Targeted gene editing by transfection of *in vitro* reconstituted *Streptococcus thermophilus* Cas9 nuclease complex

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**Dear Editor**

Cas9 protein of the Type II CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated)¹ bacterial adaptive immune system emerged recently as a promising tool for genome editing in human and other eukaryotic cells.²,³ Cas9 binds a dual crRNA (CRISPR RNA)-tracrRNA (trans-activating RNA) molecule, or an artificial single-guide RNA (sgRNA) into a functional complex that acts as an RNA-directed DNA endonuclease. It locates and binds to the target site guided by crRNA (sgRNA) while the Cas9 protein cuts DNA generating a double strand break (DSB) within the target sequence.⁴,⁵

In eukaryotic cells, DSB is repaired by “error prone” non-homologous end joining (NHEJ)⁶ or by homology directed repair (HDR)⁷ mechanisms resulting in the genome modification or insertion of new genetic information.⁸,¹⁰

Cas9 of *Streptococcus pyogenes⁵* (SpCas9) is currently used as a model system for genome editing applications. Typically, the DNA expression cassettes encoding nucleus-targeted codon-optimized Cas9 protein and sgRNAs are transfected into the cells.¹¹-¹³ The efficiency of DNA cleavage by plasmid-delivered Cas9 in eukaryotic cells depends on multiple factors, including expression vector design, transfection efficiency, cell type, recovery yield of functional Cas9 complex,¹⁴ and usually requires optimization of a set of experimental conditions. Cas9 delivery by plasmid transfection is still difficult to achieve for some hard-to-transfect cell lines including human primary cells and pluripotent stem cells.¹⁵,¹⁷

Moreover, plasmid transfection occasionally results in undesirable integration of vector plasmid into the genome and is often inefficient and stressful to cells.¹⁸ Here we report an alternative way for the Cas9-mediated genome modification in eukaryotic cells (Fig. 1A) by chemical transfection of *in vitro* reconstituted functionally active Cas9-crRNA-tracrRNA complex of *Streptococcus thermophilus* (StCas9) CRISPR3-Cas system.

The StCas9 protein bearing the nuclear localization signal (NLS) and 6xHis tag was purified from *E.coli*, and the StCas9 complex was reconstituted *in vitro* as described by Karvelis et al.¹⁹,²⁰ To enable the delivery of reconstituted StCas9 complex into CHO-K1 cells, transfection experiments were performed using a protein delivery agent TurboFect™. Alternatively, other transfection reagents like LipoFectamine® 2000 or 3000 can be used to transfect Cas9 complexes into cells (data not shown). StCas9 localization was monitored using mouse polyclonal anti-Cas9 antibodies along with FITC-labeled secondary antibodies; crRNA-tracrRNA duplex was detected via 3’-biotin labeled tracrRNA using streptavidin-coupled Qdots® 585 nm. Both StCas9 and tracrRNA were observed in the perinuclear region and within the nucleus indicating that *in vitro* pre-assembled StCas9 complexes can be efficiently delivered into mammalian cells for targeted genome modification (Fig. S1).

To monitor the DNA cleavage activity of transfected Cas9 complexes in mammalian cells, we constructed a dual reporter cassette bearing Red Fluorescent Protein (RFP) and enhanced Green Fluorescent Protein (eGFP) genes (Fig. 1B). eGFP gene contains 2 sites, T1 and T2, targeted by 2 different StCas9 complexes. The I-CreI nuclease²¹ target site was also engineered into the cassette. In the absence of Cas9, eGFP fluorescence should be observed following intron processing *in vivo*. Cas9 facilitated DSB at T1 or T2

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target site should trigger DNA repair either through NHEJ or HDR. In case of NHEJ, mutations within the eGFP gene would result in lost or diminished eGFP fluorescence. HDR, on the other hand, should result in the RFP expression due to reassembly of the RFP gene via the engineered homology arms, and enable quantification of HDR efficiency within the population of transfected cells. Integration of the dual reporter cassette into a plasmid vector generated a reporter...
amplon reannealing is required. Taken together these results demonstrate that in vitro reconstituted Cas9 complex delivered by transfection promotes generation of DSB at the target site; subsequent repair by NHEJ produces indels and inactivates the eGFP gene.

To find out whether HDR contributes to the DSB repair in the reporter plasmid, we looked at the appearance of RFP+ cells: RFP was expressed in 5–8% of cells transfected with T1 or T2 targeting StCas9 complexes, whereas no red cells were detected in cultures transfected with non-targeting Cas9 complexes (Fig. 1C). The presence of RFP positive cells was subsequently confirmed by fluorescence microscopy (Fig. 1D).

For further analysis of StCas9 cleavage activity on the chromosomal DNA we used the CHO-K1 cell line with a dual reporter cassette (Fig. 1B) integrated into the genome. The weak eGFP fluorescence signal in the engineered cell line hindered a reliable quantification of the Cas9 cleavage efficiency by monitoring the decrease of the eGFP signal by flow cytometry. The Surveyor assay, however, revealed that indel values reached 2–6% for respective chromosomal target sites (Fig. 1G). Importantly, RFP+ cells were also identified by the fluorescent microscopy indicating that DSBs are also repaired by HDR (Fig. S4). FACS analysis confirmed that about 3–4% of cells expressed RFP in reporter cell cultures (Fig. 1F). Transfection of recombinant I-CreI nuclease yielded ~4.5% of RFP+ cells, indicating that similar cleavage efficiencies can be achieved by different recombinant nucleases delivered using chemical transfection (Fig. 1G). StCas9+ cleavage specificity was further verified by deep sequencing using MiSeq system (Illumina). NHEJ-mediated indels were centered about the target site validating the cleavage specificity (Fig. S5).

To extend the study of recombinant Cas9 potential in multiplex genome modulation, a series of experiments were carried out using HEK293T cells and StCas9 complexes specific for endogenous genes DNMT3B or PPIB. The cells were transfected with each gene targeting complexes separately (single transfection) or together (dual transfection). Surveyor assay revealed that for StCas9 complex indel values for genes DNMT3B or PPIB reached 7 and 10% in single transfections or 6 and 8% in dual transfections, respectively (Fig. 1H). Transfections with the pre-assembled SpCas9 complex yielded comparable indel (%) values (Fig. S6). Overall, this data validated that in vitro pre-assembled Cas9 complexes could be successfully used for genome modulation at different genomic loci and in addition demonstrated the potential of targeting multiple loci at the same time.

In conclusion, this study demonstrates for the first time the gene editing potential of in vitro reconstituted Cas9-crRNA-tracrRNA complex delivered by a chemical transfection agent. This finding expands the arsenal of available tools for direct delivery of Cas9 nuclease into cells and opens new avenues for targeted genome modification.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental data for this article can be accessed on the publisher’s website.
References

1. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. CRISPR provides acquired resistance against viruses in prokaryotes. Science (80- ). 2007; 315:1709-12; http://dx.doi.org/10.1126/science.1138140

2. Hsu PDD, Lander ESS, Zhang F. Development and Applications of CRISPR-Cas9 for Genome Engineering. Cell 2014; 157:1262-78; PMID:24906146; http://dx.doi.org/10.1016/j.cell.2014.05.010

3. Gasius G, Siksnys V. RNA-dependent DNA endonuclease Cas9 of the CRISPR system: Holy Grail of genome editing? Trends Biochem 2013; 21:562-6; PMID:24095303; http://dx.doi.org/10.1016/j.tim.2013.09.001

4. Gasius G, Barrangou R, Horvath P, Siksnys V. Cas9 crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc Natl Acad Sci U S A 2012; 109:E2579-86; PMID:22949671; http://dx.doi.org/10.1073/pnas.1208507109

5. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012; 337:816-21; PMID:22745249; http://dx.doi.org/10.1126/science.1232033

6. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem 2010; 79:181-211; PMID:20192759; http://dx.doi.org/10.1146/annurev.biochem.052308.093131

7. Moynahan ME, Jain M. Minocid homologous recombination maintains genomic stability and suppresses tumorigenesis. Nat Rev Mol Cell Biol 2010; 11:196-207; PMID:20177395; http://dx.doi.org/10.1038/nrm2851

8. Perez-Pinera P, Ousterout DG, Gersbach CA. Advances in targeted genome editing. Curr Opin Chem Biol 2012; 16:268-77; PMID:22819644; http://dx.doi.org/10.1016/j.cpb.2012.06.007

9. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Friedman G, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Rev Genet 2011; 12:636-46; PMID:21828278; http://dx.doi.org/10.1038/nrg2842

10. Carroll D. Genome engineering with zinc-finger nucleases. Nat Biotechnol 2010; :1-8; PMID:20192759; http://dx.doi.org/10.1038/nrg2842

11. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. Science 2013; 339:823-6; PMID:23287722; http://dx.doi.org/10.1126/science.1232033

12. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013; 339:819-23; PMID:23287718; http://dx.doi.org/10.1126/science.1231143

13. Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. Elife 2013; 2:e00471; PMID:23386978; http://dx.doi.org/10.1016/j.elseid.2013.09.001

14. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agerwala V, Li Y, Fine EJ, Wu X, Shalem O, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 2013; :1-8; PMID:2330209

15. Li K, Wang G, Andersen T, Zhou P, Pu WT. Optimization of Genome Engineering Approaches with the CRISPR/Cas9 System. PLoS One 2014; 9:e105779; http://dx.doi.org/10.1371/journal.pone.0105779

16. Kim TK, Eberwine JH. Mammalian cell transfection: the present and the future. Anal Bioanal Chem 2010; 402:2469-76; PMID:20608059; http://dx.doi.org/10.1007/s00216-010-3821-6

17. Yamano S, Dai J, Moursi AM. Comparison of transfection efficiency of nonviral gene transfer reagents. Mol Biotechnol 2010; 46:287-308; PMID:20585981; http://dx.doi.org/10.1007/b102435-010-9002-5

18. Gabriel R, Lombardo A, Ares S, Miller JC, Genovese P, Kaeppel C, Nowrouzi A, Bartholomae CC, Wang J, Friedman G, et al. An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nat Biotechnol 2011; 29:816-23; PMID:21822255; http://dx.doi.org/10.1038/nb.1948

19. Karvelis T, Gasius G, Mikys V, Barrangou R, Horvath P, Siksnys V. crRNA and tracrRNA guide Cas9-mediated DNA interference in Streptococcus thermophilus. RNA Biol 2013; 10:841-51; PMID:23553277; http://dx.doi.org/10.4161/rna.24203

20. Karvelis T, Gasius G, Siksnys V. Programmable DNA cleavage in vivo by Cas9. Biochem Soc Trans 2013; 41:1401-6; PMID:24256227

21. Jurica MS, Monnat RJ, Stoddard BL. DNA recognition and cleavage by the LAGLIDADG homing endonuclease I-CreI. Mol Cell 1998; 2:469-76; PMID:9809068; http://dx.doi.org/10.1016/S1097-2765(00)80146-X

22. Guschin DY, Waite AJ, Katibah GE, Miller JC, Genovese P, Kaeppel C, Nowrouzi A, Bartholomae CC, Wang J, Friedman G, et al. An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nat Biotechnol 2011; 29:816-23; PMID:21822255; http://dx.doi.org/10.1038/nb.1948

23. Kim S, Kim D, Cho SW, Kim J, Kim J-S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res 2014; 24:1012-9; PMID:24696461; http://dx.doi.org/10.1010/g3.113

24. Ramakrishna S, Kwaku Dad A-B, Beloor J, Gopalappa R, Lee S-K, Kim J. Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. Genome Res 2014; 24:1020-7; PMID:24696462; http://dx.doi.org/10.1101/gr.171264.113