Directed Differentiation of Dopaminergic Neurons from Human Induced Pluripotent Stem Cells through Microfluidic Cell Squeeze® Delivery of Multiple mRNA Encoding Transcription Factors

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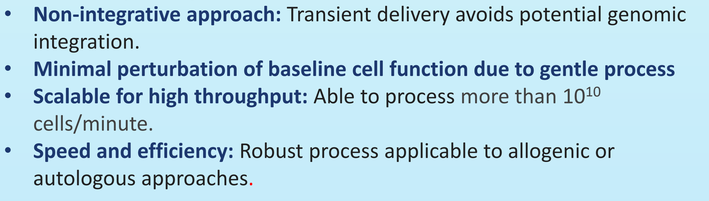
Background

Generating therapeutic cells to replace lost or diseased cells is a promising approach to treat currently intractable diseases such as Parkinson's disease. One attractive cell source for cell replacement therapies is induced pluripotent stem cells (iPSCs) since they can differentiate into a wide variety of somatic cells. However, the differentiation process through the sequential activation of key signaling pathways with small molecules is a lengthy and variable process. More recently, the forced expression of a key set of lineage-specifying transcription factors enables cell differentiation with higher efficiency, homogeneity, and speed but typically requires the use of viral or integrating vectors, which may pose safety concerns for clinical use. Cell Squeeze® technology enables non-viral, cytosolic delivery of a variety of materials while preserving cell health and limiting adverse effects on baseline gene expression. Furthermore, in preclinical studies, our technology allowed us to control the timing, intensity, and combination of transcription factor expression to create high-quality and functional cell products. In a new application of the Cell Squeeze® technology, we demonstrated the generation of dopaminergic neurons by simultaneously delivering six mRNAs encoding transcription factors into iPSCs with a single step.

Applying SQZ® Technology to Regenerative Medicine

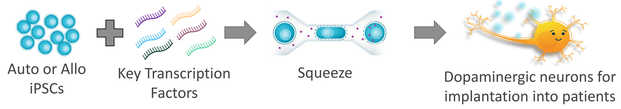
Cytosolic Delivery with Cell Squeeze® Technology[1]



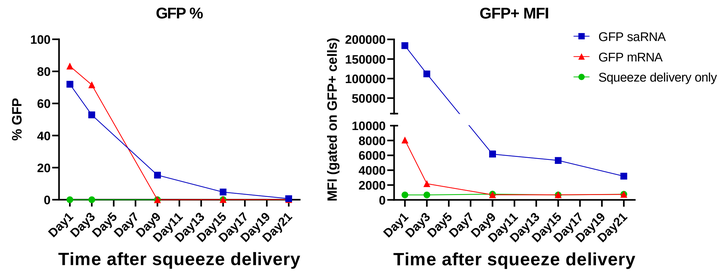
Advantages of Cell Squeeze® Technology for Directed Differentiation

- **Flexibility of cargo delivery:** Ability to control the delivery magnitude and timing of single or multiple transcription factors.
- **Non-integrative approach:** Transient delivery avoids potential genomic integration.
- **Minimal perturbation of baseline cell function due to gentle process**
- **Scalable for high throughput:** Able to process more than 10^{10} cells/minute.
- **Speed and efficiency:** Robust process applicable to allogenic or autologous approaches.

Therapeutic Vision for Cell Replacement Therapy in Parkinson's Disease

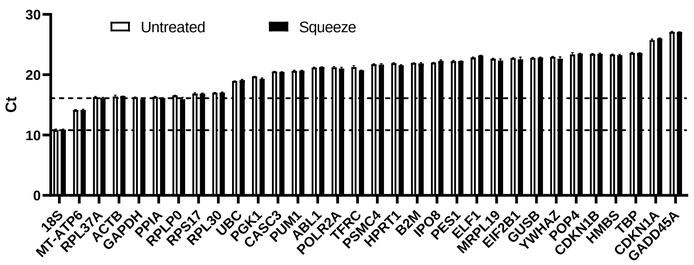


GFP saRNA Expressed at a Higher Intensity and for a Longer Period of Time vs. GFP mRNA in iPSCs

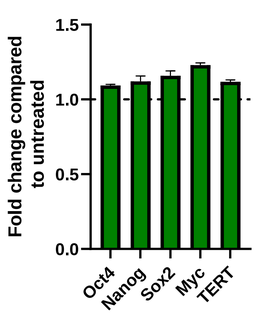


GFP self-amplifying RNA (saRNA) delivery drives a higher and longer GFP expression compared to GFP mRNA. GFP saRNA delivered iPSCs had ~20 fold higher MFI (gated on GFP+ cells) compared to GFP mRNA delivered iPSCs indicating the amplifying effect of saRNA. GFP signal was assessed by flow cytometry at day 1, 3, 9, 15 and 21.

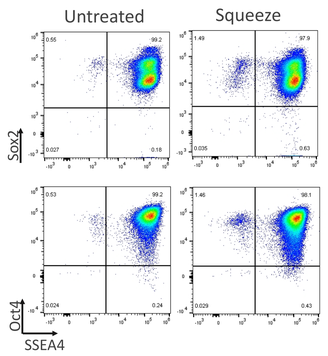
Cell Squeeze® Technology Preserves Pluripotent Stem Cell Physiological/Baseline Gene and Protein Expression *In Vitro*



No significant difference in the expression of 32 housekeeping genes between untreated and squeeze iPSCs after 24 hours. Total RNA was isolated for RT-qPCR at 24 hours post squeeze delivery.

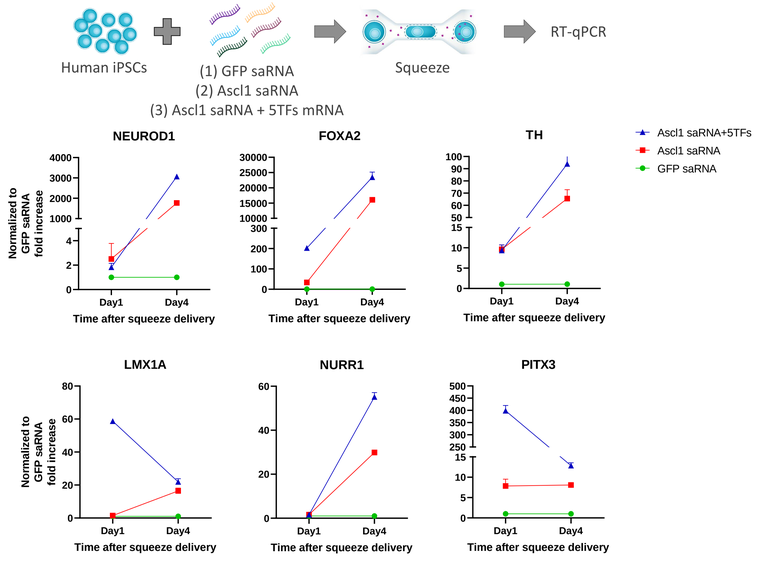


No significant difference in pluripotent stem cell marker gene expression between untreated and squeeze iPSCs after 24 hours. Total RNA was isolated for RT-qPCR at 24 hours post squeeze delivery. Relative expression of each gene was normalized to MT-ATP6. Fold change was determined between squeeze and untreated iPSCs.



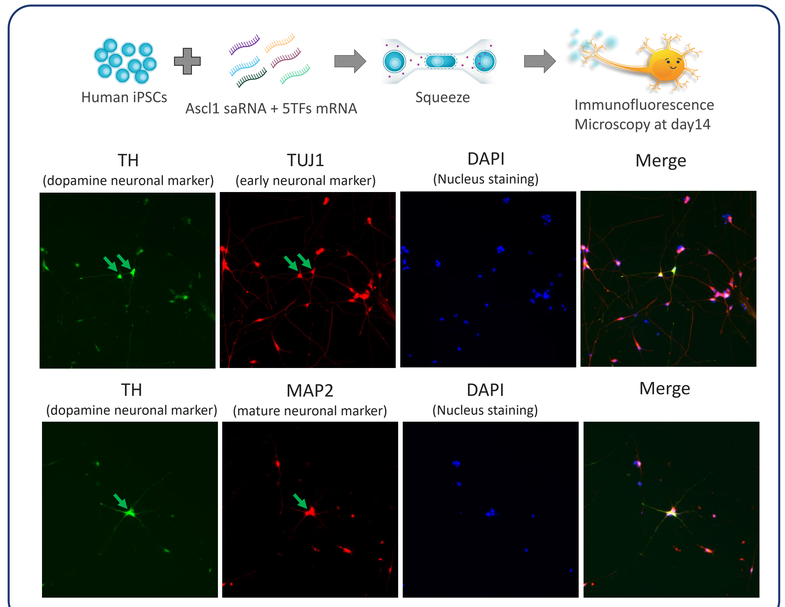
There is no obvious difference in pluripotent stem cell marker protein expression between untreated and squeeze iPSCs after 24 hours. The cells were collected to analyze pluripotent stem cell marker expression (Oct4, Sox2, SSEA4) by flow cytometry.

Ascl1 saRNA Combined with 5TFs mRNA Resulted in Rapid Activation of Target Neuronal Genes



Single squeeze of Ascl1 saRNA combined with 5TFs mRNA drive higher expression levels of downstream target genes compared to Ascl1 saRNA alone. Total RNA was isolated for RT-qPCR at day 1 and 4 post squeeze delivery. Relative expression of each gene was normalized to GAPDH. Fold increase was determined between Ascl1 saRNA delivered samples and GFP saRNA delivered sample. 5TFs: FoxA2, Lmx1a, Nurr1, Pitx3, and EN1.[2]

Single Treatment of iPSCs with Ascl1 saRNA+5TFs Generates Neurons with Mature Dopaminergic Lineage Markers



Ascl1 saRNA delivered with 5TFs mRNA generate tyrosine hydroxylase (TH) positive mature neurons at day 14. Ascl1 saRNA+5TFs mRNA-derived neurons from iPSCs all expressed early neuronal marker TUJ1 and mature neuronal marker MAP2 with high expression of dopaminergic neuronal marker tyrosine hydroxylase TH (green arrows). Cell nuclei were counterstained with DAPI. Ascl1 saRNA construct contains puromycin resistance gene PAC (puromycin N-acetyltransferase). Non-neuronal cells were removed with puromycin selection. 5TFs: FoxA2, Lmx1a, Nurr1, Pitx3, and EN1.[2]

Summary

Here, we demonstrated the rapid generation of dopaminergic neurons from iPSCs using microfluidic cell squeezing to deliver multiple transcription factors *in vitro*.

1. saRNA in iPSCs resulted in 20x increase in gene expression levels and longer expression window compared to mRNA.
2. The Cell Squeeze® process minimally altered pluripotent stem cell gene and protein expression as compared to untreated cells.
3. A single delivery of Ascl1 saRNA combined with 5TFs (FoxA2, Lmx1a, Nurr1, Pitx3, and EN1) mRNA induced a 25,000 fold increase in FoxA2 in only 4 days.
4. Ascl1 saRNA combined with 5TFs mRNA delivered cells expressed dopamine neuronal marker TH and mature neuronal markers MAP2 after 14 days.

This work demonstrates the potential of the Cell Squeeze® technology to simultaneously deliver multiple transcription factors in synthetic mRNA form to drive the differentiation of clinically relevant cell types, including dopaminergic neurons for Parkinson's Disease, which has implications for a wide variety of regenerative medicine applications.

References

1. Sharei A, *et al*. "A vector-free microfluidic platform for intracellular delivery." *PNAS*, 2013 Feb 5;110(6):2082-7
2. Ng Y, *et al*. "Efficient generation of dopaminergic induced neuronal cells with midbrain characteristics." *Stem Cell Reports*, 2021 Jul 13;16(7):1763-1776