Cryopreserved hMSCs maintain comparable *in vitro* functional activity compared to fresh hMSCs

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Abstract

Human mesenchymal stem/stromal cells (hMSC) are critical components of tomorrow's cell-based product and devices. Secretion of biomolecules by hMSC influences many biological processes and is thought to be central to the mechanism of action. Since widespread clinical use of hMSC will be facilitated by frozen storage, cryopreserved hMSC must maintain high levels of biological function upon thaw. To address this critical issue we tested the impact of cryopreservation and thawing of human bone marrow MSC (hBM-MSC) on the cells' biological function: inducible upregulation of IDO by IFN-γ and angiogenic cytokine secretion ((VEGF, HGF, TIMP-1 and -2, FGF2, and IL-8). Based on previous reports, we hypothesized that:

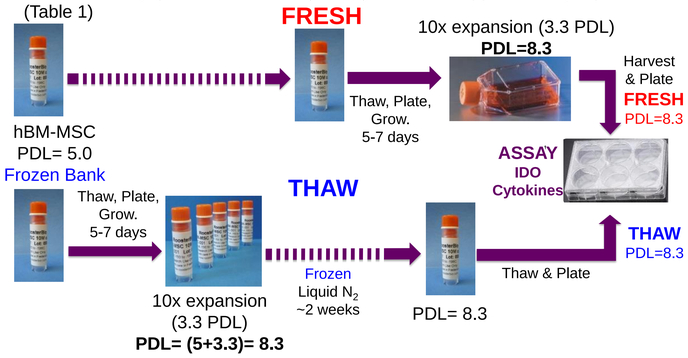
> ***Cryopreserved hBM-MSC would have diminished immunosuppression response and altered cytokine secretion immediately after thaw compared to hBM-MSC fresh from culture.***[1-3]

We compared the biological activity of hBM-MSC (RoosterBio) from 2 donors either (a) straight out of cryopreservation (THAW), or (b) cells that have been in culture for at least 5 days (FRESH) while controlling for population doubling (PDL). FRESH or THAW hBM-MSC were plated at 40,000 viable cells/cm^2, allowed 4hr attachment, and treated with vehicle or IFN-γ ± TNF-α for 24 hrs. Induction of IDO activity (immunosuppression) was assayed by measuring kynurenine in the media and cytokine secretion was measured via multiplexed ELISA (Quansys).

We report that IDO activity in both FRESH and THAW hBM-MSC was IFN-γ, and IFN-γ + TNF-α inducible and that the response was not statistically different between THAW and FRESH hBM-MSC over multiple experiments. The secreted cytokine profile was also comparable between conditions. These results are opposite of our hypothesis based on, some but not all, results in the current literature[1-4] and suggest that cryopreserved hBM-MSC produced using standardized production and cryopreservation processes and solutions can maintain *in vitro* immunological responsiveness and secreted cytokine activity immediately after thawing.

Experimental Design and Methods

Expansion and cryopreservation process keeps the PDL values of the FRESH and cryopreserved/thawed (THAW) hBM-MSC approximately equal (Table 1)



Cell growth, cryopreservation/thaw, and treatment

- FRESH Cell growth: Cells were plated at 3000/cm^2 grown in RoosterBio High Performance Media per protocol, harvested after 4-6 days, centrifuged and counted.
- THAW. Cryopreservation and thaw: hBM-MSC were resuspended in Cryostor CS5 (BioLife Solutions) at 1-3 x 10^6/ml and frozen in a CoolCell (Bioscision) to -80ºC before transfer to liquid N_2 for ~2 weeks. Thaw was done in water @ 37ºC until a small amount of ice was present. Media was added dropwise and the cells were centrifuged and resuspended in Basal Media + 2%FBS for counting.
- IFN-γ, TNF-α Treatment: FRESH or THAW cells were plated at 40,000 cells/cm^2 into 6 well plates in Basal Media + 2%FBS, allowed to attach for 4 hrs, and treated with vehicle or IFN-γ (10 ng/ml), IFN-γ/TNF-α (10 ng/ml ea.) for 24±1hr hrs. The cell supernatant was analyzed for IDO activity and cytokines.

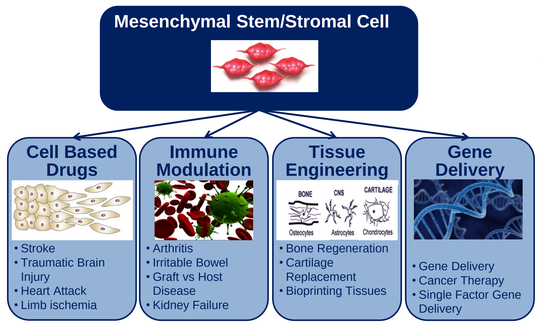
Immunomodulatory Function

- Indoleamine 2,3-dioxygenase, IDO: IFN-γ activates immunomodulatory enzyme IDO. IDO converts tryptophan → N-formylkynurenine (suppresses T-cell proliferation) and is measured by the levels of kynurenine in the culture media.
- Kynurenine (kyn): kyn concentration was measured using a spectrophotometric assay at 480nm after conversion of N-formyl-kyn to kyn and reaction with p-dimethylaminobenzaldehyde (DMBA). IDO activity is expressed as pg kyn/cell/day.

Angiogenic Cytokine Secretion

- Cytokines by ELISA: Supernatant was assayed for FGF, HGF, IL-8, TIMP-1, TIMP-2 and VEGF concentration using a MultiPlex ELISA (Quansys).

MSC are the workhorse of regenerative medicine



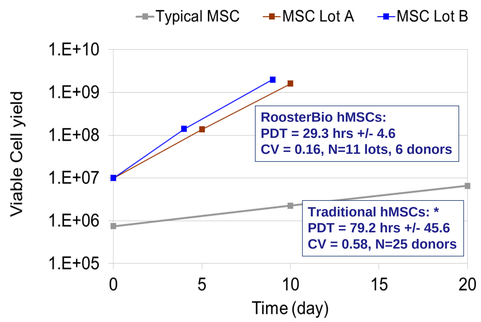
One Cell Type – Multiple Therapeutic Strategies

- 300+ clinical trials: SAFE
- Multilineage differentiation
- Immunomodulatory *in vivo*
- Function through "Secretome"

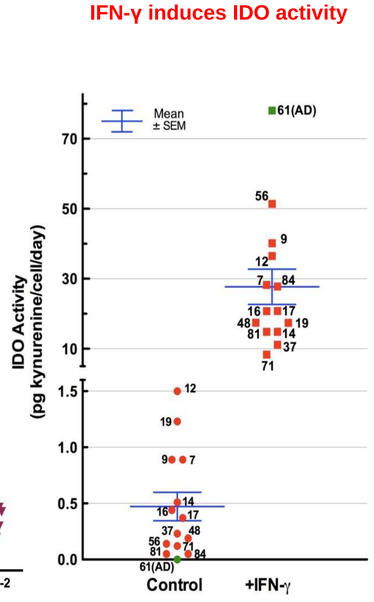
Figure adapted from: Viswanathan et al, Stem Cells & Dev.(2014)

RoosterBio hBM-MSC lots have consistently high growth rates, and a range of functional activity

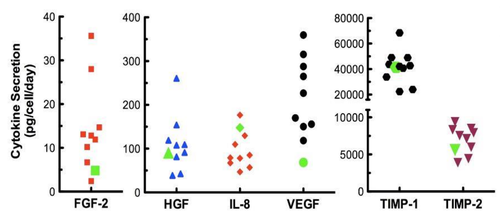
(A) Cell expansion profile



(B) Immunomodulation: IFN-γ induces IDO activity



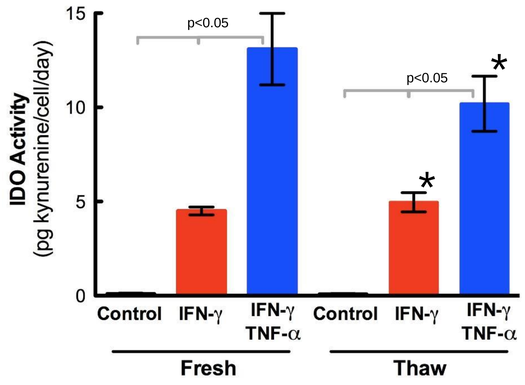
(C) Cell Potency: angiogenic cytokines



Four experiments were performed using two different cell donors

Exp.	hBM-MSC	Fresh PDL (viability)	Thawed PDL (viability)
1	BM11 (23 yo male)	9.1 (99%)	8.5 (97%)
2	BM11 (23 yo male)	7.3 (98%)	8.5 (91%)
3	BM5 (20 yo female)	11.2 (97%)	12.3 (92%)
4	BM5 (20 yo female)	11.1 (97%)	12.3 (87%)

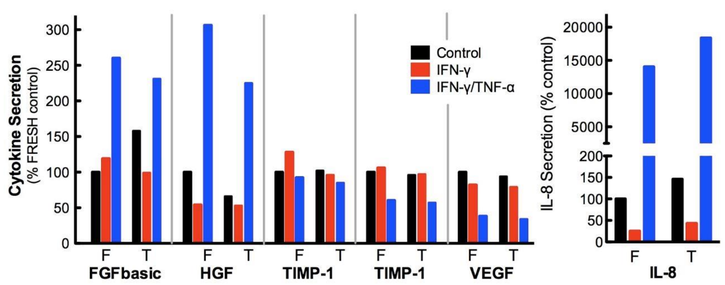
Cryopreservation Does Not Affect IDO Induction by IFN-γ ± TNF-α



Legend: Data are n=4, 2 donors (Table 1). Bars are average IDO activity +/- SEM, Significance determined via ANOVA, Bonferoni post test. Grey bars, significant difference p<0.05. *, not significant, Fresh vs Thaw for both IFN-γ and IFN-γ/TNF-α.

* not significant, Fresh vs Thaw

Cryopreservation Does Not Affect Angiogenic Cytokine Secretion Profile



Legend: Data are from experiment 2 (Table 1). For simplicity, cytokine secretion values are expressed as percentage of FRESH control (untreated, vehicle).

Conclusions

Hypothesis was false: THAW is comparable to FRESH MSCs

- IDO activity in both FRESH and THAW hBM-MSC was induced by IFN-γ ±TNF-α
- IDO induction was **NOT** statistically different between THAW and FRESH
- Basal Cytokine secretion was comparable
- Similar changes in cytokine secretion in response to IFN-γ ±TNF-α

References

1. Francois M, Copland IB, Yuan S, Romieu-Mourez R, Waller EK, & Galipeau J (2012) Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-gamma licensing. *Cytotherapy* 14(2):147-152.
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3. Moll G, Alm JJ, Davies LC, von Bahr L, Heldring N, Stenbeck-Funke L, Hamad OA, Hinsch R, Ignatowicz L, Locke M, Lonnies H, Lambris JD, Teramura Y, Nilsson-Ekdahl K, Nilsson B, & Le Blanc K (2014) Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties? *Stem cells*.
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