Original Paper

Characterization of Recombinant Adeno-Associated Viral Transduction and Safety Profiles in Cardiomyocytes

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Key Words

Adeno-associated virus • Safety profile • Gene therapy • Heart diseases • Transduction efficacy

Abstract

Background/Aims: Cardiovascular diseases (CVD) are the leading causes for human mortality. However, the effective treatment for these diseases are still lacking. Currently, gene therapy could be a potential way for efficiently treating heart diseases. The aim of our study is to analyze the transduction efficacy and safety profile of recombinant adeno associated virus (AAV) serotype 9 for cardiomyocytes *in vivo* and *in vitro*. **Methods:** We produced rAAV serotype 9 expressing enhanced green fluorescence protein (EGFP) driven by a cardiac troponin T (cTNT) promoter, and characterized its transduction efficiency in primary cultured cardiomyocytes *in vitro*, and in wild-type mouse heart tissue *in vivo*. **Results:** Our data showed that rAAV9 efficiently transduced mouse cardiomyocytes *in vitro*. Following intravenous injection, rAAV9 could efficiently and safely transduce cardiomyocytes that are involved in heart diseases. **Conclusion:** Our findings suggested that rAAV9 can efficiently and safely transduce cardiomyocytes *in vitro* and/or *in vivo*. The rAAV9 serotype vector could constitute a powerful toolbox for future gene therapy of heart diseases.

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Introduction

Cardiovascular diseases (CVD) are one of the leading causes of mortality worldwide [1]. The number of CVD caused death is expected to increase from 17.3 million in 2013 to 23.6 million by 2030 [1]. Particularly, CVD deaths represented 31% of all global deaths [1]. Heart failure and myocardial infarction are two mainly reasons for heart attack, and cardiomyocytes play very critical roles in these pathophysiological processes. Bergmann *et al.* and Senyo *et al.* showed that cardiomyocytes are renewed annually using ¹⁴C dating and imaging mass spectrometry, respectively [2-4], and that this percentage increases at least 3

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times after damage. Presumably, the replication of cardiomyocytes could be regulated by a complicated molecular network, including miRNAs, genes and epigenetic modifications.

So far, the effective treatment for CVD, especially for the heart failure and myocardial infarction, with promised prognosis is still need to be further developed. A large number of genes or non-coding RNAs were unraveled to regulate the survival of cardiomyocytes, hence, those targets could be potential candidates for effective gene therapy of CVD [5-7].

To deliver the target genes or non-coding RNAs into heart tissue, many vectors could be employed to achieve it, such as plasmid, viral vector, nanoparticles and so on. At present, the viral vectors are predominantly applied in the field of gene therapy, in particular, recombinant adeno-associated virus (rAAV) is a type of popular vector for its advantages in gene therapy applications, including low immunogenicity and genotoxicity, broad tissue tropism and high transduction efficiency *in vivo*, and long-term transgene expression [8-10]. Therefore, rAAV has been extensively evaluated in preclinical and clinical gene therapy applications for a broad range of diseases affecting many organs, including but not limited to brain, spinal cord, eyes, liver, muscle, and heart [11-14]. Even some studies explore the application of rAAV in CVD, particularly in heart diseases [5], its comprehensive evaluation on cardiomyocyte specific transduction *in vitro* and *in vivo* as well as its safety profile are still scarce.

In our study, we for the first time comprehensively investigated the efficacy and specificity of rAAV9 transduction in cardiomyocytes *in vitro* and *in vivo* using EGFP as a reporter gene driven by cardiac troponin T (cTNT) promoter. Moreover, the comprehensive safety profile of rAAV9 transduction was also evaluated by detecting mouse weight, serological parameters, and heart morphology. These findings provide critical evidences for the clinical application and basic research of rAAV in CVD.

Materials and Methods

Production of rAAV vectors

The rAAV serotype 9 vector was produced by the standard triple-transfection method in HEK293 cells as previously described [15]. The *cis* plasmid carries an EGFP-expressing cassette driven by a cardiac troponin T (cTnT) promoter.

AAV titration

Viruses were purified by ultracentrifugation over a cesium chloride (CsCl) gradient. Purified viruses were titrated both by quantitative polymerase chain reaction (qPCR) and silver staining. For silver staining, the capsid proteins of purified viruses were compared against standard capsid samples to quantitate protein-staining density. For qPCR, an EGFP-carrying plasmid was diluted to create a standard curve, and viruses were titrated using Ct values. For each serotype, two different viral preparations were tested.

Intravenous injection

C57BL/6 male mice obtained by in-house breeding were used throughout the study. For intravenous injection, each mouse was injected with 5×10^{11} genome copies (GC) of rAAV diluted in 100 μ L of sterile phosphate buffered saline (PBS). Following intravenous injection, hearts were harvested three weeks after injection under a dissection microscope.

EGFP signal analysis

Mouse hearts and livers were harvested 3 weeks after IP injection and fixed in 4% paraformaldehyde overnight at 4°C. Tissues were then sequentially dehydrated in 10%, 20% and 30% sucrose overnight at 4°C. The samples were embedded in optimal cutting temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA, USA) and stored at -80°C. Next, 8- μ m-thick cryo-sections were mounted with buffer containing DAPI, and EGFP signals were observed under a fluorescence microscope.

Serum ALT and AST assays

Blood was collected by facial vein bleeding before and at 1, 2 and 3 weeks after intravenous injection, and serum was separated using a Microtainer tube with serum separator (cat # 365967) from BD (Franklin Lakes, NJ, USA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were analyzed using an ALT colorimetric endpoint kit (cat # A526) and an AST colorimetric endpoint kit (cat # A561) from TECO Diagnostics (Anaheim, CA, USA), respectively, as per the manufacturers' instructions.

Histological analysis

Heart tissues were fixed in 4% paraformaldehyde overnight at RT, embedded in paraffin and sectioned to 4 microns thick. Sections were stained with hematoxylin and eosin (H&E), and imaged using a bright field microscope (Leica, Buffalo Grove IL, USA).

Mouse cardiomyocyte cell culture

Hearts of one-day old C57/B6 mice were harvested, and it was cut into pieces as tiny as possible. Tissues were digested with 0.05% Trypsin and collagenase II for 10 times at 37°C for 5 min. Cell suspension was filtered using a 70 µm cell strainer, and cells were cultured with DMEM supplemented with 10% fetal bovine serum (FBS) in a 96-well plate. Further, cell suspension was transferred into new wells after 45 min of culturing, and the majority of cardiomyocytes would adhere to the bottom of the plate.

Immunofluorescence staining

Mouse hearts were fixed in 4% paraformaldehyde overnight at 4°C, and then sequentially dehydrated in 10%, 20% and 30% of sucrose each overnight at 4°C. The samples were embedded in the optimal cutting temperature (O.C.T.) compound (Sakura Finetek, Torrance CA, USA), and eight micron-thick cryo-sections were permeabilized and blocked with 5% goat serum and 0.5% Triton X-100 in 1× PBS for one hour at room temperature (RT). The slides were incubated with primary antibodies against cTNT (1:100 diluted, cat # ab8295, Abcam, Cambridge MA, USA), alpha smooth muscle Actin (1:500 diluted, cat # ab5694, Abcam, Cambridge MA, USA) overnight at 4°C, and further incubated with secondary antibodies (Life Technologies) for 1 hour at RT in dark. Finally, the sections were mounted with the VECTASHIELD mounting medium containing DAPI (Vector Laboratories, Burlingame CA, USA). For the cultured cells, the cells were washed with PBS 3 times for 3 min, and fixed with 4% paraformaldehyde for 15 min. Then cells were permeabilized with 0.5% Triton X-100 in PBS for 20 min, and blocked with 5% goat serum in PBS for 30 min at RT. The primary antibodies against cTNT (1:100 diluted, cat # ab8295, Abcam, Cambridge MA, USA) were used to incubate cells at 4°C overnight. Cells were washed with PBS for 3 times for 3 min, and the incubation with secondary antibodies were performed at RT for 1 h. Finally, all solution was discarded, and the cells were mounted with 10 fold diluted VECTASHIELD mounting medium containing DAPI (Vector Laboratories, Burlingame CA, USA). Slides and cells were observed using a fluorescence microscope [16].

Statistical analysis

All data were represented as means ± the standard deviation (SD). Statistical significances for comparisons between groups and among multiple groups (> 3) were determined using a Student's paired t-test and analysis of variance (ANOVA), respectively, in Prism 7.0, and p < 0.05 was the level of significance.

Results

rAAV9-cTNT vectors specifically and efficiently transduce mouse heart other than liver

To check the efficacy and specificity of rAAV9 transduction in mouse heart, C57/B6 mice were intravenously injected with rAAV9-cTNT vectors carrying EGFP for 3 weeks (Fig. 1A). The results of frozen sectioning and microscopy showed that rAAV9 can efficiently transduce mouse hearts (Fig. 1B, EGFP panel). However, the green fluorescence cannot be detected in mouse liver (Fig. 2). These findings suggested that rAAV9-cTNT can effectively and specifically transduce mouse heart.

rAAV9-cTNT specifically transduce cardiomyocytes in vivo

To further characterize the heart cell types that were transduced with rAAV9, we performed immunofluorescence staining of mouse heart cryo-sections with antibodies against cellular markers of cardiomyocytes (cTNT), and smooth muscle cells (α -actin for smooth muscle cells). We found that rAAV9 was able to efficiently transduce cardiomyocytes (Fig. 3, the upper cTNT panel) rather than smooth muscle cells of heart tissue (Fig. 3, the lower SMA panel).

rAAV9-cTNT safely transduce mouse heart following intravenous injection

To evaluate the safety profile of rAAV9-cTNT injection, we firstly analyzed the weights of mice in the mock and treatment groups. As shown in Fig. 4, no significant difference of mouse weights was observed between the mock and rAAV9-cTNT groups. Furthermore, the effect of rAAV9 transduction on liver injury was accessed by detecting AST and ALT levels in serum, and our data showed that intravenous injection of rAAV9-cTNT did not markedly change ALT/AST levels (Fig. 5). In addition, we observed normal histology by H&E staining in mouse heart without indication of inflammation or other adverse effects following PBS or rAAV injection (Fig.

Fig. 1. rAAV9-cTNT efficiently transduce mouse heart following intravenous injection. A. Schematic timeline for i.v. injection of rAAV. B. Representative fluorescence images of mouse heart cryo-sections showing the EGFP native fluorescence following injections of PBS or rAAV9-cTNT-EGFP. I.V., intravenous.

Fig. 2. rAAV9-cTNT do not transduce mouse liver following intravenous injection. Representative images of mouse liver cryo-sections showing negative EGFP fluorescence following injections of PBS or rAAV9-cTNT-EGFP.

Fig. 3. rAAV9-cTNT specifically transduce mouse cardiomyocytes *in vivo*. The upper panel showed that rAAV9 specifically transduce mouse cardiomyocytes (positive cTNT staining). The lower panel showed that rAAV9-cTNT did not transduce blood vessels in heart (positive SMA staining). SMA, alpha actin of smooth muscle cells.

6). These results suggested that rAAV9 is a good candidate for efficient and safe delivery of genes of interest to mouse heart *in vivo*.

rAAV9-cTNT efficiently transduce mouse cardiomyocytes in vitro

To investigate the transduction efficacy of rAAV9 in cardiomyocytes *in vitro*, we firstly isolated the cardiomyocytes from 1-day old neonatal mice. Three days post-infection, the cultured cells showed strong green fluorescence, and the cells were further stained with primary antibodies against cTNT. As shown in Fig. 7, almost all cardiomyocytes were successfully transduced with rAAV9. These findings showed that rAAV9 could be a powerful *in vitro* research tool for studying heart diseases.

Discussion

In the past decades, CVD is one of the biggest threatens for human health worldwide [1]. In 2013, CVD accounting for an estimated 17.3 of 54 million total deaths, or 31.5% of all global deaths, hence, CVD obviously is the most common underlying cause of death in the world [1].

Currently, lots of drugs had been developed to treat CVD, and the interventional therapy as well as surgical methods were also applied widely. With revealing its underlying mechanism involved in heart diseases, more critical genes or non-coding RNA presented great potentials for the treatment of these diseases effectively. Therefore, gene therapy provided another route for treating heart diseases.

For the advantages mentioned above, rAAV could be an useful tool to deliver target gene or non-coding RNA into heart tissue. Our previous study showed that rAAV9 can transduce heart tissue efficiently following intraperitoneal injection at a dose of 1×10^{12} genome copy per mouse [16]. This study further investigated the

Fig. 4. Intravenous injection of rAAV9-cTNT do not change body weight of mice. Body weights of treated mice were recorded before and after 1, 2 and 3 weeks of injection.

Fig. 5. Intravenous injection of rAAV9-cTNT do not show significant difference of ALT/AST levels. Sera were collected before and after 1, 2 and 3 weeks of injection. ALT, Alanine aminotransferase, AST, aspartate aminotransferase.

Fig. 6. Intravenous injection of rAAV9-cTNT do not show significant immune cell infiltration and morphological change of heart. H&E staining showed no obvious infiltration of immune cells, and no markedly change was observed between the mock group and the treatment group.

Fig. 7. rAAV9-cTNT efficiently transduce cardiomyocytes *in vitro*. The majority of cells that showing green fluorescence were positively stained with cTNT. The white arrowhead showed the cells with positive EGFP fluorescence and cTNT staining.

efficacy of rAAV transduction in heart *in vivo* and *in vitro* using a cardiomyocyte specific promoter cTNT, and evaluated its safety profile.

Our data showed that intravenous injection of rAAV-cTNT can efficiently transduce cardiomyocytes *in vivo*, and no positive EGFP expression was detected in liver, suggesting the high transduction efficacy of rAAV9 and the high specificity of cTNT promoter. This cassette provides a great tool for delivering target genes or non-coding RNAs into cardiomyocytes. To further observe the transduction pattern of rAAV-cTNT in heart tissue, the cardiomyocyte-specific marker, cTNT, and the smooth muscle cell-specific marker, SMA, were detected using immunofluorescence (Fig. 1). The findings indicated that rAAV9-cTNT specifically transduce cardiomyocytes, who are mainly responsible for normal heart function.

After 3 weeks of intravenous injection of rAAV, the mouse weight, liver function, and heart morphology were not influenced completely (Fig. 4, 5 and 6), implying the excellent safety of rAAV mediated gene delivery. Importantly, effective rAAV transduction in cardiomyocytes *in vitro* is also very important for basic research of heart diseases. The primary cardiomyocytes could not be efficiently transfected by common transfection reagents, and rAAV can transduce cardiomyocytes efficiently *in vitro* (Fig. 7).

Heart diseases are mostly related to the dysfunction of cardiomyocytes [17]. Our study employed a cTNT promoter to investigate the specific gene expression mediated by rAAV9, and this point is very critical for the precision treatment of heart diseases. Moreover, safety profiles of liver function and cardiac histopathology indicated none adverse effects of rAAV-cTNT i.v. injection, and that is crucial for its clinical application. Also, this study explored the transduction efficacy of rAAV9 in cardiomyocytes *in vitro*, the findings *in vitro* further confirmed the excellent transduction efficacy of rAAV9 *in vivo*, suggesting the great potentials of rAAV9 mediated gene delivery in cardiomyocytes *in vitro* and *in vivo*.

Taken together, our data showed the excellent transduction efficacy of rAAV9-cTNT in cardiomyocytes *in vitro* and *in vivo* with high specificity and safety. These findings established a critical foundation for further development of rAAV-based gene therapy strategies for treating a wide spectrum of heart diseases, and also provided a powerful *in vitro* and *in vivo* research tool for studying heart diseases.

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Disclosure Statement

No conflict of interests exists.

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