Efficient Transgene Knock-In in Human iPS Cells Combined With Small Molecule-Mediated "On-Switch" Yields Clonal Populations of Engineered Tissue-Specific Cells

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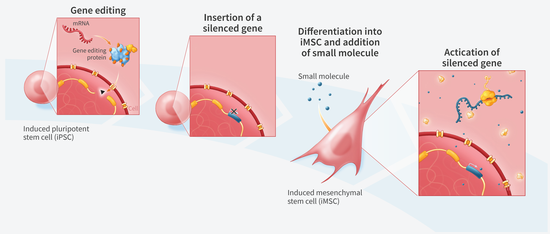
Summary

Immunosuppressive and anti-tumor properties of mesenchymal stem cells (MSCs) pose them as a promising cell-therapy platform for a diverse array of inflammatory diseases and cancer. Engineering MSCs that express therapeutic transgenes would further widen the utility of MSC therapy. This, however, would require an allogeneic MSC therapy route, since establishing well-defined, well-characterized engineered cell lines for individual patients would not be feasible. For allogeneic therapy, production of engineered induced MSCs (EiMSCs) from engineered human iPSCs (hiPSCs) through differentiation provides an ideal route, owing to the high engineering and clonal expansion potential of iPSCs. Here we present a proof of concept study, in which we establish stable clonal hiPS cell lines engineered to host a GFP-expressing cassette in the AAVS safe-harbor locus using two different promoters and differentiate the engineered hiPSCs to EiMSCs while monitoring changes in GFP expression.

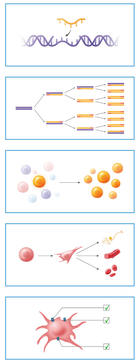


Conclusions

Data indicate that GFP expression under EF1α promoter is robust in both hiPSCs and differentiated iMSCs. Bulk sorted EF1α GFP iMSCs maintained its GFP expression for >5 weeks. Although Jet promoter was silenced at the completion of differentiation, TSA treatment increased GFP expression during differentiation, providing potentially unique application in transient upregulation of target gene during hiPSC to iMSC differentiation. Futher experiments on demetyhylation with various promoters will be necessary to find optimal expression system.

In general, our study demonstrates the feasibility of clonal EiMSC generation through hiPSCs engineered for transgene expression. We believe our ability to generate an engineered clonal MSC line in a period of just three months is a breakthrough that may help turn allogeneic MSC therapies commonplace in the future.

1 Methodology



1. **GFP transgene insertion.** Transfect hiPSCs with Ultraslice, a chromatin context-sensitive gene editing endonuclease, mRNA and single stranded DNA repair templates encoding GFP sequence under Jet and EF1α promoters.
2. **Transgene insertion verification.** Perform gDNA extraction and AAVS1 surveyor PCR to confirm ssDNA repair template integration at AAVS1 locus.
3. **Cell line generation.** Perform single cell sorting and deposition in order to obtain homogeneous cell line started from a single cell.
4. **hiPSC to iMSC differentiation.** Using Stemdiff™ Mesenchymal Progenitor kit, differentiate Jet and EF1α GFP sequence inserted hiPS cell lines to iMSCs.
5. **Surface marker verification.** Verify robust iMSC differentiation using surface marker expression at the completion of differentiation.

2 Validation of Jet and EF1α promoter activity in hiPSCs

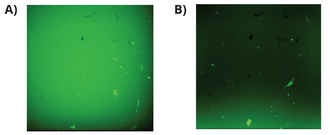


Figure 1. **GFP expression upon direct insertion of Jet and EF1α GFP transgene into iMSCs.** GFP is expressed under Jet promoter **A)** and EF1α promoter **B)** when directly inserted into the AAVS1 locus in iMSCs.

3 Jet and EF1α GFP insertion into AAVS1 locus in hiPSCs

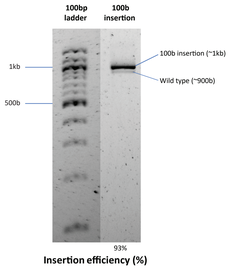


Figure 2. **Ultraslice gene ediging mRNA enables high efficiency insertion of ssDNA repair templates into AAVS1 locus in hiPSCs.** AAVS1 PCR amplicon shows that co-transfection of Ultraslice mRNA and ssDNA repair templates including noncoding 100 base sequence resulted in 93% insertion efficiency.

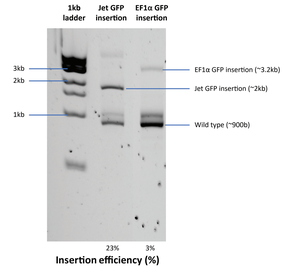


Figure 3. **High efficiency insertion of Jet and EF1α GFP sequence into AAVS1 locus.** The gel image shows AAVS1 PCR amplicon with wild type DNA band at around 900b and amplicons with Jet and EF1α GFP sequence insertion each at 2kb and 3.2kb. Efficiency of Jet and EF1α GFP sequence insertion into AAVS1 site was 23% and 3% each.

4 Validation of Jet and EF1α promoter activity in hiPSCs

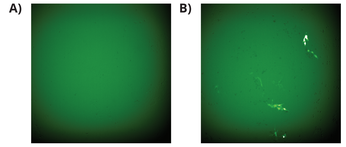


Figure 4. **Insertion of Jet and EF1α GFP transgene into AAVS1 locus of hiPSCs. A)** GFP is not expressed under Jet promoter when directly inserted into the AAVS1 locus in hiPSCs. **B)** GFP is expressed under EF1α promoter when directly inserted into the AAVS1 locus in hiPSCs.

5 Efficient isolation of biallelic transgene inserted hiPSC lines

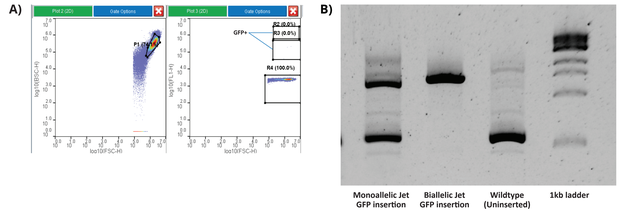


Figure 5. **Isolation of hiPSC with biallelic Jet GFP transgene insertion. A)** GFP under Jet promoter is not expressed in iPSC. Therefore, single cell was deposited into 96 well plate based on size distribution. **B)** From 3 clonal lines tested, we observed one monoallelic, biallelic and uninserted cell line. Biallelic inserted population was selected for clonal expansion.

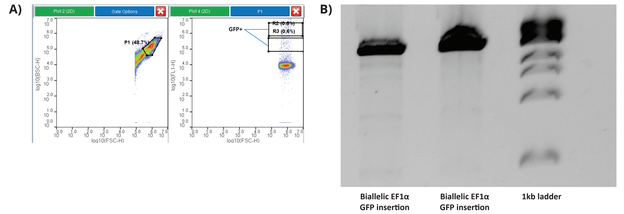


Figure 6. **Isolation of hiPSC with biallelic EF1α GFP transgene insertion. A)** GFP expressing cells were sorted into a 96 well plate for cell line development. **B)** The gel shows biallelic insertions of Ef1α GFP transgene into the AAVS1 locus which was selected for clonal expansion.

6 hiPSC to iMSC Differentiation Protocol

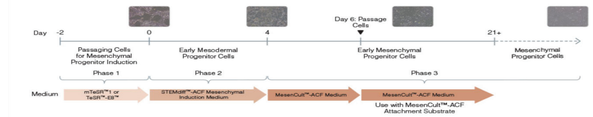


Figure 7. **Flowchart of Stemdiff™ Mesenchymal Progenitor kit protocol.**
STEMDIFF™ MESENCHYMAL PROGENITAL KIT Catalog #05240 Version 02, Stemcell™ Technologies, 2020, https://www.stemcell.com/stemdiff-mesenchymal-progenitor-kit.html#section-protocols-and-documentation

7 Jet GFP hiPSC Differentiation

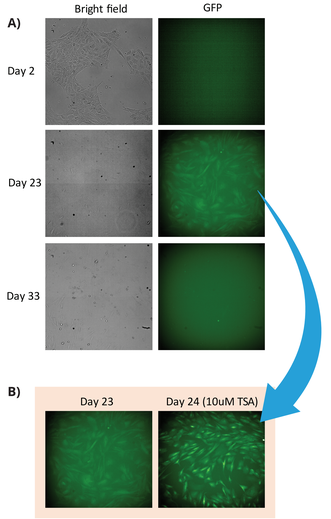


Figure 8. **Tracking GFP expression under Jet promoter during hiPSC to iMSC differentiation. A)** Bright field and GFP images were taken from Day 2 to Day 33 at which point the differentiation was completed. GFP was expressed from Day 18 to 30, but was silenced on Day 33. **B)** To reactivate Jet promoter, 10uM TSA (Trichostatin A) was treated for 24 hours to differentiating cells at Day 23. The image shows noticeable increase of GFP expression post TSA treatment, indicating that TSA reactivated Jet promoter. However, Jet promoter was silenced again at Day 33 after TSA treatment.

8 EF1α GFP hiPSC Differentiation

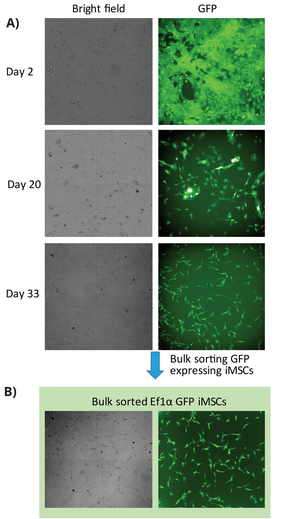


Figure 9. **Tracking GFP expression under EF1α promoter during hiPSC to iMSC differentiation. A)** Bright field and GFP images were taken from Day 2 to Day 33 at which point the differentiation was completed. GFP expression was robust until Day 20, but we started observing loss of GFP expressing cells from Day 20. By Day 33, only ~30% of differentiated iMSCs expressed GFP. **B)** In order to enrich GFP expressing population, we bulk sorted GFP positive iMSCs. Approximately 95% of iMSCs express GFP post bulk sorting for more than 5 weeks of culture.

9 Verification of iMSC surface marker expression

	Expected expression	EF1α GFP iMSC	WT iMSC
CD90	+	95%	96%
CD73	+	85%	96%
CD44	+	99%	98%
CD105	+	87%	97%
CD34	-	23%	28%
CD45	-	12%	50%
TRA1-81	-	0.9%	2%
TRA1-60	-	10%	30%
HLA-DR	-	1.4%	0.6%

Figure 10. Flow cytometry analysis shows that differentiated Ef1α GFP iMSCs exhibit similar surface marker expression pattern to wildtype iMSCs, indicating successful differentiation of transgene inserted hiPSCs to EiMSCs.

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