Targeted knock-ins with pseudovirus for the stable expression of large transgenes

We engineered integrase-deficient lentiviruses to act as vectors for the delivery of large gene knock-ins via homology-directed repair. This technology enables the non-cytotoxic, targeted insertion of difficult-to-express transgenes into genomic loci that are essential to cell survival, thereby overcoming the gene silencing that otherwise limits primary immune cell engineering.

This is a summary of:
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The problem

Technologies such as engineered receptors, clustered regularly interspaced short palindromic repeats activation (CRISPRa) or CRISPR inhibition (CRISPRi) systems, gene circuits, and cell-based vaccines have created a need for stable, long-term expression of large transgenes in primary human cells[1]. Engineered pseudovirus vectors based on lentivirus or gamma retrovirus are leveraged for semi-random insertion of genetic payloads into genomes to enable transgene expression over multiple generations. However, this methodology often succumbs to gene silencing (an umbrella term for multiple cellular processes that result in the loss of transgene expression over time)[2]. Homology-directed repair (HDR) could be leveraged to overcome gene silencing through targeted knock-in of payloads into endogenous genomic loci whose expression is essential to cell survival (termed essential genes), thereby using the natural genomic context to stabilize transgene expression. However, the most widely utilized DNA donors — adeno-associated virus (AAV) vectors[3] and naked DNA templates[4] — are restricted by limited payload size or high cytotoxicity in primary cells, which limits their use for synthetic biology and manufacture of cell-based therapies.

The solution

Integrase-deficient lentivirus (IDLV) enables reverse transcription of an encoded payload from an RNA molecule into a DNA template, which creates a useful yet low-efficiency donor for HDR. We engineered the IDLV genome to include CRISPR 'cut sites' that match the intended genomic cut site (Fig. 1). Cas9 ribonucleoprotein electroporated into cells 24 hours after transduction not only creates a double-stranded break in the genome that induces HDR, but also enables linearization and processing of the reverse-transcribed DNA template, which can greatly improve transgene knock-in efficiency. The CLIP (CRISPR for long-fragment integration via pseudovirus) method was tested in cell lines and primary human T cells to measure knock-in efficiency and toxicity compared with unmodified IDLV vectors and naked DNA donors. With CLIP, we were able to efficiently integrate large genetic payloads into an essential gene of primary human T cells, which achieved an improved duration of gene expression compared with traditional lentivirus vectors.

CLIP doubles the number of targeted integration events compared with unmodified IDLV vectors and was non-toxic in primary human T cells, unlike naked DNA donors. By targeting CLIP to *ACTB*, an essential gene encoding a component of the cellular cytoskeleton, the transgene took on the expression characteristics of the *ACTB* locus and its expression remained stable over time.

Transgene silencing was observed when large payloads of several thousand base pairs, such as Cas13d, hyperdCas12a-miniVPR (a dCas12a-based transcription activator used in CRISPRa), or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigens were delivered to cells by unmodified lentivirus. By contrast, CLIP achieved stable expression of each of these transgenes that persisted over a long period. Importantly, the CRISPRa system remained functional 31 days after its knock-in with CLIP whereas gene silencing disabled this system in cells transduced by unmodified lentivirus within only a few days. CLIP-mediated knock-in of SARS-CoV-2 antigens led to their expression for multiple weeks, which enabled presentation of antigen peptides by the major histocompatibility complex class I. These findings show that CLIP provides an efficient, low-toxicity method to stabilize large and difficult-to-express transgenes over a long period in primary human T cells.

The implications

Engineered cells are increasingly leveraged in medicine to treat diseases such as various cancers and neurodegenerative disorders. Novel approaches are urgently needed to manufacture cells with greatly improved therapeutic functions. However, most transgenes are soon silenced after they are introduced into primary cells. CLIP overcomes this challenge by ensuring that transgenes can be faithfully expressed in engineered cells. With CLIP, payloads encoding new receptors, gene modulators, multiple genes or genetic circuits can be expressed over a long period, thereby maximizing their therapeutic effects.

The efficiency of CLIP can be further improved using HDR enhancers such as non-homologous end joining inhibitors. Other genomic loci will also be explored that could maintain transgene stability and precisely modulate the degree of expression. Most excitingly, CLIP could be used to knock transgenes into conditionally active genomic locations, creating a new generation of genetic switches that leverage native gene regulation mechanisms.

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EXPERT OPINION

"CLIP yields favourable results regarding cell survival, DNA cargo size, stability of long-term transgene expression and multiplexing, while maintaining an insertion efficiency comparable to similar methods." **Jacob E. Corn, ETH Zurich, Zurich, Switzerland.**

FIGURE

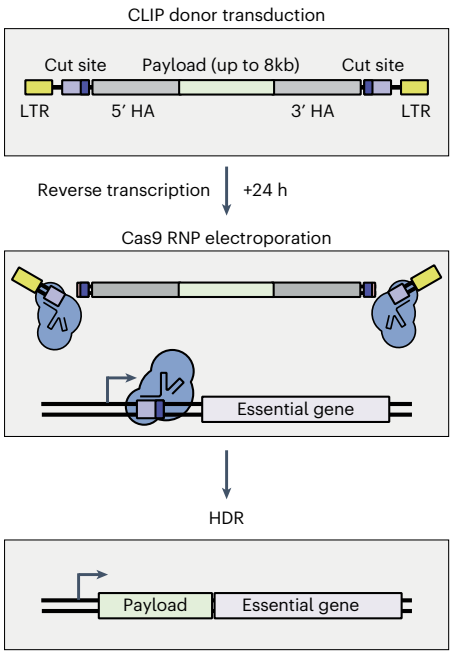

Fig. 1 | CLIP enhances the knock-in efficiency of large payloads. The CLIP methodology is based on IDLVs engineered to deliver a CLIP donor payload (top). When inside target cells, the payload undergoes reverse transcription along with the viral genome. Cas9 ribonucleoprotein (RNP) is then electroporated into transduced cells and both cuts the cell's genome and excises linearized donor DNA from the viral backbone (middle). This process facilitates efficient payload insertion via HDR (bottom). HA, homology arm; LTR, long terminal repeat. © 2023, Chavez, M. et al.

BEHIND THE PAPER

This research started with the desire to carry out CRISPRa screens to understand the drivers of primary T cell differentiation. The project quickly came to a halt when we could not obtain long-term expression of the CRISPRa system protein (which is encoded by more than 5.5 kb) no matter what transduction protocol we used. It had felt natural to place transgenes under the control of native promoters. However, mammalian promoters are not as predictable as those used in prokaryotic synthetic biology. At the same time, other groups were investigating knock-in protocols using naked DNA and AAV in primary T cells. We developed CLIP to handle large and difficult-to-express transgenes and to explore the emergent properties conferred by use of native promoters. We were excited to see that we could use CLIP to overcome the common problem of achieving stable expression of large CRISPR molecules and to understand coronavirus immunobiology. We are even more excited to see how others will use CLIP to exploit the cell's native gene regulation to create a whole new class of circuits. **M.C. & L.S.Q.**

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FROM THE EDITOR

"This method for the insertion of large genes into primary cells could facilitate the manufacturing of genetically engineered cells for therapy and vaccination." **Editorial Team, *Nature Biomedical Engineering*.**