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Introduction of Recombinant GnRH, Transmembrane Domain of PEA and Hyperthermophilic Archaea Histone (GPH) as a Novel Carrier for Gene Transfer

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***Abstract*—** *We successfully constructed and expressed a generic recombinant protein named GPH via gene engineering. GPH is made up of gonadotropin releasing hormone (GnRH), a transmembrane area of Pseudomonas aeruginosa exotoxin A (PEAII) and hyperthermophilic archaea histone (HPhA). After expression and purification, the engineered GPH protein was detected by SDS-PAGE. We then examined its transfection ability using the plasmid, pEGFP-C1 (green fluorescent plasmid). Electrophoretic mobility shift assays showed that recombinant GPH slowed down the migration rate of pEGFP-C1 in agarose gels, confirming that GPH had combined with the plasmid. Transfection experiments showed that GPH can carry pEGFP-C1 into HeLa cells to produce fluorescence. Also, genomic DNA extraction from transfected cells and PCR examination showed that recombinant GPH mediated the transport of pEGFP-C1 into HeLa cells. Immunohistochemical analysis detected GPH in transfected cells. Enzyme-linked immune sorbent assays detected GPH bound to GnRHR on the surface of HeLa cells. Taken together, recombinant GPH has the ability to bind to and transport DNA into target cells. Thus, GPH is a potential gene delivery vehicle in gene therapy.*

***Keywords*—** *Gonadotropin releasing hormone (GnRH); transmembrane area of Pseudomonas aeruginosa exotoxin A (PEAII); hyperthermophilic archaea histone (HPhA); Gene transfer carrier; Electrophoretic mobility shift assay (EMSA); Transfection.*

I. INTRODUCTION

Gene therapy refers to the replacement of disease genes with normal genes to restore gene structure and correct abnormal functions. Effective gene therapy depends on the efficient expression of exogenous genes in target cells. However, exposed exogenous genes cannot enter target cells easily and may be degraded in the body or in cells. Currently, there are two main delivery pathways for gene carriers in somatic cells. One is the viral carrier system, and the other is the non-viral carrier system. Although viral carriers have high transfection efficiencies, they may induce an immune response, and can be carcinogenic because of insertional mutagenesis[1]. In addition, the carrier capacity of viral carriers is limited and preparation titers can be low. With non-infectivity, unlimited carrier capacity, and targeting properties[1], non-viral carriers are receiving attention for the development of gene therapy.

Members of the archaea display what are generally considered to be both bacterial and eukaryotic features, which indicates a possible evolutionary relationship between eukaryotes and archaea. An impressive example of a eukaryotic-associated feature is the archaea histones. Histones, which are highly basic DNA binding proteins, are abundant and conserved in eukaryotic cells. Histone-like proteins are also conserved in archaea. The recombinant hyperthermophilic archaea histone, HphA, was expressed by a histone-like gene PHS051, from the hyperthermophilic archaea *Pyrococcus horikoshii* OT3 strain[2].

Exotoxin A of *Pseudomonas aeruginosa* asserts its cellular toxicity through ADP-ribosylation. The crystallographic structure of exotoxin A revealed a tertiary fold having three distinct structural domains: domain I is responsible for receptor binding; domain II is required for transmembrane transport; and domain III is the ADP-ribosyl transferase. These predictions have been confirmed by subsequent experiments[3-5].

Gonadotropin releasing hormone (GnRH) is a decapeptide that was originally isolated from mammalian hypothalamus. It combines with high affinity GnRH receptors in the anterior pituitary and stimulates the synthesis and release of the pituitary gonadotropins, which regulate mammalian reproduction [6]. Further studies have indicated that GnRH receptors also occur in reproductive tissues and tumors such as breast, endometrial, ovarian and prostate[7.8] in which a paracrine/autocrine role is postulated [9.10]. Hela cell is one of them[11.12].

The fusion protein, GPH, was constructed from segments of three genes, including gonadotropin releasing hormone (GnRH), the transmembrane area of *Pseudomonas aeruginosa* exotoxin A (PEAII) and the hyperthermophilic archaea histone, HPhA. H (HPhA) can bind to DNA stably and reduce DNA degradation by DNA restriction endonucleases[13.14]. P (PEAII) may transfer the fusion protein to target cells. G (GnRH) plays a key role in targeting the fusion protein to GnRH receptors on the surface of some tumor cells. GPH is a novel carrier for gene delivery potentially bearing high stability, non-infectivity and targeting properties.

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In the current study, we constructed and expressed BL21(DE3)/PET26b-PEA(Ia+II)-HPhA[15]. Then PEAIa was replaced by GnRH and expressed as GPH. The exact sequence of the full GPH is
MGSSHHHHHHGSGLVPRGSASMSDSEVNQEAKPEVKP
EVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKR
QGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHR
EQIGGQHWSYWLRPGHMAEEGGSLAALTAHQACHLPL
ETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWN
QVDQVIRNALASPGSGGDLGEAIREQPEQARLALTLAA
AESERFVRQGTGNDEAGAEFVWMMGELPIAPVDRLIG
KAGAERVSEQAAKVLAEYLEEYAIEIAKKAVEFARHA
GRKTVKVEDIKLAIKS.

We examined its transfection ability with the plasmid, pEGFP-C1 (green fluorescent plasmid). Using electrophoretic mobility shift assays (EMSAs), immunofluorescence, PCR, immunohistochemistry and enzyme-linked immunosorbent assays (ELISAs) we demonstrated that GPH can transfer pEGFP-C1 into HeLa cells.

Reagents, Plasmids and Cell Lines

The BALB/c mice used for this study were purchased from Changchun Institute of Biological Products Co. Ltd. and bred in-house. They were maintained under controlled temperature (22°C) and lighting (12L:12D), and provided with food and water *ad libitum*. Transfection reagent Lipofectamine 2000 and vector pET SUMO were purchased from Invitrogen (Carlsbad, CA, USA). SUMO protease was prepared in our laboratory. RPMI-1640 medium, fetal calf serum, penicillin and streptomycin were purchased from Gibco BRL (Gaithersburg, MD, USA). PCR product recycling kit, plasmid extraction kit, dNTP, Ex Taq DNA polymerase, T_4 DNA ligase, and DNA Marker were purchased from Takara Company (Dalian, China). Tris, phosphate buffer, imidazole, NaCl, $CaCl_2$ and $MgCl_2$ were purchased from Sigma (St. Louis, MO, USA). Synthesis of PCR primers was undertaken by Takara Company. Chromatography media was purchased from GE (Fairfield, CT, USA). Plasmid pEGFP-C1 was purchased from Biovector Company (Changsha, China). Strain BL21(DE3)/PET26b-PEA (Ia+II)-HphA, *E.coli* JM109, *E.coli* BL21(DE3) and HeLa cells (human cervical carcinoma cell strain) were maintained in our laboratory. Goat anti-mouse HRP-IgG was purchased from the AI Bo Trading Co. (Shanghai, China).

II. Methods and Materials

Construction of GPH Gene

The multifunctional fusion protein GPH contains the DNA sequences of GnRH, domain II of *Pseudomonas aeruginosa* exotoxin A (PEA), and the DNA binding domain of recombinant HPhA. To construct a plasmid encoding GPH, a DNA fragment encoding amino acids of GnRH, domain II of PEA and the DNA binding domain of recombinant HPhA was amplified by PCR using plasmid pET-PEA(Ia+II)-HPhA as template, which includes domain Ia and domain II of PEA, and the DNA binding domain of recombinant HPhA. The two oligonucleotide primer sequences were: forward primer (P1), 5′-CAGCACTGGTCCTACTGGCTGCGTCCGGGTGAGGGC GGCAGCCTG-3′ and reverse primer (P2), 5′-TCAGCTCTTAATAGCGAGC-3′. The whole sequence of GnRH, CAGCACTGGTCCTACTGGCTGCGTCCGGGT, was the upstream sequence of the forward primer. The other part of the forward primer, 5′-GAGGGCGGCAGCCTG-3′, was complementary to domain II of PEA. The reverse primer, 5′-TCAGCTCTTAATAGCGAGC-3′, was complementary to HPhA. The PCR reaction system was 20μl: P1 0.5μl, P2 0.5μl, template 1μl, dNTPs 2μl, 10 × Ex Taq Buffer 2μl, $MgCl_2$ 2μl, ddH_2O 11.8μl, Ex Taq DNA polymerase 0.2μl. Predenaturation at 94°C, 5min; denaturation at 94°C, 40s; annealing 53°C, 50s; extension at 72°C, 60s, 33 cycles; final extension 72°C, 10min.

The amplification product (GnRH-PEAII-HPhA) of 597bp was identified by 1% agarose gel electrophoresis, recovered using a recycling kit and then ligated into the vector pET SUMO by T4 DNA ligase. pET SUMO-GnRH-PEAII-HPhA was transformed by *E.coli* JM109. The positive transformants were picked and pET SUMO-GnRH-PEAII-HPhA then transformed into *E.coli* BL21 (DE3). A plasmid extraction kit was used to extract pET SUMO-GnRH-PEAII-HPhA and its integrity was confirmed by sequencing.

Expression and Purification of GPH

GPH was expressed in *E. coli* BL21 (DE3) grown at 37°C in 5ml LB medium containing 50 μg/ml kanamycin to OD_{600} = 0.5. Then 1ml was transferred to 500ml LB medium containing 50 μg/ml kanamycin and grown to OD_{600} = 0.5. Engineered bacteria were then transferred to a fermenter of 50L. The fermentation parameters were: pH 7.25, dissolved oxygen 30%, tank pressure 0.04MPa, ventilation 2.0, temperature 37°C. When $OD_{600}\approx10$, the culture was cooled to 30°C and IPTG (isopropyl-b-D-thiogalactopyranoside) was added to a final concentration of 1mM. After induction for 5 hours, bacteria were collected and cryopreserved at -20°C.

Bacteria (25g) were weighed and resuspended in 20mM Tris-Cl, pH 8.0, followed by ultrasonication. Cell lysates were centrifuged at 12000 × g at 4°C for 20 min. The supernatant was digested with DNase I (1U/ml) for 30min on ice, centrifuged at 10000 × g at 4°C for 20min. The supernatant was then precipitated with 35% ammonium sulfate, centrifuged at 10000 × g at 4°C for 20 min and the pellet was resuspended in 20mM Tris-Cl, pH 8.5. Metal chelating chromatography media Cu^{2+} (Chelating Sepharose Fast Flow) was used to purify the recombinant GPH protein. It was equilibrated with five column volumes of solution A (flow rate 1ml/min, sample loading, 1ml/min). Solution A contained 20mM Tris-Cl, 20mM imidazole, 0.5M NaCl, pH8.5. Unbound proteins were washed away by solution A. Solution B (20mM Tris-Cl, 40mM imidazole, 0.5M NaCl, pH8.5) was used to elute hybrid proteins. Solution C (20mM Tris-Cl, 300mM imidazole, 0.5M NaCl, pH8.5) was used to elute and collect the target proteins. The target protein was desalted on a Sephadex G-25 Fine column (desalting buffer: 20mM Tris-Cl, 2 mM imidazole, 0.5M NaCl, pH8.5). Desalted proteins were added to 1:100 (w:w) of SUMO protease, digested at 4°C overnight, to remove the His tag sequence expressed from the

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PET SUMO vector. Then metal chelating chromatography medium Cu^{2+} was again used to separate the His tags section. Solution A was used to balance and elute unbound proteins. Solution B was used to elute target proteins. Solution C was used to elute His tags. A Sephadex G-25 Fine column was used to desalt the pure protein GPH. The molecular weight of the fusion GPH protein was 25 Kd for SDS-PAGE detection. The purified GPH was added to 10% glycerol, packaged and stored at -80°C until used.

Cell Culture

HeLa cells were removed from liquid nitrogen, rapidly thawed in a 37°C water-bath and cultured in RPMI-1640 medium containing 10% fetal calf serum, 100U/mL penicillin and 100μg/mL streptomycin in a 5% CO_2 incubator at 37°C. The medium was replaced every 1 to 2 days, cells were digested with 0.1% trypsin and subcultured when confluent.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA, also known as the bandshift assay, has become the standard protocol for determining the potential of a DNA sequence binding to a DNA-binding protein [16.17]. Here, to analyze the ability of GPH to bind to DNA, pEGFP-C1 was used. Molar ratios of recombinant GPH to pEGFP-C1were 0.25:1, 1:1, 4:1, 6:1, and 8:1, respectively. The concentration of plasmid DNA was 17mM, volume was 0.2μl, and the concentration of GTH was 20mM. Volume was altered according to the molar ratio. At room temperature, the mixture of GPH and plasmid DNA was incubated in 20μl ddH_2O in a dark place for 40 min. Then 10μl was loaded onto a 1% agarose gel and electrophoresed for 1h, at the voltage of 2V/cm. When electrophoresis was complete, the gel was immersed in TAE buffer containing ethidium bromide, stained for 20min, visualized and photographed under UV. The control group was treated with plasmid pEGFP-C1 alone without protein GPH, other steps were the same as the experimental group.

Transfection of pEGFP-C1 Mediated by GPH

HeLa cells were cultured in 6-well plates (2ml/well) for 12h until 80% confluency. Recombinant GPH was mixed with pEGFP-C1 at a molar ratio of 4:1 in 100μl ddH_2O containing 10mM Tris-Cl and 150mM NaCl. The DNA-GPH mixture was placed at room temperature in the dark for 40min. Medium was removed from wells and cells were washed three times with serum-free and antibody-free RPMI-1640 medium. The DNA-GPH was mixed with 6mM $CaCl_2$ and 6mM $MgCl_2$ and was added to the cells. Cells were then incubated in serum-free and antibody-free RPMI-1640 medium at 37°C in a 5% CO_2 incubator for 4h. Then serum-free and antibody-free RPMI-1640 medium was replaced by RPMI-1640 complete medium. Cells were cultured and then observed the next day via fluorescence microscopy to detect transfection. Then percentage of cells expressing GFP (green fluorescent protein) was detected by scanning the grayscale value.

In the positive control group, pEGFP-C1 was transfected by general transfection reagent Lipofectamine 2000, strictly according to operating instructions; negative control groups were protein GPH alone and green fluorescent plasmid alone. Their operational methods were the same as the experimental group.

PCR Analysis for Detecting pEGFP-C1 in Cells Transfected with GPH

To detect pEGFP-C1 in transfected cells, genomic DNA was extracted 12h after stable transfection. Groups were pEGFP-C1 with GPH group, pEGFP-C1 with Lipofectamine 2000 transfection reagent positive control group, and pEGFP-C1 alone negative control group. Primers for pEGFP-C1 were: forward primer(P3), 5′-CATGCCATGGTGAGCAAGGGCG-3′; reverse primer (P4), 5′-GCGGATCCCTTGTACAGCTCGTCCATG-3′. The PCR reaction system was 20μl: P3 0.3μl, P4 0.3μl, template 1μl, dNTPs 2μl, 10 × Ex Taq Buffer 2μl, $MgCl_2$ 2μl, ddH_2O 12.2μl, Ex Taq DNA polymerase 0.2μl. Predenaturation at 94°C, 5min; denaturation at 94°C, 50s; annealing at 51°C, 50s; extension at 72°C, 120s, 33 cycles; final extension of 72°C, 10min. The PCR products were identified by 1% agarose gel electrophoresis.

Preparation and Purification of High Titer anti-HPhA Monoclonal Antibody

To obtain a primary antibody for immunohistochemical analysis, a high titer anti-HPhA monoclonal antibody was prepared and purified. Hybridoma cell lines of the anti-HPhA monoclonal antibody were retrieved from liquid nitrogen, rapidly thawed in a 37°C water-bath and cultured in RPMI-1640 medium containing 10% fetal calf serum, 100U/mL penicillin and 100μg/mL streptomycin in a 5% CO_2 incubator at 37°C. At confluency, cells were divided into culture flasks at a ratio of 1:2 or 1:3 and culture continued.

The cells were inoculated in adult BALB/c mice (2.5×10^6cells/mouse) that were pre-injected with paraffin by intraperitoneal injection. After 5 to 7 days, the mice were anesthetized by sodium pentobarbital. Ascites were collected with a disposable syringe and stored at 37°C for 1h, then at 4°C overnight. The next day, the ascites were centrifuged at 10,000 × g at 4°C for 10min. Oil on the surface was removed leaving the ascites supernatant.

After caprylic acid precipitation[18] and ammonium sulfate precipitation[19], the crude antibody was harvested and dialyzed with PBS overnight, during which the PBS was changed three times. The following day, the sample was purified by HiTrap rProtein A FF affinity chromatography. The affinity column was equilibrated with PBS (loading rate 0.8ml/min). PBS was used to wash away any unbound proteins. Target protein was eluted with 100mM Gly/HCl (pH3.5), collected into a 1/10 volume of 1M Tris-Cl (pH8.5) and mixed rapidly. The solution was then brought back to a neutral pH. Purity of antibody was assessed by 12% SDS-PAGE electrophoresis. The antibody was stored in 10% glycerol at -20°C until used.

Immunohistochemical Analysis of GPH-Mediated Transfected Cells

After cells were transfected with pEGFP-C1 and GPH for 4h, they were washed twice with PBS, mixed with 80% glacial

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acetone (1ml/well), fixed at -20°C for 20min and washed three times with PBS. Anti-HPhA monoclonal antibody (1:100) was used as the primary antibody and Goat anti-mouse HRP-IgG (1:2000) as the secondary antibody. DAB was used as a colorimetric substrate. Cells were observed under an optical microscope (400×).

The negative control group was transfected with pEGFP-C1 but without protein GPH. The other steps were the same as the experimental group.

Detection of GPH Binding to GnRHR (Gonadotropin Releasing Hormone Receptor) on the Surface of HeLa Cells by ELISA

GnRH was used as a competitor to block GPH binding to GnRHR, and TRH (thyrotropin releasing hormone) was used as control to compare with GnRH. Experimental HeLa cells were divided into eight groups (2ml/group), the concentration of GnRH was 25μM, 50μM, 75μM, 100μM, 125μM, 150μM, 175μM, and 200μM, respectively (triplicate for each concentration). After HeLa cells were treated with GnRH for 30 min, GPH was added to the wells of each group. The concentration of GPH was 20μM, and cells were treated for 30min. Then the liquid was removed. Protein anti-HPhA was used as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG (IgG-HRP) (1:2000) as the secondary antibody. ODP-H_2O_2 (pH 5.0) was used as a colorimetric substrate. OD values were detected by Xmax (US) at a wavelength of 490 nm. The control groups were the same as the experimental groups except that GnRH was replaced by TRH. All samples were pretreated in triplicate and the experiments were repeated a minimum of three times.

III. Results

Agarose Gel Electrophoresis and Sequencing of GPH Gene

To detect the GPH gene of 597bp, 1% agarose gel electrophoresis and sequencing were performed. Electrophoresis detected a fragment identical to the target fragment, shown in Fig. 1A. The sequenced gene was identical with the expected sequence, shown in Fig. 1B.

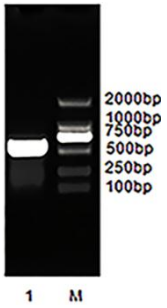

Figure 1. Analysis of GPH gene. A. 1% agarose gel electrophoresis pattern. 1: GPH gene; M: Marker. B. Sequencing result.

SDS-PAGE of Purified GPH

To detect the 25 Kd GPH, SDS-PAGE was performed. The purified GPH gives an identical electrophoresis pattern to that of the target fragment (Fig. 2).

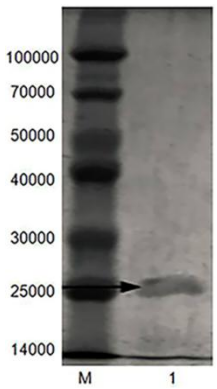

Figure 2. SDS-PAGE electrophoresis pattern of purified GPH. M: Marker; 1: Purified GPH.

Electrophoretic Mobility Shift Assay

To confirm whether GPH binds stably to DNA, EMSA was performed. As shown in Fig. 3, the mobility of pEGFP-C1 was changed according to the molar ratios of GPH to pEGFP-C1. As a control group, lane 1 was treated with pEGFP-C1 alone without GPH. From lane 2 to lane 6, the molar ratios of GPH to pEGFP-C1 were 0.25:1, 1:1, 4:1, 6:1, and 8:1, respectively. We observed that GPH had little effect on the mobility of supercoiled DNA. However, for linear DNA, the higher concentration of GPH, the slower the DNA mobility, confirming that GPH had combined with the plasmid. At the ratio of 4:1, the mobility change was distinct, so we chose 4:1 (protein GPH: plasmid pEGFP-C1) as the molar ratio of cell transfection.

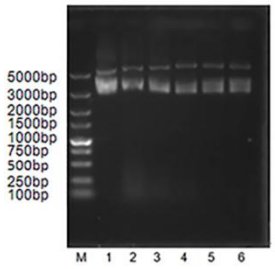

Figure 3. EMSA of different molar ratios of GPH to green fluorescent plasmid. Lane 1: Control group, treated with pEGFP-C1 alone without GPH; From lane 2 to lane6: The molar ratios of GPH to pEGFP-C1 were 0.25:1, 1:1, 4:1, 6:1, and 8:1, respectively.

GPH-Mediated Transfection of pEGFP-C1

To detect GPH binding to and transporting DNA into target cells, pEGFP-C1 was used as a marker of GPH function. A mixture of GPH and pEGFP-C1 was added into HeLa cells. A mixture of Lipofectamine 2000 and pEGFP-C1 was used as a positive control. pEGFP-C1 alone and protein GPH alone were employed as negative controls. Our data showed fluorescence expression in the GPH with pEGFP-C1 transfection group. Also, under a fluorescence microscope we found that HeLa cells appeared healthy. Although the Lipofectamine 2000 with pEGFP-C1 group also demonstrated a good transfection efficiency, there was a great deal of cell death. No fluorescence was observed in the pEGFP-C1 alone, and GPH alone negative control groups (Fig. 4A). The grayscale value also contributed to the results. As shown in

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Fig.4B, the percentage of EGFP expression in the GPH with pEGFP-C1 group was 20% higher than that in the Lipofectamine 2000 with pEGFP-C1 positive control group, while protein GPH alone and pEGFP-C1 alone negative controls showed no fluorescence.

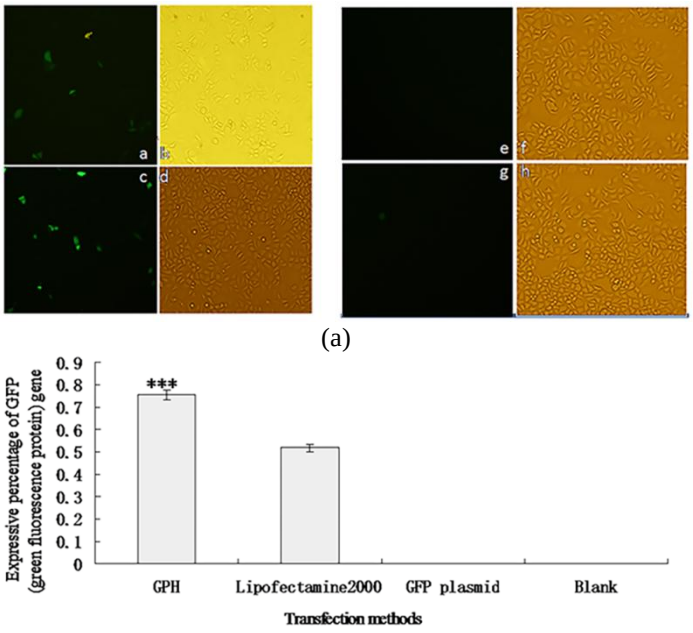

Figure 4. Transfection of pEGFP-C1 mediated by GPH. A. Immunofluorescent detection. a (fluorescence, 100×) and b (visible light, 100×): HeLa cells were transfected with a mixture of GPH and pEGFP-C1. c (fluorescence, 100×) and d (visible light, 100×): HeLa cells were transfected with a mixture of Lipofectamine 2000 and pEGFP-C1. e (fluorescence, 100×) and f (visible light, 100×): HeLa cells were transfected with GPH alone. g (fluorescence, 100×) and h (visible light, 100×): HeLa cells were transfected with the plasmid pEGFP-C1 alone. B. Percentage of GFP expression.

PCR Analysis to Detect pEGFP-C1 in Cells Transfected with GPH

To detect pEGFP-C1 in transfected cells, genomic DNA was extracted. PCR was then performed with the primers for pEGFP-C1. PCR products were identified by 1% agarose gel electrophoresis. The experimental group and the Lipofectamine 2000 positive control group produced clear bands, but the pEGFP-C1 alone negative control group did not produce a clear band (Fig. 5). Our data suggested that both GPH and Lipofectamine 2000 successfully mediated pEGFP-C1 transfer into cells.

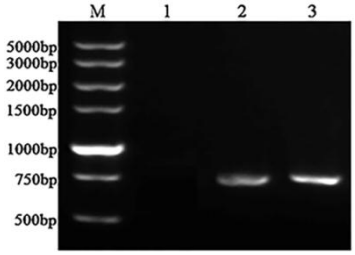

Figure 5. PCR analysis of transfected cells. Lane 1: Negative control group (pEGFP-C1 alone); Lane 2: Experimental group (GPH with pEGFP-C1); Lane 3: The positive control group (Lipofectamine 2000 with pEGFP-C1).

Purification of anti-HPhA Monoclonal Antibody with a High Titer

To prepare a high titer antibody, an anti-HPhA monoclonal antibody was cultured in the peritoneal cavity of immunodeficient BALB/c mice. After caprylic acid precipitation, ammonium sulfate precipitation and HiTrap rProtein A FF affinity chromatography, the purity was close to 100%, as tested by 12% SDS-PAGE electrophoresis (Fig. 6). This anti-HPhA monoclonal antibody can be used as a primary antibody for immunohistochemical analysis.

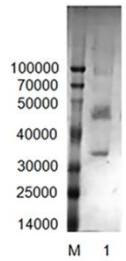

Figure 6. SDS-PAGE electrophoresis pattern of purified anti-HPhA monoclonal antibody. M: Marker; 1: Anti-HPhA monoclonal antibody.

Immunohistochemical analysis of GPH-mediated transfected cells

Immunohistochemical staining detected GPH in cells treated with GPH, suggesting that HPhA in GPH was carried into cells by GnRH and PEA II. However, GPH was not observed in cells treated without GPH. (Fig. 7).

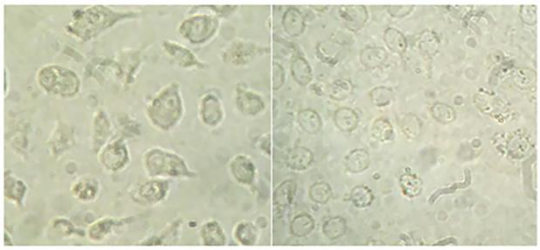

Figure 7. Immunohistochemical detection of GPH in transfected cells. a: GPH treatment group (400×). b: Without GPH treatment group (400×).

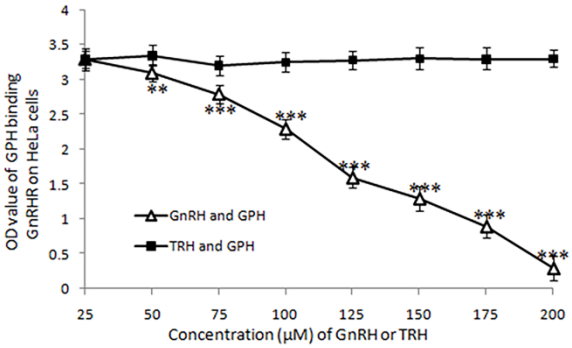

Figure 8. Inhibition curve of competitive binding of GPH, GnRH and TRH to the GnRHR. A significant difference between experimental and control groups (P<0.01andP<0.001) is indicated by "**, and ***". Error bars represent the mean± SEM of three independent experiments.

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Detection of GPH Binding to GnRHR on the Surface of HeLa Cells by ELISA

To detect the binding ability of GPH, a competition test was performed. ELISA was used to detect the OD values of GPH, which indirectly reflected the binding quantities of GPH with GnRH receptors. Fig. 8 shows the inhibition curve of competitive binding of GPH, GnRH and TRH to the HeLa cell membrane GnRHR. With the increase in GnRH concentration, the OD values of GPH binding to GnRHR were reduced. However, in the control group, the OD values of GPH binding to GnRHR on the HeLa cell surface were not affected by the treatment with TRH.

IV. Discussion

Gene therapy was a new promising technique used to treat many incurable diseases and the different strategies used to transfer DNA, taking into account that introducing DNA into the cell nucleus without degradation is essential for the success of this therapeutic technique.[20]Currently, viral vehicles are the most commonly used carriers for delivering DNA and have long been used for their high efficiency. However, these vehicles can trigger dangerous immunological responses, the production in large quantities is very difficult and expensive and the size of gene that can be delivered by the virus is limited. Consequently, the non-viral carriers are being prepared and developed since they are safe, generally causes low immune response, can be prepared easily, at low cost and in large quantities. The earliest non-viral carriers depended on the electrostatic complexation of DNA polyanions with polycations to form polyplexes and lipoplexes. Including electrostatic interaction between DNA and polycations, DNA encapsulation, DNA adsorption. In order to increase their capacity to protect the genes and decrease their size, since small size has positive effects on intracellular delivery, we constructed and expressed a generic recombinant protein named GPH which has the capacity to protect the target genes from degradating by DNase, the ability to transfer DNA stably and safely to improve these non-viral carriers.

The unique features of DNA-condensing capacity and nuclear localization signals (NLSs) make some nuclear proteins, such as histones and high mobility group (HMG) proteins, excellent gene vectors for the delivery of DNA into the cell nucleus[21.22]. In addition, their positive charge promotes binding to anionic molecules and help them to overcome the negative charge of cells that is an important barrier to cellular penetration[23]. Liu et al. had reported histone H1, H2A, H3, H4 and histone analogues as gene delivery vectors[24-31]. We took advantage of the similar characteristics of hyperthermophilic archaea histone HPhA with eukaryotic histones. Besides phylogenetical and structural similarity with the eukaryal histones, archaeal histones share the properties of eukaryal ones: are small, basic, relatively abundant in cytoplasm, toroidally wrap DNA, protect DNA from nuclease digestion, and/or form nucleosome-like structures. Pyrococcus horikoshiiOT3 strain is a hyperthermophilic archaeon with the optimum growth temperature at 98℃.The genome sequence of this organism has been completed at the National Institute of Technology and Evaluation (Tokyo, Japan). The open reading frames PHS051 (HPhA) was putative archaeal histone. The complex of the HPhA and DNA allows DNA to be protected from cleavage by the restriction enzyme TaqI at 65℃. The HPhA has inherited a remarkable thermostability and retained DNA binding activity at extreme temperature.[2]

We joined GnRH, PEAII and HPhA to construct a gene delivery carrier protein, GPH. PEAII is the transmembrane transport domain of *Pseudomonas aeruginosa* exotoxin A, which can perform DNA transfer by transmembrane transport. In recent years, GnRH binding sites have been found in ovarian, endometrial, prostate, pituitary, pancreas and some tumor cells, and GnRH analogues can inhibit the growth of these tumor cells. The tumor suppressive effect of GnRH is achieved by a mechanism independent of the pituitary gonadotropin.[7] GnRH receptors are overexpressed on the surface of certain tumor cells. GnRH plays a role in the guidance to GnRH receptors. It may carry GPH to cells overexpressing GnRH receptors, and then transfer GPH into the cells to perform the function of carrying exogenous DNA.

The expressed GPH contains the N-terminal His6 Tag and a SUMO protease substrate recognition sequence. Using metal chelating chromatography, anion exchange chromatography and Sephadex G-25 Fine column, we increased the purity of the recombinant protein to more than 90%.

To confirm the combination of GPH with DNA and to detect the concentration of GPH to transfect DNA into target cells, EMSA was used. We examined the mobility changes of GPH with pEGFP-C1 to determine the optimal concentration of GPH for pEGFP-C1 transport. The experiments showed that the transport of plasmid pEGFP-C1 into HeLa cells by GPH can be monitored by its fluorescence. PCR showed that pEGFP-C1 was present in cells transfected by GPH. With the anti-HPhA monoclonal antibody, immunohistochemical methods were performed and GPH was observed in transfected cells. With ELISA, GPH bound to GnRHR on the surface of HeLa cells was detected. Furthermore, our cell experiments showed that the transfection mediated by GPH was reliable and stable. Also, the transfer process was simple and convenient to perform.

Thus, we not only extracellularly but also intracellularly confirmed that GPH is a convenient, stable, safe carrier for targeted delivery at both gene and protein levels.

In the current study, we showed that recombinant GPH has the capability of binding to and transporting DNA into target cells. Thus, GPH may be considered as a strong gene carrier candidate and will contribute to the development of gene therapy.

About Animal Experiments

All experiments on live vertebrates were performed in accordance with relevant guidelines and regulations, and the institutional and/or licensing committee approving the experiments. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Shuang Li, Xinhai Yu, Yuan Tian, Zehong Li, Yuhuan Yue, Fuguang Zhao, Guangmou Wu, Xin Zhao, Fei Shi, and Guoli Zhang, "Introduction of Recombinant GnRH, Transmembrane Domain of PEA and Hyperthermophilic Archaea Histone (GPH) as a Novel Carrier for Gene Transfer," *International Research Journal of Pharmacy and Medical Sciences (IRJPMS)*, Volume 1, Issue 2, pp. 14-20, 2018.

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Conflict of interest

The authors declare that they have no conflict of interest.

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