Assessment of 3T3-L1 transduction using different AAV capsid variants

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Abstract

Conversion of energy-storing white adipose tissue into energy-burning beige adipose tissue, called browning, has emerged as a promising approach in the field of metabolic research and obesity treatment. Adeno-associated viruses (AAV) are widely used as viral vectors for gene delivery in eukaryotic cells. This study focused on the efficacy of transduction of AAV 2/5, 2/6, 2/8 expressing GFP in 3T3-L1 murine preadipocyte cells by live imaging microscopy using IncuCyte S3. Three transduction modes were assessed: AAVs transduction in 3T3-L1 preadipocyte cells with or without further differentiation into mature adipocyte-like cells and injection of AAVs in differentiated adipocyte-like cells. The differentiation of 3T3-L1 was induced by adipogenic IBMX-DEX-INS cocktail. AAV2/6 demonstrated the highest transduction efficiency in 3T3-L1 preadipocytes, as it was 1.5–2-fold more effective than AAV2/5 and AAV2/8 in the range of viral concentration from 2×10^4 to 8×10^4 VG/cell. AAV2/5 and AAV2/8 showed the transduction efficiency similar to each other. The expression of GFP under CMV promoter remained stable up to 20 days. The induction of 3T3-L1 differentiation in three days after AAVs transduction did not alter the GFP expression level and AAV2/6 showed the best transduction efficiency. AAV2/6 demonstrated ability to transduce mature adipocytes. Thus, AAV2/6 compared to AAV2/5 and AAV2/8 demonstrated the higher transduction efficacy in 3T3-L1 preadipocytes and mature adipocytes, which proved its usability along with AAV8 and AAV9 for gene delivery to adipocytes.

Introduction

Obesity, which has reached global epidemic, is a major contributor to the development of type 2 diabetes mellitus and is a significant risk factor for cardiovascular diseases, including coronary heart disease [1]. The expansion of adipose tissue is characterized by both hypertrophy (increased adipocyte size) and hyperplasia (increased number of adipocytes), and these processes depend on nutrient influx and the rate of adipocyte differentiation [2]. Adipocyte differentiation, also known as adipogenesis, is the process by which fibroblast-like precursor cells transform into fully differentiated adipocytes under the influence of adipogenic stimulants such as insulin and glucocorticoid agonists. This process is also influenced by an individual's genetic background.

The action of adipogenic stimuli activates transcription factors that regulate the expression of numerous genes involved in adipocyte differentiation. Among them, the nuclear receptor

PPARγ plays a key role in the adipogenic program by binding to the promoters and enhancers of adipocyte-specific genes [3]. The process of thermogenic adipocytes differentiation is determined by different PPARγ co-activactors, among which PRDM16 [4]. PRDM16 is able to stimulate the expression of uncoupling protein (UCP1). UCP1 conducts electrophoretic proton backflux to the matrix without ATP synthesis, which leads to the release of energy in the form of heat [5].

Conversion of energy-storing white adipose tissue into energy-burning beige adipose tissue, called browning, has emerged as a promising approach in the field of metabolic research and obesity treatment. Adeno-associated viruses (AAV) are widely used as viral vectors for gene delivery in mammals. We propose to use AAVs for transduction of adipose tissue cells. Therefore, it is important to use a relevant cell model to evaluate the effects of gene therapy on adipogenesis.

There exist two types of preadipocyte cell lines: multipotent fibroblasts and unipotent preadipocytes. Multipotent fibroblasts (CHEF/18, RCJ3.1, 10T1/2, 1246, Balb/c 3T3, NIH 3T3, and 3T3-Swiss albino) have the ability to differentiate into various cell types. Unipotent preadipocytes (3T3-F422A, 1246, Ob1771, TA1, 30A5, SGBS, and 3T3-L1), which have already undergone initial stages of differentiation, can continue proliferating and choose to either terminally differentiate or remain in an undifferentiated state. These lines of unipotent progenitor cells are widely recognized as suitable models for studying the molecular processes involved in the transition from preadipocytes to adipocytes. The most commonly used were derived by subcloning 3T3 fibroblasts obtained from disaggregated Swiss mouse embryos: 3T3-F422A and 3T3-L1 [6].

This study focused on the efficacy of transduction of AAV 2/5, 2/6, 2/8 in murine preadipocyte cells.

Materials and methods

3T3-L1 cell culture

Cells were grown on Dulbecco's modification of Eagle's medium (DMEM high glucose, 4.5 g/L, Paneco) with addition of 10% bovine calf serum (NBCS New Zealand origin, Gibco) and 2 mM L-glutamine (Paneco). 3T3-L1 cells were seeded onto cell culture treated plastic dishes, and incubated at 37°C in a humid modified atmosphere (95% air, 5% CO2). Cells were passaged once a week until reaching approximately 70% confluence, and the medium (DMEM+10%BCS) was replaced every 3 days. For 3T3-L1 is important to avoid cell-to-cell contact unless required by the experimental conditions.

Induction of adipogenic differentiation

Cells were cultured up to 100% confluence in the DMEM+10%BCS medium, and differentiation was induced 48 hours after reaching confluency. To induce differentiation the medium was replaced by DMEM medium with 10% fetal bovine serum (FBS South American origin, Gibco) and with the addition of 0.5 mM isobutymethylxanthine (Sigma), 1 μM dexamethasone (Sigma), 5 μg/ml insulin (Paneco), mentioned as IBMX-DEX-INS. After 48 hours of induction, the cells were cultured in DMEM with 10% FBS and 1 μg/ml insulin (adipocyte maintenance medium); the medium was replaced every 2 days.

HEK293 suspension culture

Suspension cell cultures were grown on a BalanCD HEK293 animal component-free chemically-defined medium (Irvine Scientific) in a cell concentration range between $1*10^5$ and $3*10^6$ cells/ml. HEK293 cells were cultured in plastic Erlenmeyer flasks (Corning), and incubated at 37°C in a humid modified atmosphere (95% air, 5% CO2). Cells were passaged twice a week by addition of fresh medium to reach $1*10^5$ cells/ml. Alternatively, suspension cell cultures were passaged by centrifugation with subsequent resuspension in order to remove waste products.

Adeno-associated virus production

Adeno-associated viral vectors (AAVs) were produced by transfection of suspension HEK293 cells. Suspension cell cultures were grown up to concentration $1*10^6$ cells/ml. Transfection was carried out by mixing of sequentially added plasmid DNA (pDNA) pAAV-CMV-GFP, pHelper, and corresponding pRC vector into centrifuge tube, and solution of PEI (Linear, MW 40,000, Polysciences) 1 μg/ml taken for a ratio of pDNA:PEI 1:5 into another. After pre-adding components were diluted by the serum-free medium, and the tubes were mixed by pipetting and kept at room temperature for 10 minutes. For transfection, a prepared transfection mixture with a plasmids concentration of 1.5 μg per million cells. Previously prepared transfection mixtures were added directly into the Erlenmeyer flasks using a single-channel dispenser. After this, the flasks were transferred to an incubator for cultivation and kept at 37.0 °C, humidity 70%, CO_2 content 5%, stirring 120 rpm.

After 96–120 hours past transfection chemical lysis of the cell suspension was carried out using 10% Tween 20 for 1 hours at 37.0 °C and stirring 130 rpm. After chemical lysis enzymatic treatment with Serratia marcescens endonuclease was performed in order to hydrolyze free nucleic acids. The resulting lysates were clarified by adding diatomaceous earth (0.01 g/ml of Celite HyFlo Super Cel, Roth), mixing for 5 minutes at 37.0 °C and 330 rpm, followed by sterilizing filtration of the lysates through a filter with a pore diameter of 0.22 μm. AAVs were purified using POROS CaptureSelect AAVX Affinity Resin (Thermo Fisher Scientific).

AAV transduction

After seeding the cells into a 48-well culture plate, the volume of viral eluate required was calculated (based on the cell number, in a range from 10,000 to 300,000 viral genomes per cell). Thus, to transduce 100,000 cells with 40,000 viral genomes per cell $4*10^9$ viral genomes were introduced into a well. The required volume of viral eluate was added immediately after seeding unless required by the experimental conditions.

Microscopy

1. After transduction, the culture plate was transferred to the IncuCyte S3 system for intravital cell analysis.

2. The system has selected the scan type according to schedule (Scan on schedule).

3. In the Create or Restore Vessel field, the corresponding type of vessel used (in our case, a 48-well plate manufactured by Eppendorf) and imaging channels (Brightfield and Green).

4. In the Vessel Location field, the position in the system corresponding to the position of the scanned tablet was selected.

5. In the Scan Pattern field, the wells of the plate to be analyzed and the number of microphotographs for each well of the plate (9) were selected.

6. In the Vessel Notebook field, the name of the experiment in the system, as well as the cell type, was entered.

7. In the Analysis Setup field, the following settings are selected: Basic Analyzer, GFP per cell.

8. In the Scan Schedule field, a scanning schedule was generated for the analyzed wells of the plate.

Results

Our study focused on the efficacy of transduction of AAV 2/5, 2/6, 2/8 expressing GFP in 3T3-L1 murine preadipocyte cells by live imaging microscopy using IncuCyte S3. Three transduction modes were assessed: AAVs transduction in 3T3-L1 preadipocyte cells with or without further differentiation into mature adipocyte-like cells and injection of AAVs in differentiated adipocyte-like cells. The differentiation of 3T3-L1 was induced by adipogenic IBMX-DEX-INS cocktail. AAV2/6 demonstrated the highest transduction efficiency in 3T3-L1 preadipocytes in the range of viral concentration from 2×10^4 to 16×10^4 VG/cell (fig.1). AAV2/8 showed the better transduction efficiency than AAV2/5 at high dosage (8 and 16 $\times10^4$ VG/cell) and similar efficiency at lower dosage. All AAVs serotypes have insignificant toxic activity to

3T3-L1 preadipocytes. In most studies AAV8 [7] is used for gene delivery in 3T3-L1 preadipocyte cell line. Our study showed that AAV2/6 also can be used for gene delivery in 3T3-L. Taking into account different tissue tropism of 8 and 6 AAV serotypes AAV2/6 for some application can be an option for targeted gene delivery.

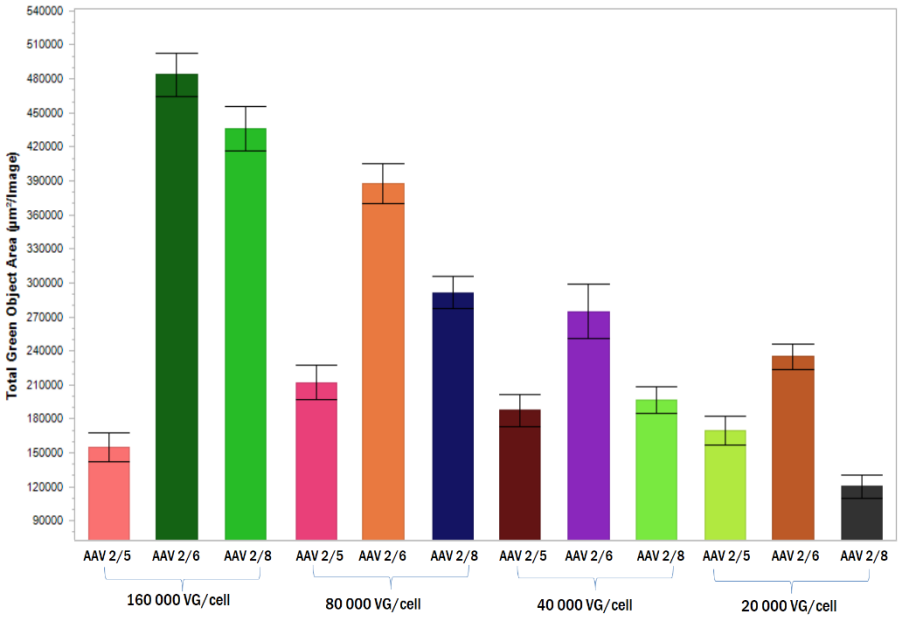

Fig. 1. Quantitation of green fluorescent objects after transduction in 3T3-L1 preadipocytes with different AAV serotypes and concentration (VG/cell - viral genomes per cell)

A transduction efficiency is the most important but not the only one criteria for choosing AAV serotype. Other one is an ability to ensure and sustain a permanent transgene expression in target cells. In our study we kept cells more than 20 days after transduction. It was observed that the expression of GFP under CMV promoter increased within 20 days after transduction. The GFP expression remained stable after reaching a peak in day 20 – 22 after gene delivery.

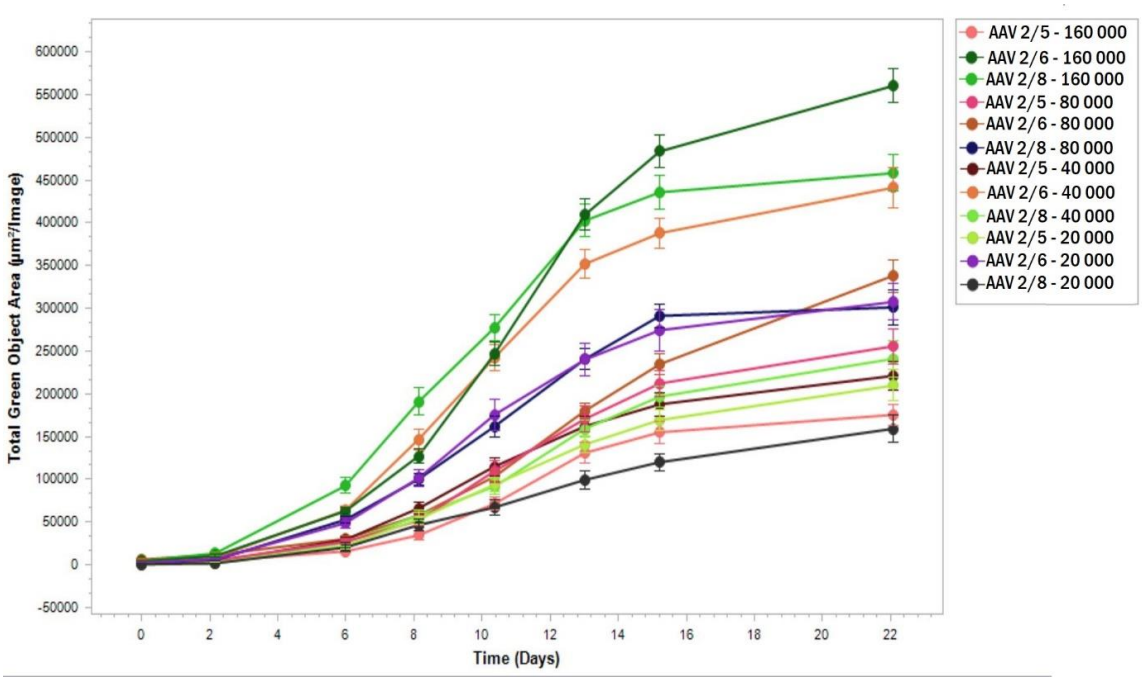

Fig. 2. Time-course quantitative analysis of green fluorescent objects after transduction in 3T3-L1 preadipocytes with different AAV serotypes and concentration (VG/cell - viral genomes per cell)

The induction of 3T3-L1 differentiation in three days after AAVs transduction did not alter significant the GFP expression level and AAV2/6 showed the best transduction efficiency (fig.3).

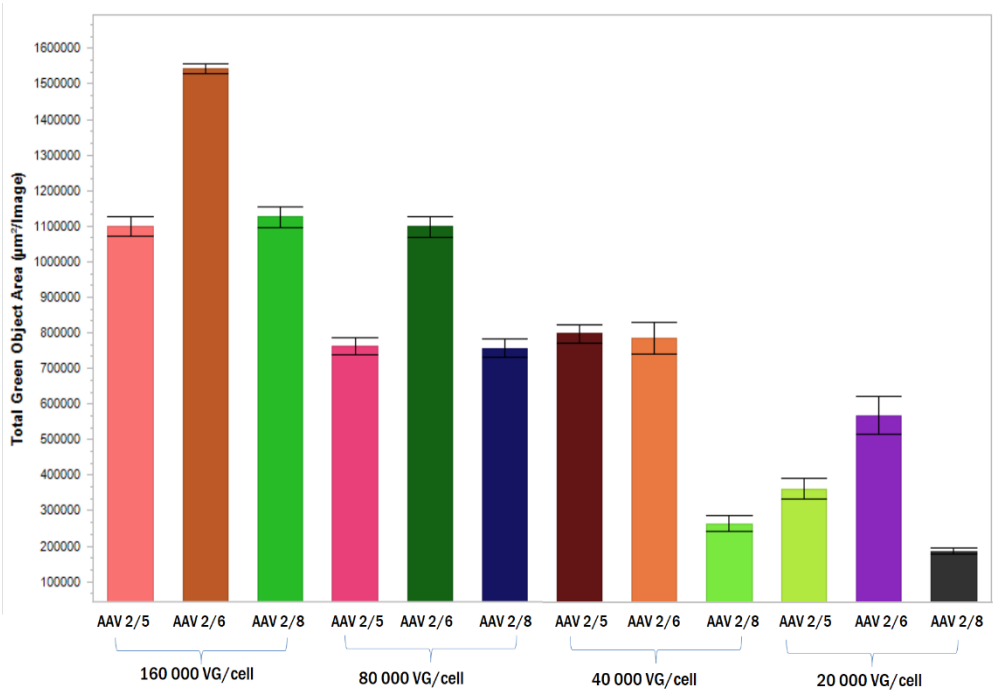

Fig. 3. Quantitation of green fluorescent objects after transduction of 3T3-L1 preadipocytes with different AAV serotypes and concentration with further differentiation (VG/cell - viral genomes per cell)

AAV demonstrated ability to transduce mature adipocytes as well (fig. 4). Differentiation of preadipocytes to mature adipocytes was initiated using IBMX-DEX-INS cocktail. Transduction was performed after a completion of differentiation process (usually it takes 10 – 14 days). AAV2/6 also showed a best transduction efficiency meaning that alteration in cell membrane and transcription profile during differentiation does not affect transduction ability.

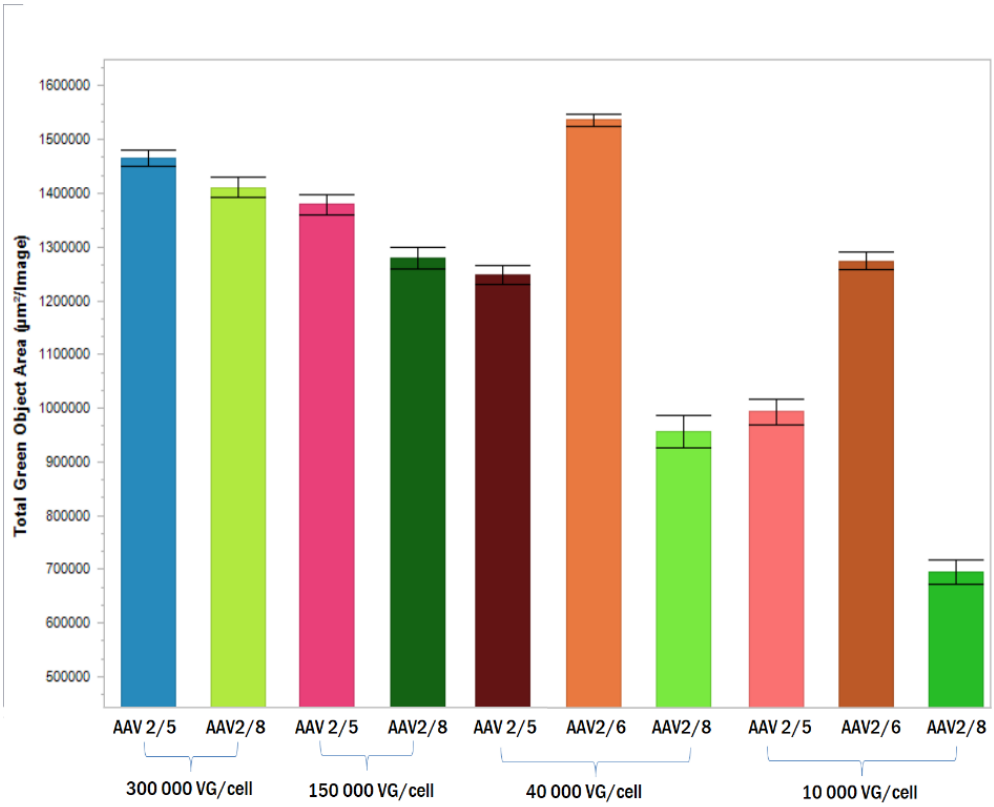

Fig. 3. Quantitation of green fluorescent objects after transduction in 3T3-L1 mature adipocytes of different AAV serotypes and concentration (VG/cell - viral genomes per cell)

Conclusion

Thus, AAV2/6 compared to AAV2/5 and AAV2/8 demonstrated the higher transduction efficacy in 3T3-L1 preadipocytes and mature adipocytes, which proved its usability along with AAV8 and AAV9 for gene delivery to adipocytes.

Acknowledgments

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