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Article

Mechanical characterization of hyaluronic acid-modified cationic liposomes for targeted deliver of ONECUT2 shRNA in hepatocellular carcinoma

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CITATION

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Abstract: Hepatocellular carcinoma (HCC) is a globally significant malignancy with high morbidity and mortality. Anti-tumor targeted drug therapy is a promising therapeutic strategy, but the strategy faces challenges related to delivery efficiency and mechanical interactions within the tumor microenvironment. In our previous study, we found that the transcription factors ONECUT2 (OC2) and CD44 receptor have important roles in HCC progression. We designed high molecular weight hyaluronic acid-modified cationic liposomes (HMW-CL) to take advantage of the binding affinity between hyaluronic acid and CD44 to deliver plasmid DNA (pshOC2) encoding a short hairpin RNA targeting OC2 to HCC cells. The results showed that the prepared HMW-CL had a uniform particle size of 179.5 nm, a moderate zeta potential of 15.8 mV, a high encapsulation efficiency of 86%, which not only protected pshOC2 from degradation but also ensured favorable mechanical stability under physiological shear stresses. Biomechanical characterization revealed that the liposomes maintained structural integrity under simulated blood flow conditions, with minimal deformation and optimal adhesion to CD44-expressing HCC cells. In vitro experiments, HMW-CL/pshOC2 liposomes were characterized by high transfection efficacy, lysosomal escape, and low cytotoxicity. They could efficiently deliver pshOC2 to cells, affecting HCC cell proliferation, migration, invasion, and triggering apoptosis. Biomechanical assays further confirmed that the liposomes altered the mechanical properties of HCC cells, reducing their stiffness and migratory capacity, which are critical factors in tumor progression. In vivo experiments, intravenous injection of HMW-CL/pshOC2 liposomes effectively reduced OC2 expression in HCC tumors and inhibited tumor growth at low toxicity with an inhibition rate of 81.77%. Our study demonstrated that OC2 may be a candidate gene suitable for HCC targeted-therapy, and our HMW-CL/pshOC2 liposomes were prepared based on the hyaluronic acid/CD44 binding strategy, with good stability, high transfection efficacy, and low cytotoxicity. Moreover, their favorable biophysical and biomechanical properties make them a promising delivery system for HCC therapy, with potential applications in modulating the mechanical microenvironment of tumors.

Keywords: hepatocellular carcinoma; biomechanics; liposome; hyaluronic acid; ONECUT2 (OC2)

1. Introduction

HCC has the third highest mortality rate in the world, and its morbidity and mortality rates are rising rapidly. Thus, HCC has emerged as a major public health problem that seriously threatens the function of the liver system [1]. Compared with traditional HCC treatments, including surgery, ablative therapy, chemotherapy. Target therapy has considerable advantages in specificity, efficacy, and side effects [2].

Meanwhile, exploring effective target gene, establishing reliable drug delivery system, and improving tissue specificity are important sources for developing novel anti-tumor targeted drugs for HCC.

Targeted drug therapy is critically dependent on the targeting of regulated genes. OC2, a member of the ONECUT family of transcription factors, is located in the human chromosome 18 [3]. As a transcription factor, OC2 widely regulated the expression of target gene, which was involved in cell proliferation, migration, adhesion, differentiation, and metabolism during the development of multiple tissues. However, the expression of OC2 is limited by tissue site and developmental stage [4,5]. OC2 plays a key role in early liver development, hepatocyte nuclear factor 1 and OC2 can promote hepatoblast growth and metastasis by regulating the expression of hepatocyte nuclear factor 4 alpha 7 and modifying the TGF-β signaling pathway [6,7]. Additionally, OC2 is strongly associated with the carcinogenesis of HCC [8] and numerous other tumors such as prostate cancer [9,10], colorectal cancer [11], and ovarian cancer [12]. Some studies have revealed some target genes of OC2 driving tumor aggressiveness. Zhang et al. reported that dysregulated microRNA-9 could upregulated OC2 expression, leading to a poor prognosis, and restoring microRNA-9 could suppress HCC cell proliferation and migration [8]. Our previous study showed that miR-6086 downregulated OC2 expression to inhibit tumor angiogenesis via the OC2/VEGFA/EGFL6 axis, and OC2 mediated tumor angiogenesis via the ZKSCAN3/VEGFA axis. Moreover, targeting OC2 could inhibit tumor growth and metastasis [13]. Given its critical role in tumor progression, we selected OC2 as a potential candidate for HCC targeted therapy.

Cationic liposomes (CLs) are commonly used drug carriers with positive charge so that these nanoparticles can be endocytosed by negatively charged cytomembrane [14,15]. Surface charge is considered one of the most crucial factors influencing cellular uptake and cytotoxicity. The positive charge facilitated the interaction of liposomes with anionic nucleic acids through their electrostatic attraction, leading to the formation of lipid-nucleic acids complexes. However, an excessively high positive charge can result in high cytotoxicity, while a low or negative charge may lead to an inability to load nucleic acids [16]. Although their transfection efficiency is lower than that of viral vectors, CLs have been developed into one of the most reliable nanocarriers for nucleic acid delivery owing to their excellent biocompatibility and low immunogenicity [17]. CLs can carry plasmid DNA encoding short hairpin RNA (pshRNA) targeting the candidate gene with a stable encapsulation rate and enter the cells. The addition of polyethylene glycol can avoid exposed degradation and mediate target gene silence [18]. CLs-based gene therapy has attracted considerable attention, however, the tissue specificity, cellular uptake and potential toxicity still limit their medical application. Therefore, it's imperative to improve the properties of CLs by surface modification [16].

Hyaluronic acid (HA) is an anionic disaccharide, has been widely employed in bioengineering and drug delivery systems due to its good water solubility, biocompatibility, and biodegradability [19]. Based on molecular weight, HA can be classified into oligo-hyaluronic acid (oHA), low molecular weight hyaluronic acid (LMW-HA), and high molecular weight hyaluronic acid (HMW-HA). These different forms of HA exhibit distinct functions upon binding to their membrane receptor CD44.

HMW-HA inhibits angiogenesis, while LMW-HA and oHA promote it. HMW-HA induces anti-inflammatory responses, while LMW-HA enhances proinflammatory effects [20,21]. Although natural HA can activate certain signaling pathways, the intensity is much lower than HA oligomers. The recognition of HA/CD44 has been applied to enhance the cell uptake and tissue specificity of nucleic acid delivery systems involving HA-modified nanoparticles and CD44 overexpressed cells, as HA/CD44 enhances receptor-mediated transcytosis [22].

It was reported that CD44 was overexpressed on the surface of HCC cells (SK-Hep1) and showed a high affinity with HA [22]. In this study, we designed and prepared hyaluronic acid modified cationic liposome (HMW-CL) for delivering pshOC2 to SK-Hep1 cells. After verifying nucleic acid encapsulation, protein adsorption, cytotoxicity, transfection efficiency, and lysosomal escape, we completely investigated the targeting effects of HMW-CL/pshOC2 lipoplexes on OC2 expression, tumor growth, and metastasis through in vitro and in vivo experiments. Our strategy based on HMW-CL/pshOC2 lipoplexes may provide promising a candidate gene and tissue-specific nucleic acid delivery system for HCC target therapy.

2. Materials and methods

2.1. Preparation of hyaluronic acid (HA)-modified liposomes and characterization

Hyaluronic acid-modified cationic liposome (HMW-CL) was prepared by freeze-drying [23]. The following raw materials (2,3-Dioleoyloxy-propyl) trimethylammonium-chloride (DOTAP, #LP-R4-117, Corden Pharma, Liestal, Switzerland), DOPE, DMG-PEG2000 (#880151P, Avanti Polar Lipids, Alabaster, AL, USA), and DSPE-PEG-cRGD (#R-9995, Ruixibio, Shanxi, China) were mixed and dispersed by ultrasound pre-frozen for 24 h, then, put it into the lyophilized machine. The lyophilized liposome powder was hydrated to obtain CL solution. The HMW-HA solution was prepared by dissolving HMW-HA powder (#Bloomage Biotech, Jinan, China) in distilled water overnight at a concentration of 1 mg/mL. The HA solution was filtered through a 0.22 μm membrane and stored at 4 ℃. HMW-HA-modified liposomes were prepared by adding HMW-HA to the CL solution with a mass ratio of 10% and incubated for 20 min. The average particle size, zeta potential, and polydispersity (PDI) of HMW-HA-modified liposomes (1 mg/mL) were measured using Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). The morphology of HMW-HA-modified liposomes was negatively stained with 1% phosphotungstic acid and imaged by transmission electron microscope (TEM, FEI).

2.2. Nucleic acid encapsulation

The HMW-CL/pshOC2 lipoplexes with different N/P ratios were determined by 1% agarose retardation assay. Picogreen dsDNA Quantitative Reagent (Yeasen, Shanghai, China) was employed to detect the pshOC2 encapsulation efficiency of CL and HMW-Cl. The fluorescence intensity of free plasmid DNA before and after rupture of 0.1% Triton X-100 was detected by a microplate reader (BioTek Instruments, USA). The HMW-CL protection to pshOC2 was evaluated by DNase I enzymolysis. The

HMW-CL/pshOC2 lipoplexes with different N/P ratios were treated with DNase I for 10 min (inactivated at 60 ℃), ruptured with 0.1% TritonX-100 and subject to 1% agarose gel electrophoresis.

2.3. Protein adsorption

Blank F-HMW-CL (high molecular weight hyaluronic acid-modified cationic liposomes without plasmid) and F-CL solution (1 mg/mL) were mixed with 1 mg/mL BSA solution, incubated at 37 ℃ for 6 h under light protection. The samples were centrifuged to separate the free BSA and nanoparticles. The supernatant protein concentration was determined via the BCA Kit (#E112-02, Vazyme, China).

2.4. Cell culture

The SK-Hep1, HepG2, and HEK293T were purchased from the Institute of Cell Research, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% Fetal Bovine Serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 100 U/mL Penicillin/Streptomycin (Gibco, Waltham, MA, USA) at 37 ℃ in an incubator with 5% CO_2.

2.5. Cytotoxicity

SK-Hep1 cells (5000 cells per well) were plated into 96-well plates and treated with 10–50 μg/mL HMW-CL and CL for 24 h. Then CCK-8 reagent was added to each well, and the absorbance at 450 nm was read by a microplate reader. The cytotoxicity of HMW-CL was indicated by the cell viability.

2.6. Transfection

HEK293T and SK-Hep1 cells (1×10^5 cells per well) were plated into a 12-well plate. Liposomes (CL) and plasmid DNA (pGPU6/GFP/Neo, GenePharma, Suzhou, China) encoding short hairpin RNA (shRNA) targeting OC2 (sense: 5'-GCCAGCTGGAAGAAATCAACA-3', anti-sense: 5'-TGTTGATTTCTTCCAGCTGGC-3') were separately diluted with Opti-MEM (Gibico, American). After 20 min of incubation at room temperature, the two diluted solutions were mixed. Subsequently, 10% high-molecular-weight hyaluronic acid (HMW-HA) was added to the mixture, then incubate for another 20-min to form the lipid complex. The formed lipid complex was then added to the 12-well plates for transfection. Finally, the transfection efficiency was examined under an Inverted Fluorescence Microscope (Carl Zeiss).

2.7. Lysosomal escape

SK-Hep1 cells (2×10^5 cells) were inoculated into confocal dishes and treated with FITC-HMW-CL/pshOC2 lipoplexes. After washing with PBS, the cell lysosomes were stained with 75 nm Lyso-Tracker Red (#C1046, Beyotime, Shanghai, China) and fixed with 4% paraformaldehyde. The samples were imaged by a confocal laser scanning microscope (CLSM, Olympus, Japan).

2.8. Western blot

Cells or tumors were lysed by RIPA solution containing PMSF. The proteins were separated by SDS-PAGE and transferred to the PVDF membrane. The membranes were blocked with 5% skim milk and incubated with primary antibodies at 4 ℃ overnight. The primary antibodies were anti-OC2 (Proteintech, 21916-1-AP), anti-Bax (CST, S41162), anti-Bcl-XL (CST, 2762), anti-E-cadherin (CST, 3195), anti-GAPDH (CST, 5174), anti-N-cadherin (CST, 13116), and anti-vimentin (CST, 5741). After incubation with secondary antibody, the membranes were exposed to ECL luminescent droplets and measured with Tanon Protein Imager. The expression of target proteins was calculated by ImageJ software.

2.9. Real-time quantitative PCR (qRT-PCR)

The total RNA was extracted via the RNA-Easy Isolation Reagent (Vazyme, Nanjing, China). cDNA was reversed via reverse transcription using HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) qRT-PCR was performed using SYBR qPCR Master Mix and the CFX96^{TM} Real-Time PCR System (Bio-Rad, Singapore). The expression of target genes were determined by the $2^{-\Delta\Delta CT}$ method and normalized to those of the control group.

2.10. Colony formation assay

SK-Hep1 cells (1×10^3 cells per well) were plated into 6-well plates and treated with F-HMW-CL (control groups), CL/pshOC2, and HMW-CL/pshOC2 (experimental groups), respectively. After 7–9 days of incubation, when the clonal clusters in control grew to 50 cells or more. The cells were fixed with 4% paraformaldehyde, then stained with 1% crystal violet and photographed.

2.11. Apoptosis assay

The cells in each treatment group were digested with 0.25% EDTA-free trypsin and resuspended in DMEM supplemented with 10% FBS at a density of 3 × 105 cells. Then, cell staining according to the instructions of the Annexin V-APC/7-AAD Apoptosis Kit (#E-CK-A218, Elabscience, China) and measured by flow cytometry.

2.12. Migration and invasion assays

For wound healing assay, SK-Hep1 cells (3×10^5 cells per well) were plated into 6-well plates. When the cell density reached 80%–90%, the cells were scratched by 200 μL pipette tip and treated with HMW-CL/pshOC2 Images were taken at 0 h, 24 h, and 48 h by using a bright-field microscope (Carl Zeiss, Thuringia, Germany) and analyzed by ImageJ software. For migration assay, SK-Hep1 cells were treated with HMW-CL/pshOC2 for 24 h, and the cells (2×10^4 cells per well) were added into the upper chamber. while 550 μL DMEM containing 10% FBS was added into the lower chamber. After 24 h, the cells were fixed with 4% paraformaldehyde, stained with 1% crystal violet, and imaged using a bright-field microscope 24 h later. For invasion assay, 50 μL of Matrigel was coated in upper chambers and SK-Hep1 cell density was adjusted to 8×10^4 cells per well.

2.13. Mouse xenograft model

Four-week-old BALB/c nude mice were euthanized by the Guidelines for the Breeding and Use of Laboratory Animals of Jinan University, and all animal experiments were approved by the Scientific Ethics and Welfare Committee for Laboratory Animals of Jinan University. Mice were routinely injected subcutaneously at the shoulder with 100 μL of treated cells (3×10^6 cells). PBS, F-HMW-CL, CL/pshOC2 and HMW-CL/pshOC2 were injected intravenously, respectively, every five days, and the tumor volume (length × width^2)/2 was measured every five days for 30 days. Mice were euthanized and tumors were harvested for immunohistochemistry, and major organs were taken for biochemical indices.

2.14. Statistical analysis

All data were presented as mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was conducted using one-way ANOVA or two-way ANOVA in GraphPad Prism 8.0 software. * $p < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation and characterization of HMW-CL

CLs were produced using a freeze-dried hydration method as previously described, and the size of nanoparticles should be controlled below 200 nm [23]. The HMW-HA solution was added to form HMW-HA-modified liposomes (HMW-CL). The TEM imaging showed that HMW-CL was spheroidal and the hydrodynamic diameter was increased from 153.1 to 179.5 nm, the zeta potential was reduced from 32 to 15.8 mV, and the PDI was increased from 0.131 to 0.239 after the addition of HMW-HA (**Figure 1**, **Table 1**). Thus, the HMW-CL had a spheroidal shape and uniform distribution after extrusion, and the slight increase of particle size indicated that the addition of HMW-HA did not induce the fusion of liposomes, in line with the standard of nanocarrier delivery.

Table 1. The particle size, zeta potential and PDI of F-HMW-CL and F-CL.

Formulations	Size (nm)	Zeta potential (MV)	PDI
F-HMW-CL	179.5	15.8	0.239
F-CL	153.1	32	0.131

F-HMW-CL, high molecular weight hyaluronic acid-modified cationic liposomes; F-CL, cationic liposomes; PDI, polydispersity index.

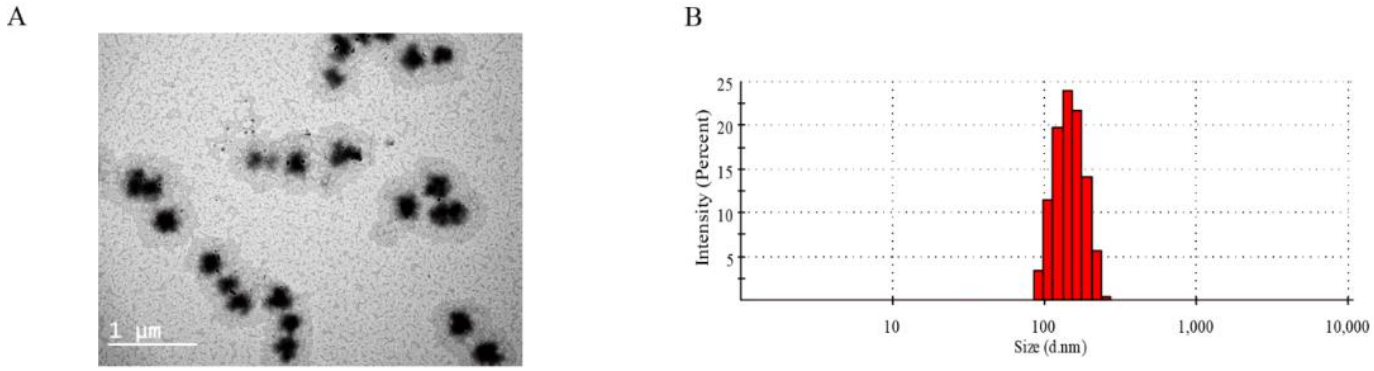

Figure 1. The physicochemical properties of HMW-CL: **(A)** TEM image of HMW-CL. scale bar: 1 μm; **(B)** The hydrodynamic diameter distribution of HMW-CL measured by zetasizer nano ZS90.

3.2. Encapsulation efficiency and protection of pshOC2 by HMW-CL

To design HMW-CL as a delivery vector for plasmid DNA, it was necessary to investigate its ability to tightly compress plasmid DNA and determine the optimal N/P ratio for efficient delivery. The agarose gel retardation assay demonstrated that, at N/P ratios exceeding 2, the plasmid remained trapped in the well, indicating complete encapsulation of pshOC2 within HMW-CL (**Figure 2A**). In contrary, for conventional cationic liposomes (CL), complete encapsulation of pshOC2 required N/P ratios greater than 3. Both CL and HMW-CL exhibited high encapsulation efficiency of pshOC2 as evidenced by fluorescence intensity of double-stranded DNA (dsDNA) at an N/P ratio of 3, with the addition of high molecular weight hyaluronic acid (HMW-HA) further enhancing this effect, achieving an encapsulation efficiency of 86% (**Figure 2B**). In addition to encapsulation, an effective nucleic acid delivery system must protect the nucleic acid from degradation by DNase in vivo. To evaluate this, DNase I was used to degrade DNA. The results revealed that CL failed to completely encapsulate pshOC2, whereas HMW-CL effectively encapsulated pshOC2 within the aqueous core of the vesicles, thereby preventing its degradation during delivery (**Figure 2C**).

Reducing non-specific protein binding is critical for prolonging the circulation time of liposomes in vivo. Given that positively charged CLs readily adsorbs negatively charged bovine serum albumin (BSA), the protein adsorption of HMW-CL was compared with that of CL. The results indicated that the adsorption rate of CL was 44%, whereas that of HMW-CL was significantly reduced to 7% (**Figure 2D**). This finding demonstrated that modification with HMW-HA effectively reduced protein adsorption on the liposomal surface, thereby enhancing the stability of nucleic acid nanocarriers in blood circulation.

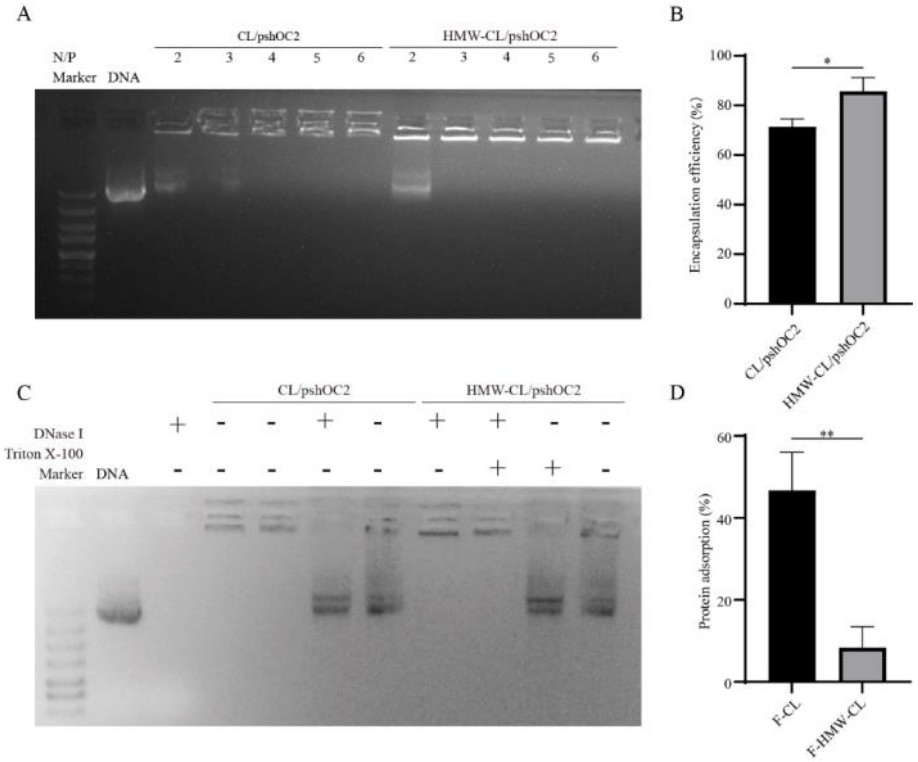

Figure 2. Nucleic acid loading capacity of HMW-CL: **(A)** The 1% agarose retardation assay of CL and HMW-CL at N/P ratios of 2, 3, 4, 5, 6; **(B)** The nucleic acid encapsulation rate of CL/pshOC2 and HMW-CL/pshOC2 at N/P ratio of 3; **(C)** The protective properties of CL and HMW-CL against pshOC2 at N/P ratio of 3; **(D)** Protein adsorption of CL and F-HMW-CL. * $P < 0.05$, ** $P < 0.01$.

3.3. Cytotoxicity of HMW-CL

HA modification of CLs effectively reduces their surface charge and cell toxicity. CCK-8 assays showed that different concentrations of HMW-HA could reduce the cell toxicity of CLs (**Figure 3A,B**). We further measured and compared the cytotoxicity of CL, HMW-CL, and Lipo2000 (**Figure 3C**). In SK-Hep1 cells, low concentrations of CL and HMW-CL (10–20 μg/mL) exhibited negligible differences in toxicity. However, when the concentration exceeded 20 μg/mL, the HMW-CL treatment group exhibited significantly lower toxicity. Furthermore, we evaluated the toxicity in 293T cells. As the liposome concentration gradually increased, there was no significant difference between the cell viability of CL and HMW-CL groups, but Lipo2000 obviously retarded the survival of 293T cells, suggesting that different cell lines had different tolerance to liposomes. The results demonstrated that, compared with the commercial transfection reagent Lipo2000, the incorporation of HMW-HA can reduce the cytotoxicity of high-dose CLs towards hepatocellular carcinoma (HCC) cells. Additionally, HMW-CLs were verified as a suitable nucleic acid delivery vector due to their low cytotoxicity.

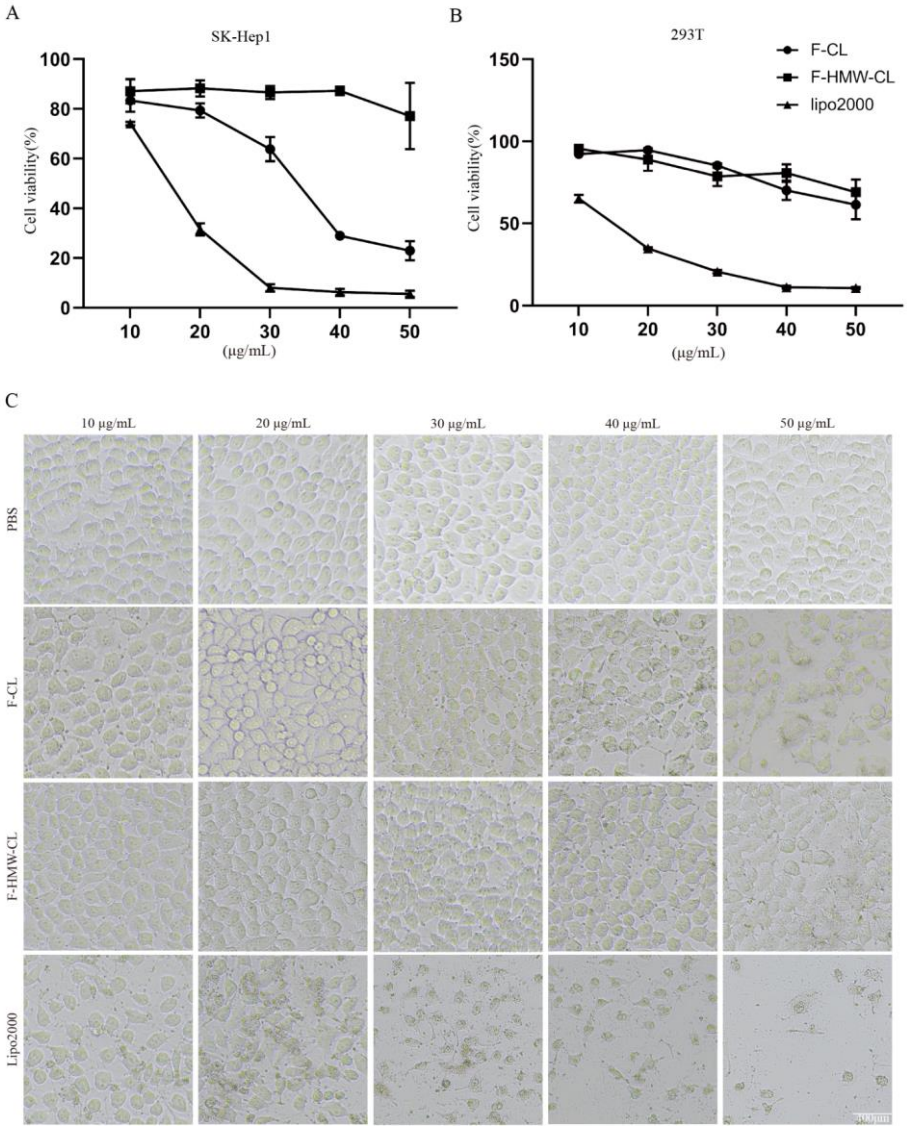

Figure 3. Low cytotoxicity of HMW-CL in vitro: **(A)** Cell viability of SK-Hep1 cells was detected by CCK-8 assay; **(B)** Cell viability of HEK293T cells was detected by CCK-8 assay; **(C)** Cell viability of SK-Hep1 cells was measured by microscopic viewing.

3.4. Transfection effects and intracellular trafficking of HMW-CL/pshOC2

Lesley et al. have demonstrated that cooperativity is the primary characteristic of the cytomembrane receptor CD44 binding to HA [24]. This cooperativity is the result of multiple binding sites on repetitive disaccharide ligands and multiple tightly packed receptors on cytomembrane surface. We modified liposomes with different molecular weights of HA to observe the transfection effects of pshOC2 lipoplexes on SK-Hep1. Fluorescence images and expression analysis indicated that the transfection efficiency of HMW-CL/pshOC2 was significantly higher than LMW-CL and close to lipo2000 in SK-Hep1 cells. Western blot assay further revealed that HMW-CL/pshOC2 could significantly down-regulate the expression of OC2 without a gradient effect on the activity of the cells (**Figure 4A–C**). The result showed that HMW-CL/pshOC2 lipoplexes could be efficiently delivered into the CD44 overexpressed HCC cells and

induce a considerable silencing effect of the target gene.

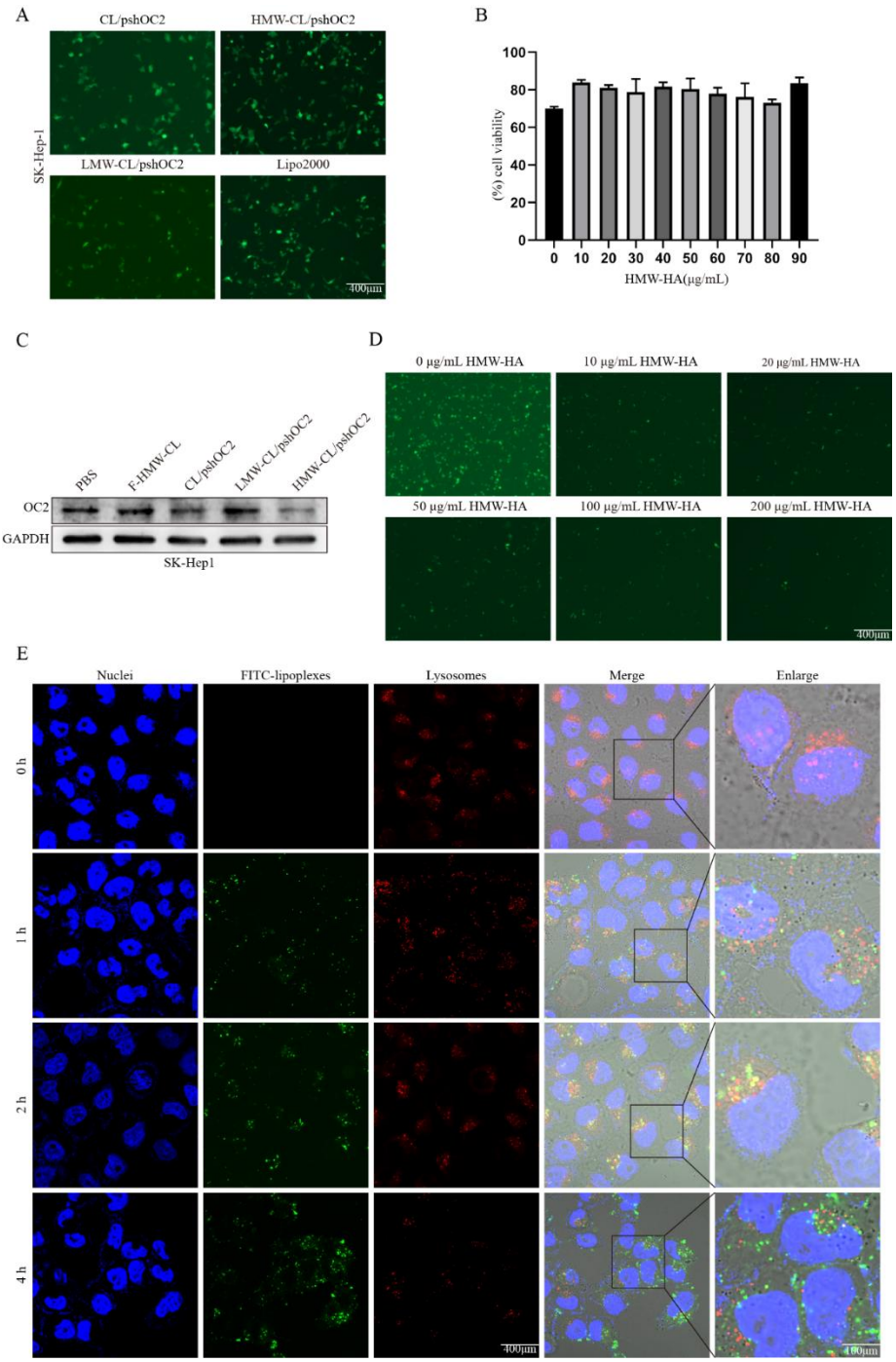

Figure 4. The cytotoxicity of HMW-HA-modified lipoplexes and transfection effects of HMW-CL/pshOC2: **(A)** The transfection efficiency of HMW-CL/pshOC2 in HCC cells was verified via fluorescence detection; **(B)** The viability of SK-Hep1 cells treated with different concentrations of HMW-HA modified liposomes was detected by CCK-8 assays; **(C)** The transfection efficiency of HMW-CL/pshOC2 in HCC cells was verified via Western blotting; **(D)** Competitive binding interaction of HMW-HA-modified lipoplexes/CD44 in SK-Hep1 cells observed by fluorescence microscope; **(E)** The pshOC2 distribution after FITC-HMW-CL/pshOC2 liposome treatment of SK-Hep1 cells for different times was detected via immunofluorescence.

In vivo, the liposome will be decomposed in lysosome. Thus, a proper nucleic acid vector should be possess the capacity to suffer and escape from the trap of

endosome and lysosome during endocytosis. To investigate the effect of the CD44 receptor on the cellular uptake of lipid complexes, free HMW-HA as an inhibitor of CD44 receptor was added before SK-Hep1 cells transfected with HMW-CL/pshOC2 lipoplexes. As the concentration of HMW-HA increased, the fluorescence associated with lipoplex uptake decreased and became scattered (**Figure 4D**). This suggested that pre-incorporation of HMW-HA preferentially binds to the CD44 receptor on the cell membrane of SK-Hep1 cells, thereby competitively blocking the uptake of HMW-CL/pshOC2 liposomes. It also indicated that HMW-HA modification contributes to the targeting of nanocarriers to HCC cells via the CD44 receptor.

To further explore the intracellular delivery of HMW-CL/pshOC2 lipoplexes. We performed CLSM analysis on SK-Hep1 cells incubated for different times. The green fluorescence gradually increased from 0 to 4 h, indicating that HMW-CL/pshOC2 lipoplexes could enter HCC cells in a large amount in a short period. The co-localization of green and red fluorescence at 1 and 2 h confirmed that the lipoplexes had been delivered to lysosomes. However, the red fluorescence attenuated a lot at 4 h, which meant the integrity of lysosomes had been broken; the lipoplexes escaped from lysosomes and gathered around nuclei to accelerate exogenous pshOC2 entering nuclei and OC2 shRNA expression (**Figure 4E**). These results demonstrated that HMW-CL/pshOC2 lipoplexes we prepared could enter HCC cells via CD44-mediated endocytosis and achieve the expected results of knockout OC2 after successful escape from lysosomes.

3.5. HMW-CL lipoplexes deliver pshOC2 inhibit HCC growth and metastasis, and promote HCC apoptosis

To further investigate the effect of HMW-CL/pshOC2 lipoplexes on the biological functions of HCC cells, it was first verified by in vitro experiments. In colony formation assays, CL/pshOC2 and HMW-CL/pshOC2 treatment groups significantly inhibited the proliferation of HCC cells, and the inhibitory effect of HMW-CL/pshOC2 was more pronounced compared with CL/pshOC2 groups (**Figure 5A**). In Transwell assay, CL/pshOC2 and HMW-CL/pshOC2 significantly inhibited the migration and invasion of HCC cells, but the inhibition rate of HMW-CL/pshOC2 was higher (**Figure 5B,C**). Furthermore, the wounding healing assay showed that HMW-CL/pshOC2 significantly reduced the migration of SK-Hep1 (**Figure 5D**). Western blotting and RT-qPCR demonstrated that HMW-CL/pshOC2 significantly reduced expression of OC2 in HCC cells, while down-regulating the expression of N-cadherin and vimentin and up-regulating the expression of E-cadherin in epithelial mesenchymal transition (EMT) (**Figure 6A,B**). These results indicated that HMW-CL/pshOC2 could efficiently deliver shOC2 into SK-hep1 cells, thereby inhibiting the proliferation and metastasis of HCC cells. Meanwhile, we also found that CL/pshOC2 and HMW-CL/pshOC2 induced apoptosis in SK-Hep1 cells. Notably, HMW-CL/pshOC2 induced a significantly higher rate of apoptosis than CL/pshOC2. (**Figure 6C**), and Western blotting and RT-qPCR also demonstrated that knockout with OC2 upregulated the expression of Bax and down-regulated the expression of Bcl-XL, which suggests that OC2 may cause HCC cell apoptosis (**Figure 6D,E**). The result also indicated that HMW-CL/pshOC2 liposomes could efficiently deliver plasmid

OC2 shRNA, silencing OC2 expression in SK-Hep1 cells, thus affecting the biological function of HCC cells.

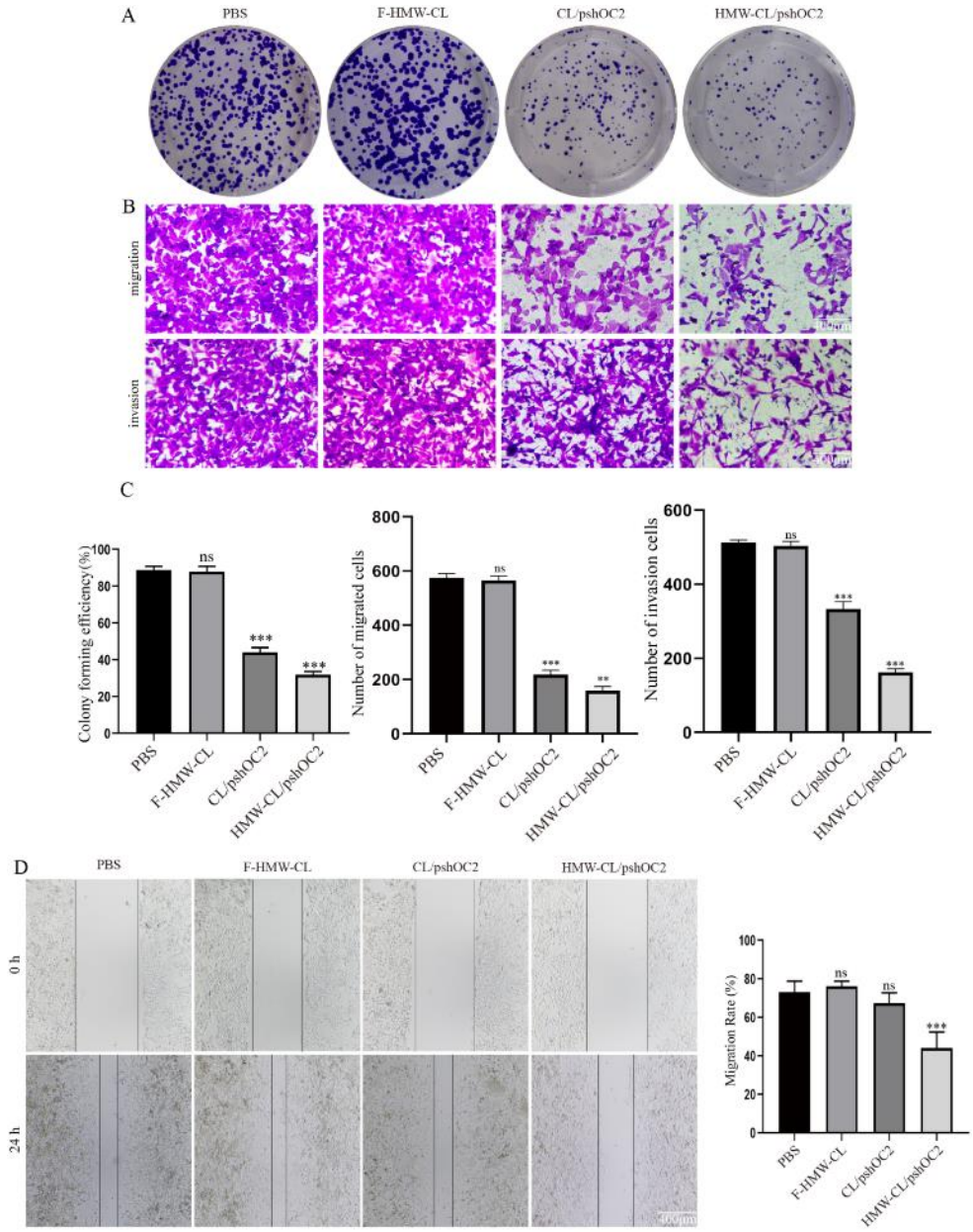

Figure 5. HMW-CL lipoplexes deliver pshOC2 inhibit HCC growth and metastasis, and promote HCC apoptosis: **(A)** The proliferation of SK-Hep1 was assessed by colony formation; **(B)** Migration and invasion of SK-Hep1 treated with PBS, F-HMW-CL, CL/pshOC2, and HMW-CL/pshOC2; **(C)** The quantification plots of proliferation, migration and invasion in SK-Hep1; **(D)** The wound healing of SK-Hep1 treated with PBS, F-HMW-CL, CL/pshOC2, and HMW-CL/pshOC2. ** $P < 0.01$, *** $P < 0.001$.

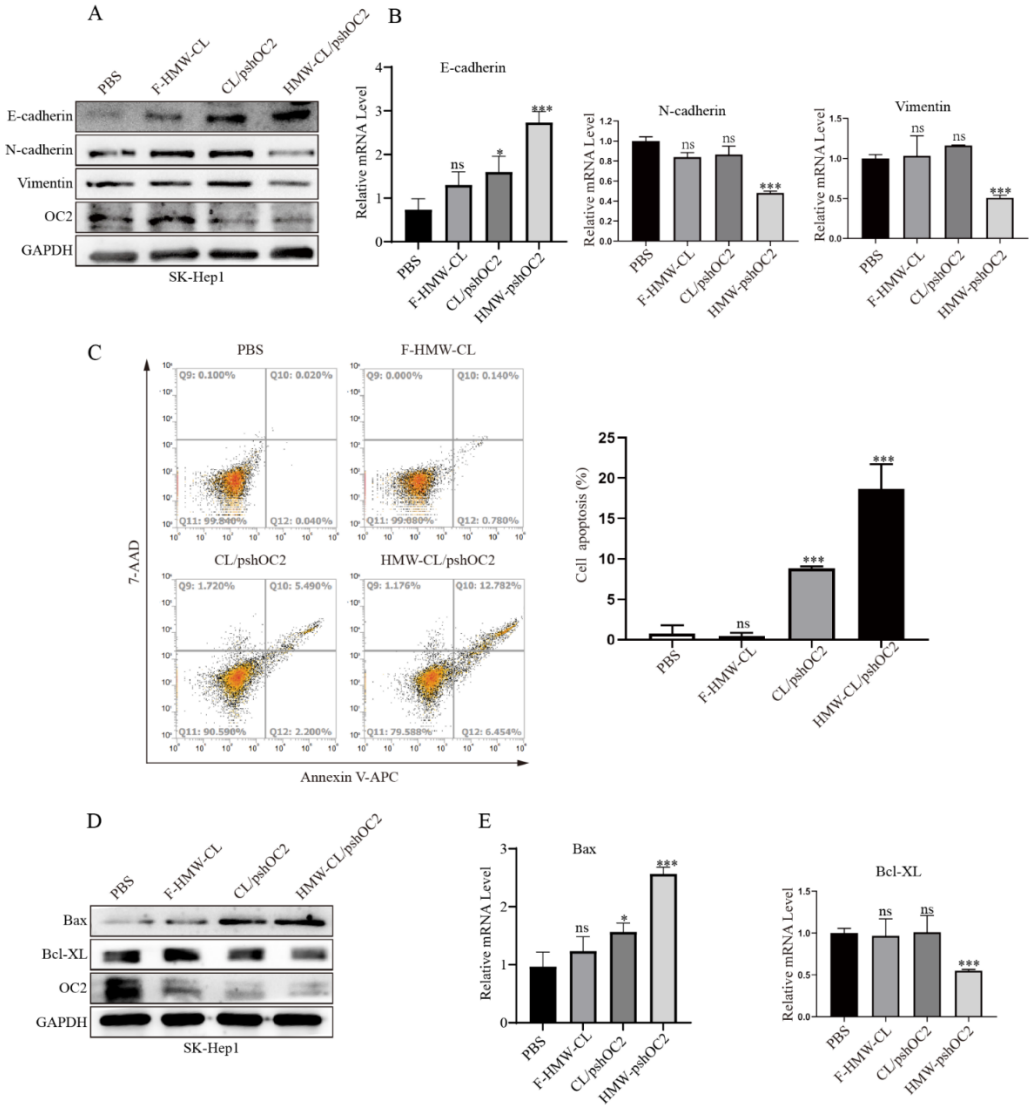

Figure 6. HMW-CL lipoplexes deliver pshOC2 inhibit HCC growth and metastasis, and promote HCC apoptosis: **(A,B)** The expression of EMT related proteins and mRNA was assessed by Western blotting and RT-qPCR; **(C)** Apoptosis results from the flow cytometry assay in SK-Hep1 cells; **(D,E)** The expression of apoptosis related factors was assessed by Western blotting and RT-qPCR. * $P < 0.05$, *** $P < 0.001$.

3.6. HMW-CL lipoplexes deliver pshOC2 to suppress the tumor growth of HCC in xenograft mouse model

In the HCC xenograft mouse model, SK-Hep1 cells were injected into the shoulder of mice, and HMW-CL/pshOC2 lipoplexes were injected through the vein. The results showed that the injection of CL/pshOC2 and HMW-CL/pshOC2 lipoplexes significantly inhibited the growth of tumors, especially in HMW-CL/pshOC2 group with an inhibition of 81.77% (**Figure 7A,C**). The results of immunohistochemistry (IHC) revealed that the expression of OC2, Ki67, and Vimentin was significantly lower in HMW-CL/pshOC2 group than in CL/pshOC2 group. The results of the TUNEL assay demonstrated that there were more apoptotic cells of HMW-CL/pshOC2 than control (**Figure 7E**), which indicated that the HMW-CL/pshOC2 lipoplexes enabled to suffering from the monitoring of blood circulation and organs, accumulated at the tumor site due to HMW-HA/CD44 binding, delivered

pshOC2 into SK-Hep1 cells, and finally, downregulated of OC2, inhibited proliferation, metastasis, and promoted apoptosis in HCC cells. The results of serum biochemical index analysis showed that no significant organ damage or lesions were observed in the HMW-CL/pshOC2-treated group compared with the control group (**Figure 8**). This further confirmed the high safety of HMW-CL/pshOC2 application in vivo.

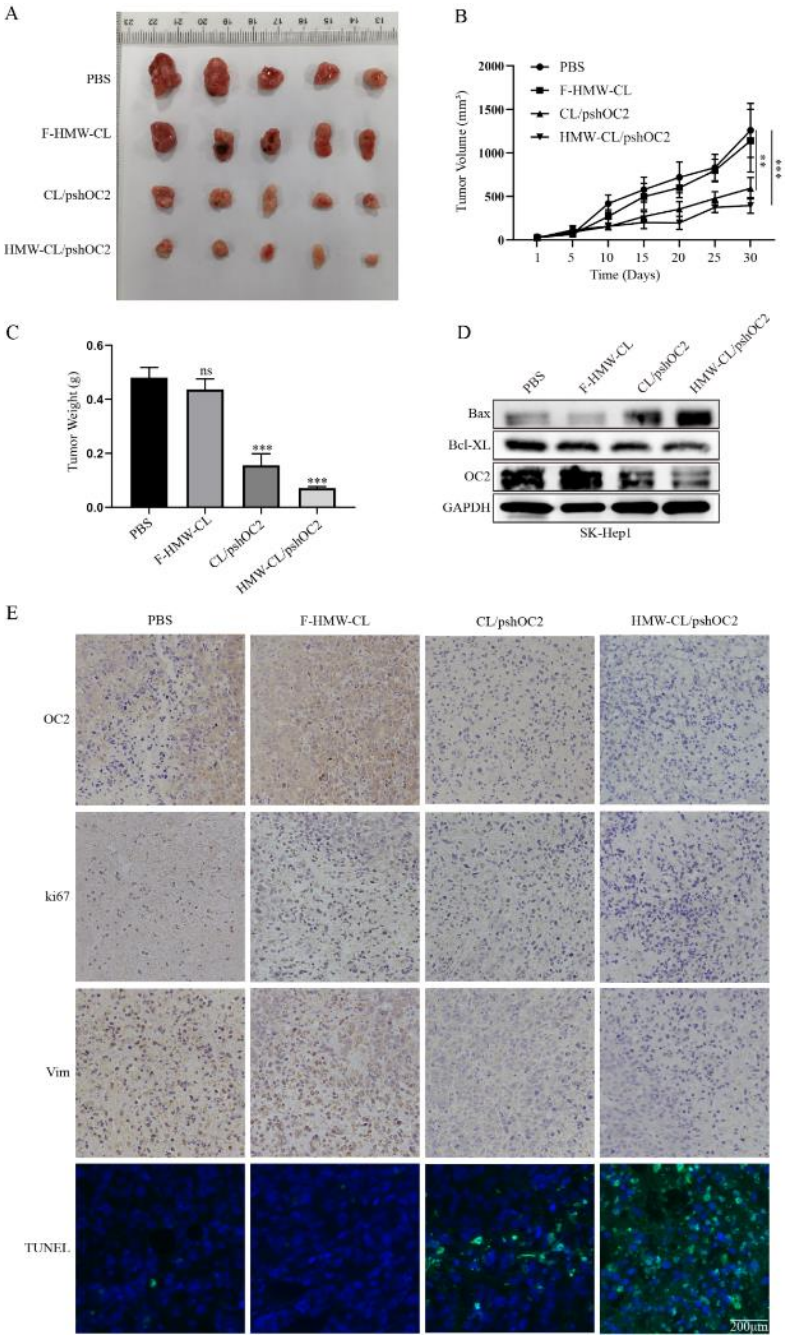

Figure 7. HMW-CL lipoplexes deliver pshOC2 to suppress the tumor growth of HCC in xenograft mouse model: **(A)** Tumor tissues were removed from the mice treated with PBS, F-HMW-CL, CL/pshOC2, and HMW-CL/pshOC2; **(B,C)** Tumor growth curve and tumor weights; **(D)** The expression of apoptosis related factors in tumor tissue was analyzed by Western blotting; **(E)** Immunohistochemistry and TUNEL for the detection of tumor growth and apoptosis. ** $P < 0.01$, *** $P < 0.001$.

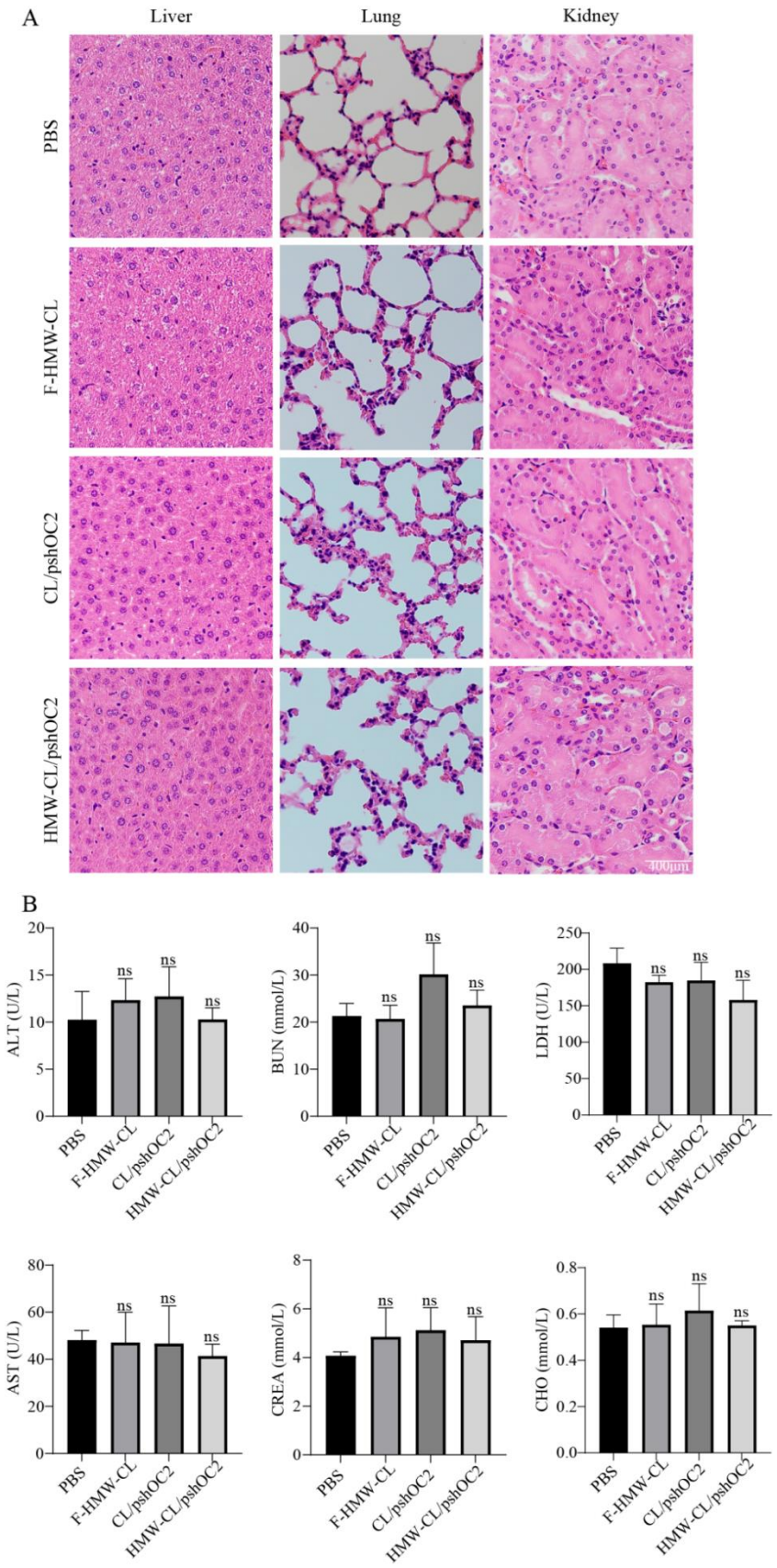

Figure 8. HMW-CL lipoplexes deliver pshOC2 to suppress the tumor growth of HCC in xenograft mouse model: **(A)** H&E staining of mouse organs treated with PBS, F-HMW-CL, CL/pshOC2, and HMW-CL/pshOC2; **(B)** The test of serum biochemical indicator.

4. Discussion

HCC is a prevalent disease globally. Currently, the main treatment modalities for liver cancer include liver transplantation, surgical resection, and radiotherapy. With the advancement of cancer drug development, immunotherapy and targeted drug therapy have been extensively applied in liver cancer treatment. Nevertheless, targeted drug therapy often encounters challenges such as drug resistance, target heterogeneity, and toxicity.

The exploration of suitable therapeutic genes is a prerequisite for effective targeted therapy in HCC patients. The studies have achieved consensus on the key roles of the transcription factor OC2 regulating downstream gene networks during the differentiation and metabolism process of liver. However, OC2 is also recognized as an oncogene due to its aberrant expression in several types of cancers [4,5,7]. In our previous study, we demonstrated that OC2 was abnormally overexpressed in HCC. In addition, Zhang et al. reported that the elevated OC2 was correlated with the poor prognosis of HCC patients after surgery [8]. Danfei et al. also revealed that the elevated OC2 promoted the tumor metastasis of HCC by upregulating FGF2 and ACLY [25]. ACLY is an ATP citrate lyase and participates in the generation of acetyl-CoA and oxaloacetate for the TCA cycle, whose activation requires AKT phosphorylation [26]. All these findings imply that OC2 may be a potential candidate for targeted therapy of HCC.

In this study, we found that hyaluronic acid-modified liposomes could better protect the shOC2 plasmid and efficiently deliver it into HCC cells to knockdown the expression of OC2. Furthermore, knocking down the expression of OC2 could inhibit the proliferation, migration, and invasion of HCC cells and promote apoptosis. Mechanistically, Western blot and RT-qPCR analyses showed that knockdown with OC2 downregulated the expression of N-cadherin and Vimentin and upregulated the expression of E-cadherin in the epithelial-mesenchymal transition (EMT) signaling pathway. It also influenced the expression of apoptosis-related marker factors, upregulating the expression of Bax and downregulating the expression of Bcl-XL. These results further suggest that OC2 can serve as a potential target for HCC treatment.

New therapeutic options are urgently needed for HCC patients. The utilization of natural compounds and nanotechnology can provide better outcomes for patients while reducing systemic toxicity and side effects. We employed high-molecular-weight hyaluronic acid as a ligand for CD44 to enhance the targeting ability to SK-Hep1 cells. In our study, we demonstrated that hyaluronic acid modification could reduce cellular toxicity, and the transfection efficiency of high-molecular-weight hyaluronic acid was significantly higher than that of low-molecular-weight hyaluronic acid. It was also observed that when the concentration of high-molecular-weight hyaluronic acid exceeded 10%, the cellular toxicity remained essentially unchanged. Considering economic benefits, we used a 10% concentration of hyaluronic acid to modify liposomes. The results showed that the 10% hyaluronic acid-modified liposomes exhibited low toxicity both in vitro and in vivo and could be effectively transfected into cells within a short period. Most importantly, the hyaluronic acid-modified liposomes we designed were able to deliver shOC2 more effectively to inhibit the

proliferation, migration and invasion of hepatocellular carcinoma cells, as well as the growth of tumors in mice and promote apoptosis. These results suggest that high molecular weight hyaluronic acid-modified liposomes are an effective delivery method for shOC2 into HCC cells.

5. Conclusion

In this study, we found that downregulation of OC2 could inhibit the proliferation, migration, and promote apoptosis of SK-Hep1 tumor cells in vitro and in vivo, indicating that OC2 may be a potential therapeutic target for liver cancer. Due to the complexity and heterogeneity of tumors, the current progress of nanocarrier-based drug delivery in liver cancer is not smooth. The HMW-HA modified liposome prepared in this study has tumor targeting and biological safety and can be used as a delivery vector for gene drugs in vivo.

In summary, pshOC2 could reduce the expression of OC2 and induce inhibitory effects on tumor growth and metastasis of HCC, so OC2 had the potential to be the candidate gene for HCC target therapy. The HMW-CL/pshOC2 lipoplexes we prepared in this study showed the advantages in suitable particle size, good stability, high transfection effects, and low cytotoxicity, which might be employed as a nanocarrier to deliver nucleic acid drugs for HCC treatment.

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Ethical approval: The study was conducted in accordance with the Declaration of Helsinki. The animal study protocol was approved by the Laboratory Animal Welfare and Ethics Committee of Jinan University (protocol code 100943 and approved on 26 October 2022).

Data availability: All data generated and analyzed during this work are included in this paper and available from the corresponding author for request.

Conflict of interest: The authors declare no conflict of interest.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. Ca Cancer J Clin; 2021.
2. Chen Z, Xie H, Hu M, et al. Recent progress in treatment of hepatocellular carcinoma. Am J Cancer Res. 2020.
3. Kreplova M, Kuzelova A, Antosova B, et al. Dose-dependent regulation of horizontal cell fate by Onecut family of transcription factors. PLoS One. 2020;15(8): e0237403. doi:10.1371/journal.pone.0237403
4. Yu J, Li D, Jiang H. Emerging role of ONECUT2 in tumors. Oncol Lett; 2020.
5. Steadman K, You S, Srinivas DV, et al. Autonomous action and cooperativity between the ONECUT2 transcription factor

and its 3' untranslated region. Front Cell Dev Biol. 2023; 11:1206259. doi:10.3389/fcell.2023.1206259
6. Li C, Xiao Y, Zhou J, et al. Knockout of onecut2 inhibits proliferation and promotes apoptosis of tumor cells through SKP2-mediated p53 acetylation in hepatocellular carcinoma. Cell. Mol. Life Sci. 2024. doi: 10.1007/s00018-024-05518-3
7. Margagliotti S, Clotman F, Pierreux CE, et al. The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. Developmental Biology; 2007.
8. Zhang J, Cheng J, Zeng Z, et al, Comprehensive profiling of novel microRNA-9 targets and a tumor suppressor role of microRNA-9 via targeting IGF2BP1 in hepatocellular carcinoma. Oncotarget; 2015.
9. Leyten GH, Hessels D, Smit FP, et al. Identification of a Candidate Gene Panel for the Early Diagnosis of Prostate Cancer. Clin Cancer Res; 2015.
10. Guo H, Ci X, Ahmed M, et al. ONECUT2 is a driver of neuroendocrine prostate cancer. Nature Communications; 2019.
11. Ranković B, Žlajpah M, et al. Epithelial-Mesenchymal Transition-Related MicroRNAs and Their Target Genes in Colorectal Cancerogenesis. Journal of Clinical Medicine. 2019.
12. Tongyi L, Yunfei Y, et al. Blockade of ONECUT2 expression in ovarian cancer inhibited tumor cell proliferation, migration, invasion and angiogenesis. Cancer Science; 2018.
13. Zhang L, Li C, Song X, et al. Targeting ONECUT2 inhibits tumor angiogenesis via down-regulating ZKSCAN3/VEGFA. Biochem Pharmacol; 2024.
14. Giulio C, Amenitsch H. Cationic liposome DNA complexes from structure to interactions with cellular membranes. European Biophysics Journa. 2012.
15. Mamta K, Diane JB, Siddhesh DP. Physicochemical characterization techniques for lipid based delivery systems for siRNA. International Journal of Pharmaceutics. 2012.
16. Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. Proc. Natl. Acad. Sci. USA; 1996.
17. Shim G, Mi-Gyeong K, Park JY, et al. Application of cationic liposomes for delivery of nucleic acids. Asian Journal of Pharmaceutical Sciences; 2013.
18. Anne S, Céline L, Pascal B, et al. Anionic polymers for decreased toxicity and enhanced in vivo delivery of siRNA complexed with cationic liposomes. Journal of Controlled Release. 2011.
19. Franco D, Barbara S, et al. Hyaluronic acid for anticancer drug and nucleic acid delivery. Advanced Drug Delivery Reviews; 2016.
20. Noble PW. Hyaluronan and its catabolic products in tissue injury and repair. Matrix Biology; 2002.
21. Deed R, Kumar P, et al. Early-response gene signalling is induced by angiogenic oligosaccharides of hyaluronan in endothelial cells. Inhibition by non-angiogenic, high-molecular-weight hyaluronan. International Journal of Cancer; 1997.
22. Kim J, Badawi M, et al. miR-221 regulates CD44 in hepatocellular carcinoma through the PI3K-AKT-mTOR pathway. Biochem Biophys Res Commun; 2017.
23. Wu SY, Liang M, et al. Development of a novel method for formulating stable siRNA-loaded lipid particles for in vivo use. Pharm Res; 2009.
24. Lesley J, Hascall V, Tammi M, et al, Hyaluronan Binding by Cell Surface CD44. J Biol Chem. 2000.
25. Danfei L, Zhang T, Xiaoping C, et al. ONECUT2 facilitates hepatocellular carcinoma metastasis by transcriptionally upregulating FGF2 and ACLY. Cell Death and Disease; 2021.
26. Migita T, Narita T, Nomura K, et al. ATP citrate lyase: activation and therapeutic implications in non–small cell lung cancer. Cancer Research; 2008.