on human umbilical vein endothelial cells (HUVEC, P4-P5) was assessed in: (i) migration assays (i.e. trans-well migration of HUVEC towards MSC-CM) and (ii) tube formation assays (i.e. *in vitro* Matrigel tube formation assay with MSC-CM and MSC:HUVEC co-cultures). ImageJ and Wimasis image systems were used for analysis. Angiogenic factors (i.e., Angiopoietin-2, EGF, Endoglin, FGF-1, Follistatin, HGF, IL-8, PIGF, VEGF-A, VEGF-C) in the MSC-CM were quantified by multiplex arrays.

Results In contrast to pediatric MSC-CM, adult MSC-CM are more effective in inducing HUVEC migration (adult vs pediatric MSC-CM percent increase compared to negative control; mean±SD: 199.1± 54.2 vs 80.81±27.6, $p<0.05$). Both adult and pediatric MSC-CM promote tube formation. Tube formation in Matrigel assays and in co-cultures was boosted by adult MSCs (adult vs pediatric MSC-CM percent increase in total length of tubes compared to negative control; Matrigel assays: 157.9±10.4 vs 136.7±15.8%, $p<0.05$; co-Cultures: 69211±7642 vs 27454±12190, $p<0.05$). The concentrations of pro-angiogenic factors in MSC-CM did not significantly differ between adult and pediatric MSCs except for an increase of PIGF in adult samples (21.07±16.4 vs 5.71±3.1 g/ml, $p<0.05$).

Conclusion Adult adipose derived human MSCs perform better than pediatric MSCs in two *in vitro* angiogenesis assays: HUVEC migration and tube formation. This could relate to age-associated differences in the MSC secretome composition.

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Modulation of fibroblast-to-myofibroblast differentiation and fibroblast migration: in vitro assessment of the anti-fibrotic effects of human adipose derived multipotent mesenchymal stromal cells

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Background & Aim Multipotent mesenchymal stromal cells (MSCs) are promising therapeutic options for a variety of diseases driven by inflammatory, vasculopathic and pro-fibrotic mechanisms. However, the anti-fibrotic effects of MSCs are not well characterized. To date, there are no recommendations on which *in vitro* assays should be used to test these effects. The development of anti-fibrotic readouts could inform the selection of MSC products to be used in studies of fibrotic diseases.

Methods, Results & Conclusion Methods: Human adipose derived MSCs at passage 4 to 6 from six adult (mean age±SD: 70.2±8.7 years) and seven pediatric (16.9±2.4 years) donors were evaluated. Resting and cytokine primed (IFNg+TNFa) MSC conditioned media (MSC-CM) were assessed in immunopotency assays (MSC-CM modulation of PHA stimulated-$CD4^+$ proliferation). The effect of MSC-CM on hTERT-immortalized human foreskin fibroblasts was tested in the following *in vitro* assays: (i) fibroblast to myofibroblast differentiation (immunostaining and Western blot for a-SMA and collagen 1); (ii) fibroblast migration (Incucyte scratch wound fibroblast cell migration); and (iii) fibroblast contraction (fibroblast-induced collagen gel contraction). Images were analyzed with ImageJ software.

Results Primed MSC-CM inhibit $CD4^+$ proliferation by 41.2% (resting vs primed, mean ± SD: 101.3±33.1 vs 58.8±23.1, n=10 per group, $p<0.01$). Resting and primed MSC-CM (n=3) decrease TGF-β1 induced fibroblast-to-myofibroblast differentiation as determined by a reduction in a-SMA levels (fold reduction of CM+ TGF-β1 to TGF-β1 treated fibroblasts: resting 4.2, primed 7.8) and type I collagen proteins (resting 2.6, primed 6.7). Resting and primed MSC-CM inhibit random fibroblast migration (relative wound density of non-conditioned vs resting MSC-CM vs primed MSC-CM at 36h, mean ± SD, 88.7 ±13.2 vs 55.3±15.7 vs 53.5±11.0, n=8). At 48h, resting and primed MSC-CM enhance collagen gel contraction by 11% both in the absence and presence of TGF-β1(n=6).

Conclusion *In vitro*, human adipose derived MSC-CM inhibit the fibroblast-to-myofibroblast switch following TGF-β1 treatment and the non-directional migration of fibroblasts. MSC-CM promote the contraction of the extracellular matrix of fibroblasts and myofibroblasts. Whether these *in vitro* assays can be used as surrogates of the *in vivo* anti-fibrotic effects of MSCs remains to be defined.

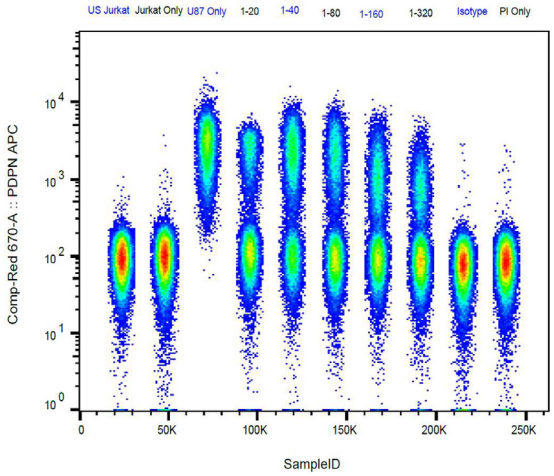

Figure 2. APC conjugated anti-PDPN antibodies were used to stain U78 (positive control) and Jurkat (negative control) cell lines. Titrations were performed at 1/20, 1/40, 1/80, 1/160 and 1/320 dilutions. Results were ran through a flow cytometer and analyzed in FlowJo.

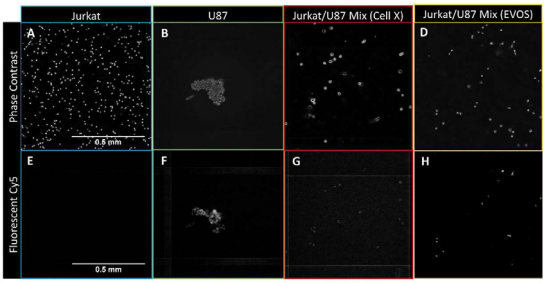

Figure 2. Jurkat only (A, E), U87 cell lines (B,F), and a mixed population were stained with APC conjugated anti-PDPN antibodies. Cells were plated then imaged using phase contrast and fluorescent channels using Cell X Technologies and an EVOS FL Auto 2 microscope to detect PDPN expressing cells.

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$CD146^+CD107a^{high}$ define a highly secretory and immunosuppressive Mesenchymal Stem/Stromal Cells subset with enhanced therapeutic effects reversing synovitis and fat pad fibrosis through M1/M2 phenotypic shift

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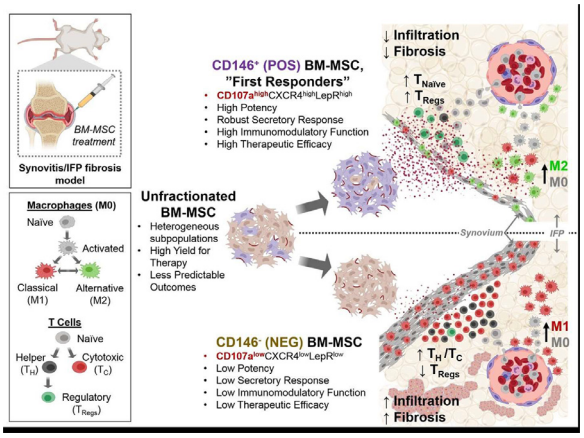
Background & Aim Mesenchymal stem/stromal cells (MSC) are highly immunomodulatory and promising therapeutic candidates for numerous clinical indications. Translational advances of cell-based

therapies require standardization, thus intrinsic heterogeneity of MSC may warrant concerns and yield inconsistent outcomes. Human bone marrow-derived MSC (BM-MSC) subpopulations have been discriminated based on their specific niche, however thorough functional analyses correlating potencies has yet to be determined.

Methods, Results & Conclusion Methods Herein, we investigated the comprehensive functionality of BM-MSC (n=8) discerned by perivascular marker CD146, inadvertently average in crude BM-MSC outcomes. Initially, inflammatory challenge (*i.e.,* priming) to crude BM-MSC, termed unfractionated (UNF), captured a baseline of signature responses including enhanced $CD146^{+}$ expression. So, UNF preparation and immunoselected subpopulations based on CD146 positivity ($CD146^{+}$ & $CD146^{-}$ cohorts) were assessed for phenotypic, transcriptional and secretome profiles, and *in vitro/in vivo* functional evaluations (immunopotency assay and reversing inflammatory and fibrotic events induced in the rat knee).

Results Both primed UNF and $CD146^{+}$ sorted cells enriched for a $CD107a^{high}$, $CXCR4^{high}$, and $LepR^{high}$ phenotype.Molecular evaluations evidenced $CD146^{+}$ cells (not the $CD146^{-}$ cohort) exhibiting a highly immunomodulatory transcriptome and secretome, with enhanced secretory capacity (determined by $CD107a^{High}$). This was functionally evident in robust immunosuppression of both stimulated PBMC and T lymphocytes while inducing significant frequencies of Regulatory T cells, providing evidence of $CD146^{+}CD107a^{high}$ BM-MSC as the distinctively potent responders to injury and inflammation. This was supported *in vivo* using a rat model of knee synovium and infrapatellar fat pad (IFP) inflammation and fibrosis, efficiently reversed following $CD146^{+}$ BM-MSC treatment (even at reduced cell doses – $1/10^{th}$) and largely failed by the $CD146^{-}$ counterpart. Mechanistically, $CD146^{+}$ cells greatly promoted a pivotal M1-to-M2 macrophage phenotypic shift within synovium and IFP further supporting local immunomodulatory effects.

Conclusion This study definitively supports that within crude BM-MSC, the $CD146^{+}CD107a^{high}$ subpopulation, deemed 'first responders' facilitate high therapeutic efficacy. By selection of the $CD146^{+}$ BM-MSC, our evidence implicates a highly translatable method that may reduce the need for high cell yields while improving outcomes.



Background & Aim Osteoarthritis (OA) is the most frequent joint disease worldwide with yet no known disease modifying drugs. In OA chondrocytes (OAC), the dysregulation of the metabolic biosensors that control mitochondrial biogenesis accounts for decreased numbers of mitochondria (MT) and reduced ATP levels in OA. Recently, our group evidenced that natural and artificial MT transfer (MitoT) from MSCs, convey potent metabolic and functional effects on target T cells, controlling organ damage and survival in an in-vivo model of inflammatory disease. In the present work, the effects of MitoT on the OAC phenotype is assessed in a coculture of MT-labelled MSCs with OAC.

Methods, Results & Conclusion MitoT was evidenced as early as 4hrs and reached 97% after 24 hours. Cytochalasin B inhibition pointed to a tunneling Nanotubes (TNT)-dependent mechanism. The impact of MitoT, isolated from other effects of the MSC secretome was assessed in OACs incubated solely with MSC derived MT. FACS analysis revealed the internalization of exogenous MT with dose-dependent uptakes of MT equivalent to MSC:OAC ratios of 1:10, 1:5 and 1:1, with averages of 20.1%, 29.4% and 91.7% $MitoT^{pos}$ target OACs, respectively. Functional effect of the MitoT was assessed in readouts including (a) immunosuppression assays, where $MitoT^{pos}$ OAC induced only a slight change in PBMCs proliferation; (b) metabolic activity measured by WST-1 demonstrating an increase in the metabolic activity of $MitoT^{pos}$ OACs; (c) ROS-regulating autophagy gene expression levels showing a significant increase in *SOD2* and *Redd1mRNA* levels in $MitoT^{pos}$ cells and (d) resistance to oxidative stress, assessed following H_2O_2 treatment, that revealed a protective effect of MitoT (15% reduction of dead cells). Furthermore, the intracellular fate of donated MT was evaluated with MT specific SNPs from the D-loop region. Using the amplification refractory mutation system (ARMS), a unique PCR product for MSC-derived MT was identified for at least 9 days in the target OACs. In conclusion, OAC were shown to be highly permissive for MT donation. The MitoT effect included an increase of metabolic activity and oxidative stress resistance that was correlated with an upregulation of *SOD-2* expression. The biodistribution and efficacy of intraarticular injection of MT are currently being investigated in an OA mouse model. ***The MitoT induced reversal of the metabolic dysfunction typical of OACs might open new avenues to the understanding and treatment of OA.***

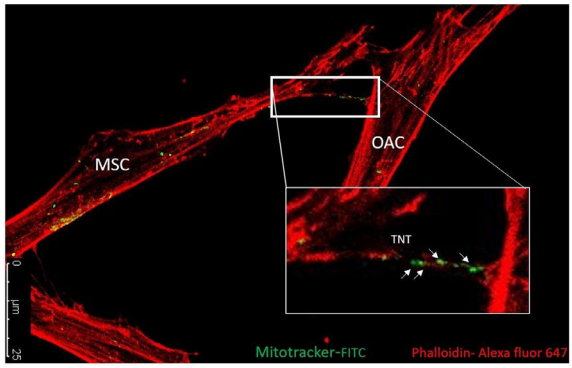


Figure 1 | Representative confocal microscopy image of mitochondria transfer (MitoT) from MSCs to osteoarthritic chondrocytes (OAC). Umbilical cord derived-MSCs were labelled with Mitotracker and cocultured with chondrocytes isolated from osteoarthritic patients. White arrows show mitochondria transiting form MSC to OAC through tunnelling nanotubes.

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Mitochondria Transfer Elicits Articular Cartilage Protection

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Protective Therapy with Mitochondria Isolated from Mesenchymal Stromal Cells in a Sepsis Model

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