Revitalizing Senescent Chondrocytes: The Protective Role of BMSCs-Apoptotic Bodies through the RAF/P38 Pathway

Ruiling Xu*[1,2,3,4], Pan Chen*[1], Junjie Wang[1], Weihong Zhu[1], Hang Lin[4], Junyong Wu[5], Zhihong Li[#1,2,3], Shiqi Xiang[#1,2,3]

[1]Department of Orthopaedics, The Second Xiangya Hospital, Central South University, Changsha, Hunan, 410011, China;
[2]Hunan Key Laboratory of Tumor Models and Individualized Medicine, The Second Xiangya Hospital of Central South University, Changsha, Hunan 410011, China;
[4]Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine
[5]Department of Pharmacy, The Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, China
Email of Presenting Author: xiangshiqi@csu.edu.cn

Disclosures: Ruilin Xu(N), Pan Chen(N), Junjie Wang(N), Weihong Zhu(N), Zhihong Li(N), Shiqi Xiang(N)

INTRODUCTION: Mesenchymal stromal cells (MSCs) are widely recognized for their regenerative capacity, making them a promising therapeutic option for modulating osteoarthritis (OA) progression. However, the underlying mechanisms remain incompletely understood. Previous studies have shown that MSCs can undergo rapid apoptosis following in vivo injection, and apoptotic exosomes of MSCs are thought to play a key role in several musculoskeletal diseases, however, the exact mechanism remains unexplored [(1)]. Furthermore, apoptotic bodies (ABs), which can be collected during the cell apoptosis process, have been suggested to influence cellular behaviors. In this context, several important questions arise: Can ABs impact the chondrocyte phenotype? Given that chondrocyte senescence is a key initiating factor in OA progression, how might MSC-derived ABs influence OA pathogenesis? Our study focuses on the potential of bone marrow-derived MSC (BMSC)-derived ABs to directly affect the phenotype of degenerative chondrocytes, thereby offering insights into their role in OA treatment.

METHODS: BMSCs were obtained from the femoral heads and the primary chondrocytes were collected from the knee joint as previously described (8 patients, 4 male, 4 female) [(2,3)]. ABs were isolated using sequential centrifugation according to previously established protocols [(4)]. 1) *Test for in-vivo implantation of BMSCs-pellets.* BMSCs were cultured in growth medium (GM) until they reached 80%-90% confluency, then centrifuged at 300g for 10 minutes to form a pellet, after 24h cultured, the cell pellets were implanted in SCID mice knee, N=5. At the 5th day, the cell pellets were collected for further analysis (TUNEL staining). 2) *Identification of ABs derived from BMSCs.* Twelve hours after STS-induced apoptosis, the culture supernatant was collected and centrifuged at 800 × g for 10 minutes to remove cellular debris. The supernatant was then subjected to centrifugation at 16,000 × g for 30 minutes to pellet the ABs, which were subsequently washed twice with filtered PBS. Protein concentration was determined using a BCA protein assay kit for quantification. A combination of TEM, SEM, NTA, Western blot (WB), confocal microscopy and nano flow cytometry was employed. Proteomics sequencing also was conducted for identify the representative markers for ABs.N=3. 3) *The assessment the functions of ABs.* The IL-1β-induced and the H2O2-induced chondrocyte senescence model were used to assess the reparative effect of ABs. Additionally, ABs were added to the chondrogenic process of BMSCs to evaluate their facilitative functions. 4) *The mechanism of ABs on rejuvenating senescent chondrocytes.* The bulk-RNA sequencing was conducted for explore the underlying mechanism of ABs on OA chondrocytes. The most relevant molecules of RAF/P38 signaling pathway were confirmed by WB. N=5.

RESULTS: As shown in **Fig.1**, after 5 days implantation in mice knee, the positive TUNEL staining grows. TEM and SEM imaging showed that ABs exhibited the typical cup-shaped or spherical morphology. NTA revealed a peak in the characteristic ABs size range of 100 - 400 nm, with a prominent peak at approximately 200 nm. Proteomics sequencing, nano flow cytometry and immunofluorescence staining confirmed that BMSC-derived ABs expressed the classical apoptotic markers. In **Fig2**, ABs (10ug/ml) could significantly increase the expression of chondrogenic makers. CCK8 and β-gal staining were performed showing the protective effect of ABs on H2O2-induced damage and chondrocyte senescence. Also, the ROS staining showed ABs could decrease the presence of reactive oxygen species. In **Fig. 3**, bulk RNA sequencing was performed to explore the underlying mechanisms. The results first highlighted the protective effect of ABs on IL-1β-induced chondrocyte inflammation. Among the enriched pathways, the Ras signaling pathway is regarded as potential downstream pathway. WB analysis further confirmed that ABs markedly upregulated RAF1 expression and phosphorylated P38 levels, while reducing AKT phosphorylation.

DISCUSSION: In this study, we observed the apoptotic process of BMSC pellets in vivo and found that BMSC-derived ABs are bioactive rather than passive byproducts. ABs enhanced chondrogenic marker expression, reduced oxidative stress, and alleviated IL-1β-induced inflammation in chondrocytes. Mechanistically, RNA sequencing and WB revealed that ABs activate the RAF/P38 pathway and inhibit AKT phosphorylation, suggesting a key role of Ras signaling in their protective effects. These findings indicate that ABs can rejuvenate senescent and inflammatory chondrocytes, offering a potential therapeutic strategy for cartilage degeneration.

SIGINFICANCE: This study demonstrates the therapeutic potential of BMSC-derived ABs in cartilage repair by rejuvenating chondrocytes, reducing oxidative stress, and modulating inflammation via the RAF/P38 pathway, offering a novel cell-free strategy for treating osteoarthritis.

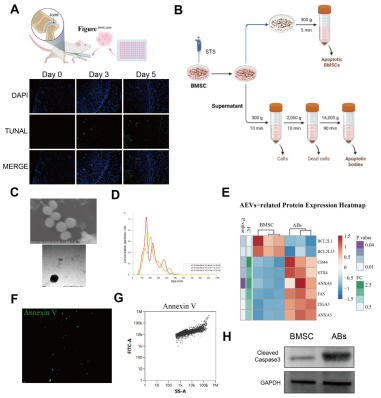

Figure 1. Isolation, characterization of BMSCs-derived ABs
(A) Tunel staining of the BMSC-pellets following 3, 5days implatation in mice knee.
(B) Schematic workflow illustrating the isolation and validation process of BMSCs-ABs.
(C) SEM and TEM images showing the spherical morphology and the characteristic cup-shaped structure of ABs.
(D) NTA revealing a round-shaped size distribution with a peak around 200 nm.
(E) Heatmap of proteomics sequencing showing the representative markers of ABs.
(F) Immunofluorescence staining further confirming Annexin V positivity in ABs. Scale bars as indicated.
(G) Nano flow cytometry analysis demonstrating Annexin V expression on the surface of ABs.
(H)Western blot analysis showing cleaved caspase-3 expression in ABs

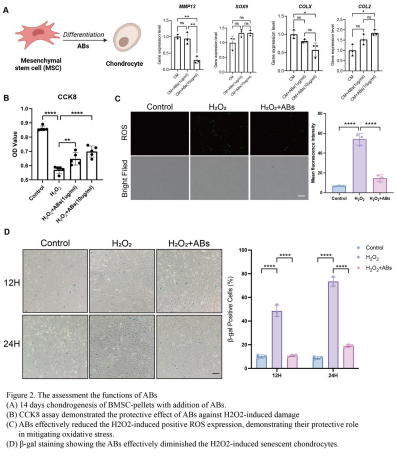

Figure 2. The assessment the functions of ABs
(A) 14 days chondrogenesis of BMSC-pellets with addition of ABs.
(B) CCK8 assay demonstrated the protective effect of ABs against H2O2-induced damage
(C) ABs effectively reduced the H2O2-induced positive ROS expression, demonstrating their protective role in mitigating oxidative stress.
(D) β-gal staining showing the ABs effectively diminished the H2O2-induced senescent chondrocytes.

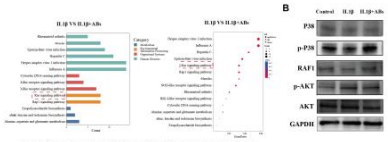

Figure 3. The mechanism of ABs on rejuvenating senescent chondrocytes
(A) RNA-sequencing data shows the RAS signaling pathway is highly relavant to the ABs intervation.
(B) WB result indicate ABs activate the RAF/P38 pathway and inhibit AKT phosphorylation.

REFERENCE: [1]Antonio Galleu et al. *Sci Transl Med,* 2017, [2]Shiqi Xiang et al. *Clin trans med*, 2022, [3]Xiurui Zhang et al. *FASEB J*, 2023,[4]Xiaoyu Miao et al. *J Transl Med*