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High-content analysis screening for cell cycle regulators using arrayed synthetic crRNA libraries

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ABSTRACT

The CRISPR-Cas9 system has been utilized for large-scale, loss-of-function screens mainly using lentiviral pooled formats and cell-survival phenotypic assays. Screening in an arrayed format expands the types of phenotypic readouts that can be used to now include high-content, morphology-based assays, and with the recent availability of synthetic crRNA libraries, new studies are emerging. Here, we use a cell cycle reporter cell line to perform an arrayed, synthetic crRNA:tracrRNA screen targeting 169 genes (> 600 crRNAs) and used high content analysis (HCA) to identify genes that regulate the cell cycle. Seven parameters were used to classify cells into cell cycle categories and multiple parameters were combined using a new analysis technique to identify hits. Comprehensive hit follow-up experiments included target gene expression analysis, confirmation of DNA insertions/deletions, and validation with orthogonal reagents. Our results show that most hits had three or more independent crRNAs per gene that demonstrated a phenotype with consistent individual parameters, indicating that our screen produced high-confidence hits with low off-target effects and allowed us to identify hits with more subtle phenotypes. The results of our screen demonstrate the power of using arrayed, synthetic crRNAs for functional phenotypic screening using multiparameter HCA assays.

1. Introduction

Gene knockout using the CRISPR-Cas9 system has recently emerged as a powerful technology for performing loss-of-function screens. CRISPR-Cas systems originated in bacteria and archaea as a form of adaptive immune response (Barrangou et al., 2007; Bhaya et al., 2011; Terns and Terns, 2011; Wiedenheft et al., 2012). One version of this system, the Cas9 RNA-directed DNA endonuclease found natively in *Streptococcus pyogenes*, has been engineered and ported into mammalian cells as a high-throughput tool for gene knockout (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). Cas9 can easily be programmed to direct a double-strand break (DSB) at almost any locus in the genome with the use of different short RNA guide sequences, either as a two-part CRISPR RNA (crRNA) and a *trans*-activating CRISPR RNA (tracrRNA) or a chimeric single guide RNA (sgRNA). The introduction of the DSB generally results in gene knockout after imperfect repair by the cells' endogenous non-homologous end joining (NHEJ) pathway (Doudna and Charpentier, 2014; Komor et al., 2017).

The rapid adoption of the CRISPR-Cas9 system for functional genomics was in part enabled by the RNA interference (RNAi) field which provided both the techniques for library construction as well as a wealth of experience on whole-genome screening using both pooled and arrayed libraries. Shortly after the discovery of CRISPR-Cas9 functionality in mammalian cells, pooled lentiviral sgRNA libraries were created (Wang et al., 2016) and used for screening thousands of genes in parallel to identify essential genes (Hart et al., 2015; Morgens et al., 2016; Wang et al., 2014). In these studies, phenotypic assays that allowed for enrichment or depletion in a cell population in a manner parallel to pooled shRNA screens were used (Agrotis and Ketteler, 2015; Barrangou et al., 2015; Kelley et al., 2016). Recently, a few smaller arrayed CRISPR-Cas9 screens have been described (Anderson et al., 2015; Hultquist et al., 2016; Tan and Martin, 2016), but the overall scarcity of arrayed CRISPR-Cas9 screens in the literature to date can be attributed to the fact that both lentiviral expressed sgRNA and synthetic crRNA libraries in an arrayed format have only just become available (Schmidt et al., 2015). The availability of these libraries has been eagerly anticipated, as a well-by-well phenotypic screening strategy is applicable to a wider variety of biological assays including endpoint assays and high content imaging, and is not limited by analysis of enrichment or depletion in a cell population.

Arrayed, expressed sgRNA phenotypic studies have been successfully applied to smaller gene sets when confirming hits from pooled

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lentiviral sgRNA screens (Hart et al., 2015; Koike-Yusa et al., 2014; Morgens et al., 2016; Schmidt et al., 2015; Wang et al., 2014; Zhou et al., 2014). Although significantly fewer arrayed lentiviral sgRNA screens (as the primary screening modality) compared to pooled sgRNA screens have been described (McCleland et al., 2016), lentiviral transduction of sgRNAs is an attractive method for screening because gene editing can be achieved in a wide variety of cell types and selection can be applied to enrich for edited cells. However, obtaining high and reproducible titers in multi-well plates can be a challenge and can lead to low transduction efficiency, the inability to perform sufficient replicate transductions, and increased experimental noise in the phenotypic readout. The difficulty of handling large quantities of virus while complying with safety regulations may also limit widespread application of this approach. Finally, the need for transduction, construct expression, antibiotic selection and phenotype visualization typically requires longer timepoints that may necessitate passaging of cells; this can be logistically challenging in high-throughput, genome-scale screens and can also result in increased experimental noise.

More recently, high-throughput arrayed screens using synthetic crRNA and tracrRNA (crRNA:tracrRNA) as the guide RNA have been published (Anderson et al., 2015; Hultquist et al., 2016; Kelley et al., 2016; Tan and Martin, 2016). Arrayed screening of synthetic crRNA:-tracrRNA delivered into Cas9-expressing cells is desirable because it is analogous to the established, relatively simple workflow for performing high-throughput siRNA screens. Specifically, algorithm-designed and chemically synthesized crRNA:tracrRNA can be reproducibly delivered into cells in multi-well format using known transfection or electroporation methods and established automation protocols. An initial concern with the use of synthetic crRNA:tracrRNA for high-throughput screening was that while the crRNA:tracrRNA can be efficiently delivered into most cells, DSBs and NHEJ-mediated editing may result in an insufficient number of cells containing a functional gene knockout to observe the biological phenotype. For screening to be successful, gene editing resulting in a functional gene knockout must be highly efficient in the overall cell population (without clonal selection), and the resulting phenotype must be clearly distinguishable from unedited cells. Even with the successful introduction of a knockout edit, observation of the phenotype relies on the turnover kinetics of both the mRNA and encoded protein and the knockout phenotype must be measurable in a short-term assay. These initial concerns have been assuaged with the publication of several examples of arrayed screens utilizing lipid delivery of synthetic crRNA:tracrRNA into Cas9-expressing cells that have demonstrated efficient editing and robust phenotypes (Anderson et al., 2015; Kelley et al., 2016; Tan and Martin, 2016). In addition, an effective arrayed screen has been reported using electroporation of synthetic crRNA:tracrRNA complexed with Cas9 protein into primary human T cells to identify new HIV host factors (Hultquist et al., 2016), clearly demonstrating that the one-gene-per-well arrayed approach in difficult-to-transfect cells is also feasible.

Here we have performed an arrayed, synthetic crRNA:tracrRNA screen with four crRNAs per gene targeting 169 genes (> 600 crRNAs) to interrogate the cell cycle process in a G1S reporter cell line. A high content assay was used in live cells to measure multiple parameters and identify cells with different cell cycle phenotypes. We first describe the HCA analysis workflow and the statistical method used for hit identification. Most hits had multiple positive crRNAs per target gene, enabling identification of target genes with high confidence, even for hits that displayed more moderate phenotypes. We used multiple strategies including gene expression analysis, confirmation of genomic insertions and deletions (indel), and validation by siRNAs to identify high confidence target genes with roles in cell cycle regulation. This study demonstrates the power of combining synthetic crRNAs libraries with HCA assays in screening for complex cellular phenotypes in an arrayed format.

2. Material and methods

2.1. Generation of G1S-CCPM-Cas9 stable cells

G1S-CCPM cells (GE Healthcare Life Sciences, Cat #67-6101-49, discontinued) were cultured in McCOYS 5A medium (GE Healthcare HyClone, #SH.30200.01) with 10% FBS, 1% L-Glutamine and 500 µg/mL Genetecin (Gibco, Ref 10131-027) and seeded at 100,000 cells per well in a 24-well plate (Costar, #3524). The cells were transduced at an MOI of 0.1 on the following day with 1.5 µL of Edit-R Lentiviral Blast-Cas9 Nuclease Particles (GE Healthcare Dharmacon, #VCAS10129) with a functional lentiviral titer of 1×10^7 lentiviral particles/mL. The cells were reseeded into a 6-well plate (Nunc, #140675) 72 hours post-transduction, and 48 h later, exposed to 10 µg/mL of blasticidin (InvivoGen, #ant-bl-05) for selection for two weeks. Cells that survived the selection were collected as a population and expanded to bank enough cells for the entire screen; these cells are referred to as G1S-CCPM-Cas9.

2.2. Transfection

G1S-CCPM-Cas9 cells were seeded in black, Greiner 96-well plates (Sigma-Aldrich, #M0562-32EA) at 2500 cells per well. The following day, the Human Edit-R Cell Cycle Regulation library stock plates (GE Healthcare Dharmacon, #GC-003200-01; Supplemental Table 1) and tracrRNA (GE Healthcare Dharmacon, #U-002000-50) were resuspended with 10 mM Tris–HCl buffer pH 7.4 buffer (GE Healthcare Dharmacon, #B-006000-100) to 10 µM. Daughter plates at 1 µM were generated from the crRNA library stock plates. The tracrRNA was further diluted to 333 nM with MEM-RS medium (GE Healthcare HyClone, #SH30564.01). For transfection in triplicate tissue culture plates, a deep-well plate was prepared with 30 µL of diluted tracrRNA (333 nM) and 10 µL of 1 µM crRNA per deep-well. DharmaFECT 4 transfection reagent (GE Healthcare Dharmacon, #T-2004-03) was diluted with MEM-RS medium (25 µL of DharmaFECT 4 transfection reagent were added to 4.98 mL of MEM-RS medium). Diluted transfection reagent (40 µL) was added to the crRNA:tracrRNA in the deep-well plates and incubated at room temperature for 20 min. After incubation, 320 µL of full growth medium was added per well to obtain a complete transfection mixture. The medium on the plated cells was replaced with 100 µL of medium with complete transfection mixture (final concentration 0.05 µL of the DharmaFECT 4 transfection reagent and 25 nM of the crRNA:tracrRNA). For repeat transfection of identified hits, crRNAs were reordered as a cherry-pick library (See Supplemental Table 2 for sequences and catalog numbers). For the siRNA transfections, individual ON-TARGETplus siRNAs were transfected per the manufacturer's protocol such that the final siRNA concentration was 25 nM and DharmaFECT 4 transfection reagent was at 0.05 µL per well of cells (See Supplemental Table 2 for catalog numbers).

2.3. Mismatch detection assay using T7EI

At 48 hours post-transfection, cells were lysed in a buffer containing proteinase K (Thermo Scientific, #FEREO0492), RNase A (Thermo Scientific, #FEREN0531), and Phusion HF buffer (Thermo Scientific, #F-518L) for 30 min at 56 °C, followed by a 5 min heat inactivation at 95 °C. PCR was completed with primers flanking the cleavage site of each gene, after which reannealing of the PCR products was performed (See Supplemental Table 3 for primer sequences). T7EI (New England Biolabs, #M0302L) was added to the PCR amplicons and incubated for 25 min at 37 °C to cleave mismatch strands. The T7EI cleavage products were separated on a 2% ethidium bromide-containing agarose gel. Gels were visualized under UV light and imaged for band intensity using ImageJ. The percent editing for each sample was estimated using the following calculation (Cong et al., 2013):