Protection of MSC from room air oxygen during isolation accelerates cell growth

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Background

The role of mesenchymal stromal/stem cells (MSC) in the cancer microenvironment is of great interest, not only for the fundamental roles that MSC play in immunity, healing, and regenerative medicine/ organoids, but also as producers of exosomes. These extracellular vesicles, which can deliver cargo to other cells, are gaining interest as tumor-homing vehicles for therapeutic agents. However, the solid tumor environment is hypoxic, and even normal tissues reside at O2 levels far below that of room air, where MSC are grown for exosome production. Exposure to supraphysioxic room air changes MSC, modulating HIF-1a which is upstream of signaling pathways related to cell proliferation, differentiation, and cell death. We previously showed that eliminating exposure of human bone marrow MSC to room air during cell culture and handling can prevent HIF-1a modulation, improve cell division rates, and reduce cell senescence in later passages.

Findings & Conclusions

- Having never experienced room air, these MSC exhibited faster cell growth at passage 8 (mean doubling time 43.2 +/- 1.2 hrs, n = 3 separate cultures) than commercially sourced MSC that had been isolated, expanded, and cryopreserved under traditional laboratory conditions (mean doubling time 128 +/- 15.2 hrs, n = 2 separate cultures).
- We concluded that protecting MSC from room air exposure, even during isolation, can improve human bone marrow MSC growth kinetics for studies of the tumor microenvironment, inflammation, and tumor-homing exosomes.

Methods

- Except for commercially sourced MSC cell lines, all cell isolation and culture operations were performed under closed, controlled conditions in Xvivo Systems (BioSpherix). Centrifuges, microscopes, and other laboratory equipment were housed inside the modules.
- Commercial human bone marrow MSC (Lonza) were cultured at constant 5% or 18% O2 (room air incubator O2 control) plus 5% CO2 in T-flasks. Media were pre-equilibrated to the proper oxygen level.
- Cell counts were with the Denovix CellDrop or manual trypan blue count.
- Cells were isolated from human cadaver pelvic bones, sourced from a licensed organ procurement organization, inside the Xvivo System for unbroken control of the cell environment. The primary bone marrow cell isolate was subjected to discontinuous centrifugal gradient separation and mononuclear cells were selected for adherence to plastic.
- To confirm MSC identity, flow cytometric analysis was done with a Cytek Aurora and the following anti-human antibody panel (Dead cell-Zombie Aqua, CD38-PACBLUE, HLA-DR-BV785, CD45-FITC, CD14-PerCP-Cy5.5, CD73-PE-Cy7, CD105-APC, CD90-APC-Cy7(Biolegend), CD11b-BUV395 (BD), CD34-PE (Thermo)).
- For differentiation, cells were cultured for 2-4 weeks in specialized media (Thermo) to differentiate them into adipocytes, chondrocytes, and osteocytes. The cultures were stained with Oil Red O, Alcian Blue, and Alizarin Red S, respectively, and assessed visually (qualitatively) for cell staining.

Conflict of Interest Disclosure: All authors are employees of BioSpherix, LLC.

Experimental Set Up

Figure 1: Full-Time Environmental Control
The BioSpherix Xvivo System® is a HEPA-filtered, closed cell incubation and processing system for Cytocentric® control of temperature, oxygen, and CO2 levels. Its modular design encloses third-party equipment for full-time control of cell environments during all cell processing operations.

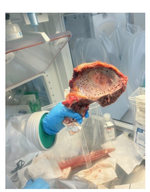

Figure 2: Isolation of Human Bone Marrow Cells Under Controlled Conditions
Human pelvic bones from two different donors, both under the age of 30, were sourced (fresh) from a licensed organ procurement organization (ConnectLife). They were transported to and imported into the Xvivo System in oxygen-proof heat-sealed mylar bags. Tiny holes were drilled into the bone interior and a Microx 4 needle oxygen probe (PreSens) was used to measure bone interior oxygen levels. More holes were drilled and the bone was perfused with DPBS (Sigma Aldrich) containing DNAse (Stemcell Technologies). The perfusate was collected in a sterile bag held open with a custom bag holder. The perfusate was subjected to discontinuous gradient centrifugation (Ficoll). Mononuclear cells were counted. Some were cryopreserved and some were placed into culture for plastic adherence selection.

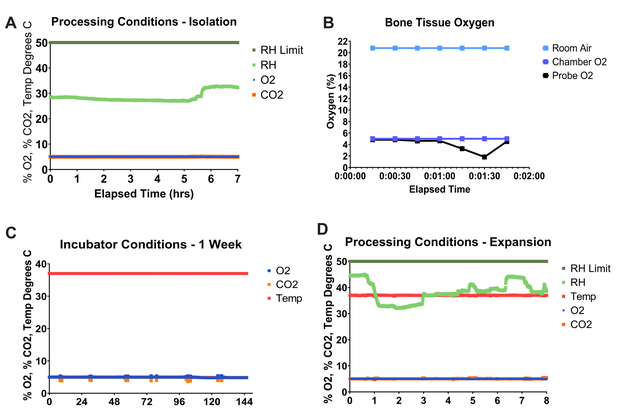

Figure 3: Stable Environmental Conditions Maintained During Handling of Human Bone Marrow-Derived MSCs Prevented Exposure of Cells to Room Air Oxygen
Utilizing the Xvivo System, conditions were controlled to physioxia for bone marrow, 5% O2 plus 5% CO2 in the processing chamber during all isolation steps including centrifugation (A). Bone marrow O2 was below 5%, confirming that room air oxygen had not permeated the bone before cell isolation (B). For cell expansion, the Xvivo System incubator (C) and processing chamber (D) was maintained at constant physioxia (typical week shown). Only changes in relative humidity (RH) were seen with incubator opening and liquid handling events.

Results

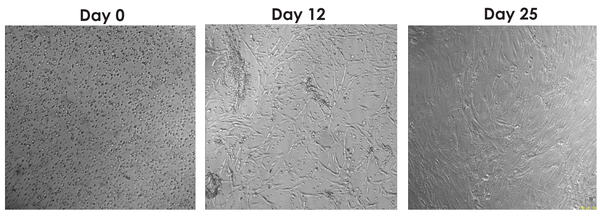

Figure 4: Cell Appearance
Bone marrow MNC were adhered and dividing well by Day 12 of culture. By Day 25, cultures were primarily MSC-like in morphological features.

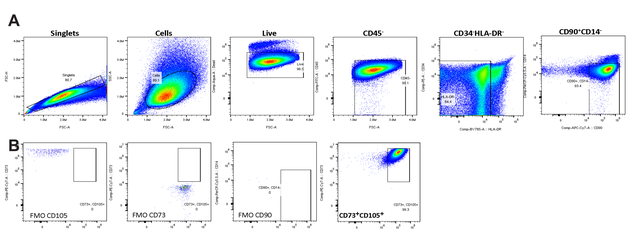

Figure 5: Adherent Bone Marrow Cell Phenotype Consistent with MSC
Flow cytometric analysis was done with a Cytek Aurora and the indicated gating pathway (A) using FMO controls (B). Representative results are shown. The cells were highly autofluorescent, but the bulk of the cells were confirmed as CD45-CD34-HLA-DR-CD14-CD90+CD105 + CD73 +, (average 79.9% +/- 9%) (n = 4, 2 separate cultures from 2 separate donors, 2 different passages each) consistent with an MSC phenotype.

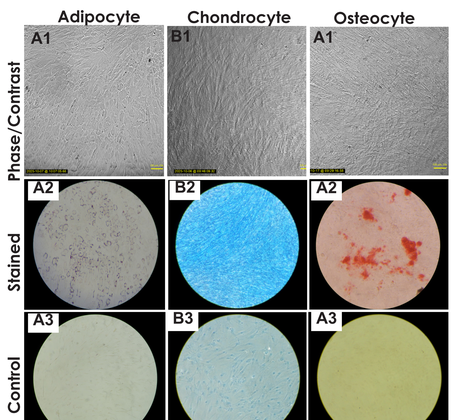

Figure 6: Differentiation of Plastic-Adherent Human Bone Marrow Mononuclear Cells
The MSC were cultured in media (StemPro) for 28 days to force differentiation into adipocyte, chondrocyte, and osteocyte phenotypes. Morphology (black/white photos, and staining with Oil Red O for fat (adipocytes), Alcian Blue for sulfated proteoglycan (chondrocytes), and Alizarin Red for calcium deposits (osteo-cytes), indicated successful differentiation of the MSC into characteristic mature cell types. Representative images from 2 separate experiments with 2 cultures (2 separate donors) are shown.

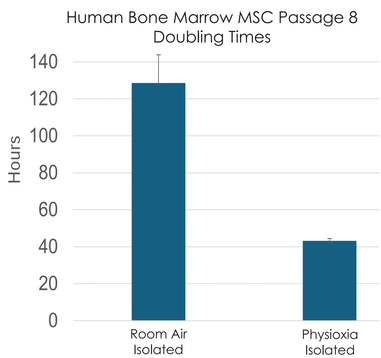

Figure 7: Better Cell Growth in Cells Isolated Under Physioxia
Cells isolated and expanded under unbroken physioxia (O2 5%) had lower doubling times than human bone marrow MSC from a commercial source (Lonza) isolated under traditional room air handling and incubation. After acquiring the cells (frozen) at Passage 2, the Room Air Isolated MSC were thawed and cultured at unbroken 5% O2/ 5% CO2. Even at Passage 8, the MSC isolated under controlled physioxia had lower doubling times (room air, n=3 separate cultures; physioxia, n = 3 separate cultures grown in 3 different cell culture media).

What does it mean?

Conclusions:

- **Human bone marrow MNC were isolated without exposing the cells to room air oxygen at any point.**
- **Plastic-adherent MNC expanded under constant physioxia had phenotype and differentiation potential characteristics consistent with bone marrow MSC.**
- **Human bone marrow MSC isolated and grown under constant physioxia had more robust growth than those isolated under traditional room air cell culture conditions (BSC and cell culture incubator), even under the same cell expansion conditions.**

Scientific Impact:

- **The artifact that is introduced by room air oxygen into MSC-based assays cultured under traditional conditions may be avoided by protecting cells from room air.**
- **Protecting MSC from supraphysiologic oxygen during cell isolation can help produce a more robust cell line.**

Future Directions:

Protecting tumor cells from room air O2 has been shown to change *in vitro* drug screening[2] and cell signalling networks[3]. This approach holds promise for protecting other cell types as well.

References

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