BMSC-Exo_1	2.4	1.96
	BMSC-Exo_2	2.7	2.03
	BMSC-Exo_3	3.7	1.97
2-1	FCSC_1	118.5	1.99
	FCSC_2	332.5	1.99
	FCSC_3	464.4	2.01
2-2	FCSC_1 + BMSC-Exo_1	562.2	2.01
	FCSC_1 + BMSC-Exo_2	576.9	2.01
	FCSC_2 + BMSC-Exo_1	434.3	2.00
	FCSC_2 + BMSC-Exo_2	599.1	2.02

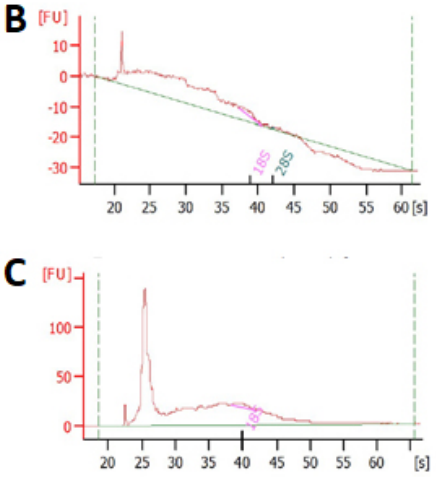

Figure 5. Preparation of exosomal RNA and FCSC RNA for Next-Generation Sequencing (NGS). (A) Concentration and quality of RNA measured by Lunatic spectrophotometer. Group 1: BMSC-Exos, Group 2: FCSCs without BMSC-Exo treatment, Group 3: FCSCs with BMSC-Exo treatment. (B and C) Representative RNA integrity and size distribution of BMSC-Exo (B) and FCSC (C) measured by Agilent 2100 bioanalyzer.

Although small RNAs of BMSC-Exos showed relatively low yield compared to that of FCSCs, RNA concentration satisfied the minimum input criteria for NEXTFLEX® Small RNA-Seq Kit (> 1 ng of total RNA in up to 10.5 µl Nuclease-free Water) (Figure 5A). The results from the Agilent 2100 bioanalyzer indicated that RNA samples were enriched with small RNA (20 - 40s) (Figure 5B). Library preparation and genome sequencing are in progress (estimate date: July 16^{th}). Obtained sequencing data will be analyzed using the Galaxy workflow platform. We will identify 10-fold changes of small RNA expression, especially miRNAs, when FCSCs were treated with BMSC-Exos, and the miRNA will be confirmed with the miRNA profile of BMSC-Exos.

4. Effects of BMSC-Exos on FCSC migration and proliferation (Aim 2)

The PKH-67-stained (green) BMSC-Exos were internalized into FCSCs and

accumulated over 90% in the cytoplasm surrounding the nucleus of FCSCs (Figure 6B and 6D). In contrast, there was no PKH-67 expression in the control (Figure 6A and 6C). The uptake ratio was dose-dependently increased (Figure 6E).

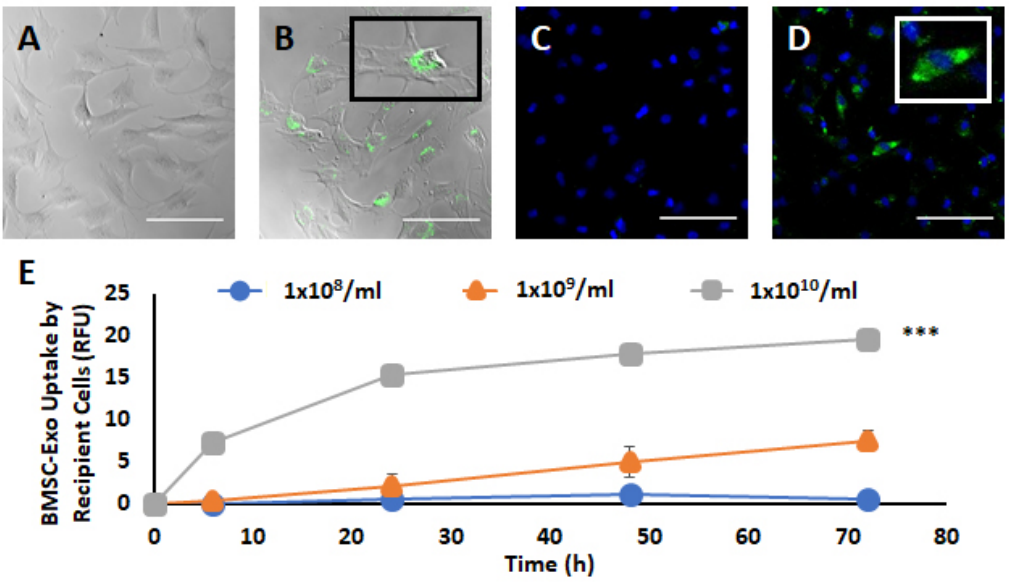

Figure 6. Uptake and internalization of BMSC-Exos in FCSCs. (A and C) FCSCs after co-incubation with PBS (untreated control) for 24 hours. (B and D) FCSCs after co-incubation with BMSC-Exos for 24 hours. (A and B) Brightfield images. (C and D) Fluorescence images. Green: BMSC-Exos labeled with PKH-67 dye. Blue: DAPI. Scale bar = 100 μm. (E) Quantified BMSC-Exo uptake ratio in FCSCs until 72 hours (n = 3, ***$p < 0.001$).

In the Transwell™ migration assay at 48 h, the number of migrated cells was significantly increased with approximately 5.5 times and 14.8 times in 1×10^9 and 1×10^{10} vesicles/ml of BMSC-Exos, respectively, compared with the control group (no BMSC-Exos treatment) (Figure 7B and 7C).

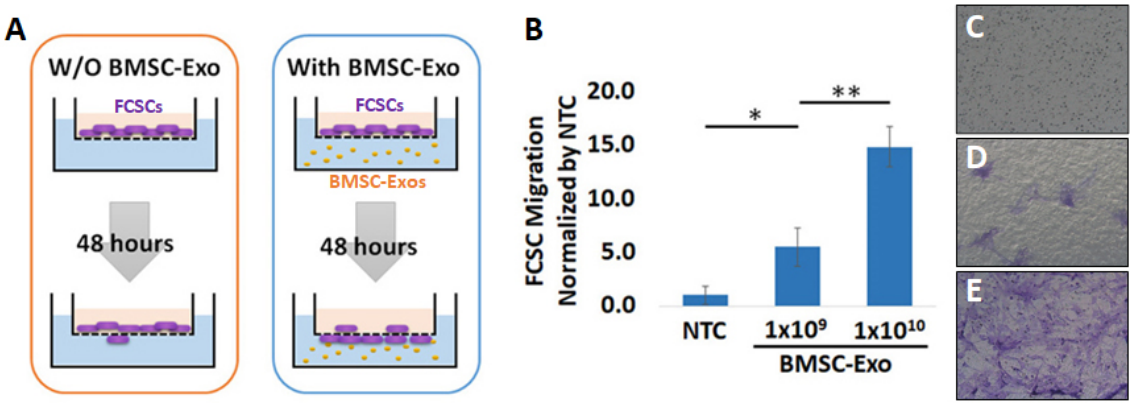

Figure 7. Effect of BMSC-Exos on FCSC migration. (A) Schematics of cell migration assay using a Transwell™ plate. (B) Quantified FCSC migration at 48 hours (n = 5, *$p < 0.05$ and **$p < 0.01$). NTC: non-treated control. The unit of BMSC-Exos: vesicles/ml. (C-E) Representative images of FCSC migration with Richardson's staining: (C) NTC, (D) $1x10^9$/ml BMSC-Exos, (E) (D) $1x10^{10}$/ml BMSC-Exos.

The result of cell proliferation assay reflected the increase of FCSC proliferation by BMSC-Exos in a dose dependent manner. The metabolic activity of FCSCs at 1×10^{10} vesicles/ml was enhanced as early as 3 days, and it was delayed to 7 days at 1×10^{9} vesicles/ml (Figure 8A). Moreover, three-times treatment of 1×10^{10} vesicles/ml BMSC-Exos enhanced FCSC proliferation up to 38% compared to one-time treatment of BMSC-Exos at day 7 ($p<0.05$) (Figure 8B).

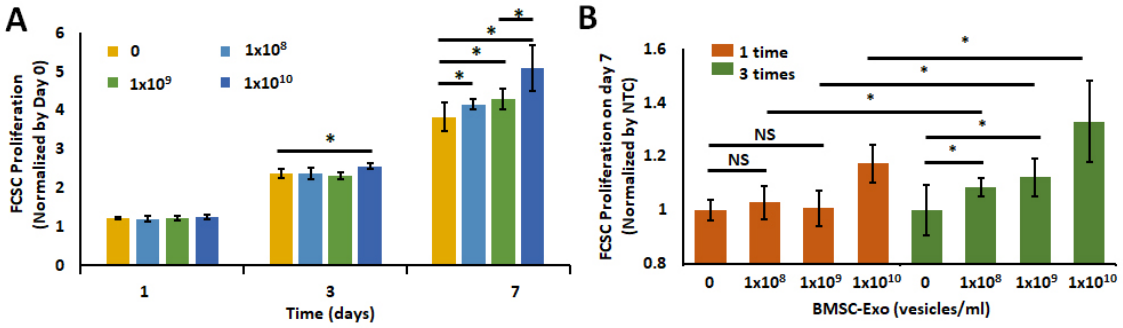

Figure 8. Effect of BMSC-Exos on FCSC proliferation. (A) Metabolic activity of FCSCs with BMSC-Exos at 1, 3, and 7 days. (B) Metabolic activity of FCSCs with one-time versus three-times treatment of BMSC-Exos at 7 days (n = 6, *p < 0.05). The unit of BMSC-Exos: vesicles/ml.

5. Effects of BMSC-Exos on fibrocartilage regeneration (Aim 2)

In comparison with other groups, BMSC-Exo induced a notable increase of PG aggregates in the FCSC pellet on day 21 (Figure 9C and 9D). Extracellular matrix (EMC) degradation resulted from PG depletion is associated with early-onset osteoarthritis. In this regard, enriched PG synthesis in FCSCs from BMSC-Exo treatment may alleviate the progression of diseases and restore the loss of the matrix. However, the amount of ECM in the BMSC-Exo group was relatively lower than the other two groups. Immunohistochemistry stains with type I and III collagens, aggrecan (ACAN), and SRY-Box Transcription Factor 9 (SOX9) are needed for further evaluation. Based on these results, we expect that BMSC-Exo treatment will show synergistic effects on PG synthesis when treated with putative growth factors (TGF-β1 and IGF-I) and finally accelerate fibrocartilage regeneration.

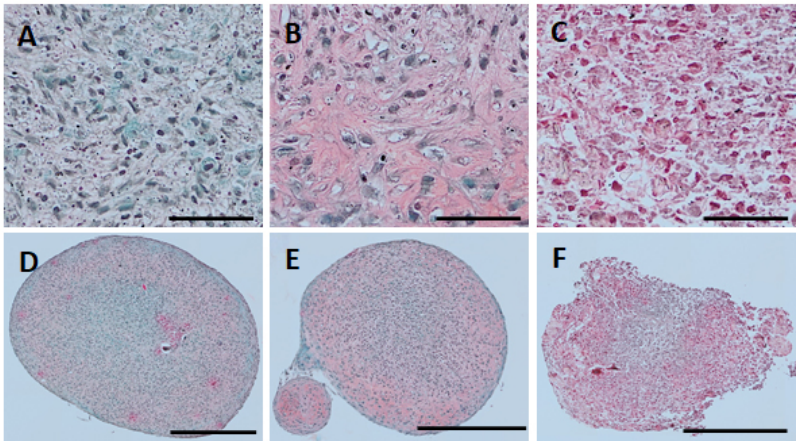

Figure 9. BMSC-Exo-mediated chondrogenic differentiation. Proteoglycan synthesis was observed at day 21 using Safranin-O/Fast-Green staining. (A and D) Non-treated control. (B and E) Positive control cultured with TGF-β1 and IGF-I. (C and F) BMSC-Exo ($1x10^{10}$ vesicles/ml). Scale bar: (A-C) 60 nm and (D-F) 400 nm. n = 3.

6. Statistical analysis

All data were expressed as the mean values with the standard deviation. Statistical significance was set at $p<0.05$ and analyzed by one-way ANOVA with the Tukey post-hoc test using SPSS Statistics software (Version 27; IBM, Armonk, NY).

7. Conclusion

Isolated bovine BMSCs and FCSCs were validated their stem cell characteristics by stem cell markers and multi-potent differentiation ability. BMSC-Exos were isolated using the SEC method which showed higher Exo yield compared to the UC method. The characterization of BMSC-Exos was confirmed by nCS1, Zeta potential, TEM, and immunoblot. The BMSC-Exos were internalized into FCSCs in a dose-dependent manner and enhanced FCSC migration, proliferation, and chondrogenic differentiation for TMJ fibrocartilage regeneration.

In order to evaluate the mechanism of Exo effects, miRNA profiling will be performed using NGS. Although we could not complete the profiling analysis, we expect to find key miRNAs for TMJ repair and regeneration. In the future, we will evaluate the effects of BMSC-Exos on *in vivo* fibrocartilage regeneration in a rabbit TMJ disc perforation model through our awarded NIH/NIDCR R03 grant.

Respond to the following questions:

1. Were the original, specific aims of the proposal realized? Yes

2. Were the results published?

We submitted a manuscript entitled, "Antioxidant and Chemotactic Effects of Subchondral Bone Mesenchymal Stem Cell-Derived Exosomes on Fibrocartilage Repair in Temporomandibular Joint," to Journal of Biomedical Science (JBMS). The manuscript was not accepted, as the journal expected the inclusion of *in vivo* animal results, which we did not plan to do. Therefore, we decided to submit to another one, the Journal of Orthopaedic Research (JOR) in the late summer of 2021. The support of AAOF will be acknowledged.

In addition, a provisional patent application has been filed by the University of Iowa Research Foundation (UIRF):

Inventors: Kyungsup Shin, Dong Rim Seol, James A. Martin, Ino Song
Title: Methods of preventative therapy for post-traumatic osteoarthritis
Provisional patent application No.: 63142451
Docket No.: 17023.249PV1
Submitted date: January 27, 2021

3. Have the results of this proposal been presented?

a. If so, list titles, author or co-authors of these presentation/s, year and locations

i) Ino Song, Dongrim Seol, James Martin, Eunmi Kim, **Kyungsup Shin**. Therapeutic Potential of Exosome in Post-Traumatic Osteoarthritis of the Temporomandibular Joint. Korean Society for Extracellular Vesicles (KSEV), Virtual conference, Jan 28-29, 2021 (The most excellent prize of oral & poster presentation).

ii) Ino Song, Dongrim Seol, James Martin, **Kyungsup Shin**. Bone Marrow Stem Cell-Derived Exosome: A Cell-Free Therapy for TMJ Repair. 2021 Departmental Senior Residents' Days. Department of Orthopedics and Rehabilitation, Carver College of Medicine University of Iowa, Iowa City, IA, Jun 18-19, 2021.

b. Was AAOF support acknowledged? Yes, all presentations and the manuscript include the AAOF support acknowledgement.

c. If not, are there plans to do so? If not, why not? We have acknowledged AAOF's supports on every presentation and manuscript. In addition, the support of AAOF will be acknowledged for a future conference and publication.

4. To what extent have you used, or how do you intend to use, AAOF funding to further your career? The outcomes from this AAOF-funded study were utilized to apply to federal funding programs (NIH and DoD) as a preliminary study. We awarded NIH NIDCR R03 grant (PI: Shin, Kyungsup: DE030166-01) entitled "A Novel Strategy of Endogenous Progenitor Cell Homing Exosomes for Condylar Fibrocartilage Repair in Temporomandibular Joint." This AAOF funding also allowed me to present our works at national and international meetings.

Accounting for Project; No leftover fund