Ultrasound-Controlled CRISPR/Cas9 System Augments Sonodynamic Therapy of Hepatocellular Carcinoma

Haohao Yin, ∇ Liping Sun, ∇ Yinying Pu, ∇ Jifeng Yu, Wei Feng, * Caihong Dong, Bangguo Zhou, Dou Du, Yan Zhang, Yu Chen, * and Huixiong Xu *

Cite This: ACS Cent. Sci. 2021, 7, 2049–2062

ABSTRACT: Sonodynamic therapy (SDT), relying on the generation of reactive oxygen species (ROS), is a promising clinical therapeutic modality for the treatment of hepatocellular carcinoma (HCC) due to its noninvasiveness and high tissue-penetration depth, whereas the oxidative stress and antioxidative defense system in cancer cells significantly restrict the prevalence of SDT. Herein, we initially identified that NFE2L2 was immediately activated during SDT, which further inhibited SDT efficacy. To address this intractable issue, an ultrasound remote control of the cluster regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) release system (HMME@Lip-Cas9) was meticulously designed and constructed, which precisely knocks down NFE2L2 to alleviate the adverse effects and augment the therapeutic efficiency of SDT. The hematoporphyrin monomethyl ether (HMME) in this system yielded abundant ROS to damage cancer cells under ultrasound irradiation, and meanwhile the generated ROS could induce lysosomal rupture to release Cas9/single guide RNA ribonucleoprotein (RNP) and destroy the oxidative stress-defensing system, significantly promoting tumor cell apoptosis. This study provides a new paradigm for HCC management and lays the foundation for the widespread application of CRISPR/Cas9 with promising clinical translation, meanwhile developing a synergistic therapeutic modality in the combination of SDT with gene editing.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of morbidity and mortality, with poor prognosis, ranking as the third cause of cancer-related deaths worldwide. 1,2 Surgical management through either resection or liver transplantation is considered to be the potentially efficacious and curative treatment for HCC, but a majority of patients with more advanced diseases would not meet the criteria for surgery. 3 Given the limitations of conventional therapeutic modalities and restrictions of liver transplantation, there is an urgency to develop additional effective strategies and remedies for the treatment of HCC. 4 Sonodynamic therapy (SDT), sonosensitizer activated under low-intensity ultrasound irradiations to generate highly toxic reactive oxygen species (ROS), has been regarded as a promising preclinical noninvasive therapeutic modality in the past decade. 5–7 Compared with phototherapies (e.g., photothermal therapy or photodynamic therapy), the high tissue-penetrating depth and cost-effectiveness allow SDT to treat HCC essentially in the body. 8–12 The scientific principle underlying SDT-mediated cytotoxicity is mainly rooted in the generation of ROS, which disturbs the intracellular redox homeostasis to damage crucial components of the cancer cells. 13,14 However, there is a comprehensive antioxidant defense system to regulate the levels of ROS and prevent the accumulation of damage induced by ROS, in which nuclear factor erythroid 2-related factor 2 (NFE2L2) is an important transcription factor that targets the antioxidant response element in the upstream regulatory regions. 15 Through controlling the constitutive and inducible expression of intracellular phase II detoxification and antioxidant enzyme genes, NFE2L2 plays a beneficial role in improving the oxidative stress status, maintaining cellular redox homeostasis and promoting cell survival. 16 Therefore, NFE2L2 possibly inhibits the efficacy of ROS-based SDT and would be a potentially vulnerable target in HCC.

RNA interference technology (RNAi) is so far mostly applied for targeted gene therapy by suppressing gene expression, 17–19 while the ephemeral nature of gene silencing limits its further application. 17,20 The cluster regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated endonuclease protein 9 (Cas9) technology is a prospective and...
powerful genome editing tool to cure diseases by reprogramming or activating specific genes in a precise way. Compared to RNAi technology, the CRISPR/Cas9 system has the following features and advantages: higher targeting accuracy, complete knocking down of the target gene, and no species restriction. Cas9 nucleases under the guidance of a single guide RNA (sgRNA) can lead to double-stranded breaks (DSBs) of the target DNA sequence, which can induce code-shifting mutations in the target gene. Since 2013, the successful application of the CRISPR/Cas9 system, for the first time, in mammalian cells has triggered a revolution in genome editing. Notably, it is still elusive to efficiently translocate the CRISPR/Cas9 system into target tissues/cells for biomedical applications due to numerous extracellular and intracellular barriers, which considerably restricts the widespread utilization of CRISPR/Cas9 in vivo. The ideal CRISPR/Cas9 delivery and release system should be suitable for the following criteria: (i) the sgRNA should be designed for precise gene targeting to avoid possible off-target effects; (ii) the vector material should be biocompatible possessing low toxicity and immunogenicity; (iii) exogenous stimuli could be introduced as a controllable switch to deliver without leakage and precisely release at targeted locations by external stimulations or microenvironmental triggers. Nonviral systems like Au nanoparticles and lipid nanoparticles as delivery vectors for the CRISPR/Cas9 system have attracted considerable interest because of the specific features such as high security and favorable load-carrying capacity. Nevertheless, the low lysosomal escape rate makes most of the nonviral materials especially for synthetic nanoparticles difficult to perform safe and effective gene editing in vivo. On this basis, it is hypothesized that the combination of SDT and CRISPR/Cas9-mediated gene editing technology would address the

---

**Figure 1.** Schematic illustration of the designed strategy of the US-mediated CRISPR/Cas9 delivery system to enhance tumor SDT performance by amplifying oxidative stress. Preparation of HMME@Lip-Cas9 nanosystem and US-controlled CRISPR/Cas9 knock down target genes (NFE2L2). The CRISPR/Cas9 nanosystem (HMME@Lip-Cas9) generates ROS to promote apoptosis under ultrasound stimulation while disrupting the membrane structure of lysosomes, thus achieving the effective escape of Cas9/sgRNA RNP from lysosomes and efficient knock down of NFE2L2 for improving the therapeutic efficacy of SDT.
current challenges in the treatment of HCC and the delivery of the CRISPR/cas9 system.

At the beginning of this study, we initially found that NFE2L2 was rapidly activated after SDT, which dissociated from Kelch-like ECH-associated protein 1 (Keap1) and rapidly translated into the nucleus, and then bonded to antioxidant response elements to maintain intracellular redox homeostasis.

---

**Figure 2.** NFE2L2 promotes proliferation of HCC cells. (a) NFE2L2 protein expression levels after different SDT treatments by the Western blotting. (b) Immunofluorescence images of NFE2L2 (red) and nuclei stained by DAPI (blue) in tumors after different treatments. Scale bars, 50 μm. (c) Tumor growth curves (n = 5, mean ± SD) of hep3B2.1-7 cells with stable knock down of NFE2L2 in a xenograft mouse model (⁎p < 0.05, ⁎⁎p < 0.01, and ⁎⁎⁎p < 0.001). (d) Statistical and (e) photographic results of tumor size (n = 5, mean ± SD) in hep3B2.1-7 cells with stable knock down of NFE2L2 (⁎p < 0.05, ⁎⁎p < 0.01, and ⁎⁎⁎p < 0.001). (f) Tumor growth curves (n = 5, mean ± SD) of hep3B2.1-7 cells with stable overexpression of NFE2L2 in a xenograft mouse model (⁎p < 0.05, ⁎⁎p < 0.01, and ⁎⁎⁎p < 0.001). (g) Statistical and (h) photographic results of tumor size (n = 5, mean ± SD) in hep3B2.1-7 cells with stable overexpression of NFE2L2 (⁎p < 0.05, ⁎⁎p < 0.01, and ⁎⁎⁎p < 0.001). (i–j) Representative immunohistochemical images of Ki67 and PCNA protein levels in (i) hep3B2.1-7 xenografts with stable knock down of NFE2L2, and (j) hep3B2.1-7 xenografts with stable overexpression of NFE2L2, scale bars, 100 μm.
ments showed that NFE2L2 promoted tumor cell growth and proliferation, which was positively correlated with tumor size. Thus, to address this issue, an ultrasound (US)-responsive CRISPR/Cas9 nanodelivery system (HMME@Lip-Cas9) was
constructed to knock down NFE2L2 and enhance the efficiency of SDT. United StatesFood and Drug Administration (FDA)-approved DLin-MC3-DMA lipid nanoparticles (Lips) were used as carriers for loading the sonosensitizers hematoporphyrin monomethyl ether (HMME) and Cas9/sgRNA ribonucleoprotein (RNP). The as-obtained US-responsive CRISPR/Cas9 system (HMME@Lip-Cas9) produced ROS, which effectively disrupted the structure of the lysosomal membrane, thus achieving efficient escape of Cas9/sgRNA RNP from lysosomes, releasing into the cytoplasm and transporting to the nucleus to exert their therapeutic effect (Figure 1). Unlike Lips that can only mediate the release of 1–4% of RNA into the cytoplasm,39,40 this system can efficiently release RNP to activate Cas9 under the spatial control of US stimulation, avoiding gene mutations in nontarget regions. This research not only systematically elucidates the cellular self-protection mechanism against ROS-induced oxidative stress and addresses the challenges on the development of SDT, but also a highly biocompatible and transfection-efficient nonviral vector has been explored for the spatiotemporally controlled transmission of the CRISPR/Cas9 system, which has a broad scope in clinical translation.

■ RESULTS AND DISCUSSION

NFE2L2 as a Poor Prognostic Marker Up-regulated in HCC Tissues after SDT Treatment. To identify the function of the NFE2L2 gene, the expressions of HCC after different treatments were measured. The NFE2L2 protein expression levels in tumors were examined by Western blotting (Figure 2a, Figure S1a, Supporting Information) and immunofluorescent analysis (Figure 2b, Figure S1b, Supporting Information), revealing that the expression of NFE2L2 in the nucleus was significantly up-regulated after SDT treatment (intravenous injection of HMME, 5 min) and highly dependent on the power density of US. Hence, to further explore the role of NFE2L2-mediated genes in HCC, the stable overexpression and knock down of NFE2L2 in hep3B2.1-7 cell lines were constructed, respectively (Figure S2–S4, Supporting Information). Cell counting kit-8 (CCK-8) assays demonstrate that cell proliferation is clearly up-regulated by NFE2L2 overexpression and is obviously down-regulated by NFE2L2 knock down in Hep3B2.1-7 cells (Figure S5, Supporting Information). In addition, two-dimensional colony formation assays indicate that NFE2L2 overexpression or knock down significantly enhances or impairs the colony formation ability of the corresponding HCC cell lines (Figure S6, S7, Supporting Information), suggesting that NFE2L2 promotes the proliferation of HCC cells. Inspired by this, we further investigated whether NFE2L2 promotes the growth of HCC in vivo. We injected HCC cells with stable overexpression or knock down of NFE2L2 into nude mice subcutaneously to construct human tumor xenograft models. Tumors from the stable knock down of NFE2L2 in hep3B2.1-7 cells-bearing mice exhibit significant growth inhibition compared to mock or negative controls (Figure 2c–e), which shows that NFE2L2 knock down reduced tumor burden compared with the control group. Tumors from stable NFE2L2-overexpressing hep3B2.1-7 cells grew faster (Figure 2f) and exhibited greater tumor mass (Figure 2g,h) than mock or vector controls. Moreover, immunohistochemical staining images and corresponding quantitative analysis results of tumor resection demonstrate lower proliferating cell nuclear antigen (PCNA) and Ki67 levels in hep3B2.1-7 tumors knocking down NFE2L2 (Figure 2i, Figure S8a, b, Supporting Information) while higher PCNA and Ki67 levels in hep3B2.1-7 tumors overexpressing NFE2L2 (Figure 2j, Figure S8c,d, Supporting Information). Taken together, these findings suggest that NFE2L2 could promote tumor cell proliferation in HCC.

Preparation and Characterization of Lips Encapsulating CRISPR/Cas9. To overcome the limitations of the current CRISPR/Cas9 delivery system, ionizable cationic DLin-MC3-DMA liposomes, a polymer applied in US FDA-approved Onpatro, were selected to encapsulate the CRISPR/Cas9 system (Figure 3a). For the most efficient genome editing, we used RNP consisting of Alt-R S.p. Cas9 nuclease in complex with Alt-R CRISPR-Cas9 sgRNA (IDT).41–43 In order to optimize the loading capacity, we prepared ionizable cationic liposomes loaded with HMME@Lip and Cas9/sgRNA at varied ratios (HMME@Lip: Cas9/sgRNA ratio) and performed agarose gel electrophoresis to determine the gene-loading capacity. The results reveal that when the HMME@Lip and Cas9/sgRNA ratio is selected at 4:1, the system exhibits an efficient and stable loading capacity for sgRNA, while a further increase of Cas9/sgRNA ratios leads to an insufficient amount of HMME@Lip, resulting in sgRNA partial degradation (Figure 3b). The monodispersed nanoparticles with a spherical shape and uniform size were obtained, as examined by transmission electron microscopy (TEM) imaging (Figure 3c,d). The diameter of HMME@Lip nanoparticles is ~170 nm (Figure 3c), and the diameter increases to ~210 nm following the combination with Cas9/sgRNA to form HMME@Lip-Cas9 (Figure 3d). The obtained composite nanoparticles are uniformly dispersed in aqueous solution, and the zeta potential measurements reveal a positive potential of 22.2 mV for liposomes. After coloading HMME and Cas9 RNP, the surface zeta potential is converted to 8.41 mV (Figure 3e). Dynamic light scattering (DLS) measurements show that the average size of HMME@Lip increases significantly following successful Cas9/sgRNA RNP loading, where the hydrodynamic diameter in HMME@Lip-Cas9 composites nanosystems was increased from the 192.6 to 243.4 nm (Figure 3f). These results all proved that Cas9/sgRNA RNP was uploaded onto the HMME@Lip composite nanoparticles successfully.

To explore the sonodynamic performance and identify the corresponding mechanism, UV–vis spectroscopy and electron spin resonance (ESR) spectra were acquired before and after US irradiation with a HMME@Lip-Cas9 nanosystem. As shown in Figure 3g,h, the UV–vis spectrum shows a special peak at 410 nm due to 1,3-diphenylisobenzofuran (DPBF) as a probe to monitor the specific singlet oxygen (¹O₂) generation. As expected, the HMME@Lip-Cas9 nanosystem efficiently generates ¹O₂ upon ultrasound stimulation, as demonstrated by a significant decrease in absorbance at 410 nm with prolonging the ultrasound time and decreases less only after ultrasound radiation treatment (Figure 3i). In addition, when 2,2,6,6-tetramethylpiperidine (TEMP) was used to track and capture the ¹O₂ characteristic ¹O₂-induced signals can be observed in the HMME@Lip-Cas9 + US group (Figure 3j), while relatively weak signals could be identified in both the HMME@Lip-Cas9 group and US group. Such results confirm that the HMME@Lip-Cas9 nanosystem acts as an effective nononosensitizer, conferring the distinct ROS production capacity. We next explored the intracellular ROS-producing capability of the as-prepared nanosystem. The total intracellular ROS production is qualitatively and semiquantitatively evaluated by employing a 2,7-dichlorodihydrofluorescin diacetate (DCFH-DA) probe under confocal laser scanning microscopy (CLSM), where the
HMME@Lip-Cas9 + US group exhibits a stronger fluorescence intensity than the HMME@Lip + US group, suggesting that the antioxidative stress of cells can be reduced, and the amount of ROS is increased by inhibiting expression of NFE2L2 (Figure 3k).
Enhanced Green Fluorescent Protein (EGFP) Disruption in Vitro. Subsequently, the internalization and trafficking processes of the HMME@Lip-Cas9 nanosystem were further observed to reveal the fundamental mechanism of transfection and Figure S9, Supporting Information). Meanwhile, flow cytometry (FCM) analysis is in line with the results of CLSM, indicating that the HMME@Lip-Cas9 + US group generates more ROS (Figure S10, Supporting Information).
within HepG2 cells, utilizing CLSM to visualize the location of Cas9/sgRNA RNP in organelles with or without US irradiation, thus revealing the fate of RNP. In the absence of US irradiation, more cyanine 3 (Cy3)-labeled Cas9 (red fluorescence) colocalized with lysosomes (green fluorescence). It is worth noting that the Cy3-labeled red fluorescent signal of Cas9/sgRNA RNP is dissociated with the lysosome of the green fluorescent signal under US irradiation. Simultaneously, Cy3-labeled red fluorescent Cas9/sgRNA RNP is detected at the nucleus, which indicates that Cas9/sgRNA RNP escapes from the endosomes and enters the nuclei (Figure 4a,b, Figure S11a,b, Supporting Information), revealing that US irradiation is essential for the lysosomal escape of RNP. Further quantitative analysis shows that the Pearson’s correlation coefficients of Cy3 and LysoTracker channels significantly decreased under US irradiation. In particular, the average Pearson’s correlation coefficient decreased to 0.42 at the sixth hour under US irradiation (Figure S11c, Supporting Information). Meanwhile, the quantification of Cy3 signals overlapping with LysoTracker and DAPI reveals that the Cy3 signals overlapping with LysoTracker decreased, while overlapping with DAPI significantly increased with the prolonged incubation time upon US treatments (Figure S11d, Supporting Information). Subsequently, nuclei were extracted and subjected to SDS-PAGE gel electrophoresis and Coomassie blue staining, and bands representing Cas9 protein appeared on the gel, especially under US treatments (Figure S12, Supporting Information). The above results further indicate that Cas9/sgRNA RNP could efficiently release from HMME@Lip nanoparticles and enter the nucleus under US irradiation. The editing capability of the HMME@Lip-Cas9 nanosystem for the target genome under US control was further evaluated. We designed and synthesized sgRNA for targeting enhancement of the green fluorescent protein (EGFP) motif coding region in the HepG2-EGFP cell line, which contained a single copy of the EGFP reporter gene and constitutively expressed unstable EGFP. Gene-editing efficiency was quantified using Western blotting, CLSM, and FCM. The Western blotting results indicate that EGFP expression is significantly reduced in the HMME@Lip-Cas9 + US group compared with other groups (Figure 4c,d). An efficient delivery of Cas9/sgRNA RNP to cultured cells treated with US exposure, bypassing the stratum corneum and translocating to the nucleus by targeted genomic editing, is confirmed by the apparent fluorescence quenching of cells treated with HMME@Lip-Cas9 after US radiation (Figure 4e,f). FCM data show that treatment with HMME@Lip-Cas9 converts 44.2% of EGFP-positive cells into EGFP-negative cells, while treatment with HMME@Lip-Cas9 + US converts 78.2% of EGFP-positive cells into EGFP-negative cells, indicating that US radiation promotes the lysosomal escape of Cas9 RNP and improves genome-editing efficiency by enhancing nuclear entry (Figure 4g). In addition, HepG2-EGFP cells treated with HMME@Lip + US still show 83.2% EGFP positivity, indicating that US treatment does not affect the expression of EGFP protein.

**HMME@Lip-Cas9 Nanosystem Induces Efficient Therapeutic Gene Editing in Vitro.** Subsequently, the potential therapeutic genome editing was explored, as a proof of concept, in which we evaluated HMME@Lip-Cas9 containing NFE2L2 sgRNA in vitro. It has been demonstrated that inhibition of NFE2L2 gene expression enhances the sensitivity of tumor cells to ROS and inhibits tumor growth. Therefore, NFE2L2 in HepG2 cells was elected for targeting, and efficient sequences targeting NFE2L2 sgRNA were designed and synthesized (Figure 5a). The biosafety of the HMME@Lip-Cas9 nanosystem was initially investigated by the standard CCK-8 assay. HMME@Lip-Cas9 nanosystem with different concentrations exhibits negligible toxicity after incubation with HepG2 cells and 293T cells for 24 h, even at a high concentration of 400 μg mL⁻¹, indicating the low cytotoxicity of the HMME@Lip-Cas9 nanosystem (Figure Sb and Figure S13, Supporting Information). Subsequently, the synergistic therapeutic potential of the HMME@Lip-Cas9 nanosystem for HepG2 cells was explored by investigating the cell viability, proliferation, and live–dead status after different treatments. After varied treatments for 14 days, NFE2L2 gene editing significantly decreased the clone formation rate because the HMME@Lip-Cas9 + US group has a lower clone formation rate than the HMME@Lip + US group, while almost no effect on cell proliferation is induced in other groups (Figure 5c,d). Furthermore, the cell viability assays reveal significant cell killing in the HMME@Lip-Cas9 + US group compared to the other groups (Figure 5e). CLSM images and semiquantitative analysis exhibit the weakest green intensity of calcine-AM (staining of live cells) and the highest red intensity of propidium iodide (PI)-stained dead cells in the HMME@Lip-Cas9 + US group (Figure 5f and Figure S14, Supporting Information). Accordingly, FCM results indicate that an 81.5% apoptosis rate was observed in the HMME@Lip-Cas9 + US group (Figure S15, Supporting Information).

The mechanism of synergistic treatment was further investigated, and Sanger sequencing known as the “chain termination method” was applied to analyze the gene-editing effect of HMME@Lip-Cas9 composite nanoparticles under US irradiation in vitro. It was observed that the mutation peak of the HMME@Lip-Cas9-transfected cell genome (NFE2L2) under US irradiation was higher than other treatments (Figure 5g, Figure S16, Supporting Information). Subsequently, the genomic DNA of the cells after different treatments was extracted, and the fragment of the target gene NFE2L2 was amplified. After T7 endonuclease I (T7E1) digestion, the HMME@Lip-Cas9 + US group displays more cleavage products than other groups, while the cleavage bands are darker or absent (Figure S17, Supporting Information). Next-generation sequencing (NGS) was applied to quantify the indel efficiency of the NFE2L2 motif, showing the results of HMME@Lip-Cas9 group achieves genomic disruption efficiency of 17.28% (Figure 5h), whereas the HMME@Lip-Cas9 + US group results in the genomic disruption efficiency of 58.77% in HepG2 cells (Figure 5i), which is significantly higher than the other groups, indicating that the genomic editing efficiency can be significantly improved by sonodynamic disruption of the lysosomal membrane (Figures S18–S23, Supporting Information). NGS also reveals that the rates of deletion and insertion mutations in the NFE2L2 locus are 44.35% and 14.42%, respectively (Figure S24, Supporting Information). Taken together, the above results further prove that the highly efficient delivery of the CRISPR/Cas9 system and knock down of the target gene locus (NFE2L2) under US irradiation are possible with the HMME@Lip-Cas9 nanosystem to reduce the resistance of cells to ROS.

**US-Activated Gene Therapy in Vivo.** Prior to in vivo efficacy testing, the biosafety was initially evaluated. The biosafety of the HMME@Lip-Cas9 nanosystem was evaluated by assaying liver function and kidney function with mice. The results of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), alkaline phosphatase, blood urea nitrogen, and uric acid urea assays indicate that neither
HMME@Lip nor targeted gene disruption causes any significant hepatorenal toxicity, revealing that the HMME@Lip-Cas9 nanosystem is biocompatible (Figure S25, Supporting Information). Hematoxylin and eosin (H&E)-stained sections of major organs (including heart, liver, spleen, lung, and kidney) further confirmed that there is almost no significant difference between the PBS and HMME@Lip-Cas9 nanosystem treatment groups (Figure S26, Supporting Information), manifesting that the

Figure 6. NFE2L2 gene editing therapy/SDT-based synergistic therapy in vivo. (a) In vivo fluorescence imaging of mice with HepG2 tumors at various time points after tail vein injection of the HMME@Lip-Cas9 nanosystem (0, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h). (b) Tumor growth curves of HepG2 tumor-bearing mice after different treatments (control, US only, Cas9/sgRNA, HMME@Lip-Cas9, HMME@Lip + US, and HMME@Lip-Cas9 + US). (c) Time-dependent tumor growth curves (n = 5, mean ± SD) after various treatments (*p < 0.05, **p < 0.01, and ***p < 0.001). (d) Tumor weight after different treatments. (e) Sanger sequencing of NFE2L2 in HepG2 tumor-bearing mice after 24 h of exposure to varying treatments. (f) HepG2 tumor-bearing mice underwent HE staining and immunofluorescence staining (TUNEL, Ki67, NFE2L2, DCFH-DA) after 24 h of different treatments. Nuclei were stained by DAPI (blue).
HMME@Lip-Cas9 nanosystem causes neglectable acute and chronic damage to major organs. We also investigated the in vivo distribution of the HMME@Lip-Cas9 nanosystem in a xenograft nude mouse model bearing HepG2 cells. As shown by in vivo fluorescence imaging (Figure 6a and S27, Supporting Information), the fluorescence signal in the tumors was obviously enhanced gradually with time and peaked at 8 h. Besides, the fluorescence signal in the tumors is apparently stronger than other organs. Subsequently, the HMME@Lip-Cas9 nanosystem is progressively cleared from the body and metabolized through the kidneys and liver.

A combination of SDT and NFE2L2 gene editing of HMME@Lip-Cas9 was tested for antitumor activity in a HepG2 cancer model. The constructed xenograft nude mouse models carrying HepG2 cells were randomly divided into six groups (n = 5) and treated differently according to
The results show that almost no significant difference in weight was observed among groups, indicating that neither the HMME@Lip-Cas9 nanosystem nor US treatment causes obvious damage to the organs (Figure S28, Supporting Information). The control, US, Cas9/sgRNA, and HMME@Lip-Cas9-treated groups show no apparent inhibitory effect on tumor growth (Figure 6b,c). Nevertheless, after 15 days of treatment, the HMME@Lip + US and HMME@Lip-Cas9 + US groups exhibit significant tumor growth retardation, and the tumors in the HMME@Lip-Cas9 + US group disappear completely without recurrence (Figure 6c, Figure S29, Supporting Information). Tumor weights are significantly reduced in the HMME@Lip + US group and HMME@Lip-Cas9 + US group (Figure 6d), which verifies the high efficacy of NFE2L2 gene editing combined with SDT. In addition, we collected tumor tissues and major organs at 24 h after the different treatments and extracted genomic DNA to explore the underlying mechanism of treatment by Sanger sequencing. The mutation peaks (NFE2L2) in the HMME@Lip-Cas9 + US group were significantly higher than that in other groups (Figure 6e, Figure S30, Supporting Information), and the mutation peaks were not detected in other organs (Figure S31, Supporting Information). Furthermore, according to the analysis of H&E-stained and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL)-stained tumor tissue sections, HMME@Lip-Cas9 + US treatment results in maximal tumor cell apoptosis and necrosis (Figure 6f, Figure S32a, Supporting Information). In addition, Ki67 antigen-labeled cell proliferation exhibits similar results, accompanied by significantly decreased positive cell proliferation after treatment with HMME@Lip-Cas9 + US (Figure 6f, Figure S32b, Supporting Information), validating the stronger antitumor effect of synergistic treatment with SDT and NFE2L2 gene editing. NFE2L2 is significantly elevated in tumor tissues of mice treated with HMME@Lip + US (Figure 6f, Figure S32c). In contrast, NFE2L2 is reduced in tumor tissues of mice treated with HMME@Lip-Cas9 + US, while ROS fluorescence is significantly higher than that of HMME@Lip + US. These results suggest that NFE2L2 is effectively genetically silenced, thereby reducing cellular tolerance to ROS and amplifying the effect of ROS treatment (Figure 6f, Figure S32d, Supporting Information).

Mechanism of Synergistic Treatment of HMME@Lip-Cas9. Finally, the mRNA profiles in control and HMME@Lip-Cas9 + US treated HepG2 tumor models were analyzed by RNA sequencing to investigate the potential therapeutic mechanism of HMME@Lip-Cas9 under US irradiation. More than 1572 genes are extensively regulated in the HMME@Lip-Cas9 + US group compared to the control group (US irradiation at a power density of 1.0 W cm−2 for 5 min), including 330 up-regulated genes and 1242 down-regulated genes ($p < 0.05$, log₂Fold-Changep > 1) (Figure 7a,b). In accordance with the gene ontology (GO) database, differential gene expression due to HMME@Lip-Cas9 under US irradiation is closely associated with the nucleic acid metabolic process, microtubule cytoskeleton, mitotic cell cycle, and cell cycle (Figure S33, Supporting Information). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway results show that HMME@Lip-Cas9 under US irradiation induces alterations in gene expression profiles associated with endocytosis, cell cycle, proteoglycans in cancer, the MAPK signaling pathway, the p53 signaling pathway and apoptosis (Figure 7c). Among the 20 most significant cellular pathways, MAPK, cell cycle, p53, and apoptotic signaling pathways are significantly enriched, which are significantly associated with the proliferation, migration, and apoptosis of tumors. According to these differentially expressed genes (DEGs), variations of p53-related genes and apoptotic-related genes after treatment with HMME@Lip-Cas9 under US irradiation are enumerated in the heat map (Figure 7d,e). Among these 12 p53-related DEGs, 7 and 5 genes are down- and up-regulated, respectively. In addition, a significantly altered gene assignment of cell cycle, MAPK, p53, and apoptotic signaling pathways after being treated with HMME@Lip-Cas9 under US irradiation is employed for Venn diagram analysis (Figure 7f). Notably, key proteins in this network including growth arrest and DNA damage inducible alpha (GADD45A), and growth arrest and DNA damage inducible beta (GADD45B) perform critical regulatory roles in multiple pathways, which are significantly up-regulated after treatment with HMME@Lip-Cas9 under US irradiation. In particular, GADD45A is a family of GADD45 genes which is an essential gene for cell growth arrest and DNA damage repair, and is an important oncogene. GADD45A, a cell growth arrest and DNA damage regulatory gene, serves in a cell damage response through different pathways, like p53 and JNK signaling pathways, to inhibit tumor cell proliferation and promote apoptosis. These results suggest that treatment with HMME@Lip-Cas9 under US irradiation may contribute to the activation of multiple signaling pathways such as cell cycle, p53, and MAPK, resulting in cell cycle arrest, inhibition of cell proliferation, and promotion of apoptosis.

CONCLUSIONS
SDT is principally based on the ROS generation for inducing cancer cell death; however, the presence of an antioxidative stress defense system to maintain oxidation—reduction homeostasis is regarded as an obstacle to enhance the efficacy of SDT. As a crucial redox-sensitive transcription factor, NFE2L2 is beneficial for alleviating oxidative stress, promoting cell survival, and maintaining cellular redox homeostasis. Therefore, NFE2L2 could be a potential target for tumor therapy. The precise knockout of target genes utilizing CRISPR/Cas9-based gene-editing technology is an efficient strategy to overcome the limitations of SDT. However, there is an urgency to address the critical questions for achieving safe and efficacious delivery of Cas9/sgRNA with controlled activation in a specific spatial region to enable tumor-specific gene editing and thus prevent undesired gene mutations in normal tissues.

Herein, we discovered that NFE2L2 was effectively activated after SDT and positively correlated with US power intensity, which promoted tumor cell proliferation and further inhibited the efficacy of SDT. Therefore, an US remote control CRISPR/Cas9 release system was constructed to address the efficacy challenge of SDT. The cationic liposomal MC3 with FDA-approved composition as carriers for codelivery of HMME and Cas9/sgRNA RNP were meticulously designed and fabricated to realize the synergistic therapeutic effects of SDT and gene editing, which achieved superior effectiveness in the management of HCC. First, the large amount of singlet oxygen produced by HMME after US irradiation induced apoptosis of cancer cells and disrupted the endosomal/lysosomal membrane structure, resulting in the lysosomal escape and release of Cas9/sgRNA RNP into the cytoplasm. Second, the released Cas9/sgRNA RNP entered the cell nucleus, recognized the target gene NFE2L2, and then efficiently knocked it down to eliminate the adverse effects of SDT and amplify the cellular oxidative stress.
level. Last but not least, this system was activated for gene editing only in tumor tissues under US irradiation, thus preventing the occurrence of gene editing in normal tissues leading to gene mutations.

In summary, US remote control of CRISPR/Cas9 release precisely knocked down NFE2L2 to implement tumor-specific gene editing. NFE2L2 gene-editing knock down reversed the limitations of SDT and amplified cellular oxidative stress levels, thus enabling synergistic SDT and gene-editing therapy. Compared with light-controlled gene-editing techniques, SDT is more suitable for the management of deep tumors such as HCC due to the higher tissue-penetration depth of US. In this study, we address the intractable problems of ROS-based SDT and provide a new vehicle for the engineering of CRISPR/Cas9 delivery systems with promising clinical translation. Also, this technology has the potential to be integrated with immuno-therapy to eliminate tumor metastasis and recurrence, and could be broadened to other diseases, such as degenerative brain diseases, genetic diseases, and inflammatory diseases.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.1c01143.

Additional experimental details, materials, figures, and methods, including HMME@Lip-Cas9 preparation, investigation of SDT effects, EGFP gene disruption assay, analysis of lysosomal escape of HMME@Lip-Cas9, in vitro cytotoxicity assay, cell clonal proliferation assay, surveyor assay for the detection of genomic modifications, NGS analysis of gene editing, in vivo toxicity assay, in vivo biodistribution and metabolism of nanosystems, in vivo synergetic multitherapy effect, and mechanism of synergistic treatment of HMME@Lip-Cas9 (PDF)

## AUTHOR INFORMATION

### Corresponding Authors

Huixiong Xu – Center of Minimally Invasive Treatment for Tumor, Department of Medical Ultrasound, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Ultrasound Research and Education Institute, Clinical Research Center for Interventional Medicine, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Shanghai Engineering Research Center of Ultrasound Diagnosis and Treatment, Shanghai 200072, P. R. China; National Clinical Research Center for Interventional Medicine, Shanghai 200072, P. R. China; Email: xuhuixiong@tongji.edu.cn

Yu Chen – Shanghai Engineering Research Center of Organ Repair, Materdicine Lab, School of Life Sciences, Shanghai University, Shanghai 200444, P. R. China; orcid.org/0000-0002-8206-3325; Email: chenyuedu@shu.edu.cn

Wei Feng – Shanghai Engineering Research Center of Organ Repair, Materdicine Lab, School of Life Sciences, Shanghai University, Shanghai 200444, P. R. China; Email: fengw@shu.edn.cn

### Authors

Haohao Yin – Center of Minimally Invasive Treatment for Tumor, Department of Medical Ultrasound, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Ultrasound Research and Education Institute, Clinical Research Center for Interventional Medicine, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Shanghai Engineering Research Center of Ultrasound Diagnosis and Treatment, Shanghai 200072, P. R. China; National Clinical Research Center for Interventional Medicine, Shanghai 200072, P. R. China

Liping Sun – Center of Minimally Invasive Treatment for Tumor, Department of Medical Ultrasound, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Ultrasound Research and Education Institute, Clinical Research Center for Interventional Medicine, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Shanghai Engineering Research Center of Ultrasound Diagnosis and Treatment, Shanghai 200072, P. R. China; National Clinical Research Center for Interventional Medicine, Shanghai 200072, P. R. China

Yinying Pu – Center of Minimally Invasive Treatment for Tumor, Department of Medical Ultrasound, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Ultrasound Research and Education Institute, Clinical Research Center for Interventional Medicine, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Shanghai Engineering Research Center of Ultrasound Diagnosis and Treatment, Shanghai 200072, P. R. China; National Clinical Research Center for Interventional Medicine, Shanghai 200072, P. R. China

Caihong Dong – Department of Ultrasound, Zhongshan Hospital, Fudan University, Shanghai 200032, P. R. China

Bangguo Zhou – Center of Minimally Invasive Treatment for Tumor, Department of Medical Ultrasound, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Ultrasound Research and Education Institute, Clinical Research Center for Interventional Medicine, School of Medicine, Tongji University, Shanghai 200072, P. R. China; National Clinical Research Center for Interventional Medicine, Shanghai 200072, P. R. China

Dou Du – Center of Minimally Invasive Treatment for Tumor, Department of Medical Ultrasound, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Ultrasound Research and Education Institute, Clinical Research Center for Interventional Medicine, School of Medicine, Tongji University, Shanghai 200072, P. R. China; National Clinical Research Center for Interventional Medicine, Shanghai 200072, P. R. China

Yan Zhang – Center of Minimally Invasive Treatment for Tumor, Department of Medical Ultrasound, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Ultrasound Research and
Education Institute, Clinical Research Center for Interventional Medicine, School of Medicine, Tongji University, Shanghai 200072, P. R. China; Shanghai Engineering Research Center of Ultrasound Diagnosis and Treatment, Shanghai 200072, P. R. China; National Clinical Research Center for Interventional Medicine, Shanghai 200072, P. R. China

Complete contact information is available at: https://pubs.acs.org/10.1021/acscentsci.1c01143

Author Contributions

H.Y., L.S., and Y.P. contributed equally to this work. H.Y., C.D., Y.C., and H.X. designed the research strategy and experiments; H.Y., J.Y., C.D., B.Z., D.D., and Y.P. performed experiments and/or analyzed the data; H.Y., W.F., H.X., Y.C., C.D., and L.S. wrote the paper; Y.C., C.D., and H.X. supervised the whole process.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We greatly acknowledge the financial support from the National Nature Science Foundation of China (Grant Nos. 81725008, 81927801, S109727836, S16727303, and S2072393), Basic Research Program of Shanghai Municipal Government (Grant No. 21JC1406002), Shanghai Municipal Health Commission (Grant Nos. 2019LJ21 and SHSLCZDZX0302), Shanghai Science and Technology Program (Grant No. 21010500100), Shanghai Science and Technology Committee Rising-Star Program (Grant Nos. 19DZ2251100 and 21QAI403100), Shanghai Municipal Science and Technology Major Project (Grant No. 2021SHZDZX0100) and the Fundamental Research Funds for the Central University.

REFERENCES

(1) Tvedten, A.; Hallouch, O.; Chernyak, V.; Kamaya, A.; Sirlin, C. B. Epidemiology of hepatocellular carcinoma: target population for surveillance and diagnosis. *Abdom. Radiol. (NY)* 2018, 43 (1), 13−25.
(2) Sun, H.; Chien, S. R.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries in 2018. *Ca-Cancer J. Clin.* 2020, 70 (3), 209−249.
(3) Cabibbo, G.; Enea, M.; Attanasio, M.; Bruschi, J.; Craxi, A.; Camma, C. A meta-analysis of survival rates of untreated patients in randomized clinical trials of hepatocellular carcinoma. *Hepatology* 2010, 51 (4), 1274−83.
(4) Bruijs, J.; Jakayama, T.; Mazaferro, V.; Chau, G. Y.; Yang, J.; Kudo, M.; Cai, J.; Poon, R. T.; Han, K. H.; Tak, W. Y.; et al. Adjutant sorafenib for hepatocellular carcinoma after resection or ablation (STORM): a phase 3, randomised, double-blind, placebo-controlled trial. *Lancet Oncol.* 2015, 16 (13), 1344−54.
(5) Guan, W.; Tan, L.; Liu, X.; Cui, Z.; Zheng, Y.; Yeung, K. W. K.; Zheng, D.; Liang, Y.; Li, Z.; Zhu, S.; Wang, X.; Wu, S. Ultrasonic Interfacial Engineering of Red Phosphorous-Metal for Eradicating MRSA Infection Effectively. *Adv. Mater.* 2021, 33 (5), e2006047.
(6) Chen, W.; Liu, C.; Ji, X.; Joseph, J.; Tang, Z.; Ouyang, J.; Xiao, Y.; Kong, N.; Joshii, N.; Farokhzad, O. C.; et al. Stanene-Based Nanoshells for beta-Elemene Delivery and Ultrasound-Mediated Combination Cancer Therapy. *Angew. Chem., Int. Ed.* 2021, 60 (13), 7155−7164.
(7) Ouyang, J.; Tang, Z.; Farokhzad, N.; Kong, N.; Kim, N. Y.; Feng, C.; Blake, S.; Xiao, Y.; Liu, C.; Xie, T.; et al. Ultrasound mediated therapy: Recent progress and challenges in nanoscience. *Nano Today* 2020, 35, 100949.
(8) Hu, H.; Feng, W.; Qian, X.; Yu, L.; Chen, Y.; Li, Y. Emerging Nanomedicine-Enabled/Enhanced Nanodynamic Therapies beyond Traditional Photonodynamics. *Adv. Mater.* 2021, 33, No. e2005062.
(9) Zhu, P.; Chen, Y.; Shi, J. Piezocatalytic Tumor Therapy by Ultrasound-Triggered and BaTiO3 -Mediated Piezoelectricity. *Adv. Mater.* 2020, 32 (29), No. e2001976.
(10) Zhang, C.; Zeng, Z.; Cui, D.; He, S.; Jiang, Y.; Li, J.; Huang, J.; Pu, K. Semiconducting polymer nano-PROTACs for activatable photo-immunomediated cancer therapy. *Nat. Commun.* 2021, 12 (1), 2934.
(11) Zhang, Y.; He, S.; Chen, W.; Liu, Y.; Zhang, C.; Miao, Q.; Pu, K. Activatable Polymeric Nanoprobe for Near-Infrared Fluorescence and Photothermal Imaging of T Lymphocytes. *Angew. Chem., Int. Ed.* 2021, 60 (11), S291−S297.
(12) Lyu, Y.; He, S.; Li, J.; Jiang, Y.; Sun, H.; Miao, Y.; Pu, K. A Photobleaching Semiconducting Polymer Nanotransducer for Near-Infrared Regulation of CRISPR/Cas9 Gene Editing. *Angew. Chem., Int. Ed.* 2019, 58 (50), S18197−S18201.
(13) Liang, S.; Deng, X.; Ma, P.; Cheng, Z.; Lin, J. Recent Advances in Nanomaterial-Assisted Combinational Sonodynamic Cancer Therapy. *Adv. Mater.* 2020, 32 (47), No. e2003214.
(14) Son, S.; Kim, J. H.; Wang, X.; Zhang, C.; Yoon, S. A.; Shin, J.; Sharma, A.; Lee, M. H.; Cheng, L.; Wu, J.; et al. Multifunctional sonosensitizers in sonodynamic cancer therapy. *Chem. Soc. Rev.* 2020, 49 (11), 3244−3261.
(27) Kleinstiver, B. P.; Pattanayak, V.; Prew, M. S.; Tsai, S. Q.; Nguyen, N. T.; Zheng, Z.; Joung, J. K. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* **2016**, *529* (7587), 490–5.

(28) Konermann, S.; Brigham, M. D.; Trevino, A. E.; Joung, J.; Abudayeh, O. O.; Barcena, C.; Hsu, P. D.; Habib, N.; Gootenberg, J. S.; Nishimasu, H.; et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **2015**, *517* (7536), 583–8.

(29) Wolter, J. M.; Mao, H.; Fragola, G.; Simon, J. M.; Krantz, J. L.; Bazick, H. O.; Oztunali, B.; Stein, J. L.; Zylka, M. J. Cas9 gene therapy for Angelman syndrome targets Ube3a-ATS long non-coding RNA. *Nature* **2020**, *587* (7833), 281–284.

(30) Zhang, C.; Pu, K. Y. Molecular and nanoeengineering approaches towards activatable cancer immunotherapy. *Chem. Soc. Rev.* **2020**, *49* (13), 4234–4253.

(31) Pan, Y.; Yang, J.; Luan, X.; Liu, X.; Li, X.; Yang, J.; Huang, T.; Sun, L.; Wang, Y.; Lin, Y.; Song, Y. Near-infrared upconversion-activated CRISPR-Cas9 system: A remote-controlled gene editing platform. *Sci. Adv.* **2019**, *5* (4), No. eaav7199.

(32) Tang, H.; Xu, X.; Chen, Y.; Xin, H.; Wan, T.; Li, B.; Pan, H.; Li, D.; Ping, Y. Reprogramming the Tumor Microenvironment through Second-Near-Infrared-Window Photothermal Genome Editing of PD-L1Mediated by Supramolecular Gold Nanorods for Enhanced Cancer Immunotherapy. *Adv. Mater.* **2021**, *33* (12), No. e2006003.

(33) Lee, B.; Lee, K.; Panda, S.; Gonzales-Rojas, R.; Chong, A.; Bugay, V.; Park, H. M.; Brenner, R.; Murthy, N.; Lee, H. Y. Nanoparticle delivery of CRISPR into the brain rescues a mouse model of fragile X syndrome from exaggerated repetitive behaviours. *Nat. Biomed. Eng.* **2018**, *2* (7), 497–507.

(34) Yin, H.; Song, C. Q.; Dorkin, J. R.; Zhu, L. J.; Li, Y.; Wu, Q.; Park, A.; Yang, J.; Suresh, S.; Bizhanova, A.; Gupta, A.; et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nat. Biotechnol.* **2016**, *34* (3), 328–33.

(35) Xu, C.; Lu, Z.; Luo, Y.; Liu, Y.; Cao, Z.; Shen, S.; Li, H.; Liu, J.; Chen, K.; Chen, Z.; Yang, X.; Gu, Z.; Wang, J. Targeting of NLRP3 inflammasome with gene editing for the amelioration of inflammatory diseases. *Nat. Commun.* **2018**, *9* (1), 4992.

(36) Liu, J.; Chang, J.; Jiang, Y.; Meng, X.; Sun, T.; Mao, L.; Xu, Q.; Wang, M. Fast and Efficient CRISPR/Cas9 Genome Editing In Vivo Enabled by Bioreducible Lipid and Messenger RNA Nanoparticles. *Adv. Mater.* **2019**, *31* (33), No. e1902575.

(37) Yin, H.; Song, C. Q.; Suresh, S.; Wu, Q.; Walsh, S.; Rhyu, L. H.; Mintzer, E.; Bolukbas, M. F.; Zhu, L. J.; Kauffman, K.; et al. Structure-guided chemical modification of guide RNA enables potent non-viral in vivo genome editing. *Nat. Biotechnol.* **2017**, *35* (12), 1179–1187.

(38) Miller, J. B.; Zhang, S.; Kos, P.; Xiong, H.; Zhou, K.; Perelman, S. S.; Zhu, H.; Siegwart, D. J. Non-Viral CRISPR/Cas Gene Editing In Vitro and In Vivo Enabled by Synthetic Nanoparticle Co-Delivery of Cas9 mRNA and sgRNA. *Angew. Chem., Int. Ed.* **2017**, *56* (4), 1059–1063.

(39) Gillieron, J.; Querbes, W.; Zeigerer, A.; Borodovsky, A.; Marsico, G.; Schubert, U.; Manygoats, K.; Seifert, S.; Andree, C.; Stoter, M.; Epstein-Barash, H.; et al. Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nat. Biotechnol.* **2013**, *31* (7), 638–46.

(40) Wittrup, A.; Ai, A.; Liu, X.; Hamar, P.; Trifonova, R.; Charisse, K.; Manoharan, M.; Kirchhausen, T.; Lieberman, J. Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown. *Nat. Biotechnol.* **2015**, *33* (8), 870–6.

(41) Zuris, J. A.; Thompson, D. B.; Shu, Y.; Guilinger, J. P.; Bessen, J. L.; Hu, J.; Maeder, M. L.; Joung, J. K.; Chen, Z. Y.; Liu, D. R. Cas9 lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat. Biotechnol.* **2015**, *33* (1), 73–80.

(42) Ramakrishna, S.; Kwaku Dad, A. B.; Beloor, J.; Gopalappa, R.; Lee, S. K.; Kim, H. Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome Res.* **2014**, *24* (6), 1020–7.

(43) Kim, S.; Kim, D.; Cho, S. W.; Kim, J.; Kim, J. S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* **2014**, *24* (6), 1012–9.