Rad51-based editing of mitochondrial DNA via CRISPR approach

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Previously, we developed a CRISPR/Cas9-based method to excise the mitochondrial mutation 15059G>A. Subsequent mitochondrial DNA analysis revealed instances of double-strand break repair via homologous recombination. Exploiting this, we aimed to edit mitochondrial DNA by inserting a "barcode" sequence of either 20 or 100 nucleotides at the mutation site 1555A>G. This was achieved using two specific nickases, D10A and H840A, facilitating double-strand breaks by targeting the leading and lagging strands, respectively. Each nickase was paired with a guide RNA, one matched to the mutation and the other to a mitochondrial DNA conserved region.

To enable efficient mitochondrial delivery of the nickases, the Cox8a signal peptide, a Mitochondrial Targeting Signal (MTS), was added to their domains, promoting cytoplasm-synthesized protein translocation into mitochondria. The delivery system comprised RNA encoding the nickases, the MTS, and a sequence for Streptavidin-SpyCatcher, forming a complex with biotinylated guide RNA for transport across mitochondrial membranes.

The barcode insertion utilized an mRNA sequence of Rad51, tagged with an MTS, and a single-stranded oligonucleotide (ssODN) bearing unique sequence AAATTTAAA. Rad51's binding to a specific ssODN sequence facilitated its mitochondrial delivery. Our integrated system, involving mitochondrially targeted nickases and Rad51, successfully delivered guide RNA and ssODN into the mitochondrion, enabling precise genome editing. We verified the integration of both 20 and 100 nucleotide sequences into the mitochondrial genome, noting higher efficiency with the 20-nucleotide sequence. Integration was confirmed through PCR and sequencing, highlighting the system's potential for precise mitochondrial DNA mutation correction.

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