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Article

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ACS Synth. Biol., **Just Accepted Manuscript** • DOI: 10.1021/acssynbio.9b00349 • Publication Date (Web): 31 Dec 2019

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A multifunction lipid-based CRISPR-Cas13a genetic circuit delivery system for bladder cancer gene therapy

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Abstract

The treatment of bladder cancer has recently shown minimal progress. Gene therapy mediated by CRISPR provides a new option for bladder cancer treatment. In this study, we developed a versatile liposome system to deliver the CRISPR-Cas13a gene circuits into bladder cancer cells. After in vitro studies and intravesical perfusion studies in mice, this system showed five advantages: 1) CRISPR-Cas13a, a transcriptional targeting and cleavage tool for gene expression editing, did not affect the stability of the cell genome; 2) the prepared liposome systems were targeted to hVEGFR2, which is always highly expressed in bladder cancer cells; 3) the CRISPR-Cas13a sequence was driven by an artificial tumor specific promoter to achieve further targeting; 4) a near-infrared photosensitizer released using near-infrared light was introduced to control the delivery system; and 5) the plasmids were constructed with three crRNA tandem sequences to achieve multiple targeting and wider therapeutic results. This tumor cell targeting lipid delivery system with near-infrared laser-controlled ability provided a versatile strategy for CRISPR-Cas13a based gene therapy of bladder cancer.

Key words: Genetic circuit delivery; CRISPR-Cas13a; liposome; gene therapy; bladder cancer

Bladder cancer (BCa) is the most common malignant urological tumor[1]. In the past decade, its incidence has been increasing every year, and it has been affecting individuals at increasingly younger ages. There are 386,000 new cases and 150,000 deaths worldwide each year[2]. The incidence ranks 9th among all malignant tumors, and 6th in male tumors. Many patients are at advanced stages of the disease when diagnosed. The recurrence rate of BCa is high and the sensitivity to radiotherapy and chemotherapy is poor. Cystoscopy and other examinations need to be repeated several times after surgery, and intravesical instillation with drugs or immunosuppressive agents are used to prevent recurrence[3]. BCa treatment is therefore the most expensive malignancy in the United States. Using traditional BCa treatments, such as chemotherapy and BCG instillation, the 5-year survival has not been significantly improved for many years[4]. Because of these statistics, new and more effective BCa treatment strategies are needed. Gene therapy is a promising cancer treatment because it includes RNA interference (RNAi) and CRISPR, which have been developed in recent years. Compared with RNAi, CRISPR is a more efficient and specific gene editing tool. The delivery of therapy gene circuits can be divided into two types, one of which is to separate the in vivo cells and treat them with gene editing in vitro, and then reinfuse them in vivo. This strategy involves adoptive immunocyte therapy, such as Chimeric Antigen Receptor T-Cell Immunotherapy (CAR-T). The other strategy is to deliver the therapeutic gene circuits directly to patients. For this strategy, there is a need for suitable carriers that can effectively deliver the circuits to the target site in the human body. The disease-related genes for therapy circuit targeting are critical to treatment effectiveness. Similar to other malignant neoplasms, there are many important biomolecules that are closely related to the development, invasion, migration, and apoptosis of BCa, such as VEGFR2, Bcl-2, and survivin[5,6,7].

VEGFR2 is always expressed in vascular endothelial cells from early fetal development[5]. It is also more highly expressed in neovascular tumor endothelia than in normal endothelia[8,9]. This biological feature means inhibition of VEGFR2 could inhibit the development of tumors during their early stages. As an important marker, VEGFR2 is minimally expressed in normal urothelial cells, while it is often highly expressed in BCa cells. In addition, invasive cells express this marker more abundantly than noninvasive cells. The increased expression of VEGFR2 in bladder carcinoma provides invasive properties to BCa cells and allows the prediction of invasive phenotypes in patients. In order to achieve targeting therapy, we chose VEGFR2 to be the target biomarker in this study. The targeting of the VEGF-VEGFR2 pathway in BCa is expected to be a new treatment strategy, complementing other standard treatments that inhibit tumor growth. The resistance to apoptosis always restricts the effects of anti-tumor therapy[10]. One of the main ways in which cancer cells evade apoptosis is to upregulate the anti-apoptotic protein of the Bcl-2 (B-cell lymphoma 2) family[11]. Bcl-2 is a key regulator in apoptosis pathways for blocking the effective oligomerization of Bax and Bak to protect against cell death[12]. The overexpression of Bcl-2 is always correlated with poor prognoses and short survival rates[13]. In BCa cells, the expression of Bcl-2 interferes with the therapeutic effects of cisplatin and Ad5CMV-p53 through the inhibition of the apoptotic pathway[14,6]. The targeting of these key apoptosis regulators to overcome apoptosis-resistance and promote apoptosis of tumor cells is a new cancer treatment strategy[15]. However, it only achieved a modest impact on patient survival in clinical trials[16]. This limited effect may be caused by the low bioavailability of oligonucleotides in the blood stream and its inability to pass through the lipid membranes. Thus, an effective targeting delivery carrier that combines Bcl-2 expression knockdown with other therapeutic strategies may provide a promising strategy for tumor treatment. Similar to Bcl-2, survivin also belongs to the apoptosis inhibitor protein family. It plays a role as a key regulator of mitosis and programmed cell death. In recent years, related studies indicated that survivin is involved in apoptosis inhibition, mitotic spindle checkpoint regulation, angiogenesis, and chemoresistance. In a clinical study involving 222 patients, survivin expression could not be detected in normal bladder specimens but was present in 64% of bladder tumors[17]. The increase of survivin expression in tumor cells enhances their invasion, metastasis and risk of

recurrence[18]. Therefore, the development of survivin inhibitors is a novel anti-BCa therapeutic strategy[7]. The potential association of Bcl-2, VEGFR2, and survivin knockdown with a decrease in cancer properties represents a new opportunity for overcoming drug resistance to BCa treatments.

CRISPR-Cas13a is a cutting-edge gene editing technology, which was first reported in 2016. Similar to the CRISPR-Cas9 system, CRISPR-Cas13a can also be used to inhibit gene expression in eukaryotic cells. However, it affects and downregulates gene expression at the transcriptional level[19]. In this study, the CRISPR-Cas13a plasmid backbone was employed for target genes knockdown in BCa cells. To achieve better inhibition, a series of candidate crRNA sequences were screened and evaluated. Our design included targeted genes with an artificially modified BCa specific promoter derived from the human telomerase reverse transcriptase (hTERT) gene to drive the CRISPR-Cas13a gene, LwaCas13a. The artificial hTERT promoter responded to and was activated by the tumor factor (E-twenty-six transcription factors), which is highly expressed in BCa cells, to drive downstream gene expression[20].

To deliver therapeutic CRISPR-Cas13a targeting and inhibiting VEGFR2, Bcl-2, and survivin plasmids, a suitable carrier was needed for bladder instillation. Many delivery systems for nucleic acids have been developed. One type is the viral carrier, which mainly includes artificial adenovirus and lentivirus carriers. However, some problems exist in viral carriers. For example, adenovirus has less than 5 kb of loading capacity and easily causes human immune rejection, which leads to treatment failure. Another important limiting factor is the low expression of adenovirus receptors in BCa cells because it is an intermediary for cell-Adv combining and is important for exerting therapeutic functions[21]. The artificial lentivirus is another type of viral carrier. The major problem of the lentiviral carrier is biosafety. It may cause unwanted mutations in the genome of host cells. In addition, the lower transfection efficiency also limits the in vivo application of lentiviral carriers. Beyond the viral carriers, nonviral carriers are another major class of gene delivery system. However, the traditional nonviral carrier lacks targeting and controlled release abilities. If treated with proper chemical modifications, these carriers possess new advantages for gene delivery. Without the limitation of the biological capacity of viruses, nonviral carriers have stronger loading capacities, being able to load multiple biomolecules simultaneously, load larger genetic editing circuits, achieve biological targeting after conjugating with ligands, and achieve better biocompatibility with biodegradable materials. In order to exploit these advantages, in the present study, we built a multi-layer component carrier to achieve graded delivery.

The nonviral carrier included cationic polymers[22], inorganic nanocarriers[23] and liposomes[24,25]. Among these, liposomes are a promising and widely-studied carrier for gene delivery. Because lipid carriers have excellent biodegradability and biocompatibility, a series of liposomal carriers are already in use in clinical trials[26]. Due to the ease of chemical modifications, lipid components could be conjugated with biofunction molecules to achieve targeted binding. The conjugated biomolecules include aptamers and peptides[27,28]. These targeted liposomes have shown efficient targeting of tumor cells via intravenous injection[28]. In recent years, liposomes have been used in clinical trials as carriers for bladder instillation therapy[29]. With the development of biomaterials, several types of liposomes with special functions have been designed and synthesized, including ones that are stable in the bladder and urine environment[30] have a urinary pH response release[31], and can be loaded with siRNA or BCG biological elements[32]. With the development of liposomes, especially the cationic lipid carriers, cationic liposomes have been used to deliver nucleic acid-based devices, such as small interfering RNA (siRNA) and plasmids[23]. In 2014, liposomes were used to deliver CRISPR-Cas9 gene editing tools and Cre recombinase into the mouse inner ear in vivo. This system achieved 90% Cre-mediated recombination and 20% Cas9-mediated genome modification in hair cells[33]. However, the current liposome systems also show some problems. For example, unstable liposome types release faster, and their release is more difficult to control, while the more stable liposome structures release more slowly, resulting in inefficient treatment. The stability and controlled release of liposomes in the urine or bladder environment are critical to

intravesical instillation. Therefore, the synthesis of targeted binding and a responsive release liposome system can achieve more effective bladder delivery therapy. The photothermal-response release liposomes are suitable for this role. Some photothermal materials, such as gold nanorods and nanoparticles, have been encapsulated into the liposome carriers to give them photoresponse-release capabilities[34]. Indocyanine Green (ICG) is an inorganic near-infrared photothermal and imaging agent that has been approved by the FDA for human in vivo applications. In this study, we introduced another commercial NIR photothermal agent, IRDye® 800CW NHS ester (IR800-NHS), modified with an NHS linker for conjugating with the amino groups of biological ligands. Conjugating the liposome with targeted ligands by chemical modification maximizes the primary targeting ability to tumor cells. After being encapsulated with the NIR photothermal agent, this liposome system further achieved a near infrared response release as a secondary targeting mechanism[35-37]. The IR800-NHS was conjugated with the nuclear localization sequence (NLS), involving the electropositive peptide to bind and guide the cargo into the nucleus for further targeting[38]. This multi-level targeting strategy created a rational bladder instillation carrier for the delivery of nucleotides or other biological components. Based on this premise, the three gene targeting plasmids were loaded into the developed liposome system, and their efficacies were tested on human BCa 5637 cells. Finally, in situ BCa model implanted in nude mice was prepared to study the in vivo biodistribution and possible biofunctions. According to different functional layers, the intelligent liposome system was divided into two components, the outer components and the inner components, to achieve the multi-level targeting (Fig. 1).

Results

The characterization and plasmid delivery test of synthesized liposome systems

The synthesized liposome delivery system was characterized by transmission electron microscopy (TEM), fluorescence microscopy, size and zeta potential, and a fluorescence spectrophotometer to verify the feasibility of this approach. TEM images of the synthesized liposomes showed complete spherical morphology with good monodispersity and uniformity of the particles (Fig. 2A). Because the particles were assembled with IR-800 dye, they could be observed with a fluorescence microscope (Fig. 2A). The conjugated IR-800 dye allowed us to trace the distribution of the liposome system in vitro and in vivo by fluorescence imaging. The particles were characterized by UV-Vis absorption, and the characteristic peaks of IR-800 (680 nm shoulder and 750 nm peak) were observed (Fig. 2B). The fluorescence spectrum of liposomes was also tested to verify the loading of IR-800 dye. An emission peak at 798 nm was clearly observed (Fig. 2B). These results indicated that the IR800-liposome composite was synthesized efficiently with multi-layer nested loading. The particles had an average diameter of 855 ± 127 nm and a zeta potential of 25.7 mV (Fig. S1). The zeta potentials of different components in liposomes were also measured (Fig. 2C).

The EGFP plasmids were then loaded into the liposomes and transferred into BCa 5637 cells for delivery test. The fluorescence microscopic imaging results showed that the liposome system could deliver the plasmids into tumor cells and led EGFP gene expression. It could also achieve targeting delivery into the nucleus to complete the intracellular delivery of each element (Fig. 2D).

The construction of mice in situ model of human BCa cell

In order to further understand the function and metabolism of the complex liposome system in the bladder environment, we constructed an in situ human BCa mice model. The bladder and tumor tissues of BCa mice were harvested for identification. We could clearly observe the tumor tissue at the edge of the bladder. The subsequent hVEGFR2 immunohistochemical (IHC) and hematoxylin and eosin staining also confirmed that the animal model had been successfully established. After abdominal dissection, visible tumor tissue was seen on the periphery of the bladder tissue. Microscopic studies revealed that there was a strong positive signal in the tumor tissue (Fig. 2E).

The NIR photothermal effect of synthesized liposomes

We initially studied the photothermal effect of liposomes in vitro and in mice (Fig. 3AB) using an 808 nm NIR laser. The results confirmed that the material had an excellent near-infrared photothermal effect and could rapidly heat the local temperature in the tube to over 8°C (Fig. 3A) or to above 42°C in the mouse bladder (Fig. 3B) in 5 min. This confirmed the near-infrared light response and controlled release strategy of the liposomes. After bladder instillation of the liposomes, a significant dye staining was observed in the tumor tissue of the model mice (Fig. 3C). Subsequently, we conducted a systematic study on the release of the liposome system after the photothermal effect via in vitro dialysis. The results showed that the release of the liposome system was significantly accelerated by near-infrared light irradiation compared with dark incubation at 37°C (Fig. 3D and Fig. S2).

The in vivo fluorescent imaging of mice model

Because of the modification using the IR800 infrared photosensitizer, the liposome system could be directly used for in vivo imaging (Fig. 3E). Fluorescence imaging showed that the fluorescence of nude mice inoculated with human BCa cells lasted for at least 7 days after bladder infusion, which was significantly higher from the 2nd day to the 7th day than that of normal nude and black mice (C57) (Fig. 3F). This also indicated that the liposome system could target hVEGFR2 expression in BCa cells for long-lasting activity.

The EGFP inhibition by CRISPR-Cas13a/liposome systems

To achieve robust Cas13a-mediated RNA knockdown, the CRISPR-Cas13a plasmid backbone was evaluated by EGFP-targeting knockdown assays. For in vitro studies, the three alternative sequences of crRNA for the CRISPR-Cas13a plasmid were prepared for the CRISPR-Cas13a/liposome system and incubated with the EGFP-transfectants. Imaging of the 5637 cells (Fig. 4A) and flow cytometry (Fig. 4B) results showed that all three crRNAs triggered knock down effects, with the No. 1 crRNA plasmid achieving the most significant effect, with >35% decreased expression (Fig. 4C). For the in vivo test, we delivered the No. 1 CRISPR-Cas13a/liposome system or negative control (NC; in which the crRNA sequence was replaced with the NC sequence) into EGFP transgenic mice by bladder instillation (Fig. 4D). Based on the bladder tissue results, a significant decrease in fluorescence signal was observed in No. 1 crRNA-treated mice. Together, the results confirmed that the LwaCas13a backbone design affected the expression of target genes at both the cellular and organismal levels.

The anti-BCa activity of CRISPR-Cas13a/liposome systems

To further investigate the possibility of the CRISPR-Cas13a/liposome system for gene therapy in BCa, we developed a suicide gene therapy for BCa based on the optimized LwaCas13a vector. The key genes relating to the development, migration, invasion and apoptosis of BCa 5637 cells were knocked down by the prepared liposomes loaded with three genes targeting CRISPR-Cas13a plasmid pHS-ACR-LW605. After 48 h of transfection, the qRT-PCR (36.5% for VEGFR2, 30.5% for survivin, and 39.6% for Bcl-2; Fig. 4E) and western blot (Fig. 4F) evaluation of the VEGFR2, Bcl-2, and survivin gene expressions indicated a significant downregulation of all three genes to varying levels after incubation with the pHS-ACR-LW605/liposome, while compared with the negative control (pHS-ACR-LW606/liposome). We therefore used this design for all further knockdown experiments. In vitro cell proliferation (Cell Counting Kit-8, CCK-8), motility (cell scratch assay), invasion (Transwell® migration assay) and apoptosis (annexin V-FITC/PI) assays indicated that the number of apoptotic cells was significantly increased (Fig. 5AB and Fig. S3, $^{**}P < 0.01$). The invasion (Fig. 5C), proliferation (Fig. 5D) and migration (Fig. 5EF and Fig. S4) of 5637 cells was significantly inhibited ($^{*}P < 0.05$) by pHS-ACR-LW605/liposome transfection compared to the control pHS-ACR-LW606/liposome (NC).

We attempted to obtain similar biological functions in vivo in BCa model mice. The prepared pHS-ACR-LW605/liposome (LW605 group) and control pHS-ACR-LW606/liposome (NC group) were perfused into the bladder of the BCa nude mice (repeat this process 3 times every other week), then collected tumor tissue after 20 days feeding to detect the changes in tumor volume and three genes expression. After measuring (Fig. 6A), the tumor volume of the LW605 treatment group (16.02 ± 3.01 mm^3) was significantly smaller than that of the NC treatment group (24.90 ± 4.65 mm^3). Tumor tissues were detected by fluorescent quantitative PCR (Fig. 6B), western blot (Fig. 6C) and IHC analysis (Fig. 6D). The expression levels of the three genes in the LW605 group were significantly lower than those in the NC group. These results indicated that the constructed CRISPR-Cas13a/liposome system could induce targeted inhibition of tumor cells in mice bladder by intravesical instillation and has the potential to be further developed for BCa gene therapy.

Discussion

In this study, we utilized the CRISPR-Cas13a plasmid backbone to inhibit target genes in BCa cells. In order to achieve robust Cas13a-mediated RNA knockdown, the CRISPR-Cas13a plasmid backbone was evaluated by an EGFP-targeting knockdown assay. This assay monitored Cas13a-mediated cleavage of EGFP transcript in 5637 cells, resulting in EGFP fluorescence reduction, which could be measured and quantified by fluorescent micro-imaging and flow cytometry. The CRISPR RNA (crRNA) for EGFP was optimized from three alternative sequences involving pHS-ACR-LW-EGFP (Table S1 and Fig. S5A). These results indicated that the biological elements from mammalian codon-optimized CRISPR-Cas13a gene (LwaCas13a) knocked down the target gene (EGFP) expression in BCa cells. LwaCas13a is an optimized CRISPR-Cas13a system from *Leptotrichia wadei* with a high cleavage activity in mammalian cells. The in vitro cleavage tests of LwaCas13a showed programmable RNA cleavage with a crRNA composed of a 28-nucleotide spacer sequence, which assisted LwaCas13a in cleaving the corresponding transcript[19]. To further investigate the biofunction of the CRISPR-Cas13a elements used in plasmids in vivo, we introduced them into EGFP transgenic mice to evaluate the target gene (EGFP) expression. In previous studies, the RNA-seq experiments indicated that the collateral RNA nonspecific degradation activity of CRISPR-Cas13a could be observed in bacteria and in vitro, but not observed in mammalian cells. Thus, the CRISPR-Cas13a induced RNA-based gene editing may not lead to nonspecific RNA splicing and an observable cell stress response at the transcriptomic level[39,40]. Furthermore, because CRISPR-Cas13a was not involved in genome editing, there was no tumorigenic and off-target risk for the CRISPR-Cas9 systems. Therefore, there is hope that it can be developed into a better gene editing tool for in vivo applications. The collateral green editing effects of Cas13a in cancer therapy with targeting EGFRVIII and PD-L1 have been reported in 2019[41]. Compared with RNAi, CRISPR-Cas13a has a significantly lower off-target effect. CRISPR-Cas13a is a more efficient and specific gene editing tool than RNAi, which makes it the preferred tool for regulating gene expression[19].

After verifying the knockdown effect on the EGFP gene, the EGFP crRNA sequences were replaced with the crRNA of other targeted genes (Table S1). The crRNA targeted to VEGFR2 mRNA was optimized from four alternative sequences (Table S1 and Fig. S5B) involving pHS-ACR-LW-VEGFR2. The optimized crRNA sequences were chosen to bind and inhibit the target transcripts (Table S1, uppercase letter sequences). In order to further achieve a multiple gene knockdown effect, we constructed the plasmid with three crRNA tandem sequences for three genes, separately driven by the U6 promoter (Fig. S5C; pHS-ACR-LW605). All these genetic elements were assembled into the same plasmid (pHS-ACR-LW605) to play triple targeting function simultaneously. A negative control pHS-ACR-LW606 (Fig. S5D) was set to replace the crRNA sequences with a non-targeting 28 nt oligonucleotide guide for specificity evaluation. The U6 promoter was an RNA polymerase III promoter, which was suitable for RNA synthesis with less than 100 nt in eukaryotic cells; meanwhile, the hTERT was an RNA polymerase II promoter that was more suitable for protein expression. The design of the constructed U6 and hTERT promoters in the same plasmid allowed for both functions and could reduce unnecessary interference. All plasmids were then separately loaded into liposomal carriers for transfer into 5637 cells.

Based on many preliminary studies, μm- to nm-sized liposomes were widely synthesized and studied. Previous studies have indicated that nanosized liposomes could have enhanced permeability and retention (EPR) effects in blood circulation and can achieve the same passive targeting as other nanoparticles. However, to effectively reach the bladder-lining cells and the non-muscle invasive bladder cancer (NMIBC) cells, the bladder instillation treatment does not require passing through the complex blood circulation. Because the nanosized EPR effect does not work, nor was it required in the present study, we did not deliberately create nanoscale-sized liposomes. Instead, we used active targeting involving chemical strategies. The liposome delivery system was assembled with inner and outer components. This feature allowed it to achieve a multi-layered

targeting capability. The outer components were constructed by tumor targeting liposomes. It has been shown that the expression level of hVEGFR2 in normal urothelial cells is almost absent, while it is highly expressed in BCa cells. VEGFR2 is an important antigenic marker molecule on the surface of BCa cells. Thus, we conjugated the monoclonal antibody to the lipids by chemical modification[27] to confer cancer cell targeting ability. The BCa targeting ligands, the thiol-modified hVEGFR2 mAbs, were conjugated to the lipid component, DSPE-PEG2000-maleimide, by the Michael addition reaction. In addition, the outer components were also used to load the inner components. Recently, increasing studies have utilized the liposome as carriers for bladder instillation therapy, which have achieved positive results. For example, after modification with polyethylene glycol, liposomes could achieve better stability in the urine environment[30]. The lipid component with acid degradation was used to produce urinary pH response release liposomes. There are also studies using modified cationic liposomes to load siRNA or BCG biotherapeutic components for BCa therapy[31]. In this study, we describe the liposome formulation suitable for bladder instillation, and the related preparation techniques were applied in the construction of the outer components. After instillation into the bladder tumor, the liposomes specifically bound and adsorbed on the BCa cell membrane by coupling the targeting ligands on the outer components. The unbound liposomes were excreted with urine. Subsequently, the tumor area was irradiated with near-infrared light to induce a photothermal effect of the inner components with a photosensitizer, causing the release of the inner components to complete the secondary targeting. The inner components were constructed using a cationic nanocarrier. The functions included release control, gene circuits loading, nuclei targeting, and a tracer for monitoring. The nucleic acid was loaded onto the cationic polypeptide portion. Together, these functional parts formed a versatile inner element system with an outer component system to form a liposome delivery system. After the inner components released and entered the tumor cells, the loaded gene circuits were translocated into the nucleus by guidance from the NLS peptide to achieve further targeting. In addition, due to the presence of the IR-800 photosensitizer on the inner components, the process could be monitored by fluorescence microscopy (near-infrared channel) to achieve the integration of treatment and treatment monitoring.

After delivered into BCa cells by liposomes, the CRISPR-Cas13a systems were expected to play anti-tumor effects. The three genes knockdown and related biofunction analysis results indicated that the codon-optimized LwaCas13a played a role in regulating the target genes, and the triple tandem U6 promoter-driven crRNA sequences were able to generate all three crRNA to activate Cas13a functions. After downregulating the Bcl-2, VEGFR2 and surviving genes, the downstream pathways were activated to achieve the therapeutic goals. In addition, we tried to investigate the in vivo biological effect using the in situ BCa mice model. The prepared liposomes loaded with pHS-ACR-LW605 or NC plasmids were instilled into bladders of mice through the urethra. However, we could not observe significant expression reduction of the three targeted genes in tumor and bladder-mixed tissues as was found in the in vitro experiments. This may be due to the proportion of human BCa cells was too small in the mixed tissue, making the differences caused by these cells difficult to detect. Among the three targeted genes, VEGFR2 is a key regulator of tumor development and invasion. The recent use of tyrosine kinase inhibitors with specificities toward VEGFR2, such as VEGFR2 blocking antibody bevacizumab, have generated expectations that these inhibitors could be effective against VEGFR2 positive tumors by targeted oncological therapy and anti-angiogenic effects[42]. In our study, VEGFR2 played dual roles as a tumor targeting marker and as a therapeutic target. The knockdown or inhibition of VEGFR2 expression in BCa cells was expected to suppress tumor development and form a logic gate circuit to reduce the binding of liposomes after the VEGFR2 expression was downregulated. This would terminate the CRISPR-Cas13a gene editing functions when they are not necessary. The other targeted genes, Bcl-2 and survivin are important BCa cells apoptosis regulators. The expression of Bcl-2 in BCa cells interferes with the effects of chemotherapy via inhibition of the apoptotic pathway[14]. Thus, after

being knocked down by the CRISPR-Cas13a tools, Bcl-2 downregulation is expected to play a role as an oncotherapy sensitizer. Similarly, survivin downregulation also helps promote programmed cell death and inhibition of the mitosis and angiogenesis of BCa cell[7]. The results of apoptosis test showed that the proportion of early apoptotic cells was significantly higher than that of late apoptotic cells, indicating that the introduced CRISPR-Cas13a therapeutic genetic circuit has a significant pro-apoptotic effect on bladder cancer cells. The targeting and knockdown of both Bcl-2 and survivin genes simultaneously could significantly accelerate the apoptosis rate of BC cells. The related pro-apoptotic effects have been widely reported[12-20]. Thus, our study provides a novel Bcl-2 and survivin biosuppressor strategy, which hopefully contributes to BCa gene therapy. This multifunctional anticancer therapeutic strategy may also play a role in other functional studies related to VEGFR2, Bcl-2, and survivin genes.

Currently, there is no mature gene therapy strategy for BCa clinical use. Using a series of studies on gene circuit delivery systems, we designed and synthesized rational delivery carriers using tumor-targeting liposomes and biocompatible polypeptides based on the biological characteristics of BCa. The clinical value of this candidate delivery strategy was subsequently screened via functional verification tests. The efficient, high capacity, multi-layered, nonviral complex system was loaded with an optimized CRISPR-Cas13a gene circuit system, which may hopefully allow us to overcome the disadvantages of traditional therapy and improve the BCa therapeutic effect.

Methods

Materials and reagents

All the CRISPR-Cas13a plasmids were constructed and synthesized by Beijing Syngentech Co., LTD. The BCa cell line 5637 was obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 μg/mL streptomycin sulfate and 100 U/mL penicillin). The NAP-10 desalting columns were purchased from GE Healthcare. The 2-iminothiolane hydrochloride (CAS 4781-83-3), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), DSPE-PEG-Maleimide (DSPE-PEG-Mal, 2 kDa), DSPE-PEG-OH (2 kDa), Cholesterol were purchased from Aladdin. 1,2-dipalmitoyl-3-trimethylammonium-propane chloride salt (DOTAP) was purchased from Avanti Polar Lipids. The inner components, that IR800 modified KKKKGRYLTQETNKVETYKEQPLKTPGKKKKGKPGKRKEQEKKKRRTR peptides were synthesized by GenScript. The mouse anti-human VEGFR2 monoclonal antibody (Human VEGFR2/KDR/Flk-1 Antibody) was purchased from R&D Systems. The primary antibodies of rabbit anti-human VEGFR2, Bcl-2, survivin, β-actin genes, and the anti-rabbit secondary antibodies were purchased from Cell Signaling Technology. TRIzol reagent was purchased from Invitrogen. The extracted RNA PrimeScript RT Reagent kit and SYBR Green PCR kit were purchased from Takara, Dalian, China. The protease inhibitor cocktail, Cell Counting Kit-8 (CCK-8), BCA protein assay kit, hematoxylin and eosin were ordered from Beyotime Institute of Biotechnology, Shanghai, China. The polyvinylidene difluoride membrane (PVDF) and nonfat milk were purchased from BioRad. The SuperSignal™ West Pico PLUS Chemiluminescent Substrate was purchased from Thermo Scientific Pierce. All the primers were synthesized from Sangon Biotech (Shanghai) Co., Ltd. Annexin V-FITC Apoptosis Detection Kit was purchased from Solarbio Life Sciences. The propidium iodide was ordered from TransGene, Beijing, China.

DNA condensation in inner components

The inner components were used to condense plasmid DNA (pDNA). The NLS peptide provides the nuclear targeting capability. The polylysine end of peptide provides positive charge for DNA adsorption. A NIR photosensitizer was modified on the peptide to achieve laser control liposome release and provide monitoring of the delivery process. The inner components were dissolved in aqueous, CRISPR-Cas13a plasmids were dissolved in 10 mM tris-HCl buffer at a concentration of 1 mg/mL, and the inner components (MW=6850, 1 mol with 86 mol nitrogen) were dissolved in aqueous, then mixed with the DNA solution at amine to phosphate (N/P) ratio of 40 (DNA 20 μg/mL, inner components 200 μg/mL), vortexed for 3 min, and incubated at 4 °C for 1 h.

Synthesis of liposome outer components

We employed the lipid formulation which could be stably dispersed in urine environment by hydration method. The DPPC/Chol/DOTAP/DSPE-PEG2000/DSPE-PEG2000-Mal with a mol ratio at 40/30/30/3/1 was dissolved in chloroform. The solvent was removed by vacuum rotary evaporation to form lipid film. High vacuum evaporation was applied overnight to remove solvent. The next day, the 2.5 mL mixtures of pDNA and inner components (DNA 20 μg/mL, inner components 200 μg/mL) were placed in the flask with above 10 μmol total lipid film and sonicated for 2 min in ice water bath. The synthesized liposomes were characterized by the TEM (JEM-1200EX, 120KV), fluorescence microscopic imaging (IX37, OLYMPUS), size and zeta potential analysis (DelsaMax CORE, BECKMAN), and fluorescent spectroscopy (F7000, Hitachi).

Synthesis of VEGFR2 targeted liposomes

The mouse anti-human VEGFR2 monoclonal antibodies (Human VEGFR2/KDR/Flk-1 Antibody, R&D Systems) were conjugated with the liposomes contained DSPE-PEG-Maleimide lipids[43]. The

mAbs were dissolved in 0.1 M borate buffer with 5 mM EDTA at a concentration of 4 mg/mL, then mixed with 2-iminothiolane at a concentration of 50 μg/mL and incubated at RT for 4 h. The thiolated mAbs were purified by NAP-10 desalting columns and conjugated to freshly liposomes at ratio of 100 μg mAbs per 1 μmol DSPE-PEG2000-Mal in PBS (pH 7.4) for overnight shock at 4 °C. Finally, the obtained mAb-liposomes were concentrated and sterilized by sterile filters

The transfection study of the liposome system in BCa cells

The synthesized liposomes (inner components plus outer components) were used to transfect an EGFP plasmid into the BCa cell 5637 for expression, and the process was monitored by fluorescence microscopy. We set liposomes (just outer components) loaded with EGFP plasmid as negative group control. For cell transfection, 5637 cells were plated in a 6-well plate with a concentration of 5×10^5 per well on the day prior to the experiment. The fifty microliters of above pDNA/liposome mixture were added into the cells with medium and incubated for 24 h at 37 °C. Then, the medium was removed, and the cells were washed with PBS for 2 times. After removed the PBS, the treated cells were then fixed in 4% paraformaldehyde solution. After 2 times PBS washing, the cells were observed by fluorescence microscopy.

The construction of mice in situ model of human BCa cell

We employed the 5637 human BCa cells to construct the in situ BCa mice model for in vivo animal studies. The 5637 cells were cultured to logarithmic growth phase. After 0.25% trypsin digestion, the cells were washed by PBS for 3 times. The cells were resuspended in 1% FBS PRMI 1640 with a concentration of 10^7 /mL. The female mice were intraperitoneally injected with 1% sodium pentobarbital physiological saline solution (100 mg sodium pentobarbital per 1 kg mice weight). After being anesthetized, urethral opening and surrounding of mice were disinfected by iodophor. The modified venous indwelling needle was used to urethral cannulation. Cut the side hose, the syringe was inserted slowly into the urethral opening of the mice with sterile paraffin oil lubrication. After successful intubation, intravenous catheter was connected to the 1 mL syringe for mice bladder perfusion operation. All mice were first lavaged with 0.1 mL PBS to wash away residual urine. Then introduced 0.1 mL 5637 cells (10^6) with matrigel, utilized the needle to puncture the bladder wall and inject slowly the cell matrigel mixture with the slow withdrawal of the needle. After two weeks feeding, the mice were sacrificed to harvest the bladder and prepare the immunohistochemical sections for detection of hVEGFR2 expression. The bladder and tumors from mice were fixed overnight in freshly prepared formaldehyde (4% in PBS), then dehydration in 70% ethanol/water and embedded in paraffin. The paraffin blocks were cut to four micrometer sections and tiled on the plus coated slides. After deparaffinized and hydrated, the slides were stained with hematoxylin-eosin (HE) by the hematoxylin and eosin according to the manufacturer's instructions. The monoclonal rabbit anti-human VEGFR2 (1: 800 in diluent) antibody was used to detect VEGFR expression. Sections were counterstained with hematoxylin and mounted for microscope evaluations.

The photothermal effect of liposome system

We employed 808 nm NIR laser to trigger the photothermal effect (FLIR® Systems, Inc.) of the synthesized liposome system and study the release efficiency by dialysis and calculating the integration of UV-vis absorbance peaks in 150 min (Fig. S2). The temperature changes were monitored under irradiation of 808 nm laser in 5 min, the in vitro studies were operated in 1.5 mL tube and the in vivo studies were operated in BC mice model and control mice. In the in vitro study, we irradiated 0.5 and 0.1 mg/mL of liposomes at 0.8 W/cm^2 power for test; In the in vivo study, liposome with concentration of 0.5 mg/mL was employed, and the NIR irradiation was performed at the power of 0.8 and 0.4 W/cm^2.

Fluorescence imaging of the liposome system after bladder infusion in mice model

We monitored the pharmacokinetics of the liposome system after bladder infusion in mice model by small animal fluorescence and bioluminescence imaging system (Xenogen IVIS 200, PerkinElmer). After fluorescence scanning by imaging system, the region of interest (ROI) area (including the bladder area) of mice imaging was selected for fluorescence intensity evaluation, and the background was subtracted. We studied 3 groups of mice model, the BC mice, control nude mice and C57 mice. Each mouse was detected fluorescence at front position in 10 days after bladder infusion of liposomes. The fluorescence detection parameters were set as Ex/Em 700/800 nm and scanned once a day.

The functional verification of the EGFP targeting CRISPR-Cas13a/liposome system

In order to test and verify the biofunction of the CRIPR/Cas13a elements and the liposome delivery system, we constructed an EGFP targeting CRIPR/Cas13a plasmid and loaded it into the liposomes to study the knock down of EGFP expression.

In vitro and in vivo experiments were introduced for biofunction verification. For in vitro study, we tried to transfect the EGFP over expression plasmids into the 5637 cells. After 48 h of transfection, the cells were divided into four groups. The three alternative sequences of crRNA for CRIPR/Cas13a plasmid were prepared to the CRISP-Cas13a/liposome system and incubated with the EGFP cells to study the EGFP knock down efficiency. The flow cytometry was used to detect the EGFP expression in the EGFP transfected cells. For in vivo test, we delivered the optimized CRIPR/Cas13a plasmid into EGFP mice by the CRISP-Cas13a/liposome system. After bladder infusion, the EGFP mice were kept feeding in 5 days, then sacrificed for bladder harvest. The obtained bladder tissues were made into frozen slides and observed by fluorescence microscopy to identify the EGFP expression in bladder tissue.

The three genes targeting CRISRP-Cas13a plasmids delivered by liposome system in BCa cells

The VEGFR2 targeting or artificial hTERT promoter, Bcl-2, survivin, VEGFR2 crRNA tandem CRISPR-Cas13a plasmids were loaded in the liposome system and transfected into the 5637 cells for biofunction identification. We used real-time Q-PCR and western blot to detect the human Bcl-2, survivin, and VEGFR2 gene expression in 5637 cells. The pDNA/liposome treated cells were incubated for 48 h and extracted RNA according to the kit instructions.

Real-time quantitative PCR

The TRIzol reagent was used to extract total RNA from BCa tumor tissues and cells. Then we synthesized the complementary DNAs (cDNAs) from the extracted RNA following the protocols. The SYBR Green PCR kit was used to perform the quantitative real-time quantitative PCR (qRT-PCR) following the manufacturer's instructions, which was carried out using the ABI 7300 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primer sequences were present as the followed, VEGFR2 primers: 5′-AGTGATGTCTGGAGCTACGGG-3′ (forward) and 5′-GTGGCTGAGGGAGGCGTT-3′ (reverse), Bcl-2 primers: 5′-GATGTGATGCCTCTGCGAAG-3′ (forward) and 5′-CATGCTGATGTCTCTGGAATCT-3′ (reverse), survivin primers: 5′-AGAACTGGCCCTTCTTGGAGG-3′ (forward) and 5′-CTTTTTATGTTCCTCTATGGGGTC-3′ (reverse), GAPDH primers: 5′-CGCTCTCTGCTCCTCCTGTTC-3′ (forward), and 5′-ATCCGTTGACTCCGACCTTCAC-3′ (reverse). The expression levels were measured by the $2^{-\Delta\Delta Ct}$ method.

Western blot assay

Cell lysates were prepared using Cell Lysis Buffer supplemented with protease inhibitor cocktail. The extracted total protein was determined by the BCA protein assay kit. The obtained 50 μg whole cell lysate was run on 8% Tris-Glycine gradient gel. Then the samples were electro-transferred to PVDF membrane and blocked the nonspecific binding in TBST buffer (0.5 mM Tris-HCl, 45 mM NaCl, 0.1% Tween-20, pH 7.4) containing 5% nonfat milk. The membranes were first incubated with rabbit anti-human VEGFR2, or Bcl-2, or survivin antibodies (1: 800 in 5% BSA/TBST solution) at 4°C for overnight, followed with washes and incubated with the horseradish peroxidase conjugated secondary antibody (anti-rabbit, in 5% nonfat milk) for an hour. Finally, the membranes were stained with SuperSignal™ West Pico PLUS Chemiluminescent Substrate. Membranes were then observed and re-probed with β-actin (1: 5000) to confirm equivalent loading and transfer of protein.

Cell proliferation assay

The effects of the designed CRISPR-Cas13a/liposome system on BCa cell proliferation were examined by Cell Counting Kit-8 according to the manufacturer's instructions. The 5637 cells were seeded in a 96-well plate (2×10^4 /well) for 12 h, then transfected the artificial hTERT promoter, Bcl-2, survivin, VEGFR2 crRNA tandem plasmid or negative control plasmid into the cells and cultured in medium. After 48 h incubation, 10 μL of CCK-8 was added to each well of the 96-well plates and incubated for 1 h. The 450 nm absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). Assays were repeated at least three times.

Cell motility assay

We employed Cell Scratch Assay to study the cell motility. The 5637 cells were seeded in 6-well plates as described above and treated with the artificial hTERT promoter, Bcl-2, survivin, VEGFR2 crRNA tandem plasmid or negative control plasmid. When achieved 100% confluents, the cells were scratched by a sterile 200 μL plastic pipette tip. After 18 h incubation, the cell migration condition in the scratch area was measured by Image J software.

Furthermore, we also performed the Transwell® migration assays to evaluate cell migration. The cells (1×10^5) were cultured in the upper uncoated culture dishes using 200 μL serum-free medium, and the lower chamber was filled with 500 μL of complete medium. Then the cells were kept in 37°C 5% CO_2 incubator for 24 h incubation. The treated cells were washed with PBS, fixed with 4% paraformaldehyde, and stained with 0.1% Crystal Violet. The filter membrane contained the invaded cells was then examined by light microscope imaging. Each chamber was added with 1 mL 33% acetic acid and shaken for 10 minutes. Then, we utilized a microplate reader to calculate the 570 nm absorbance for cell motility evaluation.

Cell apoptosis assay

We used flow cytometry assays to determine the apoptosis of BCa cells. After 48 h of transfection, the cells were harvested and washed with PBS. Then 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were co-mixed with the cells. The proportion of apoptotic cells were recorded by flow cytometry (EPICS, Xl-4; Beckman, CA, USA). The experiments were performed at least in triplicate.

The three genes targeting CRISRP-Cas13a plasmids delivered into BCa mice by liposome system

The synthetic CRISRP-Cas13a/liposomes (LW605 or LW606) were perfused into BCa model mice, the perfusion protocol was the same as before. After perfusion, the mice were kept under anesthesia for 30 min, and then the bladder area was irradiated by 0.8 W/cm^2 808 nm laser for 5 min to stimulate the release of liposomes. Then the mice were returned to the cage and kept feeding for 20 d. During the three consecutive weeks, repeat the perfusion once every week. The mice were

sacrificed after 20 d, the tumor tissues were collected to measure the tumor volume. The immunohistochemistry, western blot detection and real-time PCR analysis were also performed.

Statistical analysis

The SPSS 21.0 software (SPSS, Chicago, IL, USA) was used for statistical analyses. All quantifiable data were evaluated at least three independent experiments. The t-test or ANOVA were adapted to evaluate the group differences. The P value < 0.05 was deemed to be statistically significant ($^*P < 0.05$, $^{**}P < 0.01$).

Author contributions

J. F. and W. H. designed this study. X. L. and W. H. conceived the study and supervised this work. J. F. contributed to major experimental work. Y. H. and Y. L. assisted in the experimental and analytic work. J. F. and L. L. wrote the paper. All authors were involved and contributed to the discussion.

Funding sources

This work was supported by the Postdoctoral Science Foundation of China (2018M643312), National Natural Science Foundation of China (81772737, 81773257), Natural Science Foundation of Guangdong Province (2018A030310644, 2017B030301015, 2018B030306023), Sanming Project of Shenzhen Health and Family Planning Commission (SZSM201412018, SZSM201512037).

Conflict of interest

The authors declare that they have no competing interests.

Supporting information

Table S1. The crRNA sequences of CRISPR-Cas13a.
Fig. S1. The size and zeta potential analysis of liposomes.
Fig. S2. The release of liposomes.
Fig. S3. The in vitro cell apoptosis assays.
Fig. S4. The in vitro cell migration assays.
Fig. S5. The relevant CRISPR-Cas13a plasmids.

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Figures and Tables

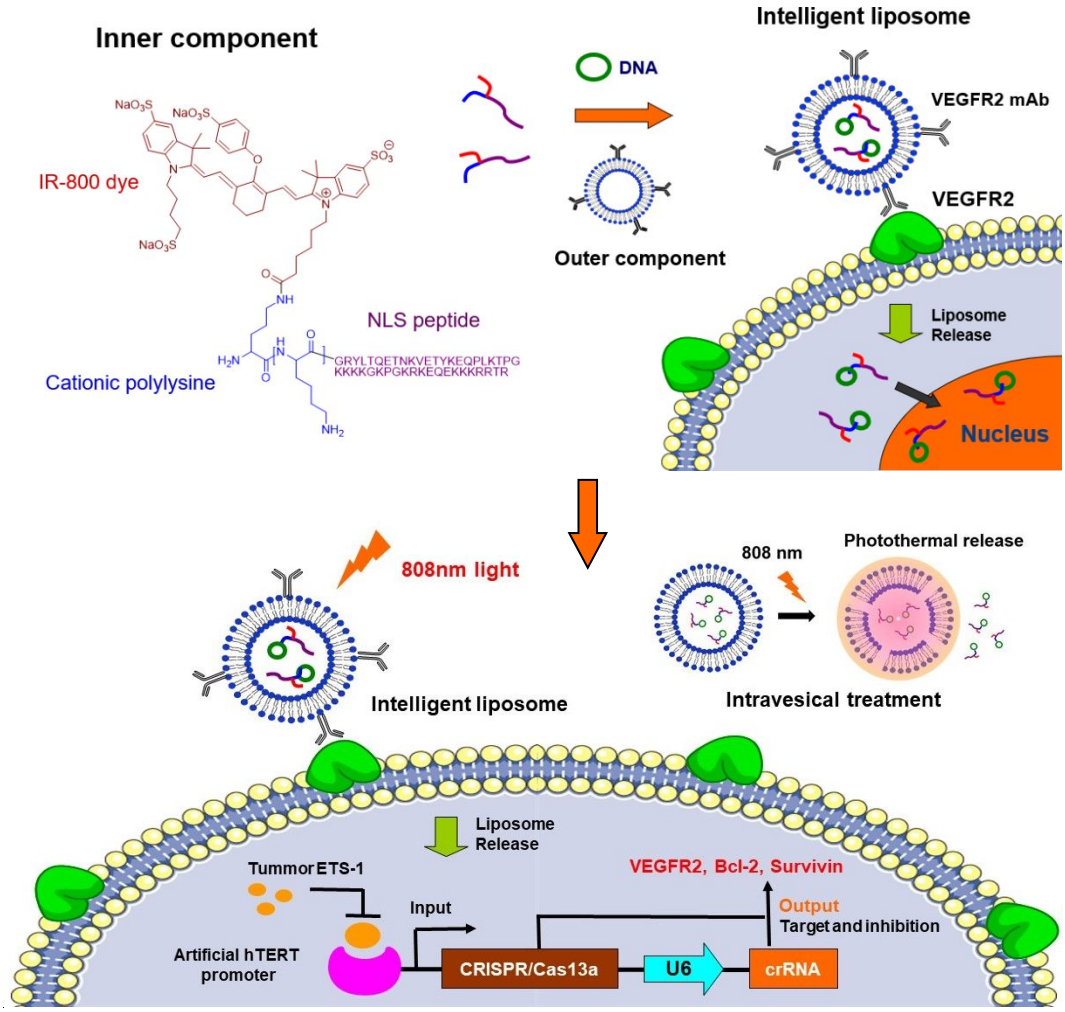


Fig. 1. Construction of the intelligent liposome delivery system.

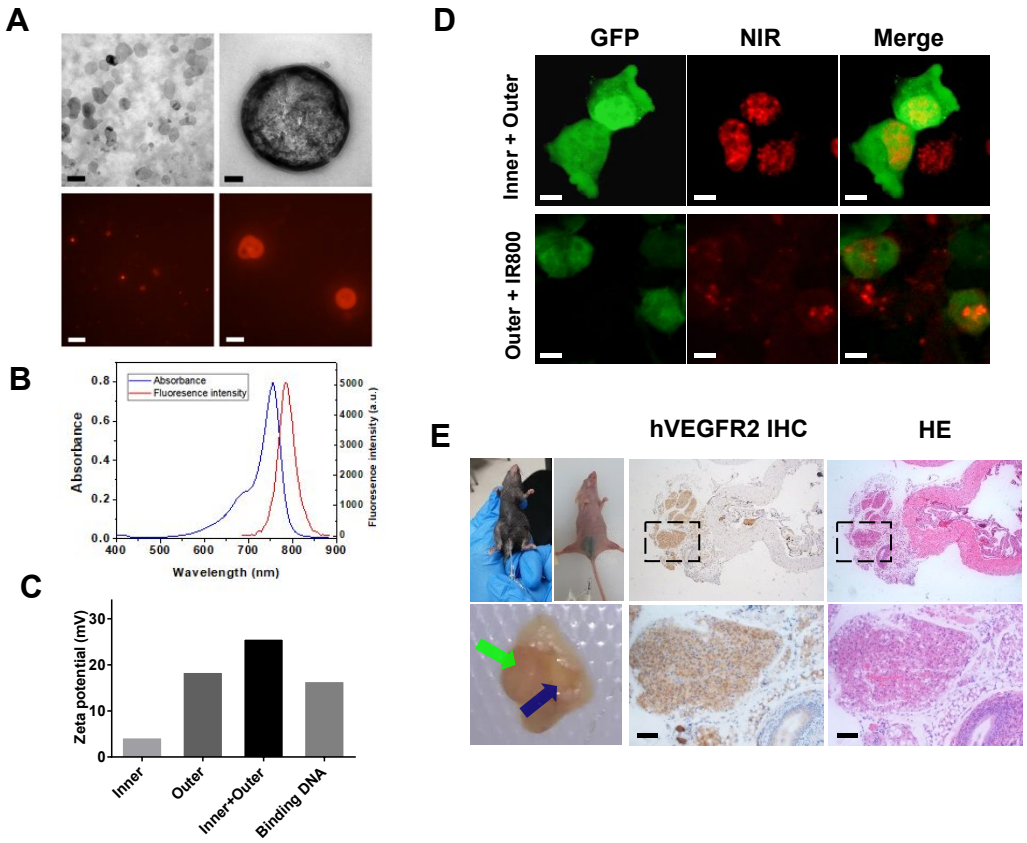


Fig. 2. Characterizations of the synthesized intelligent liposome system and constructed mice models. The synthesized intelligent liposome delivery system was characterized by transmission electron microscopy, fluorescence microscopy (A, black scale bar left is 2 μm, right is 100 nm; white scale bar left is 20 μm, right is 1 μm) and fluorescence spectrophotometry (B) to verify the feasibility and efficiency. (C) The zeta potentials of different components in liposomes were also measured. (D) The EGFP plasmids were loaded into the liposomes to transfer to 5637 BCa cells for studying the delivery effects (scale bar is 2 μm. Inner + outer are liposomes composed of inner components and outer components; outer + IR800 denotes outer components loaded with IR800 dye as control). (E) The in situ BCa mice model implanted in nude mice by bladder instillation; the mice bladders were harvested and treated with hVEGFR2 immunohistochemistry and hematoxylin and eosin staining (blue arrow is tumor tissue, green arrow is bladder tissue, dashed box is the magnified area. Scale bar is 50 μm).

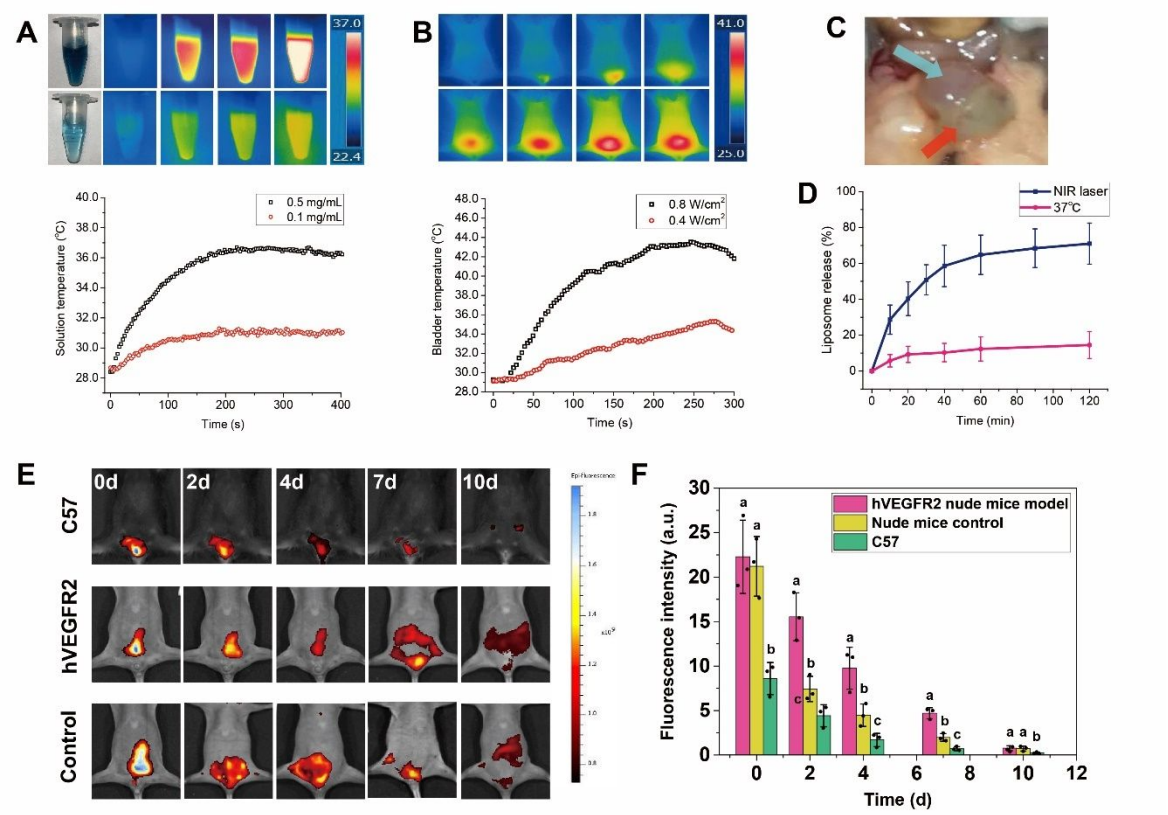


Fig. 3. The photothermal effect and in vivo imaging of the liposome system. The photothermal effect were investigated in vitro and in vivo using an 808 nm NIR laser. The thermal imaging results showed that the liposome system was rapidly heated in a tube to rise over 8°C (A) or in mouse bladders to above 42°C (B) in 5 min. After bladder instillation by the liposomes, a significant dye staining was observed in tumor tissue of the BCa model mice (C, blue arrow is normal bladder tissue; red arrow is 5637 tumor tissue). The release studies of the liposome system after treatment with near-infrared laser irradiation showed that the release of the liposome system was significantly accelerated in 150 min compared with the 37°C dark incubated group (D). The liposome system was also used for in vivo imaging (E). Fluorescence imaging indicated that the fluorescence of nude mice inoculated with human BCa cells lasted for at least 7 days after bladder infusion, which was significantly longer than that of normal nude and black mice (C57) from the 2nd day to the 7th day (F). This also indicated that the liposome system targeted the hVEGFR2 expression of BCa cells for an extended length of time (the different lowercase letters (a, b, c) represent $P < 0.05$).

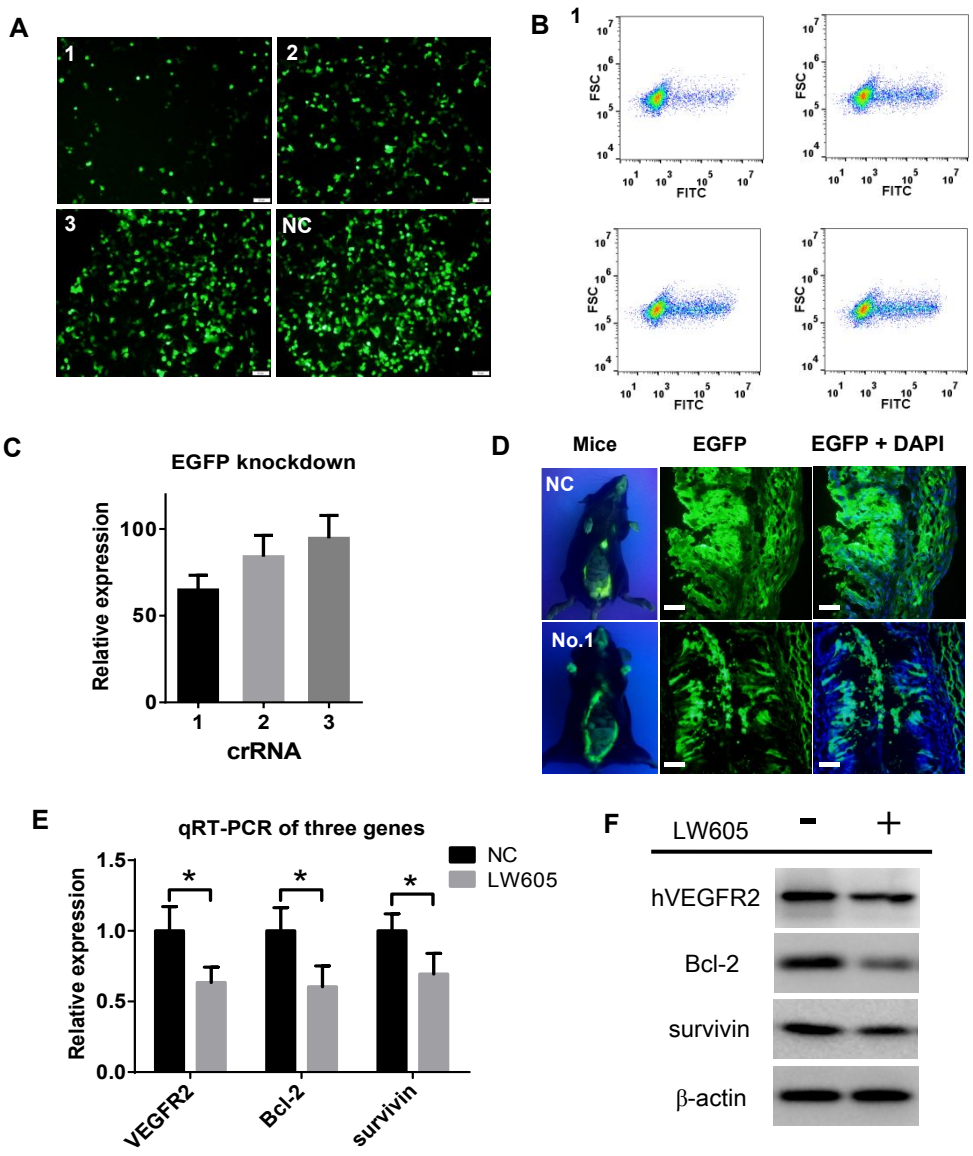


Fig. 4. The target genes knockdown by CRISPR-Cas13a/liposome systems. In order to achieve robust Cas13a-mediated RNA knockdown, the CRISPR-Cas13a plasmid backbone was evaluated by an EGFP targeting knockdown assay. For in vitro studies, the three alternative sequences of crRNA for CRISPR/Cas13a plasmid were prepared for the CRISPR-Cas13a/liposome system and incubated with the EGFP transfected 5637 cells. The imaging (A, scale bar is 50 μm) and fluorescence microimaging and flow cytometry (B) results showed that the three crRNA triggered the knockdown effects, and the No. 1 crRNA (uppercase letters in Table S1) plasmid achieved the most significant effect with a decreased > 35% (C, numbers 1, 2, 3 are the three crRNA plasmids; NC is the control). (D) For the in vivo test, the No. 1 or NC CRISPR-Cas13a/liposome system was delivered into EGFP mice by bladder instillation. A significant fluorescence signal from the frozen bladder slides showed that a decrease was also observed in No. 1 crRNA-treated mice. Subsequently, the VEGFR2, Bcl-2 and survivin genes were knocked down by the prepared liposomes loaded with pHS-ACR-LW605 plasmids (scale bar is 50 μm). The qRT-PCR (E) and western blot (F) evaluations of the gene expressions indicated a significant downregulation of all three genes after liposome treatment.

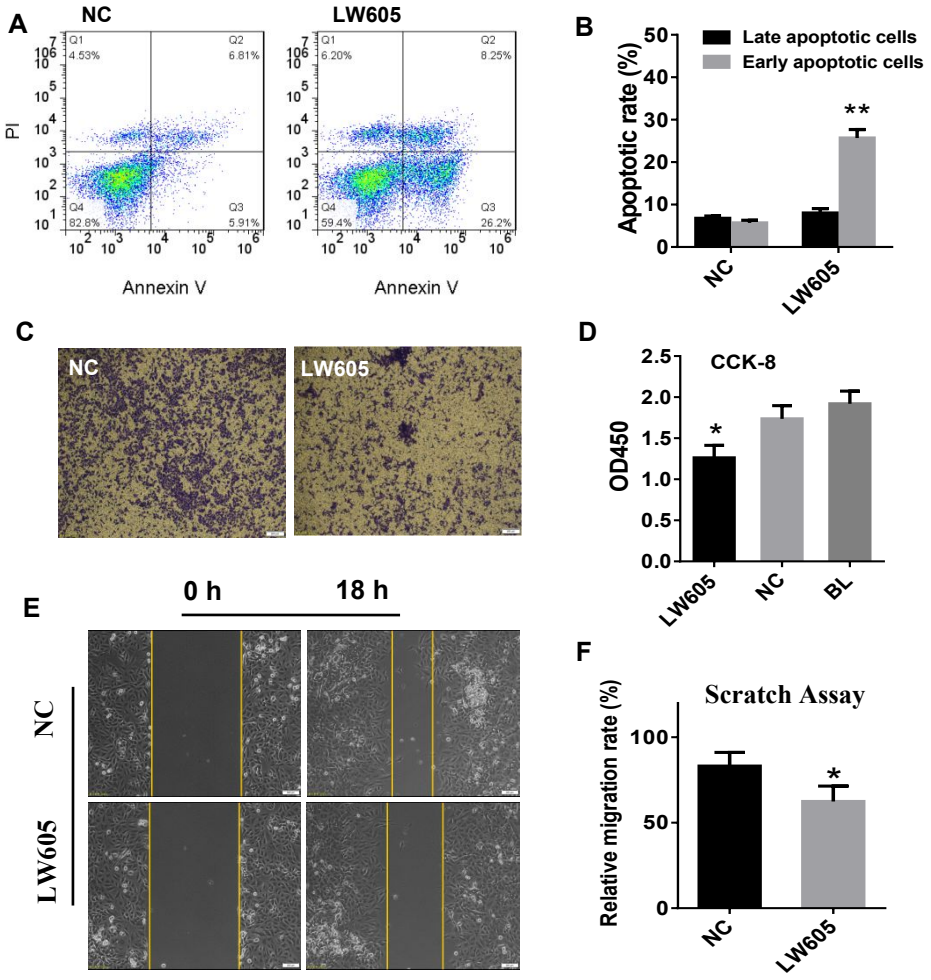


Fig. 5. The anti-BCa activity of CRISPR-Cas13a/liposome systems. In vitro cell proliferation, motility and apoptosis assays indicated that the number of apoptotic cells was significantly increased (A and B, annexin V-FITC/PI assay, NC is the negative control, LW605 is the pHS-ACR-LW605). The invasion (C, Transwell® migration assay, scale bar is 200 μm), proliferation (D, BL is the blank control without CRISPR-Cas13a/liposome system treatment; CCK-8 is the Cell Counting Kit-8) and migration (E and F, cell scratch assay, scale bar is 50 μm) of treated BCa cells were significantly inhibited by pHS-ACR-LW605/liposome transfection compared to the control group of the pHS-ACR-LW606/liposome (NC). $^{*}P < 0.05$; $^{**}P < 0.01$.

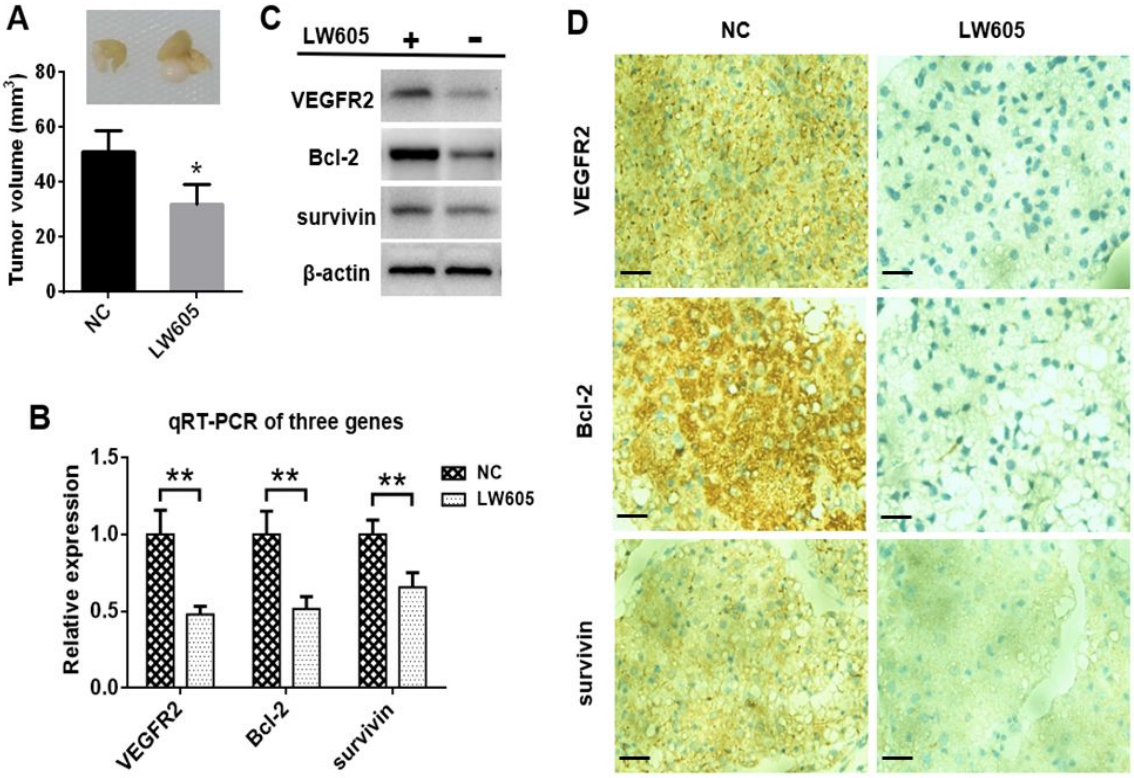


Fig. 6. The anti-BCa study of CRISPR-Cas13a/liposome systems in BCa mice. (A) The tumor tissues of BCa mice were treated with CRISPR-Cas13a perfusion for 20 days, and the difference in tumor volume was measured. The tumor volume of the LW605 group was significantly smaller than that of the NC group (LW606). (B) The total RNA of the tumor tissues was extracted for real-time PCR detection. The transcription levels of hVEGFR2, Bcl-2 and survivin in the LW605 group were significantly lower than those in the NC group. The western blot detection (C) and immunohistochemistry results (D, scale bar is 20 μm) also showed that the expression levels of the three genes in the tumor tissues of LW605 treated group were significantly lower than those in the NC group. $^{*}P < 0.05$; $^{**}P < 0.01$.

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A multifunction lipid-based CRISPR-Cas13a genetic circuit delivery system for bladder cancer gene therapy

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