Enhancing Chemotaxis of Enucleated Cells by Genetic Engineering

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Abstract

Cell-based therapies have the potential to treat a wide range of diseases, but can be limited by problems such as safety, controllability, cost, consistency, and donor heterogeneity. Genetic engineering can overcome some of these issues, especially to improve cell homing to specific diseased sites. However, engineered cells must be rendered safe for *in vivo* administration in order to gain FDA approval for clinical use. There is a critical need for novel cell-based methods to deliver therapeutic payloads *in vivo* in a safe, controllable, and predictable manner. We designed a cell-based platform in which cells are engineered with key chemotaxis receptors and then made safe by removing the nucleus. These enucleated cells (cytoplasts) are a unique method of therapeutic delivery using a safe, cell-based entity. Our proof-of-concept studies demonstrate that hTERT-immortalized mesenchymal stem cells (MSC) can be genetically engineered with cell-surface receptors CXCR4 and CCR2, be successfully enucleated, and then migrate and home to chemokines *in vitro*. Cytoplasts express stable engineered receptor levels, comparable to that of a nucleated MSC, for 48 hours after enucleation. Additionally, the engineered receptors are functional after enucleation as demonstrated by cytoplast ability to migrate towards purified chemokine ligands (CCL2 and/or SDF-1α) in a dose dependent manner in Boyden chamber chemotaxis assays. Cytoplasts can also invade through Matrigel basement membrane and be cultured in 3D collagen matrices while maintaining normal MSC morphology and cytoskeleton structure, which suggests the potential of a cytoplast to home *in vivo*. Preliminary studies indicate the potential of a cytoplast to home *in vivo* to E0771 mouse tumors, which express high levels of CCL2 and SDF-1α and elicit cytoplast migration *in vitro*. Cytoplast engineering and migration *in vitro* and *in vivo* has important clinical applications for the ability to load and deliver therapeutic cargos to specific tissues during a defined therapeutic window.

Background

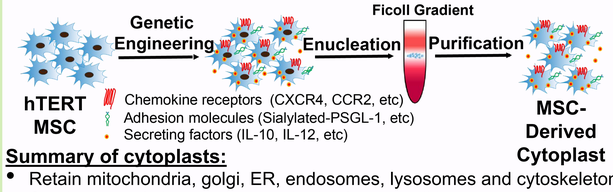
- Cells and cell-derived products are in high demand for use in clinical trials for treatment of various diseases.
- Many cell therapy clinical trials have not met desired clinical endpoints and need to be improved for precision and efficacy.
- Mesenchymal Stem Cells (MSCs) possess many important therapeutic properties that make them an ideal “off-the-shelf” allogeneic cell vehicle for therapeutic administration.
- Primary MSCs can lose expression of therapeutically important chemoreceptors in culture and senescence limits the ability to large-scale manufacture and extensively engineer these cells.
- hTERT-Immortalized MSCs can be extensively genetically engineered, expanded to a clinical scale, and bio-banked to create a homogeneous source of therapeutic product.
- However, extensive genetic manipulation is not safe for the clinic.
- We optimized a method to remove the nucleus and render the engineered cell entity safe and controllable for therapeutic use.

Current Cell Therapy	Ideal Cell Therapy
Donor dependent	Homogeneous source
Inconsistency from batch to batch	Consistent for large scale production
Limited engineering potential	Unlimited engineering potential
Uncontrollable engraftment and pharmacokinetics	Controllable and defined pharmacokinetics
Very expensive	Cost-effective
Safety concerns	Safe after administration

Our Novel Platform:

Genetically engineered MSCs remain functional and viable for 3 days after removal of the nucleus (enucleation), providing a unique and ideal therapeutic cell-based vehicle to treat human diseases (cytoplast).

Production of Cytoplasts

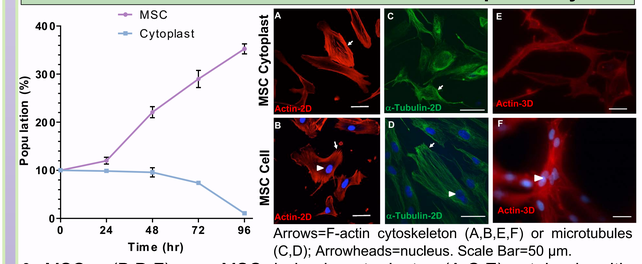


Summary of cytoplasts:

- Retain mitochondria, golgi, ER, endosomes, lysosomes and cytoskeleton.
- Survive up to 3 days under standard culture conditions.
- Survive cryopreservation and cryohibernation.
- Sense and migrate towards chemokine gradients *in vitro.*
- MSC-derived cytoplasts retain MSC surface markers: CD90+, CD44+, CD146+, CD166+, CD73+, CD105+, CD19-, and CD45-.
- Can be transfected with and translate exogenous mRNAs into functional intracellular or secreted proteins.

MSC-derived Cytoplast Capabilities

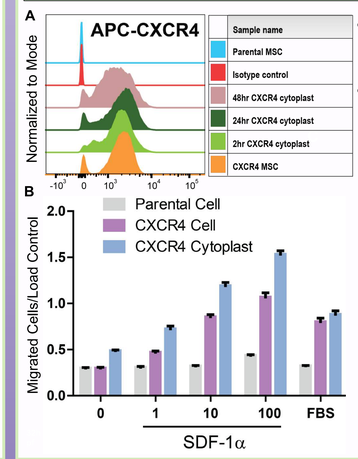
Figure 1: Cytoplasts exhibit similar cytoskeletal organization *in vitro* and are viable cell-like entities for up to 3 days.



Arrows=F-actin cytoskeleton (A,B,E,F) or microtubules (C,D); Arrowheads=nucleus. Scale Bar=50 μm.

- MSCs (B,D,F) or MSC-derived cytoplasts (A,C,E) stained with: rhodamine-phalloidin (F-actin cytoskeleton (A,B,E,F)), anti-α-Tubulin antibody (microtubule network (C,D)), and DAPI (nucleus (all panels)).
- Cytoplasts were cultured in 2D glass bottom chambers (A-D), or 3D collagen matrix (E,F) for 24 hours then imaged.

Figure 2: Engineered cytoplasts express functional CXCR4 chemokine receptors 48 hours post enucleation.

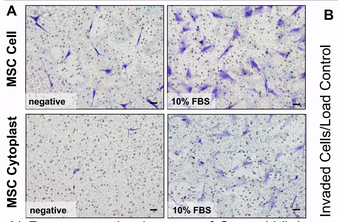


- MSCs lose CXCR4 receptor expression after *in vitro* culture expansion.
- MSCs were engineered with integrating lentiviruses to stably express the CXCR4 chemokine receptor and then enucleated.

A) Parental MSCs=non-engineered MSCs. CXCR4 expression was examined by Flow Cytometry at 2, 24, 48 hours post enucleation. Data was analyzed in FlowJo and normalized to mode.

B) CXCR4 engineered MSCs and cytoplasts migrated towards the indicated concentrations of SDF-1α (ng/ml) for 2 hours in Boyden chambers with 8μm pores. Mean ± SEM; n=10.

Figure 3: Cytoplasts can digest and invade through Basement Membrane Extract (BME).



A) Representative images of Crystal Violet-stained MSCs or cytoplasts that invaded through 8.0μm porous filters coated with BME to the underside of the membrane in 24 hours. Scale Bar=50 μm.

B) Bar graph represents the ratio of invaded cells or cytoplasts out of the total attached cells or cytoplasts as treated in (A). Mean ± SEM; n=18.

Figure 4: Cytoplasts can be engineered with multiple functional homing receptors simultaneously.

- MSCs lose key homing receptors (CXCR4 and CCR2) in culture and do not express endothelial adhesion molecules like PSGL-1 to mediate cell adhesion to inflamed blood vessel lumina.
- MSCs were engineered with integrating lentiviruses to stably express the following: CXCR4, CCR2, PSGL-1 (Triple MSC).
- Mouse E0771 breast cancer tumors have inflamed endothelia and produce substantial amounts of CCL2, the ligand of CCR2.

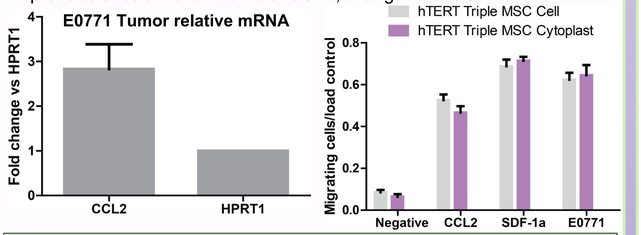
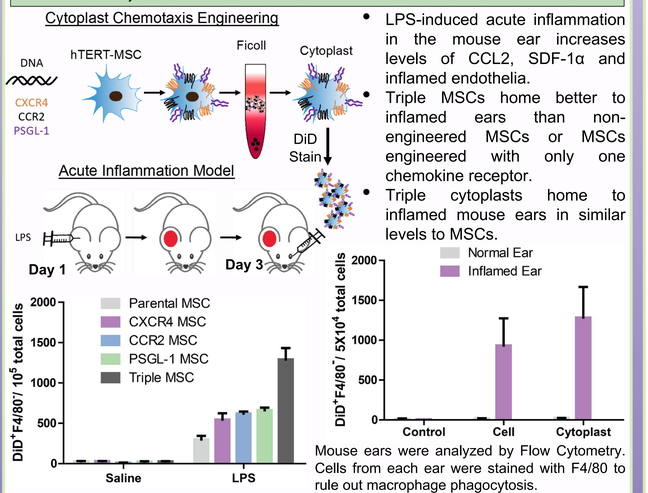


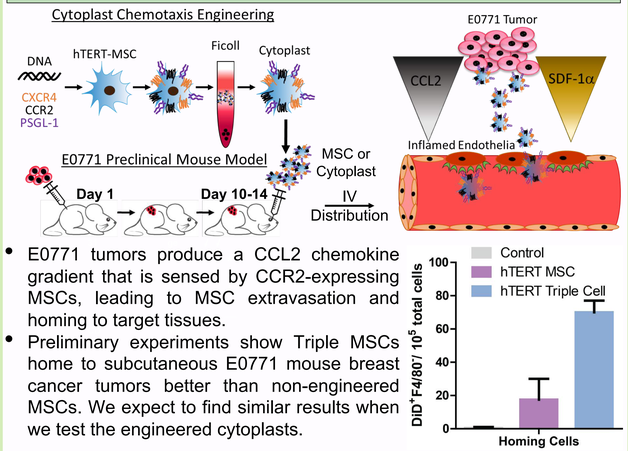
Figure 5: Cytoplasts engineered to stably express CCR2, CXCR4, and PSGL-1 home to inflamed tissue *in vivo.*



- LPS-induced acute inflammation in the mouse ear increases levels of CCL2, SDF-1α and inflamed endothelia.
- Triple MSCs home better to inflamed ears than non-engineered MSCs or MSCs engineered with only one chemokine receptor.
- Triple cytoplasts home to inflamed mouse ears in similar levels to MSCs.

Mouse ears were analyzed by Flow Cytometry. Cells from each ear were stained with F4/80 to rule out macrophage phagocytosis.

Figure 6: Triple engineered MSCs home to E0771 subcutaneous breast cancer mouse tumors.



- E0771 tumors produce a CCL2 chemokine gradient that is sensed by CCR2-expressing MSCs, leading to MSC extravasation and homing to target tissues.
- Preliminary experiments show Triple MSCs home to subcutaneous E0771 mouse breast cancer tumors better than non-engineered MSCs. We expect to find similar results when we test the engineered cytoplasts.

Conclusions

- Cytoplasts actively home towards inflammatory chemokines and growth factors commonly released by damaged tissues, including tumors, *in vitro* and *in vivo* at similar levels compared to MSCs.
- Currently, extensive engineering of primary MSCs is not feasible, cost effective, safe, or reproducible for cell therapy clinical trials due to concerns of oncogenesis or life-threatening conditions.
- The cytoplasts platform allows for extensive and multi-layered engineering, while maintaining controllability and clinically relevant safety of the cell entity.

Therapeutic Benefits of the Cytoplast Platform

- Cytoplasts can be generated from any cell type: iPSC, immortalized cells, immune cells, cancer cells, or genetically engineered cells.
- Cytoplasts are a robust cell-vehicle capable of carrying or producing significant amounts of a wide range of clinically-relevant therapeutic products: cytokines, oncolytic viruses, peptides, small therapeutic RNAs, synthetic drugs, plasmids, and gene therapy constructs.
- Cytoplast production is cost-effective and scalable for the clinic. We can generate tens of millions of cytoplasts per centrifugation, which can be cryopreserved and biobanked.

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