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Adeno-associated Virus Serotype 2 Mediated Transduction and Expression of The Human β-Globin Gene in Human Early Fetal Liver Hematopoietic Cells *

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Abstract β-Thalassemia is an inheritance anaemia disease due to the defect in β- globin gene. Gene therapy is considered to be the only method which could cure this disease. Adeno-associated virus type 2 (AAV2) has benn gaining more attention as a vector in human gene therapy for its non pathogenic character and broad host range. Although, the efficacy of recombinant AAV2 (rAAV2) in transducing human hematopoietic stem cells has been investigated by researchers, the results were varied from different laboratory. The view was proposed recently that it may be resulted from helper virus in their packaging system. Respecting this, the packaging system without helper virus was used to produce rAAV2. Human early fetal liver hematopoietic cells not only possess many superior peculiarity compared to hematopoietic cells of bone marrow or cord blood, but also the inherent β-globin gene in the cells is not expressed. Studies on the AAV2 transduction of human fetal liver hematopoietic cells and mediated expression of β- globin gene *in vivo* were performed and the potential role of AAV2 in β-thalassemia gene therapy was analyzed. The rAAV2 containing a normal human β-globin gene (rAAV2-β-globin) without helper virus contamination were produced. The viral titer, purity and the ability of mediating expression of β-globin gene were detected *in vitro*. Then, human early fetal liver hematopoietic cells were isolated and were further transducted with the rAAV2-β-globin, followed by transplantation into sublethally irradiated BALB/C nude mice to analyze the β-globin gene expression. The results showed that the high titer and purity of rAAV2-β-globin had the ability of mediating β-globin gene expression *in vitro*. In 8 recipient BALB/C nude mice, the β-globin gene expression were detected in the 2 mice marrow by RT-PCR. The results suggested that rAAV2 could transduce human fetal liver hematopoietic cells and mediate the β-globin gene expression in BALB/C nude mice, meanwhile the expression level of the gene was still rather low. It is necessary to perform further research on AAV2 biology before applying in β-thalassemia gene therapy.

Key words Adeno-associated virus type 2, β-globin gene, fetal liver hematopoietic cells, expression

Decrease or cessation of the synthesis of β-globin polypeptide caused by deletions or mutations in β-globin gene cluster results in β-thalassemia. Over the world, β-thalassemia holds the highest disease incidence among single gene inheritance diseases[1]. Up to the present, no satisfactory strategies have been found for the effective treatment of β-thalassemia, and gene therapy is the only hopeful approach to cure this disease theoretically. The replacement of normal human β-globin genes into the hematopoietic stem cells of β-thalassemia patients through viral vector is a promising therapy for the future[2].

AAV2 is deemed the most safe viral vector and possess broad host range[3,4], which resulted in its wide use in transducing multiple cell types on the study of gene therapy. Previous studies showed that the efficacy of AAV2 vectors in transducing hematopoietic stem cells existed controversies. Zhou *et al*[5]. first reported successful transduction of human $CD34^+$ cells by recombinant AAV2 vectors. While Alexander *et al*[6]. failed to observe efficient infection of these cells, and

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presumed the reported transduction had been pseudotransduction mediated by helper virus contaminants in the vector stocks. Helper virus such as adenovirus or herpesvirus have been adopted in classic methods for packaging rAAV2, but they were hard to get rid of absolutely even many kinds of means were exerted. When the vector stock contaminated with helper virus was used to transfect cells, the transduction efficiency of AAV2 could be markedly raised because of co-infection with the helper virus. Moreover, helper virus can reduplicate itself in these cells with the result that the cells could be spallated. So it is necessary to avoid helper virus contamination in rAAV2 production for safety in human gene therapy. By using AAV2 Helper-Free System packaging, and a modified purification method developed by Wu *et al.*[7], we obtained high titer and purity rAAV2-β-globin without helper virus contamination.

K562 cells, a human erythorleukemia cell line, is considered an ideal cell model for study of β-globin gene expression *in vitro*, which was due to that the β-globin gene exists in K562 cells, but is not expressed. Accordingly, the expression of β-globin gene mediated by rAAV2-β-globin was detected in K562 cells. Additionally, the effect of Hydroxyurea (HU) on increasing transduction efficiency was analyzed.

Previously, the researchers adopted bone marrow hematopoietic cells of rhesus or mice as target cells to study the β-globin gene expression *in vivo*[8~11]. To date, there are no human fetal liver hematopoietic cells to be reported for rAAV2 *ex vivo* gene transfer. The β-globin gene in early fetal is not expressed and the fetal liver hematopoietic stem cells (HSCs) have the unique biologic characteristics which are different from bone marrow HSCs[12,13]. In this study , we use human fetal liver hematopoietic cells as target cells for rAAV2 *ex vivo* gene transfer. The results showed that the β-globin gene expression was detected by RT-PCR in the 2 mice bone marrow among 8 recipient BALB/C nude mice, suggested that rAAV2 could transduce human fetal liver hematopoietic cells and mediate β-globin gene expression *in vivo*, but the level of β-globin gene expression was low. This indicated that the biological character of rAAV2 should be further researched in order to apply for gene therapy of β- thalassemia.

1 Materials and methods

1.1 Cells and plasmids

Human erythroleukemia cell line K562, was storaged in our laboratory and maintained as suspension culture in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Human embryonic kidney cells (HEK 293) was obtained from Cell Culture Center of Xiangya Medical School and maintained as monolayer culture in DMEM with 10% FBS.

The construction of a recombinant AAV plasmid containing the β-globin gene (pMT2) had been described in our previous work[10]. Plasmid pAAV2-RC and pHelper were bought from Strategene company (USA). Plasmid pAAV2-RC contained AAV rep and cap genes. Plasmid pHelper contained adenovirus-derived genes (i.e. E2A, E4, and VARNA gene).

1.2 Preparation and purification of rAAV2-β-globin

293 cells were co-transfected with three plasmid pAAV2-RC, pHelper and pAAV2-β-globin by the calcium phosphate method. After incubation 65 to 72 h at 37℃ and 5% CO_2, the cells were harvested and stored at −70℃.

The viral purified method refered to Wu *et al.*[7] Briefly, post-transfected 293 cells were treated with chloroform, then precipitated by PEG/NaCl and drew with chloroform again. Superstratum water phase was suctioned under sterile condition, which was recombinant virus by concintrated and purified. The viral titer and purity were then determined by dot- blot and SDS-10% PAGE assay as described below.

1.3 Determine the purity of the recombinant by SDS-PAGE

Purified rAAV samples were analyzed on SDS-PAGE according to Molecular Cloning. The samples were mixed with sample buffer and boiled at 98℃ for 5 min before loading.

1.4 Quantify the viral titer by dot-blot

Viral titers were determined using quantitative dot-blot analysis. Viral particles were disrupted , viral DNA as well as plasmid DNA controls were denatured in 0.5 mol/L NaOH at 65℃ . The β-globin gene fragment was used as probe and labeled by Dig-dCTP. Two-fold serial dilutions of the virus were loaded onto a nylon membrane. Having been prehybridized for 2 h, filters were hybridized at 68℃ for overnight. The

procedures after hybridization were performed according to Rocho Company instruction.

1.5 Electron microscopy

Recombinant virions purified on chloroform were negatively stained with 3% phosphotungstic acid (pH 6.0) and visualized at a magnification of 380 000 with a transmission electron microscope.

1.6 Transduction of the K562 cells with recombinant AAV2-β-globin vectors *in vitro*

K562 cells were pretreated with 15 mmol/L hydroxyurea (HU) or not, following infected by the recombinant AAV2-β-globin vector for 2 h at 37℃ at a multiplicity of infection (m.o.i.) of 50. Cells as control were mock infected. After infection for 48 h, 2×10^6 cells infected or mock infected were analyzed for presence and expression of the β-globin gene by PCR and RT-PCR.

1.7 Isolation of the human early fetal liver hematopoietic cells

Human fetal livers were obtained from 11-week-old aborted fetuses and their use was approved by donators and families. Single-cell suspension was prepared in Iscove's-modified Dulbecco's medium containing 2% fetal bovine serum. Low densities of mononuclear cells were isolated by centrifugation on Ficoll-Hypaque density gradients.

1.8 Transduction of the human early fetal liver hematopoietic cells with recombinant AAV2-β-globin vectors and transplantation into recipient mice

Human fetal liver mononuclear cells with 15 mmol/L HU pretreatment were infected with the recombinant AAV2-β-globin vector at a multiplicity of infection (m.o.i.) of 50 or mock infected for 2 h at 37℃. Following infection, cells were resuspended in Iscove's-modified Dulbecco's medium and injected intravenously via the tail vein into 8 BALB/C nude recipient mice at 6×10^6 per animal respectively, that had been sublethally irradiated with total body irradiation using 2 Gy X-ray (3 Gy/min). Bone marrow cells of the recipient were analyzed for presence and expression of the β-globin gene on 5 th, 10 th,15 th day post-transplantation, respectively.

1.9 Polymerase chain reaction (PCR) assays

DNA samples were prepared from K562cells with recombinant AAV2-β-globin vector-infected or mock-infected and analyzed following PCR amplification. The primer pair for the transduced human β-globin gene sequence included forward primer, 5′ GTGCTCGGTGCCTTTAGT 3′, specific for exon 2 coding sequence, and reverse primer, 5′ ACACCAGCCACCACTTTC 3′, specific for exon 3 coding sequence of human β-globin gene. The DNA products from the human β-globin gene amplification reactions were analyzed on 2% polyacrylamide gels.

1.10 Reverse transcription (RT) -PCR assays

Total RNA was isolated from K562 cells and bone marrow cells from each recipient mice. RNA were reverse transcribed by random primers (Stratagene) at 42℃ for 1 h, following which the cDNA was subjected to PCR analysis with the primer pair specific for human β-globin gene sequence described above. The integrity of the reverse-transcribed RNA was determined by amplification of the endogenous mouse β-actin mRNA transcripts with the specific primer . The primer pair specific for the endogenous mouse β-actin gene, 5′ TCCTCTTCC-TCCCTGGAGAA 3′ and 5′ GCTGATCCACATCT-GCTGGA 3′, yielded a 354 bp amplification product. The evidence of the human hematopoietic cells transplanting into recipient mice was provided by amplification of the endogenous human β-actin mRNA transcripts with the specific primer. The primers for amplifying the endogenous mouse β-globin mRNA transcripts were 5′ CCCAGCGG-TACTTTGATAGC 3′ and 5′ AATCCTTGCCAAG-GTGGTGG 3′.

2 Results

2.1 Identification of recombinant pMT2

The recombinant AAV2-β-globin vector used in these studies was generated as described under **Materials and methods** and is depicted schematically in Figure 1. The plasmid pMT2 is digested with *Eco*RⅠ, 1% agarose gel electrophoresis showed that the sizes of pMT2 segments were 5.9 kb and 1.4 kb, respectively, indicating that the recombinant AAV2 plasmids had been constructed correctly (Figure 1).

2.2 Determine the purity of the recombinant AAV2-β-globin by SDS-PAGE

The AAV2 capsid consists of three proteins, VP1, VP2, and VP3, which have molecular masses of 87, 72 and 62 ku, at an estimated ratio of 1∶1∶10. It was visible clearly that there are three protein bands on the PAGE gel, indicating VP1, VP2, VP3 of AAV2 (Figure 2).

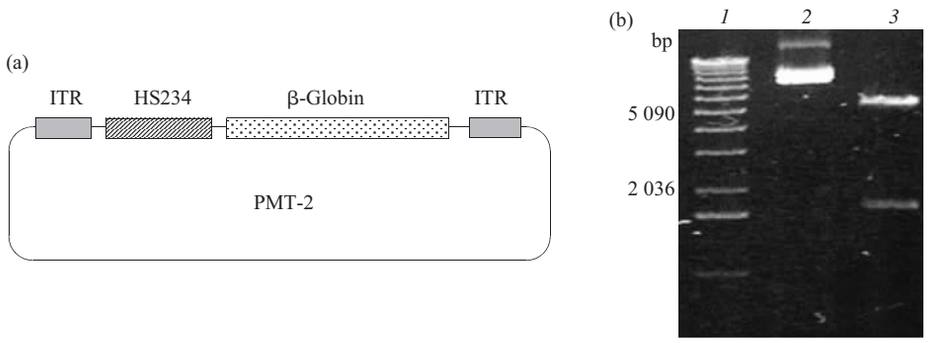

Fig. 1 The structure of recombinant AAV2-β-globin vector and restriction enzyme digestion analysis
(a) Structure of the rAAV2-β-globin vector plasmid. (b) Restriction enzyme digestion analysis of recombinant plasmid. *1*: DNA marker; *2*: Plasmid pMT2; *3*: pMT2 digested with *Eco*RⅠ.

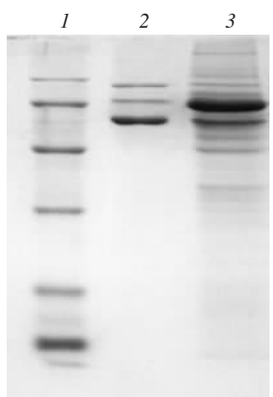

Fig. 2 SDS-PAGE analysis for purity of recombinant AAV2-β-globin
1: Protein marker; *2*: Purified AAV2(VP1, VP2, and VP3 by turns from above to below); *3*: AAV2 stock solution.

2.3 Quantify the viral titer by DNA dot-blot

Viral titer was quantified by DNA dot-blot analysis. The recombinant AAV2-β-globin titers was 3.2×10^{12} virus particles /ml (Figure 3).

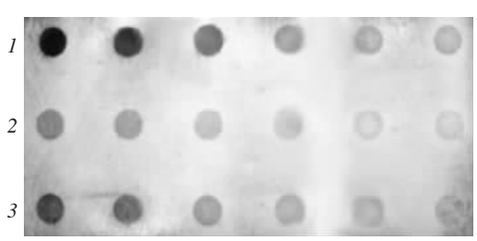

Fig. 3 DNA dot blot analysis for titer of recombinant AAV2-β-globin
Two-fold serial dilutions of equivalent amounts of viral stocks were analyzed with a Dig-dCTP labeled probe as described in **Materials and methods**. *1*: Purified β-globin fragment dilutions from 5 ng; *2*: Purified β-globin fragment dilutions from 0.5 ng; *3*: rAAV2-β-globin dilutions from 1 μl viral stock.

2.4 Electron microscopy analysis of purified rAAV2-β-globin

Recombinant virions purified on chloroform were negatively stained with 3% phosphotungstic acid (pH 6.0) and visualized with a transmission electron microscope, as previously described. The results showed that there were high titer and high purity recombinant virions in end products purified, the presence of virus particles exhibited icosahedral structures, and empty capsids were only rarely detected (Figure 4).

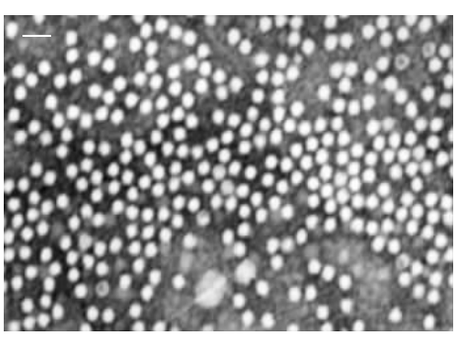

Fig. 4 Electron microscopic image of recombinant particles
The recombinant virions were purified as described in **Materials and methods**. Samples were negatively stained with 3% phosphotungstic acid (pH 6.0), and the particles were visualized at a magnification of 38 000 with a transmission 400 electron microscope (bar = 50 nm).

2.5 Presence and expression of the β-globin gene in K562 cells *in vitro*

Genomic DNA and total RNA isolated from three groups K562 cells were detected by PCR and RT-PCR analysis, respectively. The products of 1 063 bp fragment for β-globin gene were amplified from all three group cells (Figure 5).

2.6 Expression of the human β-globin gene in the human early fetal liver hematopoietic cells *in vivo*

The transcriptionally activity of the transferred human β-globin gene *in vivo* was detected. Total RNA isolated from bone marrow cells obtained

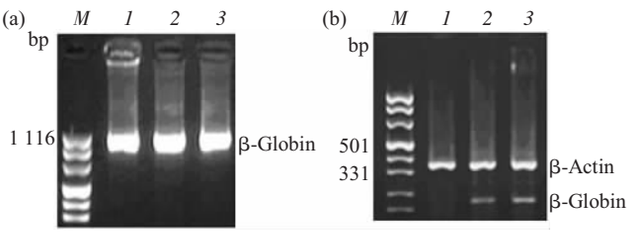

Fig. 5　Analyze the existence and the expression of β-globin gene in K562 cells using PCR and RT-PCR

(a) PCR analysis. (b) RT-PCR analysis. *M*: Puc Mix8 DNA marker; *1*: K562 cells without rAAV2-β-globin transfection; *2*: rAAV2-β-globin transfected K562 cells without HU pretreatment; *3*: rAAV2-β-globin transfected K562 cells with HU pretreatment.

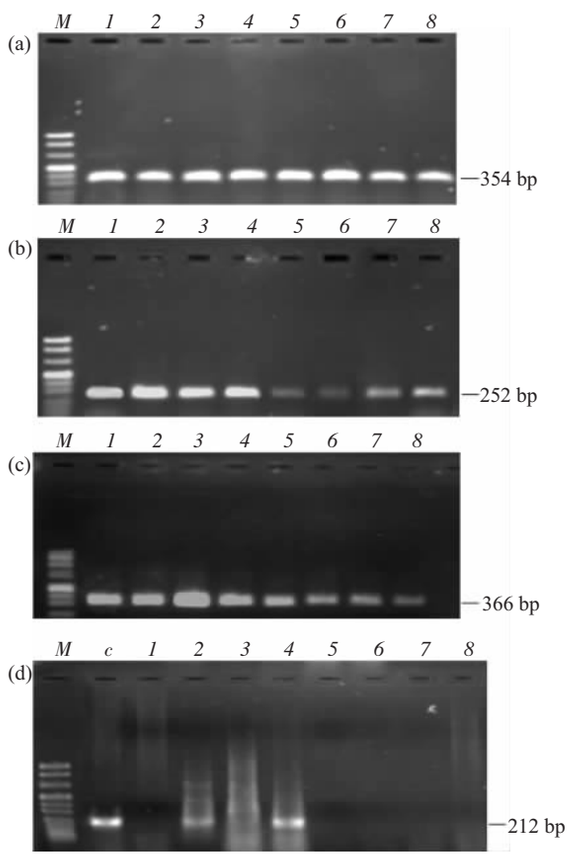

Fig. 6　RT-PCR analysis of the expression of the transduced human β-globin gene in bone marrow cells from recipient and control mice

The sizes of the RT-PCR products from the human and the mouse β-globin gene are 212 bp and 252 bp, respectively, and that from the human and the mouse β-actin gene are 366 bp and 354 bp, respectively. (a) The mouse β-actin gene transcripts; (b) The mouse β-globin gene transcripts; (c) The human β-actin gene transcripts; (d) The human β-globin gene transcripts. *M*: DNA marker; *c*: rAAV2-β-globin transfected K562 cells; *1*, *5*: Bone marrow cells of control mice; *2*～*4*: Bone marrow cells of the first set recipient animals detected on the 5 th, 10 th, 15 th day, respectively; 6～8: Bone marrow cells of the second set recipient mice detected on the 5 th, 10 th, 15 th day, respectively.

post-transplantation was subjected to RT-PCR analyses. Primers for PCR, following reverse transcription, were designed in such a way as to eliminate amplification of the mouse endogenous β-globin mRNA, as well as to distinguish amplification products from the transduced viral sequences. The RT reactions were performed using primers specific for the mouse β-actin gene as well to ensure the quality and structural integrity of RNA preparations, and primers specific for the human β-actin gene were used for evidence that human hematopoietic cells homing into BM of recipient mice as described under **Materials and methods** (Figure 6). As it can be seen, the quality of RNA was confirmed to be good using the mouse β-actin gene transcript- specific primer pair (Figure 6a). The mouse β-globin transcripts and the human β-actin gene transcripts were present in all bone marrow cells from recipient mice transplanted with the donor cells(Figure 6b, 6c). the human β-globin transcripts were present only in bone marrow cells from 2 recipient mice transplanted with the rAAV2-β-globin-transduced donor cells (Figure 6d).

3　Discussion

β-Thalassemia is a single gene inheritance disease due to the defect in β-globin gene. It has been considered that β-thalassemia is an ideal model for gene therapy research, because the localization, structure and regulation mechanism of β-globin gene have been identified. Remarkable advances have been achieved on the basis of retrovirus and lentiviral vector-mediated gene transfer into HSCs[14,15]. Although these vectors offer advantages of long-term expression, high capacity and low antivector immunity, but safety issues related to the integrating nature of these vectors caused some serious adverse events in the X-SCID trial using MLV-based vectors[16].

The AAV2-based vectors have gained attention as an alternative to the more commonly used retrovirus- and adenovirus-based vectors primarily because of the non-pathogenic nature of the wild-type (wt) AAV, and shown promise for gene therapy of cystic fibrosis and hemophilia B[17,18]. Many attempts of AAV2-mediated gene transference into hematopoietic cells *in vitro* and *ex vivo* had also been performed. But controversies exist with regard to their utility as a vector for gene transfer into human HSCs. The contaminants of helper

virus in rAAV2 virus stocks were considered as one of important reasons generally[19]. In our present study, we generated high-titer, purified virus using AAV2 Helper-Free Packaging System and a modified purification method developed by Wu *et al*. The plasmid pHelper containing the VA, E2a and E4 genes from the Ad genome was used to instead of helper virus to package the rAAV2, thereby the contamination of Ad was completely avoided. The purification method for AAV2 adopted here was proved an alternative to the established protocols, such as time consuming $CsCL_2$ gradient centrifugation, expensive iodixanol/heparin, HPLC or single-step column purification[20,21].

To investigate rAAV2 mediated β-globin gene expression, K562 cells were transfected with rAAV2-β-globin, PCR and RT-PCR were used to analyze β-globin gene expression. The results showed that the endogenous β-globin gene existed in K562 cells, but was not expressed. Our results were coincided with Maryann *et al*[22]. Meanwhile, rAAV2 mediated exogenous β-globin gene was expressed in K562 cells, and the expression level can be improved with HU pretreatment. HU was presumed that it could improve the rAAV2 transgene expression efficiency by catalyzing viral second-strand DNA synthesis and facilitating nuclear transport in previous reports[23,24].

Subsequently, the function of the vectors *in vivo* was investigated, we adopted human fetal liver hematopoietic cells to transduce and transplant without any selection in gender sublethally irradiated recipient BALB/C nude mice. Human fetal liver hematopoietic cells have the unique character that γ-globin gene instead of β-globin gene is expressed in their early stage. Therefore, they can be used to detect the ectogenous β-globin gene expression *in vivo*. It was also reported that human fetal liver contains high numbers of erythroid precursors as well as transplantable HSCs. The HSCs generated more erythroid progenitors *in vivo* than HSCs of cord blood or adult bone marrow [13], which suggested that the transduced β-globin gene expression was easier detected in human fetal liver hematopoietic cells. The results showed that the β-globin gene was expressed in 2 recipient BALB/C nude mice. They were detected on the 5 th, 15 th day post-transplantation by RT-PCR analyses, respectively, but no expression of human β-globin gene were detected in recipient mice on the 10 th day, which may relate with that the transducted cells were not homogeneous HSCs. Additionally the individual difference of recipient mice may be one of reasons. At 5 to 15 day after transplantation, peripheral blood was collected from transduced and mock-transduced mice. Hemolysates were prepared and human β-globin protein levels were analyzed after transplantation, using cellulose acetate gel electrophoresis. Unfortunately, the results showed there are no difference between transduced and mock-transduced mice (data was not shown), which suggested that the β-globin expression level was very low resulted from failure to detected it by the rough method. In this experiment, the monitoring time post-transplantation was limited only in 2 weeks, in case the switch from the production of fetal hemoglobin to that of adult hemoglobin in fetal liver hematopoietic cells happened.

In conclusion, our results showed that rAAV2 could transducted human fetal liver hematopoietic cells and mediate β-globin gene expression *in vivo*, meanwhile the expression level of the gene was still rather low. Results from many study for AAV transduction showed that the expression level of transduced gene mediated by AAV2 was lower than adenovirus or herpes simplex virus. It may related to AAV's biological characteristics and life cycle. The appropriate receptor/co-receptor expression on cell surface are necessary for AAV binding and entry cells, intracellular trafficking of AAV to the nucleus, and conversion from single stranded AAV genome to transcriptionally-active double-stranded are required for high-efficiency transduction of cells by AAV vectors. And the low efficient expression of β-globin gene in primary hematopoietic cells mediated by AAV2 vectors might be limited on the basis of that mentioned above. In spite of HU facilitating nuclear transport of AAV2, most of the virions failed to undergo uncoating, thereby leading to only a partial improvement in viral second- strand DNA synthesis and transgene expression [24]. Therefore, a more complete understanding of the virus-host cell interactions will contribute to the development of additional strategies to achieve high-efficiency transduction of these cells by AAV vectors, and will contribute to developing rAAV2 as a safe and effective gene transfer vehicle for human gene therapy.

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2型腺相关病毒转导人胎肝造血细胞及其介导的β珠蛋白基因的表达*

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摘要 β地中海贫血是一种因β珠蛋白基因缺陷导致的遗传性贫血性疾病，基因治疗是唯一有望治愈该病的方法. 2型腺相关病毒(adeno-associated virus type 2，AAV2)是一种非致病性病毒，作为一种基因治疗载体，其应用潜力日益受到关注. 目前还未见AAV2转导人早期胎肝造血细胞及其介导β珠蛋白基因在动物体内表达的实验报道. 有研究表明，AAV2转导人造血干细胞的效率，因各实验室包装和纯化rAAV2的方法不同而存在差异，其中辅助病毒的污染被认为是一重要原因. 制备了无辅助病毒污染的rAAV2，经体外检测其滴度，纯度及功能后，再转导人早期胎肝造血细胞，将被转导的胎肝造血细胞移植入受亚致死量剂量照射的8只BALB/C裸鼠体内，检测rAAV2介导的β珠蛋白基因在裸鼠体内的表达. 结果显示：制备的无辅助病毒污染的rAAV2具有较高的滴度、纯度，并能够在体外介导β基因的表达；在8只受试BALB/C裸鼠中，RT-PCR在2只BALB/C裸鼠骨髓中检测到β珠蛋白基因的表达. 提示，rAAV2能够转导人早期胎肝细胞并介导β珠蛋白基因的表达，但同时也存在表达量较低的缺点，应用于β地中海贫血的基因治疗还需要对AAV2生物学特性做深入的研究.

关键词 2型腺相关病毒，β珠蛋白基因，胎肝造血细胞，表达

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