Down-regulation of MEF2D via HBc Nanoparticle Mediated siRNA Inhibits $HepG_2$ Cell Proliferation *in vitro*

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Abstract. Objective: Recombinant hepatitis B virus nucleocapsid protein maintains the ability to form particles self-assembly. We tried to use non-virus-like particles HBc package wrapping foreign gene to protect and transfer siRNA of MEF2D gene to target position, in order to make a contribution to analyze the relationship between the expression of MEF2D gene and the proliferation of hepatocellular cells. Methods: Firstly, prepared si-MEF2D/HBc nanoparticles and transfected it into hepatocellular cells. The knockdown of MEF2D gene effects on cell proliferation was detected by MTT assay, XTT cell viability assay and CKK-8 assays. Result: The proliferation of treated hepatocellular cells were decreased significantly compared with control cells. Pretreatment of $HepG_2$ cells with si-MEF2D/HBc complex inhibited cell growth in a dose-dependent manner. Conclusion: HBc particles is one kind of promising biological nanomaterials which has presented itself as an appropriate and valuable material for other aspects of nanoparticle in future study.

Introduction

Virus-like particles (VLPs) have received increasing attention for their applications in chemistry, materials science, and medicine. They can be highly stable toward extremes of temperature, pH, and variations in solvent composition and thus, are friendly toward chemical modification, while retaining many advantageous characteristics of biological molecules. VLPs have well-controlled composition and structure, are biocompatible, and can be mutated and evolved [1]. They can potentially serve as polyvalent scaffolds for the display of nucleic acids, proteins, and other chemical moieties, and VLPs are particularly attractive as vaccines as they offer in vivo stability, trafficking to lymph nodes, and stimulation of B and T cell responses to displayed epitopes. They can also be filled with cargo to serve as delivery vehicles. Among the most commonly employed VLPs have been the coat proteins of MS2 and PP7. Here we report a set of rationally designed genetic mutations of the HBc protein that have differential effects on assembly and stability of the resulting VLPs [2]. VLPs are free of viral nucleic acid in the shell structure which is highly organized spheres that self-assemble from virus-derived structural antigens [3]. Many viral structural proteins have the ability to assemble into virus like particles automatically. VLPs are known to induce strong antibody responses in the absence of adjuvants. In addition, VIPs are able to prime cytotoxic T lymphocyte (CTL) responses in vivo. They offer a promising approach to the production of vaccines against many diseases, because their repetitive, high-density display of epitopes is often effective in eliciting strong immune responses. It is similar to natural virus like particles on the morphological structure with strong immunogenicity and biological activities. Virus like particles has been successfully used as a vaccine in clinical because of they do not contain viral genetic material and do not have infectious [4]. Simultaneously, most virus like particles have the ability to package viral nucleic acids or other small molecules, so they can be used as delivery vehicles for gene or drugs. Recombinant hepatitis B virus core protein, even with deletions at the carboxyl-terminal end or with small internal insertions, maintains the ability to form particles in *E.coli*. Core-shell or core of the mammalian hepatitis B virus core protein contains a sequence which is composed of 183 or 185 amino acid and the diameter of HBc particles infectious virus particles is about 30-34 nm, may form a T=4,T=3 icosahedral symmetry [5]. Recombinant hepatitis B virus nucleocapsid protein, even with deletions at the carboxyl-terminal end or with small internal insertions, maintains the ability to form particles. HBc particles can efficiently express, folding and self-assembly to form particles in all known homologous and heterologous expression of the system and it has good biological compatibility, no direct toxic to human cells [6]. Present study showed,

the core antigen of hepatitis B virus in particular, has been used for the presentation of various foreign epitopes. This paper tries to use HBc particles package wrapping foreign gene to protect and transfer it to target position.

Myocyte enhancer factor 2 (MEF2) is a specific transcription factor which was found in skeletal muscle tube nucleus. The family includes MEF2A、MEF2B、MEF2C and MEF2D in vertebrates. As a member of the MEF2 family, MEF2D has a binding domain and the enhancement of the transcriptional regulatory factor MCM1, whose main function is adjusted on a variety of cell survival and differentiation [7]. MEF2D is expressed abnormally in some types of cancer, proving that it has a certain impact in the occurrence and development of malignant tumor. In this study, therefore, we plan to observe the changes of cell proliferation by silencing MEF2D gene.

RNA interference (RNAi) is a new developed method for suppression of gene expression recently. This technique mediated by double-stranded RNA, can specifically block or reduce expression of the corresponding gene [8]. In cancer-targeted research, it can be used to inhibit the expression of human tumor-related genes selectively by RNAi technology, thus to inhibit the growth of tumor cells.

RNA interference technology has opened up new avenues for the study of gene function and gene therapy. Numerous therapeutic siRNAs had been used to treat diseases including cancer and genetic disorders [9]. However, siRNA cannot achieve the desired effect because of low permeability and easy to decompose. As described above, the use of siRNA is limited in certain cases. In this article, we aim to package siRNA using HBc nanoparticles.

Materials and methods

Materials

Human hepatocellular carcinoma cell lines $HepG_2$ cells were purchased from Takara Biotechnology Company. Lipofectamine 2000 was purchased from Invitrogen Company. All other reagents were of analytical grade.

Design and synthesize MEF2D-siRNA

We designed and screened the effective siRNA for gene MEF2D (Gene bank NM_001271629.1) using online design software *http://sirna.wi.mit.edu/home.php*. To ensured that the candidate siRNA targeted sites would not inhibit other human genes, we used sequence homology alignments (BLAST analysis) [10]. After designing the complete appropriate sequence, we entrusted Shanghai Sangon Biotechnology Company which is specialized in nucleic acid synthesis.

Preparation of HBc virus-like nanoparticles

HBc protein sequences with C-terminal domain codons optimized for expression in *E.coli* were used for HBc protein expression. HBc proteins were purified by ammonium sulfate precipitation and size exclusion chromatography and then stored at -80℃. Before the experiments, the HBc proteins were dissociated and separated from *E.coli* RNA by dialysis into 1.0 mol/l GnCl, 0.5 mol/l LiCl, 50 mmol/l Tris base (pH 9.5), and 5 mmol/l DTT overnight followed by separation through a sucrose 6 10/300 column.

Analysis of HBc virus-like nanoparticles by TEM

HBc VLPs were first centrifuged at 14000 rpm in 10% sucrose cushion for 10 min and then sonicated for 10 min. The step was repeated for up to six times in order to eliminate the protein aggregates and empty capsids. Then, 10 μl of the purified sample was put on a glow-discharged carbon-coated copper grid. After 10 min, the excess solution on the grid was removed with filter paper. A 10 μl portion of 2% uranyl acetate was used to stain for 10 s. Excess solution was removed by blotting with filter paper. The sample was then left to dry for at least 30 min. The assembly efficiency of VLPs was determined from the TEM images by dividing the number of complete VLPs over the total number of gold particles (involved in complete VLPs, incomplete VLPs). Images were taken from at least two independent TEM grids.

Packaged MEF2D-siRNA with HBc virus-like nanoparticles

About 40 μl solution containing HBc particles at a concentration of 20 μg/μl was mixed with an organic solvent in 50 μl HBS buffer (pH 7.4), vortexes for 30 min at room temperature. Solution containing 2 μl siRNA was added drop by drop at a concentration of 500 ng/μl under vortex

conditions [11]. Then vortex for 40 min and waited for 50 min to make full package of si-MEF2D/HBc particles in order to provide sufficient time for HBc self-assemble into nanoparticles.

Cell Cultures

Human hepatocellular carcinoma cell line $HepG_2$ cell was purchased from Takara Biotechnology Company. Cells were incubated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37℃ in 95% air/5% CO_2 atmosphere. For all experiments, cells were plated at a density of 1.0×10^5 cells/ml.

HBc/MEF2D-siRNA nanoparticles were transfected into HepG2 cells

Using Invitrogen's Lipofectamine 2000 method in accordance with its instructions. $HepG_2$ cells in the logarithmic growth phase were prepared into cell suspension with trypsin enzyme digesting technique (cell number is about 5×10^4/ml) . Cultured cells could be used for transient transfection when their cell fusion degree reached about 90%-95% in 6-well plate. Then added 4 μl si-MEF2D/HBc complex and 8 μl Lipofectamine 2000 in each well. Cells were harvested after 72 h for the cell proliferation assay.

Detection of MEF2D RNAi effects on cell proliferation

MTT assay

Cell viability was determined by the MTT assay. Briefly, 2×10^5 cell/ml of $HepG_2$ cells were incubated in 96-well microtiter cell culture plates, in the absence or in the presence of si-MEF2D/HBc, in a final volume of 100 μl. After 12 h, or indicated incubation times, 10 μl of MTT was added to each well for an additional 4 h. The blue MTT formazan precipitate was dissolved in 100 μl of DMSO and the absorbance measured at 570mn on a microplate reader [12]. Percent of cell viability = average OD of test team/average OD of control team×100%. IC_{50} means the concentration of the drug which cells have a survival rate up to 50%.

XTT assay

After verifying cell viability using trypan blue dye exclusion test by hemacytometer, cells were seeded at approximately 1×10^4/ well in a final volume of 200 μl in 96-well flat-bottom microtiter plates. After an overnight incubation, cells were treated with si-MEF2D/HBc particles while blank control group only added medium. Plates were incubated at 37℃ in a 5% CO_2 incubator for the indicated time periods. At the end of incubation, 100 μl of XTT was added to each well, and plates were incubated at 37℃ for additional 4 h. Absorbance was measured at 450 nm against a reference wavelength at 650 nm using a microplate reader.

CCK-8 assay

The viable cells were measured by Cell Counting Kit-8 (CCK-8) following the manufacturer's instruction. Briefly, 1×10^4/ well $HepG_2$ were seeded in 96-well culture plates and then treated for 24 h with si-MEF2D/HBc and blank medium. 10 μl CKK-8 working solution was added into each well and the plates were incubated for additional 4 h. The absorbance was measured at 450 nm using a microplate reader [13]. Cell viability was expressed as a percentage of the absorbance of treated cells versus untreated cells.

Statistical analysis

All experiments were carried out in duplicate and repeated at least three times. Data were expressed as mean ± standard deviation (SD). Statistical significance was evaluated using *t* test or one-way ANOVA followed by SPSS software analysis. The differences were considered statistically significant at a *P* value of <0.05.

Results

Design MEF2D-siRNA

The screened siRNA target sequences were as follows,
Sequence 1 AACCCACACTCTAGACTGAAACC;
Sequence 2 AACTGGGCATTTGCCAAAGTACC.
Two MEF2D interference sequences for the target sequences were designed as follows:
siRNA1 sense: 5'- CCCACACUCUAGACUGAAAUU -3'
antisense: 3'- UUGGGUGUGAGAUCUGACUUU -5';
siRNA2 sense: 5'- CUGGGCAUUUGCCAAAGUAUU -3'
antisense: 3'- UUGACCCGUAAACGGUUUCAU -5'.
Selected a negative control sequence that was not homologous to the target sequence of 16 consecutive bases as follows:
sense: 5'-UUCUCCGAACGUGUCACGUTT-3'
antisense: 5'-ACGUGACACGUUCGGAGAATT-3'.

Preparation and Analysis of HBc virus-like nanoparticles

HBc retains the ability to form virus-like particles both in vitro and in the cell expression system. We produced protein VLPs. In previous experiment, the virus-like HBc particles were purified successfully. In order to observe the shape of particles, utilized the TEM technology. The particles prepared in this experiment have obvious virus-like particulate. We speculated that such a virus-like particle may be more easily wrap foreign substances.

Detection of MEF2D RNAi effects on cell proliferation

Effect of si-MEF2D/HBc on the viability of human hepatoma cells $HepG_2$ was studied by MTT assay. Compared with control group, the viability of $HepG_2$ cells treated with si-MEF2D/HBc was decreased. Data showed the change of the viable cell number when treated with 50 μmol complex for 3 days. Clearly, si-MEF2D/HBc markedly inhibited the proliferation of $HepG_2$ cells.

XTT cell viability assay was used to evaluate cell viability. From the XTT viability data, a conclusion can be made that si-MEF2D/HBc acted on $HepG_2$ cells effectively, the proliferation ability of decreased significantly after reducing MEF2D gene expression.

To explore the effects of si-MEF2D/HBc on cell viability, we assessed cell viability with CKK-8 assays. As shown in Fig.1, compared with untreated cells, the viability of the cells which treated with si-MEF2D/HBc was decreased.

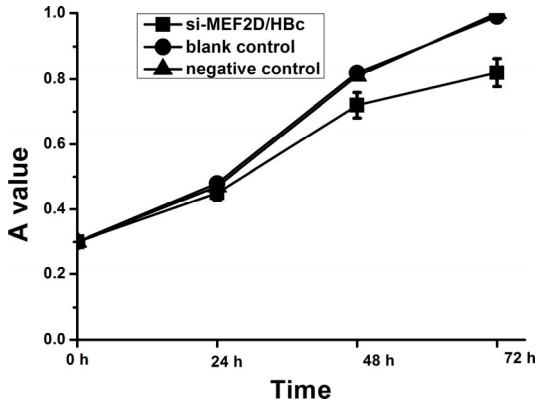

Fig.1. Effects of si-MEF2D/HBc on the proliferation of $HepG_2$ cells

Conclusion

The incidence and mortality of liver cancer are the second-highest among the cancer patients in China. Many families are suffering the pain of cancer because of the high cost of treatment and the patients' pain. Cancer became the most difficult diseases to cure in human history. Neoplastic disease was identified by its very nature as a "genetic disease", so in theory, to solve the problem of tumor at the genetic level is the best way. siRNA has highly specific gene silencing function. It can make the target genes stable silence, without affecting the normal expression of other gene, meanwhile it has many advantages, for example, characteristics of specificity, high efficiency and simplicity and so on.

VLPs are noninfectious and intrinsically very stable over wide ranges of pH and temperature, making them attractive as the assembly core for vaccines, diagnostics, and other therapeutics. Here we report the Hepatitis B virus core protein that have differential effects on assembly and stability of the resulting VLPs, which was one of the first viral proteins expressed heterologously in bacteria [14]. It can be expressed at high level not only in bacteria like *E.coli* but also in yeast and insect cells. Moreover, the heterologous expression of HBV core protein leads to the formation of core particles resembling those produced in HBV-infected hepatocytes. In addition, the core protein can be expressed also in recombinant attenuated Salmonella strains which represent potential oral vaccines. HBc virus-like nanoparticle has significant advantages as a particle-like carrier compared with other nanoparticle because of its ability to folding and assembling themselves correctly in both eukaryotic cells and prokaryotic cells. Thl and Th2 cytokines can be detected when using HBc as a carrier. Using HBc virus-like nanoparticles as a carrier to protect siRNA, its effective will last for a longer time and there is no direct toxicity to human cells. At the same time, this suggests that MEF2D plays an important role in controlling the gene expression of factors that control this important process and the dramatic effect of MEF2D on cell apoptosis anchorage independent growth and tumor growth. It is surprising that the restoration of a single transcriptional co-activator could have such a large effect on oncogenic properties of these cells. Our results have suggested that MEF2D in $HepG_2$ cells may effectively impede tumor and dissemination. Proliferation is an important step in malignant tumor growth process as one of the biological behavior of cancer. This paper transfected siRNA into liver cancer cells with HBc nanoparticles as the vector to induce RNA interference effect. In order to get more accurate results, we used MTT, XTT and CKK-8 experimental methods to detect proliferation ability of liver cancer cells. The results showed that the proliferation ability of $HepG_2$ cells decreased significantly after reducing MEF2D gene expression. It showed that HBc nanoparticles could transfer siRNA into liver cells effectively and it is one kind of promising biological nanomaterials.

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