Donor Sequence Optimization Enables Targeted Insertion of Complex Stealthing Constructs in mRNA-Reprogrammed Induced Pluripotent Stem Cells

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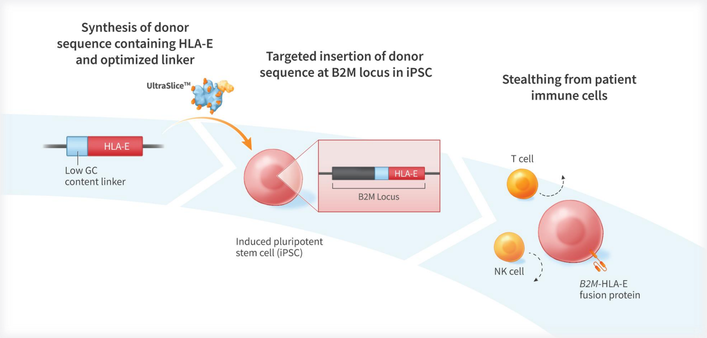
Summary

Next-generation allogeneic cell therapies derived from mRNA-reprogrammed induced pluripotent stem cells (iPSCs) can directly address many challenges associated with autologous cell therapies, including scalability, batch-to-batch consistency, and cost. Here, we describe the development of mRNA-reprogrammed iPSCs engineered to avoid elimination by host T cells and NK cells, the primary drivers of immune-mediated rejection of allogeneic cell therapies. The engineered iPSCs do not express B2M but instead express a B2M-HLA-E fusion protein. UltraSlice™ gene-editing mRNA was used to insert donor DNA containing a $(GGGGS)_4$ linker and HLA-E gene sequence upstream of the endogenous *B2M* stop codon for inducible expression of the fusion protein.



Conclusions

- Insertion efficiency of a single-stranded DNA repair template at the *B2M* locus is enhanced through codon optimization of the donor sequence.
- This work demonstrates the feasibility of generating iPSC-derived allogeneic cell therapies that have inherent potential to reduce the manufacturing complexities of autologous and donor-derived cell therapies.
- There are numerous potential therapeutic applications for cells derived from B2M-HLA-E iPSCs, as such cells will have immune-evasive properties and should therefore exhibit prolonged *in vivo* persistence and effector activity.

1. Stealthing Strategy

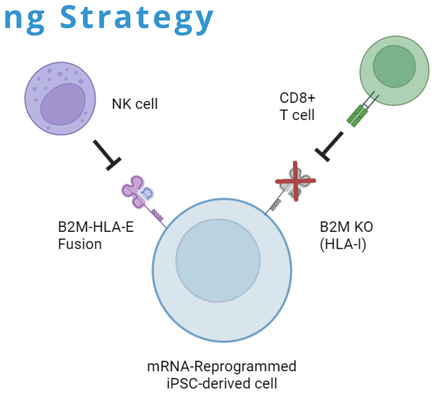


Figure 1. Targeted gene editing. To prevent recognition from CD8+ T cells, *B2M* was knocked out, resulting in the absence of endogenous HLA-I. As NK cells recognize and neutralize cells that lack HLA-I, *HLA-E* was inserted to promote NK cell evasion as it does not trigger T cell-mediated elimination.

2. Methods

Designed UltraSlice mRNA to target *B2M* exon 3 and designed donor ssDNA containing both a $(GGGGS)_4$ linker and the full HLA-E sequence

Conducted gene edits, analyzed insertion of donor sequence, and codon optimized for full insertion of the ssDNA template at exon 3 of *B2M*

Isolated iPSC colonies after editing and characterized them for B2M and HLA-I expression

3. Gene Insertion and Codon Optimization

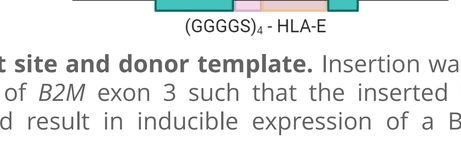


Figure 2. Target site and donor template. Insertion was targeted before the stop codon of *B2M* exon 3 such that the inserted linker and HLA-E sequences would result in inducible expression of a B2M-HLA-E fusion protein.

	Homology arm	$(GGGGS)_4$ Linker	HLA-E sequence
1)	ATCGAGACATG	GGCGGGGGAGGCTCCGGGGGAGGCGGCTCCGGCGGCGGGGGAAGTGGCGGGGGAGGATCC	GGTTCT
2)	ATC	----------	GGTTCT
3)	ATCGAGACATG	GGTGGTGGCGGCTCTGGAGGCGGTGGGTCTGGGGGTGGCGGGTCAGGTGGTGGAGGTAGT	GGTTCT
4)	ATCGAGACATG	GGTGGAGGTGGTTCAGGAGGCGGTGGATCAGGTGGAGGAGGAAGTGGAGGTGGAGGAAGT	GGTTCT

Figure 3. Truncated insertion and subsequent codon optimization. Initial insertion of the donor sequence containing the Initial Linker (1) indicated 68 missing nucleotides (2). We hypothesized that high GC content may promote secondary structure formation and lead to aberrant splicing during genomic insertion. Codon optimization was employed to reduce the GC content within the $(GGGGS)_4$ linker to 71.7% (3) and 61.7% (4). Red font indicates modified nucleotides.

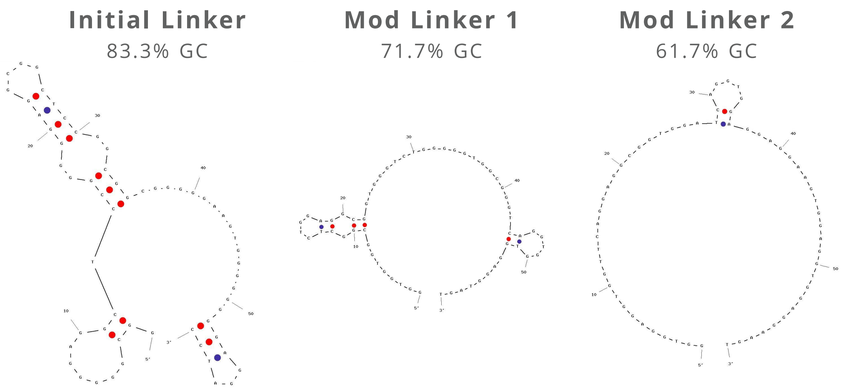


Figure 4. Theoretical secondary structures of donor DNA. Representative figures for secondary structure formation of each flexible linker variant. As the GC content decreased, fewer hairpin structures were predicted to form. Images generated using IDT OligoAnalyzer™.

4. GC Reduction Leads to Full Length Insertion of Stealthing Constructs

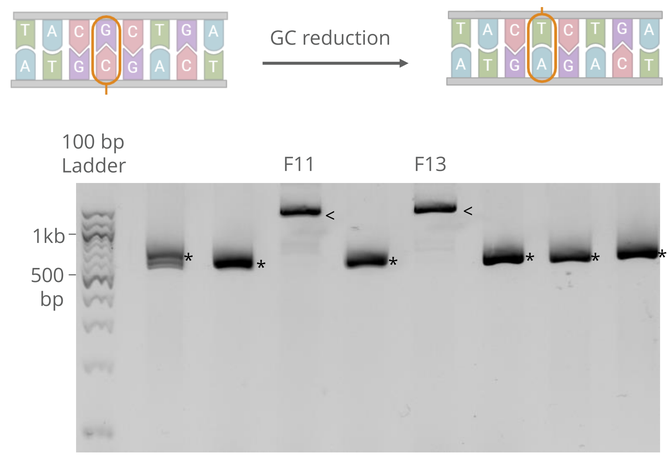
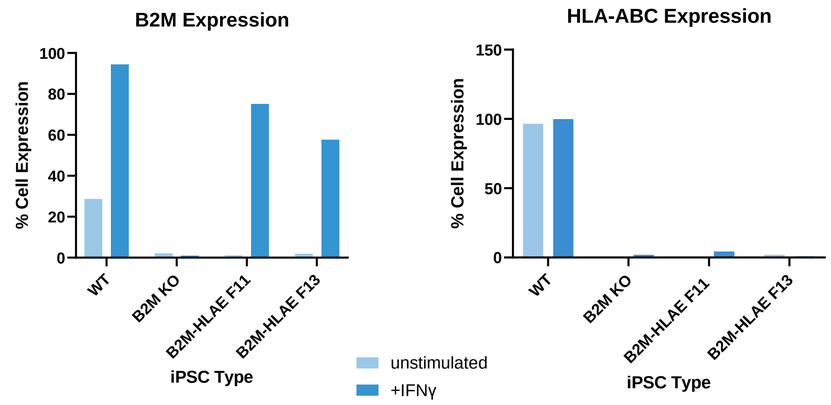


Figure 5. iPSC colonies with biallelic insertion. After electroporation with UltraSlice mRNA and the B2M-HLA-E repair template containing Mod Linker 2, edited iPSCs were single cell deposited, then single colonies were harvested and analyzed for insertion of transgene. Colonies F11 and F13 demonstrate full bi-allelic insertion (<), while the other colonies present wildtype bands (*).

5. Expression Analysis of B2M and HLA-I



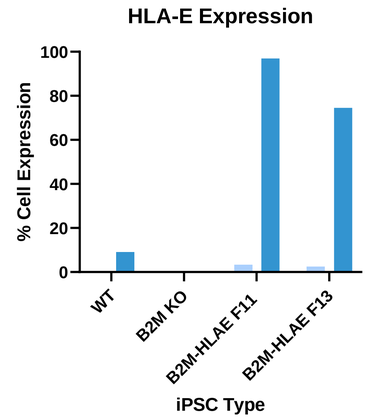


Figure 6. Surface expression of iPSCs after colony isolation. Flow cytometry of iPSCs with and without stimulation of IFNγ (50 ng/mL, 72 hr) revealed wildtype iPSCs express low levels of B2M and HLA-ABC before stimulation, both of which increase after addition of IFNγ. B2M KO iPSCs do not express B2M, HLA-ABC, or HLA-E in either case. Edited B2M-HLA-E iPSCs express B2M and HLA-E only after stimulation and do not express HLA-ABC.

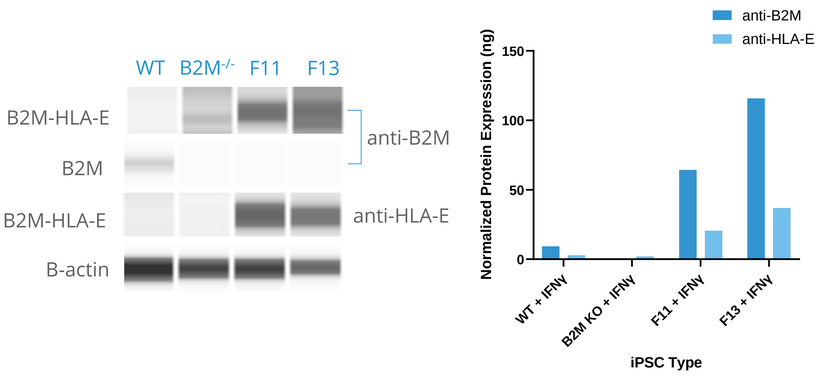


Figure 7. Expression profile of iPSCs after colony isolation. iPSCs stimulated with IFNγ (50 ng/mL, 72 hr) analyzed via Western. Wildtype iPSCs express low levels of B2M (12 kDa), while both wildtype and B2M KO iPSCs express negligible levels of HLA-E fusion protein (empirical 62 kDa). Edited clones, F11 and F13, express fusion B2M-HLA-E, which was detectable by both anti-B2M and anti-HLA-E antibodies.