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A lentivirus-free inducible CRISPR-Cas9 system for efficient targeting of human genes

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ABSTRACT

CRISPR-Cas9 is a cutting-edge tool for modifying genomes. The efficacy with which Cas9 recognizes its target has revolutionized the engineering of knockouts. However this efficacy complicates the knocking out of important genes in cultured cells. Unedited cells holding a survival advantage within an edited population can confound the knockout phenotype. Here we develop a HeLa-based system that overcomes this limitation, incorporating several attractive features. First, we use Flp-recombinase to generate clones stably integrated for Cas9 and guide RNAs, eliminating the possibility of unedited cells. Second, Cas9 can be induced uniformly in the clonal cultures using doxycycline to measure the knockout phenotype. Third, two genes can be simultaneously knocked out using this approach. Finally, by not involving lentiviruses, our method is appealing to a broad research audience. Using this methodology we generated an inducible *AGO2*-knockout cell line showing normal RNA interference in the absence of doxycycline. Upon induction of Cas9, the *AGO2* locus was cleaved, the AGO2 protein was depleted, and RNA interference was compromised. In addition to generating inducible knockouts, our technology can be adapted to improve other applications of Cas9, including transcriptional/epigenetic modulation and visualization of cellular DNA loci.

Introduction

The CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) system found in several bacteria and archaea embodies an adaptive immune mechanism that relies on faithful recognition of specific nucleic acid sequences [1–9]. Detailed investigation of the mechanism by which the CRISPR-Cas system recognizes and degrades foreign DNA sequences has fueled the emergence of a new era of genome editing [10–15]. Several clades of CRISPR-Cas mediated interference systems exist, yet Cas9 from *Streptococcus pyogenes* (SpCas9) is the most extensively used member of the Cas9 endonuclease family [16–19]. Although the natural function of SpCas9 (hereby referred to as Cas9) is to cleave infecting phage DNA, Cas9 and its accessory RNA elements have been engineered to recognize and/or cleave DNA, both *in vitro* [13,14] and in several eukaryotic model organisms [20]. Cas9 is an RNA-guided DNA endonuclease that forms a complex with a pair of RNA molecules: a guide or CRISPR RNA (crRNA) and an accessory trans-activating CRISPR RNA (tracrRNA). This ribonucleoprotein complex binds to the genomic target via Watson-Crick base pairing through information provided by the guide RNA, and Cas9 cleaves the double-stranded (ds) DNA target. For genome-editing applications, the crRNA and the tracrRNA can be fused to create a single RNA molecule termed as sgRNA [13]. The superior ability of Cas9 to recognize its DNA target, in even the most complex of eukaryotic genomes, is what qualifies Cas9 as a powerful tool for editing genomes. In addition to representing an excellent advancement in biotechnology, CRISPR-Cas9 also holds immense promise for the cure of genetically defined diseases that remain intransigent to other forms of therapy [21,22].

Although the potential of CRISPR-Cas9 in genome editing is clear, there are several other important applications of this technology. Many such applications utilize a version of Cas9 that is catalytically dead (dCas9), but fully capable of binding the DNA target in an sgRNA-dependent manner [23]. For example, dCas9 can be directed to promoters for regulating gene expression [24–28].

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dCas9 can also be tethered to chromatin-modifying enzymes to affect site-specific epigenetic changes [29]. Furthermore, dCas9 fused to fluorophores such as GFP may be used to directly visualize specific DNA sequences in living (and fixed) cells [30]; a technique which previously required cell fixation-based fluorescence *in situ* hybridization (FISH) approaches.

Establishment of gene knockouts depends on error-prone non-homologous end joining (NHEJ) of ds DNA breaks created by Cas9. Given that the major determinant of knockout efficiency is activity of Cas9, it is not surprising that this enzyme has been successful in knocking out genes in various biological contexts [31]. Although the ability of CRISPR-Cas9 technology to efficiently knockout genes is an extremely attractive characteristic, it also poses disadvantages compared to existing RNA-knockdown technologies. For example, attempts to knock out an essential gene in cultured human cells will result in selection for cells/clones that are unedited (or edited but still preserve gene function). Therefore, the measured cellular phenotype/s of the surviving cells will not be representative of a true gene knockout. Inducing Cas9 uniformly in all cultured cells can circumvent this problem and allow for the detection of an immediate knockout phenotype. Indeed, there are methods to induce Cas9 both in mouse tissues [32] and human induced pluripotent (iPS) cells [33]. However these methods require injection/transfection/transduction of guide RNAs into the cell population, leading to the caveat mentioned above. Specifically, in the case of Cas9 targeting essential genes, cells that do not receive guide RNAs and thus remain unedited have a potential growth advantage over edited cells. Finally, most of the published methods involve lentiviral-based approaches for delivering Cas9 and/or the guide RNAs [34,35]. Although advantageous in several respects, lentiviral approaches require stricter biosafety considerations that pose an additional obstacle for laboratories that are not equipped/approved to conduct such experiments.

Given that the HeLa cell line remains the most popular experimental tool for studying human gene function in cell culture [36], we set out to develop a robust methodology to apply the CRISPR-Cas9 system in this cell line. We developed a system with the ability to: (i) generate clones integrated stably with single copies of *Cas9* and guide RNA genes to eliminate the possibility of unedited cells; (ii) prevent Cas9 induction and allow for the propagation/storage of clones until the time of the knockout experiment; (iii) knockout multiple genes simultaneously; (iv) provide a simple, transient transfection-based, lentivirus-free protocol; and (v) provide an economical method for gene disruption obviating the need for repeated use of siRNA/guide RNA or transfection reagents. By using a combination of a HeLa-based clonal cell line [HeLa EM2-11 ht [37]] and a new vector that we describe in this study (pG1G2-FLAG-Cas9-F3), we have developed a method that successfully fulfills all these criteria. As proof-of-principle, we generated an inducible knockout of the *AGO2* gene in HeLa cells, and successfully shutdown RNA interference as a function of Cas9 induction. Our choice of *AGO2* knockout was driven by our inability to generate clones of this knockout using standard CRISPR-Cas9 protocols, possibly because of the importance of RNAi for cell growth and function. We believe that our newly developed system holds promise not only for the gene-editing functions of Cas9, but also for the numerous other applications of Cas9 that have emerged.

Materials and methods

Reagents and kits for molecular biology

Oligonucleotides for PCR priming, Cas9 guide RNA cloning, and Sanger sequencing were purchased from Integrated DNA Technologies. All restriction enzymes were purchased from New England Biolabs (NEB). Purification of plasmid DNA and other cloning intermediates was performed using DNA purification kits from Qiagen. Genomic DNA from cultured human cells was isolated using the GenElute Mammalian Genomic DNA Miniprep kit from Sigma. PCR reactions for cloning purposes were performed with either Pfu Turbo DNA polymerase (Agilent) or Phusion High-Fidelity DNA Polymerase (NEB) using the manufacturers' protocols. Ligations of DNA vectors with inserts were performed using the Quick Ligation Kit (NEB). Calf intestinal alkaline phosphatase (CIP) for removing the 5'-phosphate of vectors prior to ligation was purchased either from Promega or from NEB. Site-directed mutagenesis was performed using QuikChange II (Agilent). Reagents for CRISPR-Cas9 experiments are described separately below.

Sanger sequencing

The DNA sequences of all inserts as well as plasmid regions involved in site-directed mutagenesis were verified using Sanger sequencing conducted at the University of Michigan DNA Sequencing Core.

Parental plasmids

The bicistronic vector pX330-U6-Chimeric_BB-CBhhSpCas9 for site-specific genome editing in cultured human cells was a kind gift from Dr. Feng Zhang, Broad Institute of MIT and Harvard, McGovern Institute for Brain Research, and Departments of Brain and Cognitive Sciences and Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA (Addgene plasmid # 42230) [31], and was obtained upon signing a material transfer agreement (MTA). The pX333 vector that allows for cloning of two tandem U6 promoter-driven guide RNAs was a kind gift from Dr. Andrea Ventura, Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, New York, NY (Addgene plasmid # 64073) [38], and was obtained upon signing an MTA. The pBI-F3-miRNA-d1GFP-loxP-F (abbreviated here as pBi-F3) and pBI4-miRNA-d1GFP (abbreviated here as pBi4) plasmids were obtained from TET Systems GmbH & Co. KG, Heidelberg, Germany, upon signing an MTA, and have been described previously [37,39,40]. The FLAG-TPP1 plasmid and the plasmid encoding an shRNA that targets the gene coding for human TPP1 have been described previously [39,41].

Cloning of the g1/g2-inducible Cas9 F3 vector

To obtain a FLAG-Cas9 cassette driven by a doxycycline (dox)-inducible promoter, the pBI4-miRNA-d1GFP vector (6.4 kb) was first digested with *Not*I and *Xho*I. The fragment containing the dox-inducible promoter was treated with CIP and ligated to the FLAG-Cas9 insert obtained by PCR amplification of the FLAG-Cas9 fragment in the pX330 vector (containing codon-optimized *Streptococcus pyogenes Cas9* gene, a FLAG tag, and two nuclear localization sequences) to yield the pFLAG-Cas9-Bi4 plasmid.

To insert the tandem guide RNA expression cassette into the pBi-F3 backbone, the pBI-F3-miRNA-d1GFP-loxP-F vector was digested with *Bgl*II and *Stu*I. The ~5.4 kb fragment obtained after gel-purification of the digestion reaction was treated with CIP and ligated to the tandem guide RNA expression cassette insert amplified using PCR of the pX333 vector template with to yield the pG1G2-F3 plasmid.

To sub-clone the FLAG-Cas9 fragment in the pG1G2-F3 backbone, the pG1G2-F3 and pFLAG-Cas9-Bi4 vectors were separately digested with *Stu*I and *Hpa*I. The restriction digestion of pG1G2-F3 yielded a ~1.4 kb fragment and a ~4.9 kb fragment. The ~4.9 kb fragment isolated after gel purification was treated with CIP prior