Directed Evolution of Adeno-associated Virus Targeting Heart and Skeletal muscle

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Abstract

The goal of this project is to engineer gene vectors that target a single tissue type, especially striated muscles. A random library of adeno-associated virus (AAV) was constructed by shuffling the capsid genes of AAV serotypes 1 to 9, and the resulting chimeric genes were screened for muscle specificity by direct in vivo panning. Viruses expressed high in muscles were collected, and the process was repeated to construct chimeric AAV library. To further improve specificity, in vitro biopanning was done on three types of cultured cells that represent kidney, liver and muscles. For the desirable features, selection was based on infection rate, preferably high in muscle but low in liver. Using AAV2 as a standard, three reconstructed AAV were found to be expressed at higher frequencies in myotubes with comparable or lower frequencies in hepatocytes. These recombined AAV would be further selected in vivo through tail vein injection in mice to identify the virus that was the most specific to muscles.

Introduction

Gene therapy has been the frontier of modern medicine to treat a wide range of diseases, from inherited diseases, like cystic fibrosis and hemophilia, to cancer, tissue regeneration and many others (Qiao et al. 2012). The vectors for gene delivery can be divided into two groups: non-viral and viral vectors. Non-viral vectors include liposomes, naked DNA, microbubbles, nanoparticles and polymers. Some examples of viral vectors are adenoviruses, adeno-associated virus (AAV), retroviruses and lentiviruses (Luo et al. 2015). The requirements for long-term gene delivery are the efficient entry into the cells, stable vector genomes, lack of vector toxicity, and evasion of host immune systems. None of the currently available vectors meet all the

requirements, but viral vector AAV has been considered a promising candidate. In fact, 5.3% of gene-therapy-related clinical trials in the world have utilized AAV vectors mainly for the genetic diseases gene therapy (Murlidharan et al. 2014).

AAV as A Vector

AAV is non-enveloped, helper-dependent parvovirus that is about 25 nm in diameter. Its small size helps the virus to navigate through the blood stream and enter cells efficiently. AAV can transfect both dividing and non-dividing cells, so its range of application is wide (Luo et al. 2015). In comparison to adenovirus or plasmid-based gene delivery AAV has stable DNA genome, and gene expression is prolonged after a single delivery (Hammoudi et al. 2015). In fact, the longest transgene expression so far belongs to an AAV-mediated human factor IX gene in muscles that expressed for 10 years (Luo et al. 2015). Unlike other viral vectors, AAV is not known to cause any diseases, and it elicits immune response minimally (Zinn and Vandenberghe 2014). For all the above reasons, AAV is an attractive tool for gene delivery. Three scientific developments have further enabled AAV to transfer genes: the ability to cross-package AAV vectors, or to produce pseudotyped viruses (Rabinowitz et al. 2002; Gao et al. 2002), the construction of helper plasmid containing only the essence of adenovirus (Ad) that propagates AAV (Xiao et al. 1998), the identification of inverted terminal repeat (ITR) as the primary element required for productive infection and the generation of vectors (Xiao et al. 1997). These discoveries make it possible to produce recombinant AAV (rAAV) that packages therapeutic genes with minimal contamination of the wild-type virions (Murlidharan et al. 2014).

AAV for Muscle Gene Therapy

Muscular dystrophies are characterized by the progressive degeneration of multiple muscle groups. Duchnene muscular dystrophy (DMD) is a recessively inherited disease that happens 1 in 3500 male live births (Odinaka and Nwolisa 2014). It has an early on set, usually before 3 years of age, and death occurs in the mid to late twenties. DMD is caused by a mutation in the dystrophin gene on the X chromosome, leading to a lack of functional dystrophin in muscle fibers. There are no currently available cures, but various therapeutic approaches are under study. While pharmaceutical therapy uses chemical or biochemical substances to restore dystrophin or to alleviate the syndromes, gene therapy offers a more permanent solution by introducing a transgene coding full-length or partial dystrophin complementary DNA in muscles (Pichavant et al. 2014).

Muscular dystrophy gene therapy requires efficient body-wide muscle gene transfer and AAV has effective and stable transduction in striated muscles, but one challenge in AAV-mediated gene delivery is broad tissue tropism (Yang et al. 2011). AAV naturally evolve into multiple serotypes that target various cell types. For example, AAV4 targets heart and lung, and AAV 9 targets central nervous system and peripheral tissues. However, transduction of non-target organs is common in systemic gene transfer. Liver is at particular danger, because large amount of blood is filtered by the liver every moment, and what is carried in the blood is likely to stay at this filtering organ. As a result, liver is among the most commonly infected tissue in the body. This not only reduces the efficiency of transduction to specific cell populations, but presents potential safety concerns on ectopic gene expression (Luo et al. 2015; Yang et al. 2011). Since there are no natural serotypes that targets heart and muscles with high specificity, directed evolution is utilized to synthesize suitable AAV vectors.

The Construction of a Chimeric AAV Library

Xiao lab has devoted much energy to engineer gene vectors that efficiently target striated muscles after systemic delivery. Researcher Lin Yang constructed a random chimeric AAV capsid gene library by DNA shuffling which is a method of random fragmentation followed by PCR reassembly to introduce or exchange new genetic mutations (Yang et al. 2011).The capsid genes of AAV serotypes 1 to 9 were amplified and mixed in equal ratio. After the genes were cut by DNase and purified by agarose gel electrophoresis, the fragments were reassembled by DNA polymerase. The random capsid genes were incorporated into plasmid backbones containing AAV2 *Rep* gene and inverted terminal repeats. An infectious AAV library was finally produced, using the self-packaging technique developed by Müller et al (2003).

According to Yang L. et al., in vitro panning based on cultured cell lines has been used before, but gene therapy requires the AAV vectors to confront more complicated physiological environment in bodies, including serum proteins, endothelial barriers, extracellular matrix barrier and many others (2011). Thus to better mimic application environment, in vivo bio-panning was employed to assess the infection rate. The chimeric AAV was injected into adult mice via tail vein. Limb skeletal muscles and liver were collected three days after injection. Capsid genes enriched in muscles were received by PCR amplification, and the products were reshuffled and selected for a second time in vivo. The chimeric AAV library consists of capsid genes retrieved again from muscles (Yang et al. 2009). Based on the work done by Xiao lab, the goal of this paper is to select appropriate chimeric capsid genes for muscle gene therapy. Desirable AAV vectors are expected to deliver genes to cardiac or skeletal muscles efficiently, with low transfection of the liver.

Materials and Methods

Chimeric Recombinant AAV vectors

Chimeric capsids genes were incorporated into packaging plasmids. The construction of AAV reporter vector plasmids pAAV-CMV-GFP and pAAV-CMV-LacZ has been described in Qiao et al (2011). Recombinant AAV vectors were generated by triple-plasmid transfection technique (Xiao et al. 1998).

Vector Genome Copy Number Determination

Viral DNA was extracted with DNeasy blood and tissue kit (Qiagen, cat. No. 69506). Vector copy number was determined with a 7300 real-time PCR system (Applied Biosystems). TaqMan assay for AAV vector was developed as described in Qiao et al. (2012).

Differentiation of C2C12

C2C12 cells were thawed in 37^{o}C water bath, and then cultured in growth media that contained DMEM, 10% fetal bovine serum and penicillin-streptomycin. When C2C12 cells reached 50% confluence, media was changed to differentiation media that consisted of DMEM, 2% horse serum and penicillin-streptomycin.

Transduction of 293, Huh7 and C2C12 by AAV vectors

A hundred microliter of shuffled or AAV2 vectors containing either GFP or Lac-Z reporter genes were inoculated onto 293 (human embryonic kidney) and Huh7 (a liver cancer cell line). Virus was washed off after twelve hours of infection. C2C12 cells were transduced at different time points of two or three or four days after inducement of differentiation. The same

procedure was used as that for 293 and Huh7, but with vectors containing Lac-Z only, because GFP was found to be an inefficient reporter.

Infection rate analysis

Forty-eight hours after the addition of AAV, cells that were infected with GFP-carrying vectors were photographed. Cells infected with Lac-Z-carrying vectors were collected and quantitatively analyzed using Galacto-light Plus (Applied Biosystems).

Results

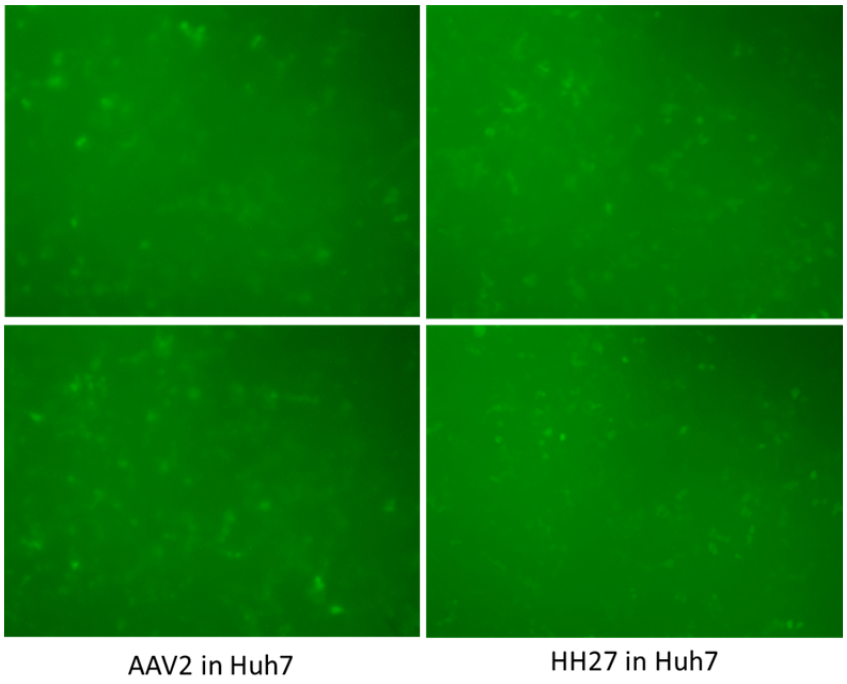

Figure 1. GFP expression of AAV2 and HH27 in Huh7. Microscopic photographs were taken 48 hours after viral infection of Huh7.

Reporter gene, GFP, was used in 293 and Huh7 cells. A few shuffled AAV appeared to infect Huh7 slightly less than AAV2, for example HH27, but the expression of GFP was so low that it was hard to tell the difference of infection determinately (Figure 1). There was too much background light and little infected cells, and the results were not very informative. GFP does not seem to be a suitable reporter gene in this case, so it was not used in the transduction of C2C12 done after that of 293 and Huh7.

Lac-Z, on the other hand, provides much more information. The protein was collected 48 hours after transduction, and measured quantitatively. The amount of Lac-Z protein was calibrated to 10^6 copies of virus. Virus genome concentrations of crude lysates were measured by dot blot. A normalized graph was constructed by dividing the amount of Lac-Z measured in cells infected with chimeric AAV by that in wtAAV2 (Figure 2).

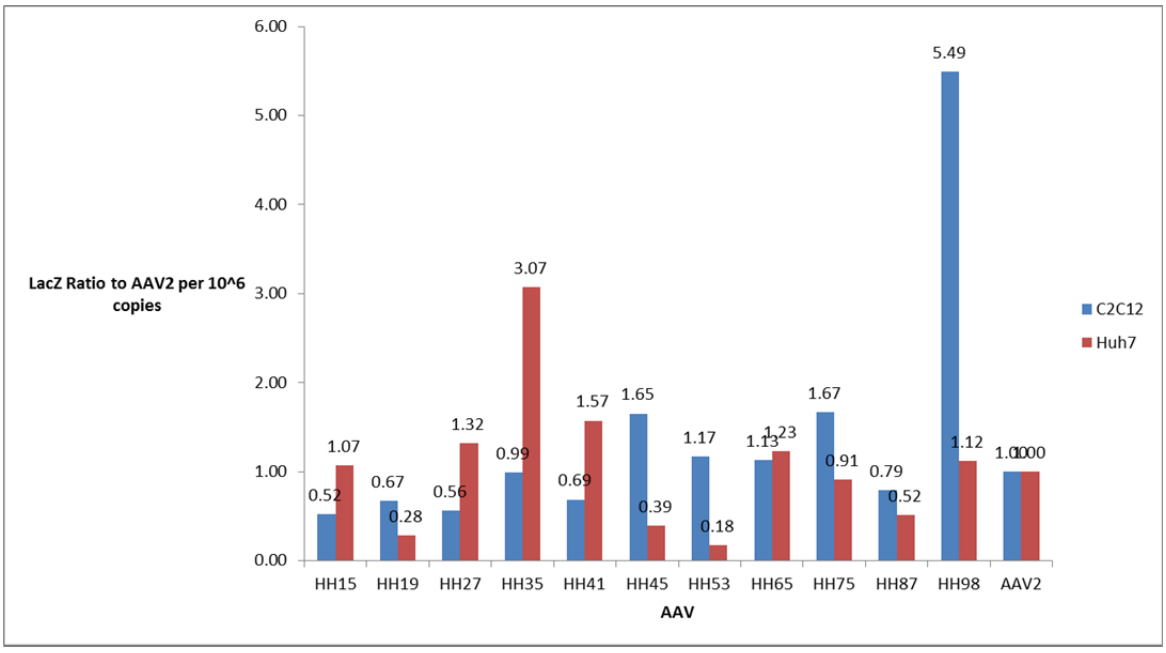

Figure 2. Lac-Z expression of twelve shuffled AAV in ratio to WT AAV2 in C2C12 and Huh7. Lac-Z proteins levels in infected cells were measured, and calibrated to 10^6 copies of virus.

Virus genome concentrations of crude lysates were measured by dot blot. The values were divided by that of AAV2 to obtain the ratios.

Since only muscle-specific AAV was desired, those vectors that infect C2C12 at a high rate, but Huh7 at a low rate were selected. Thus HH45, 53 and 98 are of particular interest. In comparison with AAV2, HH45 infects Huh7 at 39% and HH53 at 18% of the efficiency of AAV2, while their infections of C2C12 were moderately higher than AAV2, one at 1.65 and the other at 1.17. On the other hand, HH98 infected C2C12 almost six times better than AAV2 did (5.49), while kept infection of Huh7 at comparable level with AAV2 (1.12). Thus HH45, 53 and 98 were selected for further investigation.

Discussion

Through panning in cultured cells, three chimeric AAV capsid genes are selected out from a library of twelve. As the real battle ground and the verdict for AAV as a vector is inside bodies, in vivo panning will be done to determine transduction efficiency of the three candidates selected. HH45, 53 and 98 will be tested directly in mice. The capsid genes would again be used to engineer recombinant AAV that carry Lac-Z reporter genes. CB promoter will be used in the place of CMV, because the CMV promoter shuts off quickly in liver and therefore is not reliable to evaluate liver expression. Viruses will be made and injected into mice through tail-vein for a systemic delivery. Two to three weeks after infection, organs will be collected, sliced and stained to see the rate of infection of each chimeric AAV capsid genes. If HH45, 53 and 98 are expressed highly in muscles and weakly in liver, they will be suitable vectors for the treatment of muscular diseases.

Besides the HH library constructed by virus retrieval from heart and muscles, Xiao lab has other capsid libraries with different tissue focus. The same technique can be employed to develop vectors specific to other targets. By increasing specificity and de-targeting liver, the technique of directed evolution enables a broader and safer application of AAV for gene therapy.

In addition to vector selection, chimeric AAV capsid genes are also analyzed to trace the origins of each segment. The composition of capsid genes can be paired with the results from concentrated panning to discover the specific mutations that lead to the change of transduction in certain tissues. The combination of the two projects will create a table that matches mutations with desired tissue tropism, which will enable individual tailoring of AAV for gene therapy.

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