Biomaterials Science

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Biomater. Sci.*, 2025, **13**, 2082

Enhanced *in vitro* transfection efficiency of mRNA-loaded polyplexes into natural killer cells through osmoregulation

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Received 11th December 2024,
Accepted 15th February 2025

DOI: 10.1039/d4bm01661k

rsc.li/biomaterials-science

High expression of externally injected *in vitro* transcribed (IVT) mRNA in natural killer (NK) cells is a prerequisite for NK cell-mediated cell therapy. To enhance the transfection efficacy of IVT mRNA-loaded polyplexes, we exposed NK cells to a hypertonic condition during transfection, which facilitated endo/exocytosis to maintain the isotonic state of the cells. The transfection efficacy of IVT mRNA was significantly enhanced after 24 h, which was mainly due to the facilitated cellular uptake and endosomal escape of the polyplexes. Interestingly, osmotic alterations in NK cells significantly affect the expression levels of endosome-escape-related genes in ion channels. Treatment with a mild hypertonic condition exhibited negligible toxicity to NK cells, without disturbing the integrity of the cellular membranes or the innate cytotoxic abilities of NK cells against cancer cells. These results demonstrate that the hypertonic treatment of NK cells enhances the transfection efficacy of IVT mRNA to produce genetically engineered NK cells.

1. Introduction

Natural killer (NK) cells are responsible for the body's innate immune system and exhibit nonspecific cell-killing abilities without antigen recognition by antigen-presenting cells.[1] Compared with T cells, NK cells do not express T cell receptors or clusters of differentiation 3. Therefore, these patients show less immune rejection when allogeneic transplantations are performed.[2] Because of the unique advantages of NK cells, many efforts are currently being made to utilize them in cell therapy as potential alternatives to T cells. However, because of the relatively short half-life of NK cells, it is difficult to proliferate sufficient cells for *ex vivo* transfection, and it is difficult to apply them clinically as therapeutic agents because of their weaker cell-killing ability compared to T cells. To overcome the disadvantages of NK cells, various attempts have been made, such as genetic engineering,[3,4] culture optimization in bioreactor,[5] co-culture with feeder cells,[6] and development of cryopreservation media.[7,8] In particular, genetic engineering strategies, such as expressing chimeric antigen receptors, have been attempted as a major strategy to overcome the shortcomings of NK cells.[9,10]

The general protocol for expressing external genetic material during cell manufacturing involves viral transduction.[11] However, viral vectors have the potential risk of genetic mutations, in which the transferred genes are inserted at unintended sites in the host genome.[12,13] Alternatively, the use of *in vitro* transcribed (IVT) mRNA can avoid the risk of gene integration into the host genome and achieve temporary expression or suppression of the target protein. However, naked mRNAs are highly susceptible to degradation by extracellular nucleases. Negatively charged mRNA molecules exhibit low uptake efficiency by target cells.[14] Various nonviral delivery carriers, such as lipid nanoparticles,[15] polymeric nanoparticles (or polyplexes),[16,17] and inorganic nanoparticles[18] have been developed to protect vulnerable mRNA against nucleases and enhance cellular uptake. In addition to the development of novel synthetic materials, we assumed that extracellular stimuli to NK cells would improve the transfection efficiency of mRNA because the stimuli affect the regulation of cell signaling in the target cells.

Cells in the body are constantly exposed to osmotic pressure. When cells are exposed to hypotonic or hypertonic conditions, the cell shape, plasma membrane tension, and cytosolic ion levels change.[19] Importantly, cells maintain their isotonic state *via* the control of membrane tension, such as the rates of endo/exocytosis and the expression levels of various ion channels on the cellular membranes.[20,21] Particularly, when cells are exposed to a hypertonic condition, intracellular

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water rapidly moves out of the cell, causing cell shrinkage and a subsequent decrease in cell membrane tension. This reduction in membrane tension triggers endocytosis to maintain homeostasis. Therefore, we expected that the exposure of target cells to osmotic stimuli during transfection would improve the cellular uptake and translational efficacy of IVT mRNA.

In this study, we improved the transfection efficiency of IVT mRNA into NK cells by exposing them to a hypertonic condition (Fig. 1). Hypertonic culture media (330 and 500 mOsm kg^{-1} osmolality) were prepared by adding sodium chloride (NaCl) to the isotonic culture media (280 mOsm kg^{-1} osmolality). NK cells were exposed to a hypertonic condition and transfected with IVT mRNA-loaded polyplexes. We chose amphiphilic cationic polymers and polyaspartamide derivatives that efficiently associated with IVT mRNA and exhibited high mRNA translation efficacies *in vitro* and *in vivo*.[22,23] Translation efficacy of IVT mRNA, cellular uptake, and endosomal escape of polyplexes in NK cells in a hypertonic condition were compared with those in an isotonic solution to understand the underlying mechanisms. Interestingly, we found that osmotic alteration in NK cells significantly affected the expression levels of endosome escape-related genes in ion channels (*ATP6V0E1*, *ATP6V1E1*, *CLCN3*, and *CLCN5*). These results demonstrate that hypertonic treatment of NK cells enhances the transfection efficacy of IVT mRNA for the production of genetically engineered NK cells.

2. Materials and methods

2.1 Materials

Human natural killer KHYG-1 cells were purchased from AcceGen Biotechnology (ABC-TC0506, NJ, USA). Human leuke-

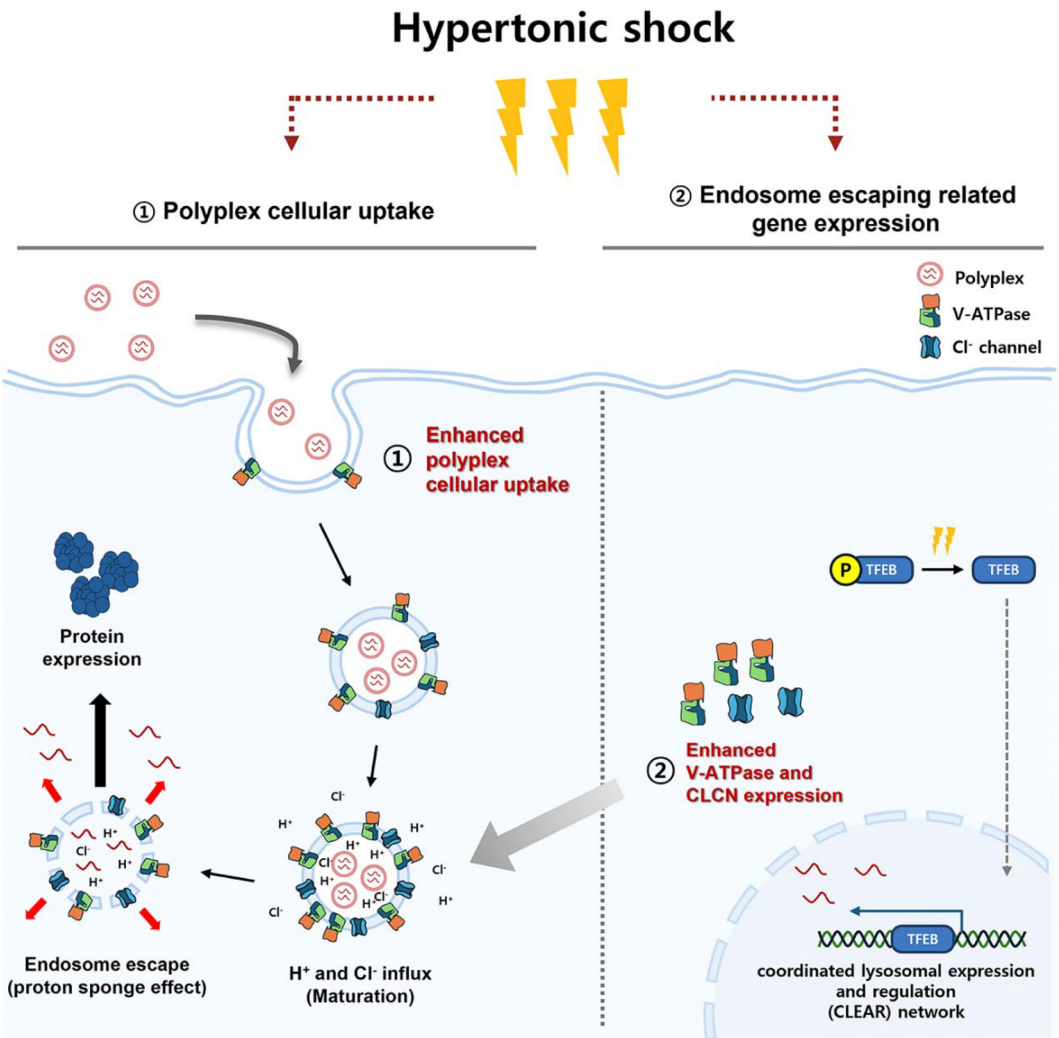

Fig. 1 Illustration of enhanced transfection efficiency of IVT mRNA *via* osmoregulation. NK cells at 330 mOsm kg^{-1} osmolality facilitates cellular uptake of IVT mRNA-loaded polyplexes *via* enhanced endocytosis. The up-regulation of endosome escape-related genes improves endosomal escape of polyplexes into cytoplasm. As a result, polyplexes arrive effectively at the cytoplasm, ultimately leading to the increase in transfection efficiency of target IVT mRNA.

mia cells (K562) were purchased from the Korea Cell Line Bank (Seoul, South Korea). Roswell Park Memorial Institute (RPMI) 1640 cell culture medium, fetal bovine serum (FBS), penicillin/streptomycin, and Dulbecco's phosphate-buffered saline (D-PBS) were purchased from Gibco (Thermo Fisher Scientific). The cell culture plates were purchased from SPL Life Sciences (Gyeonggi-do, South Korea). Interleukin-2 was bought from PeproTech (200-02, ThermoFisher Scientific). The NaCl used for osmoregulation was purchased from Samchun Chemicals (S0484, Seoul, South Korea). Cell counting kit-8 (CCK-8) was obtained from DOJINDO Laboratories (CK-04, Kumamoto, Japan). The EZ-LDH assay kit was purchased from DoGenBio (DZ-LDH, Seoul, South Korea). Luciferase assay system was obtained from Promega (E1501, WI, USA). Primers for quantitative real time PCR (qRT-PCR) were synthesized from Integrated DNA Technologies (IA, USA). PrimeScript™ RT master mix for cDNA synthesis (RR036A) and TB Green® premix Ex Taq™ II for qRT-PCR (RR820A) were obtained from Takara Bio (Shiga, Japan). Firefly luciferase (FLuc) mRNA was purchased from TriLink Biotechnologies, Inc. (L-7202; CA, USA).

2.2 Cell culture and osmoregulation

KHYG-1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, and 100 U mL^{-1} recombinant human interleukin-2 at 37 °C in a humidified CO_2 incubator (5% CO_2). For daily culture, the cells were seeded on 90 × 20 mm cell culture dish at a concentration of 1×10^5 cells per mL. Culture media were exchanged every 3–4 days. Culture media with various osmolality concentrations (330 and 500 mOsm kg^{-1}) were prepared by adding NaCl. NaCl (1.46 g and 6.43 g) was added into 1 L of 280 mOsm kg^{-1} RPMI 1640 to make 330 and 500 mOsm kg^{-1}, respectively. Osmolality of culture media was measured using a Single-Sample Micro Osmometer (Fiske® 210, MA, USA).

2.3 Synthesis of polyaspartamide derivatives and preparation of IVT mRNA-loaded polyplex

Polyaspartamide derivatives modified with diethylenetriamine (DET) and 2-cyclohexylamine (CHE) [PAsp(DET/CHE)] were synthesized as described previously.[22,23] Parent polymer, poly (β-benzyl-L-aspartate) (PBLA) was prepared by ring-opening polymerization of β-benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) with a butylamine initiator. The synthesized PBLA exhibited a degree of polymerization (DP) of 21 and a molecular weight distribution (M_w/M_n) of 1.10, as determined using ^{1}H NMR and gel permeation chromatography, respectively. PBLA was simultaneously aminolyzed with DET and CHE to produce PAsp(DET/CHE). PAsp(DET/CHE) contained nine DET and 12 CHE moieties, as confirmed by ^{1}H NMR spectroscopy. PAsp(DET/CHE) was dissolved in 10 mM HEPES buffer (pH = 7.3) for further use.

Polyplexes were formed by mixing PAsp(DET/CHE) with the FLuc mRNA. To form IVT mRNA encapsulated polyplexes, PAsp(DET/CHE) in 10 mM HEPES buffer (pH = 7.3) and FLuc mRNA in 10 mM HEPES buffer (pH = 7.3) were mixed at molar ratios of amino groups in DET unit to phosphate groups in mRNA (N/P ratio) = 7 to maintain mRNA concentration in polyplex solution (30 ng mRNA per μL). The mixture was incubated for 60 min at room temperature to form the polyplexes.

2.4 Trypan blue staining and CCK-8 assays

The viability of KHYG-1 cells in the osmoregulation medium was determined using CCK-8 and trypan blue staining assays. KHYG-1 cells were seeded in a 48-well cell culture plate (1.5×10^5 cells per mL) in cell culture media at 280, 330, and 500 mOsm kg^{-1} osmolality. After 24 h of exposure, the CCK-8 reagent was added to the culture medium. The cells were further incubated for 4 h. The absorbance was measured at 450 nm. Cell viability was calculated by normalizing the untreated samples. Trypan blue assay was performed using an automatic cell counter (SOL Inc., Seoul, South Korea). Areas measuring 10.02 mm^2 were scanned six times over a total area of 60.12 mm^2.

2.5 Lactate dehydrogenase (LDH) assay

The cytotoxic abilities of KHYG-1 cells as a function of NK cells in osmoregulatory media were determined using LDH assays. KHYG-1 cells were seeded in a 48-well cell culture plate (1.5×10^5 cells per mL) in cell culture media of 280, 330, and 500 mOsm kg^{-1} osmolality and incubated for 24 h. KHYG-1 cells were mixed with K562 cells at the cell number ratio of KHYG-1 cells to K562 cells of 3. After incubation at 37 °C for 24 h, the cell mixture was centrifuged at 600*g* for 5 min. The supernatant (10 μL) was transferred to a fresh 96-well cell culture plate. LDH reagent (100 μL) was added to each well according to the manufacturer's instructions. The mixture was allowed to react in the dark at room temperature for 30 min. The absorbance was measured at 450 nm using a microplate reader.

2.6 Luciferase assay

KHYG-1 cells were seeded in a 96-well cell culture plate (1.5×10^5 cells per mL, 90 μL) in cell culture media at 280, 330, and 500 mOsm kg^{-1} osmolality and incubated for 5 min. The cells were then transfected with polyplexes (300 ng FLuc mRNA per well, 10 μL) without exchanging the culture media. The cells were further incubated for 24 h. The cell suspension was then transferred to microcentrifuge tubes, centrifuged at 1500 rpm for 3 min, and washed twice with PBS. After the last PBS was removed, the cells were lysed using 1× Passive Lysis Buffer (Promega) diluted with ultrapure water. Lysate solution was transferred into a 96-well microplate and reacted with luciferin solution (50 μL). The photoluminescence intensity was immediately measured using a Centro LB 963 luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.7 Polyplex uptake analysis

KHYG-1 cells were seeded in a 24-well cell culture plate (2.0×10^5 cells per mL, 450 μL) in cell culture media of 280, 330, and 500 mOsm kg^{-1} osmolality and incubated for 5 min. The cells were then transfected with polyplexes (1500 ng FLuc mRNA

per well, 50 μL) without exchanging the culture media. The cells were further incubated for 1, 4, and 24 h. Cell suspension was washed with D-PBS (200 μL) five times. The cells were lysed and mRNA was collected using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from 1 μg mRNA using a PrimeScript™ RT master mix. qRT-PCR was performed using TB Green® Premix Ex Taq™ II with appropriate primers. The primer sequences are listed in Table 1. Fold changes in target gene expression levels were calculated by applying the $2^{-\Delta\Delta Ct}$ method.[24]

2.8 Polyplex stability at hypertonic conditions

The hypertonic solution used for polyplex stability analysis consisted of PBS containing 10% FBS (PBS/FBS). The osmolality of the solution was controlled using the same process used for the preparation of the hypertonic media. The hypertonic solution consisted of PBS containing 10% FBS (PBS/FBS), and the osmolality of the PBS/FBS was adjusted as described above. Polyplexes (10 μL, 300 ng FLuc mRNA) were mixed with hypertonic solution (90 μL) and the mixture was further incubated for designate incubation periods. The sample solution was purified using the RNeasy mini kit (Qiagen) to obtain intact FLuc mRNA. Half of the volume of the final eluate was mixed with 50% glycerol at a volume ratio of 1 : 1. Samples were loaded onto a 1% agarose gel and electrophoresed at 135 V for 15 min. The gels were imaged using a chemiluminescence imaging system (G:BOX Chemi XRQ; Syngene, Cambridge, UK).

2.9 Gene expression analysis

qRT-PCR was performed to determine whether the osmoregulatory media affected proton-sponge hypothesis-related gene expression. KHYG-1 cells were seeded in a 12-well cell culture plate (1.5×10^5 cells per mL, 1 mL) in cell culture medium at 280, 330, and 500 mOsm kg^{-1} osmolality and incubated for 24 h. The cells were treated with TRIzol reagent on cell plates for 5 min to isolate total cellular mRNA. Lysates were then transferred to microcentrifuge tubes and chloroform was added to the tubes. The tubes were centrifuged at 12 000*g* and 4 °C for 10 min and the supernatant was then transferred to a new microcentrifuge tube. The same volume of isopropanol was added into the supernatant and the tubes were centrifuged at 12 000*g* and 4 °C for 5 min. The supernatant was then discarded, and 75% ethanol was added to the tube prior to further centrifugation at 12 000*g* and 4 °C for 5 min. The supernatant was removed, and the tubes were air-dried. The mRNA pellet was dissolved in RNase-free water and the solution was incubated at 60 °C for 10 min. The mRNA concentration was quantified using a Nanodrop DS-11 (DeNovix, DE, USA). cDNA was synthesized from 1 μg mRNA using a PrimeScript™ RT master mix. qRT-PCR was performed using TB Green® Premix Ex Taq™ II with appropriate primers. The primer sequences are listed in Table 1. Fold changes in target gene expression levels were calculated by applying the $2^{-\Delta\Delta Ct}$ method.[24] ATPase H^+ transporting V0 subunit E1 (*ATP6V0E1*), ATPase H^+ transporting V1 subunit E1 (*ATP6V1E1*), chloride voltage-gated channel 3 (*CLCN3*), and chloride voltage-gated channel 5 (*CLCN5*) genes were selected as proton sponge hypothesis-related genes, and tubulin beta 3 class III (*TUBB3*) was selected as the housekeeping gene.

Table 1 Primer sequences for qRT-PCR

Target gene	Sequences	Size
TUBB3	F: 5′-GCTGCAAAACTTCTTCCCTCG-3′ R: 5′-CTGTCCCTGTTCAAGGGAGT-3′	120
FLuc	F: 5′-CAGGGCTTCCAGAGCATGTA-3′ R: 5′-ATCAGGGCGATGGTCTTGTC-3′	110
CLCN3	F: 5′-GGTGGGTTGAAAGCCATCCTA-3′ R: 5′-TAGAGTCAACCTGCTTCTGCC-3′	122
CLCN5	F: 5′-GACCCTTTTGTCTCCCTTCCA-3′ R: 5′-AGAGAGACAGGAGTTGATGTGC-3′	109
ATP6V0E1	F: 5′-CAGCGTGTTTTCCTTTGCCT-3′ R: 5′-TTTGTGGAGTCGGCACAGTT-3′	78
ATP6V1E1	F: 5′-CAAGAAGCCAATGAGAAAGCAGA-3′ R: 5′-GCACAAGCCGACCTTTCTCT-3′	79

2.10 Statistical analysis

Collected data are presented as mean ± SEM (standard error of the mean). Differences were considered significant when *p*-value was less than 0.05, as calculated using two-tailed Student's *t*-test.

3. Results and discussion

3.1 Effects of osmoregulation media on cell viability and cytotoxic ability of KHYG-1 cells

We chose osmolalities of 330 and 500 mOsm kg^{-1} for the hypertonic culture media because the osmolality in the thymus, a tissue in which lymphocytes develop and differentiate, is 320–330 mOsm kg^{-1}.[25] The 500 mOsm kg^{-1} condition was included as a hypertonic control to evaluate the effects of increased osmotic pressure on transfection efficiency. Cell viabilities of KHYG-1 cells in osmoregulation medium was measured using trypan blue staining and CCK-8 assays. Trypan blue molecules are impermeable to the cell membrane and can only enter cells with compromised membranes, indicating the integrity of the cellular membrane. The CCK-8 assay determines the NADH-mediated metabolic activity of living cells, in which dehydrogenase catalyzes the transfer of hydrogen from NAD^+ to NADH. KHYG-1 cells were exposed to cell culture media at 280 (isotonic), 330, and 500 mOsm kg^{-1} osmolality for 24 h, and their relative viability was calculated by normalizing to the cell viability at 280 mOsm kg^{-1} osmolality. In the trypan blue staining assay (Fig. 2A), there was no statistically significant difference in live cell counts between 280 and 330 mOsm kg^{-1} osmolality. However, the viability of KHYG-1 cells at 500 mOsm kg^{-1} osmolality was significantly reduced to approximately 80%, indicating the presence of a cellular membrane. Cell viability was measured using the CCK-8 assay (Fig. 2B). The viability of KHYG-1 cells was slightly

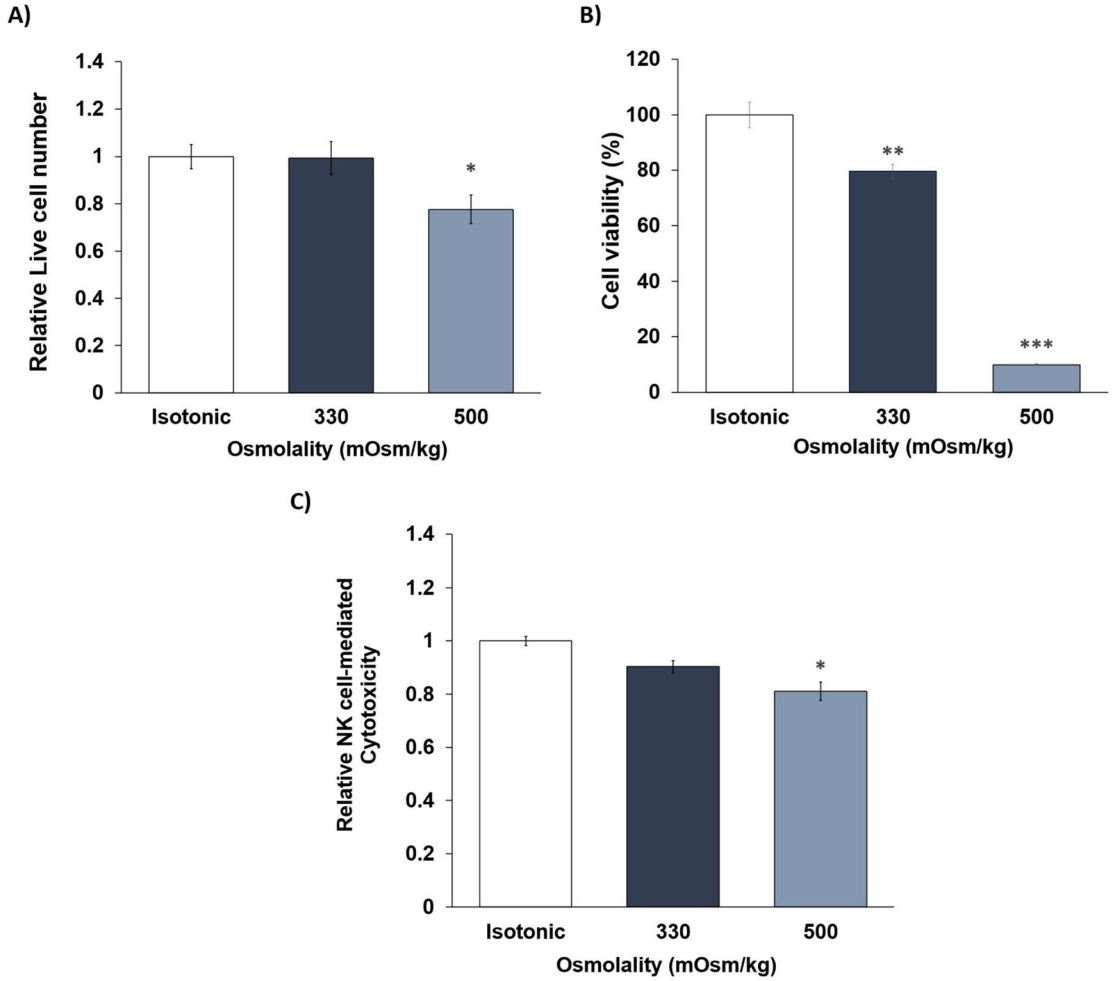

Fig. 2 Cell viabilities of KHYG-1 cells in the cell culture media with various osmolality using a (A) trypan blue staining assay and (B) CCK-8 assay. The cells were exposed to 280 (isotonic osmolality), 330, and 500 mOsm kg^{-1} osmolality for 24 h and the relative cell viability was calculated *via* normalization with cell viability in isotonic osmolality. (C) Cytotoxic abilities of KHYG-1 cells in the cell culture media with various osmolality *via* LDH assay. Released LDH from cancer cells were measured by detecting absorbance at 450 nm wavelength. All results are expressed as mean ± SE (n = 3). Statistical differences are indicated as *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.

reduced to approximately 80% at 330 mOsm kg^{-1} osmolality. However, at 500 mOms kg^{-1} osmolality, the viability dramatically reduced to 10%. This indicates that treatment with 330 mOsm kg^{-1} osmolality slightly reduced the metabolic activity of KHYG-1 cells without affect integrity of the cellular membrane.

Natural killer (NK) cells, as innate immune cells, exhibit cytotoxic effects against cancer cells through the directed release of lytic granules or by inducing death receptor-mediated apoptosis.[26] LDH assay was performed to evaluate whether exposure to osmoregulatory media affects the cytotoxicity in KHYG-1 cells. KHYG-1 cells were exposed to cell culture media of 280, 330, and 500 mOsm kg^{-1} osmolality for 24 h. Exposed KHYG-1 cells were then incubated with human leukemia K562 cells at the cell number ratio of KHYG-1 cells to target cancer cells of 3. After incubation at 37 °C for 24 h, the amount of LDH released was measured using a microplate reader (Fig. 2C). Compared to KHYG-1 cells at 280 mOsm kg^{-1} osmolality, cells at 330 mOsm kg^{-1} osmolality showed similar cytotoxic abilities. However, the cytotoxicity of KHYG-1 at an osmolality of 500 mOsm kg^{-1} was significantly reduced to approximately 80%. Altogether, treatment of KHYG-1 cells with 330 mOsm kg^{-1} osmolality did not significantly alter the viability and metabolic activities of KHYG-1 cells.

3.2 Effects of osmoregulation on *in vitro* mRNA transfection efficiency

Cationic amphiphilic polyaspartamide derivatives, PAsp(DET/CHE)s, randomly modified with diethylenetriamine (DET) and 2-cyclohexylethylamine (CHE) in the side chains forms hundred nanometer-sized polyplexes with IVT mRNA (Fig. 3A).[22,23] The DET moieties condense mRNA molecules and enhance endosomal escape from polyplexes after cellular uptake. CHE moieties tune the hydrophobicity of polymers between polyplex stability against physiological buffers, such as cell culture media or blood, and mRNA release in the cyto-

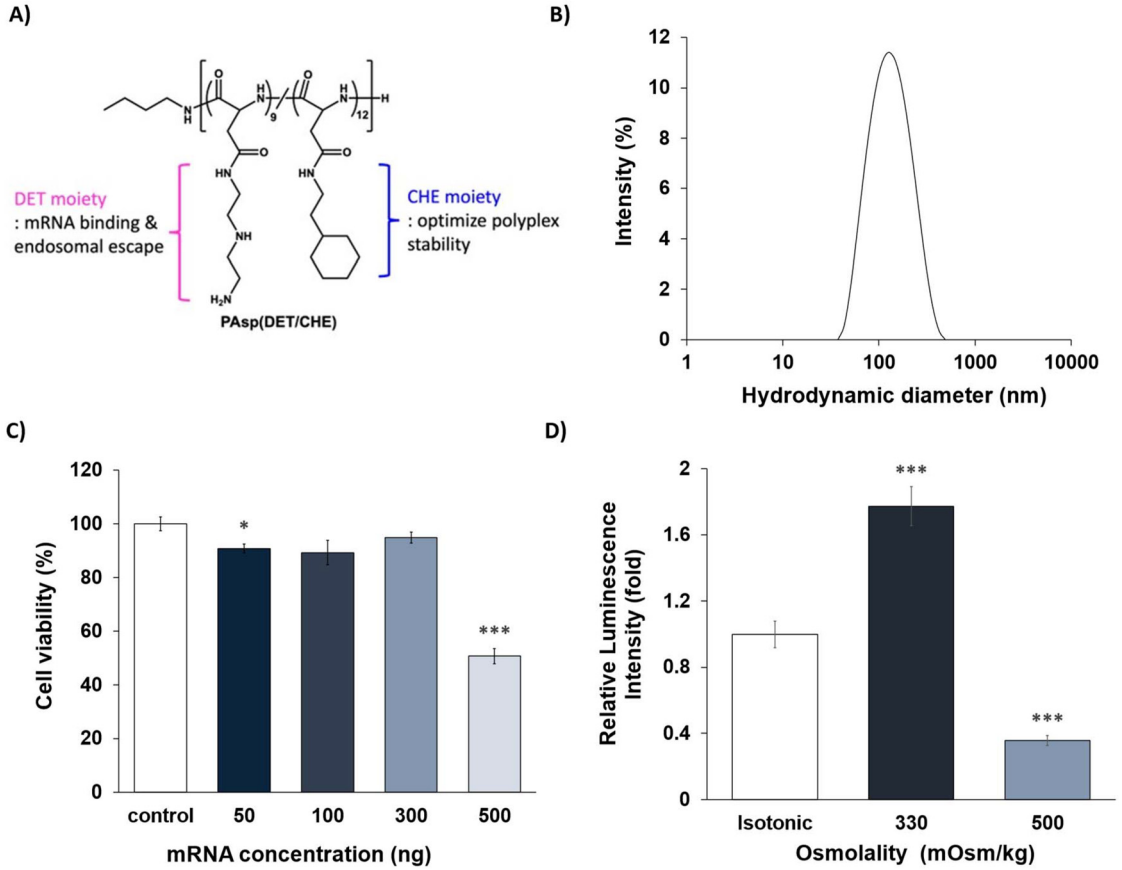

Fig. 3 (A) Chemical structure of PAsp(DET/CHE) for *in vitro* mRNA transfection. (B) Size histogram of PAsp(DET/CHE) polyplexes prepared at N/P = 7. (C) Dose-dependent viabilities of KHYG-1 cells treated with FLuc mRNA-loaded polyplexes for 24 h at 280 mOsm kg^{-1} osmolality, measured by a CCK-8 assay. (D) Luminescence intensities of KHYG-1 cells transfected with polyplexes (300 ng FLuc mRNA per well) for 24 h at various osmolality. All results are expressed as mean ± SE (n = 6). Statistical differences are indicated as *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.

plasm. PAsp(DET/CHE) significantly enhanced the mRNA delivery efficacy *in vitro* and *in vivo* with negligible toxicity.[22,23] PAsp(DET/CHE)s were associated with FLuc mRNA to form polyplexes with hydrodynamic diameters of 135 ± 17 nm and PDI of 0.20 ± 0.02, measured by a Zetasizer (Fig. 3B). Zeta potential of the polyplexes exhibited of 37 ± 6 mV, indicating highly positive surface charges. The FLuc mRNA-loaded polyplexes were evaluated to determine the optimal mRNA concentration for KHYG-1 transfection. KHYG-1 cells were transfected with FLuc mRNA at concentrations of 50, 100, 300, and 500 ng in isotonic cell culture medium for 24 h, and cell viability was measured using the CCK-8 assay (Fig. 3C). Cell viabilities after treatment with polyplexes at 50, 100, and 300 ng mRNA concentrations were 90–95%, indicating marginal cytotoxicity in KHYG-1 cells. However, after treatment with polyplexes containing 500 ng mRNA, cell viability dramatically decreased to ~50%. Therefore, we decided to transfect the FLuc mRNA-loaded polyplexes at an mRNA concentration of 300 ng into KHYG-1 cells for subsequent experiments.

The efficiency of IVT mRNA delivery by the polyplexes was determined in KHYG-1 cells at various osmolalities (280, 330, and 500 mOsm kg^{-1}). To ensure a sufficient hypertonic stimulus, KHYG-1 cells were first exposed to hypertonic culture media for 5 minutes before adding the NP buffer containing polyplexes (approximately 7 mOsm kg^{-1}), which otherwise reduces the osmolality of the culture medium. KHYG-1 cells were exposed to cell culture media at osmolalities of 280, 330, and 500 mOsm kg^{-1}. FLuc mRNA-loaded polyplexes were transfected for 24 h without exchanging the culture medium and FLuc expression levels were measured using a luminescence plate reader (Fig. 3D). Interestingly, the luminescence intensities at an osmolality of 330 mOsm kg^{-1} osmolality were 1.8-fold higher than those obtained under isotonic conditions. This indicates that osmoregulation in the culture media may increase *in vitro* mRNA transfection efficacy of polyplexes in KHYG-1 cells. In contrast, the luminescence intensity at 500 mOsm kg^{-1} osmolality decreased significantly to ~35%, probably because of the high toxicity of the culture media.

3.3 Effects of osmoregulation on cellular uptake of mRNA-loaded polyplex

When cells are exposed to hypertonic conditions, water in the cytosol flows outward. This may cause alterations in cell shape, which reduce the tension of the plasma membrane.

Numerous studies have reported that the tension in the plasma membrane regulates endocytic pathways.[27–29] Therefore, we hypothesized that NK cells exposed to hypertonic conditions enhance the cellular uptake of polyplexes *via* accelerated endocytosis, leading to high mRNA transfection efficacy. Thus, the cellular uptake of FLuc mRNA was examined using qRT-PCR to determine the amount of intact mRNA molecules in the cytoplasm. KHYG-1 cells were exposed to cell culture media at osmolalities of 280, 330, and 500 mOsm kg^{-1}. FLuc mRNA-loaded polyplexes were transfected without exchange of culture media, and total RNA was collected at 1, 4, and 24 h. FLuc mRNA levels were quantified relative to those under isotonic conditions at 1 h (Fig. 4A). Surprisingly, at all incubation times, intact FLuc mRNA levels at 330 mOsm kg^{-1} osmolality were 1.2–1.8 folds higher than those at 280 mOsm kg^{-1} osmolality. In contrast, intact FLuc mRNA levels at 500 mOsm kg^{-1} osmolality dramatically decreased by 10–60% compared to those at 280 mOsm kg^{-1} osmolality. These results matched well with the FLuc expression levels in Fig. 3D, in which KHYG-1 cells at 330 mOsm kg^{-1} osmolality exhibited 1.8-fold higher FLuc expression levels than those in isotonic conditions. Indeed, an osmolality of 330 mOsm kg^{-1} did not significantly affect cell viability (Fig. 2A and B). This indicates that osmoregulation can be used to enhance *in vitro*/*ex vivo* mRNA transfection efficacy of polyplexes in NK cells.

The cellular uptake of polyplexes at various osmolalities may be affected by the polyplex stability. Thus, time-dependent polyplex stabilities were evaluated through incubation with culture media with various osmolality at 37 °C (Fig. 4B). After 1 h of incubation, intact FLuc mRNA was detected in all the experimental groups. However, after 2 h of incubation, the intact FLuc mRNA levels at 330 and 500 mOsm kg^{-1} osmolality were lower than those under isotonic conditions. All FLuc mRNA disappeared after 4 h of incubation. These results indicate that the high cellular uptake of polyplexes at 330 mOsm kg^{-1} osmolality was not due to the high stability of the polyplex. Hypertonic treatment of KHYG-1 cells increased endocytosis, which further promoted the cellular uptake of the polyplexes. Enhanced cellular uptake in hypertonic condition was similarly observed in other type I alveolar epithelial cells, in which hypertonic exposure at 340 mOsm kg^{-1} osmolality stimulated caveolar endocytosis of cells associated with the translocation of caveolin-1 from the plasma membrane.[30] In other hands, the cells at the 500 mOsm kg^{-1} osmolality were too harsh, probably inhibiting the organization of microtubule networks and intracellular sorting.[31]

3.4 Effects of osmoregulation on endosome escape related gene expression

IVT mRNA delivery *via* nanoparticles is also influenced by the endosome escape process.[32,33] Therefore, we investigated the

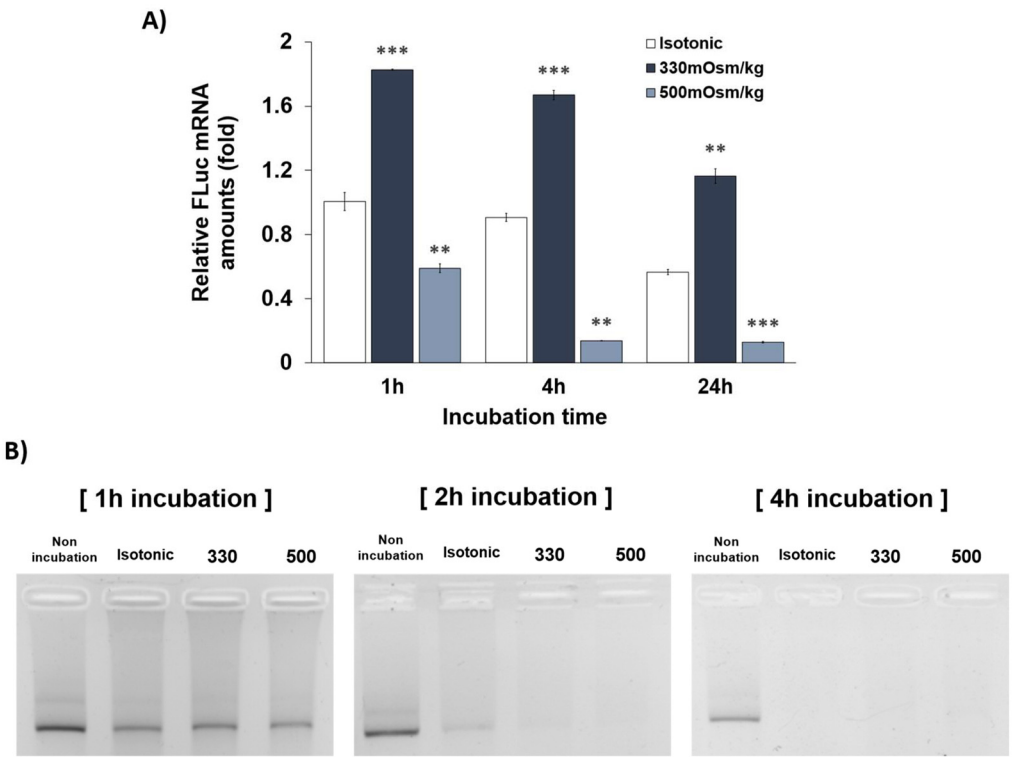

Fig. 4 (A) Time dependent cellular uptake amounts of FLuc mRNA in KHYG-1 cells. KHYG-1 cells pretreated with various osmolality were transfected with FLuc mRNA-loaded polyplexes. Total RNAs were collected, and intact FLuc mRNA amounts were measured by qRT-PCR. The results are expressed as mean ± SE (n = 3). Statistical differences are indicated as *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. (B) Agarose gel electrophoresis of FLuc mRNA after incubation of polyplex at various osmolality at 37 °C. After incubation, polyplex samples were purified with a RNeasy mini kit for mRNA release.

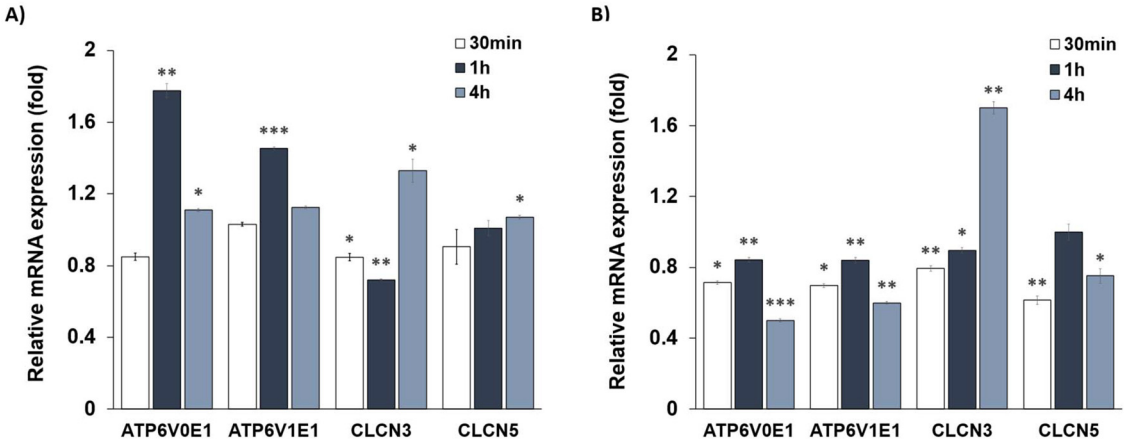

Fig. 5 Endosome escape related gene expression levels at (A) 330 and (B) 500 mOsm kg^{-1} osmolality at various incubation times. Gene expression levels at 330 and 500 mOsm kg^{-1} osmolality were normalized by those at 280 mOsm kg^{-1} osmolality. The results are expressed as mean ± SE (n = 3). Statistical differences are indicated as *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.

expression of endosome escape-related genes in KHYG-1 cells exposed to hypertonic condition. The proton sponge hypothesis is the best-known endosomal escape mechanism of polyplexes.[34,35] When transfected, polyplexes are internalized into endosomal vesicles *via* the endocytosis pathway. Afterwards, H^+ and Cl^- are transported to the endosomal lumen through the membrane bounded V-ATPases and chloride voltage-gated channels and undergo the endosome maturation process. During this process, since the deprotonated amino group in DET moiety of PAsp(DET/CHE) initially absorbs H^+, the pH in the endosome does not become acidic. Thus, additional H^+ and Cl^- continue to flow into the endosome lumen. Ultimately, high amounts of Cl^- abnormally accumulate abnormally in the endosome, inducing an osmotic imbalance. As a result, water flows in, the internal osmotic pressure increases, and the endosomal membrane becomes explosive. We selected the *ATP6V0E1* and *ATP6V1E1*, which are involved in H^+ transport to the endosome. We also selected *CLCN3* and *CLCN5*, which are involved in Cl^- transport into the endosome, as markers of the endosomal escape mechanism of the polyplexes. KHYG-1 cells were exposed to cell culture media at 280, 330, and 500 mOsm kg^{-1} osmolality for 0.5, 1, and 4 h. Total RNA was collected from KHYG-1 cells in the absence of mRNA transfection, and gene expression levels were measured by qRT-PCR. The results at 330 and 500 mOsm kg^{-1} osmolality were normalized to the gene expression levels at 280 mOsm kg^{-1} osmolality. At 330 mOsm kg^{-1} osmolality, the expression of all marker genes was significantly increased compared to that at isotonic incubation (Fig. 5A). The expression levels of *ATP6V0E1* and *ATP6V1E1* were 1.8- and 1.5-fold higher, respectively, after 1 h of incubation, probably indicating enhanced internalization of H^+ into the endosomes. The expression levels of *CLCN3* and *CLCN5* were 1.3-fold and 1.1-fold higher after 4 h of incubation, respectively. This may suggest delayed transport of Cl^- *via* chloride channels after H^+ transports. These results are consistent with the study by Tania López-Hernández *et al.*[36] in which cells were exposed to hypertonic conditions of Na^+, Na^+/H^+ exchanger 7 (NHE7) activation.[36] The initial activation leads to an increase in intracellular Na^+ and Ca^{2+} levels, along with an increase in the TFEB's trans-location activity. Consequently, the expression of lysosomal biogenesis-related genes, including V-ATPase, is regulated. The more efficient endosomal escape of polyplexes at 330 mOsm kg^{-1} osmolality also allowed the cytoplasmic transport of more mRNA, inducing higher mRNA expression levels. In contrast, at 500 mOsm kg^{-1} osmolality, most marker genes, except *CLCN3*, exhibited decreased gene expression after 4 h of incubation. This suggests that 500 mOsm kg^{-1} osmolality is a stressful stimulus for KHYG-1 cells.

4. Conclusions

The transfection efficiency of IVT mRNA-loaded polyplexes was improved by treating NK cells with NaCl-mediated hypertonic culture media. KHYG-1 cells at 330 mOsm kg^{-1} osmolality showed significantly enhanced mRNA transfection efficacy (1.8-fold) compared to cells under isotonic conditions. We identified two reasons for this enhanced mRNA translation efficacy. First, hypertonic treatment of KHYG-1 cells enhanced the cellular uptake of polyplexes, probably due to enhanced endocytosis. Second, qRT-PCR analysis revealed that hypertonic treatment of NK cells enhanced the expression of endosome escape-related genes in ion channels such as *ATP6V0E1*, *ATP6V1E1*, *CLCN3*, and *CLCN5*. Importantly, KHYG-1 cells treated with 330 mOsm kg^{-1} osmolality did not show severe toxicity without affecting the integrity of the cellular membranes and did not decrease the innate cytotoxic abilities of KHYG-1 cells. The present study, for the first time, demonstrates that external osmotic stimuli on NK cells induce the IVT mRNA transfection efficacy of polyplexes and provides

fundamental insights into the manufacture of genetically engineered NK cells.

Data availability

Data for this article are available at osf.io at **https://doi.org/10.17605/OSF.IO/UNQRP**.

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

This research was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea Government (MSIT) (RS-2023-00207801, 2021R1A2C1007668, and RS-2024-00398030) and Korean Fund for Regenerative Medicine (KFRM) grant funded by the Korea Government (the Ministry of Science and ICT, the Ministry of Health & Welfare; RS-2024-00333403).

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