

MR molecular imaging of HCC employing a regulated ferritin gene carried by a modified polycation vector

This article was published in the following Dove Press journal:
International Journal of Nanomedicine

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Purpose: Early diagnosis is essential for reducing liver cancer mortality, and molecular diagnosis by magnetic resonance imaging (MRI) is an emerging and promising technology. The chief aim of the present work is to use the ferritin gene, modified by the alpha-fetoprotein (AFP) promoter, carried by a highly safe vector, to produce signal contrast on T2-weighted MR imaging as an endogenous contrast agent, and to provide a highly specific target for subsequent therapy.

Methods: Polyethyleneimine- β -cyclodextrin (PEI- β -CD, PC) was synthesized as a novel vector. The optimal nitrogen/phosphorus ratio (N/P) of the PC/plasmid DNA complex was determined by gel retardation, biophysical properties and transmission electron microscopy morphological analysis. The transfection efficiency was observed under a fluorescence microscope and analyzed by flow cytometry. Cellular iron accumulation caused by ferritin overexpression was verified by Prussian blue staining, and the resulting contrast imaging effect was examined by MRI.

Results: The modified cationic polymer PC was much safer than high molecular weight PEI, and could condense plasmid DNA at an N/P ratio of 50 with suitable biophysical properties and a high transfection efficiency. Overexpression of ferritin enriched intracellular iron. The short-term iron imbalance initiated by AFP promoter regulation only occurred in hepatoma cells, resulting in signal contrast on MRI. The specific target TfR was also upregulated during this process.

Conclusion: These results illustrate that the regulated ferritin gene carried by PC can be used as an endogenous contrast agent for MRI detection of hepatocellular carcinoma (HCC). This molecular imaging technique may promote safer early diagnosis of HCC, and provide a more highly specific target for future chemotherapy drugs.

Keywords: molecular imaging, magnetic resonance imaging, ferritin, AFP promoter, hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is one of leading causes of death globally and is characterized by a low 5-year survival rate without improvement in recent years according to meta data.¹ This dilemma may be attributed to the absence of secondary prevention, that is, the early diagnosis of HCC. Magnetic resonance imaging (MRI) contrast agents have been widely used in the clinic to enable clearer observation of the lesions. Molecular imaging is the most promising method and is greatly significant for early diagnosis. Thus, this approach is expected to achieve early intervention for HCC and reduce its mortality.

Currently, molecular targeted contrast agents are well-studied and are almost always exogenous contrast agents, such as the gadolinium-based contrast agent, which is mainly used in T1-weighted MR imaging, and superparamagnetic iron oxide (SPIO), which is mainly used in T2-weighted MR imaging. Despite the encouraging results of their imaging and targeting capabilities, exogenous contrast agents still suffer from some drawbacks. Such as accumulation in bones and the human brain due to repeated administration which leads to potential harm especially for the latter.² In addition, being diluted in cell division or cleared in dead cells by immune cells can lead to the loss of signal or the presence of false positives on MRI.^{3,4} To remedy the shortcomings associated with exogenous contrast agents, efforts have been made in the field of molecular imaging to develop MRI reporter genes,^{5–9} especially endogenous contrast agents.

The ferritin heavy (Fth) chain and transferrin receptor (TfR) are regulators of cellular iron homeostasis in vertebrates.¹⁰ They are ubiquitously expressed inside cells and on cell membranes, respectively. Overexpression of Fth drives upregulation of TfR expression, which increases intracellular iron loading and results in a change in the signal intensity on T2-weighted MR imaging. To date, the ferritin gene has been used as a core component of endogenous contrast agents in several cell lines, particularly mesenchymal stem cells, to monitor their differentiation by MRI.^{5,6,11,12} Nonetheless, the performance of Fth in HCC diagnosis is not very well understood. In addition, TfR is overexpressed within cancer cells compared to normal cells for the purpose of obtaining iron for their rapid division and proliferation.^{13,14} On this basis, TfR of the transfected hepatoma cell is further upregulated driven by the above endogenous contrast agents. These two mechanisms work synergistically to provide a highly specific target on hepatoma cells for subsequent targeted therapy that may be performed.

Widely used targeting polypeptides play a limited role in tumor-specific diagnosis and treatment, mainly due to their targeting effect is for a certain cell line, but not a type of disease. Comparatively, alpha-fetoprotein (AFP) is expressed in at least 80% of HCC cases,¹⁵ and has been used as a serum marker for the diagnosis of HCC.¹⁶ Specific promoters can regulate targeted gene expression. Therefore, we plan to use AFP as a promoter to regulate expression of an MRI reporter gene, and allow it to function only in AFP-positive hepatoma cells.

A suitable vector plays a key role in the transfection and release processes. More importantly, early diagnostic

imaging requires the highest safety standards, since this approach will potentially be applied to healthy people. Viral vectors are under strict limitations in terms of clinical application due to their inherent immunogenicity and unpredictable mutation risk.¹⁷ Similarly, commonly used cationic liposomes and high molecular weight (HMW) cationic polymers are also plagued by toxicity. Therefore, a security vector with a satisfactory transfection efficiency is highly desired for application of MR reporter genes both in vitro and in vivo. In this study, we plan to crosslink activated beta Cyclodextrin (β -CD) with low molecular weight (LMW) polyethyleneimine (PEI) (600 Da) to synthesize PEI- β -CD (PC) as a secured vector. The addition of β -CD can enhance solubility and stability of the vector, maintain the cation density of LMW PEI to ensure the transfection efficiency, and synergize with PEI to through the cell membrane via its adhesion.^{18,19}

The purpose of this study is to use PC as a safe gene delivery vector, carrying a ferritin gene regulated by the AFP promoter, to transfect hepatoma cells for MR contrast imaging. No reports to date have investigated endogenous contrast agents for HCC carried by modified LMW PEI. Hence, our study may improve the safety of early MRI diagnosis at a molecular level and provide a highly specific target for subsequent therapy to reduce HCC mortality.

Materials and methods

PC synthesis

β -CD (2.1 g, 1.9 mmol) was dissolved in 10 ml of DMSO in a 100 ml round-bottom flask with 0.2 ml of triethylamine added as a catalyst. Carbonyl diimidazole (CDI) (2.4 g, 14.8 mmol) was dissolved in 10 ml of DMSO. The latter was added to the former solution, followed by continuous stirring with nitrogen in the dark for 3 h. PEI 600 Da (6.7 g, 11.2 mmol) was dissolved in 10 ml of DMSO and added dropwise to the above reaction system with nitrogen in the dark for 5 h. The resulting mixture was dialyzed with a dialysis tube (MW 8000–14,000) in running water for 48 h and then lyophilized for 3 days. The final PC product was collected as a white flocculent solid.

¹H NMR measurement of PC

The composition and structure of PC were characterized by ¹H NMR. First, 5 mg of LMW PEI and PC were dissolved in 0.7 ml of deuterium oxide in NMR tubes,

and 5 mg of β -CD was dissolved in 0.7 ml of dimethyl sulfoxide-d in an NMR tube. The ^1H NMR spectra were recorded using a 400-MHz spectrometer (inova 400, Varian, USA) at 25 °C.

Cell viability assay

The BEL-7402 cell line (human hepatocellular carcinoma cells, AFP positive, purchased from ATCC, USA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) in a 37 °C incubator with 5 % CO_2 . Cells were seeded into 96-well plates (Costar, Corning Corp, USA) at a density of 1×10^4 cells per well and incubated for 24 h. After rinsing with PBS, the culture medium was replaced with 200 μl of fresh media containing serial dilutions of PC, and the cells were cultured for 48 h. Then, 20 μl of a 5 mg/ml MTT stock solution was added to each well. Four hours later, unreacted dyes were removed by aspiration. A total of 150 μl of DMSO was added each well to dissolve the formazan crystals. After 10 min of low speed oscillation, the OD value was measured by a microplate reader (model 680, Bio-Rad, USA) at a wave length of 570 nm. Wells that contained only medium and cells without intervention were set as the blank and control groups, respectively.

Preparation of PC/plasmid DNA complexes

The 4.7-kb plasmid encoding green fluorescence protein (pGFP) and 4.1-kb plasmid encoding the Fth chain (pFth) were purchased from GeneChem Corporation (Shanghai, China). AFP-Fth was synthesized according to the sequence in GenBank (NM_002032), and amplified by polymerase chain reaction (PCR) using the forward primer 5'-CCATTAATAAGCTTGGTACCTGAGGAGAATATTTGTTATATTG-3' and reverse primer 5'-GCGACCGGTGAATTCGGTACGCTTTCATTATCACTGTCTCCCAG-3', which contained a KpnI restriction site. After restriction digestion with KpnI, the gene fragment was successfully inserted into the linearized vector (GV349, purchased from GeneChem Corporation, China). The recombinant vector was transformed, identified by PCR, sequenced and analyzed; then, a high-purity plasmid was obtained for the subsequent experiments. CMV-GFP was prepared with the same method.

The plasmid DNA (pDNA) stock solution was diluted to an appropriate concentration (0.2 $\mu\text{g}/\mu\text{l}$) with Tris-EDTA buffer. The PC polymer was diluted to a chosen

concentration (1.0 $\mu\text{g}/\mu\text{l}$) with pure water, which was added dropwise to the pDNA solution. The mixture was vortexed for 30 s and incubated for 30 min at room temperature. Depending on the added volume, complexes with different nitrogen/phosphorus (N/P) ratios are formed.

Gel retardation analysis and biophysical properties of PC/plasmid DNA complexes

Complexes with different N/P ratios prepared as described above were loaded onto a 1% agarose gel containing the GoldView (Sbsbio, China) nucleic acid dye. Gel electrophoresis was run at room temperature in TAE buffer at 80 V for 50 min. DNA bands were visualized by a UV (302 nm) illuminator (Alpha Imager HP, USA) to show the ability of the complexes to inhibit pDNA migration.

PC/pDNA complexes prepared at different N/P ratios were diluted to 1 ml (the concentrations of the complexes were roughly between 300–500 $\mu\text{g}/\text{ml}$). Then, the complexes were measured with the 90 Plus/BI-MAS (Brookhaven Instruments Corporation, USA) at room temperature to determine the diameters and zeta potential. Each sample was run for 200 s 4 times and analyzed in the unimodal analysis mode.

Transmission electron microscopy

The morphologies of the PC/pDNA complexes at N/P ratios of 30 and 50 were observed by transmission electron microscopy (TEM) (Tecnai, FEI, USA). The preparation process for each sample was as follows. A 10- μl complex solution was deposited onto a 200-mesh copper grid coated with carbon, and excess solution was carefully removed with filter paper. The grid was allowed to air dry at room temperature before the measurement.

In vitro gene transfection efficiency

The GFP plasmid was utilized to ascertain the efficiency of gene complex delivery using inverted fluorescence microscopy and flow cytometry.

For the in vitro transfection assay using pGFP, cells were seeded into a 24-well plate at a density of 5×10^4 cells per well in 500 μl of culture medium 24 h prior to transfection. Before the transfection procedures, HMW PEI/pDNA and PC/pDNA at N/P ratios of 10, 30 and 50 were prepared as previously described, followed by their addition and mixing with Opti-MEM medium (without serum and antibiotics) (Gibco, USA). The medium in

each well was replaced with 300 μ l of the complex-containing Opti-MEM media (1.5 μ g of pDNA per well). After incubating for 6 h, the media were replaced with 500 μ l of fresh culture medium, and the cells were returned to the incubator for 42 h. Finally, the cells were washed with PBS, and fluorescence images were obtained using a fluorescence microscope (Leica, Germany).

The cells were cultured in a 6-well plate at a density of 2×10^5 cells per well in 2 ml of culture medium for 24 h. The pDNA at a concentration of 3 μ g per well was used for transfection; the remaining transfection steps were the same as those described above. After a total transfection time of 48 h, the cells were washed twice with PBS, separated by trypsin digestion, and then collected as a cell suspension. The cells were centrifuged and resuspended in 500 μ l of PBS. The proportion of GFP-expressing cells was determined by flow cytometry (BD LSR, USA). Un-transfected cells were used to set the background.

Western blotting assay for Ferritin expression

At 48 h posttransfection, cells in a 6-well plate were lysed in 80 μ l of RIPA buffer with protease inhibitor (Sigma, USA) per well on ice for 30 min. The protein concentration was evaluated using the bicinchoninic acid assay after centrifuging and collecting the cells in EP tubes. A total of 20 μ g of protein samples with loading buffer was loaded into 12% gels for SDS-PAGE. Bands with molecular weights ranging between 15–21 kD (Ferritin) and 40–55 kD (β -actin) were transferred onto PVDF membranes for blotting. The membranes were blocked in 5% skim milk powder solution for 4 h and then incubated overnight at 4 °C with a rabbit anti-ferritin or mouse anti- β -actin monoclonal antibody (1:2000, Abcam, UK). After thoroughly washing with TBST buffer, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1: 5000, Boster, USA) for 1 h. The proteins of interest were visualized by ChemiDoc (Bio-Rad, USA) using enhanced chemiluminescent.

Immunofluorescence assay for TfR expression

Cells were cultured in a 24-well plate containing cover slips for one day before transfection. At 48 h posttransfection, the cells were washed with PBS and then fixed in 4%

paraformaldehyde (PFA) for 10 min, followed by blocking with 5% normal goat serum at 37 °C for 1 h. To examine TfR expression, the cells were incubated overnight at 4 °C with a rabbit anti-TfR monoclonal antibody (1:100, Abcam, UK) and then incubated with a DyLight 488-conjugated goat anti-rabbit secondary antibody (1:200, Boster, USA). The cell nuclei were counterstained with DAPI (1:1000, Beyotime, China). Then the cover slips were removed from the plate, fixed on slides, and examined under a laser confocal microscope (Zeiss, Germany).

Intracellular iron staining

Prussian blue staining was used to verify the effect of transfection with the PC carrying pFth on cellular iron accumulation. At 24 h posttransfection, ferric ammonium citrate (FAC, 0.2 mM, Sigma, USA) was added to the culture medium. For Prussian blue staining, the cells were washed thoroughly with PBS thrice to remove free iron, and fixed in 4% PFA for 10 min. The cells were incubated with the stain and the counterstain (Sbsbio, China) following the manufacturer's protocol, and each sample was observed under an inverted microscope (Leica, Germany).

MRI

In vitro MRI was further used to examine the effect of Fth gene transfection. At 24 h posttransfection, FAC was added to the culture medium, and the cells were incubated for 24 h. After thorough washing with PBS thrice to remove free iron, the cells (1.2×10^6) were dissociated with trypsin and fixed in 4% PFA for 10 min, followed by suspension in 10 μ l of PBS in EP tubes. Then, 30 μ l of a 1% unsolidified agarose solution was added to each tube. The cell-containing gel solution was instantly transferred into long thin glass tubes filled with agarose gel around the cell layer (thicker than the scan slice thickness) to reduce interference with the MR imaging. T2-/T2*-weighted MR images and the signal intensity value were acquired using T2 fast spin echo (FSE) and ESWAN sequences with the following parameters by a 3.0-T MR (GE Discovery MR750, USA), respectively. FSE sequence: repetition time (TR)=2480 ms, echo time (TE)=95.88 ms, flip angle (FA)=90°, and slice thickness=2.0 mm; ESWAN sequence: TR=55 ms, TE=6.02–29.59 ms (8 echoes), FA=20°, and slice thickness=2.0 mm.

Statistical analysis

All the experiments were run at least thrice. The results are presented as the mean $\pm$ standard deviation (SD) of three

measurements. Statistical calculations were computed with SPSS 10.0. Significance tests among groups were performed using the Dunnett and Bonferroni methods, and *P*-values less than 0.05 were considered statistically significant.

Results

Synthesis and ^1H NMR spectroscopy of PC

The non-viral polycation gene vector PC polymer was synthesized according to the scheme described in the Methods section (Figure 1A). β -CD was activated by CDI, followed by crosslinking with LMW PEI (600 Da) to form a polymer through the polycondensation reaction. The structure was confirmed by ^1H NMR spectroscopy (Figure 1B). The spectra of PC showed the typical proton signals attributed to the glucose units of β -CD, and a signal corresponding

to the methylene protons of PEI appeared at δ 2.5–2.8 ppm, indicating that LMW PEI was integrated into β -CD. The integral values of these characteristic proton peaks showed successful conjugation, with 1:1 molar ratio of PEI and β -CD.

In vitro cytotoxicity of PC

The in vitro cytotoxicity of PC was analyzed compared with that of HMW PEI using the MTT assay (Figure 1C). The viability of the PC group was practically 100% at the 10 $\mu\text{g/ml}$ concentration, which was significantly better than that of the HMW PEI group. Notably, at a high concentration, such as 100 $\mu\text{g/ml}$, the PC and PEI 600 Da groups suffered negligible cytotoxicity with a cell survival rate of approximately 95%. In contrast, the HMW PEI groups dropped to 30%, which showed strong dose-dependent cytotoxicity.

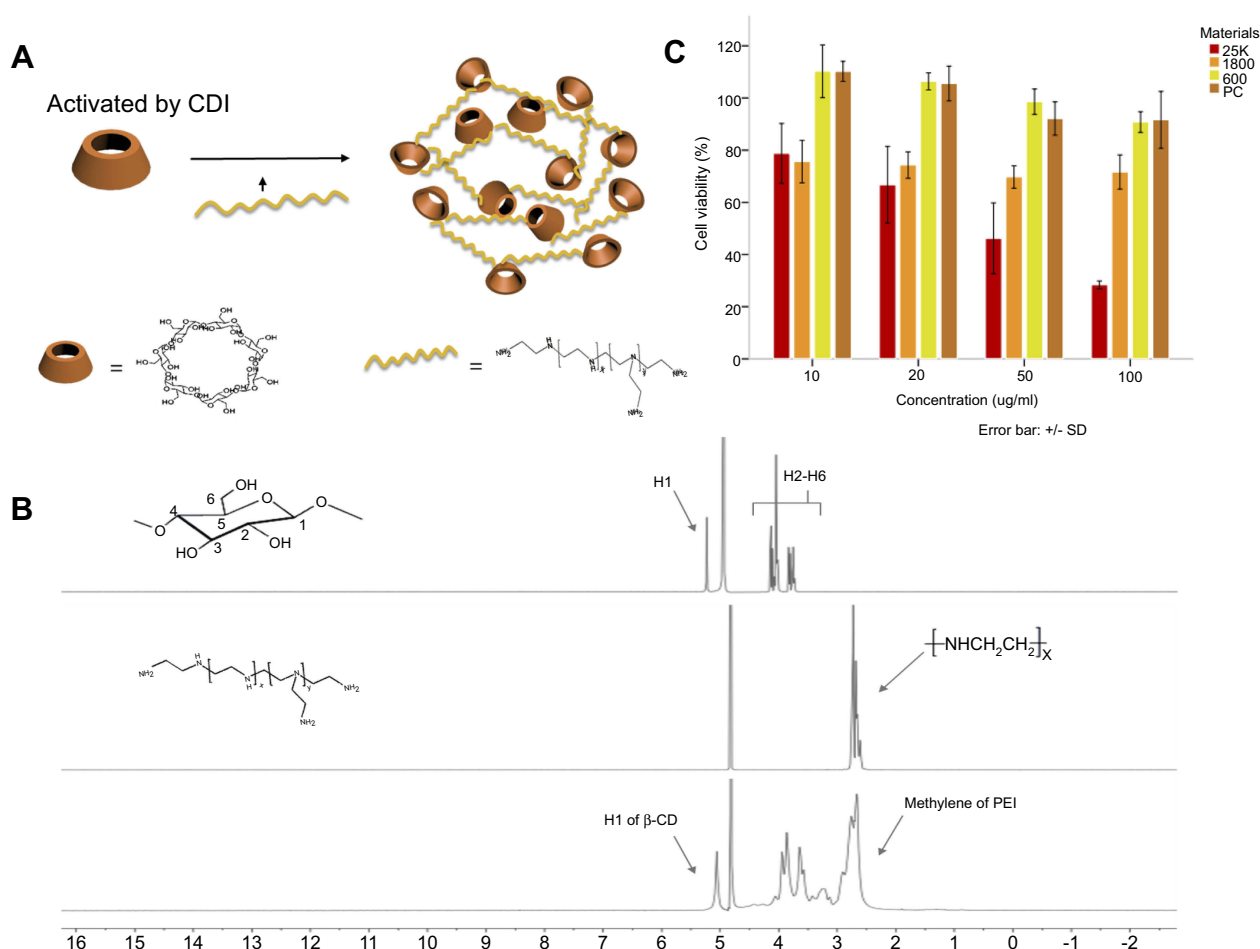


Figure 1 Synthesis, verification and cytotoxicity experiments for PEI- β -CD. **(A)** Synthetic schematic diagram of PC. **(B)** ^1H NMR spectra of β -CD, PEI (600 Da) and PC. The special peaks are marked with arrows. **(C)** Cytotoxicity of PEI with different molecular weights and PC at various concentrations. Data are expressed as the mean $\pm$ SD ($n=3$). **Abbreviations:** PC, polyethyleneimine- β -cyclodextrin; CDI, Carbonyl diimidazole.

Plasmid preparation

The 4.1-kb AFP-Fth plasmid encoding an AFP promoter and Fth chain was synthesized by GeneChem (Shanghai, China) according to the gene sequence in GenBank. The 4.7-kb CMV-GFP plasmid encoding a CMV promoter and GFP was synthesized by the same company. Unlike the AFP promoter which has cell specificity, the CMV promoter is generally applicable to eukaryotic cells.

Preparation and characterization of PC/pDNA complexes

The ability of PC to condense pDNA into nanoparticles was evaluated by agarose gel electrophoresis, the particle size, and the zeta potential assay. The electrophoretogram suggested that uninhibited free DNA would be reduced with the increasing polymer concentration, and that the cationic polymer inhibited DNA migration completely at N/P ratios of 4 and above (Figure 2A). As indicated in Table 1, the particle size of PC/pDNA decreased progressively as the N/P ratio increased from 4 to 30; at 30, the particle size was minimized within the range of the samples we set. At N/P ratios of 10 and above, the mean zeta potential remained stable within the range of 15 to 25 mV.

To further evaluate the particle size and morphology, we examined TEM images, which showed that PC condensed pDNA to form a compact near-spherical nanoparticle with a size between 100 and 200 nm at N/P ratios of 30 and 50 (Figure 2B).

In vitro gene transfection of PC/pDNA complexes

To explore the transfection efficiency of the PC/pDNA complexes, BEL-7402 (a human hepatocellular carcinoma AFP-positive cell line) and HEK-293 (a human embryonic kidney cell line) were transfected with the GFP plasmid. HMW PEI/pDNA was set as the positive control. As shown by the green fluorescence in Figure 3A, HMW PEI showed optimal gene transfection at an N/P ratio of 10 as it has been known. However, PC was optimal at an N/P ratio of 50, at which point the transfection efficiency basically reached the same level as that of HMW PEI. Strong GFP expression was detected in HEK-293 cells transfected with both vectors, which was consistent with previous research showing that the HEK-293 cell line was a commonly used tool that was more sensitive to transfection than BEL-7402 cell line.

To obtain the quantitative transfection efficiency value, flow cytometry was further used to confirm the percentage of green fluorescence-positive cells. The ratio of positive cells transfected with PC was slightly lower than that of the cells transfected with HMW PEI, as shown in Figure 3B.

Western blotting for Ferritin expression

Western blotting was used to assess whether the transfection was reflected in protein expression. The plasmid used for transfection as an MR reporter gene was the Fth chain with the AFP promoter. After transfecting the cells with HMW PEI/pDNA and PC/pDNA, the ferritin expression level showed no significant differences between these two gene delivery vectors (Figure 4A and B, $P>0.05$). However, the expression level was much higher in the BEL-7402 cells than in the HEK-293 cells because of the function of the AFP promoter.

Specific upregulation of TfR

An immunofluorescence assay was performed to confirm the different TfR expression levels within the transfected and un-transfected cells. The green fluorescence images showed that TfR was specifically upregulated in the transfected hepatoma cells, and no significant differences were observed between the two gene vectors (Figure 4C). Conversely, TfR was expressed at a low level in the un-transfected BEL-7402 cells. In the HEK-293 cells, TfR, as labelled with green fluorescence, was barely detected.

Iron accumulation

Prussian blue staining was performed to verify the ability of Fth expression to augment iron storage in transfected cells. The iron staining showed obvious blue-stained particles of accumulated iron within the transfected BEL-7402 cells treated with FAC, and no significant differences were observed between PC and the positive control (Figure 4D). In contrast, few blue granules were detected within the transfected HEK-293 cells, which are AFP-negative. Moreover, no blue-stained particles were observed in cells cultured in iron-free medium regardless of whether the cells had been transfected. In un-transfected cells treated with FAC, only a few blue particles were evident in a small fraction of the field of view. The results demonstrate that ferritin overexpression substantially increases the iron storage capacity of transfected cells in the presence of FAC.

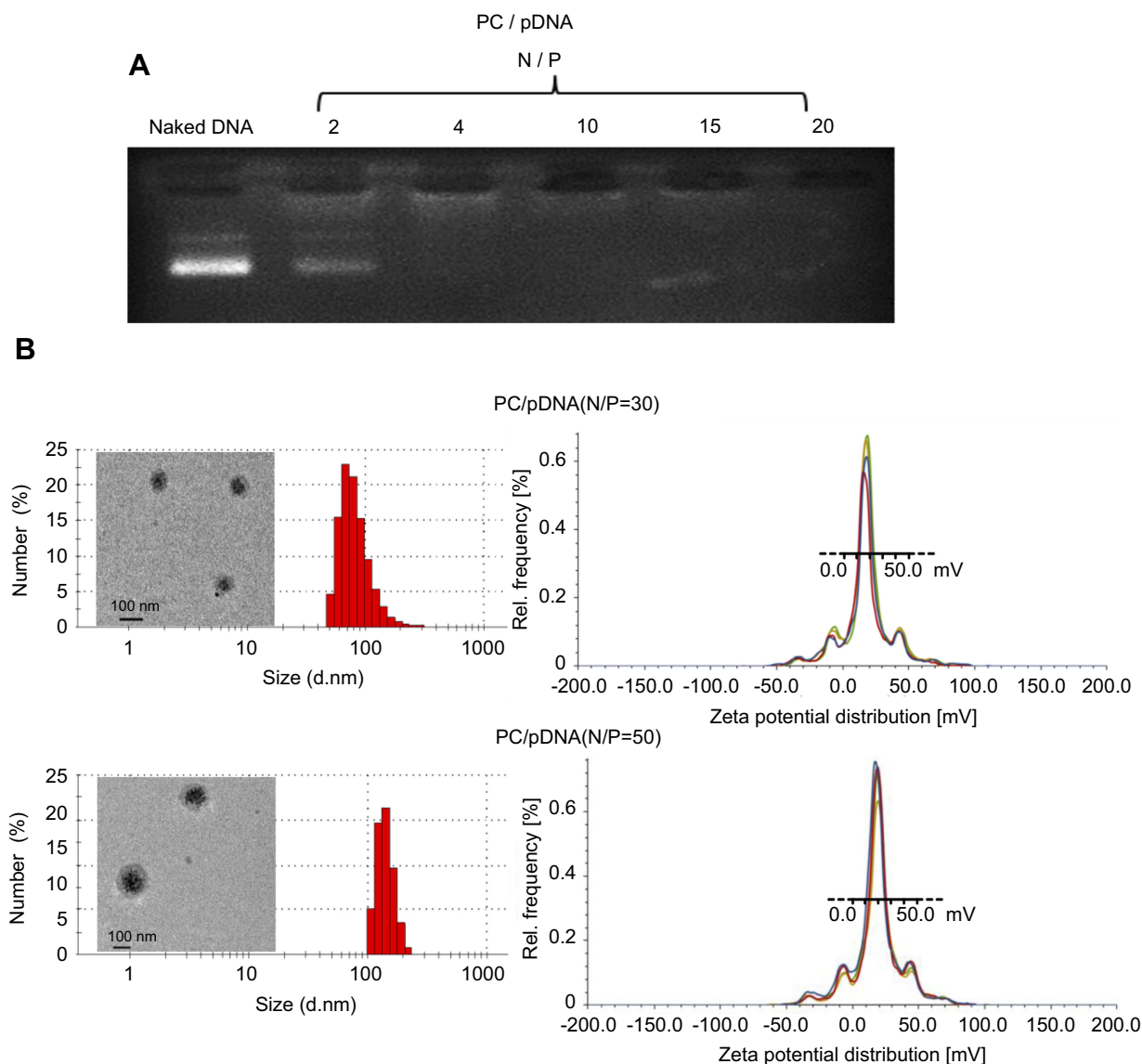


Figure 2 Characterization of the PC/Plasmid DNA complexes. **(A)** Gel retardation analysis of PC/pDNA at different N/P ratios. PC inhibited DNA migration completely at N/P ratios of 4 and above. **(B)** TEM images, particle size and zeta potential distribution of PC/pDNA complexes with N/P ratios of 30 and 50. At the above N/P ratios, the biophysical properties of the complexes are suitable for cellular uptake.

Abbreviations: PC, polyethyleneimine- β -cyclodextrin; TEM, transmission electron microscope.

Table I Diameter and zeta potential of PC/pDNA at different N/P ratios

Sample (N/P)	Mass ratio	Diameter (nm)	Pdl	Zeta potential (mv)
PC/pDNA (4:1)	0.53	225.25 $\pm$ 23.57	0.344 $\pm$ 0.02	10.89 $\pm$ 0.77
PC/pDNA (10:1)	1.33	200.25 $\pm$ 8.52	0.560 $\pm$ 0.04	15.34 $\pm$ 0.31
PC/pDNA (15:1)	1.99	189.00 $\pm$ 5.18	0.426 $\pm$ 0.02	16.86 $\pm$ 0.22
PC/pDNA (20:1)	2.66	132.75 $\pm$ 8.41	0.227 $\pm$ 0.03	23.02 $\pm$ 0.17
PC/pDNA (30:1)	3.98	97.27 $\pm$ 19.86	0.228 $\pm$ 0.05	15.58 $\pm$ 0.22
PC/pDNA (40:1)	5.31	130.75 $\pm$ 3.77	0.284 $\pm$ 0.03	19.82 $\pm$ 0.31
PC/pDNA (50:1)	6.64	143.25 $\pm$ 2.49	0.211 $\pm$ 0.02	17.94 $\pm$ 0.20

Note: The particle size and zeta potential of PC/pDNA complexes with different N/P ratios were measured. Mass ratios were determined by different N/P ratios.

Abbreviations: PC, polyethyleneimine- β -cyclodextrin; Pdl, polydispersity index.

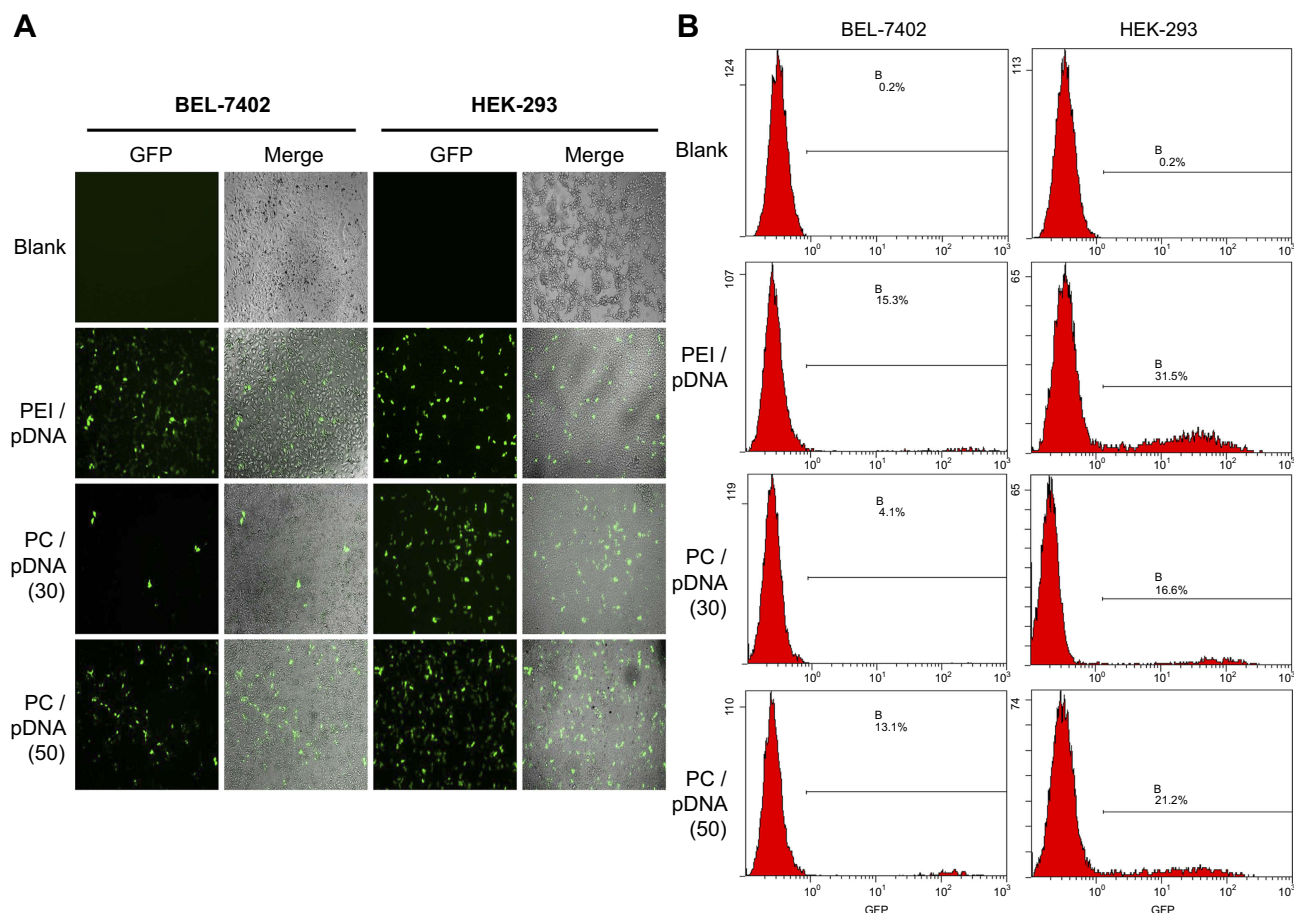


Figure 3 Optimal N/P ratio of PC/pDNA complexes for transfection. **(A)** GFP expressions in transfected Bel-7402 and HEK-293 cells are shown in inversed fluorescent images. The transfection efficiency of PC was approximate to that of HMW PEI. Cells were incubated with PC/pDNA at N/P ratios of 30 and 50 for 48 h. **(B)** Percentages of GFP-expressing cells detected by flow cytometry. The naked plasmid (Blank) and HMW PEI/pDNA at an N/P ratio of 10 were employed as the negative and positive controls, respectively.

Abbreviations: GFP, green fluorescent protein; PC, polyethyleneimine- β -cyclodextrin; HMW PEI, high molecular weight polyethyleneimine.

In vitro MR studies

MR imaging of the collected transfected cells was performed to investigate the contrast imaging effect caused by iron accumulation in hepatoma cells. When scanning the collected BEL-7402 cell samples for T2-/T2*-weighted MR imaging, a notable low signal intensity (darkening of the cell layer) was observed in the transfected samples supplemented with FAC compared to that of the untransfected samples, but this contrast was not observed in AFP-negative cells, such as the HEK-293 cells (Figure 5A and C, $P < 0.01$). The intensity of the signal loss was in good agreement with the intracellular iron accumulation measured above. No significant difference was observed between PC and the positive control, as indicated by the signal intensity value (Figure 5A and C, $P > 0.05$).

Furthermore, for the transfected BEL-7402 cells, the signal intensity was lower in samples supplemented with than without FAC (Figure 5B), which was

consistent with the previous iron accumulation experiments results.

Discussion

In this study, we innovatively reported the use of PC as a gene delivery vector, carrying the ferritin gene regulated by the AFP promoter, to transfect hepatoma cells for MR imaging. The transfection resulted in an enhanced intracellular iron storage capacity, which directly led to a change in the signal intensity on T2-weighted MR imaging. Furthermore, upregulation of TfR during the imaging process provided a highly specific target for subsequent therapy.

PC was synthesized to execute pDNA delivery in our study. No apparent cytotoxicity was observed in the group treated with PC. In contrast, HMW PEI, which is recognized as the gold standard due to its high gene transfection efficiency,²⁰ showed obvious cytotoxicity. Ping Y's and

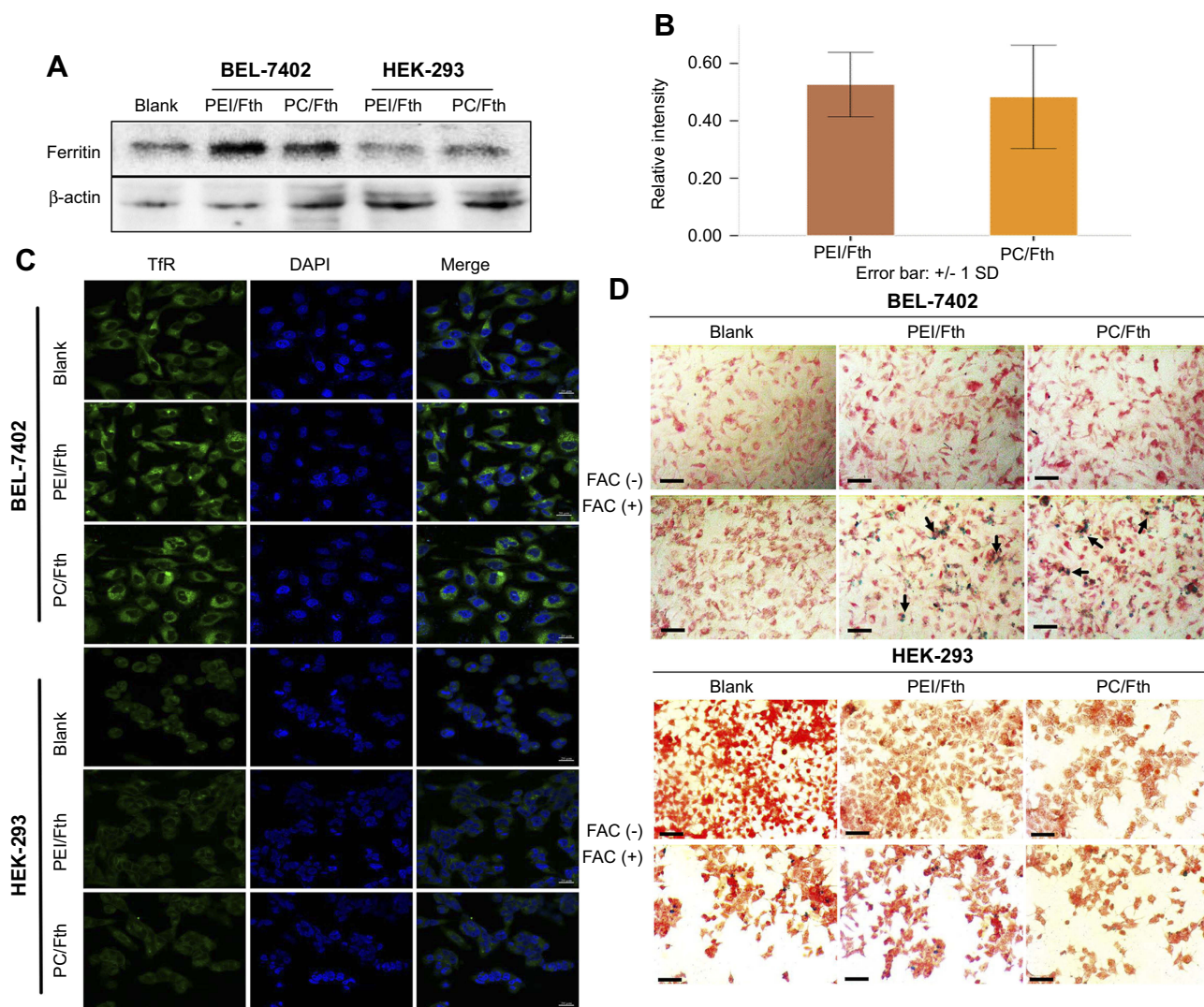


Figure 4 Ferritin expression, TfR upregulation and intracellular iron accumulation. **(A)** Western blotting assay of ferritin as the result of Fth expression regulated by the AFP promoter. Ferritin was overexpressed in AFP-positive BEL-7402 cells but not in AFP-negative HEK-293 cells. **(B)** The relative intensity of the Western blotting protein bands showed no significant differences between the PC/Fth and HMW PEI/Fth expression levels ($n=3$, $P>0.05$). **(C)** IF assay of TfR upregulation in the different groups. TfR was specifically expressed in transfected BEL-7402 cells (the fluorescence of the blank group was derived from a small amount of TfR originally expressed within tumor cells), but not in the HEK-293 cells regardless of whether they were transfected or not. Green fluorescence represents TfR, and blue fluorescence represents the cell nucleus. The scale bar represents 20 μ m. **(D)** Prussian blue staining assay. Blue-stained particles representing accumulated iron were obvious within the transfected BEL-7402 cells treated with FAC (indicated by black arrows). Few blue granules were detected in the HEK-293 cells because of FAC supplementation. The blank and HMW PEI/Fth were employed as the negative and positive controls, respectively. The scale bar represents 50 μ m.

Abbreviations: TfR, transferrin receptor; Fth, ferritin heavy chain; IF, immunofluorescence; FAC, ferric ammonium citrate.

Hong HL's studies showed similar results for different cell lines treated with HMW PEI.^{21,22} One explanation may be the higher amino group density of branched HMW PEI than linear LMW PEI.²³ Although the cationic polymer PEI has less influence on the cell structure and higher stability than the cationic liposome, the toxicity of it increases with the molecular weight.²⁴ Thus, in the current study, we introduce a biodegradable polymer CD to assemble LMW PEI and synthesize PC as a gene vector. Although its toxicity is still low, it shows a transfection efficiency similar to that of HMW PEI.²⁵ Moreover, the modification makes the carrier

system more stable and eases transmembrane transport.^{18,26} In genetic engineering studies on dendritic cells, Chen YZ et al found that the modified LMW PEI had a better transfection efficiency than Lipofectamine 2000 based on improved safety.²⁷ Hu QD et al also reported a satisfactory transfection efficiency for PEI-CD.²⁸

The cationic polymer PC and the negatively charged pDNA condense into a complex by electrostatic action.²⁹ The ability to condense pDNA into an appropriate structure is a primary prerequisite for efficient cellular transfection. The suitable particle size makes passive delivery into

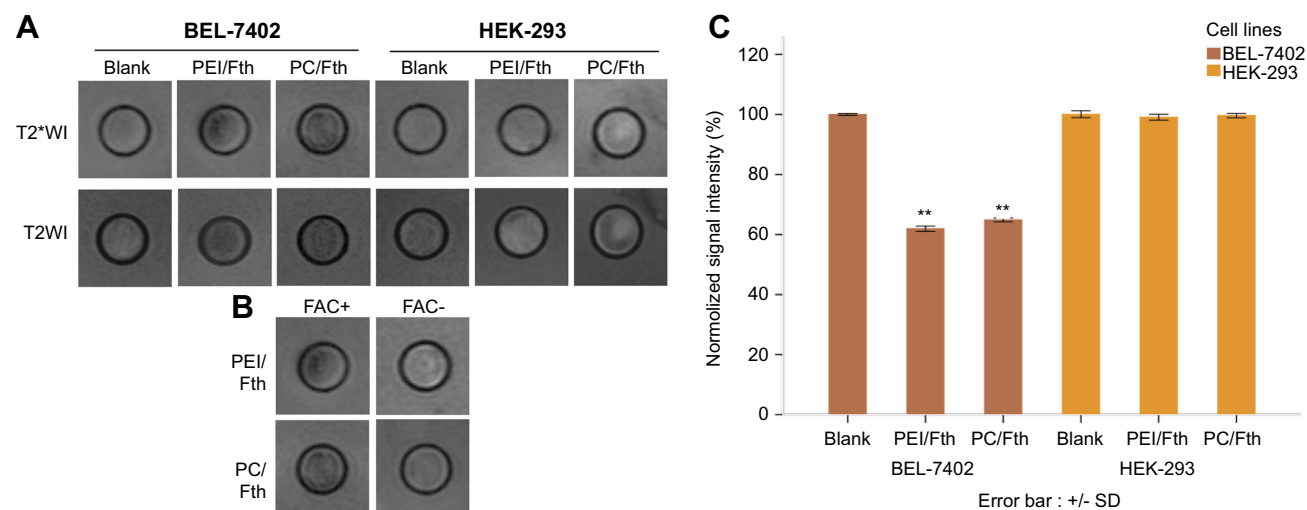


Figure 5 T2-/T2*-weighted MR imaging of the collected cell samples. **(A)** A noticeable low signal intensity was observed in transfected cells supplemented with FAC in the AFP-positive BEL-7402 cells but not in AFP-negative HEK-293 cell line. **(B)** T2*-weighted MR imaging of transfected BEL-7402 cells with or without FAC supplementation. The signal was lower for transfected cells supplemented with than without FAC. **(C)** The signal intensity analysis for the ROI (8 mm²) of each group (n=3). The blank and HMW PEI/Fth were employed as the negative and positive controls, respectively. Data are expressed as the mean $\pm$ SD. ** Significant difference between groups ($P < 0.01$). **Abbreviations:** FAC, ferric ammonium citrate; ROI, region of interest; Fth, ferritin heavy chain.

the liver easier, and the proper zeta potential makes the system stable. Therefore, we selected N/P ratios of 30 and 50 for the further transfection studies, because the particle sizes were approximately 150 nm with a compact, near-spherical morphology. Synergistic with the enhanced permeability and retention effect of the tumor, the complex would be passively targeted to HCC tissues.

Endocytosis and lysosomal escape of the PC/pDNA complex largely affect the transfection efficiency. The task of an adequate vector is to protect the plasmid from degradation and release it smoothly, to allow the plasmid to enter the nucleus. PC carrying pDNA enters cells via endocytosis through the positive surface charge and produces a buffering effect inside the endosomes to some extent, inducing the osmotic swelling and rupture of the endosomal membrane,³⁰ which is called “proton sponge effect”.

As outlined above, early diagnosis with contrast agents requires high safety standards. In attempts to use a transfection technique for HCC imaging diagnosis, PC is expected to be a safe candidate non-viral vector for gene delivery in our study. A similar transfection efficiency has been showed at an N/P ratio of 50 with much lower toxicity than that of HMW PEI; the latter approach is widely regarded as the gold standard of cationic polymers used for transfection.

The above results stimulated us to explore the effect of Fth as an MR reporter gene carried by PC in transfected cells. Previous studies have suggested that TfR expression

significantly increases with ferritin overexpression, thereby facilitating cellular iron uptake.^{31,32} The net iron contents in transfected cells increase, including iron combined with ferritin,⁷ which lead to inhomogeneity of the local magnetic field.³³ The above theories were reflected in the Prussian blue staining, and MR imaging in our report.

Observing intracellular iron accumulation after confirming ferritin and TfR expression by Western blotting and immunofluorescence, respectively, required optimal iron-containing culture medium to simulate free iron in the sera in vivo. FAC is a common supplement that is used to load ferritin-expressing cells with iron. Previous studies differ greatly in terms of a suitable FAC concentration. Nevertheless, TfR is ubiquitous on the cell surface, and the effect of iron accumulation in both the transfected and blank groups is obvious when the FAC concentration reaches 2 mM.^{8,34} This finding suggests that an excessive FAC supply will only increase background interference. Therefore, we chose a FAC concentration of 0.2 mM, which acts as a safe and efficient dose for FAC supplementation.

Consistent with previous studies,^{8,35} our work suggests that simple transfection does not result in specific intracellular iron accumulation in in vitro experiments. An extracellular iron-containing environment and intracellular ferritin-overexpression are prerequisites for satisfactory iron accumulation within cells. We used FAC in vitro to simulate non-transferrin-bound iron (NTBI) in vivo. However, TfR has a much higher affinity for transferrin-

bound iron (TBI) than NTBI, and the process of iron donation from transferrin (Tf) to cells is highly efficient.^{36,37} Furthermore, approximately 25–50% of storage iron is found in the liver of a typical adult male,³⁸ which suggests a rich iron supply in the liver. These findings led us to speculate that the signal contrast on MR images caused by Fth overexpression *in vivo* might be even higher than that *in vitro*.

The potential toxic effect of ferritin overexpression is always a focus issue. Generally, previous studies have accepted that ferritin overexpression does not influence cell viability, and has almost no effect on the cell phenotype.^{8,9,34,35} Our evidence suggests that the BEL-7402 and HEK-293 cell lines can tolerate ferritin overexpression in the presence of FAC, which is consistent with previous studies on the safety of ferritin.

In the field of MR molecular imaging, the mature contrast agents are almost exogenous, such as gadolinium-based agents and SPIO.^{39–41} Many research innovations have focused on conjugating exogenous contrast agents with a tumor-targeting polypeptide, and therefore, the MR signal intensity of the cell and tissue of interest is altered to make a comparison. Concerns about security is a current trend for contrast agents. Although exogenous contrast agents have made progress in targeting and imaging, *in vivo* deposition, especially in the brain, and metabolic toxicity constrain the limits of their application.^{3,42} However, ferritin-based endogenous contrast agents can avoid some of these limitations.

Previous studies have reported the potential of the ferritin gene as an MR reporter gene. Iordanova B et al suggested in his report that a ferritin-based MRI reporter gene could achieve *in vivo* visualization of endogenous neuroblast migration toward the olfactory bulb.¹¹ He XY et al generated this type of reporter gene in C3H10T1/2 stem cells under control of a Tet-On switch.¹² Guo RM's research demonstrated that the ferritin gene could provide sufficient MRI contrast to detect the distribution and migration of MSCs *in vivo*.⁶ However, the endogenous imaging capability of the ferritin gene has not been assessed in hepatoma carcinoma cells to date, and our results can fill this gap and provide evidence that transfection technology has prospects for application in the diagnosis of liver cancer.

TfR has been found to be abnormally overexpressed in malignant cells,⁴³ which makes it a molecular target for diagnosis and drug treatment of tumors through a receptor-mediated endocytic pathway. Mulik et al

formulated Tf-mediated nanoparticles to increase the bioavailability of the contained curcumin.⁴⁴ Han et al used Tf-modified carriers to improve the lung cancer cell-targeting effects.⁴⁵ Singh et al developed Tf-conjugated polymeric micelles to achieve better efficacy and safety in cancer treatment.⁴⁶ Nevertheless, TfR is ubiquitous on vertebrate cell membranes. Despite large differences in the expression level, undeniable potential systemic toxicity can occur in the clinic. In our study, the abnormal expression within tumor cells, which was synergistic with the overexpression caused by endogenous contrast agents, significantly upregulated TfR on the surface of target hepatoma cells, which improved the accuracy of site-specific delivery, reduced targeted drug toxicity and increased the therapeutic effect.

As mentioned above, most previous studies of contrast agents have used polypeptides to target specific tumor cells. This method is mature and has satisfactory effects on specific cell lines, but sometimes wide application for the diagnosis of a certain disease is difficult. Moreover, the toxicity and other side effects caused by off-target effects also require attention.⁷ In studies of liver cancer, the above limitations are particularly serious, because targeting a wide range of hepatoma cell lines with a specific polypeptide is difficult. Comparatively, AFP is expressed in approximately 80% of HCC cases,¹⁵ and can be used as a regulatory promoter to remedy the shortcomings associated with a single targeted polypeptide. In this study, the MR reporter gene was regulated by the AFP promoter, which was expressed only in AFP-positive cells, such as the BEL-7402 cell line, but not in AFP-negative cells, such as the HEK-293 cell line. Therefore, although HEK-293 cells are more sensitive to transfection based on GFP detection by fluorescence microscopy and flow cytometry, they are unable to express ferritin under this type of regulation. After being internalized, the ferritin gene leads to the MR signal intensity change in liver cancer tissues without significant effects on other tissues.

Our study has several limitations. Although the transfection efficiency of PC can be detected using pGFP and ferritin expression can also be observed, the sensitivity of endogenous contrast agents needs to be improved, and we await further development of a more satisfactory agent with a sharp and clear contrast signal at the lesion site. Further research will aim to modify the PC to achieve a higher transfection efficiency, and combine the plasmid with enhancers or a transcriptional amplification system to achieve adequate ferritin overexpression. Thus, the synergy between these two methods may produce a stronger hypointense signal. Another deficiency of

our study is that only one human hepatoma cell type, the BEL-7402 cell line, was investigated in our study. We speculate that other AFP-positive hepatoma cell lines will produce consistent results, but this issue needs to be verified in further studies.

Conclusion

In summary, our research reported for the first time that the ferritin gene under regulation by the AFP promoter, carried by a security non-viral vector PC, could be used to transfect hepatoma cells as an endogenous contrast agent. Then, the enhanced intracellular iron storage resulted in signal changes in the lesion area on MR images, and the specific target TfR was also upregulated during this process. Our study may improve the safety of early imaging diagnosis of HCC at a molecular level and provide a highly specific target for subsequent therapy to reduce the mortality of this intractable disease.

Acknowledgments

This study was supported in part by grants from the Natural Science Foundation of Zhejiang province (LY18H180003), and the National Natural Science Foundation of China (General Program: 81571662).

Disclosure

The authors report no conflicts of interest in this work.

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