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Short communication

Directional gene-transfer into the brain by an adenoviral vector tagged with magnetic nanoparticles

Mitsuhiro Hashimoto*, Yasuko Hisano

Hashimoto Research Unit, RIKEN BSI, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

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ABSTRACT

Adenoviral (Ad) vectors are useful for *in vivo* gene transfer into the brain. If Ad vectors are injected into the ventricle of mouse embryonic brain, Ad vectors introduce a foreign gene into neural progenitor cells on the surface of ventricle. However, Ad vectors were unable to deliver a foreign gene to a targeted region of the embryonic brain because Ad vectors evenly infected the neural progenitor cells on the surface of ventricle. Therefore, the Ad infection to the neural progenitor cells was uncontrollable. To develop a directional gene-transfer with Ad vector, we generated Ad vector tagged with magnetic nanoparticles (Ad-mag) by linking a biotinylated adenovirus vector with a streptavidin-conjugated magnetic nanoparticle. Ad-mags were attracted by magnetic force *in vitro* and *in vivo*. When Ad-mags were injected into the ventricle of mouse embryo and a strong magnet was attached to the head of the embryo, Ad-mags were attracted to the restricted direction or region where the magnet was placed. As a result, Ad-mags efficiently introduced a foreign gene into the restricted region of the brain.

1. Introduction

Adenoviral (Ad) vectors are powerful tools for gene transfer into the nervous system (Hashimoto et al., 1996; Hashimoto and Mikoshiba, 2003, 2004). When Ad vectors are injected into the ventricle of mouse embryonic brain, Ad vectors spread throughout the ventricle and then evenly infect neural progenitor cells on the surface of ventricle (Hashimoto and Mikoshiba, 2004). The progenitor cells infected with Ad vector are normally developed into neurons. Remarkably, Ad vector introduce a foreign gene into neurons in a neuronal birthdate specific manner, like a bromodeoxyuridine-labeling as neuronal birthdate analysis (Hashimoto and Mikoshiba, 2003, 2004). The neurons infected with Ad vector maintain gene expression of the transgene for a long period of time without cytopathogenic effects (Hashimoto and Mikoshiba, 2003, 2004). This technique is useful for studying the neuronal development and function because Ad vector enables us to manipulate genetically each subset of neurons that share the same neuronal birthdate. However, Ad vector was unable to introduce a foreign gene into a restricted region of the embryonic brain.

To solve this issue, we used Ad vectors tagged with magnetic nanoparticles. We hypothesized that Ad vectors tagged with magnetic nanoparticles in the ventricle of embryonic brain were controlled by magnetic force from the outside of the brain. Ad particles can be modified by biochemical techniques (Wagner et al., 1992; Smith et al., 1999; Singh and Kostarelos, 2009) because an Ad particle consists of viral DNA and a protein coat (capsid) composed of 252 capsomeres including hexons, penton bases, and fibers. Therefore, recent studies have reported several kinds of Ad vectors tagged with magnetic nanoparticles (Pandori et al., 2002; Scherer et al., 2002; Campos et al., 2004). These magnetized Ad vectors are successfully attracted to the target site by the magnetic force *in vitro*, and consequently, introduce a foreign gene into the restricted region where a strong magnet is placed. However, it has been unclear whether these magnetized Ad vectors can introduce a foreign gene into the restricted region *in vivo*, especially in the embryonic brain. To make Ad-vectors tagged with magnetic nanoparticles, we biotinylated the Ad particles by a chemical compound and linked the biotinylated Ad vectors with streptavidin-conjugated magnetic nanoparticles (Ad-mags). We found that Ad-mags were attracted to the restricted direction or region where the magnet was placed and the magnetic forces prevent the diffusion of Ad-mags from the target site. Consequently, the magnetic attraction of Ad-mags achieved effective gene transfer to a desired region of the embryonic brain, even with a relatively low amount of Ad vectors.

2. Results and discussion

2.1. Ad vector tagged with magnetic nanoparticles

The purified Ad vector AdexCAG-NL-LacZ, which expressed nuclear-targeted β-galactosidase (β-gal), was biotinylated directly using sulfo-NHS-LC-biotin, which covalently biotinylates the pri-

* Corresponding author. Tel.: +81 48 467 9678; fax: +81 48 467 9763.
E-mail address: mhashimoto@brain.riken.jp (M. Hashimoto).

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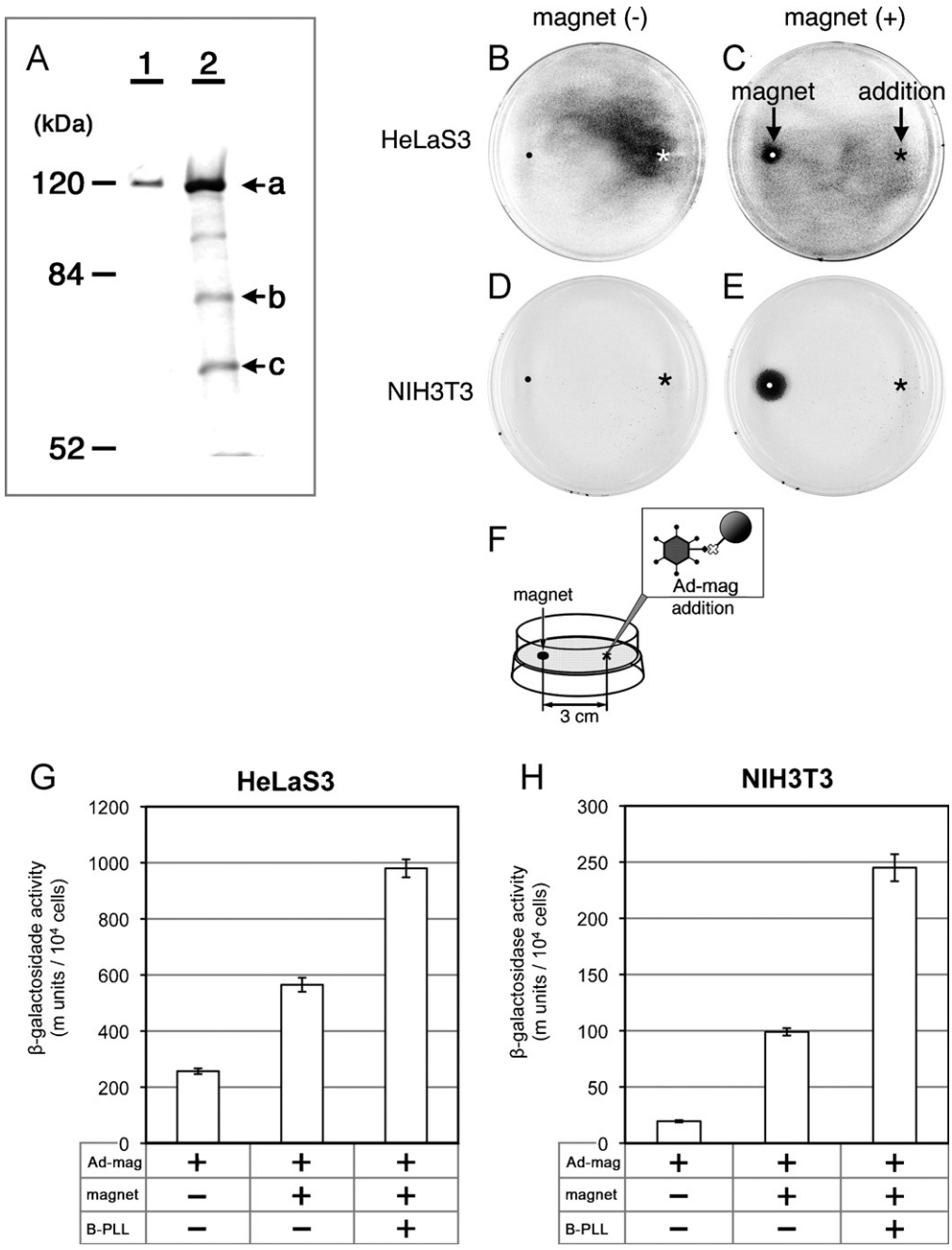

Fig. 1. Magnetic attraction of Ad-mag *in vitro* The biotinylated Ad vector was subjected to Western blotting analysis (A; lane 1, Ad vector [total 1×10^6 PFU] was biotinylated for $30 \times$ min; lane 2, Ad vector [total 1×10^6 PFU] was biotinylated for 2 h.). The biotinylated capsomeres of Ad vector are recognized as the hexon (a in A), penton base (b in A) and fiber (c in A). Molecular weights are indicated in the left side. A schematic view of this experiment is illustrated in F. Adenoviral vectors tagged with magnet nanoparticles (Ad-mags) were added into the culture medium at the addition site (the asterisks in B–F). A small magnet was attached beneath the bottom of the culture dish (the white dots in C and E). HeLaS3 (B and C) and NIH3T3 (D and E) cells were stained with Bluo-gal two days after Ad-mag was administered in the presence (C and E) or absence of a magnet (B and D). Representative results from this experiment are shown. The efficiency of gene transfer to HeLaS3 (G) and NIH3T3 (H) cells by the Ad-mag was enhanced by the magnetic force and Ad-mag coating with biotinylated polycationic polymer (B-PLL) (Means ± S.E.M., $n = 10$).

mary amines of the adenoviral capsid. After biotinylation, the biotinylated capsomeres were verified by Western blot analysis (Fig. 1A). The hexons (a in line 1, Fig. 1A) were mainly biotinylated after the treatment with sulfo-NHS-LC-biotin for 30 min. The resultant biotinylated Ad vectors were capable of introducing a foreign gene into HeLaS3 cells (Fig. 1 B). When Ad vectors were treated with sulfo-NHS-LC-biotin for 2 h (line 2, Fig. 1A), hexon (a in line 2, Fig. 1A), penton base (b in line 2, Fig. 1A), and fiber (c in line 2, Fig. 1A) were biotinylated. As a result, the excessive biotinylation of Ad vectors significantly reduced the efficiency of gene transfer to HeLa3S cells (data not shown). To optimize the efficiency of both biotinylation and gene transfer, we set the period of the biotinylation reaction for 30 min. To generate an Ad vector tagged with magnetic nanoparticles (Ad-mag: a schematic view is shown in Fig. 1F), we coupled the biotinylated Ad vectors to streptavidin-conjugated magnetic nanoparticles. A streptavidin-conjugated magnetic nanoparticle (streptavidin, 60 kDa; magnetic nanoparticle, about 50 nm in diameter) is smaller than an Ad particle (150,000 kDa and 70–80 nm in diameter, excluding the fibers). Therefore, this streptavidin-conjugated magnetic nanoparticle appeared to not prevent the infection of Ad-mags to cells.

2.2. In vitro gene transfer with Ad-mag

To examine the infectivity of Ad-mags, we inoculated Ad-mags into a culture of HeLaS3 (Figs. 1B and C) and NIH3T3 (Fig. 1D and E) cells at a multiplicity of infection (MOI) of four. The HelaS3 and NIH3T3 cells were cultured with (the white dots in Fig. 1C and E) or without (the black dots in Fig. 1B and D) a small magnet that was placed beneath the bottom of the culture dishes. Ad-mags were gently added into the culture medium (at the asterisks in Fig. 1B–F) apart from the site of magnet (Fig. 1F). Two days later, the cultures were fixed and stained for β-gal. Among HeLaS3 cells cultured without the magnet (Fig. 1B), many β-gal-positive cells around the site of addition were observed (the asterisk in Fig. 1B). In contrast, β-gal-positive cells in HeLaS3 cells cultured with the magnet (Fig. 1C) formed a compact cluster that was located to the site of magnet (the white dots in Fig. 1C), indicating that Ad-mags were attracted by the magnetic force from a distance. HeLaS3 cells show high affinity to Ad particles because a large amount of adenoviral receptors are present on the cell surface (Defer et al., 1990). In contrast, NIH3T3 cells were resistant to Ad infection because they express only a few adenoviral receptors (Seth et al., 1994). Indeed, β-gal-positive cells were rarely observed in NIH3T3 cells cultured without the magnet (Fig. 1D) compared with HeLaS3 cells cultured without the magnet (Fig. 1B). However, among NIH3T3 cell cultured with the magnet (Fig. 1E), many β-gal-positive cells were observed in a compact cluster that was predominantly located to the site of magnet (the white dots in Fig. 1E). To examine whether magnetic force influences the efficiency of Ad-mag gene transfer, we measured the β-gal enzymatic activity on HeLaS3 (Fig. 1G) and NIH3T3 (Fig. 1H) cells with or without the magnetic attraction of Ad-mag. The results indicated that magnetic force clearly increased the efficiency of gene transfer to HeLaS3 and NIH3T3 cells by 2.2- and 5.1-fold, respectively. The magnetic force strongly attracted Ad-mag to the restricted region where the magnet was placed and the magnetic attraction enhanced the efficiency of Ad-mag infection, even though NIH3T3 cells are resistant to Ad infection (Seth et al., 1994). The magnetic force keeps the Ad-mags at high concentrations in the restricted region around the magnet and then the MOI at the restricted region may increase. The magnetic attraction forces Ad-mags to bind to the cell surface, mimicking Ad receptors-mediated anchoring. Increasing the affinity of Ad particles to cell surfaces or prolonging the time that Ad particles are in contact with cell surfaces facilitates Ad infection. Consequently, the magnetic attraction of Ad-mags reduces doses of Ad vectors and facilitates the introduction of a foreign gene into a restricted region and enhances the efficiency of gene transfer into target cells even if they are Ad-resistant cells.

Streptavidin is a tetrameric protein, and each subunit binds one biotin molecule. The empty binding site of streptavidin of Ad-mag can be linked with another biotin molecule. Thus, the properties of Ad-mag appeared to be modified by linking a variety of biotinylated molecules to Ad-mag through streptavidin as a cross-linker. Cell surfaces are generally repulsive to Ad particles because both cell membranes and Ad particles are negatively charged. To circumvent this obstacle and increase the efficiency of Ad-mag gene transfer, we reversed the negative charge of Ad-mags by conjugating Ad-mags with a biotinylated polycationic polymer, poly L-lysine (B-PLL). The coupling with B-PLL further enhanced the efficiency of gene transfer into HeLaS3 (3.8-fold compared to HeLaS3 cells cultured without magnet; Fig. 1G) and NIH3T3 (12.6-fold compared to NIN3T3 cells cultured without magnet; Fig. 1H) cells. The poly-L-lysine not only enhances the affinity of Ad particles to cell surfaces but also bound to cell-surface heparins (Wickham et al., 1996; Fasbender et al., 1997). This general technique of modifying Ad-mag with a variety of biotinylated moieties has a wide range of potential applications. For instance, using biotinylated antibodies and ligands, we can generate new tropism of Ad-mags.

2.3. Directional in vivo gene transfer with Ad-mag

To develop a directional gene transfer with Ad vector, we applied this magnetic attraction of Ad-mag to *in vivo* gene transfer (Fig. 2). Ad-mags were injected into the midbrain ventricle of mouse embryos at E12.5 ($n = 14$) with glass pipette (p in Fig. 2A) and were attracted subsequently by a magnetic force from the lateral side of the head (Magnet in Fig. 2A; the large white arrows in Fig. 2B and C) over the embryonic membrane (yolk sac and amnion; em in Fig. 2A). As a control, Ad vector alone (AdexCAG-NL-LacZ) was injected into the E12.5 embryos in the same way (Fig. 2B). At E18.5, the manipulated embryos were fixed and stained by whole-mount for β-gal (Fig. 2B and C). Cells infected with the Ad vectors could be recognized as stained cells. In the control brain (Fig. 2B), many stained cells were observed in the neocortex (nc), midbrain (m) and cerebellum (cb). In the cerebellum of control brain, the stained cells formed clusters that were symmetrical around the midline (the small arrows in Fig. 2B). These clusters are formed by cerebellar Purkinje cells born on E12.5 (Hashimoto and Mikoshiba, 2003). In contrast, the stained cells in the experimental brain (Fig. 2C) were located predominantly on the side of the midbrain to which magnetic force was applied (the large white arrow in Fig. 2C). In addition, the cerebellar clusters that were formed by Purkinje cells born on E12.5 could also be observed only on one side of the cerebellar hemisphere (the small arrows in Fig. 2C). We repeatedly obtained the same result on the manipulated embryos ($n = 14$ of 14). To examine the distribution of stained cells in the experimental brain (Fig. 2C), we sectioned the experimental brain transversely on a cryostat (Fig. 2D–F). In the transverse sections, the stained cells were located only on one side of the surface of the third ventricle (Fig. 2E and F). In the dorsal midbrain, many stained cells were observed near the surface of the midbrain (the arrowheads in Fig. 2D); these were neurons infected with Ad-mag that had migrated radially to the surface of the midbrain. The surface of the lateral ventricle, the neocortex, and the hippocampus were not stained for β-gal (Fig. 2D). These results indicate that a magnetic force from the outside of the brain can attract the Ad-mags to one side of the third ventricle and prevent their diffusion to the other ventricles.

To make another type of Ad vector tagged magnetic nanoparticle, we mixed Ad vector with Viro-mag (OZ Biosciences, Marseille, France) that is a superparamagnetic iron oxide nanoparticle coated with polyethylenimine (PEI). Viro-mag is positively charged by PEI. Therefore, Ad particles that carry a negative charge bind with Viro-mag through electrostatic interaction (Scherer et al., 2002; Tresilwised et al., 2010). The complexes comprising Ad vector and Viro-mag are successfully attracted by magnetic force *in vitro* (Scherer et al., 2002) as well as Ad-mags. Accordingly, we injected Ad vector and Viro-mag complexes into the midbrain ventricle of mouse embryo at E12.5 ($n = 8$) and attracted subsequently by a magnetic force from the lateral side of the head (Fig. 2A). However, Ad vector and Viro-mag complexes did not succeed in performing directional *in vivo* gene transfer ($n = 8$ of 8). If the Ad vector and Viro-mag complexes were used, the adenovirally infected cells were observed in the whole brain (data not shown). We think that Ad vector and Viro-mag complexes are unstable in the ventricle of embryonic brain compared with Ad-mags. In fact, Tresilwised et al. (2010) indicate that the affinity between Ad particles and magnetic nanoparticles coated with PEI is reduced in 50% fetal calf serum (FCS) compared with in culture medium without FCS. Therefore, the linkage between Ad vector and Viro-mag seems to be low *in vivo*, and consequently, free Ad vectors seem to be randomly infected to neural stem cells on the surface of ventricle. In contrast, the linkage

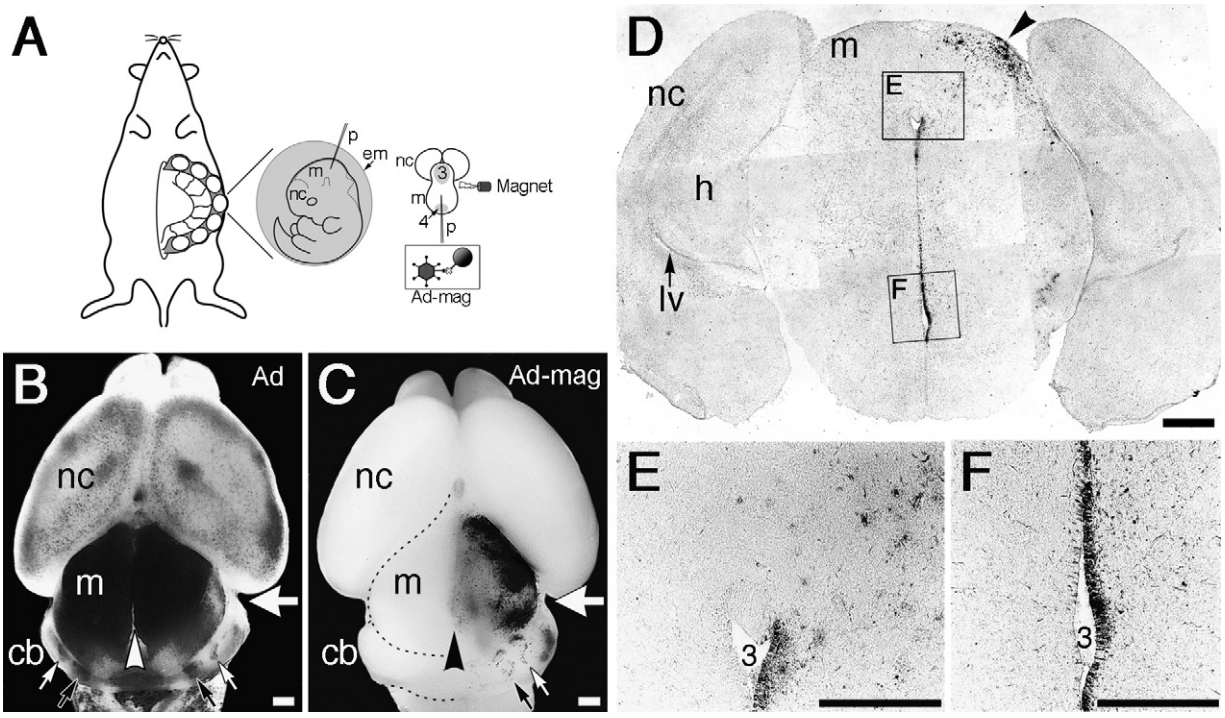

Fig. 2. *In vivo* magnetic attraction of Ad-mag in mouse embryonic brain. The schematic drawing of the adenoviral injection and the magnetic attraction is shown in A. AdexCAG-NL-LacZ (B: as a control) or AdexCAG-NL-LacZ tagged with magnet (C) was injected into the midbrain ventricle of mouse embryos at E12.5 and attracted with a magnet from the outside of the embryo ($n = 14$). The arrowheads and the large arrows in B and C indicate the site of injection and the set point of the magnet over the embryonic membrane (em), respectively. The manipulated embryos on E18.5 were fixed and whole mount stained for β-gal (dorsal view; B and C). The small arrows in B and C indicates clusters of β-gal-positive Purkinje cells in the cerebellum. D–F represents transversal sections from C. β-gal-positive cells were found mainly on the right side of the brain towards the surface of the midbrain (D, arrowhead). E (pineal recess of the third ventricle) and F (third ventricle) are high-magnification views of D. Cells on the right side of the ventricular surface was strongly stained for β-gal compared with the opposite side. 3, third ventricle; 4, fourth ventricle; cb, cerebellum; h, hippocampus; lv, lateral ventricle; m, midbrain; nc, neocortex; scale bar, 0.5 mm; $n = 12$.

between biotinylated Ad and streptabidin-mag is extremely high because the affinity between biotin and streptavidin is one of the strongest non-covalent interactions known in nature (Green, 1975). Consequently, Ad-mags are more suitable for directional *in vivo* gene transfer than Ad vector and Viro-mag complexes.

Our observations indicate that the *in vivo* magnetic attraction of Ad-mags enable us to introduce a foreign gene into a restricted region in the embryonic brain (Fig. 2), and furthermore, the birthdate-specific gene transfer by Ad vector (Hashimoto and Mikoshiba, 2003, 2004) is maintained even if Ad vector are biotinylated and coupled with streptavidin-magnetic nanoparticles (Fig. 2C). In fact, using Ad-mags, we can genetically manipulate Purkinje cells born on E12.5 in the unilateral hemisphere of the cerebellum (small arrows in Fig. 2C). Therefore, the directional *in vivo* gene transfer with Ad-mags is useful for studying the development and functions of neurons that are generated from the restricted region of the ventricular surface and also that share the same birthdate. Furthermore, the directional *in vivo* gene transfer with Ad-mags is suitable for the local examination of gene function, because the hemisphere that is genetically modified by Ad-mags (e.g. the right hemisphere of the cerebellum in Fig. 2C) is able to be compared with another hemisphere (e.g. the left hemisphere of the cerebellum in Fig. 2C) as a negative control on the same brain.

3. Materials and methods

3.1. Preparation of Ad-mag and B-PLL

Ad vector AdexCAG-NL-LacZ expresses a nuclear-targeted β-galactosidase gene under the control of the strong and ubiquitous promoter (Hashimoto et al., 1996). The Ad vectors were purified by double cesium step gradient centrifugation. After centrifugation, the intact Ad vectors were collected and dialyzed overnight in PBS containing 10% (v/v) glycerol at 4 °C. The titers (plaque forming units: PFU) of the viral stocks were determined by plaque assay on HEK293 cells. Twenty microliters of purified AdexCAG-NL-LacZ (1×10^{10} PFU/ml) were reacted with 1 nmol sulfo-NHS-LC-Biotin (Thermo Fisher Scientific K.K., Yokohama, Japan) for 30 min. The mixture was subjected to ultra-filtration with Ultrafree 0.5 (100,000 NMWL: Millipore, Tokyo, Japan). After brief centrifugation for ultrafiltration, 100 μl of PBS containing 10% (v/v) glycerol was added to each of the concentrated Ad vector samples and the ultrafiltration was repeated until the volume was concentrated to 20 μl. This sample was incubated with 40 μl of streptavidin-conjugated magnetic nanoparticles (Miltenyi Biotec GmbH, Gladbach, Germany) for 15 min on ice. Then, a complex comprising the biotinylated Ad vectors and streptavidin-conjugated magnetic nanoparticles (Ad-mag) was prepared. Another type of Ad vectors tagged with magnetic nanoparticles was prepared with mixing Ad vectors (1 μl of Ad solution) with Viro-mag that is a superparamagnetic iron oxide nanoparticle coated with PEI (1 μl of Viro-mag solution; OZ Biosciences, Marseille, France).

To construct a biotinylated poly-L-lysine (B-PLL) molecule, 0.5 mg of poly-L-lysine hydrobromide (MW 30,000–70,000; Sigma–Aldrich, St. Louis, MO, USA) was mixed with 1.25 μmol of biotin hydrazide (Thermo Fisher Scientific K.K.) and 1.25 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Thermo Fisher Scientific K.K.) in 0.1 M MES (pH 5.5) (Sigma Sigma–Aldrich) and shaken overnight. These reagents reacted with the carboxyl groups of poly-L-lysine and covalently biotinylated it. The mixture was subjected to ultrafiltration (10,000 NMWL: Millipore). One PFU of Ad-mag was mixed with about 1000 molecules of B-PLL and incubated on ice for 10 min.

3.2. Western blotting

The biotinylated Ad vector was run on 6% SDS-PAGE. The separated proteins were transferred to a PVDF membrane (Immobiron, Millipore). The membrane was blocked overnight with PBS containing 1% (w/v) skim milk and 0.1% (w/v) Triton X-100 (Sigma–Aldrich) at 4 °C. The biotinylated proteins on the membrane were visualized by an ABC kit (Vector Laboratories, Burlingame, CA, USA) by the nickel-enhanced diaminobenzidine method.

3.3. Cell culture and infection with the Ad-mag

HeLaS3 and NIH3T3 cells were maintained in DMEM (Sigma–Aldrich) supplemented with 10% FBS. Sub-confluent HeLaS3 and NIH3T3 cells placed in 6-cm culture dishes were infected with Ad-mag at an MOI (PFU/cell) of 4. These cells were cultured with or without a small and strong magnet (5 mm in diameter, 80 mT; a schematic view in Fig. 1F). Ad-mag was gently applied 3 cm from the site of the magnet (Fig. 1F). The culture dishes were kept still and incubated at 37 °C. Two days after the injection, the cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 10 min. The fixed cells were stained with Bluo-gal (Invitrogen, Tokyo, Japan) solution (Hashimoto et al., 1996) to detect β-gal activity.

3.4. Assay for β-gal

HeLaS3 and NIH3T3 cells were cultured in 24-well culture dishes. When the cells were sub-confluent, each well was infected with Ad-mag or the complex of Ad-mag and B-PLL at an MOI of 10. Some dishes were put on magnetic sheets, while others were not. One day after the infection, the enzymatic activity of β-gal in each well was measured with ONPG (Sigma–Aldrich) (Sambrook et al., 1989) and calculated with a standard curve derived from purified *E. coli* β-gal (Sigma–Aldrich).

3.5. In vivo injection

One micro-liter of Ad-mag (total 3.3×10^5 PFU) or non-biotinylated AdexCAG-NL-LacZ (total 1×10^6 PFU) was injected into the midbrain ventricle of mouse embryos at embryonic day (E)12.5 ($n = 14$) (Hashimoto and Mikoshiba, 2003, 2004). The complex comprising Ad vectors and Viro-mag (total 5×10^5 PFU) was injected into the midbrain ventricle of mouse embryos at E12.5 ($n = 8$). The neodymium–iron–boron magnet (260 mT, 1.5 mm in diameter; NeoMag K.K., Chiba, Japan) was attached to the lateral side of the embryo's head, over the yolk sac, for 3 min. Subsequently, the embryos were returned to the abdominal cavity of the dam. Six days after the injection (E18.5), the manipulated dams were sacrificed by cervical dislocation, and the surviving embryos were harvested. The embryos were fixed by cardiac perfusion with 4% PFA and stained with Bluo-gal solution. After that, the brains were transversely sectioned (20 μm) on a cryostat (Leica CM1850, Solms, Germany).

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