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**Original article**

# Human growth hormone gene expression *in vitro* following rAAV2/1-mediated gene transfer

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***Background:*** Viral vectors can produce longer-lasting effects than a recombinant protein. ***Objective:*** Develop a system of sustained GH1 (a human growth hormone gene) transfer and expression in baby hamster kidney cell (BHK-21) line using recombinant adeno-associated viral vectors pseudotyped with viral capsids from serotype 1 (rAAV2/1).
***Methods:*** The expression of GH1 *in vitro* was examined by reverse transcription polymerase chain reaction and Western blot analysis. Effects of GH1 on cell proliferation were measured by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) experiments. Mice serum IGF-1 and blood glucose, after injection of virus infected BHK-21 cells, were measured by enzyme-linked immunosorbent assays.
***Results:*** rAAV2/1-mediated GH1 gene transfer occurred effectively *in vitro* after 48 hours of transduction at 1 $10^5$ vg/mL (multiplicities of infection). MTT experiments indicated notable effects of the GH1 gene on cell proliferation *in vitro*. Subsequent animal experiment suggested that the injection of virus infected BHK-21 cells might induce increase of serum IGF-1.
***Conclusion:*** Our study shows the feasibility of rAAV2/1-mediated GH gene delivery *in vitro*, applicable for future experimental and clinical investigations.

***Keywords:*** Baby hamster kidney cell 21, growth hormone, insulin-like growth factor 1, recombinant adeno-associated virus vector

Growth hormone (GH) is an anabolic hormone that is synthesized and secreted by the somatotroph cells in the anterior pituitary [1]. It has important effects on growth in long bones, protein, lipid, carbohydrate metabolism, and metabolic functions of the liver [2]. Since recombinant human growth hormone (rhGH) was first manufactured, it has been used in many therapeutic applications, including improving muscle strength, maintaining muscle mass, mildly reducing body fat, body building, or even athletic enhancement [3, 4]. However, the use of recombinant protein products is limited by the need for repeated protein administration and costly production methods [5]. Furthermore, the structural integrity of the recombinant products generated is a major concern that genetic changes in rhGH have been linked to biological inactivates and diseases [6].

Extensive studies have demonstrated that gene therapy is capable of providing a potential solution by allowing frequent injections of expensive recombinant proteins to be replaced by the infrequent, or one-time, delivery of therapeutic genes. Genes may be directly delivered to affected cells where the therapeutic protein will be produced. Alternatively, genes may be delivered to tissues, which serve as sites for the synthesis and secretion of proteins that have effects elsewhere in the body [7, 8].

The key to a successful gene therapy is the vector system, among which adeno-associated virus (aav) has been regarded as the 'ideal' vector system. It shows great promise for human gene therapy owing to its ability to transduce dividing as well as non-dividing cells, and high transduction rates in a wide

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range of tissues. No obvious side-effects have been directly reported in many studies designed to assess the efficacy of recombinant adeno-associated virus (rAAV) vectors. rAAV vectors are generally considered safe [9].

In this study, we developed the rAAV2/1-mediated gene delivery system to introduce a high level of exogenous GH1 (a human growth hormone gene) in BHK-21 cells (a baby hamster kidney cell line). We also detected the change of serum IGF-1 and blood glucose levels in mice after injection of BHK-21 cell suspensions infected by rAAV2/1 containing GH1 gene.

## Materials and methods

### *Construction and production of rAAV2/1 vector containing GH1*

The GH1 gene was cloned from a polymerase chain reaction (PCR) product from the template of the pUC19 plasmid DNA containing the GH1gene (Xinxiang Medical University, Xinxiang, China) using the primers as we described before [10]. The GH1 DNA fragment was digested with *SalI*/EcoRI and inserted into the *SalI*/EcoRI sites of the pSNAV2.0-IRES-enhanced green fluorescent protein (EGFP) vector (AGTC Gene Technology, Beijing, China), designated as the pSNAV2.0-GH1-IRES-EGFP expression vector. Large-scale rAAV2/1 production and purification were performed as described previously [10-13].

### *Transduction of rAAV2/1 vector containing GH1 in BHK-21 cells*

BHK-21 cells were grown on monolayer cultures in Dulbecco's modified Eagle's edium-F-12 medium (DMEM, Gibco, Langley, USA) supplemented with 10% fetal calf 9 serum (Gibco, Langley, USA) and 5% $CO_2$ at 37°C. rAAV2/1 viruses bearing the GH1-containing vectors, at different multiplicities of infection (MOI) ($5.0\times10^3$, $5.0\times10^4$, and $1.0\times10^5$ vg/cell) were used to infect BHK-21 cells. The infected cells were incubated for 24 or 48 hours. Sodium butyrate 5 mM (AGTC Gene Technology, Beijing, China) was used to increase the *in vitro* transfection efficiency of rAAV2/1 vector containing GH1. Expression of the GH1 gene in BHK21 cells at different times post-infection was observed under a fluorescent microscope (Axiovert 200, G ttingen, Germany).

### *Reverse transcription polymerase chain reaction (RT-PCR) analysis*

Total RNA extracted from BHK-21 cells was analyzed in semi-quantitative RT-PCR experiments. Total RNA (5 ng) was subjected to RT-PCR using the primers [10]. Thermal cycling was performed as follows: 94°C for five minutes, 35 cycles at 94°C for 30 seconds, 57°C for 25 seconds and 72°C for 30 seconds, and a final step of 72°C for seven minutes. Six-ml aliquots of the 25-μL final PCR volume were analyzed by electrophoresis on 2.5% agarose gels. All values were normalized to the internal β-actin standard.

### *Western blot analysis*

BHK-21 cells harvested at different times post-infection were solubilized in the T-PER protein extraction reagent (Pierce, Rockford, USA) and the final protein concentrations were determined using a BCA assay (Pierce, Rockford, USA). Appropriate amounts of protein extracts were fractionated by electrophoresis in 12% SDS-polyacrylamide gels and transferred to nylon membranes. Nylon membranes containing transferred proteins were pretreated with 1.0% nonfat dried milk in 50 mM Tris (pH 8.0), and incubated overnight with a primary mouse monoclonal antibody against human growth hormone (1:800 dilution, NeoMarkers, Newmarket, UK). The membranes were washed and incubated with anti-mouse secondary antibodies and hGH immunoreactivity was visualized by exposing x-ray film to blots incubated with ECL reagent (Amersham Biosciences, Piscataway, USA).

### *3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) analysis*

A cell proliferation and cytotoxicity assay kit (Beyotime, shanghai, China) was used to detect proliferation of BHK-21 cells according to the manufacturer's instructions. Cell counting analysis was used to confirm the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assays. BHK-21 cells were seeded into 6-well plates ($5\times10^4$ cells/well) and treated as mentioned above.

Cell numbers were determined by a hemocytometer and cell proliferation rate. The cell proliferation rate was calculated using the equation:

$$\text{Cell proliferation rate (\%)} = [(OD_{\text{test samples}} - OD_{\text{Normal control}})/(OD_{\text{Normal control}} - OD_{\text{blank}})-1] \times 100\%,$$

where $OD_{test\ samples}$, $OD_{Normal\ control}$, and $OD_{blank}$ are the OD value of test samples, normal control, and blank, respectively.

### *Animal experiments*

Animal studies were performed in accordance with guidelines issued by the National Institutes of Health (NIH, Bethesda, USA) and Chinese People's Liberation Army General Hospital for the humane treatment of laboratory animals. Sixteen C57BL/6 male mice (10-12 weeks old) were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy Medical Science and Peking Union Medical College, Beijing. Ten of them were randomly selected and injected intraperitoneally with 0.5 mL BHK-21 cell suspension infected by rAAV2/1-CMV-GH1 (MOI=$1.0\times10^5$). The rest were injected with the same volume normal BHK-21 cell suspension as controls. The mice serum was collected via the aorta 72 hours after injection. At the end of experiments, all mice were killed.

### *Serum blood glucose and IGF-1*

Mice serum glucose level was measured using standard method [14]. The amounts of secreted IGF-1 (insulin-like growth factor 1) in mice serum were quantified using a series of commercial enzyme-linked immunosorbent assay (ELISA) kits. The absorbance was measured at 450 nm in a Bio-Rad Model 450 microplate reader (Bio-Rad Laboratories, Hercules, USA). Each sample was assayed in duplicate.

### *Statistical analysis*

Statistical analyses were performed using SPSS standard version 13.0. Student's t test was performed for analyzing the statistical significance ($p < 0.05$) between control and GH1 gene treated rats. One-way ANOVA was used for multiple comparisons. Data were considered statistically significant at $p < 0.05$.

## Results

### *Construction of rAAV2/1- CMV-GH1 vector*

We cloned the GH1 gene segment (677 bp, including the 651-bp cds sequence) and constructed the pSNAV2.0-GH1-IRES-EGFP expression vector, and successfully viruses bearing the rAAV2/1 vector containing the GH1 gene. **Figure 1** shows gene structure of the rAAV2 vector.

The reconstructed plasmid was confirmed by restriction enzymes, PCR and sequencing. The viruses bearing the expression construct were purified and diluted to about $1.0\times10^{12}$ vg/mL for the study.

### *In vitro* transudction of rAAV2/1-CMV-GH1 virtual particles

BHK-21 cells at greater than 50% confluence were infected with rAAV2/1 virus containing the GH1 expression vector at different MOI, and cultured in serum-free medium for 24 to 48 hours. Green fluorescence was detected under the fluorescence microscope. Strong signals were observed 48 hours post-infection in the rAAV2/1-transfected cells at $1.0\times10^5$ vg/mL (MOI) (**Figure 2**).

**Figure 3** shows the relative levels of GH1 mRNA expression and hGH expression in BHK21 cells analyzed by RT-PCR and Western blot. Interestingly, GH1 mRNA was detected 24 hours post-infection (**A)**, and the presence of the 22 kDa secreted hGH protein at different times post-infection (**B**).

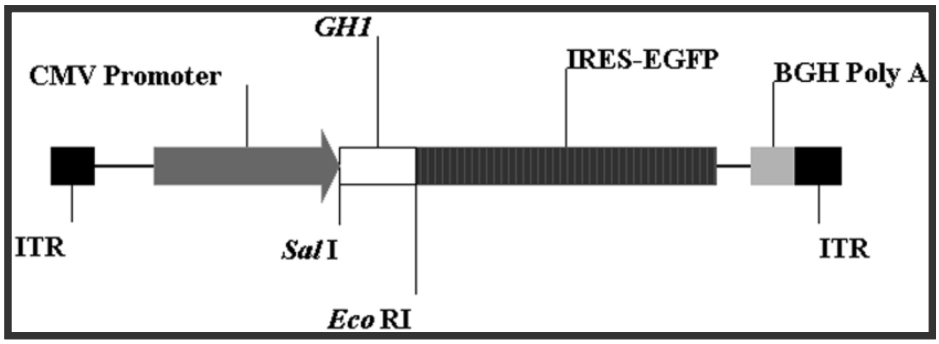


**Figure 1.** Gene structure of the rAAV2 vector containing the GH1 gene. ITR=inverted terminal repeat. The expression cassette containing the GH1 gene and EGFP gene (reporter gene) controlled by the CMV promoter and BGH poly (A) tract is flanked by AAV2/1 inverted terminal repeats.

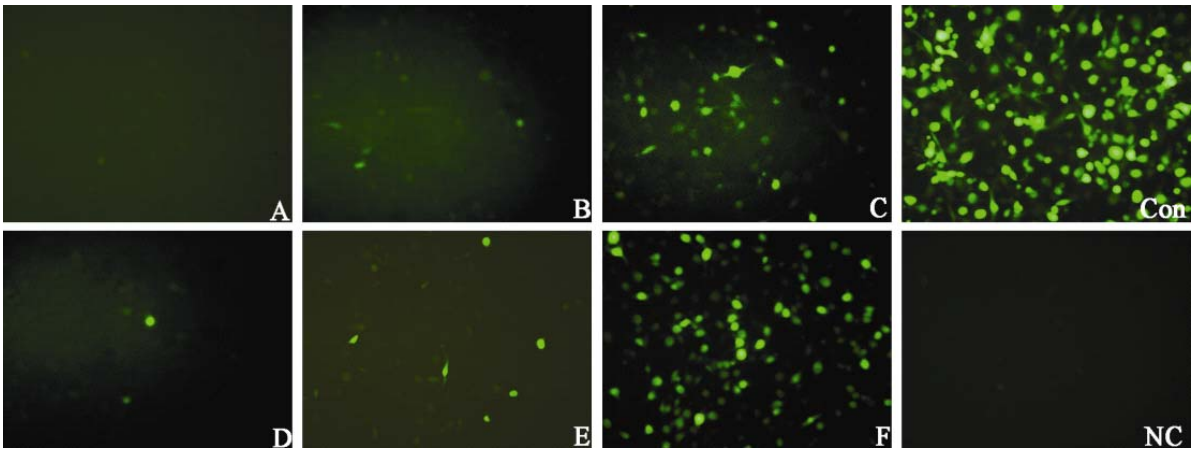


**Figure 2.** Fluorescence signals in the BHK21 cells at different MOI 24 or 48 hours after transduction. **A**: 24 hours post-infection (pi) at $5\times10^3$ vg/mL; **B**: 24 hours pi at $5\times10^4$ vg/mL, **C**: 24 hours pi at $1\times10^5$ vg/mlL, **D**: 48 hours pi at $5\times10^3$ vg/mL, **E**: 48 hours pi at $5\times10^4$ vg/mL; **F**: 48 hours pi at $1\times10^5$ vg/mL, **Con:** control (BHK-21 cells transfected with rAAV2 virus containing an EGFP expression vector 48 hours pi), **NC**: negative control.

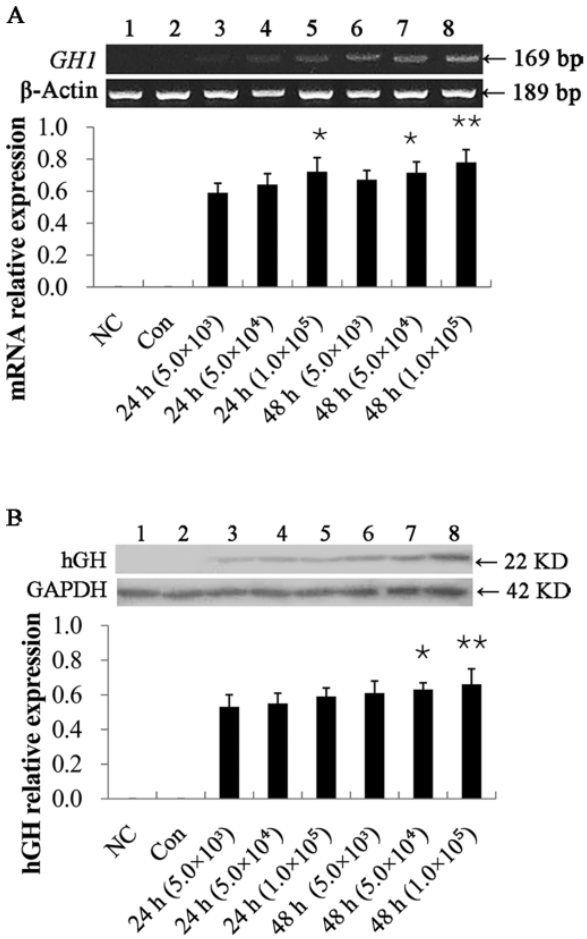


**Figure 3.** The relative levels of GH1 mRNA expression (**A**) and hGH expression **(B)** in BHK21 cells at different MOI, 24 or 48 hours after transduction. *p <0.05, **p <0.01, relative to GH1 gene expression in BHK21 cells 24 hours after transduction (MOI=$5\times10^3$ vg/mL). **1:** NC: negative control, **2**: Con: control (BHK-21 cells transfected with rAAV2 virus containing an EGFP expression vector 48 hours post-infection (ip)), **3**: 24 hours pi at $5\times10^3$ vg/mL; 4: 24 hours pi at $5\times10^4$ vg/mL; **5**: 24 hours pi at $1\times10^5$ vg/mL; **6**: 48 hours pi at $5\times10^3$ vg/mL; 7: 48 hours pi at $5\times10^4$ vg/mL; **8**: 48 hours pi at $1\times10^5$ vg/mL.

### *MTT experiments*

**Figure 4** shows the effects of rAAV2/1-mediated GH1 gene transfer on cell proliferation (**A**) and cell number (**B**). Interestingly, the effect of GH1 on cell proliferation was markable. Cell proliferation did not significantly increase in the first 24 hours post-infection at $5.0\times10^3$ vg/mL (MOI). However, there was a statistically significant proliferative effect on BHK-21 cells at $5.0\times10^4$ vg/mL (MOI). The difference was extremely significant at $5.0\times10^4$ vg/mL and $1.0\times10^5$ vg/mL (MOI) 48 hours post-infection. The proliferative effect of GH1 on cell proliferation was calculated as 20.4% (MOI=$5.0\times10^3$ vg/mL), 27.9% (MOI=$5.0\times10^4$ vg/mL), and 44.9% (MOI=$1.0\times10^5$ vg/mL) at 48 hours post-infection.

### *Serum Glucose and IGF-1 in mice*

**Figure 5** shows comparison of serum glucose and IGF-1 level between the GH1-treated and control mice. We note that the serum levels were elevated in animals injected with BHK-21 cell suspensions infected by rAAV2/1-CMV-GH1 than that in control mice but they were not significant. The mice serum concentrations of IGF-1 increased after injection of BHK-21 cell suspensions infected by rAAV2/1 virus containing GH1 expression vector increased obviously at the 72 hours after injection compared with the control mice. Single injection of BHK-21 cell suspension infected by rAAV2/1-CMV-GH1 in mice increased the serum levels of IGF-1.

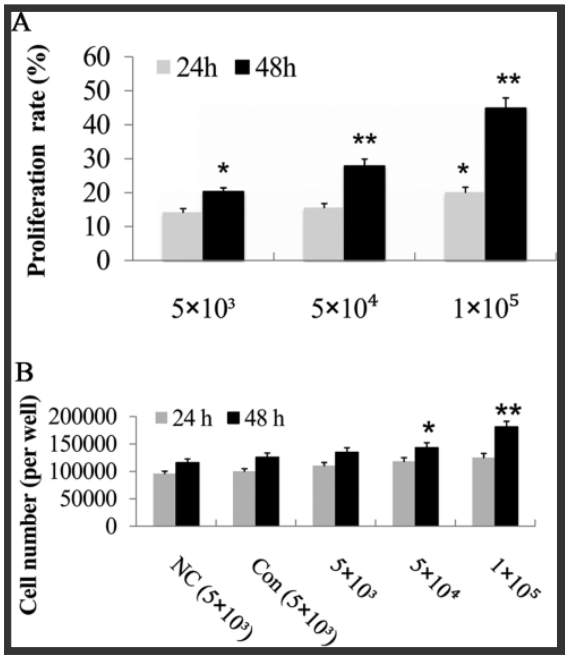


**Figure 4.** The effects of rAAV2/1-mediated GH1 gene transfer on cell proliferation (**A**) and cell number (**B**) at different MOI, 24 or 48 hours after transduction. *p <0.05, **p <0.01, relative to the level of cell proliferation 24 hours after transduction (MOI=$5\times10^3$ vg/mL). NC=negative control, Con=control.

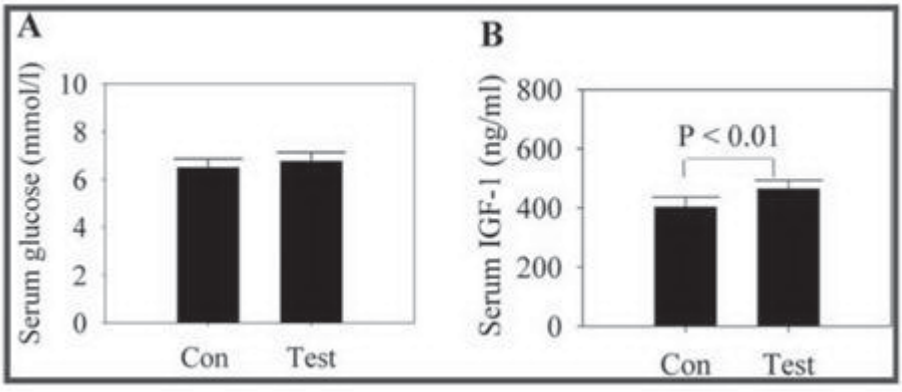


**Figure 5.** Changes in serum glucose (**A**) and IGF-1 level (**B**) between the GH1-treated (Test) and control mice (Con).

## Discussion

Recombinant GH therapy has been approved both for the child and adult GH deficient (GHD) and the AIDS-associated wasting [15]. In the present experiment, instead of injection of recombinant GH, we used GH gene delivery technique because it could provide a better long-term therapeutic effect than recombinant GH [16, 17].

Previous studies have suggested the existence of transduction delay primarily due to the uncoating efficacy of vector genomes [18]. For this transduction delay, the transduction efficiency of mammalian cells with rAAVs *in vitro* is rather low. In this study, 5 mM sodium butyrate was used to increase the *in vitro* transfection efficiency of rAAV2/1 vector containing GH1. At the MOI of $1.0\times10^5$ vg/cell, we achieved highly efficient GH1 gene transduction via rAAV2/1 in BHK-21 cells at 48 hours post-infection. The transduction efficiency is positively correlated with the MOI of the viral vector and transduction time. The MTT experiments suggested that GH1 has the remarkably proliferative effects on cell proliferation, which also followed the increase of rAAV2/1-CMV-GH1 concentration and transduction time.

GH participated in substrate metablism either directly or indirectly via insulin-like growth factor-1 (IGF-1) or the antagonism of insulin action *in vivo* [19]. GH is a principal regulator of circulation and tissue levels of IGF-1. Increases in GH concentration are generally accompanied by rises in circulating IGF-1 level [20]. In our study, the injection of BHK-21 cell suspensions transduced by rAAV2/1-CMV-GH1 viral particles induced the significant increase of serum IGF-1 in normal mice. However, the blood glucose was also elevated following the injection, which suggested the potential risk of insulin resistance *in vivo* due to the injection of cell suspensions infected by rAAV2/1 containing GH1 gene.

In conclusion, exogenous GH1 gene expression in BHK-21 cells had notable effects on cell proliferation. The injection of cell suspensions of transdcution induced the significant elevation of serum IGF-1. The gene delivery system can be used to investigate the biological functions, to evaluate the therapeutic efficacy, and to study the transcriptional regulation of GH1 and any gene of interest in vitro and in vivo. However, the increase in blood glucose suggested the potential risk of insulin resistance in vivo after GH gene therapy.

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