Research Article

Increased transgene expression mediated by recombinant adeno-associated virus in human neuroglia cells under microgravity conditions

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Received on March 30, 2016; Accepted on July 8, 2016; Published on July 31, 2016

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Abstract

The space environment has the special characteristics of radiation, noise particularity and weightlessness, all of which have adverse effects on astronauts' muscles, bones, neurons and immune system. Some reports have shown that chemotherapy and radiotherapy can increase the activity of the recombinant adeno-associated virus (AAV) which is widely used in gene therapy. In this paper, recombinant AAV2 (rAAV2) was first packaged with the enhanced green fluorescence protein (eGFP) gene and used to infect neuroglia cells including the U87 and U251 cell lines, under microgravity conditions; it was then detected by fluorescence microscopy and flow cytometry. The results show that microgravity affects the adhesion ability of cells, promotes transgene expression induced by rAAV2 and causes changes of viral infection receptors at different time points. These findings broaden the current understanding of the microgravity effects on rAAV, with significant implications in gene therapy and the mechanisms of increased virus pathogenicity under space microgravity.

Introduction

Microgravity, which is gravity or other forces caused by acceleration, is no more than $10^{-5} \sim 10^{-4}$g. Due to the balance between the centrifugal force and gravity, objects inside the orbiting spacecraft are in a close-to-zero gravity state, so the space environment is a microgravity environment. The space environment with the particularity of weightlessness has negative effects on the astronauts' muscles (Gopalakrishnan *et al*. 2010, Tesch *et al*. 2005), cardiovascular function (Platts *et al.* 2006, Stenger *et al.* 2010), metabolism (Morgan *et al.* 2012, Zwart *et al.* 2010), bones (Keyak *et al.* 2009, Smith *et al.* 2014), sensory (Lowrey *et al.* 2014) and immune systems (Crucian *et al.* 2009, Hughes-Fulford *et al.* 2015). Similarly, there is evidence showing that microgravity enhances the bacterial pathogenicity of salmonella typhimurium (Chopra *et al.* 2006, Nickerson *et al.* 2000) and the reactivation of latent virus infections, such as Epstein-Barr virus (Sonnenfeld & Shearer 2002, Stowe *et al.* 2001) as well as herpes simplex virus type 1 (Fuse & Sato 2004), but the mechanisms behind this are not well understood. In this experiment, the microgravity effects on the virus was studied via simulating a weightless environment. This can be achieved in multiple ways including drop-towers, parabolic flights of airplanes, sounding rockets or orbiting space platforms (Kiss 2014). In this study, a biaxial clinostat device, which can simulate the effect of microgravity on biology on the ground, was used to study the biological changes. The device consists of two independent rotating frames (outer and internal frame), a driving device, a bracket base and a controller, and is similar to a 3D gyrator (Hoson *et al.* 1992).

The adeno-associated virus (AAV) is a common non-pathogenic parvovirus which was discovered as a contamination in the preparation of adenovirus in 1965 (Atchison *et al.* 1965, Smith 2008). AAV is a single linear DNA virus with an icosahedron capsid but no envelope. Its diameter is about 20 nm, which makes AAV the smallest animal DNA virus. It is the only known virus which can integrate into mammalian DNA with two open reading frames (ORF) and inverted terminal repeats (ITR) (King *et al.* 2001). Recombinant adeno-associated virus (rAAV) is derived from wild type AAV by reserving the long terminal

repeat (LTR) and inserting an exogenous recombinant promoter region and the specific gene sequence which replaces the genome of AAV itself.

Gene therapy based on rAAV vectors holds promising potential in oral medication because of its good thermal stability and resistance to acid and alkali (pH 3.0-9.0), as well as the treatment of organic solvent resistance characteristics (Ma *et al.* 2005, Santiago-Ortiz & Schaffer 2016). The first human gene therapy using rAAV vectors was performed in cystic fibrosis (CF) patients (Loring *et al.* 2016) and the healing of rheumatoid arthritis and osteoarthritis (OA) using rAAV offered promise for further clinical testing (Madry & Cucchiarini 2016, Zhou *et al.* 2016). Because of the particular structure and location of the brain, rAAV-mediated gene therapy is considered difficult, but some recent studies have shown that AAV can associate with exosomes (exo-AAV) when the vector is isolated from the conditioned media of producer cells. In this case, it is more resistant to neutralizing anti-AAV antibodies and more efficient at brain-targeted gene delivery while it has also been reported to mediate brain-derived neurotrophic factor (BDNF) expression in the rat striatum (Connor *et al.* 2016, Hudry *et al*. 2016). In this experiment, rAAV was chosen because of its non-pathogenic and universality in the human body under normal gravity.

At present, 12 kinds of AAV serotypes were recognized by the International Committee on Taxonomy of viruses (Wu *et al.* 2006, Schmidt *et al*. 2008). These subtypes are mainly based on human and primate animal hosts; AAV1 - 6 is obtained from human tissue. Among AAV1-6, AAV2 is the earliest and most widely used serotype of infectious clones, because it has the widest tissue targeting abilities, including neurons, liver, skeletal muscle, retina and heart (Bartlett *et al.* 1998, Manno *et al.* 2003, 2006, Salva *et al.* 2007). Concerning the virus binding cell surface receptor, in the case of AAV1, AAV5 and AAV6, this receptor is N-linked sialic acid. The major cell surface receptor of AAV2 and AAV3 is heparan sulfate proteoglycans (HSPG), which is encoded by the heparan sulfate proteoglycan gene. It is a composite macromolecule consisting of a nuclear protein molecule and one or more glycosaminoglycans connected by glucosidic bonds. It is widely spread among mammalian cell surfaces including the cell base membrane (BM) and extracellular matrix (ECM) (Bishop *et al.* 2007). The cell surface receptor of AAV4 is O-linked sialic acid, the one of AAV8 is the laminin receptor (LamR) while the cell surface receptors of AAV7 and AAV9 - 12 are not clear at present (Summerford *et al.* 1998, 1999, Qing *et al.* 1999). The initial stage of viral infection is the viral attachment protein adsorbed to the cell surface. HSPG, as the major initial binding receptor, plays an important role in the attachment process of the AAV2 infection (Bishop *et al.* 2007). Integrin αvβ5 and fibroblast growth factor receptor 1(FGFR-1) are accessory receptors, and can enable AAV2 to attach and enter the cells through endocytosis mediated receptors (Bartlett *et al.* 1998, Perabo *et al.* 2006, Qing *et al.* 1999, Summerford *et al.* 1998, 1999).

The present study aimed to evaluate how rAAV2 responds when transfecting the human neuroglia cell line U87 or U251 and investigate the changes of rAAV pathogenicity or latency in weightlessness or microgravity conditions. The U87 and U251 cell lines, which were firstly transfected with rAAV2 encoding eGFP, were cultured under microgravity as well as normal gravity conditions to study the changes of the rAAV2 infection ability and cellular morphology. After different post-treatment time points, the infection efficiency and the transcriptional level of various viral receptors including HSPG, FGFR1 and Integrin αvβ5 were detected, in order to investigate the effect of microgravity on transgene expression, rAAV2 infection and the changes of viral infection receptors on cells.

Materials and Methods

Cell culture

U87 or U251 were cultured in DMEM (HyClone, USA) supplemented with 10% FBS, 1% NEAA and antibiotics (100 units/ml penicillin and 100μg/ml streptomycin, Beijing Solarbio company, China) at 37 °C in 5% CO_2. Cells were passaged when 80% confluent. Cells were transferred into 5 culture bottles before the experiment, one for cell counting and the others for transfection by rAAV2 encoding the eGFP gene.

RAAV2 preparation and transfection

The packaging of rAAV2, including the shuttle plasmid carrying the eGFP gene is referenced in a previous report (Ma *et al.* 2005). The transfection plasmid was extracted according to the plasmid extraction kit of the manufacturer's protocol (Tiangen biotech, Co. Ltd, Beijing, China). RAAV2 virus packaging used the calcium phosphate transfection method, according to the "AdEasy Vector System" operation protocol. HEK293 cells which were used in rAAV2 packaging were obtained by the University of British Columbia, Canada. HEK293 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) at 37 °C in 5% CO_2. 4 hours before packaging, the culture medium was replaced with 5 mL of fresh medium without antibiotics and the cell confluence was lower than 50%. The transfection plasmids were then added. After 2 days,

cells were collected and centrifuged at 1500 rpm for 5 min. The heparin column (GE company, USA) was then used to purify the packaged rAAV. The resulting concentration of the virus was about 10^{12}Gps/mL, obtained by absolute quantitative PCR analysis.

RAAV2 infection and cell culture under microgravity

RAAV2 was diluted with pure DMEM according to the multiplicity of infection (MOI), which refers to the number of virus particles per cell. When the viruses were used to infect cells, the following calculation formula was used: rAAV2 volume = the number of cells × MOI / virus titer. The rAAV2 dilution was then added into 2 of the 4 bottles and cells were cultured at 37 °C in 5% CO_2 for 1 hour, while the rest were cultured with pure DMEM.

After infection, the 4 bottles were filled up with complete culture medium; one rAAV2-infected bottle and one non-rAAV2-infected bottle was then placed into the biaxial clinostat device (developed by the Academy of Sciences Chinese space science and Applied Research Center). The other 2 bottles were placed in the normal cell culture incubator, all at 37 °C in 5% CO_2 atmosphere for three different time periods (24h, 48h and 72h). Cells were collected and analysed by flow cytometry (FACS420 Becton, USA) and a fluorescent inverted microscope (IX71, Olympus).

Real time quantitative polymerase chain reaction (RT-qPCR)

U87 or U251 cells were removed from the culture bottles by trypsinisation, following microgravity and normal gravity culture, at 24h, 48h and 72h. and were collected for RNA isolation and subsequent RT-qPCR analysis. Duplicate RT reactions (RT replicates) were performed for each RNA sample. RT controls were created from pooled samples of RNA, without reverse transcriptase or control DNA contamination of RNA. All complementary DNA (cDNA) samples were stored at -20 °C prior to analysis. The TransStart Top Green qPCR SuperMix kit (TransGen Biotech) was used for all qPCR reactions according to the manufacturer's protocol. The Applied BioRad iQ5 Real Time PCR system was used. The cycle threshold (CT) values were collected and the fold increase was calculated as following:

n-fold increase due to treatment =

$$2^{(-(CT_{\text{gene of interest}} - CT_{\text{GADPH}})\ \text{sampleA} - (CT_{\text{gene of interest}} - CT_{\text{GADPH}})\ \text{sampleB})}$$

The reaction of each primer pair (GADPH, HSPG, FGFR1, WPRE and Integrin αvβ5) is shown in Table 1; three PCR replicates were analyzed for each cDNA replicate. The primers were designed with Primer3

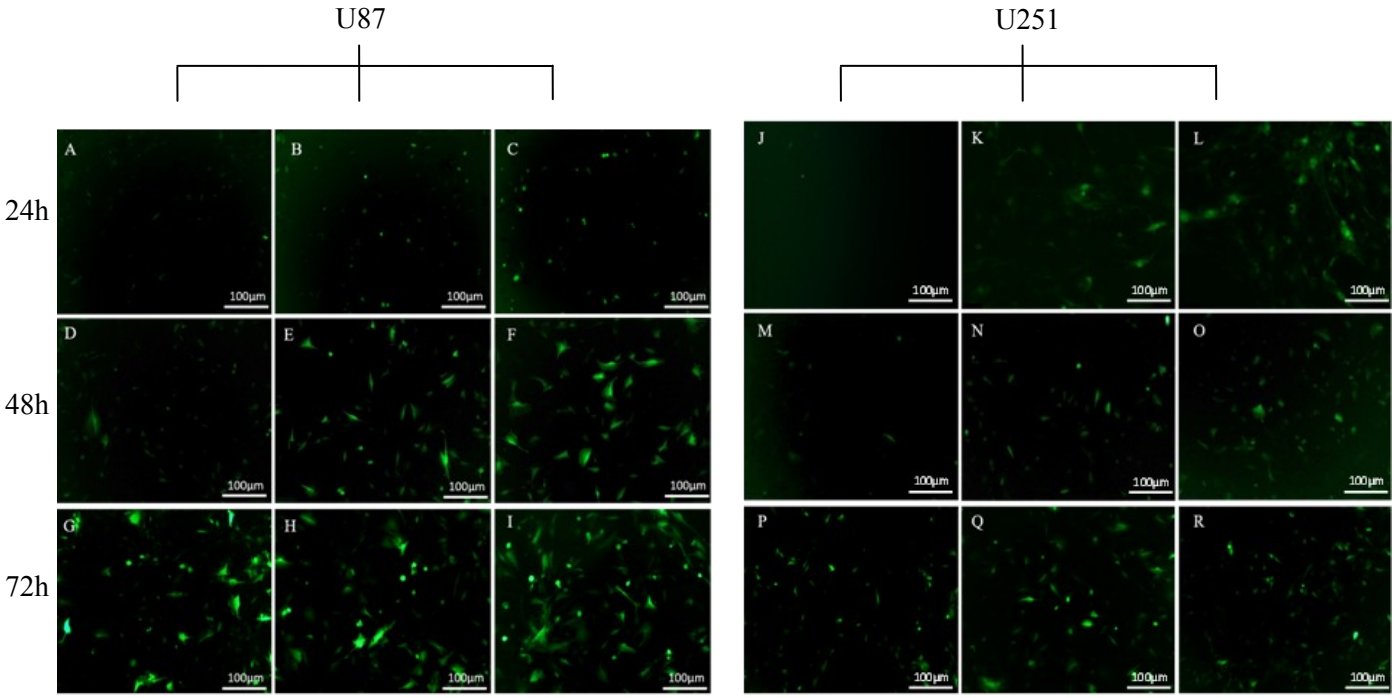


Figure 1. Comparison of the efficiency of rAAV2 infection of U87 and U251 cells at 24h, 48h, 72h in different conditions. A, D, G: The fluorescence field of U87 infected by rAAV2 in normal gravity; B, E, H: The fluorescence field of infected U87 with microgravity simulated simultaneously; C, F, I: The fluorescence field of U87 infected for 6 h first and then simulated microgravity; J, M, P: The fluorescence field of U251 infected by rAAV2 in normal gravity; K, N, Q: The fluorescence field of infected U251 with microgravity simulated simultaneously; L, O, R: The fluorescence field of U251 infected for 6 h first and then simulated microgravity (Scale bar=100μm).

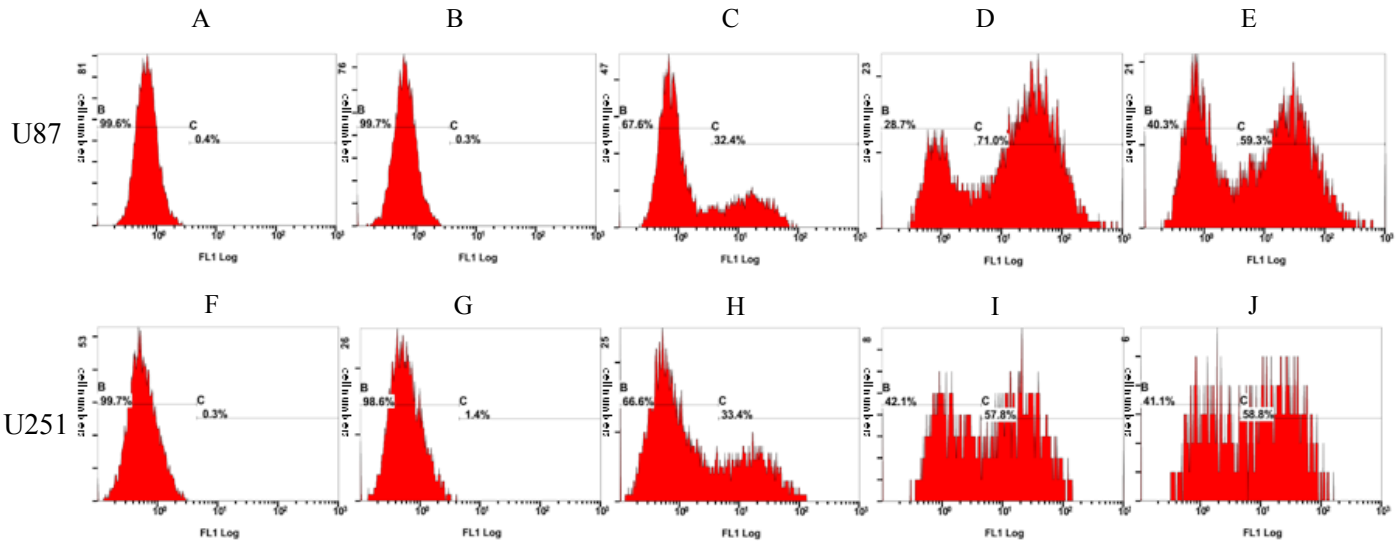


Figure 2. FACS analysis of transduction efficiency of rAAV2 in U87 and U251 cells collected 72 h post-infection. A: U87 cultured in normal gravity; B: U87 cultured in microgravity; C: U87 infected by rAAV2 in normal gravity; D: U87 infected for 6 h first and then simulated microgravity; E: U87 infected and simulated microgravity simultaneously; F: U251 cultured in normal gravity; G: U251 cultured in microgravity; H: U251 infected by rAAV2 in normal gravity; I: U251 infected for 6 h first and then simulated microgravity; J: U251 infected and simulated microgravity simultaneously. There were no positive cells in A, B, F and G compared to the others that showed different positive cell numbers.

(http://frodo.wi.mit.edu/primer3/input.htm).

Statistical analysis

Unless mentioned, the results are presented as mean ± SD of three independent experiments. Statistical analysis was performed by two-tailed t test by Excel and Origin 8 software. A p value < 0.05 was considered significant unless mentioned otherwise.

Results

Microgravity promotes the transduction of rAAV2 into neuroglia cells

To explore the relation between microgravity and rAAV2, U87 and U251 were both infected under two different treatments: either cells were infected for 6h and then went through simulated microgravity or cells were infected and went through simulated microgravity simultaneously via the biaxial rotary device. The cells were then detected at 24h, 48h and 72h via fluorescent inverted microscopy (Figure 1). The fluorescence intensity of both U87 and U251 gradually increased with time, and the weakest fluorescence intensity of U87 and U251 both appeared at 24h, while the strongest one was at 72h. These results show that there is a similar gradual increase in the transgene expression of rAAV2 in U87 and U251 with time, whether cultured through simulated microgravity (Figure 1B, C, E, F, H, I, K, L, N, O, Q, R) or normal gravity (Figure 1A, D, G, J, M, P). Interestingly, the magnitude of rAAV2 expression in microgravity was significantly higher than that in normal gravity. This suggests that microgravity might promote gene expression mediated by rAAV2 in neuroglia cells or the infection capacity of rAAV2. Meanwhile, there was no obvious difference between the fluorescence field of U87 and U251 cells that were infected and went through simulated microgravity simultaneously (Figure 1B, E, H, K, N, Q) and the ones that were infected for 6h first and then went through simulated microgravity (Figure 1C, F, I, L, O, R). This suggests that microgravity has no obvious effect on the infection capacity of rAAV2 and the difference between the microgravity and the normal gravity group is mainly caused by the improvement of gene expression mediated by rAAV2 within cells. Furthermore, the infection efficiency of rAAV2

Table 1. List of primers used for the different genes. F, forward; R, reverse.

Primer	Sequence (5'-3')
GADPH F	ACAACTTTGGTATCGTGGAAGG
GADPH R	GCCATCACGCCACAGTTTC
eGFP F	CAAGATCCGCCACAACATCG
eGFP R	GACTGGGTGCTCAGGTAGTG
HSPG F	CATGGGCTGAGGGCATACG
HSPG R	TGTGCCCAGGCGTCGGAAC
FGFR1 F	TGGAGTTCATGTGTAAGG
FGFR1 R	TCAAGATCTGGACATAAGG
Integrin αvβ5 F	CAAGAGCCGGATAGAGGACA
Integrin αvβ5 R	GTTCTGCTCCCCAAACACTT
WPRE F	TGGCGTGGTGTGCACTGT
WPRE R	AAAGGAGCTGACAGGTGGTG

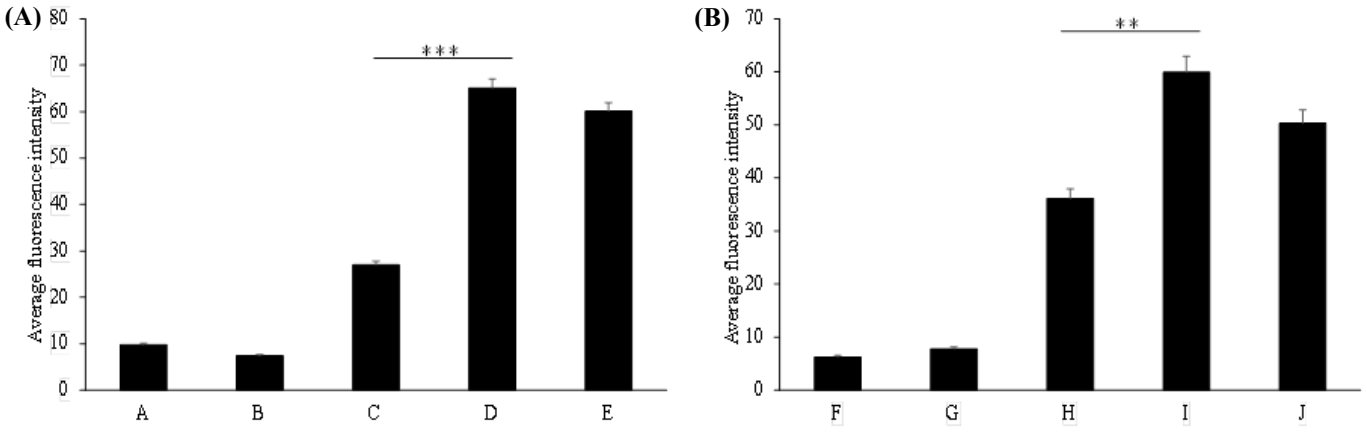


Figure 3. Analysis of average fluorescence intensity of U87 and U251 cells collected 72 h post-infection. **A)** The fluorescence intensity of U87 cells expressing eGFP. A: U87 cultured in normal gravity; B: U87 cultured in microgravity; C: U87 infected by rAAV2 in normal gravity; D: U87 infected for 6 h first and then simulated microgravity; E: U87 infected and simulated microgravity simultaneously. **B)** The fluorescence intensity of U251 cells expressing eGFP. F: U251 cultured in normal gravity; G: U251 cultured in microgravity; H: U251 infected by rAAV2 in normal gravity; I: U251 infected for 6 h first and then simulated microgravity; J: U251 infected and simulated microgravity simultaneously. Values are means ± SD (n = 3). **P < 0.01 compared with cells infected rAAV2 in normal gravity, ***p < 0.001 compared with cells infected rAAV2 in normal gravity, two tail t test.

in U87 was higher than that in U251 cells, and the transgene expression of rAAV2 in U87 and U251 gradually increased with time and was higher under microgravity compared to normal gravity conditions.

To quantify the transduction of rAAV2 in different gravity conditions, the virus infection rate of U87 and U251 was detected by flow cytometry (FACS420 Becton, USA) at 72h after rAAV2-eGFP infection (Figure 2). According to the flow cytometry results, when U87 and U251 cultured in the normal gravity group were set as their own control group, U87 and U251 positive cells, infected by rAAV2, accounted for 32.4% and 33.4% of the total cells (Figure 2C and H). This was significantly lower compared to that of U87 and U251 cells that were infected for 6h first and then went through simulated microgravity (71.0% and 57.8% respectively) (Figure 2D and I). The U87 and U251 positive cells that were infected and went through simulated microgravity simultaneously accounted for 59.3% and 58.8% of the total cells, respectively. (Figure 2E and J). These results might prove that microgravity does have a certain effect on virus infected cells. Furthermore, the results of U87 and U251 infected for 6h first and then simulated microgravity group and the results of U87 and U251 infected and simulated microgravity simultaneously group were similar, *i.e.* the proportions of positive cells over total cells were similar, which suggests that the enhancement effect of microgravity on rAAV2 entering the cell is not obvious. The data above shows infected U87 and U251 cells have an obvious improvement under microgravity and no significant difference between simulated microgravity simultaneously and 6h later.

Microgravity promotes the transgene expression mediated by rAAV2 in neuroglia cells

In order to quantify the expression of rAAV2 in cells under microgravity, the fluorescence intensity of U87 and U251 cell line expressing eGFP was detected via flow-cytometric analysis, while U87 was chosen to quantify the expression level of eGFP gene for further confirmation of the effect of microgravity on expression of rAAV2 at the molecular level. The average fluorescence intensity of U87 and U251 positive cells in the microgravity group was significantly higher compared to the normal gravity group (Figure 3A and B), which showed that microgravity might have an enhancing effect on the transgene expression mediated by rAAV2 in cells. Similarly, the expression level of eGFP gene in U87 cells that were infected by rAAV2 and went through microgravity conditions simultaneously was significantly higher compared to the normal gravity group at 24h, 48h and 72h, and was detected via a real-time fluorescent quantitative PCR instrument (Figure 4C). The proportion of U87 infected by rAAV2 and simulated microgravity simultaneously group over U87 infected by rAAV2 in normal gravity group was growing from 24h to 72h, and the proportion was highest at 48h (Figure 4D). This indicates that the fluorescence intensity of U87 infected by rAAV2 significantly increased under microgravity with time, and the efficiency of gene expression was enhanced under microgravity but decreased slightly at 72h be-

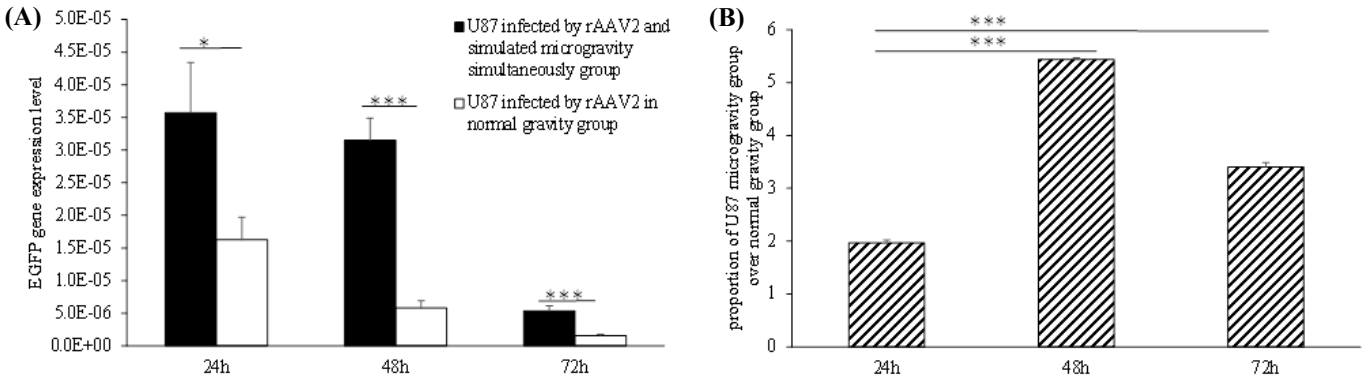


Figure 4. Effect of microgravity on the transcriptional level of transgene expression mediated by rAAV2-eGFP.A) Black bar shows U87 infected by rAAV2 and simulated microgravity simultaneously group; white bar showing U87 infected by rAAV2 in normal gravity group. B) Pattern bar showing the proportion between U87 infected by rAAV2 in microgravity simultaneously group and normal gravity group. Values are means ± SD (n = 3). *P < 0.05 compared with white bar, ***p < 0.001 compared with white bar at same point or between pattern bar at different point, two tail t test.

cause of changes in the cell death rate. All data suggests that microgravity could induce a significant transcriptional improvement after rAAV2 infection in neuroglia cells.

Microgravity affects the adhesion ability of neuroglia cells

To explore the effect of microgravity on the cell adhesion ability, U87 and U251 cells were cultured under normal gravity and microgravity conditions (Figure 5). Results show that all cells have a small number of suspension cells under microgravity, no matter whether cells were infected by rAAV2 or not (Figure 5A, B and C, D and E, F and G, H). Especially, U87 suspension cells were significantly more than U251 cells, and a portion of U87 cells actually adhered to the culture dishes with no pseudopodium and became circular while they also clustered into clusters (Figure 5B, D and F, H). The image mentioned above shows that U87 is more sensitive to microgravity and whether cells were infected by rAAV2 or not, there is no obvious effect on cell morphology. These suggest that microgravity affects the cell adhesion ability and U87 was selected to further study the effect of microgravity.

Microgravity and normal gravity induce different changes on virus receptors

To further investigate the molecular mechanism of microgravity on transgene expression induced by rAAV2, U87 cells were chosen because these cells, under microgravity, show sensitive changes in their form and in response to rAAV2 infection. The transcriptional ex-

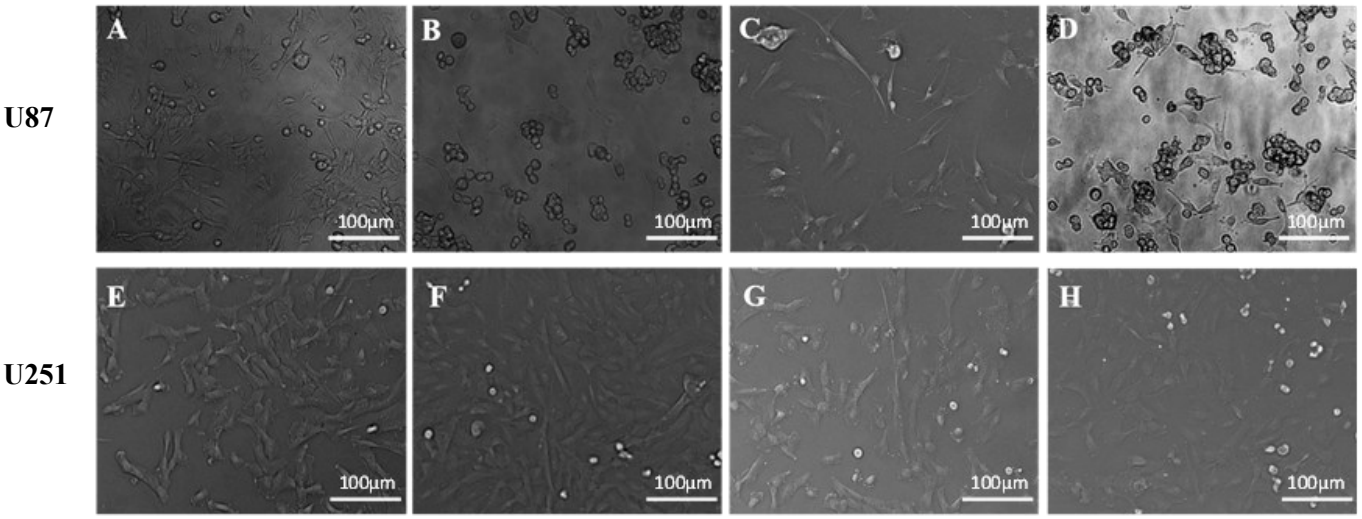


Figure 5. Effect of microgravity on the cell adhesion ability at 48h after rAAV2-eGFP infection. A: U87 cultured in normal gravity; B: U87 cultured in microgravity; C: Infected U87 cultured in normal gravity; D: Infected U87 cultured in microgravity; E: U251 cultured in normal gravity; F: U251 cultured in microgravity; G: Infected U251 cultured in normal gravity; H: Infected U251 cultured in microgravity (Scale bar=100μm).

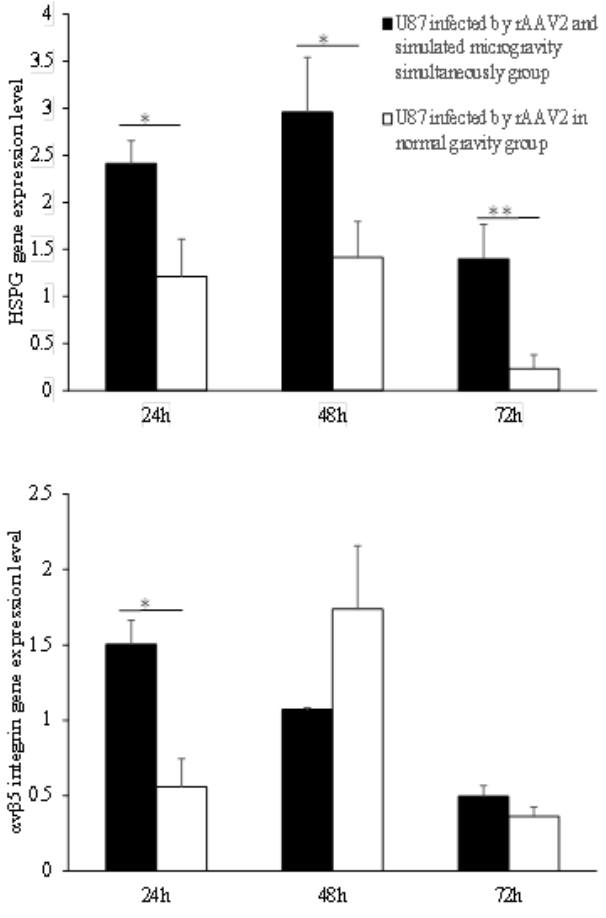
pression of virus receptors in infected cells was detected via real-time fluorescent quantitative PCR at 24, 48 and 72h (Figure 6). We analyzed the changes of HSPG, FGFR1 and Integrin αvβ5 virus receptors under microgravity.

Firstly, HSPG, as the initial binding receptor which has a major effect on the process of infection, was at an obviously higher level at 24h, 48h and 72h in U87 infected by rAAV2 under simulated microgravity simultaneously compared with U87 infected by rAAV2 in the normal gravity group (Figure 6A). This result shows that the HSPG receptor plays an important role in the expression of rAAV2 in cells and microgravity makes U87 cells become more sensitive to rAAV2 infection. Interestingly, the other virus receptors had different performances at distinct time points. The FGFR1 receptor, as a co-receptor for entering into cells, in addition to using the cell surface HSPG for attachment, had no obvious difference between the microgravity and normal gravity condition, although the expression level of FGFR1 under microgravity was lower than under normal gravity at 24h. However, the expression level of FGFR1 receptor under microgravity was significantly lower than under normal gravity at 48h and the difference disappeared at 72h (Figure 6B). Similarly, the expression level of Integrin αvβ5 receptor, which is co-activated with FGFR1, HSPG during mediated endocytosis of rAAV2 into cells, under microgravity conditions was significantly higher than under normal gravity at 24h. However, it had no obvious difference between microgravity and normal gravity condition subsequently at 48h and 72h (Figure 6C).

These might show that microgravity had various effects on different viral receptors respectively, but the change of HSPG receptor expression levels between microgravity and normal gravity was consistent at various time points, which suggests that HSPG might be a key to cells affected by microgravity.

Discussion

To investigate the effect of microgravity on the expression and infection mediated by rAAV2 on neuroglia cells, the rAAV2-eGFP gene delivery system was used to infect the U87 and U251 cell lines, which are of glioblastoma origin, under microgravity and normal gravity conditions. The results show that microgravity could change the adhesion ability of cells and promote the expression of rAAV2 (Figure 3, 4, 5), and suggests that it plays an important role on the expression and infection of rAAV2. On the other hand, the changes of



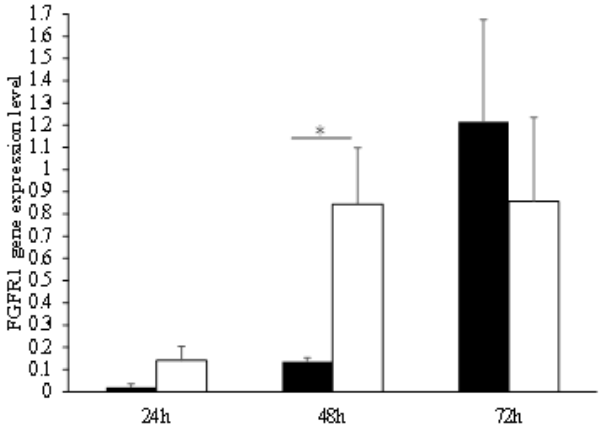


Figure 6. The effect of microgravity on expression of rAAV2 receptors. A) The expression level of HSPG receptor at 24, 48 and 72h. B) The expression level of FGFR1 receptor at 24, 48, 72h. C) The expression level of Integrin αvβ5 receptor at 24, 48, 72h. Black bar: U87 infected by rAAV2 and simulated microgravity simultaneously group; White bar: U87 infected by rAAV2 in normal gravity group, Results represent mean ± SD of 3 independent experiments. *p < 0.05, compare between black bar and white bar at same time point. **p < 0.01 compare between black bar and white bar at same point, two tail t test.

various virus receptors were detected by fluorescent quantitative PCR (Figure 6), which might show that microgravity has various effects on these receptors.

Adeno-associated virus (AAV) is a replication -defective DNA virus with relatively low host immune response, weak toxicity, and long term gene expression. In addition, AAV can integrate into the specific site of human chromosome 19, AAVS1 site (Flotte 2004, Russell 2009), and this avoids the risk of insertional mutagenesis and the occurrence of tumors caused by random integration. As a gene vector, rAAV is widely used in gene therapy (Luo *et al.* 2015, Bonten *et al.* 2013). In this experiment, U87 and U251 were infected by rAAV2 under microgravity and normal gravity and were detected at different time points. The results revealed that, even though both U87 and U251 are derived from human glioblastoma, there are different sensitivities to rAAV2 infection, suggesting that even cells from the same origin may have different rAAV2 infection sensitivities. These indicate that there are different curative effects on the same type of cells from the same part of body in gene therapy mediated by rAAV2 vectors.

There are some reports showing that microgravity can not only induce changes of gene transcription and replication levels such as Epstein-Barr virus and herpes simplex virus type 1, but also cause some immune cells, such as macrophages, T cells, to change the regulation of inflammatory cytokine expression and cell activation state (Hughes-Fulford *et al.* 2015, Stowe *et al.* 2011, Wang *et al.* 2015). Our results indicate that the ability of rAAV2 to infect neuroglia cells increased, and the expression level of rAAV2 in neuroglia cells significantly increased in the microgravity environment. This suggests that microgravity affects not only immune cells, but may also have a positive effect on rAAV or neuroglia cells, including the capacity of rAAV infection, rAAV gene expression, and the affinity of some receptors on the cell or the regulation mechanism of virus expression in cells through an unknown pathway. These factors may lead to different performances of rAAV2 in microgravity or normal gravity.

On one hand, the AAV2 type is common in the human body, which has no obvious pathogenicity under normal gravity, though, this kind of change of virus or cell under microgravity conditions makes AAV potentially pathogenic, which may have an effect on astronauts in future long-duration missions. On the other hand, the microgravity environment can also be used to change the state of pathogenic viruses in order to reduce or eliminate pathogenic factors, which provides a new direction for viral vaccine production. Previous studies have found that rAAV2 has been shown to suppress DNA replication and oncogenicity of a number of viruses including adenovirus (Casto *et al.* 1967), pox virus (Schlehofer *et al.* 1986) and human papillomavirus (HPV) (Meyers *et al.* 2001). Microgravity can be used to improve the expression of rAAV or the factors that lead to the increase of virus expression, and can be further studied to provide a new breakthrough for AAV vector mediated gene therapy.

The stabilization of the cell's morphological structure mainly relies on the cytoskeleton, which is a very active system. It can be reconstructed during a short time; even under stable conditions, polymers of cytoskeleton and free monomers are changing constantly. The original force balance is broken under microgravity, while the microfilament and microtubule polymerization and depolymerization are very sensitive to a change of gravity (Goldstein *et al.* 1992, Ingber 1999, Philpott *et al.* 1992). Studies on adherently growing human cells exposed to short-term real microgravity conditions during parabolic flights and subsequent analysis on earth have provided some evidence for cytoskeleton alteration (Grosse *et al.* 2012, Aleshcheva *et al.* 2015). Furthermore, the cell adhesion ability can be affected to a certain extent under microgravity, as the cells cultured under microgravity are more likely to remain in a suspended state while the cell attachment time is delayed (Sato *et al.* 2001). Given the importance of the cytoskeleton, its changes will directly or indirectly participate in or lead to changes in the cell cycle, signaling pathways and apoptosis response under microgravity.

In this experiment, U87 and U251, infected by rAAV2 were studied under microgravity at 24h, 48h and 72h. The number of U87 circular cells was significantly higher than that of U251; however, the circular cells are not like suspension cells which move with the movement of the liquid medium. Both U87 and U251, adhere to the culture dish with no pseudopodium and this might relate to the change of cytoskeleton under microgravity. It is worth noting that U87 cells clustered into clusters compared with U251 which had no significant clustering phenomenon under microgravity. The level of gene expression and protein expression showed a consistent trend (Fig. 3, 4), meaning that the higher the expression of a gene is, the higher the protein expression. However, taking into account the different results between U87 and U251, infected by rAAV2, in microgravity (Fig. 3, 4), the correlation between stabilization of cell morphological structure, rAAV2 infection and rAAV2 expression in cells need to be further explored. Given that there is an interaction between glial cells and immune cells in the brain (Appel *et al.* 2010), changes of nerve cell morphology and virus susceptibility might offer a new way for fu-

ture virus mediated neurological disease gene therapy.

To further investigate the causes of the affected infection ability of the virus under microgravity, HSPG, FGFR1 and Integrin αvβ5 virus receptor were chosen in this experiment. The expression level of the accessory receptors affects the endocytosis of rAAV2 entering cells. A real-time tracking experiment of a single AAV molecule showed that AAV completely entered in 100ms (Seisenberger *et al.* 2001). This shows that HSPG, FGFR1 and Integrin αvβ5 receptor play a key role in the process of rAAV2 infection. In this experiment, the expression of rAAV2 and virus receptors was detected at 24h, 48h and 72h. Our results showed that HSPG, the cell surface receptor, maintained high activity under microgravity compared with HSPG in normal gravity from 24h to 72h; however, the other receptors, namely FGFR1 and Integrin αvβ5, had different changes at different time points. The activity of FGFR1 was lower in microgravity at 24h and 48h compared to normal gravity, while there was no difference between microgravity and normal gravity at 72h. Similarly, Integrin αvβ5 receptor's activity was higher in microgravity at 24h, but there was no difference between microgravity and normal gravity at 48h and 72h. The different changes of accessory receptors (FGFR1, Integrin αvβ5) at 24h and 48h might be caused by the fact that cells couldn't adapt to the microgravity environment at the early stages. Therefore, this maladaptive situation induced a disorder of the accessory receptors; however, cells began to adapt to the microgravity environment under the extended microgravity time and the accessory receptors returned to normal levels. Interestingly, HSPG made a response in the first time when rAAV2 began to infect, although cells were not adapted to the microgravity environment at the early stages, possibly because HSPG is the main initial binding cell surface receptor of rAAV2 that is the most sensitive to virus infection. Microgravity could further improve the activity of HSPG via some pathways we do not know yet. Overall, whether microgravity affect cells themselves or rAAV2 to cause the changes on virus receptors needs to be further researched.

In conclusion, this study, which used rAAV2 mediated eGFP gene delivery to infect U87 and U251 cell line under microgravity and normal gravity condition has revealed the effect of microgravity on rAAV2 infection. The results have shown that microgravity affects the adhesion ability of cells, promotes the expression of rAAV2 in cells and causes virus receptors to have different changes at 24h, 48h and 72h. These findings may broaden our current understanding of microgravity effect on the rAAV infection progression, as a whole, with significant implications in rAAV mediated gene therapy and the mechanism of increased virus pathogenicity under space microgravity conditions.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Sun Feiyi performed all experiments, data analysis and contributed to the manuscript preparation. Ma Chengwei designed the vector, packaged recombinant AAV2 and performed the detection of flow cytometry analysis as supervised by Zhang Yaxi. Zhang Jiewen designed the primers and performed all the microgravity and real time-PCR experiments. Deng yulin, Zhang Lan and Li Yali was responsible for the conception and design of the study, data analysis and manuscript preparation. Ma Hong assisted with manuscript preparation. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by Natural Science Foundation of China (No.31200636), and was supported by Beijing Key Laboratory of brain disease to build a national key laboratory cultivation base in 2015 open plan (No.2015NZDJ01 and No.2015SJBX04).

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