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AAV Mediated β-Thalassemia Gene Therapy

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1. Introduction

β-thalassemia is one of the most common monogenic disease due to mutation or deletion in the β-globin gene on chromosome 11, inherited in an autosomal recessive fashion, with a global estimated annual birth incidence of 40,000/year[1]. The disease is particularly prevalent among Mediterranean peoples, Middle Eastern and Southeast Asians [1].

The severity of the disease depends on the production of functional β-globin chain. Mutations of β-globin gene cause reduced β-chain synthesis (β^+) lead to β thalassemia minor or intermedia, while mutations cause no β-chain synthesis (β^o) usually resulted in β-thalassemia major or Cooley's anemia[2]. Lacking of β-chain causes ineffective production of oxygen-carrying protein haemoglobin, therefore results in anemia. The relative excess of α-chains bind to the red blood cell membrane, undermine membrane, even form toxic aggregates, which aggravates anemia of patients. According to statistics, there are an estimated 80 million carriers of mutation of β-globin gene in the world[3]. The severe thalassemia is characterized by markedly ineffective erythropoiesis and severe anemia.

The treatment for β-thalassemia major usually includes lifelong blood transfusion and allogeneic hematopoietic transplantation[4]. Chronic blood transfusion often causes iron overload, accumulated iron produces tissue damage in multiple organs, so that iron chelating treatment is required to prevent iron overload damage to the internal organs in patients. To most of patients receiving the treatment, it is an expensive and inconvenience therapy for maintaining a long life.

Bone marrow transplantation is the other effective therapy, which can eliminate a patient's dependence on blood transfusions[5,6]. However, it is difficult to find the matching donors for the most of patients, which is only available for a minority of patients.

Gene therapy is one potential novel therapeutic avenue for the treatment of inherited monogenic disorder. It is a technology for correcting defective genes by introducing of the normal genes directly into patient's cells. This strategy mainly focuses on diseases caused

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by single-gene defects, such as β-thalassemia. For patients lacking a suitable bone marrow (BM) donor, gene therapy is not limited by the histocompatibility barrier and does not require immunosuppression.

The general strategy for β-thalassemia gene therapy is to obtain hematopoietic stem cell (HSC) from patient's bone marrow first, then, deliver a normal β-globin gene to patient's HSC by recombinant viral vector *in vitro*, the transfected cells will be transplanted into patients, the exogenous normal β-globin gene would be expressed in erythroid lineage cells under the regulation of the promoter, the ratio of β-chain to α-chain in red cells will be corrected in eripheralcirculation system eventually[7] (Fig1.).

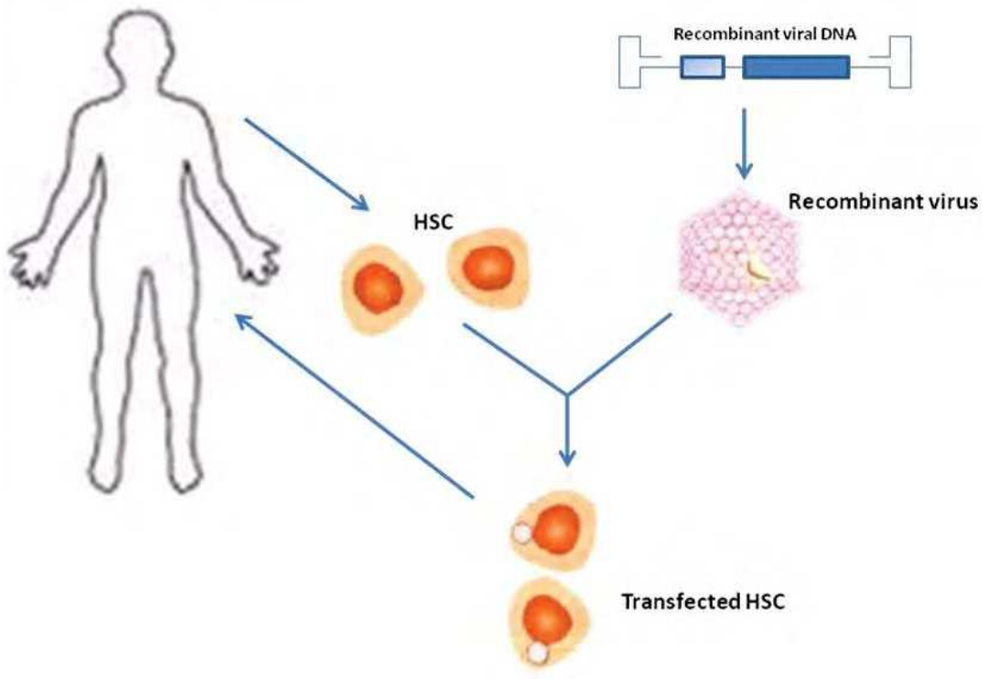
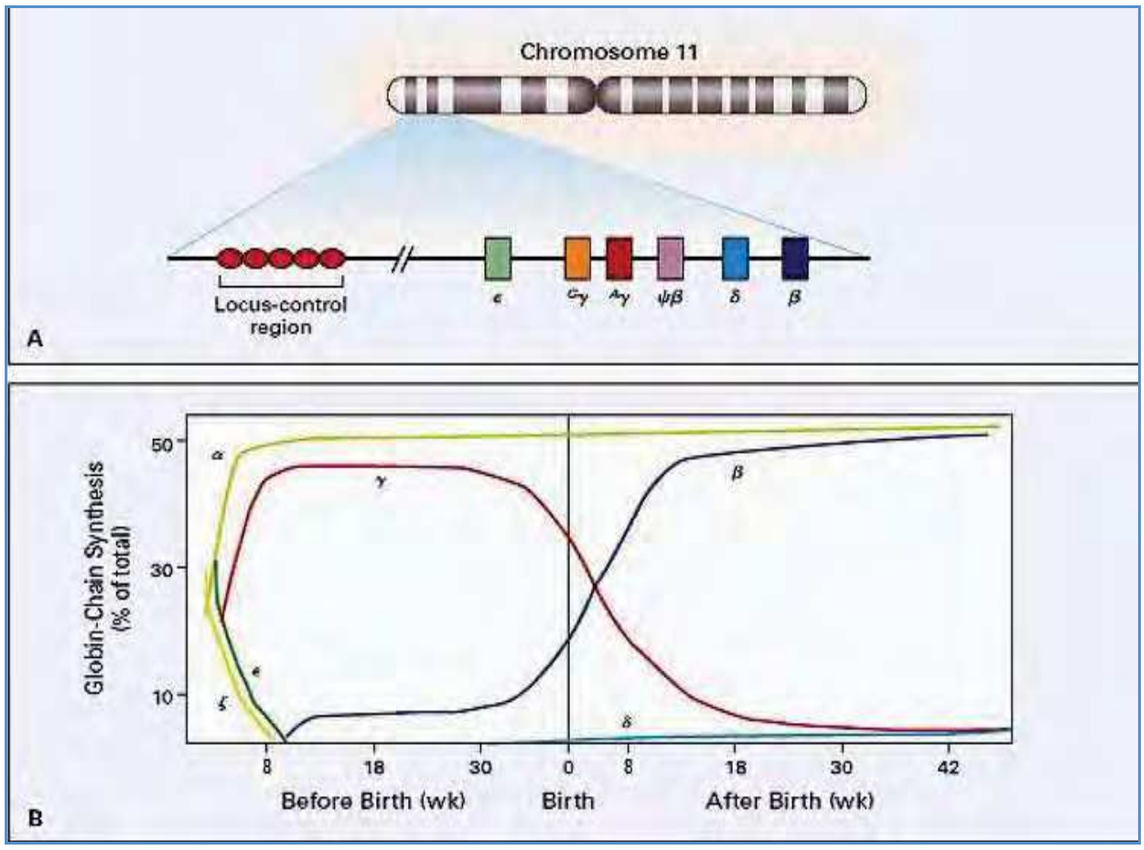


Fig. 1. The general strategy for β-thalassemia gene therapy.

To get a persistent expression of β-globin gene, $CD34^+$ cells are usually selected to be the target of gene transfer and transplantation. CD34 is considered as a maker for hematopoietic cells which possess self-renew and multiple lineage differentiation potentials, covering not only stem cells but also earlier multipotent progenitors and later lineage-restricted progenitors[8]. The success of transfecting exogenous β-globin gene into $CD34^+$ cells is the precondition of β-thalassemia gene therapy, which ensures the long term expression of the β-globin gene due to $CD34^+$ cells keeping differentiation into erythroid lineage cells, the erythroid lineage-specific expression of β-globin gene will be induced and regulated in these cells[9].

Human β-globin locus is composed of five genes which includes β, δ, $^{A}\gamma$, $^{G}\gamma$, and ε globin gene, located on a short region of chromosome 11, arranged as the sequence of 5' –ε- $^{G}\gamma$- $^{A}\gamma$- δ- β - 3'. Expression of all of these genes is controlled by single locus control region (LCR), and forms of hemoglobin expressed change during development. Genes are expressed in the order in which they are arranged in the cluster[10](Fig.2).



From Olivieri NF. The β-thalassemias. The N Engl J Med，1999，341:99-109.

Fig. 2. The β-Globin Gene Cluster on the Short Arm of Chromosome 11. **A**, the β -globin–like genes are arranged in the order in which they are expressed during development. **B**, shows the timing of the normal developmental switching of human hemoglobin.

2. Gene therapy for β-thalassemia

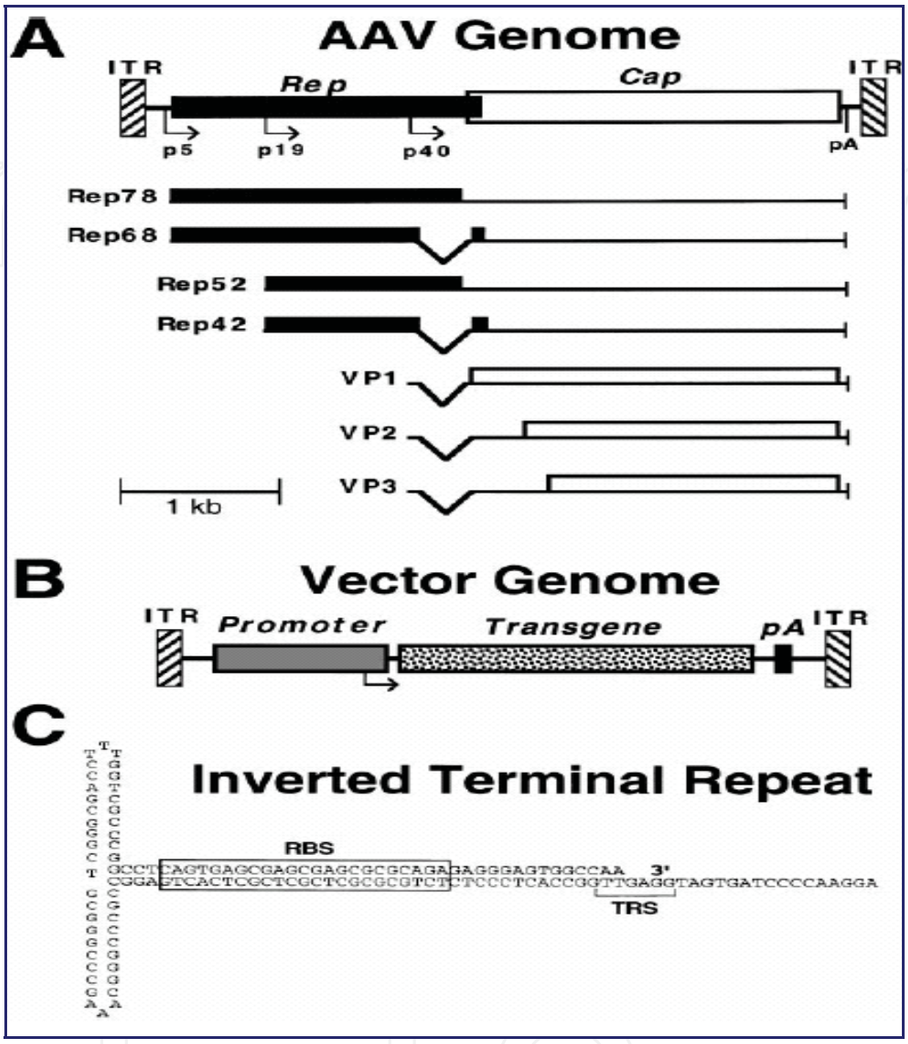
As a classic gene model for human genetics,β-globin gene has been extensively studied in the fields of gene structure, gene evolution, gene transcription and regulation. Gene therapy for β-thalassemia was started in 1980'. The retrovirus is the earliest and the most frequently used vector. It was reported in 1988 that the retrovirus (RV) containing β-globin gene successfully transfected HSC, although the erythroid lineage-specific expression of β-globin gene was low, only 1% of normal expression level[11]. It is generally considered in current studies that there is a therapeutic meaning only after the expression of exogenous β-globin gene reaches 10-20% of normal endogenous expression level. The discovering of the locus control region (LCR) in the range of 20 kb upstream of ε-gene greatly improved the erythroid lineage-specific expression of β-globin. LCR is composed of a series of hypersensitivities (HS) including HS1-HS5[12]. Sadelain et al. tried different HS combinations, reconstructed the RV vectors, got increased expression of β-globin gene, as high as 5%of normal β-globin gene expression level in mice[13]. But 4 months later, the expression of β-globin gene cannot be detected, suggested the gene silencing appeared. Gene silencing is a phenomenon that the specific gene is not expressed *in vivo* for a variety of reasons.

Studies show that the RV has the characteristics of random integrate into the host genome, while expression of β-globin gene is affected by the integrated position, which is called position effect variegation (PEV)[14]. The possible reason
for both of PEV and gene silencing is that transduced gene located in other regions outside of a normal gene locus. During the development of erythroid cells, over expressed mRNA from abnormal integrated position in chromosome may trigger specific mRNA degradation to prevent expression of the gene. Other studies also showed that gene silencing caused by RV is relative with the DNA sequences of long terminal repeats (LTR) and frame of RV virus[15].
The transduction efficiency of RV in HSC is low due to retrovirus vector only can infect dividing cells, but most of the HSCs are in quiescent stage, lacking of receptors for RV coat in HSC surface is also considered as one of the main reasons. In recent years, it was found that the random integration features of RV creates the potential risk of activating oncogenes or inactivating tumor suppressor genes, so application of RV in clinic is relatively limited[16].
The insufficiency of RV prompts people to try to develop new viral vectors for β-thalassemia gene therapy, such as lentivirus (LV), adeno associated virus (AAV),et al. The well-known lentivirus is human immunodeficiency virus－Ⅰ(HIV－1). Although LV belongs to retroviridae, it can effectively infect non-dividing cells. May et al. firstly obtained steady expression of β-globin gene in β-thalassemia mice by transducing HSCs with LV containing large fragment of LCR and β-globin gene, the expression of β-globin gene reached 10-20% of normal level, and lasted for more than 15 weeks without PEV effect, which showed preferable therapeutic action[17]. It was reported recently that a severe transfusion dependent thalassemia patient who accept β-globin gene therapy through lentivirus became transfusion independent for 21 months[18]. However, it is also noticeable that whether recombinant HIV-1 vector lost the pathogenicity completely so there will be no risk for patients to gain acquired immune deficiency syndrome (AIDS). Therefore, the safety of vector still need be monitored and valued in a long term through more experiments in vivo[19].

3. AAV mediated β-thalassemia gene therapy

Adeno-associated virus (AAV) is often found in cells that are simultaneously infected with adenovirus (Ad). However, unlike Ad, AAV does not stimulate inflammation in the host; causes a very mild immune response has a wide range of host of human and non-human cells, which can be dividing and non-dividing cells; wild AAV inserts preferentially at a specific site on human chromosome 19. AAV is not known to cause direct disease in humans and considered as the safest viral vector so far. In the absence of helper virus, recombinant AAV will stably integrate into the host cell genome, mediating the long and stable expression of the transgene. The main deficiency of AAV is the small packing capacity, only 4.5 kb[20].
AAV is a small (20 nm) replication-defective, nonenveloped virus, belongs to the genus Dependovirus, family Parvoviridae. The genome of AAV is built of single-stranded deoxyribonucleic acid (ssDNA), comprises two open reading frames (ORFs), *rep* and *cap*, flanked by inverted terminal repeats (ITRs) at both ends of DNA strand. The *rep* gene encodes 4 kinds of *Rep* proteins required for the AAV replication and rescue: *Rep* 78, Rep68, Rep52, Rep40. And the *cap* gene contains nucleotide sequences of capsid proteins: VP1, VP2 and VP3, which interact together to form a capsid of an icosahedral symmetry. The ITR

sequences comprise 145 bases each, are required in cis for efficient virus replication, integration, rescue, and encapsidation[21,22](Fig.3).



From Blood, Vol. 94 No. 3 (August 1), 1999: pp. 864-874. Adeno-Associated Virus Vectors and Hematology .

Fig. 3. Structure of wild-type and vector AAV genomes. **A,** Map of the wild-type AAV genome, including *Rep* (solid) and *Cap* (open) reading frames, promoters (p5, p19, and p40), polyadenylation site (pA), and inverted terminal repeats (ITR). The viral transcripts encoding the different *Rep* and *Cap* (VP1-3) proteins are shown below the genome. The smaller *Rep* proteins, VP2 and VP3, are translated from internal initiation sites. **B,** Map of a typical AAV vector, showing replacement of the viral *Rep* and *Cap* genes with a transgene cassette (promoter, transgene cDNA, and polyadenylation site). **C,** Secondary structure of the AAV ITR, with the locations of the *Rep* binding site (RBS) and terminal resolution site (TRS) indicated.

There have been 11 AAV serotypes identified, of which serotype 2 (AAV2) has been the most extensively examined so far[23]. AAV2 presents natural tropism towards skeletal

muscles, neurons, vascular smooth muscle cells and hepatocytes[24]. Currently, the application of AAV serotype 2 in hemophilia B gene therapy gets a promising development[25]. AAV2 is also studied in gene therapy for pulmonary cystic fibrosis, tumor and β-thalassemia. Although AAV2 is the most popular serotype in various AAV studies, it has been shown that other serotypes can be more effective as gene delivery vectors for specific tissue. Preliminary studies have demonstrated other AAV serotypes display different tissue tropisms[26]. For instance, AAV6 has a higher efficiency in infecting airway epithelial cells compare to other serotypes[27], AAV8 presents very high transduction rate of hepatocytes[28], AAV1 and 5 were shown to be very efficient in gene delivery to vascular endothelial cells[29]. The main reason causing the difference is there are distinctions among the capsid proteins of AAV serotypes, while the primary factor for virus entering into cells is the binding of capsid proteins with specific cell receptors. For example, the receptors that mediate AAV2 entering into cells are [30], fibroblast growth factor receptor and the integrin αVβ5[31,32]. So transduction efficiency of AAV serotypes is affected by distribution of specific AAV receptors in various tissues.

In 1994, Srivastava et al. first reported successful transduction of $CD34^+$ human primitive hematopoietic cells by recombinant AAV2 vectors at a relatively low vector:cell ratio of 1,000[33], indicated the potential of AAV2 in β-thalassemia gene therapy. Subsequently, AAV2 mediated transduction of $CD34^+$ cell were reported by a number of investigators [34-36]. High transduction efficiency of AAV2-mediated transgene expression in HSCs was found when the AAV2 vector particle:cell exceeded 10^6 by some groups [35,36]. A few of groups concluded that human $CD34^+$ cells were impervious to transduction by recombinant AAV2 vectors, and the transgene expression observed by others was due to 'pseudo-transduction' mediated by contaminants in the vector stocks[37], which causes people focus more on the generation of rAAV.

The helper virus or plasmid is required in production of recombinant AAV (rAAV) due to the AAV's replication deficiency characteristic. The traditional rAAV production system involves transfecting HEK 293 cells with a recombinant AAV vector plasmid and an AAV helper plasmid in the presence of a helper virus function[38,39]. The vector plasmid contains AAV ITRs and a transgene cassette. The helper plasmid contains the AAV *rep* and *cap* gene, but not ITRs. Ad is the most used helper virus, which provides adequate function in helping the replication of the recombinant AAV. However, Ad contamination is liable to occur in the latter procedures of purify of AAV. Thus, helper plasmid containing VA、E2a and E4 gene of Ad genome is developed and used in many studies[40-42].

In our study, we constructed rAAV plasmid (pMT-2) containing genomic sequences of human β-globin gene and mini-cassette of locus control region (LCR) element, as described previously. The plasmid pAAV2-RC contains AAV2 *rep* and *cap* genes and plasmid pHelpers contains adenovirus-derived genes (i.e. the E2A, E4, and VARNA genes. The pMT-2 together with pAAV2-RC and pHelper were cotransfected into HEK 293 cells to generate rAAV2-β-globin virions. The packaged rAAV2 virions were purified using a single-step gravity-flow column[43]. The purity of recombinant virions was evaluated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), and the titer of purified viral stock was determined by quantitative DNA dot-blots. The titer of rAAV2-β-globin was near 1.3×10^{10} virus particles/ml, as determined by quantitative DNA slot blots .SDS-PAGE analysis revealed that rAAV2-β-globin contained VP1, VP2, and VP3 proteins at a ratio of approximately 1:1:10, suggesting high purity of rAAV2-β-globin.

To investigate the function of rAAV2-β-globin in β- thalassemia gene therapy, we first detected rAAV2 mediated transduction and β-globin gene expression in human fetal liver hematopoietic cells from aborted fetus, as the expression of β -globin gene in early fetal has not been initiated. The results showed that rAAV2 efficiently transduced human fetal liver hematopoietic cells, and mediated expression of human β-globin gene in vivo, the detection of expression of β -globin gene was stopped at 2 weeks post transplanted considering the activation of endogenous β –globin gene. Following that, we investigated whether rAAV2 could mediate the expression of normal β-globin gene in human hematopoietic cells from β-thalassemia patients. We found that rAAV2-β-globin transduced human fetal hematopoietic cells, as determined by allele-specific PCR analysis. Furthermore, β-globin transgene expression was detected in human hematopoietic cells up to 70 days post-transplantation in the recipient mice. High pressure liquid chromatography (HPLC) analysis showed that human β-globin expression level increased significantly compared with control, as indicated by a 1.2–2.8 fold increase in the ratio of β/α globin chain.[44,45] These novel data demonstrate that rAAV2 can transduce and mediate normal β-globin gene expression in fetal hematopoietic cells from β-thalassemia patients. Our findings further support the potential use of rAAV-based gene therapy in treatment of human β-thalassemia, How to improve the transfection efficiency of AAV mediated HSC transduction, however is still an important issue.

Recent article reported that mutation of tyrosine residues on AAV2 capsid greatly enhanced transduction efficiency of AAV2 in HSC. They generated novel AAV vectors by mutating 7 tyrosine residues on AAV2 capsid to phenylalanine, respectively, named Y252,Y272,Y444,Y500,Y700,Y704 and Y730. It was showed that the transduction efficiency of Y444F was 8-11 times higher than wt AAV2, next followed by Y500F and Y730F. Furthermore, the combination of mutations Y444 + Y500F+Y730F showed even more increased transduction efficiency (4 times) compare to Y444F. The similar effect also was observed when the tyrosine residues on AAV6 capsid was mutated to phenylalanine. They discovered that increased efficiency is relative with phosphorylation of tyrosine residues on AAV capsid. Tyrosine residues exposed on AAV capsid surface could be phosphorylated by epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) on cell surface, which has no effect on the steps of AAV entering into cells.[46,47] However, phosphorylation of tyrosine residues on AAV capsid consequently triggered degradation of ubiquitin and proteasomal when AAV was present in cell plasma, which further caused the AAV degradation. The degradation of AAV is successful avoided by mutation of tyrosine residues on AAV2 capsid to phenylalanine, thus improved transduction efficiency of AAV. Base on these encouraging results, we are trying to improve AAV transduction efficiency in HSC by mutating the single or combination of tyrosine residues on AAV capsid after analysis of sequence of AAV capsid protein, in order to facilitate the use of AAV in transduction of hematopoietic stem cells, and provide an effective therapeutic way for β-thalassemia gene therapy.

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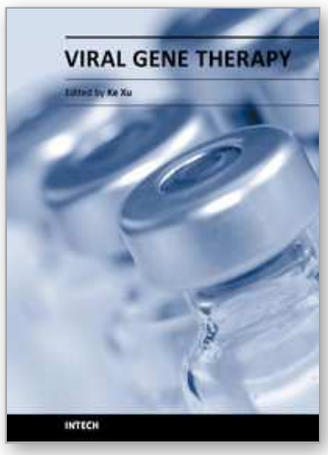
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The development of technologies that allow targeting of specific cells has progressed substantially in recent years for several types of vectors, particularly viral vectors, which have been used in 70% of gene therapy clinical trials. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. This book is designed to present the most recent advances in viral gene therapy

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