Title

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Journal

Proceedings of the National Academy of Sciences of the United States of America, 112(51)

ISSN

0027-8424

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Publication Date

2015-12-22

DOI

10.1073/pnas.1520883112

Peer reviewed

Synthetic CRISPR RNA-Cas9–guided genome editing in human cells

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Contributed by Don W. Cleveland, October 26, 2015 (sent for review September 13, 2015)

Genome editing with the clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 nuclease system is a powerful technology for manipulating genomes, including introduction of gene disruptions or corrections. Here we develop a chemically modified, 29-nucleotide synthetic CRISPR RNA (scrRNA), which in combination with unmodified transactivating crRNA (tracrRNA) is shown to functionally replace the natural guide RNA in the CRISPR-Cas9 nuclease system and to mediate efficient genome editing in human cells. Incorporation of rational chemical modifications known to protect against nuclease digestion and stabilize RNA–RNA interactions in the tracrRNA hybridization region of CRISPR RNA (crRNA) yields a scrRNA with enhanced activity compared with the unmodified crRNA and comparable gene disruption activity to the previously published single guide RNA. Taken together, these findings provide a platform for therapeutic applications, especially for nervous system disease, using successive application of cell-permeable, synthetic CRISPR RNAs to activate and then silence Cas9 nuclease activity.

genome editing | CRISPR/Cas9 | synthetic crRNA | scrRNA

The bacterial type II clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated (Cas) system is composed of a dual RNA-guided Cas endonuclease complex that is capable of sequence-specific nucleic acid cleavage (1–3). The CRISPR-Cas system was discovered in bacteria and is a natural defense mechanism to protect against invading pathogens (3–5). In the type II system, the Cas9 protein recognizes the complex of a 42-nucleotide CRISPR RNA (crRNA), which provides DNA specificity by Watson–Crick pairing with the sequence adjacent to a *p*rotospacer *a*djacent *m*otif (PAM) and an 80-nucleotide transactivating crRNA (tracrRNA), which binds to crRNA (6). These dual RNA molecules bind to Cas9 protein, and the three-component complex has been shown to mediate site-specific DNA double-stranded breaks in vitro and in mammalian cells. A single 102-nucleotide guide RNA (sgRNA), constructed as a fusion of crRNA and tracrRNA, was shown to enhance double-stranded break activity compared with the initial two RNA system (6–8). In mammalian cells the ensuing double-stranded break is repaired either by mutagenic nonhomologous end joining (NHEJ), a process that results in insertions or deletions (indels) leading to gene disruptions, or by homologous recombination where introduction of an exogenous donor template can result in precise insertion of a user-defined sequence. The CRISPR-Cas9 system is advantageous over other engineered nucleases including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) because of its ease of use, low cost, multiplexing capabilities, and equal or greater on-target DNA cleavage activity (8, 9).

Genome editing using ZFNs, TALENs, or CRISPRs has transformed biomedical research because of the unique ability to manipulate expression of mammalian proteins and RNAs through gene disruption and tagging as well as modulation of gene expression (10–14). Many in vitro successes of engineered nucleases, especially with the multiplexing capabilities of CRISPR-Cas9, stimulated proof-of-principle studies in animal models (12, 15–17), including the ability to produce mice in which modification of both alleles of a target gene can be made in one generation (15). Additionally, a recently reported transgenic mouse in which the Cas9 protein is made constitutively provides a model for many biological applications upon delivery of sgRNA [e.g., with adeno-associated virus (AAV)-mediated gene delivery and subsequent transcription to produce the sgRNA] (18, 19). Nevertheless, despite early successes in animal models, application of genome editing is still nascent as a potential therapeutic approach in humans (20) with practical, ethical, and safety concerns still to be solved (21). One of the technical challenges is delivery of the Cas9 protein and guide RNA(s) to recipient cells (17). Recently, Hendel et al. (22) demonstrated that chemically synthesized 100-mer sgRNAs with minimal modifications can be used for genome editing in vitro by cotransfection with either a DNA plasmid or mRNA encoding Cas9. Other challenges include identifying and limiting potential off-target cleavage (23–27) and controlling persistent nuclease activity (24, 25, 28).

We now report development of a chemically synthesized, 29-nucleotide synthetic CRISPR RNA (scrRNA) suitable for transient, therapeutic delivery. Using rational chemical design, we identify scrRNA phosphorothioate (PS) backbone modifications and 2′-fluoro (2′-F), 2′-*O*-methyl (2′-*O*-Me) and *S*-constrained ethyl (cEt) substitutions that increase metabolic stability (29, 30) and binding affinity of scrRNA to tracrRNA while enhancing their ability (relative to unmodified crRNA) to mediate gene editing in human cells. These synthetic CRISPR RNAs provide a platform for therapeutic applications of genome editing, including use of two scrRNAs for mediating activation and then silencing of Cas9 nuclease activity in human cells.

Significance

Genome editing with nucleases that recognize specific DNA sequences is a powerful technology for manipulating genomes. This is especially true for the Cas9 nuclease, the site specificity of which is determined by a bound RNA, called a CRISPR RNA (crRNA). Here we develop a chemically modified, 29-nucleotide synthetic CRISPR RNA (scrRNA) and show that it can functionally replace the natural crRNA, producing enhanced cleavage activity at a target DNA site with apparently reduced off-target cleavage. scrRNAs can be synthesized in a commercially feasible manner today and provide a platform for therapeutic applications.

Author contributions: M.R., M.A.M., T.P.P., E.E.S., C.F.B., and D.W.C. designed research; M.R. and M.A.M. performed research; M.R., M.A.M., and T.P.P. contributed new reagents/analytic tools; M.R., M.A.M., T.P.P., E.E.S., C.F.B., and D.W.C. analyzed data; and M.R., M.A.M., T.P.P., E.E.S., C.F.B., and D.W.C. wrote the paper.

Conflict of interest statement: M.R., T.P.P., E.E.S., and C.F.B. are employees of Isis Pharmaceuticals, Inc. D.W.C. is a consultant for Isis Pharmaceuticals, Inc.

Freely available online through the PNAS open access option.

See Commentary on page 15536.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1520883112/-/DCSupplemental.

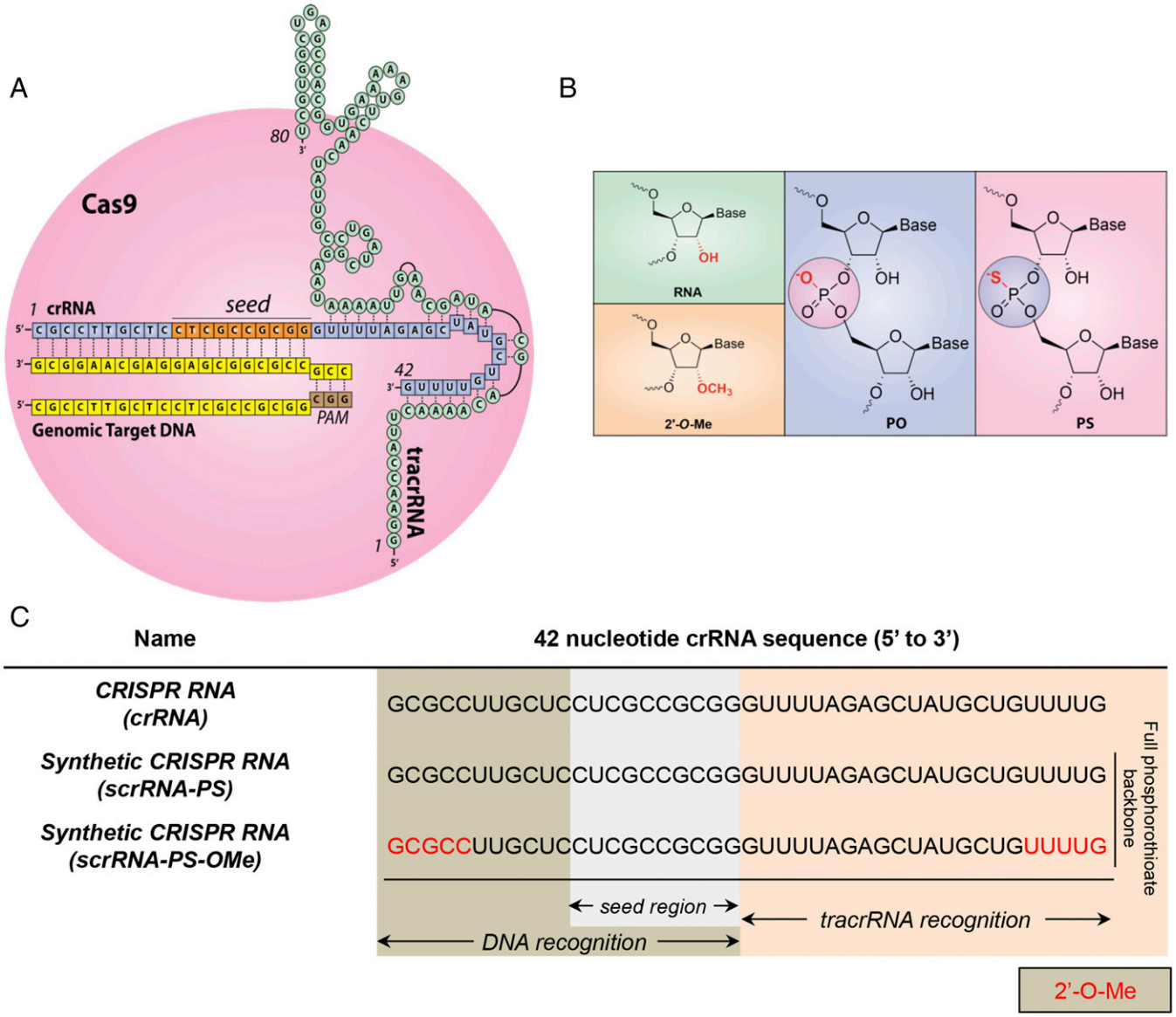


Fig. 1. Sequence recognition and structure of synthetic CRISPR RNA. (*A*) Schematic illustration of DNA recognition by CRISPR-Cas9. Cas9 (pink circle) recognizes the complex of crRNA (blue and orange squares) and tracrRNA (green circles) and binds to its complementary 20-nucleotide DNA target (yellow boxes) adjacent to a 3′-PAM (brown squares). The crRNA seed sequence (orange squares) is the 10 nucleotides that recognize DNA closest to the PAM sequence. Dashed lines between nucleotides indicate direct base pairing. (*B*) Structure of modified nucleotides incorporated into scrRNA. Native RNA (green box) is substituted at the sugar 2′-position with *O*-Me (orange box). The native phosphate backbone (blue box, pink circle) is substituted with a sulfur at a nonbridging oxygen (pink box, blue circle). (*C*) Full sequence of *LDLR*-specific crRNA unmodified (crRNA), complete PS substituted backbone (scrRNA-PS), or complete PS substituted backbone with 2′-*O*-Me (scrRNA-PS-OMe).

Results

Production of a scrRNA with Chemical Modifications for Targeted Gene Disruption in Human Cells. Activation of the natural CRISPR-Cas9 system requires hybridization of a 42-nucleotide crRNA to an 80-nucleotide tracrRNA with both RNAs bound to the Cas9 protein (Fig. 1*A*). RNA is highly unstable in vivo as a result of a variety of ribonucleases present in serum and tissues. Specific chemical modifications have greatly increased half-lives and allowed for their use in many therapeutic platforms (29, 30). For development of a scrRNA, we first tested the activities of Cas9 protein/tracrRNA when combined with crRNA or with a scrRNA in which PS modifications (31) were incorporated (i.e., substitution of sulfur for one of the nonbridging oxygen atoms in the phosphate backbone) (Fig. 1*B*). The PS modification is known to improve stability to nucleolytic degradation (31).

We designed synthetic CRISPR RNAs to human low-density lipoprotein receptor (*LDLR*) as either unmodified (crRNA) or PS backbone-modified (scrRNA-PS) (Fig. 1*C*). We first compared gene disruption of uniformly PS-modified scrRNA to native RNA (as outlined in Fig. 2*A*). Briefly, cells were transfected with plasmids expressing RNAs encoding Cas9 or tracrRNA. After 24 h, cells were subsequently transfected with either crRNA or scrRNA. CRISPR activity was assessed 48 h later by a Surveyor assay (12, 15–17, 32) where the genomic DNA from transfected cells was (*i*) used as a template to generate amplicons from the CRISPR target sequence (amplified by PCR); (*ii*) melted; (*iii*) slowly reannealed to allow formation of homo and heteroduplexes; (*iv*) incubated with the surveyor nuclease that recognizes and cleaves DNA mismatches (a result of insertions or deletions from repair of the double-stranded break by NHEJ);

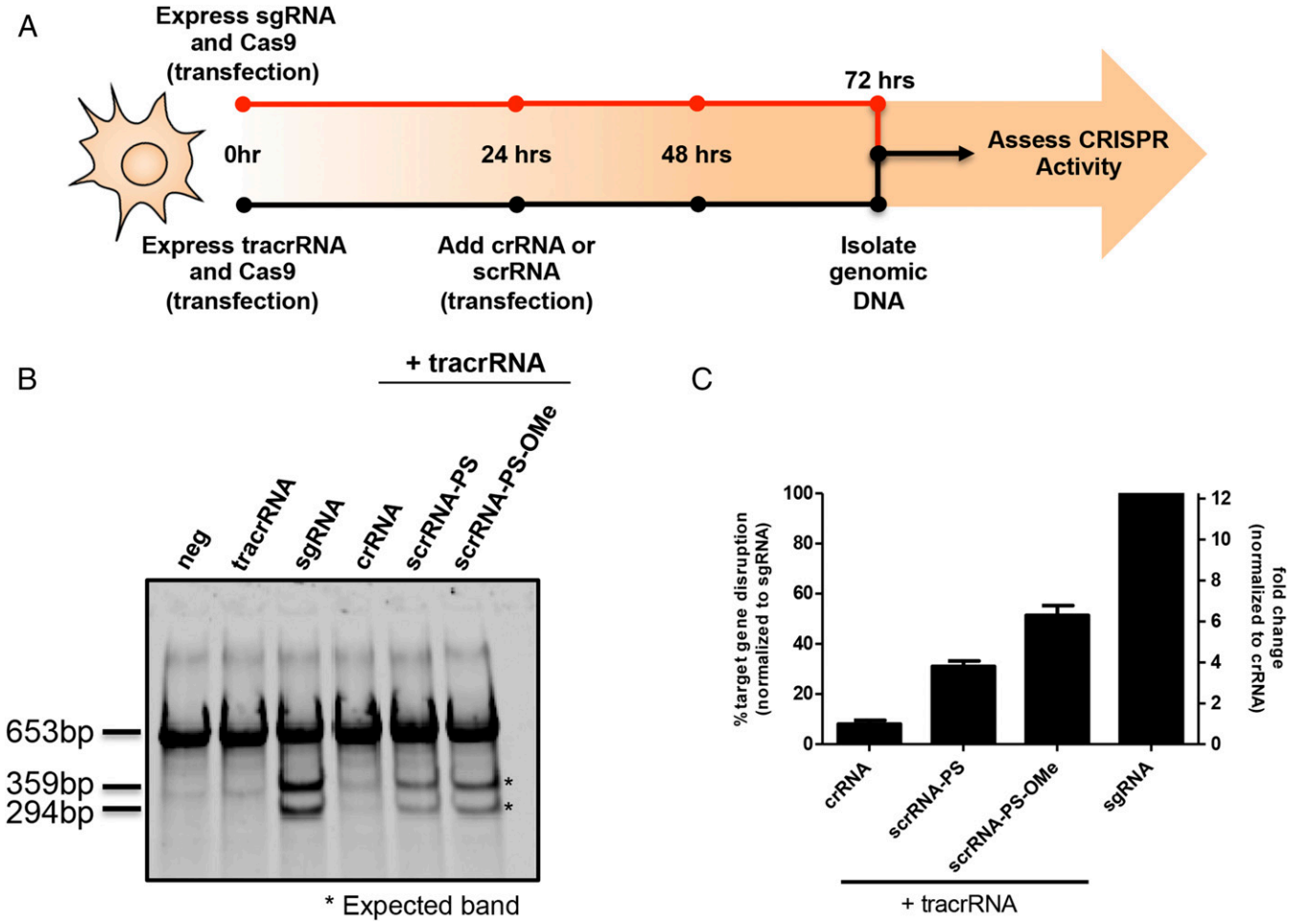


Fig. 2. scrRNA mediates gene disruption in human cells. (*A*) Schematic outlines the experimental design for assessing activity of scrRNA in cells where the red line is the time line for the parallel transcription-produced sgRNA transfection and the black line for crRNA or scrRNA transfection. (*B*) Representative surveyor nuclease assay of genomic DNA isolated from transfected cells as outlined in *A* where "neg" indicates no transfection and "tracrRNA" indicates transcription-produced Cas9 and tracrRNA alone. (*C*) Graph shows quantification of *B* normalized to sgRNA (100%).

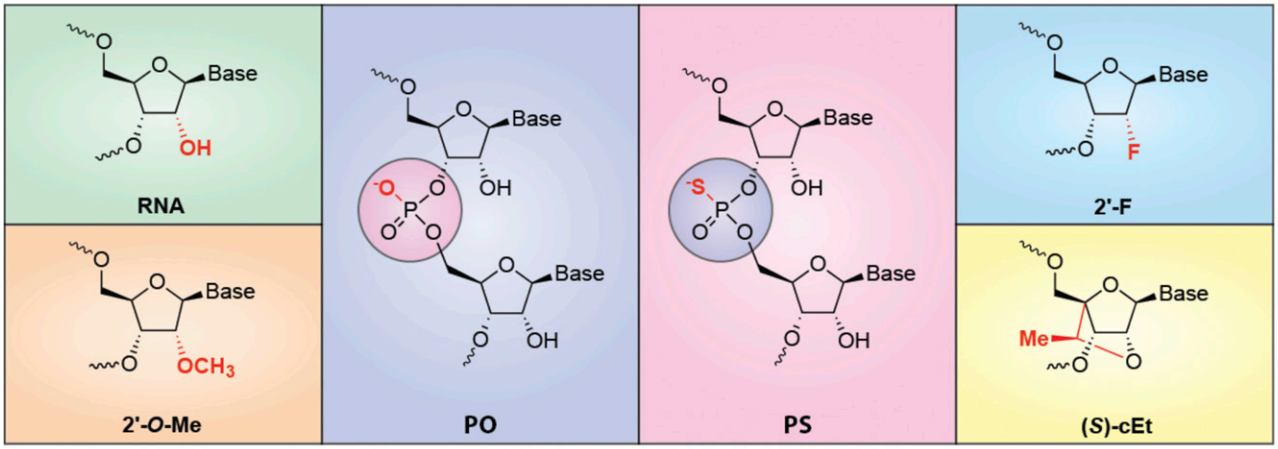
and (*v*) separated using standard electrophoresis (Fig. 2*B*). The efficiency of disruption of the *LDLR* alleles was measured by direct comparison with activity measured in a parallel assay 72 h after expression by DNA transfection of both the Cas9 protein and a highly active, 102-base sgRNA. scrRNA with scrRNA-PS showed significant gene disruption (33% of the level of cleavage by Cas9/sgRNA) and a greater than fourfold activity compared with unmodified crRNA (Fig. 2 *B* and *C*). (We note that this is a relative normalization to Cas9 activation produced by sgRNA, which is produced by continuous transcription from the active H1 promoter, whereas the scrRNA is limited by the amount of modified RNA transfected into cells.)

Several modifications at the 2′-position of the sugar ring, such as 2′-*O*-Me, forces RNA to adopt an energy-favorable conformation that increases Watson–Crick binding affinity due to the proximity of the 2′-substituent and the 3′-phosphate, thus improving nuclease resistance (33). We therefore generated scrRNA with five 2′-*O*-Me–modified nucleotides at both the 5′- and 3′-termini with a fully substituted phosphorothioate backbone (scrRNA-PS-OMe) (Fig. 1*C*). These combined modifications produced an additional improvement in CRISPR-Cas9–mediated gene disruption activity, yielding a sevenfold increase relative to unmodified crRNA and reaching nearly half (48%) of the activity achieved with sgRNA. As expected, no activity was observed in cells in the absence of crRNA/scrRNA or tracrRNA alone (Fig. 2*B*, lanes 1 and 2).

Additional 2′ Sugar Modifications to scrRNA Can Enhance Gene Disruption. Recognizing that our initial scrRNA demonstrated a sevenfold higher target gene disruption activity compared with native crRNA, we next tested if further enhanced activity could be produced with replacement of the 2′-OH of the sugar with a 2′-F group or replacement of the ribose sugar with the bicyclic nucleotide-cEt (Fig. 3*A*). Both of these modifications are known to increase binding affinity to RNA and DNA. The 2′-F binding is largely energetically driven by the electronegative substituent (33). Alternatively, affinity, as well as stability, can be increased with the use of constrained bicyclic analogs like the cEt substitution that links the 2′ and 4′ positions of the ribose sugar (34). Using the same experimental design as in Fig. 2*A*, we first tested whether scrRNA with an 2′-F substitution of a single position at the 5′- (F01), 3′- (F02), or both- (F03) termini mediated gene disruption, this time using scrRNAs targeted to the vascular endothelial growth factor A (*VEGF-A*) gene locus. All three 2′-F–modified scrRNA showed significant target gene disruption, retaining 18–29% of the activity shown by transcription-produced sgRNA (Fig. 3*B*; Figs. S1 and S2).

Because 2′-F–modified terminal nucleic acids supported gene disruption activity at the *VEGF-A* locus, we tested the effects of adding 10 consecutive 2′-F–modified nucleotides at the 5′-end of the scrRNA or 7 alternating 2′-F RNA modifications at the 3′-termini (Fig. 3*B*, F04 and F05). Activities were comparable but

A

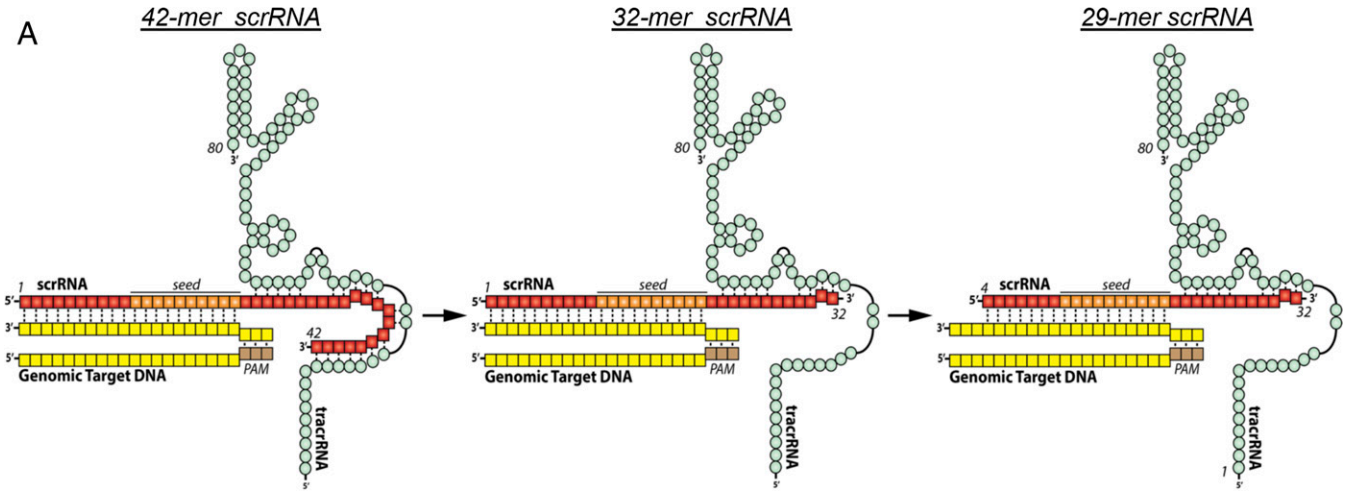


B

Name	scrRNA sequence (5' to 3')	% gene disruption (normalized to sgRNA)
F01	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUUG	19
F02	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUUG	29
F03	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUUG	18
F04	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUUG	14
F05	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUUG	18
F06	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUUG	<1
FC01	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUTG	75
FC02	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUTG	<1
M01	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUUG	40
FMC01	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUTG	65
FMC02	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAUGCUGUUUTG	<1

Fig. 3. 2′-F and cEt substitutions of scrRNA enhance NHEJ-mediated gene disruption. (*A*) Structures of modified nucleotides incorporated into scrRNA including 2′-*O*-Me, 2′-F, or cEt substitutions. (*B*) Full sequence of 42-mer *VEGF-A* targeting scrRNAs with substituted nucleotides indicated by green (2′-F), red (2′-*O*-Me), and blue (2′-cEt) letters. Far right column (% gene disruption) is quantification from surveyor nuclease assay using genomic DNA from transfected cells normalized to sgRNA (100%). Data represent average from two or more replicate experiments.

did not demonstrate substantial improvement over single 2′-F–modified scrRNAs. Introduction of three 2′-*O*-Me modifications at the 5′-end mediated gene disruption to ~40% of the sgRNA produced by transcription (Fig. 3*B*, M01).



B

Name	scrRNA sequence (5' to 3')	% gene disruption (normalized to sgRNA)
	32-mers	
FC-32-01	GGUGAGUGAGUGUGUGCGUGGUUUTAGAGCTA	42
FM-32-01	GGUGAGUGAGUGUGUGCGUGGUUUUAGAGCUA	<1
	29-mers	
FMC-29-01	GAGUGAGUGUGUGCGUGGUUUTAGAGCTA	64
FC-29-01	GAGUGAGUGUGUGCGUGGUUUTAGAGCTA	42
FC-29-02	GAGUGAGUGUGUGCGUGGUUUTAGAGCTA	107
FC-29-03	GAGUGAGUGUGUGCGUGGUTUTAGAGCTA	<1
MC29-01	GAGUGAGUGUGUGCGUGGUUUTAGAGCTA	71
C-29-01	GAGUGAGUGUGUGCGUGGUUUTAGAGCTA	67
MC-29-02	GAGUGAGUGUGUGCGUGGUTUTAGAGCTA	<1
C-29-02	GAGUGAGUGUGUGCGUGGUTUTAGAGCTA	<1
FM-29-01	GAGUGAGUGUGUGCGUGGUUUUAGAGCUA	<1

Fig. 4. Truncated scrRNAs mediate gene disruption comparable to transcription-produced sgRNA. (*A*) Step-wise synthesis of 32-mer scrRNA (3′ 10-nucleotide truncation of scrRNA) and 29-mer scrRNA (additional 5′ 3-nucleotide deletion of scrRNA). scrRNA is represented by red squares (seed sequence as orange squares) and tracrRNA by green circles. DNA is represented as yellow boxes. (*B*) Full sequence of *VEGF-A*–targeting truncated scrRNAs with colors indicating type of 2′ modification (as in Fig. 3). All truncated scrRNAs are full PS substituted backbone. The percentage of gene disruption is quantified from the surveyor nuclease assay using genomic DNA from transfected cells normalized to transcription-produced sgRNA (100%). Data represent average from two or more replicate experiments.

The 3′ region of the scrRNA is known to interact with tracrRNA; consequently, we hypothesized that addition of high-affinity cEt modifications at the 3′-end of scrRNA could improve gene disruption activity through the addition of modifications that stabilized RNA interactions. Indeed, we found that these substitutions added to either the 2′-F or 2′O-Me–modified scrRNA

further enhanced activity (Fig. 3*B*, FC01 and FMC01) with FC01 producing 75% of the gene-editing activity of the transcription-produced sgRNA and ∼65% for scrRNA FMC01 (Fig. 3*B*).

Recently, Jiang and colleagues (35) reported the crystal structure of Cas9 bound to sgRNA. From this, they determined that a 10-nucleotide RNA "seed" sequence that binds DNA nearest the PAM sequence is essential for Cas9 target DNA recognition. This finding suggests that mismatch or deletion in this region could greatly affect "on-target" activity. In addition to mismatches or deletions, we found that this region is sensitive to chemical modifications with enhanced hybridization affinity. scrRNAs with chemically modified nucleic acids in the seed region (F06, FC02, and FMC02) resulted in reduction of activity for cleavage at the *VEGF-A* locus to a level below the detection limit of our surveyor assay, regardless of modifications used throughout the rest of the scrRNA (Fig. 3*B*).

Identification of a Minimal 29-mer scrRNA Retaining High Activity in Gene Disruption. Because addition of 2′-sugar modifications increased stability and affinity-enhanced scrRNA activity, we further hypothesized that addition of specifically placed high-affinity nucleosides at the 3′-end of the scrRNA, which interacts with the tracrRNA, would allow truncation of the scrRNA from the 3′-end while retaining the ability to mediate gene disruption. Correspondingly, we designed several scrRNAs in which we removed 10 nucleotides from the 3′-end of scrRNA (positions 33–42) (FC-32–01) (Fig. 4*A*). FC-32–01 has a similar chemical substitution pattern to the most active scrRNA FC01 (Fig. 2*B*). Remarkably, FC-32–01 demonstrated efficient gene disruption at the *VEGF-A* locus, resulting in ∼42% maximal activity compared with sgRNA and almost 60% of the activity of the comparable 42-mer scrRNA (Fig. 3*B* and Fig. S3 *A* and *B*). As in the case with the 42-mer scrRNA, multiple substitutions in the seed region (FM-32-01) resulted in loss of activity (Fig. S3 *A* and *B*).

It has been previously shown that CRISPR-Cas9 specificity can be enhanced by reduction of the 20-nucleotide DNA specificity sequence to 17 nucleotides without affecting overall on-target activity (36). We therefore tested whether cleavage activity at the *VEGF-A* locus was retained after further shortening the scrRNA by an additional 3-nucleotide truncation from the 5′-end, thereby producing a 29-mer scrRNA (Fig. 4*A*). Remarkably, FC-29–01, synthesized with the same chemistry as FC-32–01 (Fig. 4*B*) yet 3 nucleotides shorter, displayed an equivalent activity to mediate gene disruption at the *VEGF-A* locus relative to its 3-base longer variant (FC-32-01), an activity level 42% of the transcription-produced 102-nucleotide sgRNA (Fig. S3 *A* and *B*). FC-29–02, with an identical chemistry to FC-29–01, but without a modified nucleotide in the seed sequence, enhanced activity to equal that of transcription-produced sgRNA and exceeded by more than twofold the activity of FC-32–01 and FC-29–01, demonstrating that even one modified nucleotide in the seed region can greatly affect activity.

Additional scrRNA variants FMC-29–01, MC29-01, and C-29–01 (Fig. 4*B*; Fig. S3 *A* and *B*; Fig. S4) were synthesized with the same modifications 3′ of the seed sequence but varying alternating modifications 5′ to the seed sequence. These variants retained ∼63–71% activity compared with sgRNA. Not unexpectedly, extensive modification in the seed region (e.g., FM-29-01) produced scrRNAs that were completely inactive (Fig. 4*B*). It is predicted that FMC-29–01 would have enhanced activity, similar to or better than FC-29–02, if synthesized without a modified nucleotide in the seed sequence (Fig. 4*B*). cEt modifications at position 20 of several scrRNAs (FC-29–03, MC-29–02, and C-29-02) produced complete loss of activity (Fig. 4*B* and Fig. S4).

Synthetic CRISPR RNA Activity at Predicted *VEGF-A* Off-Target Sites. Because we had identified scrRNAs (e.g., FC01, FMC01, FMC-29–01, MC-29–01, C-29–01, and FC-29-02) with 50–100% on-target activity relative to transcription-produced sgRNA in stimulating Cas9-dependent cleavage of the *VEGF-A* gene in cells, we tested the relative selectivity of that scrRNA-dependent activity on target DNAs containing 1- or 2-base mismatches with the scrRNA. We examined off-target cleavage within the MAX gene at chromosome position 14q23 (36), which carries an 18- of 20-nucleotide match within the *VEGF-A* gene targeted by FC01 (Fig. S5*A*). Remarkably, using the surveyor nuclease assay, we determined that FC01 produced a fourfold reduction relative to the corresponding sgRNA in cleavage of the MAX locus (Fig. S5*A*). Correcting for its retention of three-fourths the level of nuclease activity for cleavage of the *VEGF-A* gene target, this yielded an overall threefold decrease in off-target:on-target cutting mediated by FC01 relative to the corresponding sgRNA (Fig. 3*B*). Despite a shortened 17-bp DNA recognition domain including a 1-nucleotide mismatch to the MAX locus (Fig. 4*B*), FC-29–01 also demonstrated a 1.6-fold decrease in off-target:on-target nuclease activity at this locus (Fig. S5*A*) (4-fold reduced cleavage at MAX combined with retention of 40% of sgRNA activity at *VEGF-A*). Most interestingly, FC-29–02 was even more selective for the *VEGF-A* target locus. It produced a fourfold reduced level of off-target MAX cleavage to on-target *VEGF-A* activity while maintaining activity at *VEGF-A* equal to that of transcription-produced sgRNA (Fig. 4*B*). The cleavage activity of the scrRNAs at three additional predicted *VEGF-A* off-target sites (36) was also compared with off-target cleavage by sgRNA. This revealed that, compared with the off-target:on-target activity of sgRNA, the scrRNAs were (*i*) greater than fivefold reduced at chromosome 5q14.3 (Fig. S5*B*), (*ii*) slightly reduced at the SLIT1 gene (chromosome 10 q24.1), and (*iii*) similar to sgRNA at chromosome 22q13.1 (Fig. S5*C*).

Discussion

Genome engineering using CRISPR-Cas9 is a valuable tool for manipulating mammalian genes in cell culture as well as animal models and holds the potential for therapeutic applications in humans. In this report, we have provided characterization of scrRNA and demonstrated that (*i*) a PS-modified backbone throughout the scrRNA can mediate high levels of gene disruption; (*ii*) addition of 2′-*O*-Me or 2′-F to >5 terminal nucleotides at 5′, 3′, or both ends enhances this activity; (*iii*) cEt substitution of nucleotides in the tracrRNA-binding region further increases activity; and (*iv*) truncation of the 42-mer scrRNA to a 29-mer scrRNA retains high levels of gene disruption activity. Furthermore, multiple modified nucleotides in the DNA-binding seed sequence completely abolish this activity, indicating the importance of this region for target recognition.

A 100-mer sgRNA delivered by nucleofection and synthesized with 2′-*O*-Me, 2′-*O*-Me 3′phosphorothioate, or 2′-*O*-Me further modified with the neutral thiophosphonoacetate substitution (37) modifications of the three terminal 5′ and 3′ nucleotides has been reported to yield sgRNA capable of mediating genome editing against three target genes in vitro and in human primary T cells and CD34+ hematopoietic stem and progenitor cells (22). Our synthetic CRISPR RNA approach with short scrRNAs overcomes many of the multiple technical limitations that preclude the use of synthetic sgRNAs for routine cell culture and in vivo therapeutic applications. Advantages of the scrRNA approach include the following: First, due to the stepwise synthesis of chemically modified oligonucleotides, the synthesis complexity, yields, and purities of 100-mer sgRNAs severely limit the utility of the synthetic sgRNA approach. In contrast, the 29-mer scrRNAs described here can be chemically synthesized at high efficiency, on an industrial scale, and in a commercially feasible manner today. In addition, all three modifications described in this article have been broadly used in animal studies and are in approved therapeutic products or in clinical trials, demonstrating broad efficacy and safety. Second, to reach high-enough levels in

cells, a partially modified synthesized sgRNA (22) is likely only useful ex vivo, requiring transient transfection or nucleofection. In contrast, prior experience has established that fully chemically modified oligonucleotides (PS backbone) when injected systemically (33, 38) or infused into the cerebral spinal fluid (CSF) to target cells in the central nervous system (39–41) are rapidly distributed out of plasma and taken up by cells in many tissues or out of CSF to neurons and nonneurons throughout the nervous system. Additionally, in many cases such single-stranded oligonucleotides are freely taken up by cells in culture (42). The scrRNAs described here (with similar modifications as the previously studied diffusible oligonucleotides) are expected, although not yet tested, to have similar properties with the potential to activate Cas9-dependent cleavage without transfection. Third, the most active scrRNA reported here has a uniformly modified PS backbone and terminally modified nucleotides, both of which protect against exo- and endo-nuclease degradation (33, 43). The 100-mer synthetic sgRNAs (with only terminal modifications) are almost certain to be much more susceptible to nucleolytic degradation. They would also be predicted to activate immune cells through interaction with Toll-like receptors (44). And fourth, we have demonstrated that activity at several predicted off-target sites was reduced with a 29-mer scrRNA that maintains on-target activity equivalent to transcription-produced sgRNA (Fig. S5).

The properties we have established for a modified 29-nucleotide scrRNA enable development of strategies for gene disruption or editing either in cell culture or in animals, the latter activated by infusion of scrRNA into the periphery or nervous systems of mice or humans. Coupled with the known free uptake into cells and tissues of similarly modified short oligonucleotides (33, 38–42), scrRNAs enable gene inactivation or modification, provided that the target cells already express Cas9 and tracrRNA (which have no cleavage activity in the absence of a crRNA or scrRNA). scrRNA with the backbone and side-chain modifications established here will very likely result in dose-dependent, high rates of gene-editing events over time. One specific strategy (outlined in Fig. 5) that is enabled by discovery of effective 29-base scrRNAs would be to exploit a delivery vector (such as AAV) to drive expression of Cas9 and the tracrRNA in transduced cells in either peripheral tissues (17, 45, 46) or within the nervous system (47). Target gene inactivation would be achieved by error-prone NHEJ following Cas9-dependent target locus cleavage activated by free uptake of the scrRNA after injection. Gene correction, rather than inactivation, could be achieved by homology-driven repair after Cas9-mediated target locus cleavage by providing a DNA template with a corrected sequence of the target gene on the transduced AAV. A major advantage of the use of exogenously added scrRNA is that it allows not only controlled activation of Cas9 activity, but also controlled silencing (*i*) through natural decay of the scrRNA; (*ii*) by injection of an oligonucleotide that is freely taken up by cells and is complementary to the scrRNA or tracrRNA (48), thereby inhibiting their ability to activate Cas9; or, and perhaps most attractively, (*iii*) by injection of an scrRNA to induce cleavage of the gene encoding Cas9, thereby eliminating chronic synthesis of the Cas9 nuclease.

Finally, it is expected, although not yet tested, that scrRNA will also be applicable to other CRISPR/Cas9 technologies including gene transcription, inhibition, and activation (CRISPRi/a) (49) and genomic loci visualization (50, 51).

Materials and Methods

scrRNA Synthesis. Synthetic scrRNA was synthesized using a reported procedure (52). In brief, scrRNA were synthesized on a solid-phase DNA/RNA synthesizer using the 2′-*O*-TBDMS RNA phosphoramidites according to the reported protocols. 2′-F, 2′-*O*-Me, cEt, and 2′-*O*-MOE phosphoramidites with exocyclic amino groups protected with benzoyl (Bz for A and C) or isobutyryl (ibu for G) protecting groups were used for the synthesis of the RNA chimera. A solution (0.12 M) of the phosphoramidites in anhydrous acetonitrile was used for the synthesis. Oxidation of the internucleosidic phosphite to the phosphate was carried out using *tert*-butyl hydroperoxide/acetonitrile/water (10:87:3) with a 10-min oxidation time. Phosphorothioate linkages were introduced by sulfurization with a 0.1-M solution of xanthane hydride in 1:1 pyridine/CH_3CN for a contact time of 3 min. The overall coupling efficiency of all modified phosphoramidites was more than 97%. Oligoribonucleotide-bearing solid supports were heated with aqueous ammonia/ethanol (3:1) solution at 55 °C for 6 h to deprotect the base labile protecting groups. The 2′-*O*-TBDMS group was removed using a mixture of triethylamine trihydrofluoride/1-methyl-2-pyrrolidinone/triethyl amine mixture at 65 °C for 6 h. After deprotection, all of the scrRNAs were isolated by HPLC on a strong anion exchange column and subsequently desalted using HPLC on a

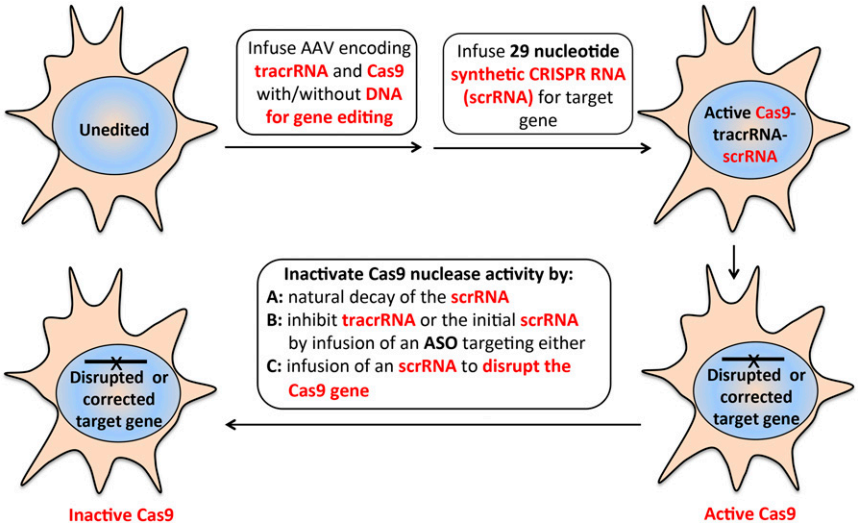


Fig. 5. A model for in vivo gene disruption or editing with a pair of 29-nucleotide synthetic CRISPR RNAs transiently activating and then silencing Cas9 nuclease activity. AAV-mediated delivery of DNA encoding tracrRNA and Cas9 in vivo followed by injection of 29-mer scrRNA to a target gene. Gene inactivation would be achieved by error-prone DNA repair. Gene correction could be achieved by homology-driven repair after Cas9-mediated target locus cleavage by providing a DNA template with a corrected sequence of the target gene on the transduced AAV. Nuclease activity can be inactivated by (*A*) natural decay of the scrRNA, (*B*) inhibition of tracrRNA or the initial scrRNA by infusion of an ASO targeting, or (*C*) infusion of an scrRNA to disrupt Cas9 gene.

reverse-phase column to yield scrRNAs. The scrRNAs were characterized by ion-pair HPLC-MS analysis.

Cell Lines and Transfection. HEK293T cells (ATCC) were cultured in DMEM (Life Technologies) supplemented with 10% FBS (Omega Scientific), 25 mM Hepes, and Antibiotic-Antimycotic (10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of Fungizone). Cells were transfected in six-well plates at 40% confluency using 2 μg of plasmid encoding tracrRNA and Cas9 protein or control plasmid encoding sgRNA and Cas9 protein using lipofectamine 3000 (Life Technologies). Twenty-four hours after DNA transfection, cells were washed one time with PBS and transfected with 5 μL of 74-μM scrRNA complexed with 15 μL of RNAiMAX (Life Technologies).

Surveyor Assay. The surveyor assay was performed according to the manufacturer's instructions (Integrated DNA Technologies). Briefly, 48 h following transfection, the *VEGF-A* or *LDLR* locus was amplified by PCR from 100 ng of genomic DNA using Q5 polymerase (NEB) and *VEGF-A* primers (forward: 5′-TCCAGATGGCACATTGTCAG-3′ and reverse: 5′-AGGGAGCAGGAAAGTGAGGT-3′) or *LDLR* primers (forward: 5′-GGAGACCCAAATACAACAAATC-3′ and reverse: 5′-CTAGACTCCGTCTCAAAGAAG-3′) in a 50-μL reaction. Twelve microliters was then denatured by heating to 95° C and slowly reannealed using a stepwise temperature-gradient program in a T100 thermocyler (Bio-Rad). The reannealed samples were incubated with Cel-1 nuclease (Surveyor kit; Integrated DNA Technologies) for 1 h at 42° C. Products were separated on a 10% (vol/vol) TBE acrylamide gel and stained with SYBRgold for visualization. Quantification was performed using ImageJ software where indel incidence was calculated using the following formula: indel (%) = 100 × [1-(1-fraction_cut)^0.5], where fraction_cut = (sum of the intesity of the cleaved product)/(sum of intesity of cleaved product and undigested PCR amplicon), and fraction_uncut = (b + c)/(a + b + c).

ACKNOWLEDGMENTS. This work was supported by Grants R01-GM 074150 and R01-NS27036 from the National Institutes of Health (NIH) to D.W.C., who receives salary support from the Ludwig Institute for Cancer Research. M.A.M. was supported by a postdoctoral fellowship from the NIH (F32-GM109657).

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