Genotoxicity screening and miRNA profiling and of extracellular vesicles derived from human infrapatellar fat pad mesenchymal stem/stromal cells

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Introduction

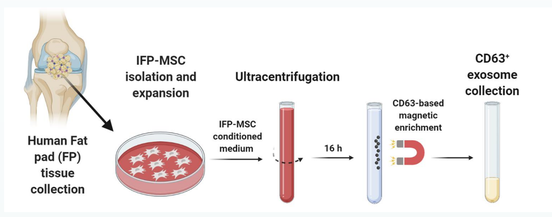
Mesenchymal stem/stromal cells (MSC) have garnered attention for their potential in regenerative medicine due to their immunomodulatory and anabolic properties[1]. Extracellular vesicles (EVs) serve as vehicles for cellular export products, including lipids, proteins, and RNAs (mRNAs and miRNAs), and can modulate the function of other cells at proximal or distal sites[2]. The immunomodulatory, angiogenic, and anabolic capabilities of MSC-EVs are well documented through cargo analysis and preclinical studies, supporting their use instead of MSC in clinical trials [3-9]. Recent studies have shown that EVs may enhance tissue regeneration and could therefore function as a cell-free alternative in injury healing. However, their cargo molecules have not been fully characterized, whereas the genetic toxicity of MSC-EVs remains unknown.
Our results showed that MSC-EVs from infrapatellar fat pad (IFP) tissue are not genotoxic and therefore do not activate a DNA damage response pathway in U2OS target cells. In parallel, IFP MSC-EVs possess distinct miRNA immunomodulatory profiles. Reactome analysis of miRNAs highly present in EVs showed their involvement in the regulation of six gene groups in target cells, particularly the immune system.

Objectives

- To study the genotoxic potential of IFP MSC-EVs by employing *in vitro* cell-based assays, using human osteosarcoma (U2OS) cells.
- To evaluate the role of the miRNAs involved in Infrapatellar Fat Pad-(IFP) Mesenchymal Stem Cell (MSC) derived Extracellular Vesicles (EVs).

Experimental design - Methodology

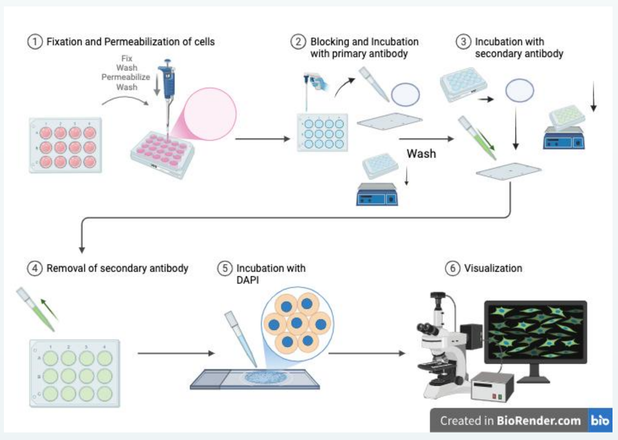
Human IFP-MSCs were extracted from non-arthritic patients (2 male and 2 female) undergoing elective knee arthroscopy and cultured in vitro up to passage 2 under standard conditions. IFP MSC-EVs were isolated from IFP-MSC conditioned medium by step-wise ultracentrifugation method and CD63-immunomagnetic purification. Quantity and size determination was performed by nanoparticle tracking analysis (NTA) (NanoSight NS300) and their presence was validated by CD9 expression in CD63+-gated particles by flow cytometry.



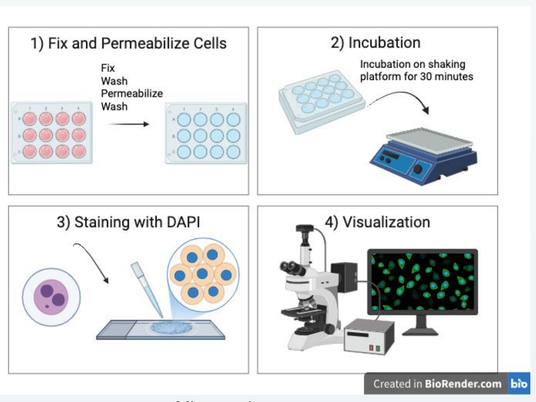
- Genotoxicity testing:

Human U2OS cells were used as a cellular model to assess the genotoxic effects of these EVs. Two cell-based assays were employed:

1. γH2AX immunofluorescence assay: cells were stained for a phosphorylated version of Histone 2AX (H2AX) at Ser139 (γH2AX), which serves as a marker of DNA breaks.
2. Micronucleus assay: cell nuclei were stained with DAPI to assay the presence of DNA fragments (micronuclei) that may come as a result of DNA damage.



γH2AX assay



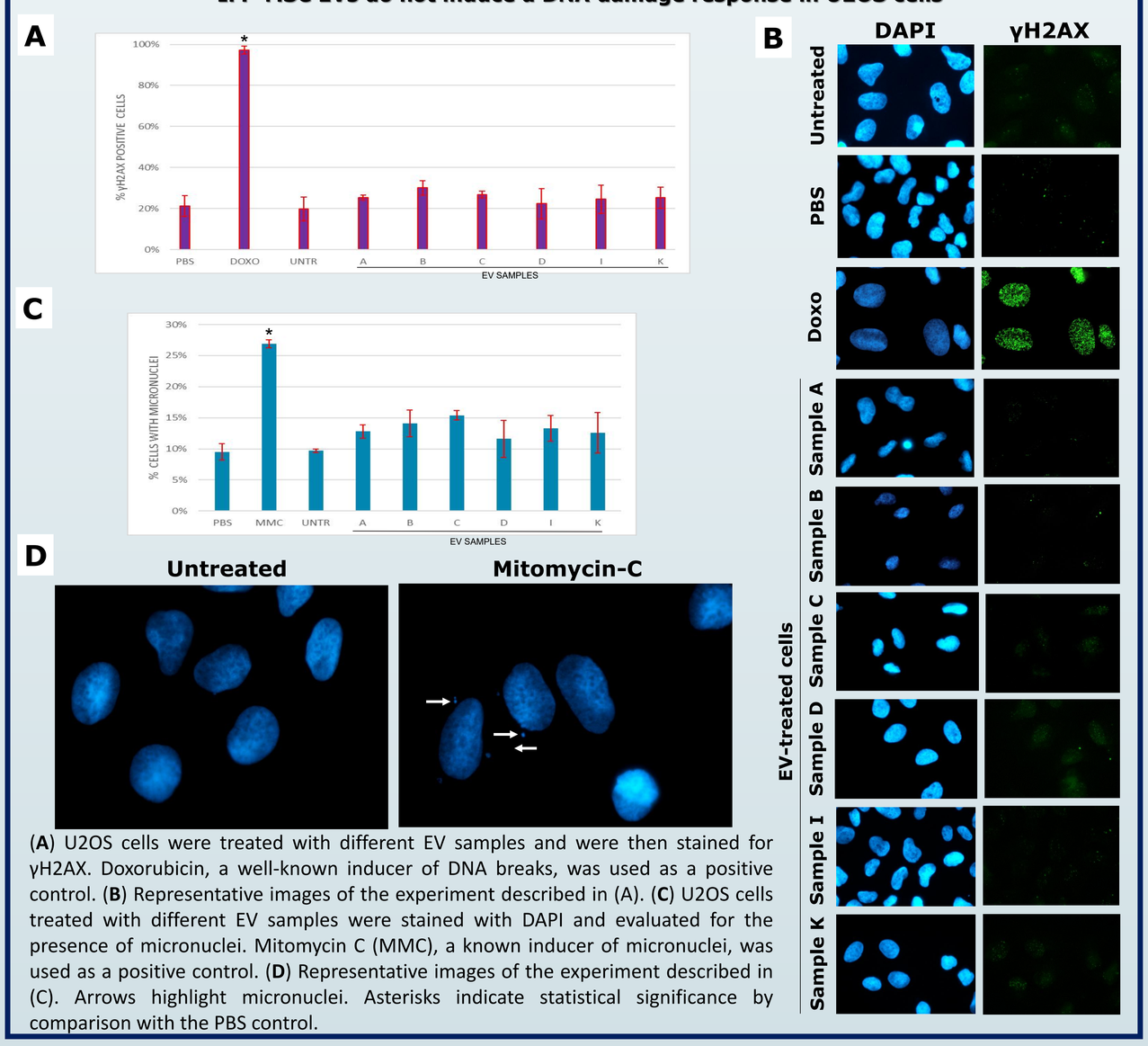
Micronucleus assay

- miRNA profiling:

We investigated human IFP MSC-EVs miRNA signature using human MSC exosome 166 miRNA qPCR array (GeneCopoeia). Growth factor- & inflammation-related exosomal cargo was assessed using multiplex protein arrays (RayBiotech). Putative miRNA interactomes were generated using a miRNet centric network visual analytics platform (https://www.mirnet.ca/). Values (with 34 cycles cut-off point) were represented in a topology miRNA-gene interactome network using force atlas layout and hypergeometric test algorithm. InfoMap algorithm was also used to identify miRNAs with higher node degree that act as hubs in a gene network.

Results

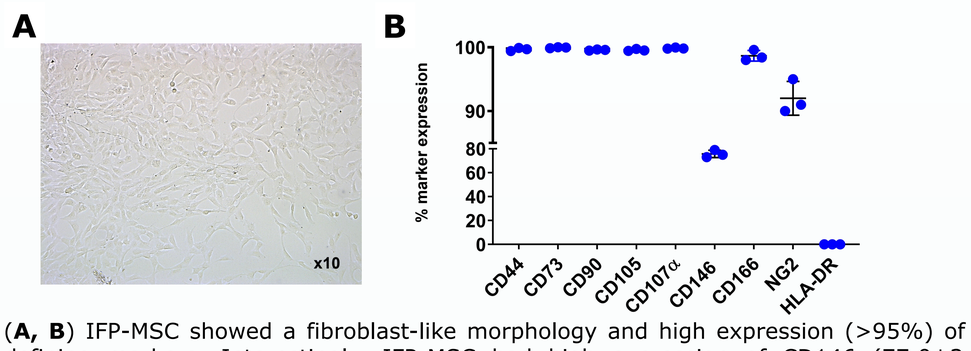
IFP-MSC EVs do not induce a DNA damage response in U2OS cells



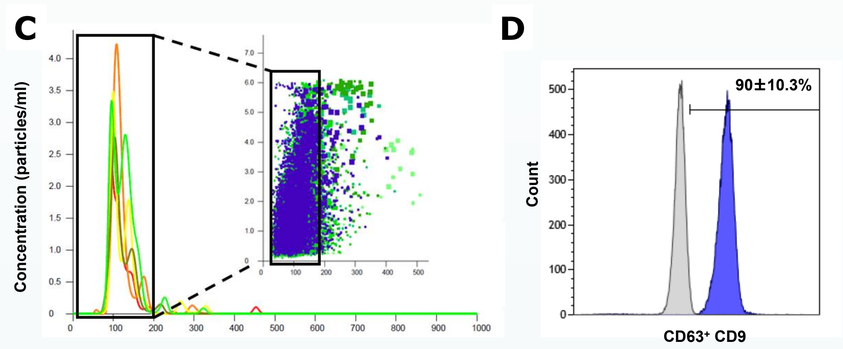
(**A**) U2OS cells were treated with different EV samples and were then stained for γH2AX. Doxorubicin, a well-known inducer of DNA breaks, was used as a positive control. (**B**) Representative images of the experiment described in (A). (**C**) U2OS cells treated with different EV samples were stained with DAPI and evaluated for the presence of micronuclei. Mitomycin C (MMC), a known inducer of micronuclei, was used as a positive control. (**D**) Representative images of the experiment described in (C). Arrows highlight micronuclei. Asterisks indicate statistical significance by comparison with the PBS control.

Results

IFP-MSC EVs show <200nm in diameter and showed high purity

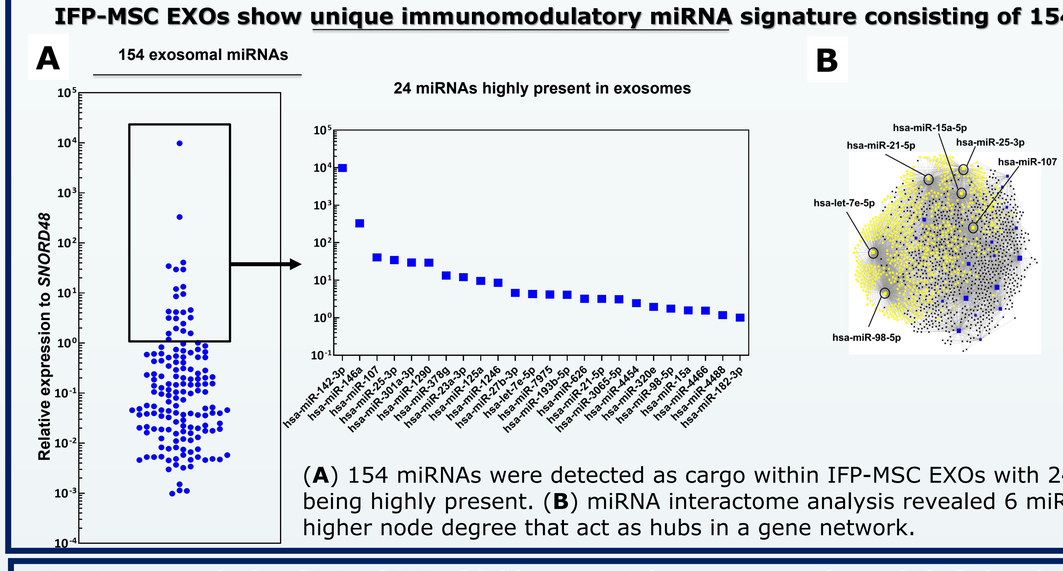


(**A, B**) IFP-MSC showed a fibroblast-like morphology and high expression (>95%) of common MSC-defining markers. Interestingly, IFP-MSC had high expression of CD146 (77.0±3.0%) and NG2 (92.5±2.7%) pericytic markers, whereas showed absence of human leukocyte antigen HLA-DR (n=3).



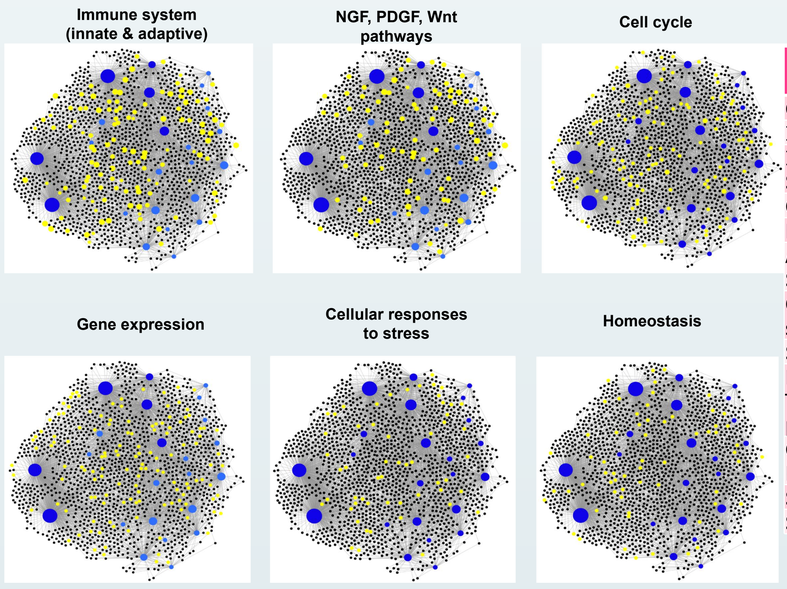
(**C**) Nanoparticle tracking analysis showed that isolated vesicles from IFP-MSC conditioned medium are <200nm diameter size, consistent with the known size of exosomes. (**D**) CD63⁺-selected exosomes showed purity by high positivity for exosome marker CD9.

IFP-MSC EXOs show unique immunomodulatory miRNA signature consisting of 154 miRNAs

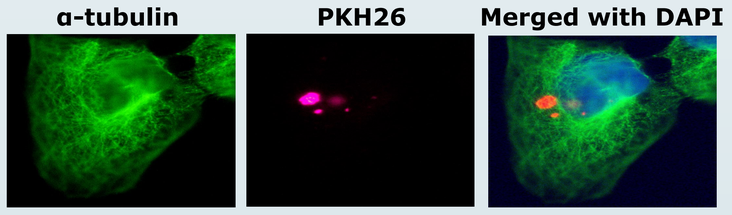


(**A**) 154 miRNAs were detected as cargo within IFP-MSC EXOs with 24 miRNAs being highly present. (**B**) miRNA interactome analysis revealed 6 miRNAs with higher node degree that act as hubs in a gene network.

Reactome analysis of 24 miRNAs highly present in exosomes showed their involvement in the regulation of six gene groups related to the: immune system, NGF/PDGF/Wnt pathways, cell cycle, gene expression, cellular responses to stress, and homeostasis.



Top Reactome hits	Number of Genes
Gene Expression	134
Immune System	123
Innate Immune System	79
Cell Cycle	67
Hemostasis	63
Adaptive Immune System	62
Cellular responses to stress	55
Signalling by NGF	52
NGF signalling via TRKA from the plasma membrane	41
Generic Transcription Pathway	41
Signaling by PDGF	40
Signaling by Wnt	40



Verification of EV cellular uptake. PKH-26 labeled IFP-MSC EVs (red) were added in U2OS cells, and 16 h later, cells were fixed and stained using an antibody against alpha-tubulin (green).

Conclusions

- IFP-MSC EVs show vigorous miRNA immunomodulatory profiles without inducing a genotoxic effect.
- Analysis of EV miRNAs suggest roles in immunity, as well as gene expression and the cell cycle.
- Based on the strong immunomodulatory impact of IFP-MSC EVs, we propose that these EVs may constitute a sustainable cell-free alternative to MSC-based therapeutics.
- Further studies are required to assess the long-term cellular impact of IFP-MSC EVs before clinical application.

References

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