OPEN ACCESS Freely available online

Advancements in Genetic Engineering

ISSN: 2169-0111

Commentary

CRISPR-Cas9 Mediated Multiplex Gene Editing in Hematopoietic Stem Cells for Inherited Immunodeficiency Correction

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DESCRIPTION

The application of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) technology has revolutionized therapeutic approaches for monogenic disorders affecting the hematopoietic system. This investigation demonstrates the efficacy of multiplex gene editing strategies targeting multiple genomic *loci* simultaneously within CD34+ Hematopoietic Stem Cells (HSCs) isolated from patients with Severe Combined Immunodeficiency (SCID). The experimental protocol employed dual guide RNA (gRNA) constructs designed to facilitate precise double-strand break formation at target sites within the *IL2RG* gene, commonly mutated in X-linked SCID, while simultaneously introducing corrective sequences through Homology-Directed Repair (HDR) mechanisms.

Inherited immunodeficiencies, including Severe Combined Immunodeficiency (SCID) and Chronic Granulomatous Disease (CGD), result from mutations in genes critical for immune cell development and function. These conditions leave patients highly susceptible to infections and often require bone marrow transplantation or lifelong supportive care. Hematopoietic Stem Cells (HSCs), which give rise to all blood and immune cells, offer a promising target for curative gene therapy. However, many immunodeficiencies involve mutations in multiple genes or require the coordinated modulation of several pathways to restore full immune competence. In this context, CRISPR-Cas9-mediated multiplex gene editing has emerged as a powerful and precise approach to correct complex genetic defects in HSCs.

The CRISPR-Cas9 system allows targeted gene editing by inducing site-specific double-strand breaks, which are then repaired by endogenous cellular mechanisms. Multiplex editing extends this capability by enabling the simultaneous modification of multiple genes within a single editing event. In HSCs, this approach can be used to correct disease-causing mutations, disrupt negative regulatory elements, or insert therapeutic transgenes. When combined with optimized delivery methods and *ex vivo* expansion techniques, multiplex editing preserves the self-renewal and multilineage potential of HSCs, allowing for durable correction following autologous transplantation.

This strategy holds immense therapeutic potential for treating inherited immunodeficiencies at their root cause, minimizing immune rejection and eliminating the need for donor matching. This introduction explores the methodology, advantages, and clinical promise of CRISPR-Cas9-mediated multiplex gene editing in hematopoietic stem cells for the correction of inherited immune disorders.

Primary human $CD34^+$ cells were isolated from bone marrow aspirates using Magnetic-Activated Cell Sorting (MACS) and maintained in serum-free expansion medium supplemented with Stem Cell Factor (SCF), Thrombopoietin (TPO), and FLT3 ligand. Ribonucleoprotein (RNP) complexes consisting of Cas9 protein, chemically modified gRNA, and single-stranded Oligodeoxynucleotide (ssODN) templates were delivered *via* electroporation using optimized pulse parameters. Post-editing analysis revealed successful HDR-mediated correction in 23.7% of treated cells, with off-target analysis using whole-genome sequencing confirming minimal unintended modifications.

Flow cytometric analysis demonstrated restoration of functional IL-2 receptor gamma chain expression on the cell surface, with subsequent in vitro differentiation assays confirming the ability of corrected HSCs to generate T, B, and NK cell lineages. Long-Term Culture-Initiating Cell (LTC-IC) assays validated preservation of stem cell characteristics following genetic modification. Notably, edited cells maintained normal karyotype and exhibited no signs of malignant transformation over extended culture periods.

CONCLUSION

This study establishes a robust platform for multiplex CRISPR-Cas9 editing in primary human HSCs, demonstrating therapeutic potential for treating inherited immunodeficiencies. The high correction efficiency, minimal off-target effects, and preserved stem cell functionality support clinical translation of this approach. Future investigations will focus on optimizing delivery methods and evaluating long-term safety profiles in

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Received: 28-Feb-2025, Manuscript No. MAGE-25-38109; **Editor assigned:** 03-Mar-2025, PreQC No. MAGE-25-38109 (PQ); **Reviewed:** 17-Mar-2025, QC No. MAGE-25-38109; **Revised:** 24-Mar-2025, Manuscript No. MAGE-25-38109 (R); **Published:** 31-Mar-2025, DOI: 10.35841/2169-0111.25.14.408

Citation: Gilani R (2025). CRISPR-Cas9 Mediated Multiplex Gene Editing in Hematopoietic Stem Cells for Inherited Immunodeficiency Correction. Adv Genet Eng. 14:408.

relevant animal models before advancing to human trials. The multiplexing approach enabled simultaneous correction of compound heterozygous mutations, significantly improving therapeutic efficiency compared to sequential editing strategies.