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Adenovirus enhancement of transferrin–polylysine-mediated gene delivery

(DNA transfection/gene therapy/transferrin receptor/polylysine/endocytosis)

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ABSTRACT **Gene transfer may be accomplished by the receptor-mediated endocytosis pathway using transferrin–polylysine conjugates. For some target cells, however, gene transfer by this vector is extremely limited, despite the presence of the appropriate surface receptors, a phenomenon attributed to lysosomal degradation of endosome-internalized conjugate–DNA complexes. To enhance DNA escape from the cell vesicle system and thus augment gene transfer by this route, we have used the capacity of adenoviruses to disrupt endosomes as part of their entry mechanism. Adenoviral infection augmented levels of gene transfer by transferrin–polylysine conjugates in a dose-dependent manner: levels of gene transfer of >2000-fold above baseline were achieved. Use of the adenovirus in this context allowed enhanced levels of gene transfer in a variety of target cells, including cell lines otherwise refractory to gene transfer by transferrin–polylysine conjugates. This augmentation was based on adenoviral-mediated vesicle disruption, a process independent of viral gene expression. Thus, the development of specific mechanisms to effect release from the endosome in combination with gene transfer by the receptor-mediated endocytosis pathway will increase the utility of this delivery system by allowing high levels of gene expression in target cells.**

Foreign DNA introduced into eukaryotic cells must traverse multiple cellular barriers to enter the nucleus where heterologous gene expression can be effected. Various DNA-mediated gene-transfer methods efficiently accomplish transit of the initial barrier, the cell membrane; however, the specific mechanisms involved in this transfer are frequently associated with significant cell toxicity (1, 2). To circumvent this limitation, strategies have been devised to subvert physiological mechanisms of macromolecular transport to achieve gene transfer. In this regard, DNA delivery has been accomplished by means of the receptor-mediated endocytosis pathway (3, 4). This strategy of gene transfer employs bifunctional molecular conjugates consisting of a cognate moiety for a cell-surface receptor that is covalently linked to a DNA-binding moiety. When the cognate domain is recognized by the appropriate cell-surface receptor, the conjugate is internalized by the receptor-mediated endocytosis pathway, cotransporting any foreign DNA that is complexed to the DNA-binding domain.

Although this method can achieve high levels of gene expression in selected contexts, in many instances gene expression is limited despite the presence of an appropriate surface receptor on the target cell. It has been hypothesized that this limitation is a consequence of lysosomal targeting of the endosome-internalized conjugate–DNA complexes (5, 6). To increase the fraction of DNA that would escape degradation and be expressed within the nucleus, agents to inhibit lysosomal enzymes have been used. By employing this strategy, augmented expression of transferred DNA can be demonstrated. However, this response is highly idiosyncratic; selected lysosomatropic agents successfully augment gene expression, whereas others actually inhibit it (6, 7). Additionally, the response to a given agent can be highly variable among different target-cell lines (7).

In the present study, we have used an alternative strategy to augment gene transfer by the receptor-mediated endocytosis pathway. By using the capacity of adenoviruses to disrupt endosomes as part of their normal mechanism of target-cell entry, we hypothesized that cointernalized conjugate–DNA complexes would be released into the cell cytoplasm and, thus, avoid lysosomal degradation. When we infected cells with a replication-incompetent adenovirus, we observed very significantly enhanced gene transfer by transferrin–polylysine conjugates in a variety of cell lines. Notably, this augmentation was shown to depend upon adenoviral mechanisms of endosome disruption, consistent with the concept that entrapment within the vesicle system of the cell is a major limitation to gene transfer by the receptor-mediated endocytosis pathway. Thus, the development of strategies to augment escape from the endosome will likely increase the applicabilities of gene transfer by this route by allowing high-level expression of transferred genes in target cells.

MATERIALS AND METHODS

Preparation of Transferrin–Poly(L-Lysine)–DNA Complexes. Human transferrin–poly(L-lysine) conjugates with an average chain length of 190 lysines (hTfpL190B) were prepared, as described (7). The specific ligation was accomplished through modification of the transferrin carbohydrate moiety (8). The DNA plasmid pRSVL containing the *Photinus pyralis* luciferase gene under control of the Rous sarcoma virus long terminal repeat enhancer/promoter (9) was used as a reporter gene. Conjugate–DNA complexes were prepared by dilution of 6 μg of pRSVL DNA in 350 μl of HBS (150 mM NaCl/20 mM Hepes, pH 7.3) followed by addition to 12 μg of hTfpL190B diluted in 150 μl of HBS. Complexes were allowed to form for 30 min at room temperature before addition to cells.

Preparation of Adenoviruses. Adenovirus dl312, a replication-incompetent strain deleted in the Ela region (10), was provided by T. Shenk (Department of Molecular Biology, Princeton University). Propagation of the virus was on the Ela trans-complementing cell line 293. Large-scale preparation was exactly as described (11). Purified virus was resuspended in storage buffer (100 mM Tris, pH 8.0/100 mM NaCl/0.1% bovine serum albumin/50% glycerol) and maintained in aliquots at −70°C. Determination of virion concentration was accomplished by UV spectrophotometric analy-

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Abbreviation: DMEM, Dulbecco's modified Eagle's medium.

sis of extracted viral genomic DNA using the formula that one absorbance unit (A_{260}) equals 10^{12} viral particles per ml (12).

Cells and Media. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM)/5% heat-inactivated fetal calf serum/penicillin at 100 international units per ml/streptomycin at 100 μg/ml/2 mM glutamine. WI-38, MRC-5, and KB cells were grown in Eagle's minimal essential medium /10% heat-inactivated fetal calf serum/penicillin at 100 international units/ml/streptomycin at 100 μg/ml/10 mM nonessential amino acids/2 mM glutamine. CFT1, a cystic fibrosis respiratory epithelial cell line, was from J. Yankaskas (Division of Pulmonary Diseases, University of North Carolina). These cells were grown in F12-7X medium as described (J. Yankaskas, personal communication). For gene-transfer experiments, cells were grown in 6-cm tissue-culture plates until ≈50% confluent (5×10^5 cells). Medium was removed, and 1 ml of DMEM or Eagle's minimal essential medium/2% fetal calf serum was added. Conjugate–DNA complexes were added followed immediately by adenovirus dl312 ($0.05–3.2 \times 10^4$ particles per cell) or a comparable volume of viral storage buffer (1–80 μl). Plates were returned to the incubator (5% CO_2, 37°C) for 1 hr, after which 3 ml of complete medium was added. After an additional 24 hr, incubation cells were harvested for analysis of luciferase gene expression. For CFT1, cells were grown in F12-7X medium lacking human transferrin supplement for 4 hr before and during gene-transfer experiments. For sequential binding experiments, HeLa cells were equilibrated at 4°C in 1 ml of DMEM/2% fetal calf serum. Conjugate–DNA complexes were added, as before, and plates were incubated at 4°C for 2 hr. After this period, the plates were washed extensively with ice-cold DMEM/2% fetal calf serum followed by addition of 2 ml of the same. Adenovirus dl312 or viral storage buffer was administered, and the cells were allowed to warm up gradually before being returned to the incubator for an additional 24 hr. After this incubation, cells were harvested for analysis of luciferase gene expression. Preparation of cellular extracts, standardization of protein content, and analysis of luciferase activity were as described (4).

RESULTS

Evaluation of the Effect of Adenoviral Infection on Gene Transfer by Transferrin–Polylysine Conjugates. Initially, the effect of increased viral dosage on the capacity of a fixed amount of conjugate–DNA complex to effect gene transfer was examined. In this analysis, increased amounts of added adenovirus resulted in a corresponding increase in the levels of gene transfer achieved by the transferrin–polylysine conjugates (Fig. 1). This adenoviral-mediated augmentation of gene transfer plateaued at $\approx 1 \times 10^4$ particles per cell, a figure corresponding to the approximate number of adenoviral receptors on the surface of each HeLa cell (13). Levels of luciferase expression up to 2000-fold over baseline transfer with transferrin–polylysine conjugate alone were achieved with the higher number of viral particles. In a second set of experiments, the capacity of limiting amounts of conjugate–DNA complex to achieve gene transfer was examined in the presence of a static dose of adenovirus dl312. Adenoviral infection-augmented gene transfer by transferrin–polylysine conjugates over a broad range of DNA dosages (Fig. 2). The maximal level of gene expression effected by the conjugate–DNA complexes alone was achieved by 100-fold less DNA when adenoviral infection was used to augment delivery.

Determination of the Mechanism of Adenoviral Augmentation of Gene Transfer by Transferrin–Polylysine Conjugates. The effect of adenoviral infection on gene transfer was examined for both uncomplexed DNA and DNA that had been complexed with polylysine or transferrin–polylysine conjugates (Fig. 3*a*). By this analysis, adenoviral infection

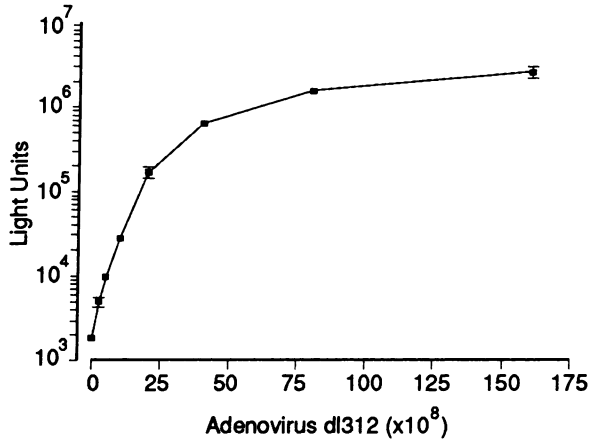

FIG. 1. Effect of adenoviral infection on gene transfer by transferrin–polylysine conjugates. Viral dosage effect: for complex formation 6 μg of luciferase-encoding plasmid DNA (pRSVL) was mixed with 12 μg of human transferrin–polylysine conjugate (hTfpL190B). Conjugate–DNA complex plus variable amounts of the replication-defective adenovirus dl312 ($0.05–3.2 \times 10^4$ viral particles per cell) were added to HeLa cells. Cell lysates were standardized for total protein content and analyzed for luciferase enzyme activity. Results are expressed as "light units" per 50 μg of total cellular protein. Data are the mean of two to four separate experiments; error bars represent SEM.

did not significantly augment transfer of naked, uncomplexed DNA. In marked contrast, transfer of DNA complexed to polylysine or transferrin–polylysine conjugates was augmented by adenoviral infection. This effect was, however, much greater for the transferrin–polylysine conjugates. Because the polycation portion of the conjugate molecule not only serves to attach transferrin to DNA, but also effects significant structural changes in the DNA (15), these experiments could not differentiate whether the observed effect was on the basis of enhanced fluid-phase endocytosis of the polycation-condensed DNA or augmented delivery of receptor-bound conjugate–DNA complex. To distinguish between these possibilities, sequential binding experiments were done (Fig. 3*b*). Binding of transferrin–polylysine–DNA or polylysine–DNA complexes at low temperature without internalization allowed removal of excess complex in the fluid phase before adenoviral infection (16). When administered in this fashion, delivery of the receptor-bound transferrin–polylysine–DNA complexes was significantly augmented by

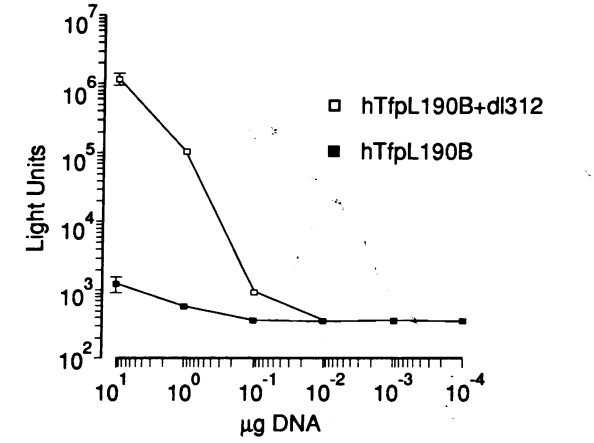

FIG. 2. Conjugate–DNA-complex dosage effect. Logarithmic dilutions of conjugate–DNA complexes, formed as in Fig. 1, were added to HeLa cells with or without a fixed dosage of adenovirus dl312 (1.0×10^4 viral particles per cell). Analysis of luciferase activity was as before.

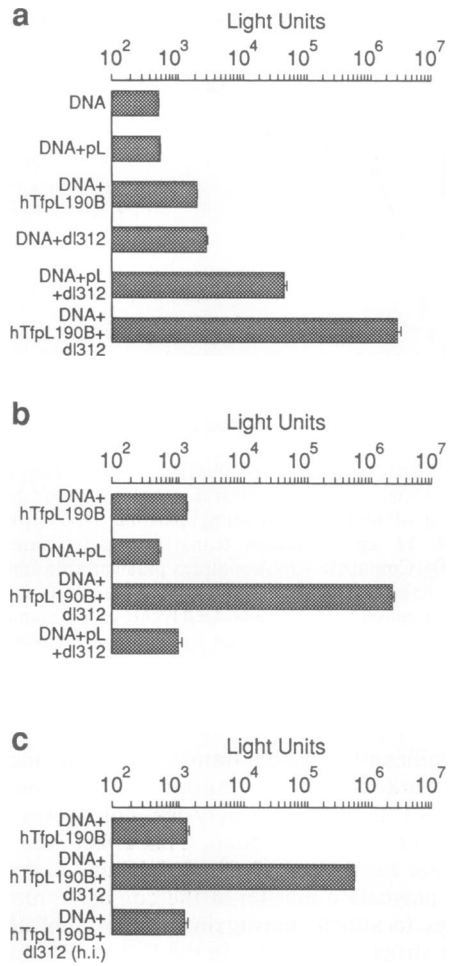

FIG. 3. Adenoviral augmentation of transferrin–polylysine-mediated gene transfer occurs via the receptor-mediated endocytosis pathway. (*a*) Effect of adenoviral infection on transfer of complexed DNA. Complex formation reactions were set up containing 6 μg of pRSVL DNA without transferrin–polylysine conjugate (DNA), 6 μg of pRSVL DNA plus 6 μg of unconjugated $(lysine)_{270}$ (DNA + pL), or 6 μg of pRSVL DNA plus 12 μg of human transferrin–polylysine conjugate (DNA + hTfpL190B). The above were added to HeLa cells with or without added adenovirus dl312 (dl312) (1.0×10^4 viral particles per cell). Cell extracts were prepared, standardized for total protein, and analyzed for luciferase enzyme activity as before. (*b*) Effect of adenoviral infection on transfer of receptor-bound DNA. Conjugate–DNA complexes (DNA + hTfpL190B) or polylysine–DNA complexes (DNA + pL) were bound to HeLa cells without internalization by incubation at 4°C. Unbound complex was removed before addition of adenovirus dl312 (1.0×10^4 viral particles per cell) or a comparable volume of viral storage buffer. Subsequent incubation was at 37°C to permit internalization of bound DNA complexes and adenoviruses. Analysis of luciferase enzyme activity was as before. (*c*) Effect of adenoviral entry on gene transfer by transferrin–polylysine conjugates. Conjugate–DNA complexes containing 6 μg of pRSVL DNA plus 12 μg of transferrin–polylysine (DNA + hTfpL190B) were added to HeLa cells with 1.0×10^4 adenovirus dl312 particles (dl312) or a similar amount of adenovirus dl312 that had been heat inactivated (dl312 h.i.). Heat inactivation was done by incubating at 45°C for 30 min (14). Analysis of luciferase enzyme activity was as before.

addition of adenoviral particles, whereas the polylysine–DNA complexes were not. Thus, it is the entry of DNA by the receptor-mediated endocytosis pathway that is specifically enhanced. Next, we analyzed the specific adenoviral function mediating enhanced gene transfer by the receptor-mediated endocytosis pathway (Fig. 3*c*). Mild heat treatment of virions does not alter the ability of adenoviruses to bind to target-cell membranes but does ablate their capacity to disrupt endosomes after internalization (14). Thus, the distinct effects of viral binding and viral entry could be separately evaluated. In this analysis, heat inactivation of the adenoviruses completely abolished their ability to enhance receptor-mediated gene transfer. This result suggests that it is the capacity of the adenoviruses to disrupt endosomes as part of their entry mechanism which specifically effects enhancement of gene delivery by transferrin–polylysine conjugates.

Effect of Adenoviral Infection on Gene Transfer by Transferrin–Polylysine Conjugates in Selected Cell Lines. The presence of transferrin receptors on target cells is necessary, but not sufficient, to allow gene transfer by transferrin–polylysine conjugates. Cell-specific factors relating to the fate of endosome-internalized conjugate–DNA complexes appear an extremely important determining factor in the levels of gene transfer achievable by this route (7). In this regard, selected cell lines were examined for both gene transfer by transferrin–polylysine conjugates and augmentation of receptor-mediated gene delivery by adenoviral infection (Fig. 4). CFT1 cells showed moderate levels of luciferase gene expression after treatment with transferrin–polylysine–DNA complexes. This level of gene expression was significantly augmented by infection with adenovirus dl312. In marked contrast, KB cells treated with the transferrin–polylysine conjugates exhibited levels of luciferase gene expression barely above background levels, despite the presence of transferrin receptors (17). Treatment with adenovirus dl312, however, allowed expression of readily detectable luciferase levels. Similarly, HeLa cells exhibited levels of gene expression just above background levels with conjugate–DNA complexes. These levels of gene expression could also be augmented by adenoviral infection; however, this effect was much greater than for KB cells. Because HeLa cells and KB cells possess approximately the same number of surface receptors for adenovirus (18), it is likely that this difference in augmentation reflects the number of transferrin receptors characteristic of each cell type. In marked contrast to these findings, however, WI-38 and MRC-5, cell lines that support adenoviral infection very poorly (19), showed very little augmentation with dl312 over levels achieved by the conjugate–DNA complexes alone. Adenoviral infection thus appears to augment gene transfer by conjugate–DNA complexes in instances where gene transfer by the receptor-mediated endocytosis pathway appears feasible, as for CFT1 cells, and also in some instances where transfer by this route appears ineffective, as for HeLa and KB cells. The level of augmentation achieved varies significantly among different

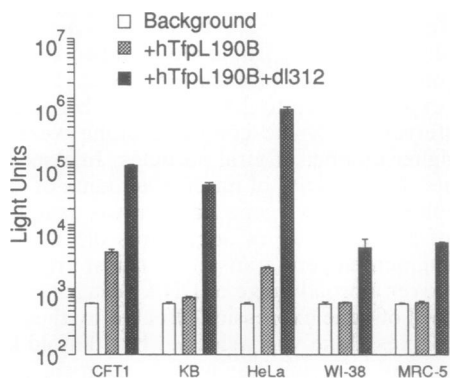

FIG. 4. Effect of adenoviral infection on gene transfer by transferrin–polylysine conjugates in selected cell lines. Conjugate–DNA complexes (6 μg of pRSVL plus 12 μg of hTfpL190B) were added to CFT1, KB, HeLa, WI-38, and MRC-5 cells with or without adenovirus dl312 (1.0×10^4 viral particles per cell). Cell lysates were analyzed for luciferase enzyme activity as before.

target cells, suggesting that this effect is a function of both the number of adenoviral cell-surface receptors characteristic of each cell type, as well as the number of receptors for transferrin.

DISCUSSION

The development of strategies to accomplish gene transfer by the receptor-mediated endocytosis pathway has established a vector system with special features and potentials. The ability to accomplish gene delivery by means of a physiologic pathway has many advantages including (*i*) a nontoxic mechanism of transiting the eukaryotic cell membrane, (*ii*) the capacity to administer DNA on a repetitive or continuous basis, and (*iii*) the possibility of cell-specific targeting (7). In addition, because the bifunctional molecular conjugate gene-transfer vehicles are synthetically derived, the capacity to prepare large amounts of these agents is feasible.

Gene-transfer efficiencies comparable or superior to those obtained with conventional DNA-mediated gene transfer vectors have been achieved using transferrin–polylysine conjugates (6, 7). The utility of this vector system in a given context, however, is not predicted solely by the presence of the appropriate cell-surface receptor, as extremely limited gene transfer is noted for many target cells that possess transferrin receptors (5, 6). It has been suggested that the internalization of the conjugate–DNA complexes by means of receptor-mediated endocytosis targets them for cellular pathways resulting in lysosomal degradation (5, 6). On this basis, it was reasoned that inhibition of lysosomal enzymes would increase the fraction of DNA surviving transition through the vesicle system of the cell. In this regard, augmentation of gene transfer by transferrin–polylysine conjugates has been demonstrated by the cotreatment of target cells with chloroquine, a lysosomatropic agent with pleotropic subcellular effects. Unexpectedly, however, other agents employed to inhibit lysosomal degradative functions, such as monensin, were not effective in augmenting gene transfer, and, in fact, functioned as inhibitors (6, 7). These conflicting findings raise important questions as to whether the chloroquine effect is exclusively on a lysosomatropic basis and, thus, whether strategies to inhibit lysosomal enzymes will be generally useful.

The goal of allowing internalized conjugate–DNA complexes to escape the endosome before lysosomal targeting led us to explore the utility of adenoviral infection as a means to augment gene transfer by this route. Adenoviruses enter cells in a fashion analogous to the conjugate–DNA complexes; after binding to specific cell-surface receptors the virions are internalized by the receptor-mediated endocytosis pathway (18, 20). Unlike the conjugates, however, the adenovirus possesses a specific mechanism to disrupt the endosome, allowing escape to the cell cytosol; changes in endosome pH during internalization expose hydrophobic domains of the adenoviral capsid proteins, permitting them to interact with the vesicle membrane in a fashion that ultimately disrupts its integrity (21, 22). Once within the cell cytoplasm, uncharacterized mechanisms effect delivery of the virions to the host-cell nucleus, where the viral genomic DNA is replicated.

As a consequence of their specific entry mechanisms, viruses elicit major alterations in the cell membrane and vesicle system of target cells. These changes have been shown to be associated with altered cellular macromolecular transport. In this regard, Fernandez-Puentes and Carrasco (23) noted enhanced transport of administered protein toxins in picornavirus-infected cells, an effect they attributed to altered membrane permeability. Enhanced delivery of macromolecules was also observed by means of the receptor-mediated endocytosis pathway concomitant with adenoviral infection (14, 16, 24). An important aspect of these studies was the observation that adenoviruses enter cells by the receptor-mediated endocytosis pathway and that they are cointernalized with other receptor-bound macromolecules. Thus, adenoviral-mediated disruption of the internalized endosome augments release of its heterogeneous contents, enhancing delivery to the cell cytosol of other receptor-bound cognates (14, 16, 24).

We wondered whether the capacity of adenovirus to disrupt the endosome would enhance gene delivery by the receptor-mediated endocytosis pathway. Coinfection with adenovirus resulted in very significant augmentation of gene transfer using transferrin–polylysine conjugates. The fact that a replication-defective adenovirus could elicit this augmentation of gene transfer is consistent with the concept that this phenomenon is based on the capacity of the virion to bind target cells and be internalized and not on viral replicative functions. Furthermore, the exposure of comparable quantities of adenovirus dl312 to a K-562 cell line constitutively expressing a Rous sarcoma virus long terminal repeat–luciferase gene revealed no detectable change in luciferase activity, whereas the adenovirus can function to enhance delivery of pRSVL/polylysine–transferrin conjugates in the parental K-562 line (data not shown). This result demonstrates that the adenovirus enhancement of luciferase gene expression is functioning at the level of gene delivery and not a transactivation phenomenon at the gene expression level. These findings establish that gene transfer by transferrin–polylysine conjugates is functionally limited by the absence of specific mechanisms to accomplish conjugate–DNA-complex release from the cellular vesicle system. In the present study, this mechanism of endosome release was provided by capitalizing on the specific entry functions of the adenovirus mediating vesicle disruption. In this regard, it is noteworthy that subversion of the highly efficient entry mechanisms of the adenovirus is the rationale for the development of recombinant adenoviruses as vectors to transfer heterologous genes (25). The employment of these agents, however, has been limited by intrinsic constraints on their construction; relatively short heterologous DNA segments of <6 kilobases can be incorporated into the recombinant adenoviral genome (26), and expression of these introduced genes is obligated by means of adenoviral regulatory signals (27). The use of adenoviruses in a facilitative fashion, as in the present study, offers the advantages of efficient adenoviral entry in combination with the advantages of gene transfer by transferrin–polylysine conjugates; DNA segments of up to 15 kilobases, and possibly larger, can be transferred, and there is no constraint on the functional design of the introduced DNA.

Note Added in Proof. We recently demonstrated that UV-inactivated adenovirus dl312 also functions to enhance receptor-mediated gene delivery.

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