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RESEARCH PAPER

Magnetic Nanoparticles Enhance Adenovirus Transduction *In Vitro* and *In Vivo*

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ABSTRACT

Purpose Adenoviruses are among the most powerful gene delivery systems. Even if they present low potential for oncogenesis, there is still a need for minimizing widespread delivery to avoid deleterious reactions. In this study, we investigated Magnetofection efficiency to concentrate and guide vectors for an improved targeted delivery.

Method Magnetic nanoparticles formulations were complexed to a replication defective Adenovirus and were used to transduce cells both *in vitro* and *in vivo*. A new integrated magnetic procedure for cell sorting and genetic modification (i-MICST) was also investigated.

Results Magnetic nanoparticles enhanced viral transduction efficiency and protein expression in a dose-dependent manner. They accelerated the transduction kinetics and allowed non-permissive cells infection. Magnetofection greatly improved adenovirus-mediated DNA delivery *in vivo* and provided a magnetic targeting. The i-MICST results established the efficiency of magnetic nanoparticles assisted viral transduction within cell sorting columns.

Conclusion The results showed that the combination of Magnetofection and Adenoviruses represents a promising strategy for gene therapy. Recently, a new integrated method to combine clinically approved magnetic cell isolation devices and genetic modification was developed. In this study, we validated that magnetic cell separation and adenoviral transduction can be accomplished in one reliable integrated and safe system.

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INTRODUCTION

Since their discovery, more than 50 different serotypes of human adenoviruses (Ads) have been isolated. The *Adenoviridae* family further comprises multiple non-human serotypes (1). Historically, Ads are among the first viral gene vector systems to be developed, and are now routinely used for *in vitro* and *in vivo* gene delivery (2). Among their advantages, the up-to-37 kb high cloning capacity and the ability to transduce both quiescent and dividing cells are the two main factors making Ads the vectors of choice for gene delivery applications (3). Furthermore, Ads generally present a low potential for oncogenesis as they do not insert their genome into the host DNA, as opposed to retro- or lentiviral vectors for instance (4). Because of their beneficial features, these viruses are used in one-quarter of all gene therapy trials (http://www.wiley.co.uk/genetherapy/clinical). However, clinical use of Ads in combination with systemic, non localized administration is hampered by adverse reactions, including thrombocytopenia (5), acquired immune response mediated by cytotoxic T lymphocytes against viral and/or transgene products (6) and a potentially life-threatening systemic cytokine syndrome due to activation of innate immunity (7). In addition, the limited ability to confine the vector to its site of action and prevent its spreading to non-target tissues also contributes to compromised therapeutic efficiency. Other limitations include therapeutically suboptimal transduction levels in cell types deficient in Coxsackie-Ads receptor (CAR) (8,9). Indeed, adenoviral transduction efficiency is highly dependent upon the expression level of CAR on the target cell surface (10) : low CAR expression levels result in low concentration of Ads on the cell surface, which decreases its gene expression efficiency. Therefore, even if

Ads vectors hold great promise in the development of effective gene transduction technology, their applications have been limited by the fact that the vectors cannot enter into cells lacking the expression of virus-associated receptors (11). This limitation can be overcome by the use of high vector titers (12), which may in turn increases the risk of adverse dose-dependent effects (13–15). Other disadvantages include a lack of potential for long-term transgene expression, the inherent hepatic tropism of intra-vascularly administrated Ads which leads to hepatotoxicity at higher doses (16) and precludes targeted delivery to alternative organs or disease sites, and induction of a strong cellular and humoral immune response, with potentially toxic inflammatory response.

The needs to improve Adenoviral efficiency while minimizing associated risks are becoming increasingly urgent in gene therapy. Several solutions have been proposed such as the development of targeted Ads vectors (17) or the generation of Ads vectors with novel viral tropism that can overcome the limited infectivity associated with deficiency of viral receptor (18). Nevertheless, genetic engineering of Ads vectors is still difficult to develop routinely and at low cost. One solution to improve safety of adenoviral gene therapy is to reduce the total injected viral dose while maintaining or enhancing their transduction capacities. The Magnetofection method has been developed to overcome biological barriers to efficient gene delivery by using nucleic acid or viral vectors associated with superparamagnetic nanoparticles (MNPs) (19,20). The MNPs/vector complexes formed by electrostatic and hydrophobic interactions are concentrated onto cells/tissues within minutes by the influence of an external magnetic field gradient generated by specific permanent magnets (Fig. 1a). Over the years, association of viral vectors (generally lenti- or retroviruses) and MNPs have been successfully used for (Table I) : 1/enhancing transduction such as in hematopoietic cells (21) or primary T cells (22), 2/lowering the multiplicity of infection (MOI) while improving the efficiency (23), 3/targeting gene delivery (19), 4/synchronizing infection (24,25) or 5/enlarging viral tropism (26). Magnetofection has also been successful with other viruses such as Paramyxovirus, Polyomavirus, Rhabdovirus, Baculovirus, flavivirus, alpha and herpes viruses (26–31).

Application of magnetic nanoparticles for enhancing adenovirus and adeno-associated virus infectivity has been previously reported using adenoviruses conjugated to microbeads by specific ligand-ligand interactions (5,32–34). However such assemblies required lengthy chemical modifications of the viral vectors and/or the micro particles which limit their applicability. On the other hand, non specific assembly of viral vectors and MNPs by electrostatic and hydrophobic interactions is a versatile strategy because it does not require further manipulation of the particles and/or virus. Combined with an external magnetic field, these magnetic adenovirus complexes increased transduction efficiency and transduced cells expressing little or no CAR receptor (19,35–37). Similarly, this approach was also used to enhance the oncolytic potency of replicating adenoviruses (37). A 10-fold enhancement of the oncolytic potency and 4 orders of magnitude in virus progeny formation was reported *in vitro* and *in vivo*. The *in vivo* efficacy of these adenovirus/MNPs complexes has also been demonstrated in other models to target and improve local gene delivery (19,34). In the same way, the feasibility of *ex vivo* gene therapy has been demonstrated with viral gene delivery (38). However, the implementation of cell therapy approaches requires powerful methods for cell separation and genetic modification in an affordable efficient and safe manner that could be amenable to automation in a closed system. Recently, a new technology (Magselectofection) for *ex-vivo* cell separation and transduction in a single standardized procedure has been developed (39). This integrated magnetic cell sorting and transduction (i-MICST™) technological platform combines a clinically approved magnetic cell separation technique with Magnetofection and allows transducing cells with viral vectors in a one-step procedure during cell sorting (Fig. 1b). Using this approach, efficient lentivirus-mediated transduction was reported in T cell lines, mouse and human enriched hematopoietic stem cells and human umbilical cord mesenchymal stem cells (39).

Despite the various successes of the Magnetofection technology with adenoviruses, optimization of the MNPs formulation to improve stability of the magnetic complexes, increase viral capture by the MNPs and achieve higher transduction efficiency is still required. In this study, we explore the use of a novel MNPs formulation (AdenoMag) for magnetically enhanced delivery of Ads. Vectors based on adenovirus serotype 5 (Ad5) have been chosen as gene delivery vehicles due to their well characterized biology, large transgene capacity and efficient gene transfer to both dividing and non-dividing cells (40). The gene transfer efficiency of AdenoMag/Ad5 was studied in cultured cells and *in vivo*. We showed that this approach improves adenovirus infectivity, accelerates the infection kinetics and allows transducing non permissive cells. In addition, we demonstrated that *in vivo* application of this strategy allows a focal targeting of infection by magnetic force into E15 rat brain embryos. Finally, we developed a MNPs formulation (Viro-MICST) optimized for i-MICST™ technology.

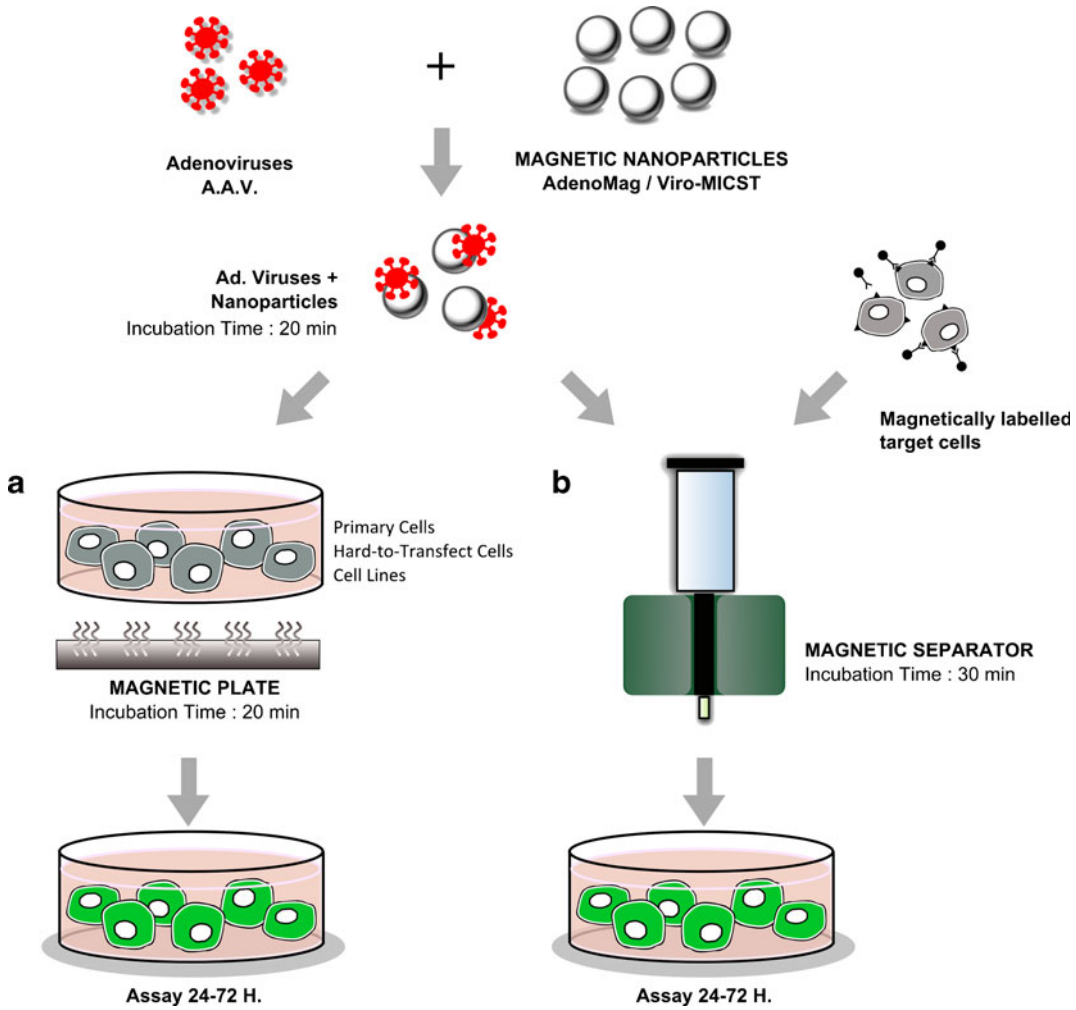

Fig. 1 Magnetic assisted Adeno- or Adeno-associated viruses transfection and Integrated Magnetic Immuno Cell Sorting and Transduction procedure (i-MISCT™). Adeno-associated or Adenoviruses solutions are mixed with magnetic nanoparticles (AdenoMag or Viro-MICST, OZ Biosciences). Complexes are allowed to form during a 20 min incubation time at room temperature. Once formed, the complexes are added onto cell cultures (Primary cells, Hard-to-transfect cell lines, cell lines…) and incubated on a magnetic field for 20–30 min (**a**). The permanent rare earth magnet attracts the magnetic nanoparticles attached to adenoviral particles. After incubation period, a washing step procedure is possible that permit to synchronize infection and/or to lower toxicity. For i-MICST procedure (**b**) complexes are added on a MS magnetic cell separation column from Miltenyi. MicroBeads-labeled cells are loaded and allowed to infiltrate the column. After 30 min incubation onto the Adenovirus/Viro-MICST modified column, cells are flushed and cultivated until evaluation of the transfection.

Table I 10th More Relevant and/or Recent Publications on Magnetofection™/Virus Association – *In Vitro*

Targeted cells	Origin/description	Virus types	References
CD4+ T lymphocytes	Human PBMC	HIV Retrovirus	Sacha *et al.*, 2010 (44)
CD4+ T lymphocytes	Macaque PBMC	SIV Retrovirus	Minang *et al.*, 2010(65)
CD8+ T lymphocytes	Human PBMC	HIV Retrovirus	Greene *et al.*, 2010 (66)
CD8+ T lymphocytes	Macaque PBMC	SIV Retrovirus	Sacha *et al.*, 2010 (67)
U87 cell line	Glioblastoma	HIV Retrovirus	Berro *et al.*, 2009 (68)
U937 cell line	Leukemic monocyte lymphoma	HIV Retrovirus	Payne *et al.*, 2010 (69)
Mesencephalic cells	Cerebellum, spinal cord	Retrovirus	Mizuhara *et al.*, 2010 (70)
Neurons	Ventral mesencephalons	Retrovirus	Nakatami *et al.*, 2010 (71)
16HBE14o cell line	Bronchial epithelium	Lentivirus	Orlando *et al.*, 2010 (23)
Hepatocytes	Liver	Adenovirus	Wang *et al.*, 2009 (72)

MATERIALS AND METHODS

Cells and Virus

Human cervical carcinoma (HeLa), Human Embryonic Kidney (HEK-293T), Mouse fibroblasts (NIH-3T3) and Rat glioma (C6) cell lines were cultured in DMEM (Lonza) supplemented with 10% Foetal Bovine Serum (FBS), 2 mM final L-Glutamine, 100 units/ml penicillin and 100 µ/ml streptomycin (Lonza). Murine macrophage cell line (Raw 264.7) was maintained in RPMI (Lonza) with 10% FBS, 50 IU/ml penicillin G, 50 µg/ml streptomycin, and 2 mM glutamine. Human Microvascular Endothelial Cell line 1 (HMEC-1) was grown in MCDB-131 (Invitrogen) supplemented with 10% FBS, 2 mM final of L-Glutamine and recombinant human epidermal growth factor, 10 ng/ml (Peprotech) and hydrocortisone, 5 ng/ml (Sigma-Aldrich), 100 units/ml penicillin and 100 µ/ml streptomycin. Primary Human Umbilical Vein Endothelial Cells (HUVEC) were kindly given by Dr F. Anfosso (INSERM UMR-608, Marseille) and were cultured in EBM-2 (LONZA) supplemented with Singlequots and 5% FBS. HUVECs were used no later than passage 4. Human Mesenchymal Stem Cells (MSC), kindly provided by Dr. M. Murphy (REMEDI, National University of Ireland, Galway) were grown in complete Alpha-MEM (Lonza) with 1 ng/mL fibroblast growth factor (Peprotech). All cell lines were cultured in 75 cm² flasks at 37°C in a humidified incubator with 5% CO2 atmosphere, and trypsinized at 80% confluency in order to maintain an exponential division rate before transduction assays. Infection experiments were performed in 96-well and 24 well-plate. Neurons from 18 days rat embryos were dissociated using trypsin and plated at a density of 70 000 cells cm−2 in minimal essential medium (MEM) supplemented with 10% NU serum (BD Biosciences), 0.45% glucose, 1 mM sodium pyruvate, 2 mM glutamine, and 10 IU ml−1 penicillin–streptomycin as previously described (41). Neuronal cultures were plated on coverslips placed in 35 mm culture dishes.

Type 5 replication-deficient (ΔE1/E3) Adenovirus encoding Green Fluorescent Protein and β-Galactosidase under the control of a cytomegalovirus (CMV) promoter (Ad-GFP and Ad-LacZ, respectively) were purchased from Vector Biolabs. Aliquots of 1×10^{10} IFU/ml viral stock corresponding to 5×10^{11} VP/ml were diluted extemporary in PBS in order to reach the desired Multiplicity Of Infection (MOI). No medium change was performed after infection. Where indicated, polybrene (Sigma-Aldrich) was added to viral suspension at a final concentration of 8 µg/mL.

AdenoMag/Adenoviruses Complexes Formation and Infection

Infections of the different cell lines and primary cells with Ad-GFP or Ad-LacZ by Magnetofection were performed according to AdenoMag instruction protocol (OZ Biosciences). Briefly 10 µL of viral suspension corresponding to the desired MOI were mixed with different volumes of AdenoMag in 100 µL of DMEM without FBS. Complexes of virus and magnetic beads were allowed to be formed during 20 min at room temperature (RT). After incubation, the magnetic complexes were dispatched in a drop wise manner onto the cells; cells were then incubated for 30 min on the super magnetic plate (OZ Biosciences) at 37°C, 5% CO2. Magnetic plate was then removed and cells were cultured under classic conditions until the end of experiments.

Capture Experiments

For Adenovirus capture experiments, AdenoMag reagent was added to 100 µL of viral suspension corresponding to 10^6 infectious viral particles and complexes were allowed to form during 20 min at RT. Tubes containing magnetized adenovirus were pelleted on a MagID device (OZ Biosciences) during 10 min. Supernatants were saved for controlling capture experiments and pellets were washed two times with 100 µL PBS before magnetic particles re-suspension in 100 µL PBS.

Infection Assays Measurement

Fluorescent Microscopy

Cells were washed, and fixed with 4% paraformaldehyde (PFA). GFP expression was observed under Nikon Eclipse 2000 inverted fluorescence microscope equipped with a digital camera. Images were taken with the same contrast settings for luminosity and contrast. Images presented represent three independent experiments.

Flow Cytometry

Cells were washed two times with PBS and detached with a Trypsin/EDTA 0.2% solution before being fixed with 400 µL of PFA. Cells were then analysed by flow cytometry on a FACS Scan (BD Biosciences). Ad-LacZ infected cells were used as a negative control for the living-gated cell population to determine the % of positively Ad-GFP infected cells.

Fluorescence Intensity

After infection, cells were washed two times and culture medium was replaced with PBS after 15 min fixation. Intensity of infected cells expressing the green fluorescent protein (GFP) was measured in a plate reader (CytoFluor Series 4000, PerSeptive Biosystems) with 485/20 nm excitation and 530/25 nm emission lights. Experiments were performed in triplicate with three determinations per condition.

Expression of β-galactosidase

The expression of β-galactosidase in cells infected by Ad-LacZ was assessed with the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining kit (OZ Biosciences). For quantitative assay, β-galactosidase expression levels were analysis by using CPRG β-galactosidase assay kit (OZ Biosciences) according to the manufacturer protocol.

Washing procedures

30 min after infection of Adenovirus + or - MNPs, cells were maintained on the magnetic plate and the medium was changed by fresh pre-warmed medium after two PBS washes.

In Vivo Experiments

Wistar rats (Janvier) were mated, cared for, and used in INMED's facilities in agreement with European Union and French legislation. Timed pregnant rats [embryonic day 15] were anesthetized with a mixture ketamine/xylazine (at 100 and 10 mg/kg, respectively). The uterine horns were exposed, and the third ventricle of each embryo was injected via pulled glass capillaries and a microinjector (Picospritzer II; General Valve Corporation) with Fast Green (2 mg/ml; Sigma) combined with Ad-GFP, at a concentration of 10^7 infectious particles alone or complexed to AdenoMag MNPs. Briefly, 10 µl of viral particles, 15 µl AdenoMag and 2 µl of fast green were mixed, then 2 to 3 µl of the mixture were injected to each brain. When injected with AdenoMag, a 1 Tesla magnet was applied for 30 s following injection on one side of the embryo cranium. After surgery, the uterine horns were replaced and post-surgical treatment was administrated to the mother. Brains from E17 rats (2 days post surgery) were fixed with PFA 4%. Brain sections (100 µm) were obtained with a vibratome (Leica) and permeabilized for 30 min at room temperature (RT) in PBS–Triton X-100 (0.3%)–goat serum (5%). After permeabilization, slices were incubated overnight at RT with mouse anti-GFP (1:300, A-6654, Life technology). The slices were rinsed three times in PBS and incubated for 2 h at RT in 488-conjugated goat anti-mouse antibodies (1:1000, A-11001, Life technology). Experiments were repeated two times with sections from five different brains. Images were acquired with an Olympus Fluorview-500 confocal microscope (4 ×, 10 ×, 20 ×; 1.0 NA). For this, we focused on neurons visualized with the GFP antibody (Alexa 488 fluorescence). Fluorescent images of GFP were then acquired.

Integrated Magnetic Immuno Cell Sorting and Transduction (iMICST) Procedure

The Integrated magnetic immuno cell sorting and transduction procedure was realized according to Sanchez-Antequera *et al.* (39). 1×10^6 Mesenchymal Stem Cells were suspended in 100 µL complete medium and magnetically labelled for 20 min at 4°C with 20 µL anti-CD105 MicroBeads (Miltenyi) according to the manufacturer protocol. In the meantime, adenovirus used at a MOI of 1 was mixed with different volumes of Viro-MICST MNPs (OZ Biosciences). When needed (according to the 60 µL void volume of the MS column), the Ad-GFP/Viro-MICST mixture was concentrated with the MagID device (OZ Biosciences); the supernatants were withdrawn after sedimentation and complexes were re-suspended in 60 µL of complete medium. MS columns (Miltenyi) were then loaded with the magnetized adenoviruses and positioned within the MACS separator magnet (Miltenyi). MicroBead-labelled cells, suspended in 500 µL of complete medium were then loaded on the modified column and allowed to infiltrate. After complete diffusion of the cells, the columns were washed with 2 × 1 mL of cell culture medium and incubated onto the MACS for 30 min at RT to allow the magnetically-driven infection. Subsequently, columns were removed from MACS magnet and cells were flushed from column according to the manufacturer protocol before being cultivated in standard culture conditions until evaluation of magnetically-assisted infection. Reporter gene expression analysis was performed 48 h after transduction with adenoviral vector.

RESULTS

Magnetofection Increases Infection in Cells

To analyse whether magnetic nanoparticles can enhance adenoviral transduction we used a type 5 Adenovirus carrying a CMV promoter-driven eGFP expression cassette. We determined the percentage of GFP positive cells in different

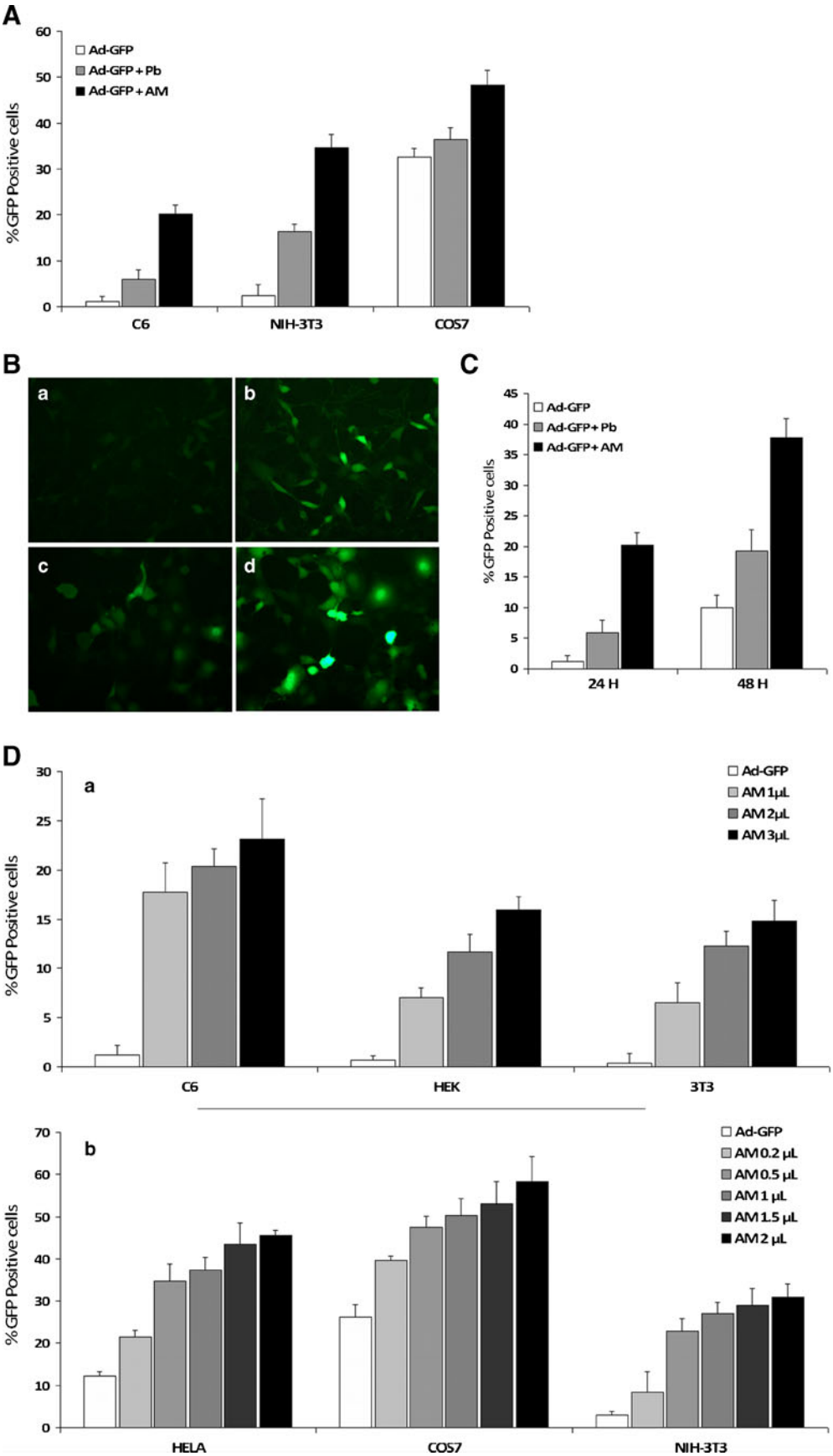

Fig. 2 Infection increased by AdenoMag in several cell lines. Magnetofection induces an augmentation in% GFP + cells. (**A**) C6, NIH-3T3 and COS7 cells were infected with Ad-GFP alone (Ad-GFP), Ad-GFP with 8 μg/mL Polybrene (Ad-GFP + Pb), or with complexes of Ad-GFP and 2 μL of AdenoMag (Ad-GFP + AM) at a MOI of 1 under a magnetic field. The percentage of infected cells was determined 24H after infection by flow cytometry. (**B**) Representative images of NIH-3T3 (**a**, **b**) and COS7 (**c**, **d**) cells infected with Ad-GFP alone (**a**, **c**) or Ad-GFP + 2 μL AdenoMag (**b**, **d**). Photos were taken under fluorescence microscope 24H after infection. (**C**) For C6 cells,% GFP + cells after infection with Pb or AM was measured after 24 and 48 H of infection. (**D**) C6, HEK-293 and NIH-3T3 (**a**) were infected with complexes of Ad-GFP and 0 to 3 μL of AdenoMag at a MOI=0.5. HeLa, COS7 and NIH-3T3 (**b**) were infected with complexes of Ad-GFP and 0 to 2 μL of AdenoMag at a MOI=1 for 24 H.

cell lines with an MOI of 1 (Fig. 2). It is now well established that incubation of cells with Ads in the presence of polycations increases the efficiency of infection and gene transfer (42). Therefore, adenoviral particles were incubated with polybrene (8 μg/μL) as a positive control of infection enhancement. All cell lines tested (C6, NIH-3T3, COS7) showed an increase in infection when 2 μL AdenoMag was complexed with adenoviral particles compared to adenovirus alone and to adenovirus mixed with polybrene (Fig. 2A). Moreover, MNPs were able to raise transfection efficiency in cells lacking CAR receptor as well as permissive cells (Fig. 2B). 24 h post-infection, the two non-permissive cell lines (C6-Glioblastoma and NIH-3T3) showed very low infection level even in presence of polybrene, whereas addition of AdenoMag allowed enhancing infection efficiency more than 10 times. On permissive cells (COS7), polybrene slightly improved infection compared to virus alone. However, Magnetofection increased significantly the number of infected cells by at least 15% at very low MOI. Noteworthy, infection enhancement was still measurable 48 h after infection: about 40% of C6 cells were infected with complexes of Ad5/AdenoMag at MOI of 0.5, as compared to only 10% infection with Ad5 alone and 20% with Ad5/polybrene (Fig. 2C). To further analyse the efficiency of MNP-assisted transduction, several doses of nanoparticles were tested (Fig. 2D). As a result, the number of infected cells increased proportionally to the volume of magnetic nanoparticles added, even at MOI as low as 0.5. This experiment showed the ability of Magnetofection to dramatically enhance adenoviral infection at low MOI when compared to virus alone or polybrene adjunction. Moreover magnetofection technology allowed lowering the MOI used while keeping a high percentage of infected cells.

Magnetofection Increases Level of Viral Transgene Expression

Even if determining the percentage of transduced cells is a remarkable indicator of vector efficiency, linear correlation with protein expression is not straightforward. Depending on the application pursued, not only a high percentage of positive transduced cells is required but also within each infected cell, a high amount of produced protein might be needed. In order to assess the ability of AdenoMag to enhance both percentage of infected cells and viral transgene expression, two replication-defective adenovirus type 5, Ad-GFP and Ad-LacZ, were mixed with MNPs for evaluating GFP and β-galactosidase (β-Gal) expression in several

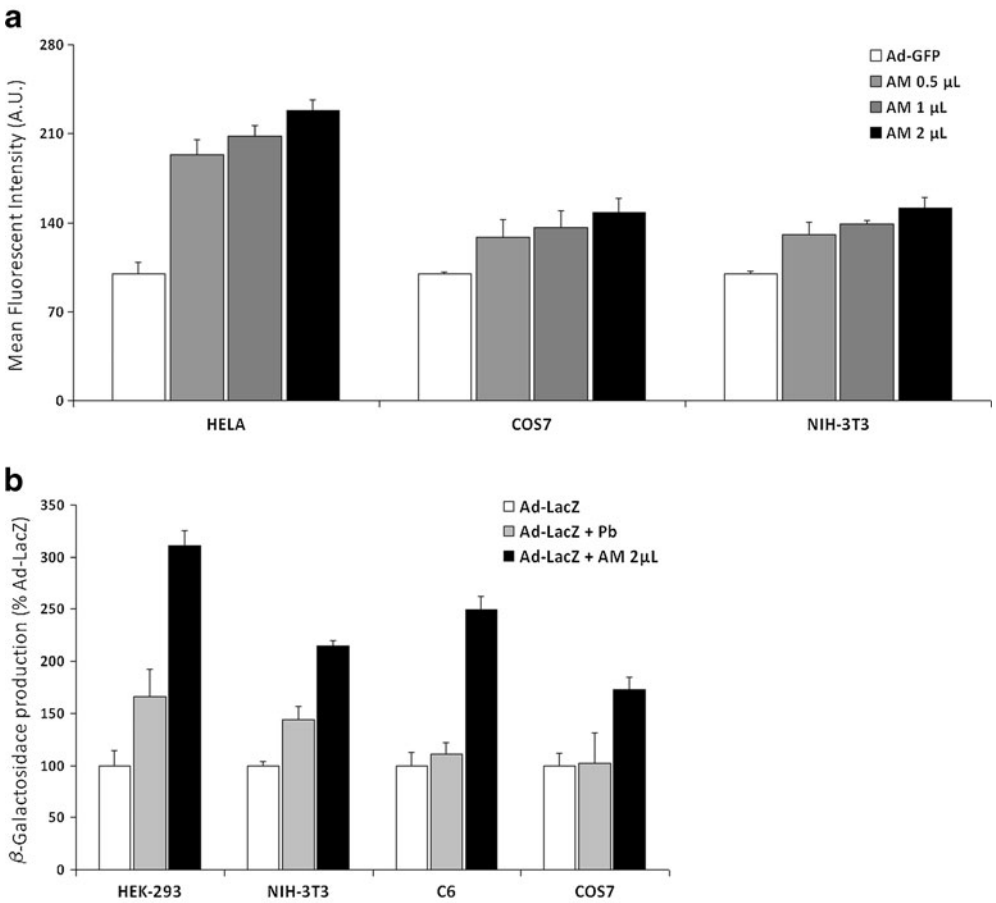

Fig. 3 Viral transgene expression increase with AdenoMag. Magnetofection induces a raise in transgene expression. (**a**) Cell lines were infected with Ad-GFP mixed with 0 to 2 μL AdenoMag under magnetic field. Fluorescence intensity was determined 24 H after infection by cytofluorimetry and expressed as% of Ad-GFP GFP protein expression. (**b**) Beta-Galactosidase expression was assessed by CPRG analysis after 24H of infection with Ad-LacZ, Ad-LacZ with Polybrene, 8 μg/μL (Ad-LacZ + Pb) or complexes of Ad-LacZ and 2 μL of AdenoMag (Ad-LacZ + AM). Results presented here are expressed as a percentage of AdLacZ beta-galactosidase expression.

cell lines. Various amounts of AdenoMag (0 to 2 µL) were combined to Ad-GFP at a MOI of 1 and added to permissive and non-permissive cells (Fig. 3). First, using HeLa, COS and the non-permissive NIH-3T3 cell line, a dose-dependent increase in GFP expression was observed as before that correlates with the increase of the percentage of infected cells (Fig. 3a). Then, to assess the effect of Magnetofection on protein production, we used Ad-LacZ adenovirus to quantitatively measure the amount of transgene produced with CPRG β-galactosidase assay kit (Fig. 3b). Results were normalized to Ad-LacZ with no additive (100% β-Gal production). We showed that 1) even if polybrene achieved a slight amplification in number of GFP positive cells, it did not succeed in producing higher amount of protein; 2) AdenoMag significantly enhanced β-Gal production in permissive and non-permissive cells up to 300%. Intracellular β-Gal protein production was also monitored histochemically by microscopy using X-Gal staining kit in NIH-3T3 and HeLa cells. With virus alone, low level of cell staining was obtained, indicating that the protein expression is not sufficient for a correct detection whereas with magnetized adenovirus high level of staining was achieved indicating a large protein production (data not shown). The results confirmed the ability of magnetic nanoparticles to enhance protein production in a dose-dependent manner in both permissive and non-permissive cells. Altogether these results linked the capacity of magnetic nanoparticles to increase both the number of infected cells and transgene expression.

Virus Capture and Magnetic Field Effect on Adenovirus Infection Efficiency

Recombinant adenoviruses used in vaccines and gene therapy require complex and time consuming processes in order to maintain viral infectivity, high titers while also permitting to concentrate the viral sample for delivery (43). Since AdenoMag MNPs are capable of improving adenoviral

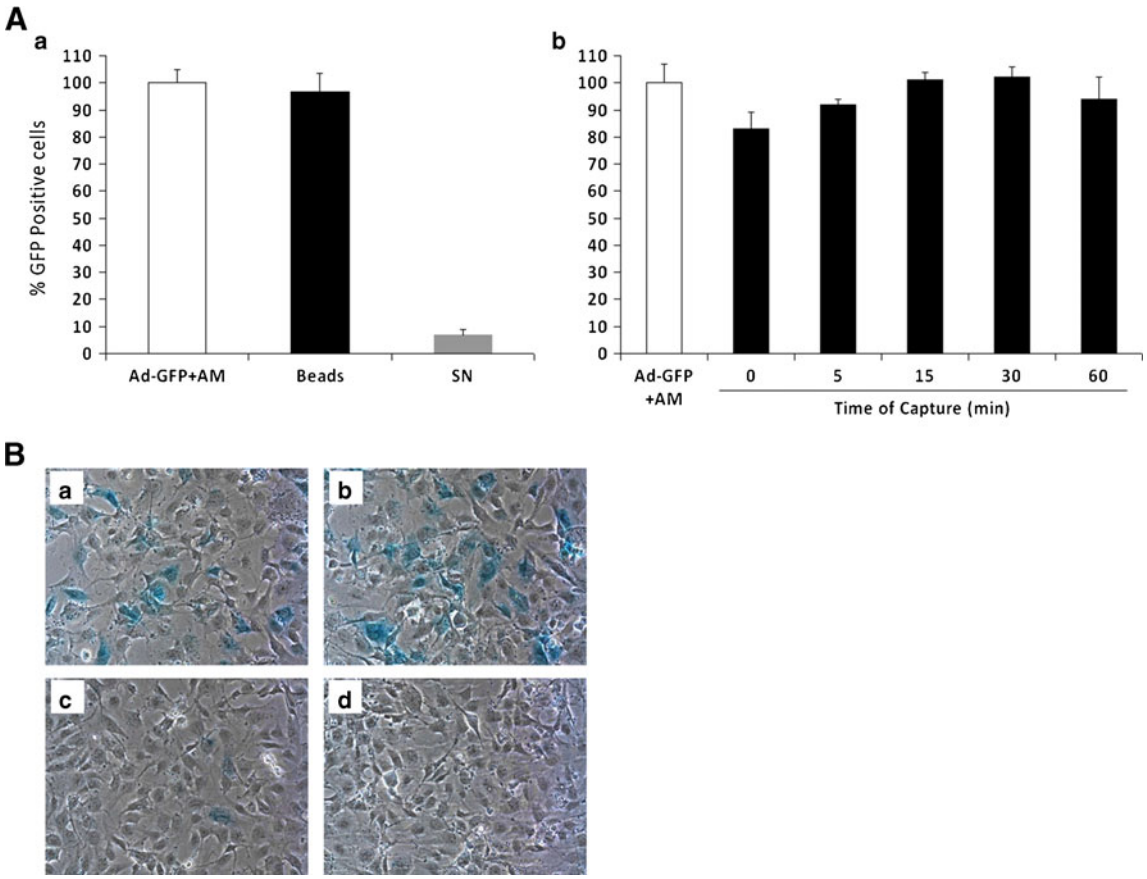

Fig. 4 Adenoviral capture efficiency with Magnetofection. Magnetic nanoparticles allows to efficiently capture adenoviral particles in supernatants. (**A**) Adenoviral particles were captured with 20 µL AdenoMag reagent for 20 min (**a**) or for different times, from 0 to 60 min (*b*). Supernatants (SN) were saved and pellets were suspended in PBS (Beads) before infection. Cos-7 (**a**) and HeLa (**b**) cell lines were infected either with Ad-GFP + 2 µL AM as infection control, with beads or SN. The percentage of infected cells was determined 24H after infection by flow cytometry. (**B**) 10 µL (**a**, **c**) and 20 µL (**b**, **d**) of AdenoMag were used to capture Ad-LacZ adenovirus for 20 min. After capture, Beads were suspended in PBS and tested for infectious capacity (**a**, **b**) in parallel of saved supernatants (**c**, **d**) on HMEC-1 cell line. 24H post infection, cells were fixed, stained with X-Gal kit (OZ Biosciences) and observed under light microscope.

infectious properties, we determined their capacity to capture adenovirus as a rapid and efficient way of concentration and purification. Pellets of magnetically-captured adenovirus were tested against supernatants for infection in COS-7 cell line (Fig. 4A). Results were normalized to magnetofection-assisted Ad-GFP infection (100% infection) and showed that pellets of captured adenovirus induced more than 96% infection where as supernatants only induced ~5% infection (Fig. 4Aa). Moreover, results on HeLa cells showed that most of the viral particles are captured as soon as the reagent is added to the viral suspension and that maximum of binding efficiency occurs between 15 and 30 min (Fig. 4Ab). Dose response in capture efficiency was also investigated on HMEC-1 cells (Fig. 4B). Pellets of magnetically-captured viruses induced an increase in protein production proportionally to the amount of AdenoMag added whereas infectivity of the supernatants showed a decreasing trend. We next assessed the importance of the magnetic field on the infection enhancement mediated by AdenoMag. To this end, cells were inoculated with Ad-GFP and AdenoMag and in presence or not of magnetic field for 30 min (Fig. 5). In the CAR lacking NIH-3T3 cells, the absence of magnetic field, induced less than 10% of GFP + cells whereas in the presence of the magnetic field up to 35% of cells were infected. Even on permissive cells, application of magnetic field enhanced the infected cells number (Fig. 5a). These results showed the stringent requirement of a magnetic field for Magnetofection action on adenovirus infection especially for CAR-negative cells. Finally, the effect of medium change on infection efficiency was also investigated. Cells were infected with Ad5 alone or Ad5/AdenoMag for 30 min, and a medium change was then performed while keeping the cells in the presence of the magnetic field (Fig. 5b). As expected, this washing step dramatically reduced the efficiency of Ad-GFP alone since unbound adenoviruses are removed during the washing procedure. In contrast, the magnetic field underneath the culture plate permits to keep the entire magnetic viral clusters onto the cell surface and maintains high level of infectivity mediated by AdenoMag when the medium change is performed. Altogether, these results demonstrated that 20 µL AdenoMag were sufficient to efficiently capture 10^6 infectious viral particles within minutes, demonstrating that self-assembling of magnetic vectors by electrostatic and hydrophobic interactions is a powerful way of binding viruses to magnetic beads. Moreover, the presence of magnetic field specifically designed to induce a correct attractive gradient is necessary for a proper magnetofection technique leading to a rapid accumulation of viral cluster at the cell surface.

Magnetic Nanoparticles Accelerate Infection Kinetics

Kinetic studies are of high importance when studying the time course of infection in immunology and vaccine-related studies fields (44). Thus, we studied the accelerating effect of magnetofection on infection of various cells using Ad-GFP (Fig. 6). C6, COS-7, NIH-3T3 and HEK-293T cells (Fig. 6Aa–d) where infected with Ad-GFP or Ad-GFP + AdenoMag. The percentage of GFP positive cells from 2 to 14 h post infection was analyzed. A rapid increase in GFP positive cells compared to control (Ad-GFP alone) was detected as soon as 6 h post infection. Moreover, total fluorescence intensity was monitored by flow cytometry (Fig. 6Ba–b) and visualized at the end of the kinetics under fluorescence microscope (data not shown) and confirmed the shortening of magnetically-transduced cells detection. Altogether, the results showed that protein expression was enhanced and thus earlier detected. This effect could result from acceleration in the infection process or from a higher level of gene dosage reaching the nucleus due to increased likelihood of encountering and entry of adenoviral particles by magnetic nanoparticles.

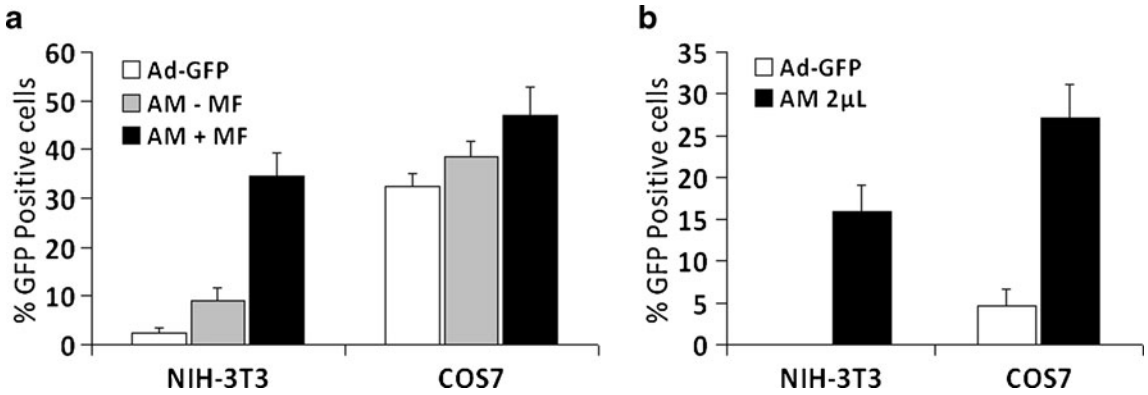

Fig. 5 Magnetic Field (MF) and medium change effect on AdenoMag infectivity. (**a**) NIH-3T3 and COS7 cell lines were infected at a MOI of 1 with Ad-GFP alone (Ad-GFP), with Ad-GFP mixed with AdenoMag in absence of magnetic field (AM – MF) or with Ad-GFP mixed with AdenoMag in presence of a 30 min incubation on a magnetic plate device (AM + MF). The percentage of GFP + cells was determined after 24H infection by flow cytometry. (**b**) For medium change, cells were infected with Ad-GFP or Ad-GFP mixed with AdenoMag at a MOI of 0.5. After 30 min infection, a medium change was performed while keeping the cells on the magnetic plate device. The percentage of GFP + cells was determined by flow cytometry 24H after infection.

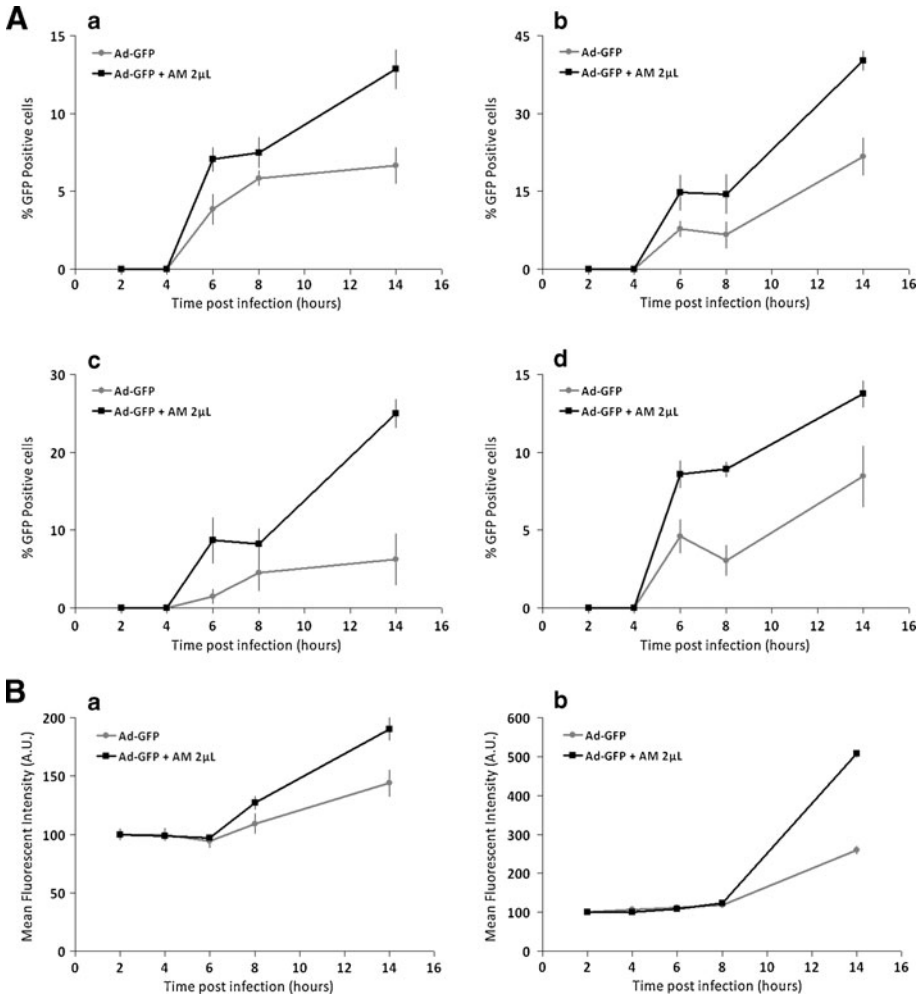

Fig. 6 Infection kinetics with AdenoMag. Magnetofection accelerates the infection process in terms of infectivity. (**A**) C6, COS-7, NIH-3T3 and HEK-293T cell lines (respectively **a**, **b**, **c** and **d**) were infected with Ad-GFP or Ad-GFP mixed with 2 μL of AdenoMag at a MOI of 1. 2H, 4H, 6H, 8H and 14H after infection, cells were harvested and% GFP + cells was measured by flow cytometry. (**B**) Representative kinetics of transgene expression in C6 (**a**) and COS-7 (**b**) cell lines during the same time course of infection with and without AM (2 μL). Results are given as a% of GFP expression at 2H.

Infection of Hard-To-Infect Cell Lines and Endothelial Primary Cells with AdenoMag

In order to evaluate the ability of Magnetofection to enhance infection on hard-to-infect cells at low MOI, HMEC-1, Raw 264.7 and CHO cells were inoculated with several MOI of Ad-GFP and varying quantities of MNPs (Fig. 7). Infection was enhanced in all cell types, particularly in HMEC-1 where the increase reached up to 6-fold for MOI of 0.5 or 1 (Figs. 7Aa and 8Ab). Human primary endothelial cells are also very hard-to-transduce, because of their intrinsic physiology and low level of CAR receptor expression (45). Even under these conditions, we confirmed the positive effect of the magnetofection on Ad-GFP adenovirus infection on these primary cells (Fig. 7B). These results confirmed the previous observations; the percentage of infected cells increased in a dose-dependent manner with AdenoMag (Fig. 7B) regardless of the MOI used (Fig. 7C). Altogether these results showed that Ad magnetically guided onto non permissive cells or low-CAR expressing cells induced high percentage of transduced cells through Ad independent pathways.

Magnetofection Induces Adenoviral Infection in Neurons *In Vitro* and *In Vivo*

Even if gene therapy vectors based on herpes simplex virus, adenovirus, lentivirus and adeno-associated virus have been developed for neurosciences applications there is still a gap between bench and clinical trials (46). In order to move forward, we focused on the potential of Magnetofection to improve adenoviral mediated gene delivery within the nervous system (Fig. 8). *In vitro* data first confirmed that AdenoMag complexed to Ad-GFP increased the infection potential of Ad in a dose-dependent manner (Fig. 8A). In contrast, Ad alone failed to transduce a large number of hippocampal neurons. As noted previously, addition of AdenoMag not only increased the number of infected cells but also induced a larger amount of transgene expression. These data clearly highlighted the synergistic effect of

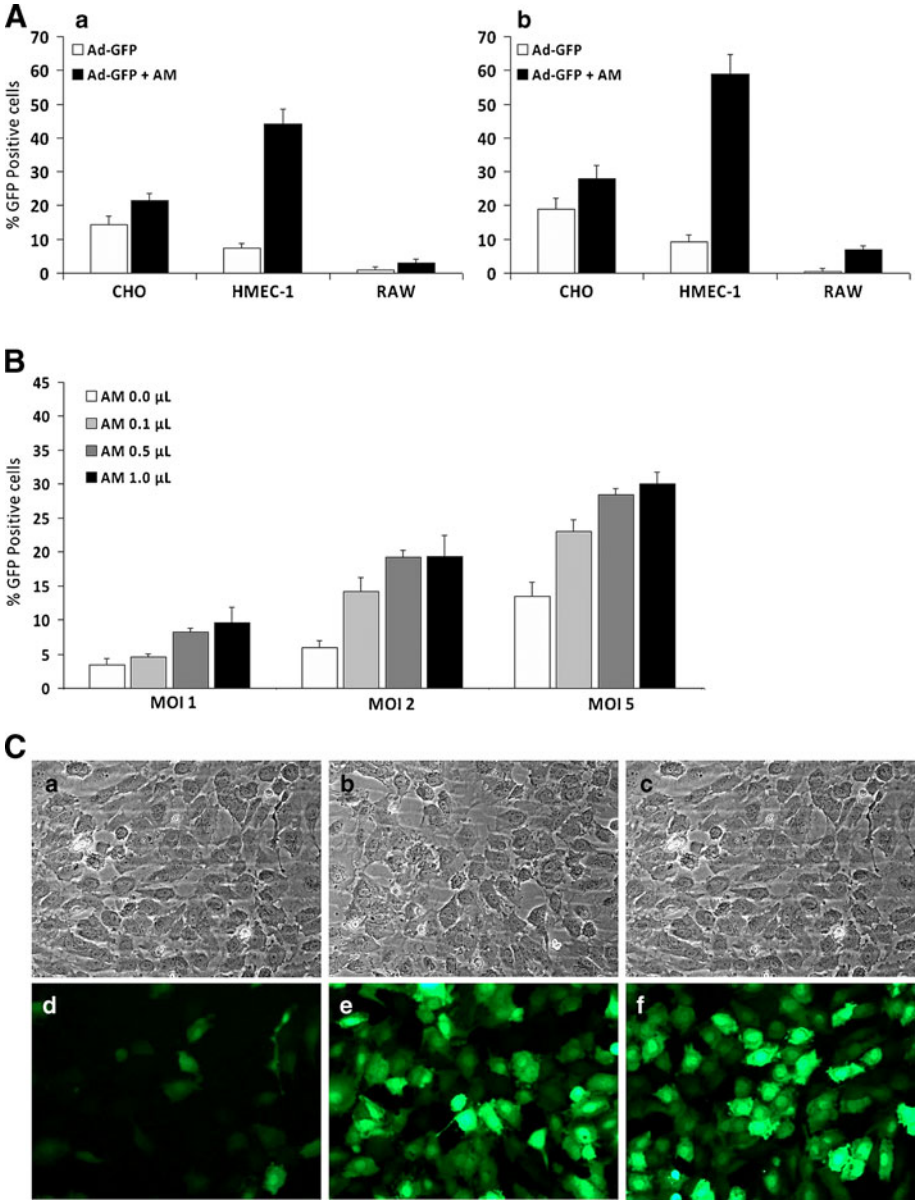

Fig. 7 Infection of adenovirus-non-permissive cells. (**A**) Cell lines CHO, HMEC-1 and RAW cells were infected with a MOI of 0.5 (**a**) and 1 (**b**) with Ad-GFP alone or Ad-GFP mixed with 2 μL AdenoMag. 24 H post infection, % of infected cells was measured by flow cytometry. (**B**) Primary human umbilical endothelial cells (HUVEC) were infected with Ad-GFP at MOI of 1, 2, and 5 in presence of 0 to 1 μL of AdenoMag.% Infected HUVEC was determined by flow cytometry 48 H after infection. (**C**) Representative images of HUVEC infected with MOI of 5 in presence of 0 (**d**), 0.5 (**e**) and 1 μL (**f**) of AdenoMag. Photos were taken under white field (**a**, **b** and **c**) and fluorescence (**d**, **e** and **f**) after 48 H infection.

AdenoMag to improve Ad efficiency while using low viral doses which is critical for gene therapy. This was further demonstrated by *in vivo* application in brain of rat embryo. Ad-GFP complexed or not to MNPs were injected into E15 rat-embryos third ventricle and magnetically-guided to one side of the brain (Fig. 8B). Results demonstrated that magnetic adenoviral vectors are compatible with *in utero* application and can infect cells efficiently. Indeed, with the control conditions, (2 to 3 µL of 10^7 active viral particles/µL per brain), the infected cells were only found near the injection site all around the third ventricle. When Ad particles were combined with AdenoMag and injected into the same ventricle under the influence of a magnetic field, infection was not only improved but also guided to a confined area corresponding to the magnet location. This magnetic targeting opens new avenues to *in vivo* brain gene therapy applications.

Integrated Magnetic Immuno Cell Sorting and Transduction (i-MICST) Procedure Using Adenoviral Vector

Magselectofection has been recently described by Sanchez-Antequera *et al.* as an integrated method of magnetic separation and genetic modification of target cells. It has been used with a very high efficiency to infect magnetically immuno-purified cells with magnetic vectors (39). However, most of the work has been performed with lentiviral and non-viral vectors. In this study we have specifically developed new MNPs formulation adapted to cell sorting column

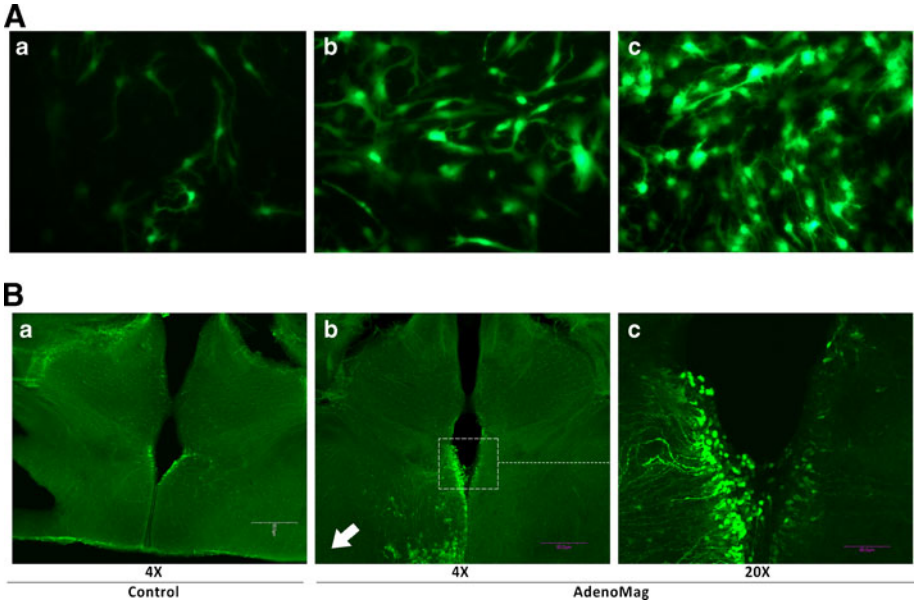

Fig. 8 Magnetofection induced adenoviral gene modification in neurons *in vitro* and *in vivo*. (**A**) Rat hippocampal neurons (DIV 7) were infected with a GFP adenovirus at a MOI of 1 (**a**) complexed with 1 μL (**b**) or 5 μL (**c**) AdenoMag reagent. 24 H% of infected cells was monitored under fluorescence microscopy. (**B**) Brains harvested two days after surgery, 100 μm coronal sections were made and immunostained with a GFP antibody and finally revealed with 488-Alexa conjugated secondary antibody. (**a**) Picture of the third ventricle of a E17 rat embryos, the adenovirus-injected cells are located on both side of the ventricle; cells are mainly restricted to this region indicating that the injection took place in this part of the brain. There are no cells among the other brain regions. (**b**) Combination of adenoviral particles with AdenoMag, in this situation, the infected cells are located on one side of the ventricle due to the 30 s magnet-application. The white arrow indicates the direction of the applied magnetic field. At higher magnification, the region defined by the doted square (**c**) shows significantly increased quantities of cells on the magnet-exposed side compared to the non-exposed side.

and adenoviral capture. This novel MNPs formulation, called Viro-MICST, was used to transduce cells during magnetic cell separation in one closed and integrated system (Fig. 1b). The complexes of Ad-GFP and Viro-MICST MNPs were formed at RT for 20 min before loading onto an MS column (Miltenyi). Ad-GFP was used at a MOI of 1 with various amounts of MNPs. Thereafter, MSC cells were purified on the modified MS column with CD105 Microbeads. The results showed that the purified MSC cells were infected in a cell number dependent manner (Fig. 9a). We have compared magnetic nanoparticles from Sanchez-Antequera with Viro-MICST particles used in this paper and the results were similar or better especially in terms of magnetic nanoparticles stability (data not shown). With a MOI of 1, both percentage of infected cells and transgene expression level increased proportionally to the number of cells purified on the column (Fig. 9a). The efficiency of transduction on this system is also dependent on the quantity of MNPs used (Fig. 9b). Moreover results obtained with optimal cell number (up to 5×10^6 cells can be sorted on MS column according to Miltenyi) presented the same percentages of infected cells (data not shown). These results confirmed that this magnetically assisted gene delivery can be combined with magnetic cell sorting in one safe system that is clinically approved.

DISCUSSION

The Magnetofection technology comprises the association of nucleic acid vectors with superparamagnetic iron oxide nanoparticles under the application of a magnetic field (47). Numerous examples have shown that Magnetofection was successful in enhancing plasmid DNA delivery, viral infection and siRNA mediated gene silencing (36). Based on this technology, we developed a novel dedicated MNPs formulation for adenoviruses applications. AdenoMag, bearing proprietary cationic biodegradable components confers to the MNPs the ability to bind adenoviruses and to enhance their infectious properties. The principle of Magnetofection for enhancing adenoviral gene transfer is simple and comprises easy steps as depicted in Fig. 1: Magnetic nanoparticles are first mixed with the adenovirus of choice; once formed, complexes of adenovirus and magnetic nanoparticles are added to the cell culture and attracted by an external magnetic field. In this paper, we reported that magnetic nanoparticles/adenoviruses complexes bypassed the CAR receptor uptake pathway allowing non permissive cells infection. The natural infectivity of the virus is governed by the presence or absence of receptors (such as the CAR) to which the virus needs to bind. In the absence of receptors, the virus will not infect, unless it is modified with

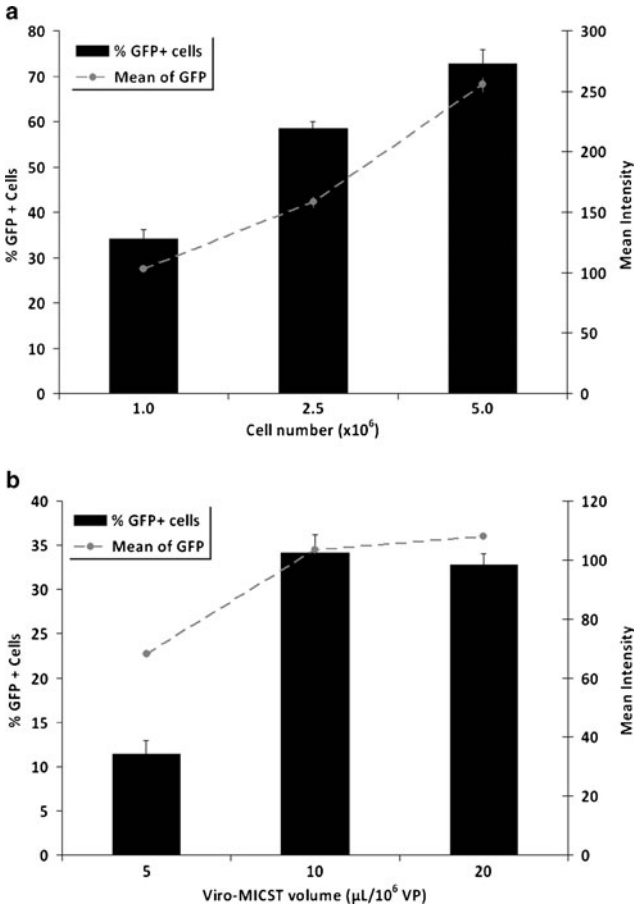

Fig. 9 Viro-MICST infection efficiency on Mesenchymal Stem Cells retained on Magnetic Column. (**a**) Several densities of MSC were labelled with CD105 MicroBeads and infected on magnetic separation column with MOI 1 of Ad-GFP mixed with 10 μL Viro-MICST. (**b**) 1×10^6 MSC were then infected with a MOI 1 of Ad-GFP mixed with several volumes of Viro-MICST. 96H post infection,% of infected cells (black bar-left scale) and mean intensity (dot line-right scale) were measured by flow cytometry.

additional binding entity. Magnetic nanoparticles provide this function in a simple manner and Magnetofection can enforce infection even in the absence of virus receptors. As a result, MNPs allow improving cells transduction in terms of the overall number of infected cells and the transgene expression level per cell. Thus, magnetic targeted delivery of adenoviral vectors can greatly enhance their therapeutic potential as lower dose (MOI) is required, which in turn permit higher specificity and reduction of deleterious off-target side effect. Moreover, acceleration of the kinetic course of infection could be useful in avoiding adenovirus inactivation and bypassing immune response.

Adenoviruses and to a lesser extent Adeno-associated viruses (AAV) remain the most commonly used and studied gene therapy vectors (48). These vectors however still encounter often hazardous issues which have so far hampered the development of successful gene therapy strategies. For example : 1/Transient expression and sub-optimal delivery, 2/vector dissemination causing strong immune response (5–7), 3/impossibility for cells lacking CAR receptors to be genetically modified (11), 4/dose dependent effects due to high vector titers (12). Clinical trials aiming at ischemia (49,50), cystic fibrosis, haemophilia or heart disease (51,52) have been conducted but limitations were met due to the vector physiology. Furthermore physiological barriers for *in vivo* delivery of adenovirus have been described. Indeed, erythrocytes can alter adenovirus blood circulation profile and reduce their extravasation and infectivity (53). Adenoviruses were also shown to interact with the coagulation factor X and the complement binding protein-4 that induced liver addressing (54,55), subsequent Kupffer cells uptake and virus inactivation (56). In this context, PEG shielding of adenovirus has been used to bypass liver scavenging (57) but this can alter transduction efficiency (58). In this way, Magnetofection allows minimizing the interaction of adenovirus with blood components and cells because of the nanoparticles shielding. In that way, vectors are less accessible to competing binding factors. Moreover, a rapid guidance by external magnetic field would prevent from cell scavenging and allow to direct virus to the site where vectors are needed.

Ideally the goal of cancer therapy and any other gene therapy would be to selectively target and destroy tissue or repair gene without spreading to the surrounding healthy tissues. There is thus a critical need for guiding and confining the genetic vector in the vicinity of the tumour or on the site where genes need to be repair. What is easily possible in traditional cell culture becomes a real challenge when dealing with entire tissue or organism. Directing molecules to the site where they are needed remains difficult without invasive methods and immune reactions due to systemic spreading are still an issue to deal with. Throughout the past decade, several experiments using plasmids

Table II *In Vivo* and *Ex Vivo* Viral Transfection Mediated by Magnetofection

Model	Species	Origin/description	Virus types	References
In vivo	Mouse	Brain	Adenovirus	Hashimoto *et al.*, 2011 (34)
	Mouse	Stomach	Adenovirus	Scherer *et al.*, 2002 (19)
	Rat	Aorta	AAV	Burdorf *et al.*, 2007 (73)
Ex vivo	Mice	Aorta	Lentivirus	Hofmann *et al.*, 2009 (74)
	Mouse	Liver hepatocytes	Lentivirus	Wang *et al.*, 2009 (72)

or viruses have shown that Magnetofection offers a number of key advantages for the *ex vivo* and *in vivo* gene targeting. First, high cellular uptake is reached within minutes. In addition, targeted and confined gene expression is made possible by magnetic focusing of MNPs at the desired site of action in a non invasive manner. In a pioneer study, Jahnke *et al.* explored the feasibility of Magnetofection-assisted gene therapy in cats with fibrosarcoma (59). Since then, some studies have pinpointed the utility and the versatility of magnetically assisted viral vector for *ex vivo* and *in vivo* delivery (Table II). Some works report the guidance of aerosols to specific regions of the lung using an external magnetic field. This aerosol approach comprised magnetically responsive nanoparticles and DNA. "Nanomagnetosols" were generated with nebulizers and used magnetism to direct magnetizable aerosol droplets specifically to desired regions of the lung (60). This approach overcomes the natural deposition mechanism of inhaled aerosol droplets in the lungs that only allows targeting on the central airways or lung periphery but not local regions in the lung. A two-fold higher dose of plasmid DNA was found in the magnetized right lung than in the unmagnetized left lung, thus opening a way to gene therapy by magnetofection. Delivery of genes to the brain and spinal cord across the blood–brain barrier (BBB) has not yet been totally achieved (61). Commonly, *in utero* electroporation is applied on embryonic rats for transfection after DNA injection in the lateral ventricle of embryos (62,63). Also, another report showed that the AAV-9 virus injected intravenously could bypass the BBB and target cells of the central nervous system (64). In order to enhance transduction and bypass current barriers, magnetic nanoparticles associated with lentivirus have been used to concentrate and target viral transduction in the brain. Preliminary results showed a significant enhancement of gene expression compared with standard infection and most importantly a magnetic targeting. Indeed, a GFP-lentivirus injected in the facial vein of rat embryos can be targeted in some of the cortex zone, depending on the magnet location on the skull surface (38). In a recent study, Hashimoto *et al.* have tagged an adenoviral vector with magnetic nanoparticles via biotin-streptavidin interaction and magnet-mediated directional gene-transfer into the brain was reported. (34). However, this study requires chemical modifications of the adenovirus and the magnetic nanoparticles. AdenoMag nanoparticles can stably bind viruses by electrostatic and hydrophobic interactions and thus, avoiding the needs of further particles and/or virus modifications. In this paper, we demonstrated that assembled "magnetic adenovirus" can be targeted and concentrated *in vivo* under the influence of a magnetic field. Moreover, transduction efficiency was also improved. This approach combines the efficiency of a virus with fine magnetic targeting and represents a promising gene therapy approach.

A very recent promising gene therapy approach, Magselectofection, has been defined as a versatile integrated procedure for cell sorting and genetic modification (39). Previous work used essentially non-viral or lentiviral based magnetofection for gene delivery integrated to cell sorting column and settled the basement for novel efficient way of genetically manipulate the cells. We thus associated this promising procedure with adenoviral capacity for gene delivery and showed that combining i-MICST with adenoviral potential holds great promise for genetic modification. Moreover i-MICST, taking advantages of the well-established MACS® system depicted as compatible with clinical experiments, did not impair the performance of cell sorting and recovery nor the function, viability, differentiation and transplantation potential of the cells. Toxicity of iron-based MNPs has been extensively studied showing that the total biocompatibility of both iron core and coating molecules ensure optimal gene transport efficiency with minimal toxicity (36). In this way, translated to the clinical setting, adenoviral magnetic targeting would be a promising strategy using all the advantages of the adenoviral vector. It could provide a mean for controlling the biodistribution of the vector and may therefore be instrumental for improving both the safety and efficiency of the current experimental gene therapeutic methods.

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