Effects of Enhancers and Coating Substrates on the Transgene Expression Mediated by Branched Polyethylenimine

Wen-Chi Tseng,[1] Tsuei-Yun Fang,[2] Kuang-Wei Chang,[1] Chien-Hsiang Tang,[1] Ling-Yu Su[1]

[1]*Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan*
[2]*Department of Food Science, National Ocean University, Keelung, Taiwan*

Received 2 December 2008; accepted 22 March 2009
DOI 10.1002/app.30466
Published online 2 July 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Gene delivery is an important step in the preparation of cell arrays. A highly efficient delivery system is required to modulate the cellular functions for analysis. In this study, branched polyethylenimine (bPEI), a very potent and cost-effective polymer for transfection, was used as a primary carrier for gene delivery. The percentage of cells expressing green fluorescent protein was used to indicate the transfection efficiency. Various substrates were coated on slides for the examination of cell growth and transfection efficiency. Different enhancers, such as polyethylene glycol, dextran, and gelatin, were combined with the transfection reagent in an attempt to increase the transfection efficiency. For the slides coated with gelatin type B that could provide better cell growth, the transfection efficiency was observed to strongly depend on the amount of plasmid contained within the transfection reagent in a dosage response. In the absence of any enhancers, the efficiency reached 20%, whereas in the presence of polyethylene glycol of MW 2000 or gelatin type A, the efficiency could be further enhanced to around 70%. When bPEI was replaced with dextran-grafted bPEI, a more efficient carrier for transfection in cell culture, very minimal levels of GFP positive cells were detected, suggesting that different factors might exist in transfecting cells in culture and those in microarrays for PEI-based vectors to achieve high efficiency. This study showed that using bPEI combined with an enhancer as a gene delivery system could efficiently modulate cellular functions for the preparation of cell arrays. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 2221–2225, 2009

Key words: biological applications of polymers; drug delivery systems; polyamines

INTRODUCTION

Gene delivery provides an attractive approach to modulate cellular functions by increasing the expression of the delivered gene or by suppressing the expression of a specific endogenous gene through the delivered small interfering RNA[1] and has been applied to cure the effects of acquired and inherited diseases in a straightforward manner by adding, correcting, or replacing genes.[2–4] Recently, gene delivery has been extended to the preparation of cell arrays that allow parallel assays in a short time for efficient high-throughput assays.[5–15]

The process of establishing cell arrays generally consists of the following steps.[5,6,10,15] The transfection reagents are first embedded or immobilized inside substrate by way of an arrayer, which can deliver nanoliters of the reagents onto a defined spatial spot on a slide. Then cells are layered on the top of the transfection reagents. A further incubation of the cells under a standard culture condition is required for the cells to grow on the substrate and allows the nucleic acids to be delivered into the cells. Because each immobilized spot contains the transfection reagent that is prepared with a specific gene, each individual cluster of cells grown on the top of the reagent can express different specific genes in parallel. Therefore, each cluster of cells exhibits different cellular functions that can be used for cell-based functional analysis.

Gene delivery is a pivotal step in preparing cell arrays. Two major systems exist for gene delivery: viral and synthetic vectors, of which the latter generally uses synthetic polymers or lipids to deliver nucleic acids.[3,4,14] Compared with viral vectors, synthetic vectors have the advantage of simplicity and versatility for preparing the transfection reagents in parallel to be used in fabricating cell arrays. However, the transfection efficiency of synthetic vectors is generally lower than that of viral vectors. Within each single spot, the low efficiency will result in only a small fraction of the cells whose cellular

Correspondence to: W.-C. Tseng (tsengwc@mail.ntust.edu.tw).

Contract grant sponsor: National Science Council, Taiwan; contract grant number: NSC-97-2221-E-011-001-MY3.

functions are modulated and thus could impair the performance of a cell array because a less extent of modulated cellular functions might fail to provide sufficient signals for detection.

The transfection procedures used in preparing cell arrays are different from those typically used in transfecting the cells in culture. The strategies developed for improving the efficiencies of synthetic vectors might become inapplicable in preparing cell arrays. Currently it is rarely reported whether there exist some specific considerations that might affect the efficiencies of synthetic vectors used in arrays other than those already known in the typical transfection procedures. In this study, branched polyethylenimine (bPEI),[16] a very potent transfection reagent, coupled with a plasmid encoding green fluorescent protein (GFP) were used in the preparation of cell arrays because bPEI is commercially available and more cost-effective than cationic liposomes. Different coating materials and enhancers were examined for their effectiveness in improving the transfection efficiency, which was indicated by the GFP positive percentages of each cell cluster. A dextran-grafted bPEI was used in place of unmodified bPEI to investigate the differences of typical transfection and cell arrays.

MATERIALS AND METHODS

Materials

Agar, agarose, gelatin type A (gel-A), gelatin type B (gel-B), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, MO). Polyethylene glycols of molecular weight 2000 (PEG-2K) and 20,000 (PEG-20K) were from Merck (Darmstadt, Germany). Dextran of molecular weight 1500 (dex-1.5k) was from Fluka Chemie (Buchs, Switzerland), and dextran of molecular weight 20,000 (dex-20k) was from Amersham-Pharmacia (Piscataway, NJ). bPEI (MW 25,000) was obtained from Aldrich (Milwaukee, WI). bPEI grafted with dex-1.5k (dex-PEI) was synthesized as previously described. All chemicals were used as received without further purification. Water was deionized by a Milli-Q water purification system (Millipore, Bedford, MA). The pEGFP-C1 vector containing a mutated GFP gene driven by a cytomegalovirus promoter was from Clontech (Palo Alto, CA). Plasmid purification kits were purchased from Qiagen (Valencia, CA). Antibiotics of penicillin-streptomycin-amphotericin and fetal bovine serum were obtained from Hyclone (Logan, UT).

Plasmid preparation

The pEGFP-C1 vector was amplified in *Escherichia coli* DH5α by a standard procedure and purified by a Qiagene plasmid purification kit according to the manufacture's instructions.

Slide coating

Glass slides were precleaned using the Piranha solution overnight. A solution containing 1% agar, 1% agarose, or 0.2% gelatin was incubated at 60°C in a water bath with constant shaking until the solution became completely clear. A piece of glass slide was coated by immersing in the above solution for at least 1 min followed by air-drying at 30–50% humidity. The coated slide was then sterilized by immersing in 70% ethanol for 10 min and dried under a laminar flow hood before the application of transfection reagents.

Preparation of transfection reagent

bPEI or dex-PEI was used as a DNA carrier to prepare the transfection reagent as previously described. The desired amounts of PEI were diluted in 200 μL of dilution buffer (20 m*M* HEPES, 5.2% glucose, pH 7.0) and added into an equal volume of dilution buffer containing 40, 80, 240, and 360 μg DNA, respectively. The charge ratio of PEI to DNA in the transfection reagent was maintained at 9, except as specified elsewhere. After equilibrium was reached by allowing the mixture to stand at room temperature for 20 min, another equal volume of dilution buffer containing various concentrations of different enhancers was incorporated into the mixture. The enhancers included dex-20K, dex-1.5K, PEG-20K, PEG-2K, gel-A, and gel-B. The reagents were dispensed into a 96-well plate. A manual slide arrayer (VP Scientific, San Diego, CA) was used to spot the transfection reagent from the 96-well plate onto a coated slide. A total of four different spots were produced on each slide for the same composition of transfection reagent.

Cell culture

CHO-K1 cells (a Chinese hamster ovary cell line, ATCC CCL-61) were maintained at 37°C, 5% CO_2, and 100% humidity in complete medium containing DMEM supplemented with 10% fetal bovine serum and 1% antibiotics of penicillin–streptomycin–amphotericin. Cells were harvested from the flasks and seeded onto the coated slides containing the spots of transfection reagents at 20,000 cells/cm^2.

Quantitation of transfection efficiency

After exposure to the transfection reagent for 36 h, the slides were washed with PBS twice and then fixed in the presence of PBS containing 1% of

paraformaldehyde for 15 min. The fixed cells of each spot were visualized under a fluorescence microscope (Olympus IX 70, Tokyo, Japan), and the photographs of the cells were taken by a digital camera (Olympus C-4040, Tokyo, Japan) under a magnification of 10 × 20. The digitized photos were analyzed by using Scion Image software (Frederick, MD). Transfection efficiency was expressed as the percentage of the cells expressing GFP and calculated by dividing the number of the green cells by the number of the total cells within the spot.

RESULTS AND DISCUSSION

Cell growth on the coated slide

The state of cellular proliferation determines the efficiency of transfection mediated by nonviral vectors because mitosis is an essential event for transgene expression. The cell number of each spot was used to indicate the condition of cell growth on the coating materials. Cells grew slightly faster on the gel-B–coated and agar-coated slides than on the noncoated slide, whereas poor growth was found on the agarose-coated slides [Fig. 1(A)]. When the spot contained 0.3 mg/mL plasmid, higher transfection efficiency was found on the slides with a coating that could provide a better growth condition except the noncoated slides. The percentages of the cells expressing GFP were around 20% and 8% for the gel-B–coated and agar-coated slides, respectively [Fig. 1(B)]. The noncoated slide showed an almost undetectable level of transgene expression due to its incapability to hold the transfection reagents on the slide. We examined cell growth and transfection efficiency on the slides coated with several biopolymers including agar, agarose, alginate, gel-A, gel-B, pectin, and polyhydroxybutyrate, among which only gel-B, agarose and agar could provide better cell attachment and yield more consistent transfection efficiency (data not shown).

Effects of enhancer and DNA dosage

Because the gel-B–coated slides provided a better condition for cell growth and higher transfection efficiency, the effects of enhancer and DNA dosage on the transfection efficiency were conducted on the gel-B–coated slides by varying the plasmid amounts in the transfection reagents and adding various concentrations of different enhancers into the transfection reagent. The GFP-positive percentages exhibited a function of the applied plasmid and responded mainly in a dose-dependent fashion (Fig. 2). When the applied plasmid was below 0.1 mg/mL, the transfection efficiency was at very minimal levels and hardly improved by the incorporation of any

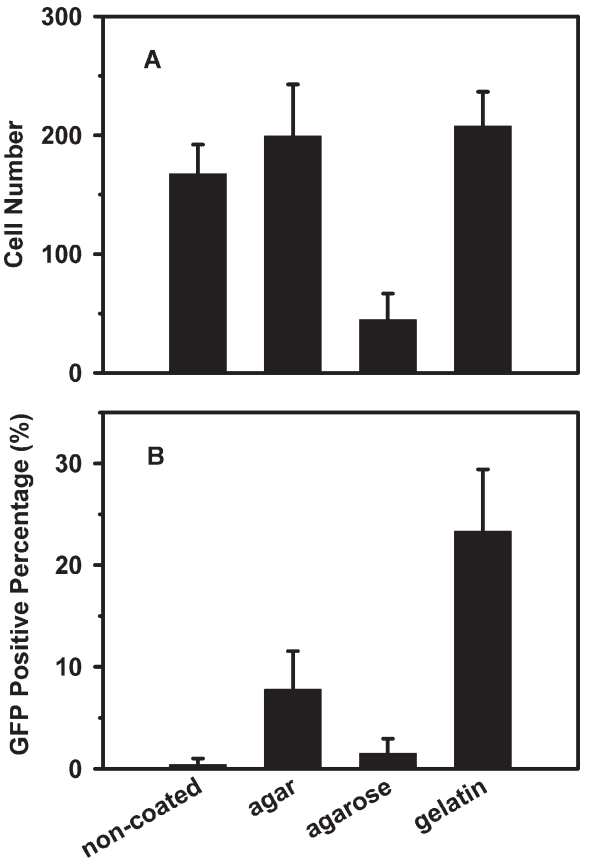

Figure 1 Effect of coating substrate on transfection efficiency and cell growth. A manual microarrayer was used to spot DNA-PEI complexes onto the slides coated with agar, agarose, and gel-B. The cell number within the spot indicated a condition of cell growth as shown in panel A. The percentage of GFP-positive cells indicated transfection efficiency, as shown in panel B (n = 3 independent preparations).

enhancers into the transfection reagent. Besides, not every spot contained the cells expressing GFP, indicating a very heterogeneous pattern of transgene expression at a low level of applied plasmid. In the absence of any enhancer, the transfection efficiency was enhanced to around 20% when the applied plasmid increased to 0.3 mg/mL and remained at the approximate level when the applied plasmid reached 0.45 mg/mL, as shown by the dashed line in Figure 2. A further increase of plasmid level to 0.6 mg/mL was used in an attempt to further increase the efficiency, but such an increase resulted in large aggregates of DNA–polymer complexes, which tended to precipitate during spotting.

The incorporation of enhancers could promote the transfection efficiency under most conditions, and the efficiency increased in proportion to the applied plasmid for certain enhancers. Using PEG-2K was most effective and almost tripled the efficiency to above 60%. The transfection efficiency was highly reproducible and independent of both the PEG-2K concentrations from 0.25% to 1.0% and the plasmid

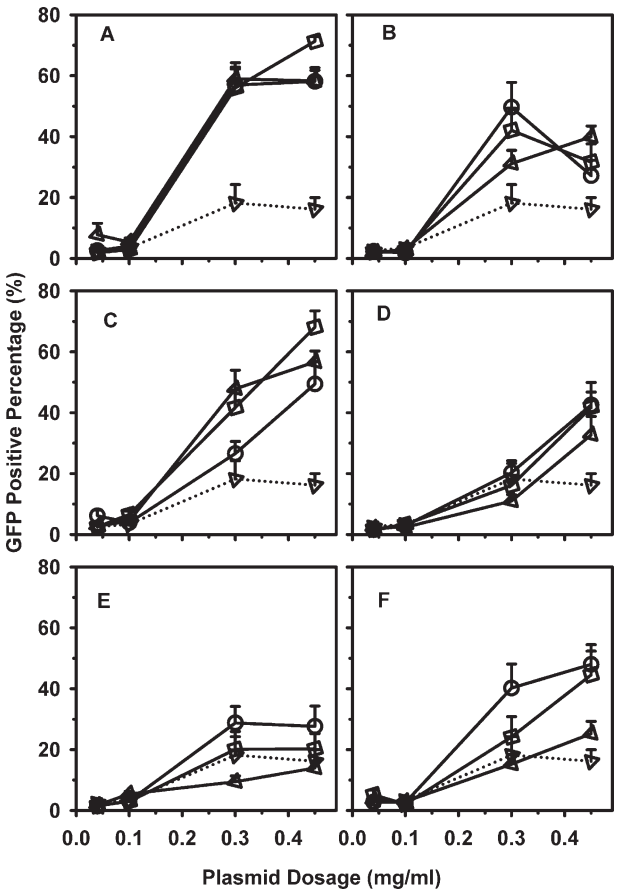

Figure 2 Effect of enhancer on transfection efficiency. Different enhancers were included into the transfection reagent before spotting onto gel-B–coated slides. The dashed line in all panels shows the efficiency in the absence of any enhancers. Panel A shows the effect of PEG-2K at 1% (Δ), 0.5% (□), and 0.25% (○). Panel B shows the effect of PEG-20K at 1% (Δ), 0.5% (□), and 0.25% (○). Panel C shows the effect of gel-A at 0.05% (Δ), 0.025% (□), and 0.0125% (○). Panel D shows the effect of gel-B at 0.05% (Δ), 0.025% (□), and 0.0125% (○). Panel E shows the effect of dex-1.5K at 1% (Δ), 0.5% (□), and 0.25% (○). Panel F shows the effect of dex-20K at 1% (Δ), 0.5% (□), and 0.25% (○) (n = 3 independent preparations).

level from 0.3 to 0.45 mg/mL, except that an optimal PEG-2K concentration of 1.0% existed to achieve a high transfection efficiency of 72% at a plasmid level of 0.45 mg/mL [Fig. 2(A)]. When PEG-2K was replaced with PEG-20K, the transfection efficiency was not a dose function of applied plasmid, with high efficiencies occurring at a level of 0.3 mg/mL. The efficiency decreased as the enhancer concentrations decreased from 1% to 0.25% at the plasmid level of 0.3 mg/mL but became less dependent of the enhancer concentrations at the plasmid level of 0.45 mg/mL [Fig. 2(B)]. When the transfection reagents contained gel-A, the transfection efficiency increased proportionally to the levels of applied plasmid irrespective of the enhancer concentrations [Fig. 2(C)]. The efficiency of using gel-A depended on both the plasmid level and enhancer concentration [Fig. 2(C)]. A high efficiency of 68% was observed at an optimal concentration of 0.025% of gel-A and a plasmid level of 0.45 mg/mL [Fig. 2(C)]. On the other hand, using gel-B was effective to improve transfection efficiency only at a plasmid level of 0.45 mg/mL, and the improvements seemed to be independent of the enhancer concentrations [Fig. 2(D)]. Unlike PEG-2K and PEG-20K, low-molecular-weight dextran, dex-1.5K, was a less effective enhancer in promoting transfection efficiency than high-molecular-weight dextran, dex-20K [Fig. 2(E,F)]. Using dex-1.5K only slightly improved the transfection efficiency and maintained a similar dose–response function of the applied plasmid and the transfection efficiency similar to that without any enhancer. For using dex-20K, the transfection efficiency increased as the applied plasmid amount and was doubled to 50% at a plasmid level of 0.45 mg/mL and a dex-20K concentration of 0.25% or 0.5%.

Both PEG-2k and gel-A were very effective enhancers in cell array. PEGs of low molecular weight have been widely used in the preparation of hybridoma and protoplast because they can facilitate cellular fusion. In cell arrays, the PEG might also facilitate the cellular entry of vectors when the cells settled on the substrate and began to grow. Gelatin showed very interesting properties. Gel-A and gel-B differ in the manufacturing processes during which gel-A and gel-B received an acid and alkaline pretreatment, respectively. When used as substrate, gel-B provided better cell growth than gel-A; however, when used as an enhancer, gel-B was less efficient than gel-A.

Use of dextran-grafted bPEI for transfection

Numerous studies have demonstrated that the transfection efficiency of bPEI could be improved through chemical modifications, such as the attachment of a pendant group onto bPEI. We previously reported that the GFP-positive percentages of CHO cells by a typical transfection procedure could be enhanced by twofold when bPEI was conjugated with dextran of molecular weight 1500 at a low degree of grafting.[17] The dex-PEIs were used to prepare the reagents for cell arrays in an effort to improve the efficiency (Fig. 3). However, the dex-PEI failed to enhance the efficiency and even resulted in less GFP-positive percentages than unmodified bPEI. Incorporating an enhancer into the reagents was attempted to enhance the efficiency. The enhancer that showed great effectiveness for bPEI lost the ability to improve the efficiency of dex-PEI at different charge ratios.

Although the dex-PEI was shown to be a more efficient carrier for gene delivery than unmodified bPEI in typical transfection, the dex-PEI failed to

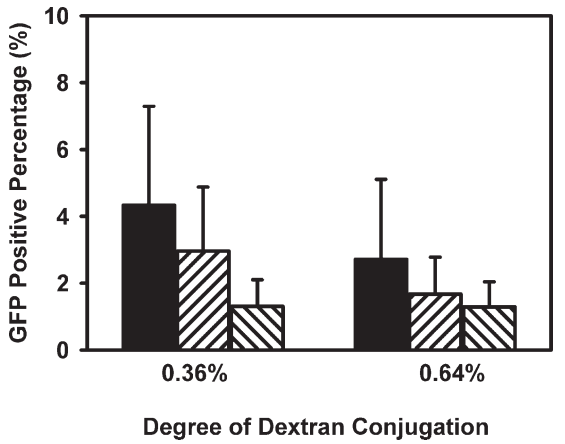

Figure 3 Transfection efficiency of using dex-PEI in the preparation of cell microarrays. PEI with two different degrees of dex-1.5K conjugation was used in preparing cell arrays. Compared with the reagents without any enhancers (solid bar), no enhancements were observed for the percentages of GFP-positive cells mediated by the transfection reagents containing PEG-2K (///) and gel-A (\\\) as enhancers.

transfect cells in cell arrays, suggesting that the requirements of vectors for high efficiency were different. The dex-PEI formed a looser structure of vector in comparison to unmodified bPEI.[17] Because in the typical transfection procedures cells have grown on the substrate already, the vectors can enter the cells after the attachments onto the cell membrane from the bulk solution. However, in cell arrays, because the vectors are immobilized within the substrate first, the entry of vectors into the cells requires the cells to settle down from the medium and then to attach on the substrate. The cellular entry of vectors requires proteoglycan, which might be partially digested by trypsin when the cells were harvested and prepared to seed onto cell arrays. It takes time to restore the proteoglycans on the cell membrane. For the vectors formed by dex-PEI, the loose structure tended to release DNA in a short period of time and could not be retained within the substrate long enough for the utilization of the recovered proteoglycans to enter the cells. The observations indicated that the capability of forming tighter complexes with DNA is requisite for the carriers to be used in cell arrays. Linear PEI consisting of linear repeating units was examined for its efficacy as a gene delivery vehicle in cell arrays. Despite that it has been shown to be a very efficacious carrier for gene delivery in cell culture and animals, using linear PEI completely abolished the appearance of GFP-positive cells (data not shown) presumably also due to the formation of loose structures with DNA.[18,19] All the effective enhancers in cell arrays failed to improve any efficiency when a typical transfection process was carried out in cell culture, indicating the differences in the transfection mechanisms of cell arrays and cell culture.

CONCLUSION

Branched PEI is a highly efficient agent that could be applied in the preparation of cell arrays. The efficiency of cells expressing the delivered gene exhibited strong dependence on the amount of DNA that was immobilized on the substrate. In the presence of PEG 2K or gelatin A, more than 70% of the cells grown on the top of each spot could express the delivered gene at a DNA concentration of 0.45 mg/mL. The high efficiency could allow a substantial extent of changes in the cellular function of the cells on the arrays and thus provide significant signals for detection and analysis.

References

1. Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. Nature 2001, 411, 494.
2. Crystal, R. C. Science 1995, 270, 404.
3. Mulligan, R. C. Science 1993, 260, 926.
4. Miller, A. D. Nature 1992, 357, 455.
5. Sturzl, M.; Konrad, A.; Sander, G.; Wies, E.; Neipel, F.; Naschberger, E.; Reipschlager, S.; Gonin-Laurent, N.; Horch, R. E.; Kneser, U.; Hohenberger, W.; Erfle, H.; Thurau, M. Comb Chem High Throughput Screen 2008, 11, 159.
6. Fiebitz, A.; Nyarsik, L.; Haendler, B.; Hu, Y. H.; Wagner, F.; Thamm, S.; Lehrach, H.; Janitz, M.; Vanhecke, D. BMC Genomics 2008, 9, 68.
7. Erfle, H.; Neumann, B.; Liebel, U.; Rogers, P.; Held, M.; Walter, T.; Ellenberg, J.; Pepperkok, R. Nat Protoc 2007, 2, 392.
8. Bailey, S. N.; Ali, S. M.; Carpenter, A. E.; Higgins, C. O.; Sabatini, D. M. Nat Methods 2006, 3, 117.
9. Vanhecke, D.; Janitz, M. Oncogene 2004, 23, 8353.
10. Silva, J. M.; Mizuno, H.; Brady, A.; Lucito, R.; Hannon, G. J. Proc Natl Acad Sci USA 2004, 101, 6548.
11. Delehanty, J. B.; Shaffer, K. M.; Lin, B. Anal Chem 2004, 76, 7323.
12. Baghdoyan, S.; Roupioz, Y.; Pitaval, A.; Castel, D.; Khomyakova, E.; Papine, A.; Soussaline, F.; Gidrol, X. Nucl Acids Res 2004, 32, e77.
13. Kumar, R.; Conklin, D. S.; Mittal, V. Genome Res 2003, 13, 2333.
14. Bailey, S. N.; Wu, R. Z.; Sabatini, D. M. Drug Discov Today 2002, S113, 7.
15. Ziauddin, J.; Sabatini, D. M. Nature 2001, 411, 107.
16. Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. Proc Natl Acad Sci USA 1995, 92, 7297.
17. Tseng, W. C.; Tang, C. H.; Fang, T. Y. J Gene Med 2004, 6, 895.
18. Kunath, K.; Von Harpe, A.; Fischer, D.; Petersen, H.; Bickel, U.; Voigt, K.; Kissel, T. J Controlled Release 2003, 89, 113.
19. Wightman, L.; Kircheis, R.; Rossler, V.; Carotta, S.; Ruzicka, R.; Kursa, M.; Wagner, E. J Gene Med 2001, 3, 362.