Tumor-microenvironment activated duplex genome-editing nanoprodrug for sensitized near-infrared titania phototherapy

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Abstract
Near-infrared (NIR)-light-triggered nanomedicine, including photodynamic therapy (PDT) and photothermal therapy (PTT), is growing an attractive approach for cancer therapy due to its high spatiotemporal controllability and minimal invasion, but the tumor eradication is limited by the intrinsic anti-stress response of tumor cells. Herein, we fabricate a tumor-microenvironment responsive CRISPR nanoplatform based on oxygen-deficient titania (TiO₂-x) for mild NIR-phototherapy. In tumor microenvironment, the overexpressed hyaluronidase (HAase) and glutathione (GSH) can readily destroy hyaluronic acid (HA) and disulfide bond and releases the Cas9/sgRNA from TiO₂-x to target the stress alleviating regulators, i.e., nuclear factor E2-related factor 2 (NRF2) and heat shock protein 90α (HSP90α), thereby reducing the stress tolerance of tumor cells. Under subsequent NIR light illumination, the TiO₂-x demonstrates a higher anticancer effect both in vitro and in vivo. This strategy not only provides a promising modality to kills cancer cells in a minimal side-effects manner by interrupting anti-stress pathways but also proposes a general approach to achieve controllable gene editing in tumor region without unwanted genetic mutation in normal environments.

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1. Introduction

Near-infrared (NIR) photo-triggered cancer treatments (photothermal therapy) and photothermal therapy (PTT) have attracted extensive interest due to their remarkable effect on synergistic cancer therapy. Compared with conventional strategies, for example, chemotherapy, near-infrared light-activated therapy demonstrates reduced side effects, minimal invasive nature, and higher spatiotemporal selectivity for precisely controlled promising oncotherapy. However, phototherapy would trigger a stress response in living cells, which serves as a survival mechanism to relieve the induced oxidative/heat stress and damage for improving survival of cancer cells in harsh microenvironment. Such survival pathways are generally mediated by several proteins including nuclear factor E2-related factor 1 (NRF2), and hypoxia-inducible factor 1 (HIF-1), heat-shock protein 90 (HSP90), and hypoxia-inducible stress related proteins (HSP90α and NRF2), leading to the reduced tolerance of tumor cells to photothermal and reactive oxygen species (ROS). Therefore, local heat and oxidative stress subsequently generated by TiO$_2$ NPs under mild 808 nm laser irradiation can obtain desirable antitumor efficacy. This work provided a new method to improve the combination therapeutic effects of PTT and PDT against cancer, which had the great promising potential for further application in the field of synergistic therapy based on CRISPR-Cas9 gene editing system enhanced PTT/PDT.

2. Materials and methods

2.1. Materials

Titanium butoxide (TBOT) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). The acetone and ethanol were acquired from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). Acetic acid, ethylene glycol, sodium borohydride (NaBH$_4$), 3-aminopropyl triethoxysilane (APTES) were obtained from Aladdin Co., Ltd. (Shanghai, China). The 1,3-diphenylbenzofuran (DPBF) was purchased from Solarbio (Beijing, China). The sgRNA sequence, 3,3’-dithiodipropionic acid-di(N-succinimidyl ester) (DSP), and BCA protein assay kit were purchased from Sangon Biotech (Shanghai, China). Calcein-AM and PI were obtained from Yeasen Biology (Shanghai, China). DMEM media were acquired from Gibco (New York, USA). Fetal bovine serum (FBS) was obtained from ExCell Bio (Shanghai, China). CCK-8 reagent, Hoechst, and dichlorodihydro fluorescein-acetoacetate (DCFH-DA) were acquired from Beyotime (Shanghai, China).

2.2. Characterization

The transmission electron microscope (TEM) images and high-resolution transmission electron microscopy (HRTEM) images of nanoparticles were detected with an EMXplus (Bruker, Germany). X-ray spectra were detected with an XUV 3600 (SHIMADZU, Japan). FTIR spectra were conducted on PHI5000 VersaProbe from ULVAC-PHI, Japan. X-ray diffraction (XRD) patterns were obtained on XTRA from Thermal Scientific. X-ray photoelectron spectroscopy (XPS) was conducted on PHIS000 VersaProbe from ULVAC-PHI, Japan. Zeta potentials of all samples were conducted by electrophoretic light scattering (Brookhaven Instruments Corporation, Holtsville, NY, USA). The UV–Vis–NIR spectra were recorded using a UV3600 (SHIMADZU, Japan). Fetal bovine serum (FBS) was obtained from ExCell Bio (Shanghai, China). CCK-8 reagent, Hoechst, and dichlorodihydro fluorescein-acetoacetate (DCFH-DA) were acquired from Beyotime (Shanghai, China).
2.3. Synthesis of TiO2 and TiO2-x

A mixture of 1 mL TBOT and 50 mL ethylene glycol was magnetically stirred for 8 h. Then, the resulting mixture was added to a mixture of 1 L acetone, 4 mL H2O, and 800 mL glacial acetic acid for a 2-h stirring. After that, the precipitates were rinsed several times with DI water and ethanol, and the precipitates were kept in an oven at 50 °C for overnight drying. The products were white TiO2 NPs.

200 mg TiO2 NPs powder and 200 mg NaBH4 were ground thoroughly in a mortar. The mixture was transferred into a tube furnace, and mild reduction progress was conducted at 350 °C (heating rate of 10 °C/min) for 90 min in a nitrogen atmosphere. Afterwards, the product was washed with the mixture of DI water and ethanol three times to remove unreacted NaBH4. At last, the desired TiO2-x NPs were synthesized successfully.

2.4. Construction of sgRNA

The sgRNA used in this research was prepared due to the in vitro transcription kit (Inovogen). The necessary forward primer containing targeted genes was acquired from Sangon Biotech. The sgRNA sequences target sites were as follows: sgRNA (NRF2), 5′-TGGAGGCAAGATATAGATCT-3′; sgRNA (HSP90a), 5′-ATTC CCTGTCACTGCGTCAC-3′; scrambled sgRNA, 5′-GCACTAC-GAGCTAACTCA-3′.

2.5. Synthesis of TiO2-x@Cas9-NH@HA

TiO2-x NPs (10 mg) were mixed with APTES (200 µL) and stirred for 24 h. Then, the product was washed three times with DI water/ethanol mixture. After that, TiO2-x@APTES NPs were dispersed in an aqueous medium and were added dropwise to DSP solution (5 mmol/L) dissolved in DMSO. The mixture was stirred magnetically for 2 h and was washed with DI water. TiO2-x@DSP NPs were dispersed in 20 mmol/L HEPES Buffer and ready for use. The complexed sgRNA (1 µg sgRNA (NRF2) and 1 µg sgRNA (HSP90α) in 10 µL) was added to the Cas9 protein (8 µg in 10 µL) in 80 µL of nuclease-free Cas9 buffer (20 mmol/L HEPES, 150 mmol/L KCl, 10% glycerol, pH 7.5). The mixture was allowed to react for 10 min at room temperature. Cas9/sgRNA complex was added to the TiO2-x@DSP NPs solution for a 2h reaction, generating TiO2-x@Cas9-NH NPs (100 µg/mL). Finally, 100 µg of HA was added to the TiO2-x@Cas9-NH solution, followed by stirring for half an hour. The TiO2-x@Cas9-NH@HA products were obtained by centrifugation.

2.6. Photothermal property

An infrared thermal image recorder (Testo 869) was employed to characterize the photothermal performance of TiO2-x, by recording the temperature changes during 808 nm laser irradiation. TiO2-x aqueous solution at different concentrations (0, 25, 50, 100, and 200 µg/mL) were exposed to 808 pump laser irradiation at laser power densities of 1.5 W/cm². Furthermore, the temperature elevation of TiO2-x dispersed in DI water at a concentration of 200 µg/mL as irradiated by an 808 nm laser at various power intensities (0.5, 0.75, 1.0, 1.25, and 1.5 W/cm²) was characterized. Photonothermal stability of TiO2-x NPs was evaluated by periodic laser irradiation for four cycles (laser on for 10 min and off for 10 min).

2.7. Extracellular ROS detection

1,3-Diphenylisobenzofuran (DPBF) as a ROS molecular probe was used to detect the generation of singlet oxygen (1O2). DPBF dissolved in ethanol (0.5 mg/mL) was added into a TiO2 or TiO2-x aqueous solution (1 mL, 100 µg/mL). Next, the mixture was treated by 808 nm laser irradiation (1.5 W/cm²) for 4 min under stirring in the dark. The changes in DPBF were evaluated by
measuring the absorption intensity at 410 nm in UV−Vis absorption spectra.

2.8. Cell culture and biocompatibility assay

Human melanoma cell line A375 was obtained from American Type Culture Collection. The A375 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO2 with the temperature of 37 °C. To evaluate the in vitro biocompatibility of TiO2-x or TiO2-x@Cas9-NH@HA NPs, a typical CCK-8 viability assay was conducted. Different concentrations of TiO2-x or TiO2-x@Cas9-NH@HA (0, 25, 50, 100, 200, and 400 μg/μL) were incubated with A375 cells seeded in 96-well plates for 24 h, and then CCK-8 reagent (10 μL/100 μL DMEM) was added into the plates to characterize the absorption at a wavelength of 450 nm after 2 h on a microplate photometer.

2.9. Surveyor assay

After being treated with formulations, the DNA genomes of A375 cells were isolated and harvested with FastPure® Cell/Tissue DNA Isolation Mini Kit (Vazyme). The 2 × Phanta Max Master Mix (Vazyme) was applied to amplify sgNRF2-targeted Tissue DNA Isolation Mini Kit (Vazyme). The 2 of A375 cells were isolated and harvested with FastPure Phanta Max PCR program [(95 °C for 30 s; 60 °C for 30 s) for 35 cycles and (72 °C for 5 min)] was used. Then, we purified the amplified product and used the T7 Endonuclease I Kit (Vazyme) to detect the frequency of indels. The digested DNA was analyzed using 2% agarose gel electrophoresis. Indel formation efficiencies were calculated by Image J.

2.10. Western bolt analysis

Whole-cell lysates were prepared from variously treated cells using RIPA buffer containing protease inhibitors. The whole cell lysates were collected and quantified by the BCA protein assay. The same quantity of protein lysates was separated by SDS-PAGE on 10% SDS acrylamide gels and transferred onto a PVDF membrane. The membranes were incubated with primary antibodies against NRF2 and HSP90α respectively (1:1000 diluted) overnight at a refrigerator at 4 °C. Then, the membranes were incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. GAPDH (1:1000 dilution) was set as a loading control.

2.11. Intracellular ROS detection

A375 cells were plated into a 12-well plate at a density of 1 × 104 cells per well for 12 h and co-incubated with different formulations (100 μg/mL) at 37 °C for 6 h. After that, the cells were washed three times with PBS to remove noninternalized NPs. DCFH-DA (20 μmol/L) in DMEM was added to wells for a 30 min incubation in the dark. Then the A375 cells were irradiated with 808 nm light at the energy density of 1.5 W/cm² for 5 min. The cells were then observed by a fluorescence microscope, and intracellular green fluorescence was examined and recorded.

2.12. In vitro cytotoxicity assays

(i) The A375 cells were preseeded in 96-well plates (8 × 103 per well) and treated in various formulations. After a 24 h incubation, the cells were treated with NIR (1.5 W/cm², 5 min) or without NIR irradiation. Then cells were incubated in the incubator for another 24 h. We evaluated cell viability by the CCK-8 assay. (ii) The A375 cells were preseeded in 24-well plates (5 × 104 per well) and treated in various formulations. After a 24 h incubation, the cells were treated with NIR irradiation (1.5 W/cm², 5 min) or without NIR irradiation. Then cells were incubated in the incubator for another 6 h. After that, the cells were washed three times with PBS and stained with Calcein-AM and PI. Then the cells were washed with PBS three times and observed under a fluorescence microscope. (iii) For apoptosis analysis by flow cytometry, A375 cells were seeded in 24-well plates for 24 h. The YF 488-annexin V and PI Apoptosis Kit were employed and the results were acquired by a flow cytometer.

2.13. Distribution of nanoparticles in vivo and antitumor assessment

All animal procedures were approved and compiled with the guidelines of the Institutional Animal Care and Ethics Committee of Nanjing University. A375 cells were injected subcutaneously into the flank region of male BALB/c nude mice (4~5 weeks old) which bought from GemPharmatech (Jiangsu, China). Then Cy7 covalently modified NPs were intravenously injected into the nude mice bearing A375 xenograft tumor. After anesthetized by isoflurane, images of the mice were captured with Near-infrared optical imaging technology at the time of 3, 6, 12, 24 and 48 h post injection, respectively. Meanwhile, the fluorescence intensity of major organs (hearts, livers, spleens, lungs, and kidneys) and tumor issues ex vivo was observed with the imaging instrument at each time point. To evaluate the antitumor efficacy of TiO2-x@Cas9-NH@HA, A375 tumor xenograft bearing nude mice were randomly divided into seven groups (n = 4), which intravenous injection with PBS (with NIR), TiO2-x@Cas9-NH@HA (without NIR), TiO2-x (with NIR), TiO2-x@HA (with NIR), TiO2-x@Cas9-N@HA (with NIR), TiO2-x@Cas9-N@HA (with NIR), TiO2-x@Cas9-NH@HA (with NIR), respectively. After 24 h of injection, 808 nm laser irradiation (1.5 W/cm² for 10 min) was exposed to tumor region. Tumor volume and weight of mice were recorded every other day. Tumor volume was calculated as Eq. (1):

Volume (mm³) = 0.5 × (Length × Width²)  (1)

After the last treatment, the mice were euthanized and the tumors and major tissues were harvested. The resected tumors and major organs were fixed with 4% paraformaldehyde and prepared into tissue sections for hematoxylin and eosin (H&E) staining. The tumor slices were also for immunohistochemistry and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining with the manufacturer’s instructions.

2.14. Statistical analysis

All results were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to analyze the data. The level of significance between two groups of the data was analyzed based on the Student’s two-tailed t-test (**P < 0.05; ***P < 0.01; and ****P < 0.001).
3. Results and discussion

The monodisperse white titania (TiO$_2$) with the size of about 100 nm was synthesized via ethylene glycol-mediated control hydrolysis process of tetrabutyl titanate (TBOT) according to the previous report (Fig. 2A)$.^43$ After being reduced by NaBH$_4$, the white titania can be converted into black titania (TiO$_2$-$\times$) with an anatase crystal phase (Fig. 2B and C). To endow GSH-stimulus responsibility, the TiO$_2$-$\times$ was modified by (3-aminopropyl) triethoxysilane (APTES) to obtain amine groups (TiO$_2$-$\times$-$\text{NH}_2$), and a disulfide bond containing linker [3,3-dithiodipropionic acid-di (N-succinimidyl ester), DSP] was employed to bridge the resulting TiO$_2$-$\times$-$\text{NH}_2$ and Cas9 RNPs (TiO$_2$-$\times$@Cas9-NH). Finally, hyaluronic acid (HA) was functionalized onto TiO$_2$-$\times$@Cas9-NH for targeting tumor (Fig. 2D, and Supporting Information Fig. S1). Such titania exhibited a strong EPR signal at a $g$ value of 2.003 (Fig. 2E), indicating the existence of oxygen vacancies. Besides, we examined the element-valence status before and after reduction by X-ray photoelectron spectroscopy (XPS). No significant signal was observed in the recorded spectra, and two intense peaks at $\omega$465 and $\omega$459 eV, assigned to Ti 2p$_{1/2}$ and Ti 2p$_{3/2}$ in both forms of titanium oxide (Fig. 2F‒H, Supporting Information Fig. S2). The zeta potential analysis confirmed the successful synthesis of products at each step (Fig. 2I). And the characteristic peak of polysaccharide at 1078 cm$^{-1}$ in the Fourier transform infrared (FTIR) spectroscopy further suggested the coating of HA (Supporting Information Fig. S3).

Generally, strong absorption in the near infrared region is the key to therapeutic reagents for NIR-phototherapy. As shown in Fig. 3A and Supporting Information Fig. S4, TiO$_2$-$\times$@CasNH@HA NPs dispersed solution show strong absorption in the near infrared and visible regions. To clarify the high near infrared absorption, diffuse reflectance spectroscopy (DRS) was applied, and the results demonstrated that an enhanced absorbance in the Vis–NIR region was obtained in TiO$_2$-$\times$ (Supporting Information Fig. S5). According to the calculation of Kubelka–Munk function$,^44$ the band gap values of TiO$_2$ and TiO$_2$-$\times$ were 3.40 and 1.80 eV, respectively (Supporting Information Figs. S6 and S7), which was a symbol of the red shift of absorbed light, and suggesting a higher photothermal conversion effect of TiO$_2$-$\times$. As anticipated, the solutions can readily be heated by TiO$_2$-$\times$, exposed to 808 nm laser irradiation (Fig. 3B), while no obvious temperature change was obtained by TiO$_2$ (Supporting Information Fig. S8). In addition, the temperature change was highly dependent on TiO$_2$-$\times$ concentration and power density (Fig. 3C and D). To test the photothermal conversion efficiency, temperature change curve of TiO$_2$-$\times$ solution was recorded under continuous irradiation. From Fig. 3E and F, the photothermal-conversion efficiency of TiO$_2$-$\times$ was calculated to reach 40.1%. Moreover, the increase in temperature for the TiO$_2$-$\times$ remained almost robust after 4 cycles (Fig. 3G), indicating a good stability of photothermal conversion.

Importantly, oxygen-deficient of TiO$_2$-$\times$ also endowed effective ROS generation ability in a broad range of light wavelengths.

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As shown in Fig. 3H, the absorption of 1,3-diphenylbenzofuran (DPBF, an ROS probe) significantly decreases after treatment of TiO$_2$-x under NIR irradiation (1.5 W/cm$^2$, 4 min), which implied that TiO$_2$-x can effectively produce singlet oxygen ($^1$O$_2$) to oxidized DPBF. To highlight the superiority of TiO$_2$-x, the ROS production abilities under 808 nm NIR laser irradiation were studied, as compared with that of TiO$_2$. After 4 min of laser irradiation (1.5 W/cm$^2$), a more significant amount of $^1$O$_2$ in TiO$_2$-x group was observed than that of TiO$_2$, as expected (Supporting Information Figs. S9 and S10).

To examine the GSH-responsiveness of the nanoplatform, the in-vitro CRISPR release profiles were performed under exposure to different concentrations of GSH. Firstly, the protein on TiO$_2$-x was labeled with FITC, and after incubating with 10, 1, and 0 mmol/L GSH, the releasing protein in the supernatant was recorded by a fluorescence spectrometer. As shown in Supporting Information Figs. S11–S13, with the increasing concentrations of GSH, the rate of change for fluorescence signal accelerates, suggesting the cleavage of the disulfide bond contributed to CRISPR release from TiO$_2$-x. To further confirm the GSH-triggered RNP detachment, bicinchoninic acid (BCA) analysis was applied, and the results show that ca 80% of RNP was released from TiO$_2$-x within 12 h incubation in 10 mmol/L GSH (Supporting Information Fig. S14).

Next, we investigated the targeted gene editing performance of TiO$_2$-x@Cas9-NH@HA in vitro. First, the internalization of TiO$_2$-x@Cas9-NH@HA in GFP stably expressing human melanoma cell line (A375 cells) was detected by confocal laser scanning microscopy (CLSM). With the high concentration of glutathione (GSH, 2–10 mmol/L) in tumor cells, the disulfide bonds connecting Cas9 RNPs and TiO$_2$-x, NPs could be cleaved, resulting in the release of Cas9 RNPs from TiO$_2$-x and obvious signal (red) in the nuclei (Fig. 4A). Two days later, T7 Endonuclease I (T7EI) assay was performed to quantify the frequency of mutations. The results indicate that the HSP90 and NRF2 gene loci were successfully edited with a high mutation frequency in TiO$_2$-x@Cas9-N@HA/NIR, TiO$_2$-x@Cas9-H@HA/NIR, and TiO$_2$-x@Cas9-NH@HA/NIR group (Fig. 4B). To further confirm the results, the protein levels of HSP90 and NRF2 were also evaluated in A375 cells treated with various formulations using Western blotting. As shown in Fig. 4C, HSP90 and NRF2 protein of the tumor cells (TiO$_2$-x@Cas9-NH@HA/NIR) are lower than that of the control groups. DNA sequencing of the target also showed two representative indels and insertions nearing the protospacer adjacent motif (PAM, Fig. 4D), further confirming the CRISPR/Cas9-mediated HSP90 and NRF2 knockout.

To confirm the generating ROS in response to NIR irradiation in living cells, we employed 2,7-dichlorofluorescein diacetate (DCFH-DA). As shown in Figs. 3B and 3D, the fluorescence intensity of DCFH-DA increased significantly upon exposure to 808 nm laser (1.5 W/cm$^2$), which indicated the generation of ROS. The results suggested that TiO$_2$-x could effectively generate ROS upon NIR irradiation.

Figure 3  In vitro photothermal and photodynamic effects of TiO$_2$-x. (A) UV–Vis–NIR absorption spectra of TiO$_2$-x at different concentrations (B) photothermal images of water and TiO$_2$-x (100 µg/mL) upon exposure to an 808 nm laser (1.5 W/cm$^2$). (C) temperature increase of TiO$_2$-x at various concentrations (DI Water, 25, 50, 100, and 200 µg/mL) under 808 nm laser (1.5 W/cm$^2$) for 10 min. (D) temperature elevation of TiO$_2$-x (200 µg/mL) at various power densities (0.5, 0.75, 1.0, 1.25 and 1.5 W/cm$^2$) under 808 nm laser (1.5 W/cm$^2$) for 10 min. (E) heating and cooling curve of the aqueous dispersion of TiO$_2$-x (200 µg/mL) under 808 nm NIR irradiation (1.5 W/cm$^2$) for four cycles. (F) Linear time data versus lnθ acquired from the cooling period of the TiO$_2$-x aqueous dispersion. (G) Heating and cooling curve of the aqueous dispersion of TiO$_2$-x (200 µg/mL) for four cycles under 808 nm laser irradiation at a power intensity of 1.5 W/cm$^2$. (H) UV–Vis spectra of TiO$_2$-x, solution containing DPBF exposed to an 808 nm laser (1.5 W/cm$^2$) for a different duration.
(DCFH-DA) to evaluate the level of intracellular ROS. As shown in Fig. 4E, no green signal was observed in A375 cells after being treated with NIR or TiO$_2$-x@Cas9-NH@HA NPs only. By contrast, we can readily discover a green fluorescence in the cells after treatment with TiO$_2$-x/NIR and TiO$_2$-x@HA/NIR. Since more TiO$_2$-x@HA can get in cells with the assistance of HA coating, fluorescence signals of TiO$_2$-x@HA/NIR group were stronger than TiO$_2$-x/NIR group. Notably, we have discovered that green fluorescence in TiO$_2$-x@Cas9-N@HA/NIR and TiO$_2$-x@Cas9-NH@HA groups were strongest among all groups, which probably resulted from the successful gene editing mediated by TiO$_2$-x@Cas9-N@HA or TiO$_2$-x@Cas9-NH@HA NPs and the edited NRF2 failed to regulate the intracellular ROS level of A375 cells.

Encouraged by the aforementioned results, we further evaluated the anticancer effect of our nanoplatform in vitro (Fig. 5A). The cytotoxicity of TiO$_2$-x and TiO$_2$-x@Cas9-NH@HA nanoparticles was tested by the standard cell counting kit 8 (CCK-8) assay. As shown in Fig. 5B, various concentrations of TiO$_2$-x and TiO$_2$-x@Cas9-NH@HA solution (0, 25, 50, 100, 200, and 400 μg/mL) without NIR irradiation show no obvious cytotoxicity, indicating that TiO$_2$-x and TiO$_2$-x@Cas9-NH@HA demonstrated favorable biocompatibility as therapeutic nanagents and CRISPR-Cas9 delivery system. Next, we investigated the in vitro cytotoxicity of TiO$_2$-x, TiO$_2$-x@HA, TiO$_2$-x@Cas9-H@HA, TiO$_2$-x@Cas9-N@HA, TiO$_2$-x@Cas9-NH@HA exposed to 808 laser (1.5 W/cm$^2$, 5 min, Supporting Information Fig. S15). The results demonstrate low phototherapy efficiency could be achieved, i.e., about 70.5% and 63.6% cell viability were found in TiO$_2$-x and TiO$_2$-x@HA groups, respectively (Fig. 5C). After combining with CRISPR, TiO$_2$-x@Cas9-H@HA (only targeting HSP90α) and TiO$_2$-x@Cas9-N@HA (only targeting NRF2) groups enhanced the anticancer effect, and exhibited lower viability to A375 cells, about 40.1% and 36.4%, respectively. As anticipated, TiO$_2$-x@Cas9-NH@HA group, which targeted HSP90α and NRF2 at the same time, showed a much higher cell-killing effect, indicating that our gene editing strategy had significantly improved the in vitro therapeutic effect of PTT and PDT. Besides, the gene editing-mediated PTT and PDT were analyzed by flow cytometry apoptosis protocol based on the AnnexinV-FITC and PI assay, as well as calcein-AM (stained live cells) and PI (stained dead cells) staining (Fig. 5D and E).

Finally, we built xenograft model of A375 tumor-bearing BALB/c mice to evaluate the synergistic therapeutic effect of the resulting nanoplatform in vivo (Fig. 6A). First, the biodistribution of TiO$_2$-x@Cas9-NH@HA was evaluated. With increasing time,
TiO₂₉-NH₂@Cas9-NH@HA progressively accumulated in the tumor tissues, the highest fluorescence signal appeared at 12 h and was still detained after 48 h (Fig. 6B). As shown in Fig. 6C, a much stronger fluorescence was detected in the tumor tissues after intravenous injection (i.v.), which laid a foundation for the following therapy. Notably, an enhanced significant Cy7 fluorescence intensity was observed in the tumor after intravenous administration of TiO₂₉-NH₂@Cas9-NH@HA, indicating the relatively higher tumor-targeted delivery after HA coating (Supporting Information Fig. S16). Next, the mice were randomly divided into seven groups via intravenous injection of different drugs after the tumors grew to ca. 80 mm³. Drawing on previous experiment results in vitro, NIR laser light (1.5 W/cm²) for 10 min was used to achieve desired temperature changes of mild-PTT (43 °C)/PDT every two days (Supporting Information Fig. S17). As provided in Fig. 5D, the tumor volumes in both control groups (PBS/NIR and TiO₂₉-NH₂@Cas9-NH@HA without NIR) show a tendency of rapid increase, indicating the limited suppression efficacy of these treatments in vivo. In contrast, TiO₂₉-NH₂@Cas9-NH@HA/NIR displayed the strongest inhibition of tumor growth among all groups, and almost eliminated tumor in mice, which benefited from the synergistic gene editing/photodynamic/photothermal effect. Similar results were obtained via tumor weight and the photograph of isolated tumors collected from the euthanized mice (Fig. 6E and F). Importantly, the mice body weight of TiO₂₉-NH₂@Cas9-NH@HA/NIR treatment was not observed any abnormal changes (Fig. 6G), and the H&E staining of major organs tissue slices were observed no obvious abnormality nor appreciable organ damage (Supporting Information Fig. S18), demonstrating the low toxicity of various treatments. In addition, the tumors were collected and sliced for further analysis. As shown in H&E staining (Supporting Information Fig. S19), a remarkable inhibition of tumor growth was observed in TiO₂₉-NH₂@Cas9-NH@HA group from the highly decreased cell density in the tumor tissues. We can obtain a similar phenomenon from TdT-mediated dUTP nick-end labeling (TUNEL) analysis (Supporting Information Fig. S20) and Ki67 antibody staining, as well (Supporting Information Fig. S21). These results demonstrate

**Figure 5** In vitro PTT/PDT-based synergistic cancer therapy. (A) Schematic illustration for tumor cells with HSP90α and NRF2 depletion by TiO₂₉-NH₂@Cas9-NH@HA undergoing an NIR light irradiation and then the cell viability analysis was performed. (B) CCK-8 assay for the cytocompatibility assessment on A375 cells treated with TiO₂₉-NH₂@Cas9-NH@HA at elevated concentrations (0, 25, 50, 100, 200, and 400 μg/mL) without NIR light irradiation. (C) CCK-8 assay for the cytotoxicity assessment on A375 cells treated with PBS/NIR, TiO₂₉-NH₂@Cas9-NH@HA, TiO₂₉-NH₂@Cas9-H@HA/NIR, TiO₂₉-NH₂@Cas9-N@HA/NIR, and TiO₂₉-NH₂@Cas9-NH@HA/NIR. (D) Flowcytometry apoptosis assay of A375 cells. Q1, dead cells; Q2, late apoptotic cells; Q3, early apoptotic cells; and Q4, normal cells. (E) The representative fluorescent images of A375 cells after different treatments. Green, calcein-AM; Red, PI. Scale bar = 400 μm. The mean value was analyzed using t-test (n = 3). ***P < 0.001; ns, not significant.
that the combination involving both TiO$_2$-based PDT/PTT and gene editing gained a greater therapeutic effect than other single strategies alone. To further study the combined therapy, HSP90$\alpha$ and NRF2 immunohistochemistry (IHC) staining of tumoral tissue sections were monitored, and the group with the treatment of TiO$_2$-@Cas9-NH@HA/NIR exhibited the lower expression of HSP90$\alpha$ and NRF2, which indicated its effective disruption of HSP90$\alpha$ and NRF2 (Fig. 6H), confirming successful gene editing in vivo. Furthermore, the mutation analysis showed similar results (Supporting Information Figs. S22–S24). Furthermore, we introduced the addition of another comparison group which used scrambled sgRNA to support the conclusion (Supporting Information Fig. S25). Compared with the TiO$_2$-@Cas9-S@HA/NIR (scrambled sgRNA only) group, the TiO$_2$-@Cas9-SH@HA/NIR and TiO$_2$-@Cas9-SN@HA/NIR groups (HSP90$\alpha$ or NRF2 + scrambled sgRNA, respectively) displayed an obvious suppressive effect on tumor development. Notably, the TiO$_2$-@Cas9-NH@HA/NIR group indicated the strongest tumor suppressive effect, which strengthened our previous conclusion that duplex genome-editing sensitized phototherapy can kill cancer cells most effectively.

4. Conclusions

In summary, we developed a tumor-microenvironment responsive nanotherapeutic platform for mild PTT/PDT. In the system, CRISPR system was covalently linked on TiO$_2$ by a GSH-responsive disulfide bond, and HSP90$\alpha$ and NRF2 genes were chosen to edit by Cas9 for sensitizing tumor cells to heat and ROS. Our results demonstrated significant synergistic therapy efficacy that have been achieved both in vitro and in vivo under 808 nm laser irradiation, and no need to maintain hyperthermia temperature (>$50^\circ$C) and high ROS generation level. This method provides a versatile strategy for gene editing-mediated synergistic therapy with maximizing efficacy and minimizing side-effects, which displayed great potential for clinical translation in the future.

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Author contributions

Zekun Li and Yongchun Pan contributed equally to this work. Xin Han and Yujun Song conceived the idea and designed the experiments. Zekun Li and Yongchun Pan performed the majority of experimental work. Xiaowei Luan performed TEM characterization of materials. Zekun Li, Shiyu Du, Yayao Li, Chao Chen, Hongxiu Song, Yueyao Wu and Qin Xu performed the in vivo experiments. Zekun Li and Yongchun Pan wrote the manuscript with feedback from all the authors. Xiaoxiang Guan, Yujun Song and Xin Han supervised the project.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.asphb.2022.06.016.

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