Connexin 43 Mediates Mitochondrial Transfer from Human Mesenchymal Stromal Cells to Chondrocytes

Rebecca M. Irwin[1], Matthew A. Thomas[1], Megan J. Fahey[1], María D. Mayán[2], James W. Smyth[3], Michelle L. Delco[1]
[1]Cornell University, Ithaca, NY, [2]INIBIC, A Coruña, Spain, [3]Fralin Biomedical Research Institute at Virginia Tech Carilion, Roanoke, VA
rmi8@cornell.edu

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INTRODUCTION: Mesenchymal stromal cells (MSCs) have recently shown promise for treating degenerative diseases like osteoarthritis (OA), but the mechanisms remain unclear. Evidence suggests MSCs can rescue injured/stressed cells by donating healthy mitochondria,[1] highlighting a potential therapeutic strategy for treating OA. Our group recently documented MSC to chondrocyte intercellular mitochondrial transfer in cell culture and injured cartilage tissue,[2,3] but the mechanism of transfer is unknown. Connexin 43 (Cx43), a gap junction protein, is critical for mitochondrial transfer in non-orthopaedic, highly oxidative cell types, but has not been evaluated in chondrocytes. GJA1, the gene encoding Cx43, undergoes alternative translation to produce isoforms with both channel (43k isoform) and protein trafficking roles (20k isoform). Notably, Cx43 is strongly upregulated in OA.[4] Here, our goal was to investigate the role of Cx43 in MSC to chondrocyte mitochondrial transfer. We hypothesized that (1) MSC-chondrocyte mitochondrial transfer would occur through direct cell-cell contact, and (2) the magnitude of mitochondrial transfer would be dependent on Cx43 expression in MSCs, where MSC Cx43 knockdown would decrease mitochondrial transfer, and overexpression would increase transfer.

METHODS: *Human cell lines:* Human immortalized chondrocytes (T/C-28a2) and human MSCs (RoosterBio) were cultured using standard methods. GJA1 siRNA (ThermoFischer) was used to knockdown Cx43 expression in MSCs (Stealth RNAi was used as a negative control). Cx43 was overexpressed in MSC using GJA1 lentiviral transduction, as previously described (empty lentivirus backbone served as a control).[5] Transduced cells were selected with Blasticidin. Knock down and overexpression of Cx43 was confirmed using Western Blot. *Fluorescent Labeling:* Chondrocytes were transduced for mCherry cytoplasmic fluorescence and MSC lines were transduced for GFP mitochondrial fluorescence using lentiviral vectors (Vectalys). *Mitochondrial Stressor:* tert-Butyl hydroperoxide (t-BHP) was used to induce oxidative stress in the chondrocytes, which was assessed using JC-10, a mitochondrial polarity indicator. *Co-Cultures:* Chondrocytes were seeded on 12-well plates and cultured in OptiMEM +/- t-BHP (12 μM) for 24 hrs. Afterwards, MSCs were added in a 1:2 MSC:chondrocyte ratio and co-cultured for 24 hrs. Cells were either fixed in 4% PFA and stained for actin (Phalloidin) and Cx43 or analyzed using flow cytometry to quantify transfer. *Mitochondrial Transfer Quantification:* Mitochondrial transfer was quantified using flow cytometry, where single-color controls were used to create a quadrant gate, and events positive for both mCherry and GFP were considered transfer events. *Stats:* A one-way ANOVA was used to compare the effect of t-BHP on chondrocyte mitochondrial polarity (JC-10). T-tests were used to compare mitochondrial transfer between MSC populations.

RESULTS: After 24 hrs of co-culture, confocal imaging identified chondrocytes that took up MSC mitochondria, indicated by GFP fluorescence within the chondrocyte cell body (Fig 1A). Actin extensions protruding from MSCs contained mitochondria and were in direct contact with adjacent chondrocytes that had taken up MSC mitochondria (Fig 1B). Notably, MSC mitochondria within actin extensions co-localized with Cx43 immunostaining (Fig 1C). t-BHP induced mitochondrial dysfunction in a dose-dependent manner, where concentrations >12 μM had significantly higher mitochondrial depolarization compared to the unstimulated control ($p<0.05$, Fig 2A). In co-cultures, chondrocytes stressed with 12 μM t-BHP for 24 hrs had a significantly higher magnitude of mitochondrial transfer compared to unstimulated chondrocytes ($p<0.05$, Fig 2B). MSC Cx43 expression was associated with the magnitude of mitochondrial transfer to chondrocytes, as knocking down Cx43 significantly decreased transfer, while Cx43 overexpression significantly increased transfer ($p<0.05$, Fig 3).

DISCUSSION: This is the first study to quantify mitochondrial transfer from MSCs to chondrocytes in human cells and identify Cx43 as a key mediator of mitochondrial transfer. Cx43 is a multi-functional protein with roles in intercellular communication (43k full length protein) and is involved in protein and mitochondrial trafficking within cells (20k isoform).[6] Our finding that MSC Cx43 expression was associated with the rate of mitochondrial donation to chondrocytes suggests Cx43 is a critical mediator of mitochondrial transfer from MSCs to chondrocytes. Chondrocytes stressed with t-BHP had increased mitochondrial depolarization and transfer events, suggesting mitochondrial stress/damage may initiate transfer in cartilage, as described in other tissues.[1,3] This increase in mitochondrial transfer to stressed chondrocytes may involve the release of chondrocyte stress signaling molecules, such as damage associated molecular patterns (DAMPs), to recruit MSC rescue during co-culture.[7] We have previously shown that MSC-chondrocyte mitochondrial transfer can occur through extracellular vesicles,[2] and here we show evidence of transfer involving direct cell contact mediated by Cx43 (Fig 1BC). While the specific mechanisms are still under investigation, MSC mitochondrial donation requires mitochondria to be translocated within MSCs and between cells, which may be mediated by the truncated 20k isoform of Cx43 (GJA1-20k), as GJA1-20k has been shown to traffic proteins and mitochondria along the actin cytoskeleton.[6]

SIGNIFICANCE: This is the first study to identify Cx43 as a critical mediator of MSC-chondrocytes mitochondrial transfer. This understanding can be used to exploit the Cx43-mediated mechanism of mitochondrial transfer to engineer MSC-based regenerative therapies for treating cartilage defects and OA.

REFERENCES: [1] Yao+ *Stem Cell Rep* 2018 [2] Thomas+ *Front Bioeng Biotechnol* 2022 [3] Fahey+ *Sci Rep* 2022 [4] Mayan+ *Am J Pathol* 2013 [5] James+ *Mol Biol Cell* 2018 [6] Shimura+ *eLife* 2021 [7] Reigger & Brenner *In J Mol Sci* 2020

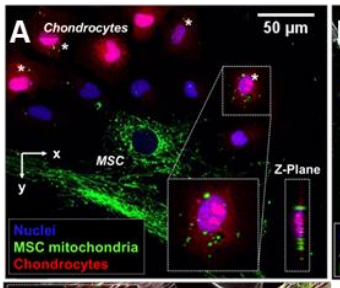

Figure 1: Evidence of MSC to chondrocyte mitochondrial transfer through direct cell contacts involving Cx43. (A) Chondrocytes (red, mCherry cytoplasm), have taken up MSC mitochondria (green). (B) MSC mitochondria travel along actin cellular extensions (grey) to adjacent chondrocytes (arrows), and (C) co-localize with Cx43 (red, arrows).
* Indicates transfer event

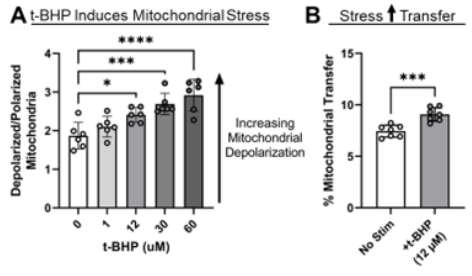

Figure 2: Chondrocyte Mitochondrial Stress Increases Transfer. (A) t-BHP induces mitochondrial dysfunction where t-BHP concentrations > 12 μM increase the relative number of depolarized mitochondria. (B) Chondrocytes +t-BHP prior to co-culture have increased magnitude of transfer ($p<0.001$).

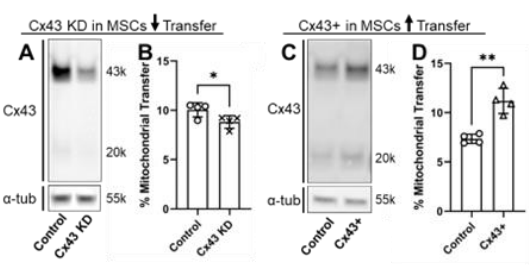

Figure 3: MSC Expression of Cx43 Regulates Magnitude of Mitochondrial Transfer. (A) siRNA decreased Cx43 expression in MSCs compared to control (Stealth RNAi). (B) Mitochondrial transfer was lower in co-cultures with Cx43 knock down MSCs ($p<0.05$). (C) Lentiviral transduction increased Cx43 expression in MSCs compared to control. (D) Mitochondrial transfer was higher in co-cultures with Cx43 overexpression MSCs ($p<0.01$).