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The Mechanical Agitation Method of Gene Transfer for *Ex-Vivo* Gene Therapy

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1. Introduction

Gene therapy is a therapeutic method used to treat diseases by altering genes within a patient's cells. The concept of gene therapy emerged as molecular biology evolved from the mere discipline of studying DNA molecules to the scientific art of virtually manipulating the genes of cells. Explosive worldwide research was conducted after the first introduction of the concept of gene therapy into the scientific community. The original aim was to directly modify patient genes through *in vivo* gene therapeutic approaches. However, DNA molecules introduced into the body by *in vivo* gene therapy are delivered at a very low frequency into terminally differentiated tissue cells, which typically do not have the capability of self-renewal (Tenenbaum et al., 2003). Because of the short-lived nature of *in vivo* gene therapy, a defective gene in patients is only temporarily corrected by *in vivo* gene therapy (Kaloss et al., 1999). The development of gene therapeutic methods in which the corrected patient gene remains permanently has been actively pursued.

Ex vivo gene therapeutic methods have been considered as alternative options to gene therapy to overcome the short-lived nature of the corrected genes of *in vivo* gene therapy. In *ex vivo* gene therapy, the surgically removed adult stem cells, such as mesenchymal stem cells or hematopoietic stem cells, are typically cultured in a laboratory apparatus. The therapeutic DNA molecules are introduced into the isolated cells, and these transfected cells are then introduced into the patients. By using adult stem cells in *ex vivo* gene therapeutic methods, the corrected genes that are introduced are, in most cases, expressed permanently once they are corrected properly because the adult stem cells have the capability of self-renewal (Dube & Denis, 1995; Muller-Sieburg & Sieburg, 2006; Tseng et al., 2006; Nehlin & Barington 2009). In *ex vivo* gene therapy, genetic manipulation is conducted in a lab outside of the body. However, normal somatic cells, including adult stem cells, do not propagate indefinitely and are vulnerable to epigenetic modification. Therefore, long-term cultures of somatic cells isolated from the body are very difficult to sustain (Beyer & Da sliva, 2006; Tonti & Mannello, 2008). This means that the long-term culture of adult stem cells in *ex vivo* gene therapy should be avoided as much as possible. Therefore, it is absolutely necessary to deliver therapeutic DNA molecules into isolated cells immediately with high efficiency.

In current gene transfer protocols, gene delivery vehicles containing therapeutic DNA molecules make only limited contacts with their target cells by passive diffusion, thereby limiting the chances of gene delivery. In our lab, we developed a very efficient method to deliver therapeutic genes to adult stem cells based on mechanical agitation (Park et al., 2009).

In this method, mechanical agitation of the gene delivery vehicles containing cell suspensions increases the movement of gene delivery vehicles and target cells, resulting in an increase in contact between them. The application of our mechanical agitation method to the gene delivery process of *ex vivo* gene therapy, both in transfection and transduction, has increased the gene transfer efficiency more than that of any other previously known gene transfer protocol.

2. Basic principles of current gene therapeutic approaches

Gene therapy is classified as somatic gene therapy or germ line gene therapy. The application of current molecular genetic techniques used during the manipulation of transgenic or knock-out animals would definitely make gene therapy possible in virtually any type of germ line. However, all civilized societies in the world currently legally prohibit any attempts to genetically modify embryos. Thus far, gene therapy essentially implies somatic gene therapy. Compared to the easy genetic manipulation of embryonic stem cells, the genetic manipulation of somatic cells, including adult stem cells, is limited such that none of the somatic gene therapies are used practically thus far. Therefore, it is not surprising that the main quest of current gene therapy is to improve the efficiency of genetic manipulation in gene therapy, and the future success of gene therapy depends on the efficiency of genetic manipulation.

Genetic manipulation in gene therapy can be achieved by two different approaches: direct genetic manipulation of somatic cells in the body and genetic manipulation of autologous cells outside of the body. These two different strategies for gene delivery are termed *in vivo* and *ex vivo*, respectively. In the *in vivo* strategy, therapeutic genes are delivered into cells *in situ* using a variety of vectors to produce therapeutic proteins in specific sites in the body. In *ex vivo* gene therapy, genetically modified autologous cells are surgically implemented into the body. The *ex vivo* and *in vivo* gene therapies both have positive and negative aspects. Although gene therapy has been a very hot topic in biomedical science for several decades, it is still in its infancy, and a number of hurdles must be overcome to achieve the practical application of gene therapy to patients.

2.1 *In vivo* gene therapy

In vivo gene therapy is a process in which a therapeutic gene is delivered through a vector directly into the target cells of patients to produce a therapeutic effect that prevents or treats diseases (Fig. 1). Theoretically, once an ideal gene delivery vehicle for a therapeutic gene transfer is developed, the *in vivo* gene therapy should involve a very simple procedure: the injection of a solution containing the gene delivery vehicle into the body. Because of this potentially easy treatment procedure in clinics, *in vivo* gene therapy is considered the preferred gene therapeutic method than *ex vivo* gene therapy. However, *in vivo* gene therapy has a basic and fundamental problem in the delivery of therapeutic genes to target cells: the low efficiency of gene transfer.

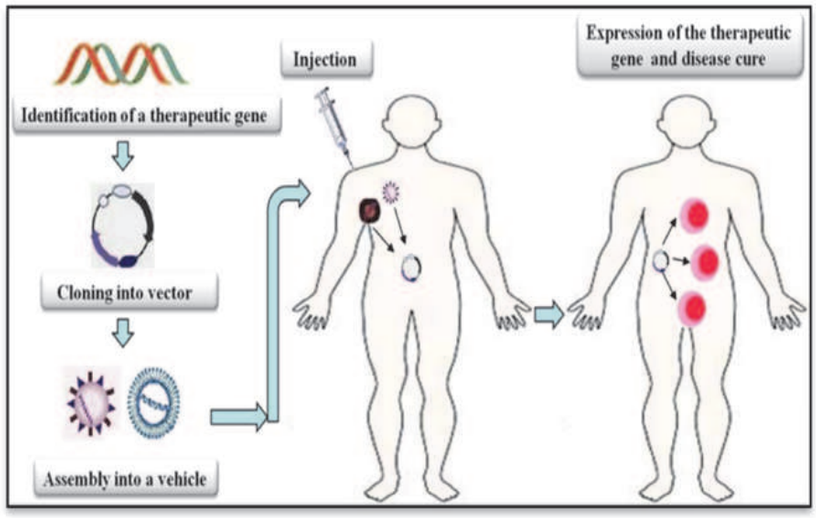


Fig. 1. Strategies for *in vivo* gene therapy. *In Vivo* gene therapy involves introduction of therapeutic DNA directly into the patient body. The DNA is introduced by cell–specific direct injection into tissue in need. Once inside the body and in contact with the specifically targeted cells, the inserted DNA is incorporated into the tissue cells where it encodes the production of the needed protein.

The bottleneck in development of *in vivo* gene therapeutic methods has been the development of an efficient method for delivery of a therapeutic gene into the target cells of the body. The main reason for poor gene delivery efficiency in *in vivo* gene therapy is rooted to the nature of the body. The cells in the body are typically surrounded by an extracellular matrix that usually provides structural support to the cells in addition to performing various other important functions (Fig. 2). The main constituent of the body is the extracellular matrix, not cells (Suki & Jason, 2008). For example, collagen proteins, which are one of the components of the extracellular matrix, constitute approximately 25-35% of the protein content of the entire body, implying that the extracellular matrix occupies the main volume of the body (Khan et al., 2009). The injected gene delivery vehicles must pass through the extracellular matrix to deliver therapeutic proteins into target cells in *in vivo* gene therapy. However, because the extracellular matrix spatially occupies such a large portion of the body, there is an unsolvable limitation for efficient gene transfer in *in vivo* gene therapy.
In addition to the low efficiency of gene delivery, *in vivo* gene therapy has another problem. The gene transfer vector is obligatorily exposed to the immune system of the body. This exposure causes an immune response that blocks gene delivery entirely. Overall, the potential immune response is another factor contributing to the low efficiency of gene delivery in *in vivo* gene therapy. Therefore, development of an ideal gene delivery vehicle for *in vivo* gene therapy is so extremely challenging that, until now, none of the *in vivo* gene therapeutic methods have not a satisfactory result.

2.2 *Ex vivo* gene therapy

In *ex vivo* gene therapy, cells are removed from a patient, maintained in culture to introduce a therapeutic gene into the cells, and then transplanted into the patient (Fig. 3). The role of the transplanted cells, which are genetically modified, is to deliver a recombinant gene

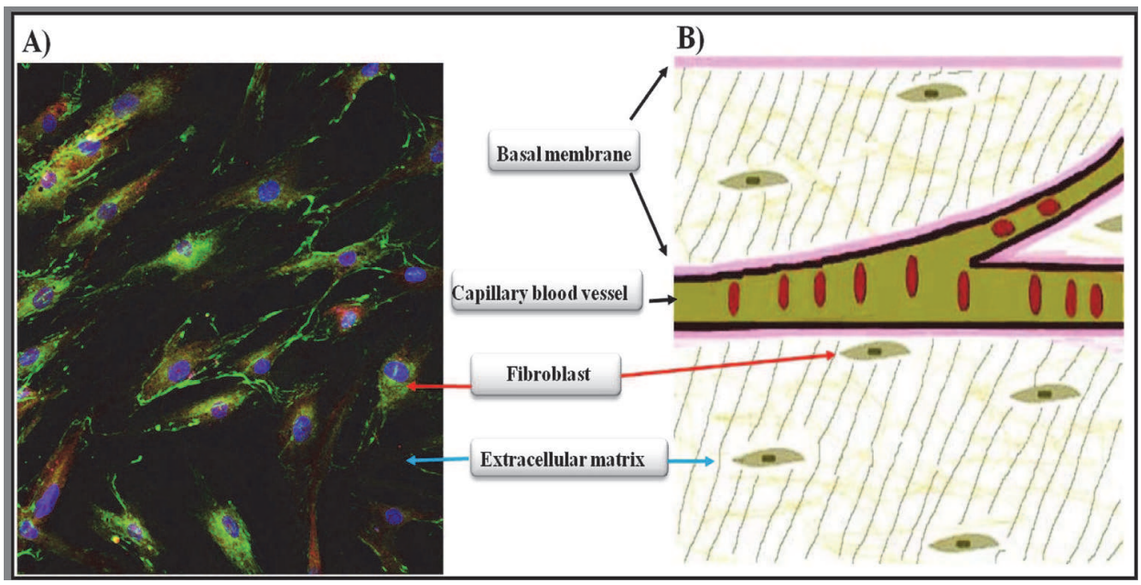


Fig. 2. Typical Anatomical Structure of Connective Tissue. A) The Confocal Microscopic Image of a Mouse Connective Tissue. B) Schematic Illustration Depicting Extracellular Matrix. The extracellular matrix (ECM) is the extracellular part of animal tissue that usually provides structural support to the animal cells in addition to performing various other important functions. The extracellular matrix is the defining feature of connective tissue in animals.

product into the patient's body. The genetically modified cells are not required to reconstitute a particular organ or tissue for the purpose of reimplementation of the cells in a location where the cells were originally obtained. For example, genetically modified hepatocytes harvested from one liver lobe may be re-infused throughout any part of the liver of patients in *ex vivo* gene therapy.

The main disadvantage of *ex vivo* gene therapy is that it requires the surgical removal of cells from the body and transplantation of the cells back to the body. These surgical steps are very painful. However, *ex vivo* therapy has several advantages over *in vivo* gene therapy. First, the efficiency of gene transfer into the targeted cells is very high compared to *in vivo* gene therapy because gene delivery is performed under controlled, optimized conditions. Second, the transduced cells can be enriched if the vector has a selectable gene marker. Third, the immunological side effects that are caused by gene delivery vehicles in *in vivo* gene therapy are usually minimized in *ex vivo* gene therapy.

2.3 *Ex-vivo* gene therapy as a practical option to correct a defective gene permanently

In vivo gene therapy introduces the therapeutic genes directly into the patient by intravascular injection. Because this approach is much simpler and less technically demanding than *ex vivo* gene therapy, which requires two surgical steps, the science of *in vivo* gene therapy has been preferentially developed. However, as discussed above, the nature of the mammalian body imposes innate, unsolved problems for *in vivo* gene therapy to achieve gene expression at therapeutically effective levels. The limitation of *in vivo* gene therapy for practical application is mainly due to low efficiency gene transfer. Because genetic manipulations are conducted in a lab in *ex vivo* gene therapy, the application of

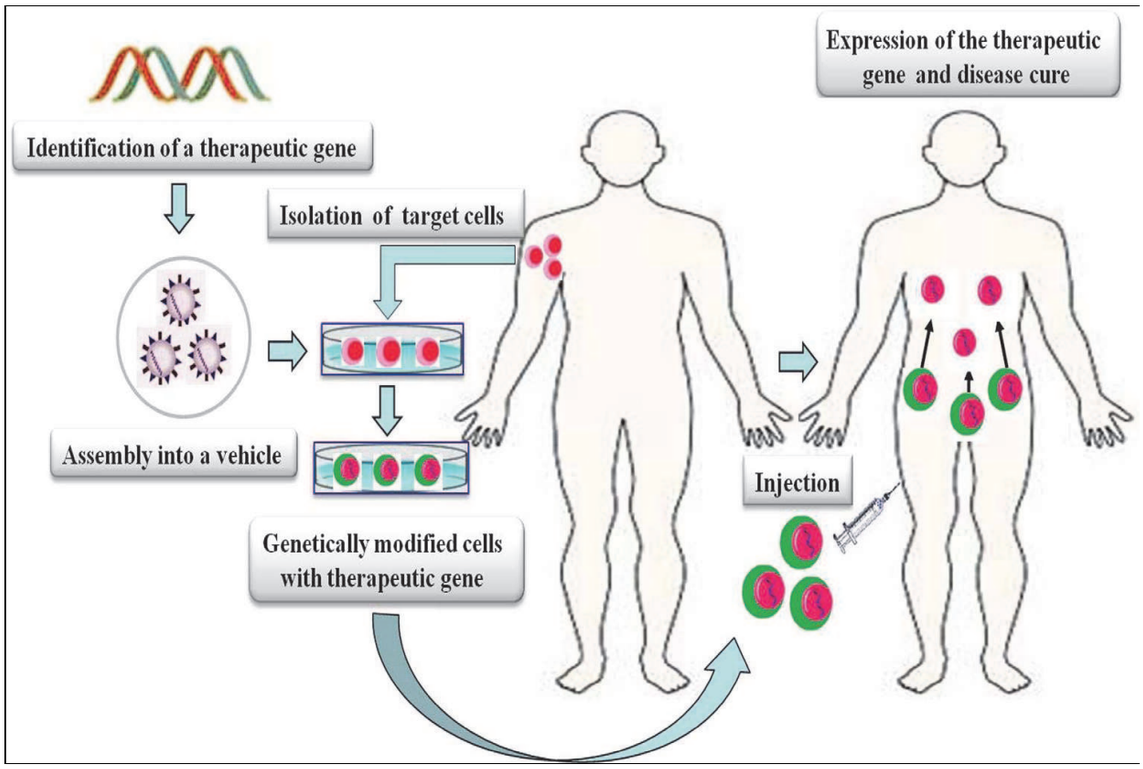


Fig. 3. Strategies for *Ex Vivo* Gene Therapy. *Ex vivo* gene therapy is performed with the genetic alterations of patients target cells happening outside of the body in as culture. Target cells from the patient are infected with a recombinant virus containing the desired therapeutic gene. These modified cells are then reintroduced into the patient body, where they produce the needed proteins that correspond to the inserted gene.

current molecular biological techniques can solve the low efficiency of gene transfer. *Ex vivo* gene therapy has the potential to ultimately solve this major problem of gene therapy, and it could be practically used in clinics. However, normal somatic cells, including adult stem cells, do not only propagate well in a typical cell culture environment and are also vulnerable to epigenetic modification (Islam et al., 2007; Martinez-Climent et al., 2006), requiring that the transfer of therapeutic genes to the isolated cells be performed as soon as possible. Therefore, one of the key factors for the success of *ex vivo* gene therapy is to deliver therapeutic DNA molecules into isolated cells promptly with high efficiency. If these problems could be solved successfully, the *ex vivo* technique could be practically applied to patients in the near future.

3. Current methods for gene delivery in *ex vivo* gene therapy

As the name implies, the success of gene therapy depends on introducing therapeutic genes into target cells with high efficiency. Since Friedmann and Roblin formulated the concept of gene therapy in 1972 (Friedmann & Roblin, 1972), the biggest challenge in gene therapy has been the development of a method to deliver therapeutic genes to target cells with high efficiency. Although gene delivery in *in vivo* gene therapy is much easier than in *ex vivo* gene therapy, gene delivery into primary cells of *in vitro* cell cultures is also quite difficult.

Typical efficiencies of gene delivery to primary cells are 5-10% in most current methods (Cai et al., 2002; Ding et al., 1999; Eiges et al., 2001; Lakshmipathy et al., 2004; Peister et al., 2004), which is not high enough for satisfactory *ex vivo* gene therapy. Because of this, many different methods of gene delivery have been developed using primary cells for *ex vivo* gene therapy. Generally, gene delivery methods can be divided into two categories, viral and non-viral.

3.1 Viral methods for gene delivery in *ex vivo* gene therapy

Viruses have evolved specialized molecular mechanisms to efficiently transport their genomes into cells. Viral vectors have developed by taking advantage of the molecular mechanisms of the virus to deliver exogenous DNA into target cells. Currently, viral vectors are frequently used molecular biology tools for delivery of genetic material into cells of a living organism (*in vivo*) or in cell culture (*in vitro*). Viral vectors are tailored to their specific applications but share a key property. Because viral vectors are essentially created from pathogenic viruses, they are modified to minimize the pathogenic properties of the original viruses. This usually involves the deletion of a portion of the viral genome that is critical for viral replication. Such viral vectors can efficiently infect cells but once the infection has taken place, they cannot replicate. The viral vectors require helper genes to provide the missing proteins for production of new virions. Replication of viral vectors is usually conducted in packaging cells that were engineered with helper genes. Therefore, viral vectors can only replicate in packaging cells and exist solely to deliver exogenous DNA to target cells where the viral vector cannot replicate.

Table 1 summarizes the types of viral vectors currently developed. In general, viral vectors are very efficient in terms of gene delivery into target cells. However, viral vectors have common problems including the following: (i) a limited DNA capacity, (ii) expression of viral genes, (iii) initiation of the antiviral immune response, (iv) reversion to a replication competent state and (v) decreasing expression over time.

Viral vectors can be classified as DNA or RNA viral vectors. DNA viral vectors are derived from viruses such as adenovirus or herpes virus, which carry their genetic material in the form of DNA. Because these viral vectors persist as an extrachromosomal element after delivery into target cells, the viral vectors remain only temporarily. One advantage of the episomal presence of the vectors is that the gene expression level is high. Because exogenous genes cannot stay indefinitely, these vectors are not suitable for *ex vivo* gene therapy. Currently, these vectors are mostly used for *in vivo* gene therapy.

Retroviral and lentiviral vectors are examples of RNA viral vectors that are replicated in target cells via reverse transcriptase to produce DNA from their RNA genomes. The DNA is then incorporated into the host's genome by an integrase. Thereafter, the virus replicates as part of the host cell's DNA, permitting long-term expression of the exogenous gene and ensuring transmission of the exogenous gene to the progeny of transduced cells. Therefore, these vectors are suitable for *ex vivo* gene therapy in which permanent gene expression is required in *in vitro* cell culture. These vectors are also widely used for *in vivo* gene therapy.

3.2 Non-viral methods for gene delivery in *ex vivo* gene therapy

Gene therapy was originally devised for the treatment of inherited genetic diseases, such as hemophilia and cystic fibrosis. However, the realm of gene therapy has been expanding to develop strategies for cancer, infectious diseases like HIV, and various complex diseases,

Vector	Genetic material	Packaging capacity	Tropism	Inflammatory potential	Vector genome forms	Main Advantages
Retrovirus	RNA	8 kb	Dividing cells	Low	Integrated	Persistent gene transfer in dividing cells
Adenovirus	dsDNA	7.5 kb	Dividing and non-dividing cells	High	Non-integrated	Extremely efficient transduction of most tissues
Adeno-associated virus	ssDNA	< 5kb	Dividing and non-dividing cells	Low	Non-integrated (90%) Integrated (>10%)	Non-inflammatory; non-pathogenic
Lentivirus	RNA	8 kb	Dividing and non-dividing cells	Low	Integrated	Persistent gene transfer in most tissues
Herpes virus	dsDNA	> 30 kb	Dividing and non-dividing cells	High	Non-integrated	Large packaging capacity; strong tropism for neurons

Table 1. A comparison of different viral vectors used for gene therapy

such as diabetes, dementia and hypertension. Genetic manipulations for these diseases are more complicated than genetic manipulations for the treatment of inherited genetic diseases. This means that current gene therapies need to deliver DNA, RNA, siRNA, or antisense sequences that alter gene expression within a specific cell population to manipulate cellular processes and responses. Viral vector-mediated gene deliveries are by far the most effective means of DNA delivery. However, the recombinant vector containing the therapeutic gene has to be packaged with viral coat proteins to make gene delivery possible, meaning that viral vector-mediated gene deliveries are limited to a DNA molecule of a certain size because the viral coat proteins have a limited DNA carrying capacity. Other than the physical limitation of viral vector-mediated gene deliveries, there are more limitations, such as immunotoxicity caused by viral coat proteins, restricted targeting of specific cell types, and recombination. Therefore, non-viral gene deliveries have been a very popular research topic, and many interesting and creative methods have been developed. The efficiency of gene delivery (*i.e.* transfection efficiency) is crucial to the success of non-viral gene deliveries. Various non-viral gene delivery methods currently developed could be classified into two groups: physical gene delivery methods and chemical gene delivery methods.

3.2.1 Physical gene delivery methods

Physical gene delivery methods are methods for transferring DNA molecules from the surrounding medium into cells. Naked DNA (*i.e.,* an uncomplexed form of DNA) is used in

physical gene delivery methods. The easiest method to deliver genes into cells is to draw naked DNA into a microneedle and then inject the microneedle into cells to transfer the naked DNA directly to the cells. Though gene transfer efficiency by this method is very efficient, the method is very slow and laborious. The main drawback of this method is that microinjection can be only performed on one cell at a time, which means that this approach cannot be used for typical gene therapeutic approaches. The approach is limited to use for gene delivery into germ-line cells to produce transgenic organisms.

Currently, the most popular physical methods for gene delivery into cells are electroporation and sonoporation. The cellular membrane is punctured by an electric pulse (electroporation) (Neumann et al., 1982) or ultrasonic wave (sonoporation) (Yizhi et al., 2007). The pores in the cellular membrane are only temporarily formed, and DNA molecules pass through during the short period of time when the pores open. These methods are generally efficient and work well across a broad range of cell types. However, a high rate of cell death limits their use, especially in gene therapy. These methods are widely used for gene delivery of immortal cells in which cell viability is not a critical issue during gene transfer.

Another popular method for physical gene delivery is the use of particle bombardment. In this method, gold particles (gene gun) (Gan et al., 2000) or magnetic particles (magnetofection) (Scherer et al., 2002) are coated with naked DNA. In the gene gun method, the DNA-coated gold particles are shot into the cell using high pressure gas, and the particles pass through the cellular membrane to introduce the particles inside the cells. In the magnetofection method, a magnet is placed underneath the tissue culture dish to attract DNA-coated magnetic particles. Then, the DNA-coated magnetic particles come into contact with a cell monolayer to introduce the particles inside the cells. These methods yield reasonably high efficiency gene transfers, but do not yield better efficiencies compared to other non-viral gene transfer methods, despite the requirement for expensive equipment. Also, it is quite difficult to control the DNA entry pathway, and the metal particles in the cells following gene transfer could negatively affect cells. Therefore, these methods are not widely used.

3.2.2 Chemical gene delivery methods

Because DNA cannot pass through cellular membranes alone, various chemicals have been designed to aid the transfer of therapeutic genes into cells. The chemicals used in chemical gene delivery function to enhance the stability of the DNA molecule, to increase the efficiency of cellular uptake and intracellular trafficking, or to alter the distribution of the transferred DNA in the cells. These methods are very successful in terms of transferring genes into cells and are currently the most widely used methods. Also, chemical gene delivery methods are the easiest and most effective among various non-viral gene delivery methods developed thus far.

The most well-studied and effective approach for non-viral gene delivery is the use of cationic lipids. Positively charged cationic lipids naturally bind to negatively charged DNA in solution to condense DNA so that the DNA molecules and cationic lipids form complexes called lipoplexes. After lipoplexes are formed, the positively charged cationic lipids of the lipoplexes interact with cell membranes to allow cells to take up the lipoplexes by endocytosis. In typical cell physiology, endosomes that are formed as the result of endocytosis will fuse with lysosomes to degrade the lipoplexes containing the DNA. An

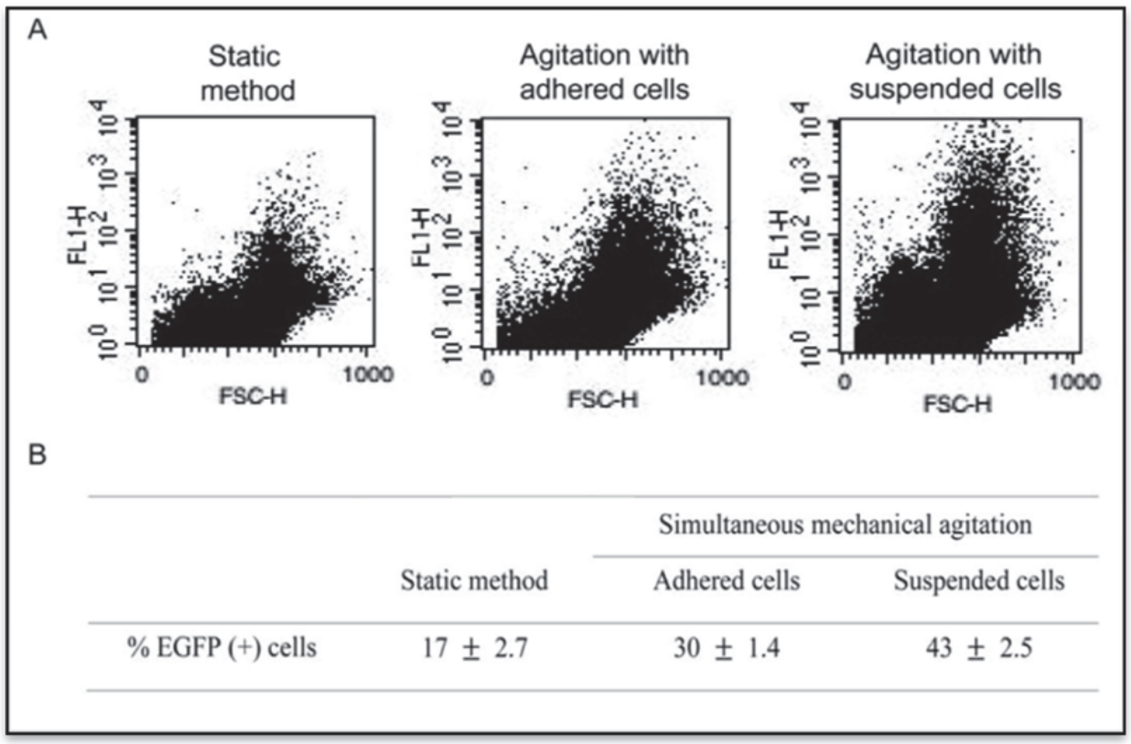
exogenous gene in the lipoplexes would not have a chance to be released into the cytoplasm for gene expression if the endosomes are stable. Therefore, helper lipids are added to form lipoplexes to facilitate the endosomal escape of the exogenous gene (Herringson et al., 2009a, 2009b; Savva et al., 2005). This approach is very successful because it increases the transfection efficiency dramatically. There are various combinations of cationic lipids and helper lipids available. More than 40 products are commercially available for cationic lipid-based gene delivery, including LipoTAXI (Agilent Technologies), Lipofectamin™ (Invitrogen), NanoJuice® (Merck), Transfectam® (Promega), and LipoJet ™ (SignaGen Laboratories). The cationic lipid-based gene delivery shows a very high transfection efficiency of up to 90 in *in vitro* cell culture. Because the cationic lipid-based lipoplexes are not stably maintained in the blood, these methods are best for *ex vivo* gene therapy. However, cationic lipid-based lipoplexes show a very poor transfection efficiency with primary cells, such as stem cells, indicating that new methodological developments are required for the practical application of *ex vivo* gene therapy.

Other than cationic lipids, several different positively charged materials are used as a base material for non-viral DNA delivery, such as cationic polymers (Segura & Shea, 2001), cationic peptides consisting of poly-L-Lysine (D'Haeze et al., 2007; Mullen et al., 2000; Niidome et al., 1997), or other types of cationic proteins (De Lima et al., 1999; Jean et al., 2009; Lam et al., 2004; Lee et al., 2003; Oliveira et al., 2009; Vighi et al., 2007). These approaches produce DNA carrying complexes that are more stable. However, the transfection efficiency of this method is not better than cationic lipid-based lipoplexes. Therefore, most of these methods are designed for *in vivo* gene therapy.

4. Application of the mechanical agitation method to *ex vivo* gene therapy

One of the main obstacles for the application of adult stem cells in *ex vivo* gene therapy is the low efficiency of gene transfer to these cells. For example, electroporation or transfection in mesenchymal stem cells yields 5-10% gene delivery efficiency (Cai et al., 2002; Ding et al., 1999; Eiges et al., 2001; Lakshmipathy et al., 2004; Peister et al., 2004). Therefore, improved gene delivery methods would potentially be very beneficial for the practical application of *ex vivo* gene therapy in patient care. In current gene transfer protocols, virus particles or lipoplexes passively diffuse through the liquid culture medium to reach their target cells, which are layered on the bottom of a culture dish (Chuck & Palsson, 1996). Because the virus particles or lipoplexes contact the target cells by passive diffusion, increasing the chance of contact between virus particles or lipoplexes and their target cells would increase the chance of gene transfer and to promote higher transfer efficiencies. One simple way to increase contact between viruses or lipoplexes and target cells is through mechanical agitation. Based on this hypothesis, we developed a mechanical agitation method for retroviral transduction of primary cells or transfection by lipoplexes (Park et al., 2009). In this method, we simply implemented a step in which virus-containing or lipoplexes-containing cell suspensions are agitated to increase the movement of viruses or lipoplexes and target cells with the purpose of generating more frequent contact between them. Suspended target cells have a better chance of making physical contact with virus particles or lipoplexes than adherent target cells because of the possibility for three-dimensional contact between the cells and viruses or lipoplexes. The simple addition of the mechanical agitation step to the conventional transduction or transfection protocol increased gene transfer efficiency two-fold above the current rates these protocols (Fig. 4). In the following

section, we describe one example of retroviral transduction using our mechanical agitation protocol.



B

	Static method	Simultaneous mechanical agitation	
		Adhered cells	Suspended cells
% EGFP (+) cells	17 ± 2.7	30 ± 1.4	43 ± 2.5

Fig. 4. A Typical Example of Application of Machanical Agitation Method to Transduction of EGFP-Carrying Retrovirus (Park et al., 2009). (A) The representative FACS plots of rat mesenchymal stem cells after transduction with Retro-EGFP using the static method (left panel), mechanical agitation of viruses with adhered cells (middle panel) and simultaneous mechanical agitation of retroviruses with suspended cells (right panel). (B) Numerical representation of the transduction efficiencies of EGFP retrovirus under the static protocol versus the new agitation protocol. The transduction efficiency is defined as the percentage of cells expressing EGFP as measured using FACSCalibur. The mean percentage of GFP-positive cells is presented as the average of three independent transduction experiments (+/- SEM, n=3).

4.1 Materials

1. Packaging cell line PT67 (Clontech).
2. pCAG-EGFP expression vector (Addgene, USA) and pMSCVneo retroviral vector (Clontech, USA).
3. Lipofectamine 2,000 (Invitrogen).
4. Rat mesenchymal stem cells (isolated by flushing the femurs of two-month-old female SD rats (Damool Bioscience Co., Korea).
5. DMEM/ High glucose (Hyclone, USA) with 10% FBS (Hyclone), 2 mM L-glutamin (Invitrogen, USA), 100 µg/ml streptomycin, and 100 U/ml penicillin (Sigma, USA).
6. Selection drug, G418 (Sigma) and Polybrene (Calbiochem, USA).
7. Rat mesenchymal stem cell characterization kit (Millipore,USA).

8. TrypLE Express (Invitrogen).
9. Carl Zeiss LSM510 Meta microscope.
10. 0.45 μm cellulose acetate filter (Millipore).
11. Rocker (SLS4, Seoulin, Korea).
12. Incubator at 37°C under 5% CO_2.
13. 24-well plates and 96-well plates.
14. E-Max micro-well reader (Molecular Devices, USA).
15. Fluorescence microscope (TE2000-S, Nikon, Japan).
16. FACSCalibur instrument (Becton Dickinson, USA).

4.2 One example protocol of retroviral transduction into mesenchymal stem cells

1. Trypsinize pure rat mesenchymal stem cells (0.1 ml/cm^2) for 3 min at 37°C.
2. Adjust the cell suspension to contain 5×10^5 cells/ml.
3. Mix a 1 ml aliquot of the trypsinized rat mesenchymal stem cells directly with 1 ml virus stock in the presence of 6 μg/ml polybrene (Calbiochem, USA).
4. Seed the mixing solution in a six-well plate (Falcon, USA).
5. Mechanically agitate the plate containing the mixture of rat mesenchymal stem cells and virus on a rocker (SLS4, Seoulin, Korea) at 20 rpm for 50 min while incubating at 37°C under 5% CO_2.
6. Incubate the plate at 37°C under 5% CO_2 for 24 h.
7. Replace the supernatant containing virus particles with fresh growth medium.
8. Observe EGFP fluorescence in the transduced rat mesenchymal stem cells with a fluorescence microscope (TE2000-S, Nikon, Japan) after an 86 h incubation.

5. Conclusion and prospect

Since the first clinical trial of gene therapy in 1990, 1703 gene therapy clinical trials have been completed as of March of 2011 (http://www.wiley.com/legacy/wileychi/genmed /clinical/). However, little progress has been made since the first gene therapy clinical trial, and therefore, the Food and Drug Administration of the United States has not yet approved any human gene therapy for actual patient treatments. Although current gene therapy is still in the experimental stage, *ex vivo* gene therapeutic approaches show a great potential to treat monogenic genetic diseases as shown in the clinical trial results of adenosine deaminase deficiency and familial hypercholesterolemia (Cappelli et al., 2010 & Kassim et al., 2010). The clinical trial results of these diseases were encouraging to continually pursue *ex vivo* gene therapy.

Primary cells, including adult stem cells, have limited self-renewal ability and are vulnerable to epigenetic modification (Dube & Denis, 1995; Muller-Sieburg & Sieburg, 2006; Tseng et al., 2006; Nehlin & Barington, 2009). The long-term culture of primary cells is not possible. Therefore, it is absolutely necessary to deliver therapeutic DNA molecules into isolated cells promptly with high efficiency. However, the transfer efficiency of exogenous DNA into primary cells is very low (Beyer & Da sliva, 2006; Tonti & Mannello, 2008). The gene transfer efficiency into primary cells is several fold less than those of cell lines in current gene transfer methods. This means that improvement of the transfer efficiency of exogenous DNA into primary cells is the first obstacle for the practical use of for *ex vivo* gene therapy.

The mechanical agitation method discussed here led to a higher efficiency gene transfer, either in transfection using lipoplexes or transduction, than that of any current static transduction method. This method can potentially be applied to a variety of current transduction or transfection protocols with slight adjustments to the agitation time and speed. We believe that this protocol will contribute to various *ex vivo* gene therapies and *in vitro* gene transfer experiments for primary cells.

6. Acknowledgement

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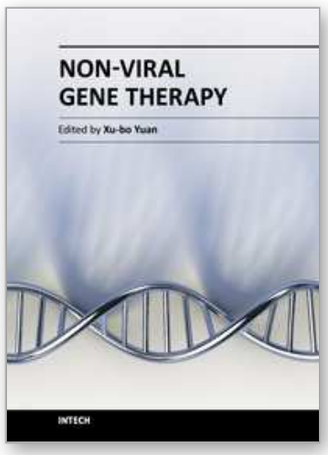

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This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

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