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Supplementary Materials for

Supramolecular nanosubstrate–mediated delivery system enables CRISPR-Cas9 knockin of hemoglobin beta gene for hemoglobinopathies

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Materials

Chemical reagents and solvents were purchased from Sigma (Missouri) and used as received without further purification unless otherwise noted. For cell culture: the 8-well rectangular culture plates were purchased from Thermo Fisher Scientific (Massachusetts). Fetal bovine serum (FBS), OPTI-1640 and OPTI-MEM were obtained from Thermo Fisher Scientific (Gibco Life Technology, Massachusetts). For Indel analysis: DNA extraction kit was purchased from Qiagen (QIAamp® DNA Mini Kit, Hilden, Germany). EnGen® Mutation Detection kit was purchased from NEB. Lipofectamine CRISPRMAX agent and Lipofectamine 3000 agent were both purchased from Thermo Fisher Scientific. The methods for Lipofectamine CRISPRMAX and Lipofectamine 3000 both followed the protocols from the Thermo Fisher Scientific website. Cas9 Nuclease: EGFP-Cas9 nuclease was purchased from GenScript (New Jersey), while Cas9 protein was purchased from Thermo Fisher Scientific (Massachusetts). The Donor DNA HBB/GFP-plasmid was purchased from GeneCopoeia (Knock-in ORF donor clone for HBB, DC-I0513-SH01, Maryland). K562 3.21 (SCD) cell line was provided by Prof. Kohn's group at UCLA.

Characterization methods and settings

Dynamic light scattering (DLS) and Zeta potential were measured on a Zetasizer Nano instrument (Malvern Instruments Ltd., United Kingdom) equipped with a 10-mW helium-neon laser (λ = 632.8 nm) and a thermoelectric temperature controller. Measurements were taken at a 90° scattering angle.

Cell imaging studies were performed on a Nikon TE2000S inverted fluorescent microscope with a cooled charge-coupled device (CCD) camera (QImaging, Retiga 4000R), X-Cite 120 Mercury lamp, automatic stage, and filters for five fluorescent channels (W1: 325-375 nm, W2: 465-495 nm, W3: 570-590 nm, W4: 590-650 nm, and W5: 650-900 nm). Fluorescence intensities were measured by a Fujifilm BAS-5000 microplate reader. Microscopy-based image cytometry was used to detect the cellular uptake performances under different conditions. After different

treatments, the cells were harvested and the GFP signal was quantified by NIS-Elements imaging software. The “cellular uptake %” was determined based on the fluorescence intensities (>250, compared with control). About 1000-2000 cells were analyzed per sample.

Scanning electron microscopy (SEM) images were obtained using a TS-5136MM (TESCAN, Czech Republic) scanning electron microscope at an accelerating voltage of 20 kV. Samples dispersed at an appropriate concentration were cast onto a glass sheet at room temperature and sputter-coated with gold.

Transmission electron microscopy (TEM) images were recorded using a Philips CM 120 electron microscope, operating at an acceleration voltage of 120 kV. The TEM samples were prepared by drop-coating 2 μL of sample suspension solutions onto carbon-coated copper grids. Excess amounts of solution were removed by filter papers after 45 s. Subsequently, the samples were negatively stained with 2% uranyl acetate for 45 s before TEM studies.

Preparation of Adamantane-Grafted Silicon Nanowire Substrates (Ad-SiNWS)

We fabricated SiNWS *via* a wet chemical etching process. First, the surface of the silicon substrate was made hydrophilic according to the following procedure: the silicon wafer was ultrasonicated in acetone and ethanol at room temperature for 10 and 5 min, respectively, to remove contamination from organic grease. Then, the degreased silicon substrate was heated in boiling piranha solution (4:1 (v/v) H_2SO_4/H_2O_2, Safety warning: piranha solution is very dangerous, being both strongly acidic and a strong oxidizer. Solution that is no longer being used should never be left unattended if hot. It should not be stored in a closed container. Piranha solution should not be disposed of with organic solvents.) and RCA solution (1:1:5 (v/v/v) $NH_3/H_2O_2/H_2O$) each for 1 h. Subsequently, the silicon substrate was rinsed several times with deionized water. Then, the clean silicon substrate was used in a wet chemical etching process. An etching mixture consisting of deionized water, 4.6 M HF, and 0.2 M silver nitrate was used at room temperature. The etching duration was dependent upon the required length of the nanowires. After etching, the substrate was immersed in

boiling *aqua regia* (3:1 (v/v) HCl/HNO_3, Safety warning: *aqua regia* solution is very dangerous, being both strongly acidic and a strong oxidizer. Solution that is no longer being used should never be left unattended if hot. It should not be stored in a closed container. The *aqua regia* solution should not be disposed of with organic solvents.) for 15 min to remove the silver film. Finally, the substrate was rinsed with DI water and dried under nitrogen and was then ready for surface modification. The surface modification of the SiNWS was processed with 4% (v/v) 3-aminopropyl trimethoxysilane in ethanol at room temperature for 45 min. Then, the SiNWS were treated with the 1-adamantane isocyanate (1.0 mM) in DMSO for 30 min. The modified Ad-SiNWS were then washed with DMSO twice to remove excess 1-adamantane isocyanate. The substrates were rinsed with DI water three times and stored at 4 ^{o}C before cell seeding.

Stoichiometric calculations for determining the amount of Cas9•sgRNA and HBB/GFP-plasmid encapsulated into each Cas9•sgRNA⊂SMNP and HBB/GFP-plasmid⊂SMNP, respectively

The total number of Cas9•sgRNA⊂SMNPs, n_{vector} , was calculated using the equation:

$$n_{vector} = \frac{m_{total\ vectors}}{m_{vector}} = \frac{m_{total\ vectors}}{\frac{4}{3}\pi r^3 \rho} \qquad (1)$$

where $m_{total\ vectors}$ is the total mass of Cas9•sgRNA⊂SMNPs (75.8 μg), r is the radius of SMNP vector (120 nm), ρ is the density of SMNP vector (1.1 g/cm^3). By calculation, n_{vector}=8.4×10^{10}.

The total number of Cas9 protein, n_{cas9} was determined *via* the equation:

$$n_{cas9} = \frac{m_{total\ cas9}}{M_{cas9}} N_A \qquad (2)$$

where $m_{total\ cas9}$ is the total mass of Cas9 protein (3.0 μg), M_{cas9} is the molecular weight of Cas9 protein (160 kDa), N_A is the Avogadro constant (6.02×10^{23}). By calculation, n_{vector}=1.1×10^{13}.

The number of Cas9•sgRNA encapsulated into each Cas9•sgRNA⊂SMNP, $n_{cas9/vector}$ was found to be:

$$n_{cas9/vector} = \frac{n_{cas9}}{n_{vector}} \approx 130 \quad (3)$$

The total number of HBB/GFP-plasmid⊂SMNPs, n_{vector} was calculated using the equation:

$$n_{vector} = \frac{m_{total\ vectors}}{m_{vector}} = \frac{m_{total\ vectors}}{\frac{4}{3}\pi r^3 \rho} \quad (4)$$

where $m_{total\ vectors}$ is the total mass of HBB/GFP-plasmid⊂SMNPs (50.4 μg), r is the radius of SMNP vector (140 nm), ρ is the density of SMNP vector (1.1 g/cm^3). By calculation, n_{vector} =3.2×10^{10}.

The total number of HBB/GFP-plasmid, $n_{plasmid}$ was determined using:

$$n_{plasmid} = \frac{m_{total\ plasmid}}{M_{plasmid}} N_A \quad (5)$$

where $m_{total\ plasmid}$ is the total mass of HBB/GFP-plasmid (1.0 μg), $M_{plasmid}$ is the molecular weight of HBB/GFP-plasmid (10 kp≈6600 kDa), N_A is the Avogadro constant (6.02×10^{23}). By calculation, $n_{plasmid}$=9.1×10^{10}.

The number of HBB/GFP-plasmid encapsulated into each HBB/GFP-plasmid⊂SMNP, $n_{plasmid/vector}$ was:

$$n_{plasmid/vector} = \frac{n_{plasmid}}{n_{vector}} \approx 3 \quad (6)$$

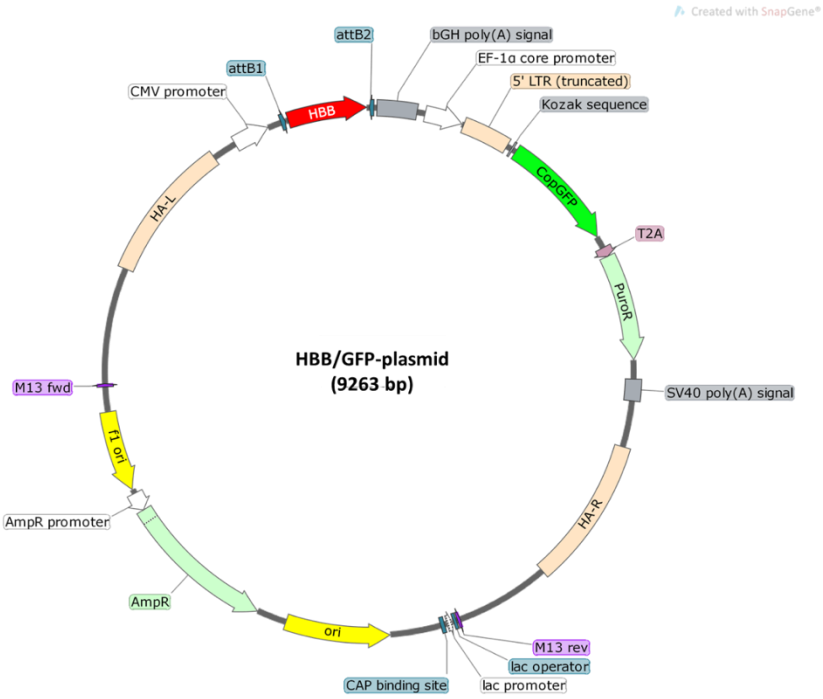

Fig. S1. Detailed map of HBB/ GFP-plasmid.

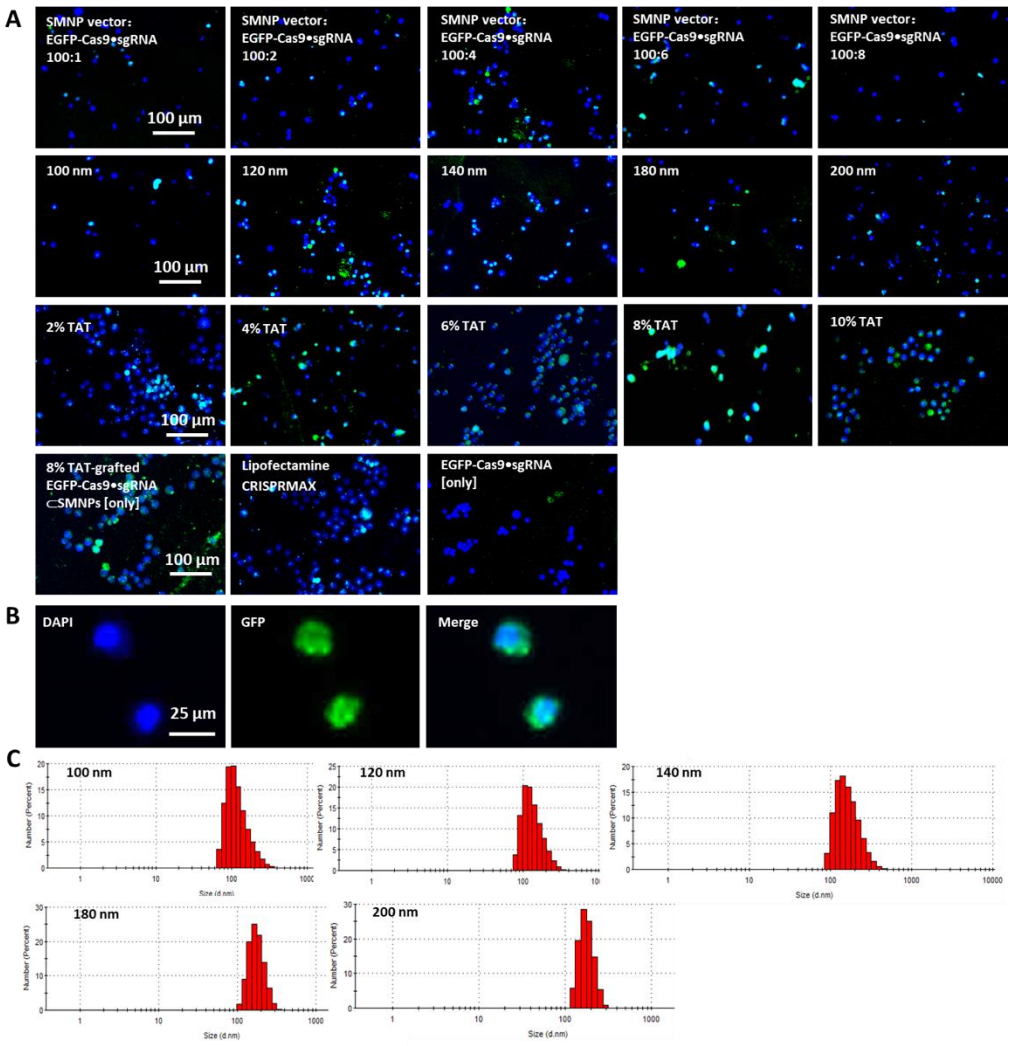

Fig. S2. The combined SMNP/SNSMD strategy for delivering EGFP-Cas9•sgRNA into K562 3.21 cells. (A) Representative fluorescence images of K562 3.21 cells treated by EGFP-Cas9•sgRNA⊂SMNPs with 15 formulations. Three control studies, *i.e.*, K562 3.21 cells treated with EGFP-Cas9•sgRNA⊂SMNPs (no Ad-SiNWS), Lipofectamine CRISPRMAX agent (encapsulated EGFP-Cas9•sgRNA), and EGFP-Cas9•sgRNA (without SMNP vectors) were conducted in parallel. **(B)** The enlarged image of the K562 3.21 cells treated by 8%-TAT-grafted Cas9•sgRNA⊂SMNPs shows that EGFP-Cas9 was successfully delivered and accumulated into the cell nucleus after 24 h. **(C)** Hydrodynamic sizes of the resulting EGFP-Cas9•sgRNA⊂SMNPs were determined by dynamic light scattering (DLS).

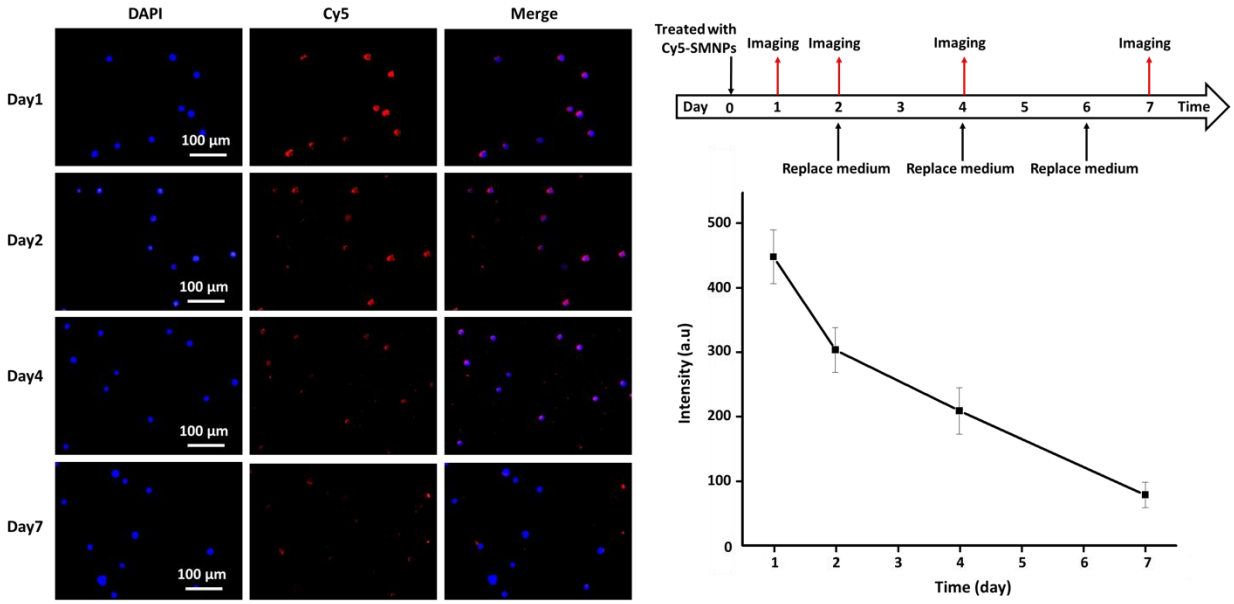

Fig. S3. Representative fluorescence images and fluorescence intensities of K562 3.21 cells treated by Cas9•sgRNA⊂Cy5-SMNPs at different times.

Top 5 Off-Target (OT) Loci

Target site	sgRNA sequence (w/ PAM)	Chromosome	Position	Direction	MMs	PCR Product
OT_1	GGGGCaACTAGaGACAGGAaGGG	Chr8	22778067	R	3	703
OT_2	GGtGCCACTAGGcACAGGAgCGG	Chr8	143802949	F	3	618
OT_3	tGGGCCACTAtGGACAGGAaTGG	Chr12	108187898	R	3	523
OT_4	GaGGCCACcAGGGACAGGcTGGG	Chr5	170084408	R	3	613
OT_5	GGGGtacCTAGGGtCAGGATGGG	Chr8	23551868	F	4	603

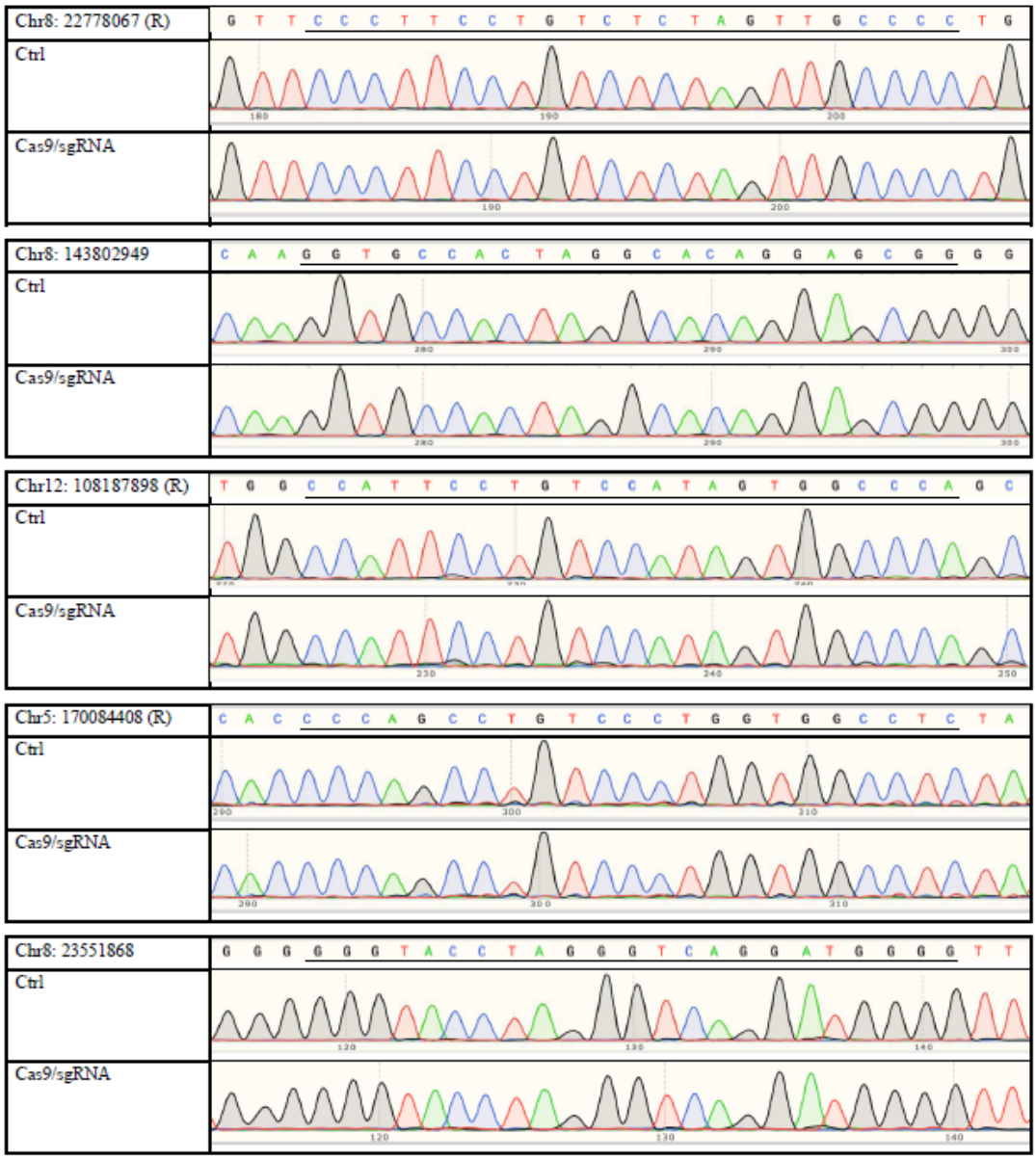

Fig. S4. Off-target effect analysis in K562 3.21 treated by Cas9•sgRNA⊂SMNPs. Detailed potential off-target sites for the Cas9•sgRNA-AAVS1 were predicted using online software and Sanger sequencing of PCR products amplified and sequenced. Mismatches (MMs) for up to five potential off-target sites were selected for analysis. Red indicates a mismatch with the targeted sequence. Note that there were no off-target sites with 1 MM. Four potential off-target sites with 3 MMs and one potential off-target site with 4 MMs were identified in coding regions.

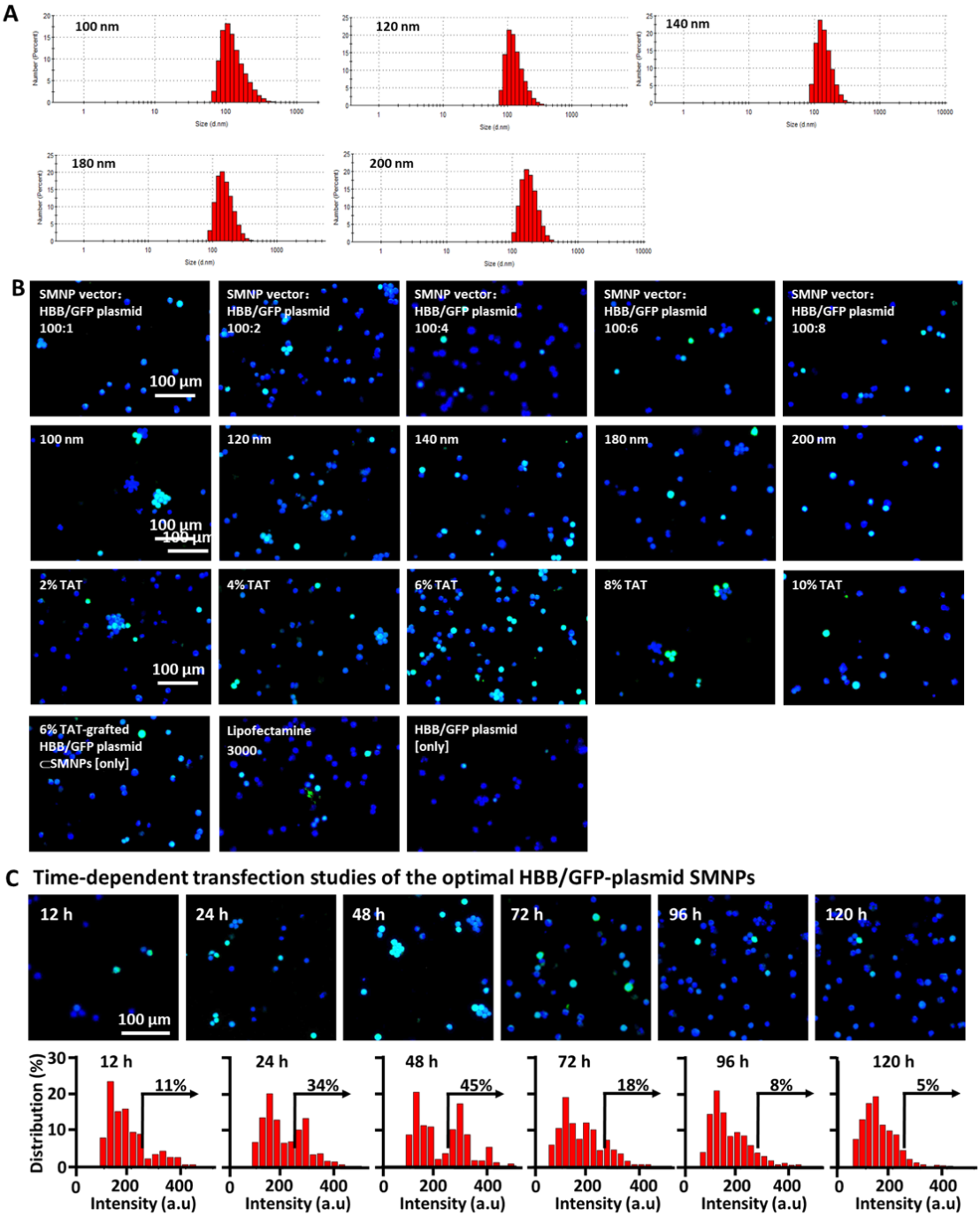

Fig. S5. The combined SMNP/SNSMD strategy for delivering HBB/GFP-plasmid into K562 3.21 cells. (A) Hydrodynamic sizes of the resulting HBB/GFP-plasmid⊂SMNPs with different sizes. **(B)** Representative fluorescence images of K562 3.21 cells treated by HBB/GFP-plasmid⊂SMNPs with 15 formulations. Three control studies, *i.e.*, K562 3.21 cells treated with HBB/GFP-plasmid⊂SMNPs (no Ad-SiNWS), Lipofectamine 3000 agent (encapsulated HBB/GFP-

plasmid) and HBB/GFP-plasmid (without SMNP vectors) were conducted in parallel. **(C)** Serial fluorescent micrographs of the K562 3.21 cells taken at 12, 24, 48, 72, 96 and 120 h after their treatment by HBB/GFP-plasmid⊂SMNP *via* the combined SMNP/SNSMD strategy. Histograms of EGFP-Cas9 uptake in individual K562 3.21 cells at 12, 24, 48, 72, 96 and 120 h.

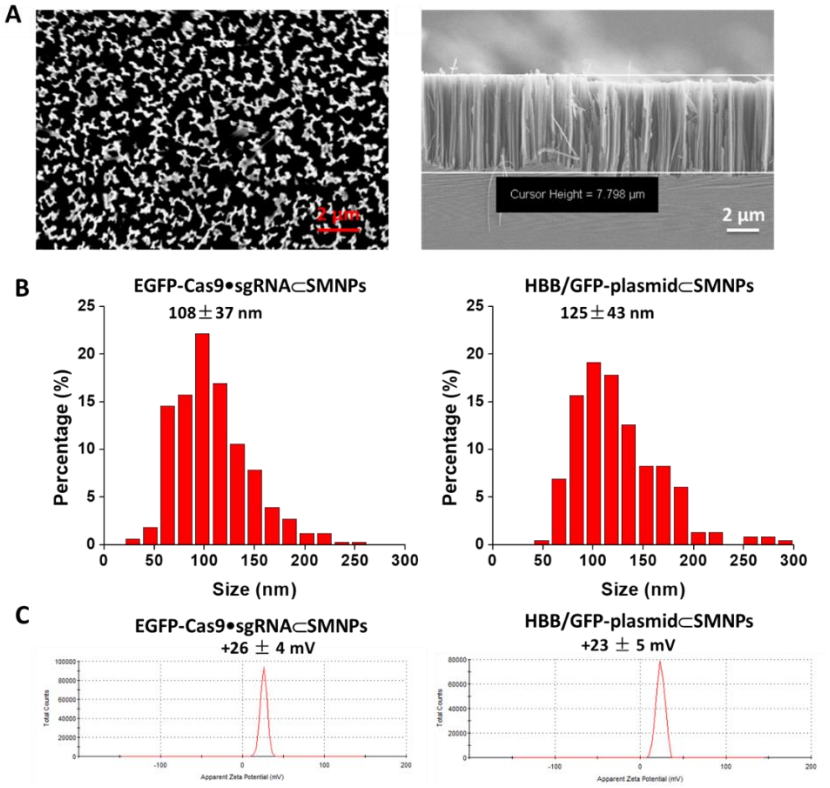

Fig. S6. Characterization of the Ad-SiNWS and SMNPS. (**A**) Scanning electron microscope images of the Ad-SiNWS, which were prepared from wet-etching followed by covalent functionalization of Ad. The diameters and lengths of Ad-SiNWS are ca. 100-150 nm and 5-10 μm, respectively. **(B)** The size distributions of the resulting optimal EGFP-Cas9•sgRNA⊂SMNPs (108±37 nm) and HBB/GFP-plasmid⊂SMNPs (125±43 nm) were checked by SEM and TEM. Nano Measure 1.2 software was used to make statistics of the size distribution of SMNPs in the TEM and SEM images. More than 200 SMNPs were counted in each sample. **(C)** The surface-charge densities of SMNPs were determined by zeta potential measurements in PBS buffer solution,

which showed that the zeta potentials of 120 nm 8%-TAT-grafted Cas9•sgRNA⊂SMNPs and 140 nm 6% TAT-grafted HBB/GFP-plasmid⊂SMNPs were +26 ± 4 mV and +23 ± 5 mV, respectively.

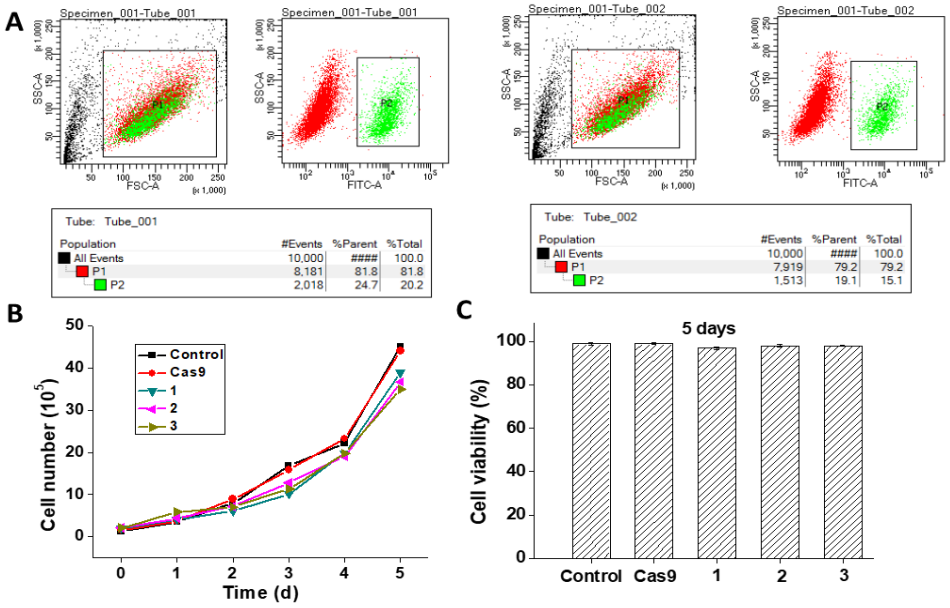

Fig. S7. GFP knockin efficiency, cell growth and cell viability of K562 3.21 cells treated by Cas9•sgRNA⊂SMNPs and HBB/GFP-plasmid⊂SMNPs. (A) Quantitative analysis of GFP knockin efficiency of the K562 3.21 cells harvested after the three-round SMNP treatments checked by flow cytometry. Two samples were tested in parallel at the same time. **(B)** Cell growth and **(C)** cell viability of K562 3.21 cells treated by Cas9•sgRNA⊂SMNPs and HBB/GFP-plasmid⊂SMNPs from 1 to 3 rounds.

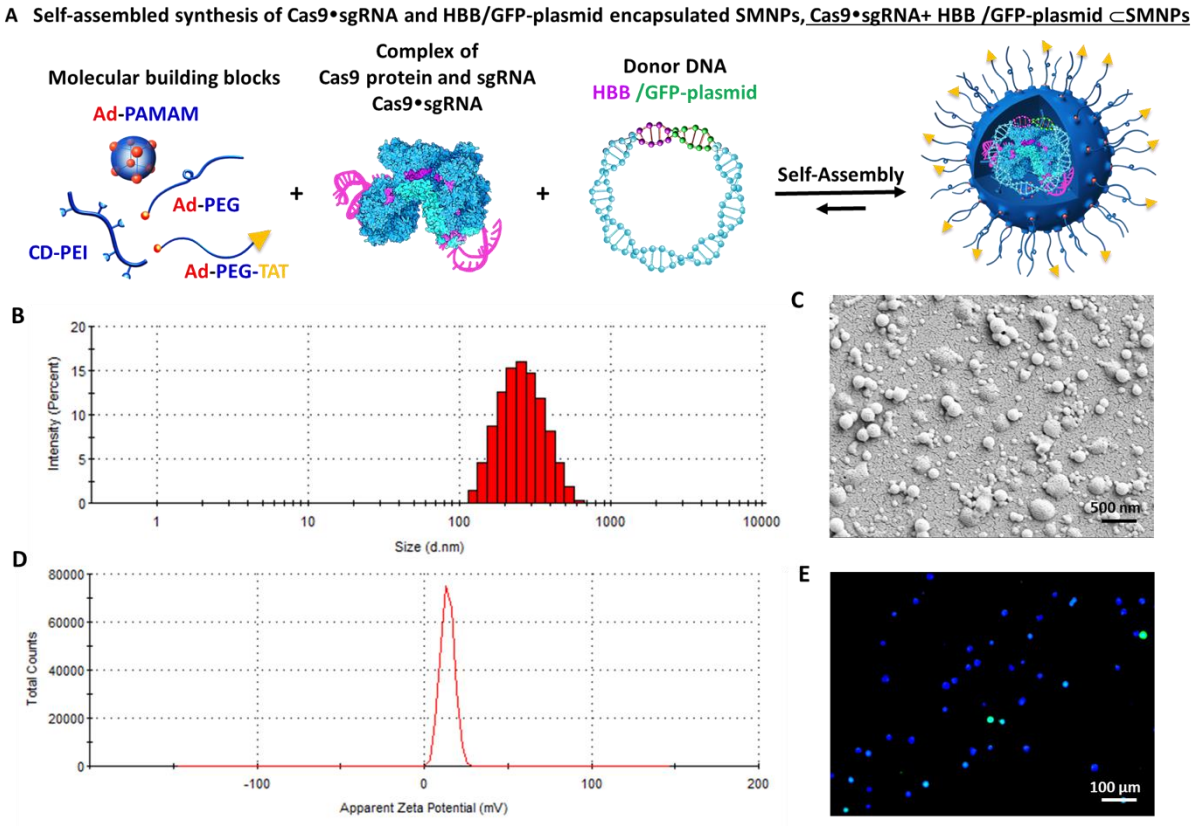

Fig. S8 Delivery of Cas9•sgRNA+HBB/GFP-plasmid⊂SMNPs into K562 3.21 cells. (A) A self-assembled synthetic approach for the preparation of Cas9•sgRNA+HBB/GFP-plasmid⊂SMNPs. **(B)** Hydrodynamic size of the resulting co-encapsulated SMNPs by dynamic light scattering (DLS). **(C)** Scanning electron microscopy (SEM) image of co-encapsulated SMNPs. **(D)** The zeta potential of co-encapsulated SMNPs. **(E)** Fluorescence image of the K562 3.21 cells harvested 10 days after co-encapsulated SMNP treatments.

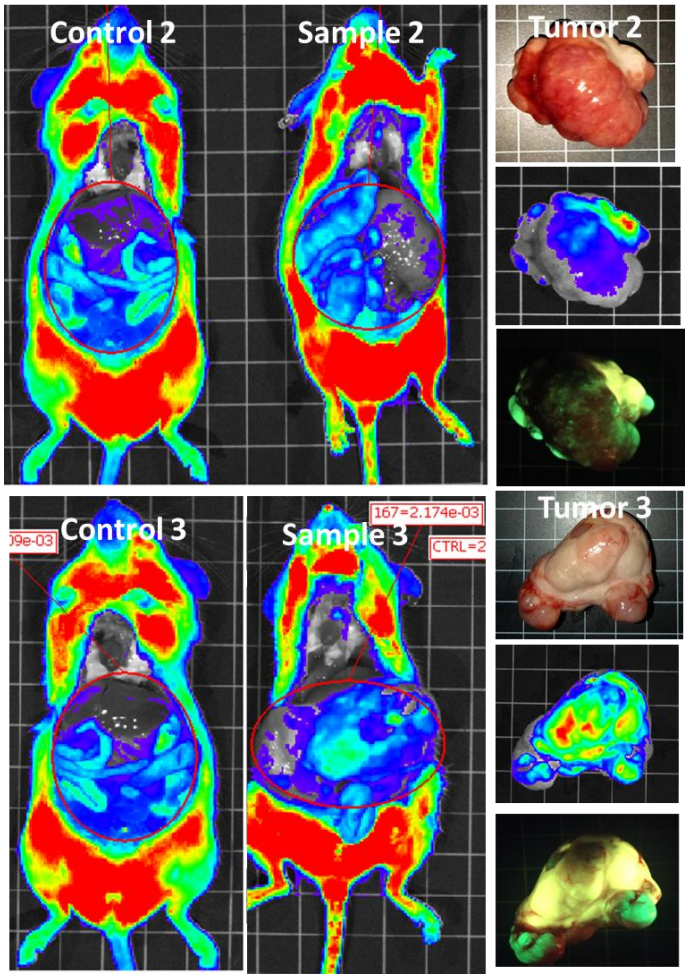

Fig. S9. Analysis of the proliferative potential of HBB/GFP-knockin K562 3.21 cells *in vivo*. HBB/GFP-knockin K562 3.21 cells were introduced into athymic nude mice via intraperitoneal injection to test the cells' *in vivo* growth potential. IVIS spectrum images taken post-injection of control and HBB/GFP-knockin K562 3.21 cells. Control did not form tumor (left panel). Images of the harvested tumors at the end of the experiment in Sample 2 (top right panel) and Sample 3 (bottom right panel). (Photo Credit: Photographer Name: Shih-Jie Chou. Photographer Institution: Division of Basic Research, Department of Medical Research, Taipei Veterans General Hospital, Taipei, Taiwan. Institute of Pharmacology, School of Medicine, National Yang-Ming University, Taipei, Taiwan).

Table S1. Comparison of knockin efficiencies between this study and other studies.

Study	Cell line	Cell type	Cas9 system	Delivery method	Pathway	Size of knockin gene	Knockin efficiency
This Study	K562	Suspension cell	Cas9 RNP	Non-virus	HDR	3.7 kb	21%
A. Paix, *et al.* (*34*)	HEK293T	Adherent cell	Cas9 RNP	Electroporation	HDR	1.0 kb	17.9%
						0.7 kb	23.5%
X. He, *et al.* (39)	SMMC-7721	Adherent cell	Cas9/gRNA plasmid	Electroporation	NHEJ	4.6 kb	18.2%
					HDR		2.5%
	LO2	Adherent cell			NHEJ		14.3%
					HDR		5.0%
X. Yao, *et al.* (37)	HEK293T	Adherent cell	Cas9 mRNA/gRNA	Virus	HMEJ	0.8 kb	18%
J. Zhang, *et al.* (35)	HEK293T	Adherent cell	Cas9/gRNA plasmid	Virus	HDR	0.7 kb	21.2%
X. Yao, *et al.* (36)	Mouse hepatocyte	Adherent cell	Cas9/gRNA plasmid	Virus	HMEJ	0.7 kb	20.5%
J. Song, *et al.* (38)	Rabbit embryos	N/A	Cas9 mRNA/gRNA	Microinjection (with an HDR enhancer, RS-1)	HDR	2.0 kb	26.1%