Exosomes of Osteogenic Mesenchymal Stromal Cells Enhance Osteogenesis *in vitro*

Zijun Zhang[1], Sharada Paudel[2], Lew Schon[1]
[1]Mercy Medical Center, Baltimore, MD, [2]Frederick National Laboratory of Cancer Research, Frederick, MD
zzhang@mdmercy.com

Disclosures: Zijun Zhang (N), Sharada Paudel (N), Lew Schon (Zimmer Biomet)

INTRODUCTION: Exosomes are extracellular lipid-bilayer vesicles carrying signaling mRNA and proteins and conducting intercellular communication via cell penetration, invasion, and fusion events. The working hypothesis of this study was that osteogenic differentiated mesenchymal stromal cells (MSCs) produce exosomes with enhanced osteogenic induction signals.

METHODS: Bone marrow and subcutaneous fat tissue from 7 donors (average age 55, five female and 2 male) were used for isolation of bone marrow-derived MSCs (BM-MSCs) and adipose tissue-derived MSCs (AT-MSCs; approved by MedStar Health Institutional Review Board). BM-MSCs were isolated with a density-gradient method and AT-MSCs using an enzymatic digestion protocol established previously [1,2]. Both BM-MSCs and AT-MSCs were characterized by MSC cell markers and tri-lineage differentiation.

Osteogenic differentiation of BM-MSCs: When BM-MSCs reached 70% confluence, osteogenic differentiation medium was introduced. The osteogenic medium consisted of Dulbecco's modified Eagle medium, 10% fetal bovine serum (FBS), 10 mM β-glycerophosphate, 100 nM dexamethasone, 50 µg/ml L-ascorbic acid 2-phosphate, and 100 ng/ml human recombinant bone morphogenetic protein 2. BM-MSCs were cultured in osteogenic medium or regular medium, as control, for 10 days. Osteogenic differentiation was confirmed by positive Alizarin red staining of sampled flasks.

Isolation of exosomes: Prior to exosome isolation, osteogenic and non-osteogenic BM-MSCs were rinsed with phosphate-buffered saline (PBS) and continued culture with corresponding osteogenic or regular medium, containing exosome-free FBS, for two days. The medium of 3 T-75 flasks of the same cultures was combined and centrifuged at 2,600 ×g for 10 minutes to remove cellular debris, followed by centrifugation at 10,000 ×g for 30 minutes to remove microvesicles. To isolate exosomes, the medium was centrifuged at 100,000 ×g for 2 hours. The resultant pellet was resuspended and washed once in PBS, and re-pelleted by centrifugation at 100,000 ×g for 2 hours. Once isolated, exosomes were resuspended in PBS.

Exosome characterization: Exosomes were quantified by measuring protein content, using bicinchoninic acid assay. Western blot for CD63, heat shock protein 70 (HSP70), and flotillin-1 was performed for exosome validation. Samples of exosomes were also examined with transmission electron microscopy (TEM) for size distribution.

Inducing osteogenic differentiation with osteogenic exosomes: AT-MSCs were seeded in 24-well plates at a density of 1,000 cells per well. The following 4 types of media, containing 10% exosome-free FBS, were applied: 1) non-osteogenic regular medium; 2) osteogenic differentiation medium; 3) osteogenic differentiation medium with regular exosomes produced by non-osteogenic BM-MSCs (5ug/ml); 4) osteogenic differentiation medium with osteogenic exosomes produced by osteogenic BM-MSCs (5ug/ml). Each medium condition was set up in duplicate or triplicate. AT-MSCs were cultured for 3 weeks, and the medium was changed twice a week. Alizarin red staining was used to detect osteogenic differentiation. Alizarin red stain was extracted from cell culture with 10% acetic acid. The absorbances of the culture samples and Alizarin red standards were measured in a microplate reader at 405 nm. Amount of Alizarin red in each culture groups were analyzed with one-way ANOVA, followed by *post hoc* Tukey's test.

RESULTS SECTION: A total of 5 batches of BM-MSCs were used for this study. From 3 T-75 flasks of osteogenic or non-osteogenic BM-MSCs, 6-15x10^6 (average 10.6x10^6) cells were used for exosome isolation and produced 10.7-30.8µg (average 21.2µg) exosomes. All exosome samples were positive of CD63, SHP70 and flotllin-1 (Fig 1A). TEM showed that the size of the isolated exosomes was in a range of 17-52.6 (average 32.9) nm (Fig 1B).

After three weeks of culture, AT-MSCs in regular medium showed no sign of osteogenic differentiation (Fig 2A). There were sporadic spots of calcium deposition in the AT-MSCs cultured in osteogenic differentiation medium and the cultures with non-osteogenic exosomes. Osteogenic differentiation, as shown by Alizarin red staining, was most abundant when AT-MSCs were cultured with osteogenic differentiation medium and osteogenic exosomes. By colorimetric quantification, Alizarin red concentration was 12 mM in the AT-MSCs cultured with regular medium (Fig 2B). It increased to 95 mM when osteogenic differentiation medium was applied, and further increased to 125 mM with the use of osteogenic differentiation medium and non-osteogenic exosomes. The greatest Alizarin red concentration among all the study groups was 304 mM when the osteogenic differentiation medium supplemented with osteogenic exosomes ($p < 0.05$).

DISCUSSION: Exosomes isolated from osteogenic and non-osteogenic BM-MSCs did not differ in quality (assessed by TEM morphology) and quantity (by protein content). MSCs carry certain traits of their tissue origin, owing to either the impurity of cell population or influence from tissue environment. To examine the osteogenic induction capacity of osteogenic exosomes, this study used AT-MSCs, which don't differentiate into an osteogenic lineage in regular cultures. By visual observation, as well as colorimetric analysis, of Alizarin red staining, cultures of AT-MSCs demonstrated varied degrees of osteogenic differentiation within different osteogenic induction medium. While exosomes isolated from non-osteogenic BM-MSCs exerted enhanced osteogenic induction capacity, the osteogenically differentiated BM-MSCs-produced exosomes nearly tripled the amount of calcium deposition from the AT-MSCs cultured with non-osteogenic exosomes.

SIGNIFICANCE/CLINICAL RELEVANCE: Osteogenesis is fundamental for fracture and bone graft healing. This study further maximized the osteogenic induction capacity of BM-MSCs-produced exosomes and, therefore, is relevant to many challenging conditions facing orthopaedic surgery.

REFERENCES: 1. Paudel S et al. Regen Med. 2019;14(3):199-211. 2. Zhang Z et al. Anat Rec (Hoboken). 2021;304(7):1582-1591.

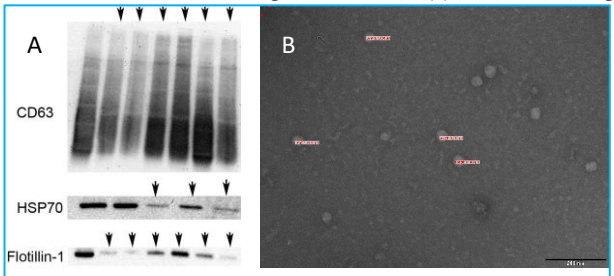

Fig 1. A: western blot. CD63, Heat Shock Protein 70 (HSP70) and Flotillin-1 are expressed in exosomes (lanes marked with arrows, unmarked lanes are cell lysate controls) isolated from BM-MSCs. B. BM-MSCs-produced exosomes imaged under TEM (bar = 200 nm).

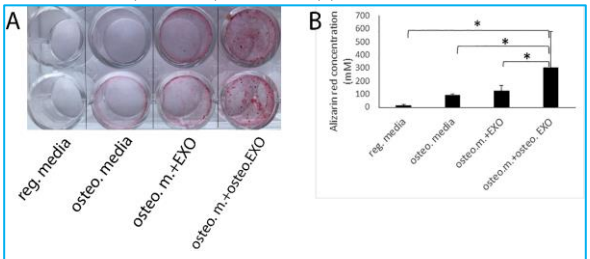

Fig 2. A: Alizarin red staining of AT-MSCs after cultured for 3 weeks in 1) regular medium; 2) osteogenic differentiation medium; 3) osteogenic differentiation medium with BM-MSCs produced exosomes; and 4) osteogenic differentiation medium with osteogenic differentiated BM-MSCs produced exosomes. B: Quantification of Alizarin red for mineral deposition in cultures.