Selection of Adeno-Associated Virus Serotypes For Gene Therapy In Krabbe Patient IPSCs-Derived NSCs

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Short Report

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Abstract

Krabbe disease is caused by the mutation or deficiency of galactocerebrosidase (GALC) enzyme, which is located in the lysosome and hydrolyzes the galactolipid substrates like psychosine. Psychosine would accumulate abnormally in the myelin forming cells and result in demyelination in the nervous systems with the clinical symptoms of spastic paraparesis and seizures. Adeno-associated virus (AAV) is a well-established and safe viral vector for gene delivery. However, effective AAV serotype for the transduction of the human neural stem cells (NSCs) has not been identified. Here, we screened a variety of AAV serotypes to transduce NSCs-related disease model induced by Krabbe patient induced pluripotent stem cells (iPSC) differentiation. It has been found that AAV2 has a higher transduction effect for NSCs, and AAV2 carrying *GALC* gene rescued the GALC enzymatic activity of Krabbe NSCs. Our findings established Krabbe patient iPSCs-derived NSCs as a new model for study the pathogenesis of Krabbe disease, and also demonstrated the potential of using AAV2 as a vector in gene therapy for Krabbe disease, which also proved the potential of AAV in devising gene therapy strategies for the treatment of genetic neurodegenerative diseases.

Introduction

Krabbe disease, also known as globoid-cell leukodystrophy (GLD), is a rare inherited neurodegenerative disease resulted from the deficiency or mutation of galactocerebrosidase(GALC), a critical enzyme present in lysosomes(Wenger, Rafi et al. 1997, Wenger, Rafi et al. 2000). GALC is needed for the lysosomal hydrolysis of galactosylated sphingolipids, e.g., galactosylceramide and galactosylsphingosine (psychosine)(Wenger, Sattler et al. 1974). GALC deficiency leads to the accumulation of psychosine, a cytotoxic lipid especially damaging to oligodendrocytes and Schwann cells(Potter and Petryniak 2016). The abnormal accumulation of psychosine in GALC-mutated cells leads to the progressive death of cells involved in myelination, resulting in dysmyelinating and affecting both the central and peripheral nervous systems. As a result, the nervous system has some clinical symptoms, such as spastic paraparesis, seizures and blindness(Orsini, Kay et al. 2016, Wasserstein, Andriola et al. 2016, Pannuzzo, Graziano et al. 2019).

Human induced pluripotent stem cells (iPSCs) are powerful tools that act as models for human disorders. They can differentiate into various cell types and thus offer in vitro models of disease, such as NSCs(Gorba and Conti 2013, De Filippis, Zalfa et al. 2017, Szlachcic, Wiatr et al. 2017). NSCs are a series of cells, and there are characterized by the ability of self-renew with the undifferentiated state and differentiate into specialized neural cells. NSCs can act as basic medical research models for some mental illnesses lacking suitable mouse or primate models. NSCs also are ideal research tools for a majority of disease even though lots of mammalian models have been described previously as these animal models couldn’t fully recapitulate characteristic features of human disease(De Filippis, Zalfa et al. 2017). In addition, using some reliable methods for gene delivery and expression in NSCs will promote the development of regenerative medicine.

Adeno-associated virus (AAV) has the advantages of low immunogenicity and high efficiency in infecting a wide range of dividing and non-dividing cells, and is stably integrated into the host genome of target cells to support strong and persistent transgene expression, which makes it potential to be widely used in research and clinical fields(Wang, Tai et al. 2019, Li and Samulski 2020). AAV belongs to the genus *Dependovirus* of the family *Parvoviridae*. AAV genome is a 4.7 kb single stranded DNA, which contains two large open-reading frames (ORFs) flanked by inverted terminal repeats (ITRs). The two ORFs encodes proteins essential for AAV replication and assembly. The capsid has an icosahedral symmetry and includes three important proteins VP1, VP2 and VP3(Srivastava, Lusby et al. 1983). There are lots of wild AAV serotypes have been described so far, and there are distinguished from the capsid amino acid sequence(Gao, Zhong et al. 2011). AAV have been used to infect a broad range of cells, such as embryonic stem cells, hematopoietic stem cells, fibroblasts, tumor cells, etc. However, there are few reports on the infection of NSCs derived from patient iPSCs.

In this study, we aimed to screen AAV serotypes that can effectively deliver genes to the NSCs derived from Krabbe patient iPSCs. We hypothesized that the ideal model for AAV serotypes selection should be human origin and also exhibit disease-related phenotypes. Krabbe patient iPSCs derived NSCs (K-NSCs) provide a reproducible *in vitro* model for studying human/ viral vector interactions. We verified the effect of AAV gene therapy *in vitro* and demonstrated the potential of using some AAV serotypes as vectors in human NSCs research, which could explain the pathogenic mechanism of certain disease such as Krabbe disease, and also proved the potential of AAV applied in gene therapy *in vivo*.

Results

Krabbe patient iPSCs-derived NSCs(K-NSCs) were obtained as described in the Methods. iPSCs can be successfully induced into NSCs within 7 days (Fig. 1a). K-NSCs are positive for neural stems marker NESTIN and stem cells marker SOX2, but negative for another stem cells marker OCT4(Choi, Kim et al. 2014). The results of immunofluorescence staining (Fig. 1b and c) indicated that we successfully induced K-NSCs, and the K-NSCs were contributed to further explain the mechanism and medicinal researches of Krabbe disease.

The AAV was obtained using methods described in the Methods. There are 9 AAV serotypes used in this study. All AAV serotypes successfully expressed the three vital capsid VP1-3(suppl. Figure 1). We choose AAV-PHP.eB, which was described as having strong infectivity for the nervous system(Chan, Jang et al. 2017), as the candidate AAV to infect K-NSCs at different MOIs. As the amount of virus used increased, the expression level of GFP relatively increased as well (suppl. Figure 2, A and B). The results of immunostaining shown that K-NSCs still maintained NESTIN expression, which indicated that AAV-PHP.eB infection couldn't change the undeferential state of K-NSCs (Fig. 2).

In order to screen AAV serotypes that can more efficiently infect NSCs. K-NSCs were infected by different AAV serotypes in the same titer (10^9 vg). The GFP protein expression level was evaluated 48 hours after infection. The results show that AAV2 has the highest efficiency to infect K-NSCs, AAV-PHP.eB and AAV-

DJ as well as AAV1 also shown higher infected efficiency than other serotypes used in this research (Fig. 3a and b). Furthermore, we cloned the *GALC* coding sequence into the AAV vector (Fig. 3c), and used AAV2 to carry the *GALC* gene to infect K-NSCs. The results shown that overexpression of GALC protein by AAV2 delivery increased enzyme activity compared to mock infection with AAV2-GFP virus (Fig. 3 ,d and e).

Discussion

Due to ethical restrictions, human nerve cells can't be obtained directly, so the pathogenesis of many neurological diseases is difficult to explore. NSCs can differentiate to a majority of neuronal cell types, including neurons, glia, astrocytes and oligodendrocytes, which are crucial for mammalian brain development, learning and memory. The study of the mechanism of quiescence, proliferation and self-renewal of NSCs is vital to explain the nervous system development and neurogenesis or mental disorders. This study established a neural stem cell model by inducing Krabbe patient-derived iPSCs into NSCs for the study of Krabbe disease.

Among the widely used viruses, AAV has emerged as an attractive vector for gene therapy as it is non-pathogenic and has a broad-host and tissues range. AAV could infect a variety of dividing and non-dividing cells, such as fibroblast and neurons as well as some tumor cell lines(Hendrie and Russell 2005, Rogers, Hao et al. 2008). It's reported that AAV infected some stem cell types with low efficiency, such as mouse and human embryo stem cells and rat NSCs(Hughes, Moussavi-Harami et al. 2002, Smith-Arica, Thomson et al. 2003, Jang, Koerber et al. 2011). There are numerous proposed reasons for the low infected efficiency for stem cells. Intracellular transport pathways that may distinct from different cell types may as barriers for AAV transduction. For example, translocation of AAV to the nucleus is an important rate-limiting step for AAV transduction for certain cell types such as murine fibroblasts and HeLa and KB cells(Hansen, Qing et al. 2000).Additionally, non-capsid related limitations exist. For instance, double-stranded DNA synthesis with the single-stranded D sequence-binding protein phosphorylation can influence AAV-mediated targeted gene expression, but the barrier mentioned here has been circumvented using self-complementary vectors(Hansen, Qing et al. 2000, Wang, Ma et al. 2003, Hirsch, Green et al. 2010). The potential and easily accepted reason is that stem cells lack some crucial receptors in the surface, such as heparan sulfate proteoglycan (HSPG), which plays a vital role in AAV recognition and affinity(Jang, Koerber et al. 2011). Insertion of several short peptides into the exterior surface of AAV substantially increased the delivery efficiency(Jang, Koerber et al. 2011, Kotterman, Vazin et al. 2015, Kremer, Cerrizuela et al. 2021). More importantly, the previously reported NSCs with low AAV transduction efficiency are mainly derived from rodent or human embryos, which indicted that species and time-related heterogeneity may lead to distinct infection efficiency.

Here, we used Krabbe patient iPSCs-derived NSCs named K-NSCs as the Krabbe disease model and screening platform to select some widely used AAV serotypes. It's found that AAV2 has the highest infected efficiency, of which the mechanism we proposed was that K-NSCs have the AAV2 serotype recognition and binding sites. The exact sites in the K-NSCs as well as the function mechanism for AAV2

binding and entry should need further studies. We also used AAV2 serotype carrying the Krabbe disease associated gene *GALC* to infect K-NSCs and concluded that AAV2-*GALC* could infect K-NSCs and rescued the GALC enzyme activity.

Conclusion

In this study, K-NSCs derived from Krabbe patient were used as a platform to screen a variety of commonly used wild AAV serotypes. It was found that AAV infected NSCs at different MOIs could not change the stem cell status of NSCs and AAV2 was selected as a potential AAV serotype that effectively transduced K-NSCs *in vitro*. By carrying the *GALC*, AAV2 restored GALC enzyme activity in K-NSCs. The selected AAV candidate revealed its potential utility in further studies on the pathogenic mechanism of Krabbe disease and novel gene therapy or NSCs replacement therapy applications.

Abbreviations

AAV: Adeno-associated virus; **NSCs**: Neural stem cells; **GFP**: Green fluorescence protein; **iPSCs:** Induced pluripotent stem cells; **K-NSCs**: Krabbe patient iPSCs derived NSCs; **GALC**: Galactocerebrosidase; **GLD**: Globoid-cell leukodystrophy; **MOI**: Multiplexity of infection; **RT-qPCR**: Real-time quantitative PCR; **HSPG**: Heparan sulfate proteoglycan; **ORFs**: Open-reading frames; **ITRs**: Inverted terminal repeats.

Statements & Declarations

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Competing interests

The authors have declared that no competing interest exists.

Authors' contributions

GT carried out the molecular studies and drafted the manuscript. CC and SY helped with the experiments design and data interpretation. YL and WW conceived of the study, participated in its design, and wrote the manuscript. YZ help performing new analyses and drafting the revision of manuscript. The authors read and approved the final manuscript.

Data Availability

The authors confirm that all data underlying the findings are fully available.

Ethics approval

This study is approved by medical research ethics committee of the China-Japan Friendship Hospital, Beijing, China. Approval number:2018-102-k74

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

Not applicable.

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Figures

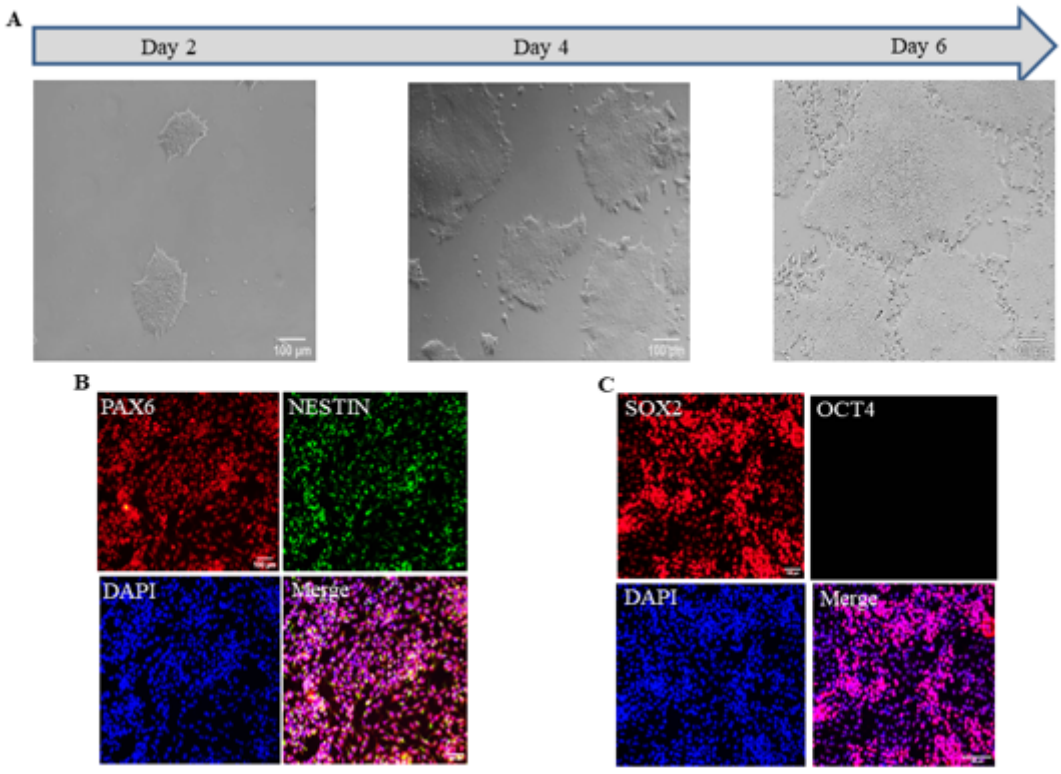

Figure 1

Differentiation of K-NSCs derived from K-iPSCs cells. **A,** Timeline of differentiation of K-NSCs. **B and C,** Representative images of K-NSCs stained with anti-PAX6(red), anti-Nestin(green), anti-SOX2(red) and anti-OCT4(green) antibody, nuclei stained with DAPI (blue). Scale bar 100 μm.

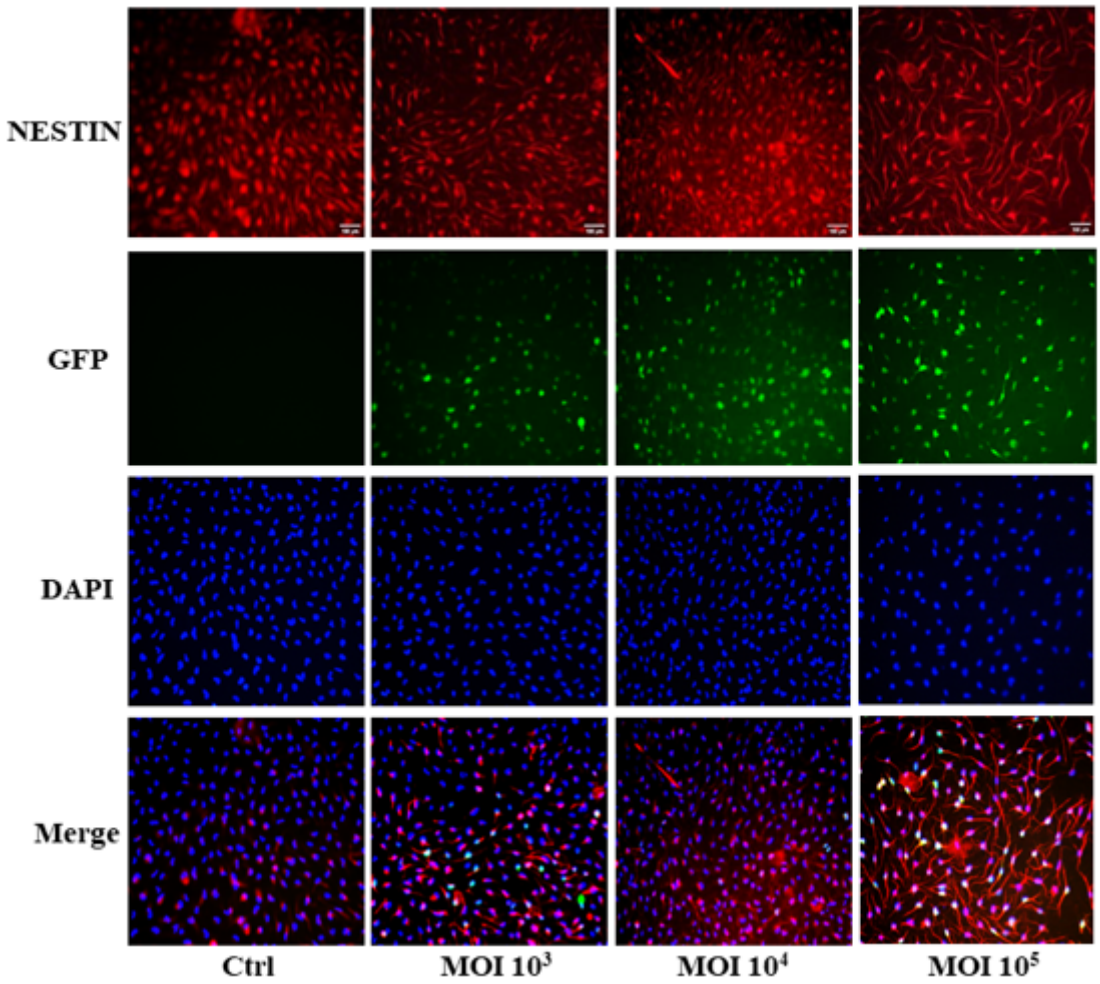

Figure 2

Native GFP fluorescence and immunostaining nestin in K-NSCs 48h after AAV-PHP.eB transduction. K-NSCs infected by AAV-PHP.eB at different MOIs. Immunostaining revealed that the infected K-NSCs cells were positive for NESTIN(red), a neural progenitor cell marker. nuclei stained with DAPI (blue). Scale bar 100 μm.

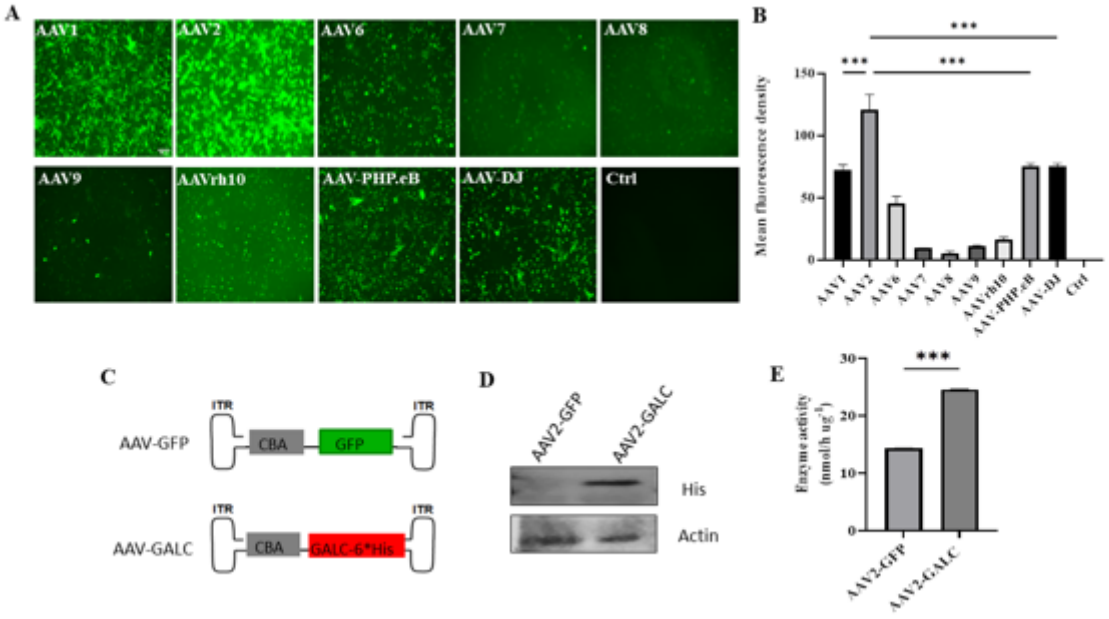

Figure 3

AAV2 infected K-NSCs with higher efficiency. **A,** Native GFP fluorescence in K-NSCs 48h after different AAV serotypes infection at the MOI of 10^5. Scale bar 100 μm. **B**, Mean fluorescence density of GFP in K-NSCs infected by different AAV. ***P < 0.001. **C,** Schematic representation of AAV-containing GFP and human GALC was subcloned under the control of the CBA promoter. **D,** Western analysis of infected K-NSCs with the antibody against His and β-actin. **E,** AAV2-GALC virus infection increased the GALC enzyme activity of K-NSCs. ****P* < 0.001.

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