Published in final edited form as:

Nat Biotechnol. 2016 November 28; 35(1): 17–18. doi:10.1038/nbt.3753.

Failure to detect DNA-guided genome editing using *Natronobacterium gregoryi* Argonaute

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DNA-guided DNA cleavage using Argonaute-family proteins was previously reported but of limited practical utility due to the requirement for supraphysiological conditions, such as high temperature^{1,2}. Recently, Gao *et al.*³ reported an Argonaute protein isolated from *Natronobacterium gregoryi* (NgAgo) as a genome engineering tool for editing the human genome. After transfecting human cell lines with a plasmid DNA encoding NgAgo and a 24nucleotide, 5'-phosphorylated single-strand guide DNA (gDNA), they showed gene editing at endogenous targets. Here, we report the results of three different groups (Cathomen *et al.*, Supplementary Fig. 1; Ekker *et al.*, Supplementary Fig. 2; and Kim *et al.*, Supplementary Fig. 3), independently attempting to reproduce the original findings of Gao *et al.*³, specifically focusing on the evidence for DNA edits in cultured human cell lines. We synthesized the same 5'-phosphorylated gDNA sequences and used the NgAgo vector provided by Gao *et al.*³ via Addgene (Cambridge, MA) to transfect the same cell lines, and analyzed the genomic DNA for signs of gene editing. Controls confirmed efficient delivery

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Competing financial interests

S.C.E. is a co-Founder of Lifengine Technologies, Inc.; T.C. is a consultant for TRACR Hematology; J.-S.K. is a co-founder and shareholder of ToolGen, Inc.

of both plasmid DNA and gDNAs as well as expression of the NgAgo protein. Despite various attempts to optimize NgAgo-mediated genome editing in three of the reported cell lines, no evidence of successful editing of endogenous target sequences was detected.

Gao *et al.*³ used an episomal enhanced green fluorescent protein (EGFP) marker gene as a target to optimize the NgAgo platform. We used the same experimental setup to disrupt an episomal EGFP expression plasmid in HeLa and HEK293T cells, but failed to detect any statistically significant decrease in EGFP expression (Supplementary Fig. 1a). Moreover, we were not able to detect any significant disruption of an integrated EGFP marker gene in human U2OS cells using either the NgAgo-encoding plasmid obtained from Addgene or another plasmid, generated by subcloning the NgAgo sequence in a mammalian expression vector (Supplementary Fig. 1b,c). In contrast, cells transfected with plasmids encoding an RNA-guided nuclease based on SpCas9 and a specific guide-RNA (gRNA) decreased EGFP expression 14-fold in the episomal assay in HeLa and HEK293T (not shown) cells and knocked out EGFP in ~80% of transfected U2OS cells, demonstrating efficient delivery of, and endonuclease expression from, plasmid DNA in these cell lines.

Gao *et al.*³ demonstrated the ability of NgAgo to robustly edit the human genome under physiological conditions by testing several targets on *DYRK1A* gene in four human cell lines.³ Of these four, we used HEK293, HeLa and K562 cells and transfected them with NgAgo expression plasmid and five gDNAs corresponding to G5, G6, G10, G12 and G13 in the original report. To test for NgAgo-induced gene editing, genomic DNA was extracted from transfected cells, PCR amplified and subjected to TIDE (tracking of indels by decomposition) analysis⁴. In all samples (N=3 per gDNA, for a total of 45 samples), analysis of the DNA chromatogram failed to show any evidence of robust DNA sequence alterations above the detection threshold (2%) (Supplementary Fig. 2a). Given that a gene editing efficiency of >20% was reported with NgAgo for these guides in the original report³, this detection threshold would be sufficient to identify comparable gene editing activity. Assuming a normal distribution of the efficiency of generating insertion/deletion (indel) mutations in these samples, the probability that none of these would show any evidence for *de novo* indels is $p < 0.0001$. In addition, parallel sequence analysis of 45 subcloned colonies from HeLa cell samples also failed to show any evidence of indels (data not shown). These independent replicates fail to detect any NgAgo-induced editing despite clear evidence of cellular delivery of each of the listed components for this system.

Gao *et al.*³ reported successful gene editing on other human loci, including *EMX1*, *GATA4*, and *GRIN2B* (Supplementary Fig. 3a). We delivered gDNA and NgAgo expression plasmids to HEK293 cells or HeLa cells either by lipofection (Supplementary Fig. 3b–e) or electroporation (Supplementary Fig. 3f–j). We also added Mg^{2+} to the culture medium because NgAgo requires this cation for catalytic activity (Supplementary Fig. 3k). T7E1 (T7 endonuclease I) assay⁵ and targeted deep sequencing (NCBI SRA: SRX2161446) was applied to detect NgAgo-induced mutations. NgAgo failed to generate indels at any of the four analyzed loci (*DYRK1A*, *EMX1*, *GATA4* and *GRIN2B*), with frequencies above sequencing error rates (~0.1%). Moreover, we were not able to detect signs of gene editing at the *CCR5* locus neither by T7E1 assay⁵ nor by TIDE analysis⁴ (Supplementary Fig. 1c,d).

In contrast, SpCas9 nucleases, which were used as controls in these experiments, induced indels at frequencies that ranged from 3% to 73% (Supplementary Figs 1d, 3b–k).

To confirm that the used gDNA molecules were chemically phosphorylated at the 5' end, mass spectrometry (Supplementary Fig. 3m) was performed. To confirm efficient co-delivery of plasmid DNA and the putative guide DNA, we co-transfected 3'-Alexa Fluor 594-tagged gDNA bearing the same targeting sequence as G5 along with the EGFP expression plasmid (pEGFP-N1) driven by a cytomegalovirus (CMV) promoter. As the expression of NgAgo modified at its N terminus with a nuclear localization signal (NLS; NLS-NgAgo) is also driven by a CMV promoter, we used EGFP expression as a surrogate marker of NgAgo in these cells. At 4, 12, and 24 hours post-lipofection, HeLa cells and HEK293 cells were fixed with paraformaldehyde and the coverslips mounted on slides. Strong red fluorescence corresponding to Alexa Fluor 594 was observed inside the cells by 4 hours and persisted for at least 24 hours (Supplementary Fig. 2b). The distribution of red fluorescence was diffuse within cells at 4 hours, but later became more punctated. As expected, some EGFP fluorescence was observed by 4 hours, with a much stronger signal detected at 12 and 24 hours posttransfection. Delivery of NgAgo plasmid was also confirmed by PCR assays (Supplementary Fig 2c). To confirm expression of NgAgo in transfected cells, the extent of RNA or protein expression was determined either by qualitative reverse transcription PCR (RT-PCR; Supplementary Fig. 1e), immunoblot analysis of hemagglutinin (HA)-tagged versions of NgAgo (Supplementary Figs. 1f,3n), or flow cytometry of a DsRed-tagged version of NgAgo (Supplementary Fig. 1f). Taken together, these data suggest that plasmid DNA and gDNA are efficiently delivered to the human cell lines used here and that all the tested cells are capable of expressing NgAgo.

On the basis of the above data, we conclude that in conditions designed to replicate those in Gao *et al.*³, co-delivery of plasmid DNA encoding NgAgo and a 5'-phosphorylated single-strand gDNA alone is insufficient to induce gene editing at the indel frequencies in cultured human cells reported in the original study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all members of our labs who made valuable contributions in trying to establish NgAgo-based genome editing.

This work was supported by grants from the German Federal Ministry of Education and Research (BMBF-01EO0803 to T.C.), American Heart Association (16PRE30470004 to H.A.), the National Institutes of Health (GM63904 to S.C.E.), the Mayo Foundation (to H.A., S.N., Z.L., and S.C.E), and the Institute for Basic Science (IBS-R021-D1 to J.-S.K).

References

1. Swarts DC, et al. DNA-guided DNA interference by a prokaryotic Argonaute. *Nature*. 2014; 507:258–261. [PubMed: 24531762]
2. Swarts DC, et al. Argonaute of the archaeon *Pyrococcus furiosus* is a DNA-guided nuclease that targets cognate DNA. *Nucleic Acids Res*. 2015; 43:5120–5129. [PubMed: 25925567]

3. Gao F, Shen XZ, Jiang F, Wu Y, Han C. DNA-guided genome editing using the *Natronobacterium gregoryi* Argonaute. *Nat Biotechnol.* 2016; 34:768–773. [PubMed: 27136078]
4. Brinkman EK, Chen T, Amendola M, van Steensel B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* 2014; 42:e168. [PubMed: 25300484]
5. Kim HJ, Lee HJ, Kim H, Cho SW, Kim JS. Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res.* 2009; 19:1279–1288. [PubMed: 19470664]