

Effect of cryopreservation on A172 and U251 glioma cells infected with lentiviral vectors designed for CRISPR/Cas9-mediated knockdown of aquaporin-8

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Abstract

The gene editing technology CRISPR/Cas9 is presently applied in several fields. Here, we routinely infected A172 and U251 cells with lentiviral vectors in which aquaporin-8 (AQP8) was knocked down using CRISPR/Cas9. Cryopreservation did not significantly alter the viral infection efficiency, but it affected the expression of AQP8 protein and its mRNA in infected cells compared with non-cryopreserved samples. The expression of AQP8 and its mRNA in cryopreserved infected cells that were recovered did not significantly differ from that in the blank and negative controls. This finding indicated that the lentiviral vector lost viability due to cryopreservation at low temperatures and failed to release the *AQP8* gene-targeting guide RNA in the infected cells, or the guide RNA was released, but underwent changes that caused it to malfunction in CRISPR/Cas9-mediated *AQP8* gene knockdown. This finding might provide a new direction for the prevention and treatment of viral infections.

1. Introduction

The CRISPR/Cas9 gene editing technology is a convenient and efficient means of precisely and site-specifically modifying a specific sequence of a gene of interest, and it has been widely used. Cryopreservation is a means of temporarily storing cells at low temperatures to prevent sustained growth. Various types of cells transfected with specific plasmids or infected with specific viruses can be cryopreserved for later use. However, cryopreservation might not be applicable to all types of cells. Here, we constructed an aquaporin-8 (AQP8)-knockdown viral vector using CRISPR/Cas9 and then used it to infect A172 and U251 cells. We incidentally found that cryopreservation was unfavorable for AQP8 expression in the infected cells.

2. Results

2.1 Virus construction and sequencing

Figure 1A shows the construction of the lentiviral vector Lenti-sgRNA-tag, which we used to recognize the target gene sequence, and the lentiviral vector Lenti-cas9-puro, which was used to knockdown AQP8. The presence of the target gene in the vectors was confirmed by sequencing (Shanghai Genechem Co., Ltd., Shanghai, China). Figure 1B shows the sequence underlined in red.

Fig. 1. Vector GV371 and lentivirus.

a, Vector GV371 with components linked in order of (a) U6-sgRNA-SV40-EGFP and (b) Lenti-cas9-puro. **b**, Sequence of sgRNA.

2.2 Viral infection rate of cryopreserved versus non-cryopreserved glioma cells A172 and U251

We counted cells with and without cryopreservation in bright and fluorescent fields, then determined viral infection in A172 and U251 cells using the ratio of the fluorescent to bright field data. The abundance of GFP expression and viral infection rates did not significantly differ between A172 and U251 AQP8-knocked down cells with and without cryopreservation and negative control cells.

Table 1. Viral infection rates

Data are shown as means \pm standard deviation (SD).

Figure 2. Viral infection efficiency assessed by microscopy using GFP fluorescence.

a, Bright field. **b**, Infected cells in the fluorescent field. Blank control (a), negative control (b), non-cryopreserved cells with *AQP8* knocked down (c), cells infected and then cryopreserved for 48 hours (d) and 1 (e), 2 (f) and 4 (g) weeks.

2.3. Protein expression of AQP8 in virally-infected A172 and U251 cells with and without cryopreservation

Western blots (Figure 4) revealed significantly lower AQP8 expression (gray values) in A172 and U251 cells with AQP8-knockdown that were not cryopreserved than in the blank and negative controls ($P < 0.001$). The expression of AQP8 in infected, cryopreserved, and recovered cells did not significantly differ from the two controls regardless of the duration of cryopreservation.

Figure 3. Western blots of AQP8 protein expression in lentivirus-infected A172 and U251 cells with or without cryopreservation.

A. Changes in AQP8 protein band in A172 cells. B. Comparison of AQP8 expression among different groups of A172 cells. C. Changes in AQP8 protein band of U251 cells. D. Comparison of AQP8 expression among different groups of U251 cells. Blank control (a), negative control (b). Non-cryopreserved cells with AQP8 knockdown (c). Cells infected then cryopreserved for 48 hours (d) and 1 (e), 2 (f), and 4 weeks (g). $*P < 0.001$, non-cryopreserved cells with AQP8-knockdown vs. the other six groups.

2.4. Detection of *AQP8* mRNA expression in A172 and U251 cells with and without cryopreservation using RT-qPCR

Figure 4 shows significantly lower *AQP8* mRNA expression in A172 and U251 cells with AQP8-knockdown without cryopreservation than in the blank and negative controls ($P < 0.001$). The expression of *AQP8* mRNA did not significantly differ between infected, cryopreserved, and recovered cells compared with the two control groups, regardless of the duration of cryopreservation.

Figure 4. Detection of *AQP8* mRNA expression in cryopreserved versus non-cryopreserved cells using QT-qPCR.

A, Amplification (A) and **B**, dissociation (B) curves of *AQP8* mRNA in A172 cells. **C**, Amplification (C) and **D**, dissociation (D) curves of β -actin RNA in A172 cells. **E**, Amplification (E) and **F**, dissociation (F) curves of *AQP8* mRNA in AU251 cells. **G**, Amplification (G) and **H**, dissociation (H) curves of β -actin mRNA in U251 cells. Relative expression of *AQP8* mRNA in **I**, A172 (I) and **J**, U251 (J) cells. * $P < 0.001$, cells with AQP8-knockdown without cryopreservation vs. the other six groups.

3. Discussion

In this study, the CRISPR/Cas9 gene editing technology was used to construct two viruses, GV371 (U6-sgRNA-SV40-EGFP) and Lenti-cas9-puro (Puro represents puromycin used for screening uninfected cells). Sequencing showed that the construction was successful (as shown in Fig. 1). The cell lines U251 and A172 were infected with the AQP8 knockdown viruses and routinely cultured to observe the effect of AQP8 on glioma growth and cell proliferation. However, in the process of using cryopreserved cells, we accidentally observed a certain phenomenon. First, the stable cells were frozen for 48 hours, 1 week, 2 weeks, and 1 month and then resuscitated for culture. The experimental results of virus infection rate showed that the virus infection efficiency was kept above 90% compared with that in the inactive storage group of the negative control group (as shown in Table 1). As shown in Fig. 2, no abnormal changes occurred, indicating that with the extension of freezing time, the virus was still in the cell and had a certain ability to replicate. However, western blotting and RT-qPCR results obtained during the assessment of virus infection (Figs. 3 and 4) showed that stably infected cells without cryopreservation could inhibit the expression of AQP8 mRNA and protein, which was consistent with our expectation. Second, cells stably infected with AQP8 knockdown virus were frozen for different time periods (48 hours, 1 week, 2 weeks, and 1 month). After resuscitation, the expression of GFP in stably infected cells was not significantly changed, but the expression of AQP8 mRNA and protein in cells was not significantly different from that in the control group, suggesting that the sgRNA targeting the AQP8 gene sequence did not play the role of target recognition or that the Cas9 protein could not cut the AQP8 gene sequence. It is not clear whether the virus lost the ability to release the specific AQP8 gene-targeting sequence in cells after cryopreservation or whether the sequence was released but its original function was lost.

Mammalian cell lines have become important tools for a wide range of biomedical, biotechnological, and cancer research *in vitro*^[1, 2]. The sustained proliferation and growth of cultured cells are often temporarily

inhibited for subsequent studies. Cell samples can be stored for a long time by cryopreservation in a liquid nitrogen-cooled environment. Dimethyl sulfoxide (DMSO) mixed with fetal bovine serum (FBS) at different ratios can serve as a cell cryopreservation medium to decrease the freezing point of the intracellular solution. Moreover, slow freezing allows water to slowly permeate cells, which reduces ice crystal formation and thus avoids cell damage. The biological metabolism of living cells is significantly reduced, and enzymatic and chemical reactions stop under cryopreservation, thus fulfilling the experimental requirement for temporary inhibition of cell growth. Cell culture technology has matured and is now widely applied. Here, we confirmed the reliability of culture technology by routinely culturing the U251 and A172 cell lines to meet our experimental requirements.

The applications of lentivirus-based siRNA and CRISPR/Cas9 technologies have widened to include exploratory studies of cancer cell lines. These technologies rely on the ability of lentivirus to deliver siRNA and CRISPR/Cas9 systems into specific cancer cells at a super-high infection rate. These systems are used to modify specific genes to achieve over-expression or knockdown of a specific protein in cancer cells. This allows for subsequent observation of the growth, proliferation, and invasiveness of cancer cells under changed expression of a target protein. Here, the sgRNA system was delivered via lentiviral vectors into glioma cells (A172 and U251) that had been previously infected with the lentiviral particles dCAS9-VP64 and CAS9 and selected by puromycin, which led to the knockdown of *AQP8* in the two cell lines. Thus, the reliability of the recombinant virus was confirmed. Notably, *AQP8* knockdown disappeared in the A172 and U251 cells recovered after 48 h to 1 month of cryopreservation. Cryopreservation is now an established conventional method for preserving target cell lines infected with lentivirus. For example, Chen et al. used a GILT-knockdown lentivirus to infect U87 human glioma cells, and cryopreserved them^[3]. However, the present findings revealed that cryopreservation is not suitable for the intracellular survival of the virus targeting the *AQP8* gene, and this has also not been reported so far. Our findings suggested that the *AQP8* gene-targeting guide RNA might be a key factor in affecting lentiviral survival at low temperatures.

The CRISPR/Cas9 technology is associated with off-target risk, that is, a restriction enzyme recognizes and cleaves a target site while also cleaving the DNA sequence at a similar site^[4], resulting in off-target events and uncontrollable mutations. The off-target risk is also a main limiting factor of the wide application of CRISPR technology. An off-target detection technology called genome-wide off-target analysis by two-cell embryo injection was established in 2019^[5]. However, due to the limitations of our experimental conditions, we were unable to determine whether off-target events had occurred during lentiviral vector-mediated knockdown of the *AQP8* gene in cryopreserved cells, and these issues should be addressed in further investigations.

In summary, cryopreservation decreased the potency of an *AQP8*-knockdown virus in stably infected U251 and A172 cell lines. This finding might provide a new direction for the prevention and treatment of viral infections.

4. Methods

4.1 Cell lines and groups

The A172 and U251 glioma cell lines were infected with a lentivirus carrying CRISPR/Cas9, after which we investigated whether AQP8 knockdown differed between cells with and without cryopreservation. We created two control groups: blank (cells without viral infection) and a negative control (cells infected with empty vectors), and the following five experimental groups: non-cryopreserved A172 and U251 cells infected with CRISPR/Cas9-carrying lentivirus and cultured under normal conditions, and A172 and U251 cells infected with CRISPR/Cas9-carrying lentivirus and cryopreserved for 48 hours and 1, 2, or 4 weeks.

4.2 Construction and identification of the dual-lentiviral-vector CRISPR/Cas9 system

An *AQP8* gene-targeting sgRNA sequence was designed, based on which single-stranded DNA oligos were synthesized and purified using PAGE (Shanghai Genechem Co., Ltd., Shanghai, China), then annealed to form double-stranded DNA fragments with sticky restriction sites at the 5' and 3' ends. The double-stranded DNA fragments were ligated to the Lenti-sgRNA-tag, GV371 (U6-sgRNA-SV40-EGFP). The ligation product was transformed with TOP10 competent cells, and positive clones were confirmed by colony PCR and sequenced to obtain a lentiviral knockdown plasmid expressing sgRNA (5'-caccgCTGCACAAACCGTTCGTACC-3'). The CRISPR/Cas9 lentiviral vector was Lenti-cas9-puro. The virus was sequenced at Shanghai Genechem Co., Ltd.

4.3. Cell culture and viral infection

4.3.1 Cell culture

The glioma cells A172 and U251 (Shanghai Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in complete DMEM (Gibco Laboratories, Gaithersburg, MD, USA) containing 10% FBS (Cellcook Biotech Co., Ltd., Guangzhou, China) supplemented with 5 mg/mL of penicillin-streptomycin (NCM Biotech, Newport, RI, USA). The optimal viral titer for infection was $\geq 1 \times 10^8$ transduction units(TU)/mL determined using conventional plaque assays.

4.3.2 Evaluation of the viral infection rate

Glioma cells A172 and U251 with good growth status were selected one day before viral infection and evenly spread on six-well plates until reaching 20–30% confluence. On the first day of infection, Lenti-cas9-puro was added to the corresponding groups of cells to reach the desired viral titer, then the resulting mixture was routinely cultured for 3 days, followed by puromycin screening to select cells for further infection with Lenti-sgRNA-tag. Forty-eight hours later, GFP expression was monitored using

fluorescence microscopy. Cells were counted separately in bright and fluorescence fields (both at 50× magnification). The viral infection rate, calculated as the ratio of fluorescence to bright field data, was determined as > 80%, which was sufficient for subsequent experimentation.

4.4 Cryopreservation and recovery

4.4.1 Cryopreservation

Infected cells were washed twice with PBS, suspended in trypsin (1 mL), and incubated in T25 cell culture flasks at 37° for 1 minute to detach cells from the internal surface of the flasks. Thereafter, complete medium (1 mL) was added to stop digestion, then the suspended cells were pelleted in 15 mL tubes by centrifugation 800 rpm for 5 minutes. Cell pellets (20×10^4 cells/mL) were uniformly mixed with 1:9 ratio of DMSO: FBS as a cryopreservative, rapidly transferred to disposable cryopreservation tubes and cooled at -20°C for 2 hours, stored overnight at -80°C then placed in liquid nitrogen for 48 hours or 1, 2 or 4 weeks.

4.4.2 Recovery

The cryopreserved cells were rapidly transferred from the liquid nitrogen to a water bath until the tube temperature reached 37°C. Cell suspensions in 15 mL centrifuge tubes were separated by centrifugation at 800 rpm for 5 minutes, then the supernatant was discarded. Cell pellets were resuspended in complete medium and routinely cultured in T25 flasks.

4.5 Western blotting

Cells were harvested and lysed in lysis buffer containing protease and phosphatase inhibitors (both from Beyotime Biotechnology, Shanghai, China) followed by centrifugation to remove cell debris. Lysates were then mixed with SDS protein loading buffer, and the proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Non-specific protein binding on the membranes was blocked using Blocking Solution (Beyotime Biotechnology) for 20 minutes, after which the blots were incubated with 1:1,000-diluted anti-AQP8 (Abcam Plc., Cambridge, UK) and anti-β-actin (Signaling Technology, Danvers, MA, USA) antibodies at 4°C overnight, followed by incubation with a 1:5,000-diluted horseradish peroxidase-conjugated secondary antibody (Earthox Co., Ltd., San Francisco, CA, USA) for 1 h. Proteins of interest were visualized using ultra-sensitive ECL color developing solution (4A Biotech, Beijing, China).

4.6 RT-qPCR

The total RNA extracted from each group of cells using RNA extraction kits (Omega Bio-Tek Inc., Norcross, GA, USA) was reverse transcribed into first-strand cDNA as described by the manufacturer (Toyobo Co., Ltd., Osaka, Japan) and amplified by RT-PCR using a LightCycler system (Roche Diagnostics, Basel, Switzerland) with SYBR Primescript RT-qPCR kits. Sequences of the upstream primer and downstream primers (5'3') (Sangon Biotech, Shanghai, China) were as follows:

AQP8: TGCCATCAATGAGAAGACAAAG and ATCTCCAATGAAGCACCTAATG, β -actin:

AGAAAATCTGGCACCACACCT and GATAGCACAGCCTGGATAGCA. The PCR conditions were: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s. Data were analyzed using the $2^{-\Delta\Delta C_t}$ method, and *β -actin* was the internal reference gene.

4.7 Statistical analysis

All data are expressed as means \pm standard deviation (SD) and were statistically analyzed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Groups of two were compared using Student t-tests, and multiple groups were compared by one-way analysis of variance (ANOVA). All experiments were conducted in triplicate.

Declarations

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Source data are provided with this paper.

Acknowledgment

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Competing interests

The authors declare no competing interests.

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Tables

Table 1. Viral infection rates

Cells	Control	Negative control	Knocked down not cryopreserved	Cryopreserved 2 days	Cryopreserved 1 week	Cryopreserved (2 weeks)	Cryopreserved (4 weeks)
A172	0.0	94.0±0.1	94.8±0.2	93.4±0.1	91.5±0.3	92.8±0.4	92.4±0.3
U251	0.0	91.7±0.2	94.3±0.4	92.3±0.3	91.1±0.3	90.9±0.2	93.5±0.1

Figures

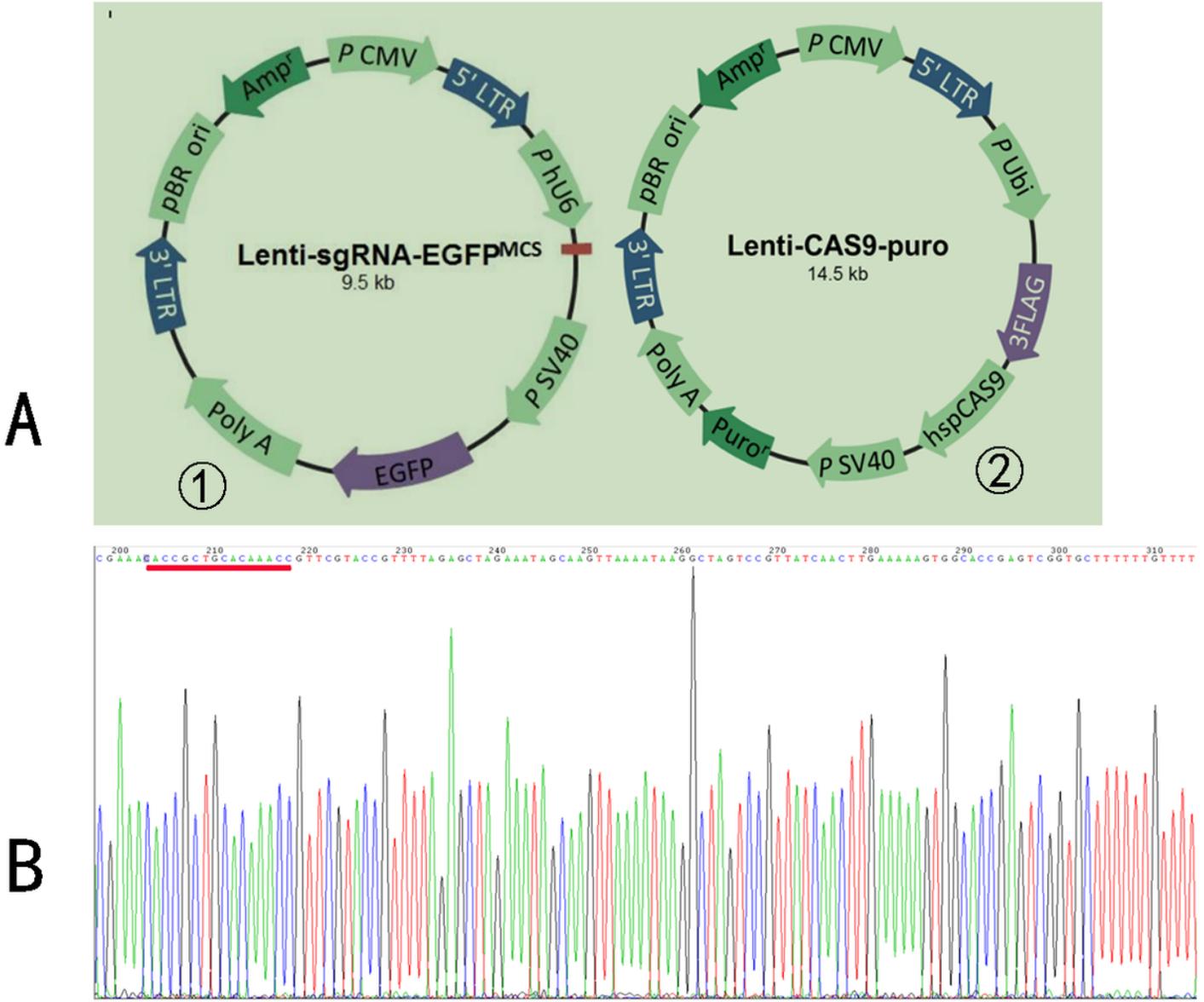
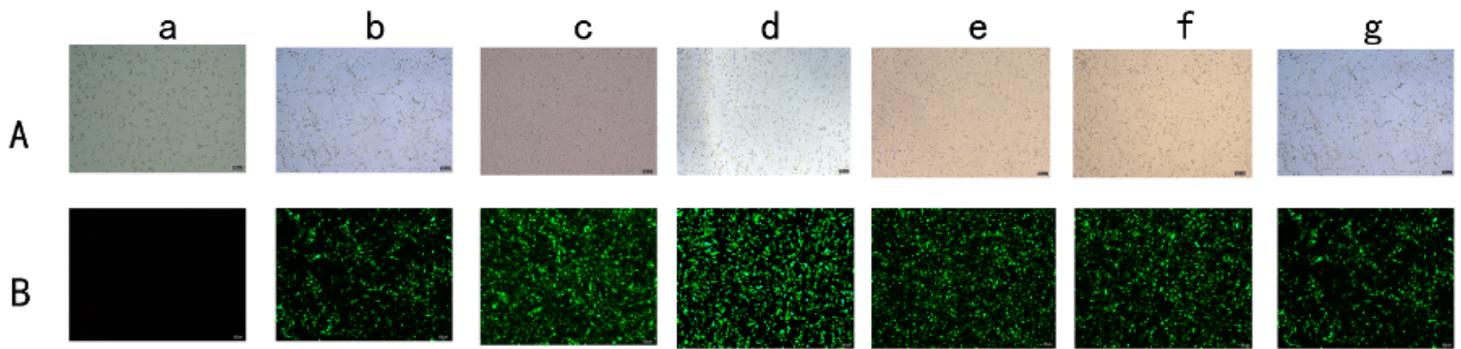


Figure 1

Vector GV371 and lentivirus. a, Vector GV371 with components linked in order of (a) U6-sgRNA-SV40-EGFP and (b) Lenti-cas9-puro. b, Sequence of sgRNA.

A172cells



U251cells

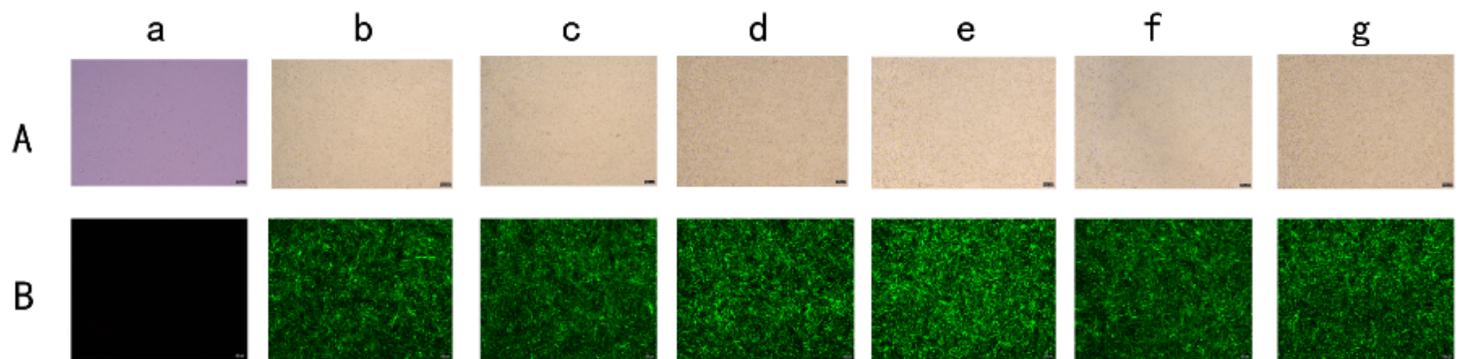


Figure 2

Viral infection efficiency assessed by microscopy using GFP fluorescence. a, Bright field. b, Infected cells in the fluorescent field. Blank control (a), negative control (b), non-cryopreserved cells with AQP8 knocked down (c), cells infected and then cryopreserved for 48 hours (d) and 1 (e), 2 (f) and 4 (g) weeks.

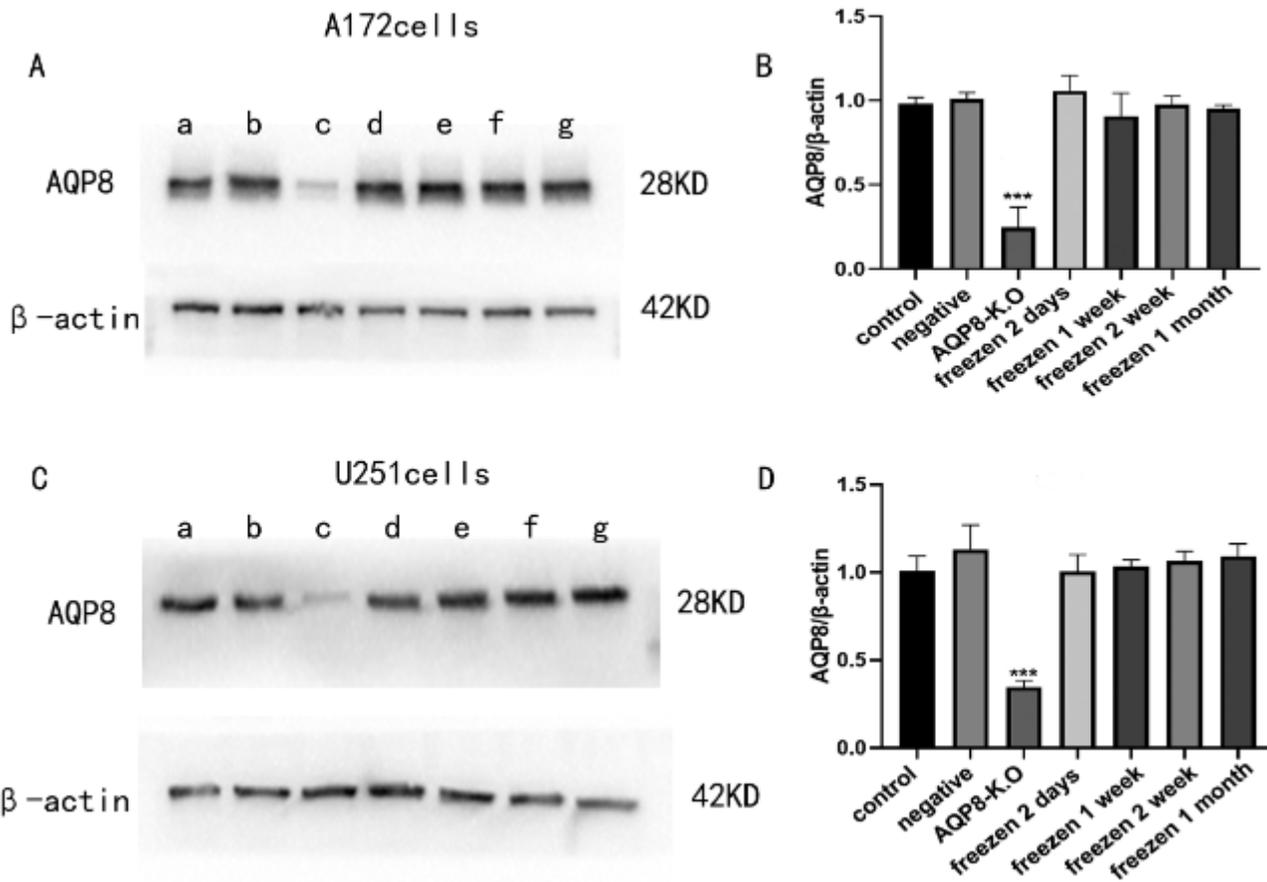


Figure 3

Western blots of AQP8 protein expression in lentivirus-infected A172 and U251 cells with or without cryopreservation.

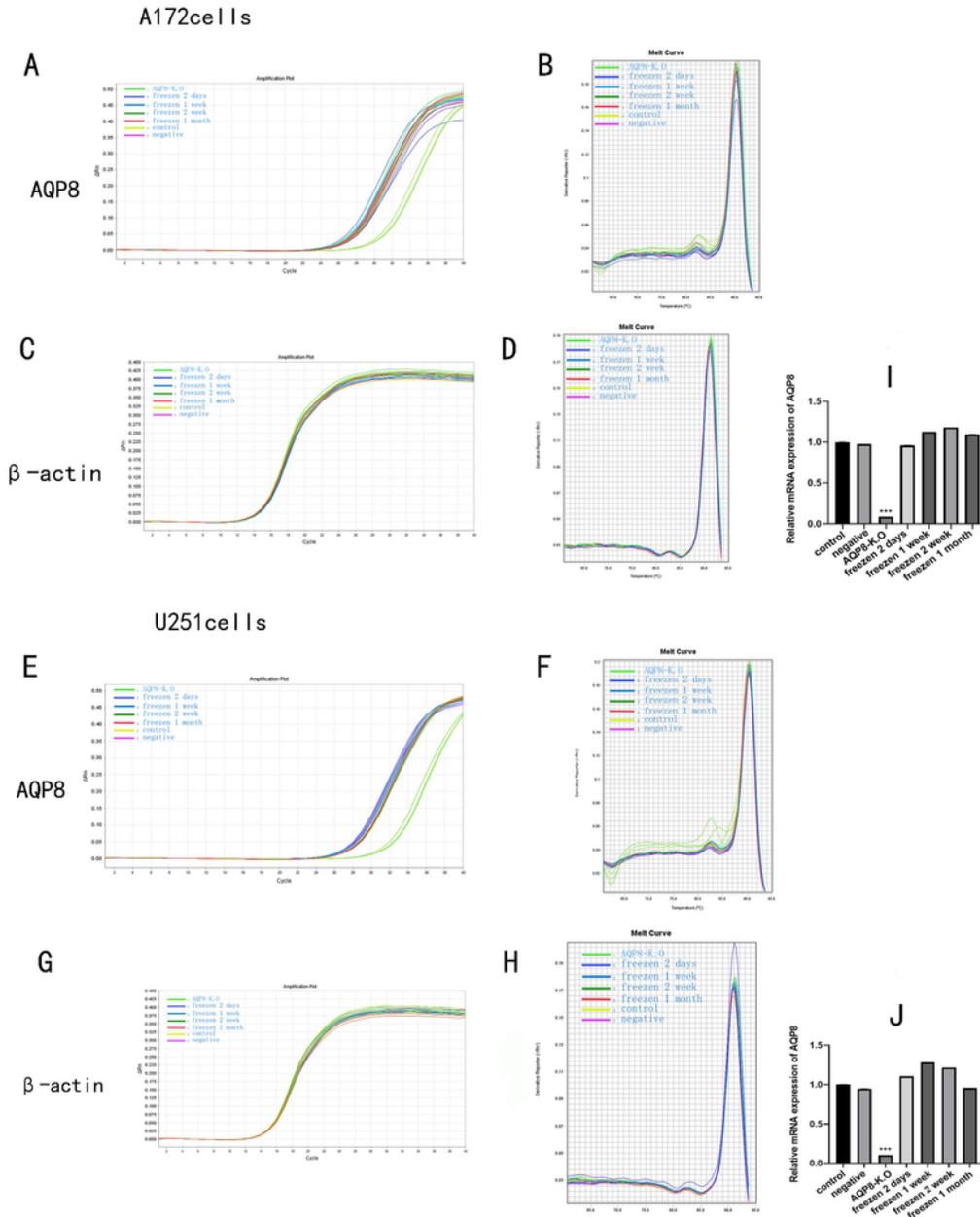


Figure 4

Detection of AQP8 mRNA expression in cryopreserved versus non-cryopreserved cells using QT-qPCR. A, Amplification (A) and B, dissociation (B) curves of AQP8 mRNA in A172 cells. C, Amplification (C) and D, dissociation (D) curves of β-actin RNA in A172 cells. E, Amplification (E) and F, dissociation (F) curves of AQP8 mRNA in AU251 cells. G, Amplification (G) and H, dissociation (H) curves of β-actin mRNA in U251

cells. Relative expression of AQP8 mRNA in I, A172 (I) and J, U251 (J) cells. *P <0.001, cells with AQP8-knockdown without cryopreservation vs. the other six groups.

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