Magnetically enhanced adeno-associated viral vector delivery for human neural stem cell infection

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Gene therapy technology is a powerful tool to elucidate the molecular cues that precisely regulate stem cell fates, but developing safe vehicles or mechanisms that are capable of delivering genes to stem cells with high efficiency remains a challenge. In this study, we developed a magnetically guided adeno-associated virus (AAV) delivery system for gene delivery to human neural stem cells (hNSCs). Magnetically guided AAV delivery resulted in rapid accumulation of vectors on target cells followed by forced penetration of the vectors across the plasma membrane, ultimately leading to fast and efficient cellular transduction. To combine AAV vectors with the magnetically guided delivery, AAV was genetically modified to display hexa-histidine (6xHis) on the physically exposed loop of the AAV2 capsid (6xHis AAV), which interacted with nickel ions chelated on NTA-biotin conjugated to streptavidin-coated superparamagnetic iron oxide nanoparticles (NiSINPs). NiSINP-mediated 6xHis AAV delivery under magnetic fields led to significantly enhanced cellular transduction in a non-permissive cell type (i.e., hNSCs). In addition, this delivery method reduced the viral exposure times required to induce a high level of transduction by as much as to 2–10 min of hNSC infection, thus demonstrating the great potential of magnetically guided AAV delivery for numerous gene therapy and stem cell applications.

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1. Introduction

The advancement of stem cell technologies requires the elucidation of molecular or environmental cues that can regulate the properties of self-renewal, the capacity to expand stem cells, and differentiation into specialized cell lineages. However, developing tools that can mediate the precise control of stem cells remains a challenge. Delivering genes that encode transcription factors or regulatory molecules can be a powerful strategy to circumvent the challenges associated with current stem cell technologies because it can serve as an efficient tool to modulate stem cell fates by the up- or down-regulation of specific genes [1–4]. Critical issues, however, still remain in the development of efficient and safe gene delivery vectors that can be utilized in stem cell applications.

Current approaches to mediate gene delivery to stem cells can be classified as plasmid- or virus-based gene delivery. Although plasmid-based gene delivery has great advantages in terms of safety and the ability to carry large genes, this method may have limitations in the stimulation of stem cells due to its low stem cell transfection efficiencies [5]. Alternatively, viral vectors, which typically offer high infectivity and stable gene expression, have been extensively utilized to manipulate stem cells [6,7]. However, there exist major concerns about the use of viral vectors that are primarily based on retroviruses and lentiviruses for a variety of stem cell applications; these concerns are primarily based on insertional mutagenesis [8,9]. Therefore, the transient expression of regulatory signals to manipulate stem cells may be desirable, which can relieve safety issues associated with insertional mutagenesis. Adenovirus-mediated vectors have demonstrated efficient gene delivery and transient gene expression in stem cells, but virally encoded factors that can cause immune responses may hinder the widespread use of adenoviral vectors for stem cell applications [10,11].

Alternatively, adeno-associated virus (AAV), which is being broadly explored in clinical trials, may satisfy the safety and efficiency criteria for use as a gene delivering vector in stem cell research. AAV is a parvovirus with a 4.7 kb single-stranded DNA genome containing two genes (rep and cap), and AAV has the ability to induce high delivery efficiencies in a broad range of both dividing and non-dividing cells [12–14]. Additionally, one of the great advantages of AAV for stem cell applications is that AAV vector genomes can mediate homologous recombination with target sequences in a cellular genome at efficiencies that are 103–104
higher than plasmid constructs [15,16], thus increasing its potential utility in numerous stem cell applications, such as gene correction, precise stem cell regulation, and the creation of disease models. Unfortunately, AAV has intrinsic properties that prevent it from efficiently delivering genes to the majority of stem cell types [17,18]. Therefore, the development of AAV vector-based delivery systems that are capable of efficient stem cell infection is a prerequisite to investigate stem cells using this vector.

In this study, the enhancement of AAV-mediated gene delivery to stem cells by employing magnetic fields that act on viral vectors associated with superparamagnetic nanoparticles was attempted. Streptavidin-coated superparamagnetic iron oxide nanoparticles (SPIONs) were used to form complexes with AAV vectors, and the SPIONs were additionally conjugated with biotin-nitrilotriacetic acid (NTA) to allow nickel ion chelation (Fig. 1). To associate AAV vectors with the NiStNPs, a hexa-histidine (6xHis) tag was inserted into a physically exposed loop of the wild type AAV2 capsid (i.e., amino acid position 587). Magnetic nanoparticle-mediated AAV delivery was demonstrated to be an effective tool to enhance gene delivery efficiency in human neural stem cells (hNSCs), a cell type that is non-permissive to AAV, thus demonstrating the great advantages of magnetically guided AAV delivery for numerous potential stem cell applications, such as gene correction, stem cell regulation, or the creation of disease models.

2. Materials and methods

2.1. Superparamagnetic nanoparticles conjugated with Ni-NTA-biotin

Streptavidin-coated superparamagnetic iron oxide nanoparticles (SPIONs; fluidMAG-Streptavidin) were purchased from Chemicon (Berlin, Germany). The hydrodynamic diameter of a SPION was approximately 100 nm, and the binding capacity of 1 mg of SPIONs was approximately 80 pmol of biotinylated protein, according to the manufacturer’s specifications. The SPIONs were diluted in aqueous solution containing 40 mM NaCl and subsequently agitated gently with the excess amount of biotin-nitrilotriacetic acid (biotin-NTA, 240 pmol; Biotium, Hayward, CA) and nickel chloride (100 mM; Sigma–Aldrich, St. Louis, MO) for 2 h to generate Ni-NTA-biotin-streptavidin-SPION complexes (NiStNPs). The presence of Ni-NTA-biotin on the streptavidin SPIONs was confirmed using Fourier-Transform Infrared (FT-IR) spectroscopy (Spectrum 100, Perkin Elmer, USA).

2.2. Cell culture

AAV 293 cells (Stratagene, La Jolla, CA) were utilized for viral packaging and were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO₂. HEK293T cells and human fetal neural stem cells (hNSC, 13 weeks of gestational age) were cultured to analyze the transduction efficiencies. HEK293T cells were grown in DMEM with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO₂. Human NSCs, which were derived from the telencephalon (HFT13) as previously described [19], were cultured in DMEM/F12 (Invitrogen) containing N-2 supplement, 20 ng/mL of fibroblast growth factor 2, 8 μg/mL of heparin (Sigma–Aldrich, St. Louis, MO), and 10 ng/mL of leukemia inhibitory factor (LIF; Chemicon, Temecula, CA).

2.3. Production and purification of 6xHis-AAV vectors

Two AAV vectors were produced in this study: i) 6xHis AAV vectors for immobilization on the NiStNPs and ii) wild-type AAV2 vectors as a control. Hexa-histidine (6xHis) residues were utilized as moieties to allow the AAV vectors to interact with the nickel ions that were chelated to the NTA-biotin-streptavidin-coated SPIONs (NiStNPs). The hexa-histidine-coding plasmid (pX22 His6) and pX22 were constructed as described in a previous study [20]. Hexa-histidine residues were inserted at position 587 in the amino acid sequence, corresponding to physically exposed loop domain: - QLRGNLHHHHHHRSQA- (wt AAV2: 583LQRGNRQA-). Recombinant 6xHis-AAV vectors carrying CDNA encoding green fluorescent protein (GFP) driven by a cytomegalovirus (CMV) promoter were packaged using the transient transfection method. Briefly, an equal mass (17 μg) of three plasmids, including an AAV helper plasmid (i.e., pX22 His6), the CMV GFP vector plasmid containing inverted terminal repeats (ITR) (pAAV CMV GFP SFN), and an adenoaviral helper plasmid (Stratagene), were transfected via calcium phosphate into AAV 293 cells [21]. Because the heparan sulfate proteoglycan (HSPG) binding domain was preserved upon hexa-histidine insertion, the resulting viral vectors were harvested as previously described [22] and purified using heparin column chromatography [23]. For purification using a heparin column, the crude lysate of 6xHis-AAV vectors was loaded onto a 1 mL of HiTrap heparin column (GE Healthcare, Pittsburgh, PA) that had been previously equilibrated with Tris buffer (50 mM, pH 7.5) containing 150 mM NaCl. The column was washed with 3 mL of 150 mM NaCl, and elutions were performed with 3 mL of 1 M NaCl. The viral fractions were desalted and buffer exchanged into phosphate-buffered saline (PBS)/0.01% Tween 20 using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA) according to the manufacturer’s instructions. DNase-resistant genomic titers were determined by quantitative PCR (QPCR; Mini Opticon, Bio-Rad, Hercules, CA).

2.4. Preparation of AAV/NiStNP complexes

The resulting 6xHis-AAV vectors (2 × 10⁹ viral genomic particles) were diluted in 50 μL of serum-free DMEM and mixed gently with the Ni-NTA-biotin-streptavidin-SPION nanoparticles suspended in 50 μL of serum-free DMEM with different quantities of nanoparticles (i.e., 0.02, 0.2, 2, or 4 μg). The mixture was additionally agitated for 120 min at room temperature, and an additional 200 μL of serum-free DMEM was added to reduce the aggregation of 6xHis-AAV/NiStNP complexes prior to addition to the cell culture medium for infection. To examine the association of AAV vectors with the NiStNPs, after magnetic sedimentation (NeodyMagTEK Magnet 1170–1250 mT; BSM magnet, Berlin, Germany), the number of genomic particles of unbound AAV vectors in the supernatant was determined by quantitative PCR (QPCR).

2.5. Characterization of AAV/NiStNP complexes

The presence of Ni-NTA-biotin on the streptavidin-coated SPIONs was confirmed by Fourier-Transform Infrared (FT-IR) spectroscopic measurements (Spectrum 100, Perkin Elmer, USA). Hydrodynamic particle sizes were determined with 3 mL of 1M NaCl. The viral fractions were desalted and buffer exchanged into phosphate-buffered saline (PBS)/0.01% Tween 20 using Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA) according to the manufacturer’s instructions. DNase-resistant genomic titers were determined by quantitative PCR (QPCR).
by dynamic light scattering using a Zetasizer (Zen3600, Malvern Instruments Ltd., Worcestershire, United Kingdom). Additionally, the conjugation of Ni-NTA-biotin and the immobilization of 6xHis AAV onto the streptavidin-coated SPIONs were visualized using transmission electron microscopy (TEM) (JEM-2011, JOEL Ltd., Tokyo, Japan) at the Yonsei Center for Research Facilities. Each sample (NiStNPs only, 6xHis AAV/NiStNPs) was prepared by dipping a carbon-coated grid into the sample followed by drying overnight at room temperature, and TEM images were taken under 200 kV with 400000x magnification.

2.6. Transduction assay

To test the infectivity of SPION-mediated AAV delivery, the AAV/NiStNP complexes (2 × 10^14 viral genomic particles) carrying cDNA encoding GFP driven by the CMV promoter were added on top of the medium containing cells (HEK293T: 4 × 10^4 cells/well and hNSCs: 1 × 10^4 cells/well) grown on 24-well plates (TCP) in the presence or absence of a magnetic field (MagneTOctor plate 24, Chemicon, Berlin, Germany). Each well was exposed to the magnetic field for 2, 10, or 120 min, and cell culture media containing AAV (AAV2, 6xHis AAV)/NiStNP or AAV vectors only (AAV2, 6xHis AAV) was replaced with fresh media at the same time. The percentage of GFP-expressing cells out of the total number of cells was determined at 48 h post-infection using flow cytometry at the Yonsei University College of Medicine Medical Research Center (Becton Dickinson FACS Caliber).

2.7. Internalization and inhibition of AAV-nanoparticles in the cytoplasm

Fluorescently labeled streptavidin-SPIONs (nano-screenMAG-Streptavidin: excitation at 578 nm, Chemicon, Berlin, Germany) were utilized to visualize the internalization of AAV/NiStNP complexes within the cytoplasm. To image the fluorescence, cells were trypsinized immediately after exposure to AAV/NiStNP complexes for 2, 10, or 120 min, re-plated onto new 24-well TCPs, and grown for additional 24 h prior to fluorescence imaging (Eclipse Ti-S, Nikon, Tokyo, Japan) and for 48 h prior to flow cytometry analysis. The number of GFP-expressing cells was quantified using flow cytometry to assess the capability of SPION-mediated AAV delivery under magnetic guidance for rapid internalization into the cytoplasm compared with non-SPION-mediated delivery (i.e., virus only) or SPION-mediated delivery without magnetic forces. Additionally, to test the inhibition of the energy-dependent endocytosis pathway for magnetically guided AAV delivery, hNSCs were exposed to the 6xHis AAV/NiStNP for 2, 10, or 120 min at 4 °C, and the percentage of GFP-expressing cells was compared with that at 37 °C. After viral exposure at 4 °C, the supernatant was removed at designated time points (2, 10, and 120 min), and the cells were vigorously washed twice and cultured for additional 48 h at 37 °C prior to flow cytometry analysis.

2.8. Cell viability

To analyze the viability of the cells infected by the 6xHis AAV/NiStNP complexes, metabolically active cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich), and colorometric changes were detected using a spectrophotometer (NanoDrop 2000; Thermo Scientific, West Palm Beach, FL) relative to the positive control (i.e., virus only delivery of 5 × 10^6 genomic MOI for HEK293T and 2 × 10^4 MOI for hNSCs). Each cell (HEK293T, hNSC) was infected by AAV only (AAV2, 6xHis AAV), NiStNP only, or AAV/NiStNP complexes and harvested after 48 h. Briefly, 0.1 volume of MTT solution relative to the volume of the medium containing cells (20 μL of 2.5 μg/μL in 200 μL medium) was dispensed into each well, and the cultures were incubated for 4 h at 37 °C. Supernatants were collected and mixed with dimethyl sulfoxide (DMSO; EMD Biosciences, Gibbstown, NJ), and spectrophotometric absorbances (550 nm) were measured using a NanoDrop 2000 (Thermo Scientific, West Palm Beach, FL). Finally, the absorbance values were normalized to the absorbance value of the positive control, in which cells were cultured with AAV only for the same durations.

2.9. Statistical analysis

All the conditions were performed in triplicate, and experimental data were expressed as mean ± standard deviation (SD). Statistical analyses between conditions (i.e., Student’s t-test) were performed using the JMP software package (SAS Institute, Inc., Cary, NC, USA).

3. Results and discussion

3.1. Characterization of AAV/NiStNP complexes

We developed a strategy to immobilize genetically modified AAV vectors (i.e., 6xHis tagged AAV) onto superparamagnetic iron oxide nanoparticles (SPIONs), as schematically illustrated in Fig. 1. Specifically, we inserted a 6xHis tag into a physically exposed loop of the AAV2 capsid (i.e., amino acid position 587), as previously...
described [20], which enabled the immobilization of the AAV on a surface displaying nickel ions chelated by biotin-nitrilotriacetic acid (biotin-NTA) moieties bound to a streptavidin-coated surface [24]. Similarly, the same 6xHis AAV vectors were bound to streptavidin-coated SPIONs conjugated to biotin-NTA-Ni (NiStNPs). Genomic titers of 6xHis AAV vectors, produced by the transient transfection method followed by heparin column purification, were slightly lower (2.2 × 10^{12} vg (viral genomes)/mL) than those of the wild type AAV2 (3.8 × 10^{12} vg/mL) generated by the same method. The 6xHis AAV vectors, including crystal structures, heparin affinity, and infectious units, compared with wild-type AAV2 have been previously characterized in detail [20].

Prior to immobilizing the 6xHis AAV vectors on the NiStNPs, successful conjugation of biotin-NTA onto the streptavidin-coated nanoparticles for the subsequent chelation of Ni ions on the NTA was confirmed using Fourier-Transform Infrared spectroscopy (FT-IR) analysis (Fig. 2A). Characteristic peaks appearing upon the addition of Ni-NTA-biotin to the streptavidin-coated iron oxide nanoparticles were observed in the regions of 3025–2918, 2078, and 1600–1370 cm⁻¹. The stretching vibration of the C=O occurring at approximately 1600 cm⁻¹ confirmed the presence of the C=O bond within the tetrahydridomidazalone ring fused with the tetrahydrothiophene ring of biotin [25]. Additionally, the peaks at 1495 and 1449 cm⁻¹ corresponded to the N–H bending and the ring vibrations of the tetrahydridomidazalone-tetrahydrothiophene ring of biotin, respectively [25]. Regions 1370–1600 and 2900–3000 cm⁻¹ of the spectrum, assigned to the NTA-amine [26], confirmed the presence of NTA. Because the presence of nickel ion cannot be identified in the mid-infrared region, successful chelation of nickel ions on the NTA was verified by its specific

Fig. 3. Metabolic activities of HEK293T cells (A) and hNSCs (B) upon exposure to virus only, magnetically guided 6xHis AAV vectors. The MTT assay was performed to measure the metabolic activities (i.e., cell viabilities) of cells treated with different quantities of NiStNPs under various viral exposure times (2, 10, and 120 min). The symbols * and ** indicate statistically significant differences compared with those that were treated with virus (6xHis AAV) only for 24 h (P < 0.05 and P < 0.01, respectively).
interactions with the 6xHis-tagged proteins (i.e., AAV vectors) on the nanoparticles.

The 6x-histidine displayed on the viral capsid led to the binding of the 6xHis AAV vectors to the NiStNPs, with high efficiencies ranging from 98.1% to 99.5%. In contrast, the absence of the histidine residues (i.e., AAV2 vectors) resulted in binding capacities less than 30%, revealing the specific interactions between the 6x-His AAV vectors and the nickel ions chelated on the nanoparticles (Fig. 2B). Because the majority of the 6xHis AAV vectors (>98%) successfully bound to the NiStNPs, the separation of the unbound 6xHis AAV vectors prior to infection was not necessary. The average amount of NiStNP-bound AAV2 vectors slightly increased with increased quantities of NiStNP, possibly indicating non-specific interactions between the vectors and the nanoparticles.

An increased quantity of nanoparticles led to an increase in the hydrodynamic particle size of the 6xHis AAV/NiStNP complexes, ranging from 104.60 nm to 161.89 nm (Fig. 2C). Similarly, the hydrodynamic diameters of the nanoparticles alone increased in conjunction with an increased quantity of nanoparticles (i.e., 85.75 nm–126.30 nm), implying that the larger diameter of the virus/NiStNP complexes may simply be a result of the agglomeration of nanoparticles. Interestingly, formulations of complexes using AAV2 resulted in larger nanoparticles ranging from 135.98 nm to 188.67 nm, presumably due to loose binding of AAV2 to the NiStNPs or agglomerations of the AAV2/NiStNP complexes caused by non-specific interactions. Transmission electron microscopy was used to visualize the conjugation of Ni-NTA-biotin and the immobilization of 6xHis AAV vectors on the streptavidin-coated SPIONs (Fig. 2D and E). The NiStNPs displayed a spherical morphology with small agglomerated particles on the surface, which were expected to be Ni-NTA-biotin-streptavidin tags on the iron oxide nanoparticles (Fig. 2D). The particle sizes were approximately 100–120 nm, which is consistent with the dynamic light scattering analysis shown in Fig. 2C. After immobilizing the 6xHis AAV vectors, the icosahedral shape of the 6xHis AAV attached to the NiStNPs (the arrow in Fig. 2E) was observed, confirming the successful formulation of the 6xHis AAV/NiStNP complexes prior to cellular transduction.

3.2. The effect of SPION-mediated gene delivery on cell viability

Next, we assessed the influence of SPION-mediated AAV delivery under magnetic forces on the metabolic functions of cells by comparing cells exposed to the 6xHis AAV/NiStNP complexes with or without magnetic forces: (A) representative fluorescence images showing GFP-expressing HEK293T cells (with 2 μg NiStNPs). The scale bar indicates 100 μm. (B) The percentage of GFP-expressing HEK293T cells out of total number of cells at 2 days post-infection. The genomic MOI (i.e., genomic viral particles/cell) was 5 x 10⁴. Note that the symbols * and ** indicate statistically significant differences compared with infection by 6xHis AAV/NiStNPs without magnetic forces and compared with infections by 24 h-exposure to 6xHis AAV only, respectively (P < 0.05). However, no increased transduction as compared with 24 h-exposure to 6xHis AAV only was observed in HEK293T infection.

Fig. 4. Cellular transduction of HEK293T cells by AAV2/NiStNP complexes vs. that by 6xHis AAV/NiStNP complexes with or without magnetic forces: (A) representative fluorescence images showing GFP-expressing HEK293T cells (with 2 μg NiStNPs). The scale bar indicates 100 μm. (B) The percentage of GFP-expressing HEK293T cells out of total number of cells at 2 days post-infection. The genomic MOI (i.e., genomic viral particles/cell) was 5 x 10⁴. Note that the symbols * and ** indicate statistically significant differences compared with infection by 6xHis AAV/NiStNPs without magnetic forces and compared with infections by 24 h-exposure to 6xHis AAV only, respectively (P < 0.05). However, no increased transduction as compared with 24 h-exposure to 6xHis AAV only was observed in HEK293T infection.
with those that were treated only with the virus for 24 h (Fig. 3A and B). Human neural stem cells mostly maintained metabolic activity upon exposure to a small quantity (<2 µg) of magnetically guided 6xHis AAV/NiStNP complexes (Fig. 3B), but exposure of HEK293T cells to even a small quantity (0.02–0.2 µg) of nanoparticles yielded significantly reduced cellular metabolic activities compared with positive controls (i.e., virus only) (Fig. 3A). At the highest quantity of nanoparticles (4 µg), substantial reductions in the viability of hNSCs were observed, possibly due to the cytotoxicity of the nanoparticle itself or the presence of a greater number of internalized nanoparticles within the cytoplasm [27]. The presence of magnetic forces did not influence the cell viability of either cell type.

3.3. Enhanced infection with 6xHis AAV/NiStNP complexes

SPION-mediated 6xHis AAV delivery under magnetic forces significantly enhanced the cellular transduction of HEK293T cells compared with SPION-bound 6xHis AAV delivery without magnetic forces (Fig. 4A and B). However, due to the intrinsic ability of AAV2 vectors to induce high transduction efficiencies in HEK293T cells, magnetically guided 6xHis AAV vectors demonstrated only slightly improved or comparable transduction efficiencies compared with AAV2 vectors alone or with AAV2/NiStNP complexes. Additionally, NiStNP-mediated 6xHis AAV delivery induced a lower transduction efficiency than conventional transduction with AAV2 vectors, that is, a 24-hour exposure to viral vectors prior to medium replacement. The genomic multiplicity of infection (MOI) employed for infecting the HEK293T cells was 5 × 10^4. The magnetically guided 6xHis AAV vector delivery enhanced cellular transduction in a NiStNP dose-dependent manner. Upon infection with AAV2 vectors, magnetically guided delivery did not exhibit any trends of cellular transduction with various amounts of nanoparticles, but infectivities gradually increased as the exposure times increased, as expected. Non-specific interactions between AAV2 and the NiStNPs might result in limited binding to or easy detachment from the nanoparticles, consequently leading these two species (i.e., AAV2 vs. nanoparticles) to act independently within the magnetic fields.

The effect of magnetic guidance of 6xHis AAV/NiStNP complexes (genomic MOI: 2 × 10^4) on cellular transduction was maximized when infecting non-permissive cell types, such as hNSCs (Fig. 5), which is consistent with previous studies [28–30]. Compared with non-magnetically guided viral infection and 24-hour viral exposure prior to medium replacement (i.e., conventional transduction), magnetically guided 6xHis AAV vectors yielded 2–5 fold and approximately 2 fold enhanced infectivities, respectively (Fig. 5A and B). The levels of cellular transduction mediated by magnetically guided 6xHis AAV delivery (~50%) are comparable to those obtained by a previous study in which a novel AAV vector (i.e., AAV

![Cellular transduction of hNSCs by AAV2/NiStNP complexes vs. that by 6xHis AAV/NiStNP complexes with or without magnetic forces: (A) representative fluorescence images showing GFP-expressing hNSCs (with 2 µg NiStNPs). The scale bar indicates 100 µm. (B) The percentage of GFP-expressing hNSCs out of the total number of cells at 2 days post-infection. The genomic MOI (i.e., genomic viral particles/cell) was 2 × 10^4. The symbols * and ** indicate statistically significant differences compared with infection by 6xHis AAV/NiStNPs without magnetic forces and compared with infection by 24 h-exposure to 6xHis AAV only, respectively (P < 0.05).]
r3.45) with enhanced properties for NSC infection was developed by directed evolution [16]. Importantly, the magnetically guided AAV delivery system induced similar cellular transduction in hNSCs even with a 4-fold decreased viral genomic MOI (2 × 10^4 MOI vs. 1 × 10^5 MOI). Significantly improved gene correction frequencies within the NSCs were achieved with comparable transduction efficiencies (~50%) in the previous study; therefore, we can expect a future in potential stem cell applications of the magnetically guided 6xHis AAV delivery system, such as in gene targeting, stem cell regulation, and creation of disease models.

Fig. 6. Internalization of 6xHis AAV/NiStNPs into the cytoplasm and GFP expression exclusively produced by the internalized viral vectors. Prior to visualizing the internalization and quantifying GFP expression, cells exposed at different viral exposures (2, 10, and 120 min) were vigorously rinsed with PBS, trypsinized, and finally replated onto fresh 24-well dishes. Representative images of the presence of fluorescently labeled NiStNPs (with 2 μg) within the HEK293T (A) and hNSC (B) cytoplasm. To visualize the cells effectively, bright field images taken to show cell morphologies were merged with the fluorescence images of the NiStNPs (red color). The scale bar indicates 50 μm. The percentage of GFP-expressing HEK293T cells (C) and hNSCs (D) after the internalization of viral vectors. The symbols * and ** indicate statistically significant differences compared with infections by 6xHis AAV/NiStNPs (with the same quantity of NiStNPs) without magnetic forces (P < 0.05 and P < 0.01, respectively).
While AAV2 infection with or without NiStNP complexation resulted in low cellular transduction (<13%) without demonstrating improvement or reduction in transduction, NiStNP-guided 6xHis AAV infection induced gradually enhanced transduction under the influence of the magnetic field as the viral exposure time increased up to 10 min. However, as the quantity of NiStNPs increased, cellular transduction decreased, presumably due to the cytotoxicity of the higher amounts of NiStNPs, as shown in Fig. 3. Interestingly, upon infection with 6xHis AAV/NiStNPs complexes, even in the absence of magnetic forces, the longest exposure (i.e., 2 h) of 6xHis AAV/NiStNPs to the hNSCs led to a comparable enhancement of cellular transduction with magnetically guided 6xHis AAV delivery approaches.

Importantly, the magnetically enhanced viral gene delivery approach significantly reduced the viral exposure times required for a high level of cellular transduction, especially in a non-permissive cell type. Conventional transduction using viral vectors typically requires a 24-hour viral exposure to maximize the efficiency of gene delivery. However, for NiStNP-mediated 6xHis AAV delivery under a magnetic field for hNSC infection, exposure times of only a few minutes (2–10 min) were sufficient to yield 2–3.5 fold improved transduction compared with the conventional transduction method (i.e., 24-hour exposure). After a 10 min viral exposure of hNSCs under the magnetic field, further enhancement of cellular transduction was not observed (Fig. 5B), indicating that a 10 min viral exposure under the magnetic field was fully sufficient for hNSC infection. The enhanced transduction of a non-permissive cell type even when using a short viral exposure time may be due to the rapid sedimentation of the full vector dose on the target cells followed by the forced penetration of the vector across the plasma membrane with the aid of the NiStNPs acted upon by the magnetic field [31–33].

3.4. Internalization of 6xHis AAV/NiStNP

Substantial barriers to AAV infection of hNSCs may include surface binding, intracellular trafficking, and/or nuclear entry [34,35]. AAV2 utilizes heparan sulfate proteoglycan as its primary receptor [36], and this molecule is present on every cell type in organisms with tissue organization, possibly including neural stem cells [37,38]. Hexa-histidine AAV vectors, which still exhibit heparin affinity [20], also demonstrated low delivery efficiencies in hNSC transduction, as did wild-type AAV2 (Fig. 5). A recent study demonstrated that surface modification of AAV2 vectors by directed evolution significantly improved transduction efficiencies for hNSCs, implying that key blocking steps against AAV delivery in hNSCs may be related to the external surface of the AAV vectors [16]. Therefore, the low transduction efficiency of AAV2 and 6xHis AAV for hNSCs may be caused by the reduced level of HSPG on the hNSCs or by the requirement of an alternate secondary receptor to be internalized, both of which are related to the entry of viral vectors across the plasma membrane. Consequently, the application of a method to cross the cellular membrane is expected to be a key strategy in enhancing cellular transduction in hNSCs. Consistently, the rapid accumulation of viral vectors on the hNSCs followed by direct penetration across the cellular membrane by the magnetically guided gene delivery approach resulted in significantly improved cellular transduction.

Magnetically guided 6xHis AAV/NiStNP complexes were internalized rapidly compared with non-magnetically delivered complexes, ultimately resulting in a high level of transduction. To verify the rapid internalization of 6xHis AAV/NiStNP complexes under magnetic fields, each cell type (HEK293T, hNSC) was exposed to the complexes for designated time points (i.e., 2, 10, and 120 min) and then trypsinized and replated onto a fresh dish. Trypsinizing cells provides a typical way to remove the majority of proteins, including surface-bound viral vectors, on the cellular membrane. Trypsinization can be used to investigate the effect of the entry of 6xHis AAV/NiStNP complexes via magnetic forces on the enhanced cellular transduction. The presence of fluorescently labeled nanoparticles within cells was visualized at 24 h post-infection. Additionally, gene expression exclusively produced by the internalized viral vectors was quantified using flow cytometry at 48 h post-infection. Consequently, large amounts of fluorescently labeled nanoparticles, which were delivered under the magnetic field, were detected within the cytoplasm as the exposure time increased (Fig. 6A and B). The advantageous effect of rapid internalization by magnetic guidance on cellular transduction was not substantial in a permissive cell type (HEK293T), but it was maximized in non-permissive hNSCs (Fig. 6C and D). Additionally, a 10 min viral exposure under the magnetic field during hNSC infection caused a dramatic increase in internalization followed by transduction compared with non-magnetically guided 6xHis AAV delivery (Fig. 6D). A large quantity of 6xHis AAV vectors was rapidly internalized into the hNSCs as the result of the magnetic guidance of NiStNPs under magnetic fields, with approximately 5–100 fold enhanced GFP expression compared with the internalized AAV2 vectors (Fig. 6B and D). An approximate 2 fold increase in GFP expression for hNSCs shown in Fig. 5 compared with gene expression demonstrated in Fig. 6 may have resulted from the removal of surface-associated viral vectors, specifically non-internalized viral vectors, upon trypsinization for replating onto a new tissue culture dish.

The entry of magnetically guided 6xHis AAV particles was independent of the energy-dependent endocytosis pathway, as demonstrated by the fact that cellular transduction by magnetically guided 6xHis AAV vectors at 4 °C was comparable with that at 37 °C (Fig. 7). Therefore, these results suggest that direct penetration of viral vectors by magnetic forces can be a key factor in enhancing cellular transduction in non-permissive cell types, consistent with the results of previous studies [28,30,39,40].

4. Conclusion

We reported a highly efficient AAV-mediated gene delivery system for the transduction of non-permissive cell types, such as
hNSCs, by employing magnetic forces. Magnetically guided 6xHis AAV delivery induced rapid internalization for the subsequent significantly enhanced cellular transduction. The successful establishment of an AAV delivery system with enhanced abilities to efficiently infect stem cells will provide a powerful means to regulate stem cell functions. Thus, this technique will have numerous applications that range from basic stem cell biology to stem cell therapy or the development of disease models.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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References