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High content analysis platform for optimization of lipid mediated CRISPR-Cas9 delivery strategies in human cells ☆

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ABSTRACT

Non-viral gene-editing of human cells using the CRISPR-Cas9 system requires optimized delivery of multiple components. Both the Cas9 endonuclease and a single guide RNA, that defines the genomic target, need to be present and co-localized within the nucleus for efficient gene-editing to occur. This work describes a new high-throughput screening platform for the optimization of CRISPR-Cas9 delivery strategies. By exploiting high content image analysis and microcontact printed plates, multi-parametric gene-editing outcome data from hundreds to thousands of isolated cell populations can be screened simultaneously. Employing this platform, we systematically screened four commercially available cationic lipid transfection materials with a range of RNAs encoding the CRISPR-Cas9 system. Analysis of Cas9 expression and editing of a fluorescent mCherry reporter transgene within human embryonic kidney cells was monitored over several days after transfection. Design of experiments analysis enabled rigorous evaluation of delivery materials and RNA concentration conditions. The results of this analysis indicated that the concentration and identity of transfection material have significantly greater effect on gene-editing than ratio or total amount of RNA. Cell subpopulation analysis on microcontact printed plates, further revealed that low cell number and high Cas9 expression, 24 h after CRISPR-Cas9 delivery, were strong predictors of gene-editing outcomes. These results suggest design principles for the development of materials and transfection strategies with lipid-based materials. This platform could be applied to rapidly optimize materials for gene-editing in a variety of cell/tissue types in order to advance genomic medicine, regenerative biology and drug discovery.

Statement of Significance

CRISPR-Cas9 is a new gene-editing technology for "genome surgery" that is anticipated to treat genetic diseases. This technology uses multiple components of the Cas9 system to cut out disease-causing mutations in the human genome and precisely suture in therapeutic sequences. Biomaterials based delivery strategies could help transition these technologies to the clinic. The design space for materials based delivery strategies is vast and optimization is essential to ensuring the safety and efficacy of these treatments. Therefore, new methods are required to rapidly and systematically screen gene-editing efficacy in human cells. This work utilizes an innovative platform to generate and screen many formulations of synthetic biomaterials and components of the CRISPR-Cas9 system in parallel. On this platform, we watch genome surgery in action using high content image analysis. These capabilities enabled us to identify formulation parameters for Cas9-material complexes that can optimize gene-editing in a specific human cell type.

1. Introduction

Genomic medicine involving gene-editing to correct disease causing mutations or insert other genetic sequences into patients' cells is a growing area of biomedical research [1–6]. Recent

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gene-editing technologies utilize nucleases to generate a DNA double strand break (DSB), or "cut," in genomic DNA at a desired location [7–11]. CRISPR-Cas9, an emerging gene-editing technology, exploits a modified bacterial immune defense mechanism termed CRISPR (clustered regularly interspaced short palindromic repeats) that cuts DNA at specific sequences.

The engineered CRISPR-Cas9 system encompasses two essential components: 1) an endonuclease, Cas9; and 2) a short, single-guide RNA (sgRNA) that forms a ribonucleoprotein (RNP) complex with Cas9 and targets endonuclease activity to a specific sequence in the genome [12,13]. Interaction of an approximately 20 nucleotide (nt) sgRNA sequence to the complementary genomic DNA increases the residence time of the Cas9-sgRNA complex at that specific genomic locus (Fig. 1A and B), enabling Cas9 nuclease to create targeted DSBs. However, the Cas9-sgRNA complex can associate with and cut the genome at other "off-target" sites [14,15]. Decreasing off-target mutagenesis has been achieved by titrating the quantity of Cas9 and sgRNA delivered [16,17] and by engineering the specificity of CRISPR-Cas9 components [18–20]. While RNP engineering efforts have increased efficiencies of editing and reduced off-target mutagenesis, a significant bottleneck in genomic medicine remains in effectively delivering these engineered components to human cells.

Employing CRISPR-Cas9 gene-editing requires cellular delivery and subsequent nuclear translocation of RNP complexes or plasmid DNA and RNAs that encode the two components of the system. Initial CRISPR-Cas9 experiments with human cells used electroporation of plasmids encoding Cas9 and sgRNAs driven by constitutive promoters [9,10]. Viral delivery strategies have also been employed [21], although non-viral delivery strategies are typically preferred over viral delivery. This is because viruses can integrate into the genome causing insertional mutagenesis, which is problematic for many research and clinical applications.

A common method for delivering Cas9 components to cells makes use of synthetic biomaterials that form lipid nanoparticles containing DNA, RNA, or pre-formed RNP complexes [22–24]. These methods involve encapsulation or complexing of nucleic acid cargo through interactions between the negatively charged phosphate backbone of the cargo and the positively charged lipid head groups. In the case of CRISPR-Cas9 RNPs, the negative charge of the sgRNA allows the RNP complex to be encapsulated by the cationic lipids [16]. Cellular uptake and subcellular trafficking can be mediated by endocytosis and macropinocytosis, although exact mechanisms are poorly understood and may vary widely across transfection reagents [25]. Direct comparison of liposomal based transfection of a Cas9-encoding plasmid to Cas9-encoding mRNA indicated that mRNA increased editing efficiencies and lowered off-target mutagenesis in many human cell lines [17]. However, each human cell line required different doses and formulations of lipid nanoparticles or complexes. Despite these challenges, lipid-based delivery of mRNA encoding Cas9 provides an attractive route to achieving precise editing in human cells.

Optimizing CRISPR-Cas9 gene-editing with RNA in human cells is a complex multiparametric space. For each cell line in research and each patient in the clinic, screening of many different formulations of the mRNA/sgRNA/material complex will likely be required for optimal editing [23,24]. Combinatorial synthesis and assessment of non-viral transfection agents for delivering plasmid DNA [26] and siRNA [27,28] have yielded successful strategies both *ex vivo* and *in vivo* [29]. To our knowledge these platforms have not been applied for optimizing CRISPR-Cas9 delivery. Indeed, there are limited platforms available to rapidly screen synthetic biomaterial transfection agents and optimize the amount, ratios, and chemical modifications to both components of the CRISPR-Cas9 system. Thus far, optimization has relied on low-throughput endpoints assays on samples homogenized from 10^4 to 10^6 cells [16,17]. Such cell population-averaged measurements of DSB formation utilize DNA sequencing or an endonuclease assay (e.g., SURVEYOR [30] or T7E1 [31]) of genomic DNA. These assays can be difficult to standardize [32,33] and do not monitor the cargo trafficking and editing processes *in situ*. Thus, the development of new materials with these low-throughput assays can be slow, and the delivery kinetics involved in optimized strategies are poorly defined.

Here we describe a high throughput method to optimize non-viral gene-editing strategies. Our platform exploits image cytometry and high content image analysis (HCA) in combination with a customized microcontact printed cell substrate [34,35] to simultaneously monitor non-viral delivery and editing in human cells. We use established image analysis methods [36] and design-of-experiments (DOE) based statistical techniques [37,38] to screen existing liposomal delivery materials and maximize gene-editing outcomes. Dynamic tracking of Cas9 protein expression, subcellular localization and gene disruption within subpopulations of cells suggests that the cell number and the level of Cas9 expression within 24 h of delivery are important predictors of editing. This platform enables the rapid screening of material mediated CRISPR-Cas9 gene-editing strategies in human cells.

2. Materials and methods

2.1. H2B-mCherry reporter construction and culture

A constitutively expressed histone 2B-mCherry reporter transgene was integrated into the genome of human embryonic kidney (HEK) 293T cells via CRISPR-Cas9 gene-editing. The H2B-mCherry plasmid was generated by cloning the H2B-mCherry sequence (Addgene #20972) into the EGFP sequence of the AAV-CAGGS-EGFP plasmid (Addgene #22212). This plasmid was electroporated with plasmids encoding human codon optimized Cas9 (Addgene #41815) and sgRNA targeting the *AAVS1* locus (Addgene #41818) into HEK 293T cells. Populations containing the transgene were purified through puromycin selection and clonal isolation followed by sequencing and fluorescent imaging to confirm mCherry expression.

HEK H2B-mCherry cells were maintained at 37 °C and 5% CO_2 in growth media composed of DMEM (Thermo Scientific), 10% v/v FBS (Thermo Scientific), 2 mM L-Glutamine (Thermo Scientific), and 50 U/mL Penicillin–Streptomycin (Thermo Scientific). Media was changed every 2 days. Cells were passaged 1:40 every 4–5 days once 80–90% confluent with 0.05% Trypsin–EDTA (Thermo Scientific) onto tissue culture polystyrene (TCPS) plates (Thermo Scientific) coated overnight with a sterile 0.1% (w/v) gelatin A (Sigma) in water solution. At least two passages prior to experimental treatment, cells were transitioned to "imaging media" containing FluoroBrite DMEM (Thermo Scientific) supplemented with 10% (v/v) FBS and 2 mM L-Glutamine. Specific culture volumes and media change regimens during experimentation are described in Section 2.9.

2.2. Single-guide RNA (sgRNA) design, synthesis and characterization

Coding sequences for genomic targets were obtained from NCBI Gene (www.ncbi.nlm.nih.gov/gene) and imported into an online sequence management tool (Benchling, www.benchling.com). Potential sgRNA sites were identified using Benchling's online genome-editing design tools. For mCherry reporter targeting, sgRNAs were designed against the mCherry coding sequence. sgRNA sequences were selected based on high on-target [39] and low off-target [40] ranking. In general, three sgRNAs were screened for each locus (sequences available in Appendix, Table A.1). Initial

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