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Highly Efficient Gene Delivery into Transfection-Refractory Neuronal and Astroglial Cells Using a Retrovirus-Based Vector

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Abstract Introduction of foreign genes into brain cells, such as neurons and astrocytes, is a powerful approach to study the gene function and regulation in the neuroscience field. Calcium phosphate precipitates have been shown to cause cytotoxicity in some mammalian cells and brain cells, thus leading to low transfection efficiency. Here, we describe a retrovirus-mediated gene delivery method to transduce foreign genes into brain cells. In an attempt to achieve higher gene delivery efficiency in these cells, we made several changes to the original method, including (1) use of a new packaging cell line, Phoenix ampho cells, (2) transfection of pMX retroviral DNA, (3) inclusion of 25 mM chloroquine in the transduction, and (4) 3–5 h incubation of retroviruses with target cells. The results showed that the modified protocol resulted in a range of 40–60% gene delivery efficiency in neurons and astrocytes. Furthermore, these results suggest the potential of the retrovirus-mediated gene delivery protocol being modified and adapted for other transfection-refractory cell lines and primary cells.

Key words: Retrovirus-mediated gene delivery, neuronal cell, astroglial cells, retrovirus-based vector

Expression of foreign genes in cultured mammalian cells has often been performed to define gene function and regulation. Introduction of foreign genes into mammalian cells involves, in general, complex formation of expression vector DNA with gene delivery vehicles, binding to the cell surface, uptake of the complex into the cell, translocation to the nucleus, and unpacking the DNA for transcription [9, 16]. There are a number of *in vitro* gene delivery techniques available [2, 4], however, the efficiency of these techniques varies considerably between cell types, cell lines, and primary cells. Efficient gene delivery in one cell type or one cell line or one type of primary cells may not predict a similar situation in another cell line or another cell type or another type of primary cells [10, 12]. The calcium phosphate precipitation transfection has been considered as the simplest, most reproducible, and most inexpensive method for transient gene expression in the research laboratory settings. However, calcium phosphate precipitates have been shown to cause cytotoxicity (direct cell killing) in some mammalian cells, including epithelial cells [1, 11] and brain cells such as neurons and astrocytes [14], thereby leading to low transfection efficiency and sometimes even to misleading results [6, 17]. Alternatively, a more expensive gene delivery approach, such as lipid-mediated transfection, has also been exploited in cells that are not or less transfectable by the calcium phosphate precipitation method [7]. Nevertheless, the lipid-mediated transfection often requires empirical optimization of the ratios of lipid versus DNA, incubation time, and with or without serum [5], in order to achieve an ideal transfection efficiency. Here, we report an adapted retrovirus-mediated strategy that gives rise to 40–60% gene delivery efficiency in those calcium phosphate precipitation transfection-refractory cells. The method described herein is fast, inexpensive, and easy to perform, and results in higher levels of transient gene expression, as well as stable integration of gene expression cassettes for long-term expression.

The original retrovirus-mediated strategy developed by Onishi *et al.* [8] is based on a pBabeX-derived retroviral vector pMX, and has been successfully utilized for gene delivery in cells of hematopoietic origin that are refractory to commonly used gene delivery methods, such as the calcium phosphate precipitation transfection and electroporation [3, 8]. In general, the strategy consists of preparation of retrovirus stock by using a packaging cell line and transduction of target cells with recombinant retroviruses (see Table 1). Compared to an average 30% gene delivery efficiency in

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Table 1. The modified retrovirus-mediated gene delivery protocol.

I. Preparation of recombinant retroviruses

1. Plate the packaging Phoenix Ampho cells at a density of 1.2×10^6 cells per 60-mm dish in 3 ml of regular cell culture medium (RM), i.e. DMEM containing 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine, 24 h prior to transfection. This will give rise to approximately 70–80% confluency prior to transfection the next day.
2. Transfect the packaging cells with retroviral DNA. Prepare the transfection cocktail in a sterile plastic tube by mixing 7.5 μg of retroviral DNA, 18 μl of 2 M $CaCl_2$, and H_2O in a total of 180 μl, adding 180 ul of 2× HBS (50 mM HEPES, 10 mM KCl, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.05) in a dropwise manner, and incubating on ice for 20 min.
3. Replace the cell culture medium with fresh RM containing 25 μM chloroquine, and apply the transfection cocktail onto the cells in a dropwise manner, followed by a brief gentle agitation of the dish to ensure uniform distribution of the precipitates.
4. Replace the cell culture medium with fresh RM twice, first at 8–10 h after transfection, and second at 16–24 h after the first medium change; harvest the supernatant 16–24 h after the second medium change, pass it through a 0.45 μm sterile filter, and save it as the retrovirus stock.

II. Transduction of target cells with recombinant retroviruses

1. Plate astrocytes and neurons at a density of 0.2×10^6 and 0.5×10^6 per well on a 6-well cell culture plate 12–18 h prior to retroviral transduction.
2. Replace the cell culture medium with the recombinant retrovirus stock supplemented with 4 μg/ml of polybrene (no difference noted between 4 μg/ml and 8 μg/ml), and incubate the cells with the viruses for 3–5 h.
3. Remove the retroviruses, and incubate the cells in fresh RM for 24–48 h before analysis for gene expression.

hematopoietic cells, this method gave rise to only about 5% efficiency at best in non-hematopoietic and highly transfection-refractory brain cells (see Table 2) [13, 15]. In an attempt to achieve higher gene delivery efficiency in these cells, we made several change of the original method, including (1) the use of a new packaging cell line, Phoenix ampho cells, (2) transfection of pMX retroviral DNA by the calcium phosphate precipitation method, (3) inclusion of 25 mM chloroquine in the transduction, and (4) 3–5 h incubation of retroviruses with target cells.

To demonstrate the feasibility of the modified strategy described, we constructed a pMX-GFP reporter retroviral DNA, in which the green fluorescence protein gene (*gfp*) was cloned into the backbone of pMX, linearized by *Eco*RI and *Not*I digestion. The expression of green fluorescence protein (GFP) allowed an accurate and sensitive determination of gene delivery efficiency by either flow cytometry and/or fluorescence microscopy. The modified protocol is detailed in Table 1. Briefly, we transfected Phoenix Ampho cells (ATCC, Manassas, VA, U.S.A.) with pMX-GFP DNA by using the calcium phosphate precipitation method. We also included the pMX retroviral backbone DNA as a control. We next harvested the cell culture supernatant as recombinant retrovirus stock, and infected neurons and astrocytes with the retroviruses. Then, the gene delivery efficiency was determined by flow cytometry and fluorescence microscopic analysis for GFP expression. We tested several neuronal and astroglial cell lines, as well as primary brain cells, in our studies. The results showed that the modified protocol resulted in a range of 40–60% gene delivery efficiency in neurons and astrocytes, as demonstrated in one commonly used neuron cell line rat pheochromocyoma PC12, and two astroglial cell lines U373.MG and U87.MG (Fig. 1, Table 2). More importantly, the cell viability MTT assay revealed no cell death or cytotoxicity in all the cells transduced with pMX.GFP recombinant

Table 2. Comparison of various methods in the gene delivery efficiency of brain cells.

Methods	Gene delivery efficiency (%)[a], cell line		
	PC12	U373.MG	U87.MG
Calcium phosphate precipitate method	7.6±2.9	4.2±2.2	5.5±3.5
Modified retrovirus-mediated gene delivery[b]	58.3±9.6	41.1±8.7	45.8±6.9
--- packaging cell line BOSC23	44.1±10.3	32.4±7.7	38.2±7.9
--- without 25 mM chloroquine	15.5±4.7	16.1±9.2	12.9±8.3
--- without 4 μg/ml polybrene	26.1±9.7	22.6±5.9	19.7±6.8
--- 10 h incubation of retroviruses	16.7±6.3	22.6±5.6	18.1±5.4

[a]Gene delivery efficiency (%) was determined by flow cytometry.

[b]This method was performed according to Table 1.

Results of three separate experiments are presented.

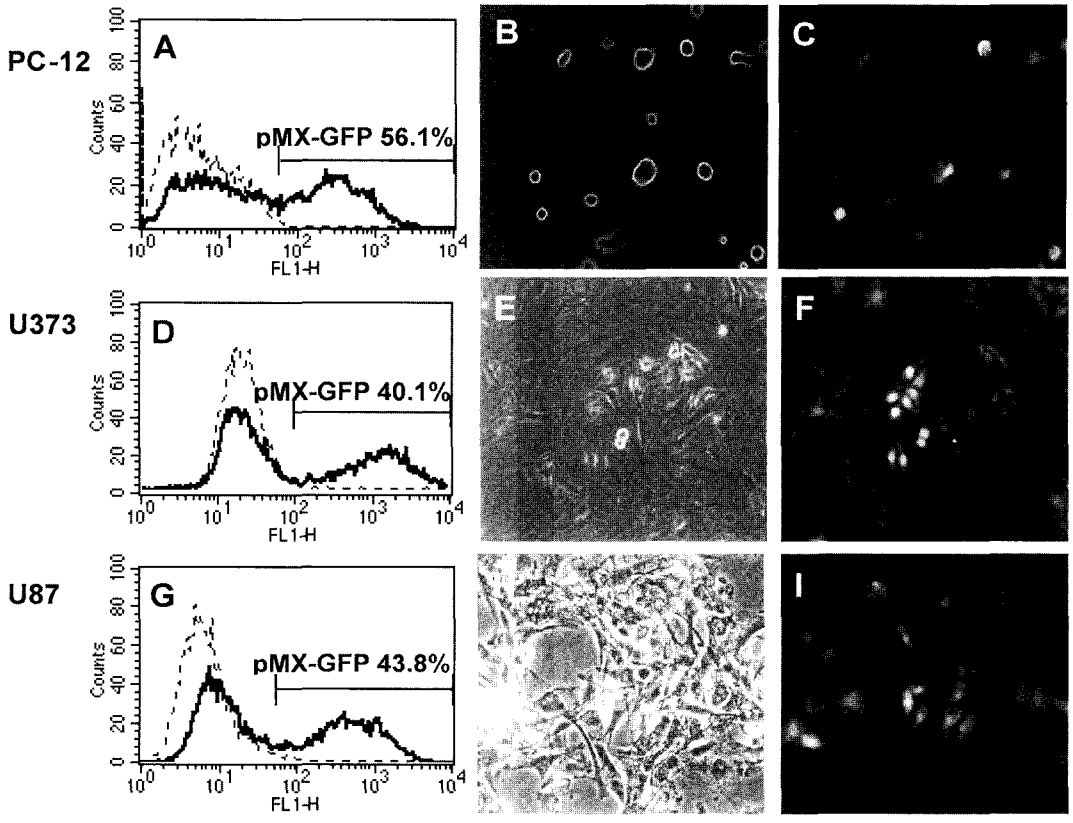

Fig. 1. GFP expression in PC12, U373.MG, and U87.MG cells by pMX-GFP retroviruses.
PC12 cells were maintained in DMEM, containing 5% heat inactivated fetal bovine serum, 10% horse serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 4 mM L-glutamine, at 37°C, in 10% CO_2 incubator; U373.MG, U87.MG, and Phoenix ampho cells were maintained in DMEM, containing 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, at 37°C, in 5% CO_2 incubator. All the cells were purchased from ATCC (Manassas, VA, U.S.A.). pMX.GFP (solid line) and pMX (broken line) recombinant retroviruses were made by transfection of Phoenix ampho cells, and used to transduce PC12 (panels A-C), U373.MG (panels D-F), and U87.MG cells (panels G-I). GFP expression was determined by flow cytometry (panels A, D, and G) and fluorescence microscopic analysis (panels B, E, and H for bright field images; C, F, and I for FITC images). All images were captured and processed by using a digital fluorescence imaging microscope Axiovert M200 (Carl Ziess, New York, NY, U.S.A.).

retroviruses or pMX recombinant retroviruses (data not shown).

In summary, we have adapted a retroviral-mediated system for delivering foreign genes into brain cells. Because of its high efficiency and no cytotoxicity, this system would allow cost-effective and accurate assessment of gene function and regulation, as well as the effects of specific genetic deficiencies in cell lines of brain origin and brain primary cells. In addition, the ability of this system to stably introduce genes into cells would also allow rapid generation of stable cell lines. Furthermore, these results suggest the potential of the retrovirus-mediated gene delivery protocol after being modified and adapted for other transfection-refractory cell lines and primary cells.

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