Gene Transfer Technique

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Abstract: This article is describing the nine principle techniques for the gene transfection, which are: (1) lipid-mediated method; (2) calcium-phosphate mediated; (3) DEAE-dextran-mediated; (4) electroporation; (5) biolistics (gene gun); (6) viral vectors; (7) polybrene; (8) laser transfection; (9) gene transfection enhanced by elevated temperature, as the references for the researchers who are interested in this field. [Nature and Science. 2005;3(1):25-31].

Key words: DNA; gene; technique; transfer

1 Introduction

Gene transfer is to transfer a gene from one DNA molecule to another DNA molecule. Gene transfer represents a relatively new possibility for the treatment of rare genetic disorders and common multifactorial diseases by changing the expression of a person's genes (Arat, 2001). In 1928, Griffith reported that a nonpathogenic pneumoccocus strain could become pathogenic when it was mixed with cells of heat-killed pathogenic pneumoccocus, which hinted that the pathogenic genetic material could be transformed from the heat-killed pathogenic pneumoccocus to the nonpathogenic strain (Griffith, 1928). This is the first report for gene transfer observation. However, the transforming substance was not identified in these experiments. Up to 1944, Avery et al demonstrated that deoxyribonucleic acid (DNA) was the transforming substance (Avery, 1944). In 1952, Hershey and Chase showed that DNA was the only material transferred during bacteriophage infection, which suggested that the DNA is the genetic material (Hershey, 1952).

The basic technique for introducing DNA into E. coli have inspired procedures for the introduction of DNA into cells from a wide variety of organisms, including mammalian cells. Genetic engineering of food is the science which involves deliberate modification of the genetic material of plants or animals. Introduction of DNA into plants is of great agricultural potential and medical importance (Campbell, 1999; Uzogara, 2000; Lorence, 2004).

The gene transfer methods normally include three categories: 1. transfection by biochemical methods; 2. transfection by physical methods; 3. virus-mediately transduction. The gene transfer results can be transient and stable transfection.

Gene therapy can be defined as the deliberate transfer of DNA for therapeutic purposes. Many serious diseases such as the tragic mental and physical handicaps caused by some genetic metabolic disorders may be healed by gene transfer protocol. Gene transfer is one of the key factors in gene therapy (Matsui, 2003), and it is one of the key purposes of the clone (Ma, 2004).

Gene transfer can be targeted to somatic (body) or germ (egg and sperm) cells. In somatic gene transfer the recipient's genome is changed, but the change will not be passed on to the next generation. In germline gene transfer, the parents' egg and sperm cells are changed with the goal of passing on the changes to their offspring. Germline gene transfer is not being actively investigated, at least in larger animals and humans (Bordignon, 2003; Umemoto, 2005).

2 Transient and Stable Transfection

2.1 Transient transfection

In transient transfection, the transfected DNA is not integrated into host chromosome. DNA is transferred into a recipient cell in order to obtain a

temporary but high level of expression of the target gene.

2.2 Stable transfection

Stable transfection is also called permanent tran sfection. By the stable transfection, the transferred DNA is integrated (inserted) into chromosomal DNA and the genetics of recipient cells is permanent changed.

3 Transfection Methods

Generally, there are 9 ways for gene transfer: (1) Lipid-mediated method; (2) Calcium-phosphate mediated; (3) DEAE-dextran-mediated; (4) Electroporation; (5) Biolistics; (6) Viral vectors; (7) Polybrene; (8) Laser transfection; (9) Gene transfection enhanced by elevated temperature (Sambrook, 2001).

3.1 Lipid-mediated method

This method can be used for both transient and stable transfection, and it can be used for adherent cells, primary cell lines, and suspension cultures. For the following protocol, the Lipofectamine Reagent from Invitrogen Corporation will be used. Lipofectamine Reagent is a 3:1 (w/w) liposome formulation of the plycationic lipid 2,3-dioleyloxy-N-[2(sperminecardoxido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water (Catalogue Number 18324, Invitrogen Corporation, Carlsbad, California, USA) (Hawley-Nelson, 1993; Shih, 1997).

(1) Put about 40,000 cells per well of a 24-well plate in 0.5 ml of the appropriate complete growth medium (add 10% serum if it needs).

(2) Incubate the cells at 37^{o}C in a CO_2 incubator until the cells are 50-80% confluent (about 20 hours, depending on the cells).

(3) Dilute 3 μg DNA into 25 μl medium without serum for each well and mix.

(4) Dilute 3 μl Lipofectamine Reagent into 25 μl medium without serum for each well and mix.

(5) Combine diluted DNA (Step 3) and Lipofectamine Reagent (Step 4) and incubate at room temperature for 30 min. In this step the DNA-liposome complexes are formed.

(6) Replace the medium in the cells with 0.2 ml transfection medium without serum.

(7) Add 0.15 ml medium without serum to the tube containing the complexes for each well.

(8) Incubate the cells with the complexes for about 10 hours at 37^{o}C in a CO_2 incubator. The incubating time will be flexible by the cell type.

(9) Add 0.4 ml growth medium containing double the 2× normal concentration of the serum without removing the transfection mixture.

(10) Replace the medium with fresh, complete medium at 20 hours following the start of transfection if continued cell growth is required.

(11) Assay cell extracts for transient gene expression at 24-72 hours after transfection, depending on the cell type and promoter activity.

(12) To obtain stable transfectants, passage the cells 1:10 into the selective medium after 72 hours of transfection for the reporter gene transfected.

3.2 Calcium-phosphate mediated

To get a better description, the following protocol is using the human interleukin-2 gene transfer into cultured rat myocytes as the example manual.

3.2.1 Rat heart muscle cells are primarily cultured:

(1) Adult rats are sacrificed by decapitation with a decapitator.

(2) Rat hearts are moved out and left atria are isolated under sterile condition.

(3) Tissue is transferred to a fresh sterile phosphate buffered solution (PBS) and rinse.

(4) Transfer to a second dish and dissect off unwanted tissue such as fat or necrotic material and chop finely with crossed scalpels to about 1 mm cubes.

(5) Transfer by pipette (10 – 20 ml with wide tip) to a 15-ml sterile centrifuge tube.

(6) Wash by resuspending the pieces in PBS, transfer the chopped pieces to the trypsinization flask, and add 1 ml trypsin solution (0.25%) per 100 mg tissue. Incubate the tissue in trypsin solution for 12 hours at 4^{o}C then wash with PBS for 3 times.

(7) Add 1 ml trypsin solution (0.25%) per 100 mg tissue, with 1 mg/ml elastase and 1 mg/ml collagenase then stir at about 200 rpm for 30 min at 36.5^{o}C.

(8) Allowing the pieces to settle, collect supernatant, centrifuge at approximately 500 g for 5 min, resuspending pellet in 10 ml medium with 10% serum (FBS) (Gibco BRL Life Technologies, Inc., Grand Island, NY, USA), and store cells on ice.

(9) Add fresh trypsin to pieces and continue to stir and incubate for a further 30 min. Repeat steps 6 – 8 until complete disaggregation occurs or until no further disaggregation is apparent.

(10) Collect and pool chilled cell suspensions, and count by hemocytometer.

(11) Dilute to 10^6 per ml in growth medium and seed as many flasks as are required with approximately 2 x 10^5 cells per ml or set up a range of concentrations from about 10 mg tissue per ml.

(12) Put into CO2 incubator with 36.5oC.

(13) Culture medium used is Medium 199 with 10% FBS. All the solutions used contain 0.1 mg/ml of anti-biotic ampicillin (Sigma, St Louis, MO, USA).

3.2.2 Bacteria Culture (Sambrook, 1989; Frederick, 1992)**:**

(1) Growth of *E. coli*: Dissolve *E. coli* in 0.3 ml LB plus tetracycline (2 mg/ml) medium, transfer it into a tube containing 5 ml LB plus tetracycline (2 mg/ml) medium, 37°C overnight, then freeze it at -70°C.

(2) Harvesting E. coli:

A. Streak an inoculum across one side of a plate. Resterilize an inoculating loop and streak a sample from the first streak across a fresh part of plate, then incubate at 37°C until colonies appear (overnight).
B. Transfer a single bacterial colony into 2 ml of LB medium containing tetracycline (2 mg/ml) in a loosely capped 15-ml tube. 37°C overnight with vigorous shaking.
C. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at 12,000g for 30 seconds at 4°C in a microfuge. Store remainder at 4°C.
D. Remove the medium by aspiration.

(3) Lysis of E. coli and purification of plasmid:

A. Resuspend *E. coli* pellet in 100 µl of ice-cold Solution I (50 mM glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0).
B. Add 200 µl of freshly prepared Solution II (0.2 N NaOH, 1% SDS), inverting the tube rapidly 5 times. Do not vortex. Store at 4°C.
C. Add 150 µl ice-cold Solution III (5 M potassium acetate 60 ml, glacial acetic acid 11.5 ml, H_2O 28.5 ml), on ice for 3-5 min.
D. Centrifuge at 12,000g for 10 min, at 23°C .
E. Pour supernatant into QIAprep column (silicon gel column, Qiagen Company, USA).
F. Centrifuge at 12000g for 1 min and discard flow through.
G. Wash the column with 0.75 ml PE buffer (55 ml of 5 mM Mops-KOH, pH 7.5-7, 0.75 mM NaCl plus 220 ml of ethanol).
H. Centrifuge 1 min at 12000g and discard flow through.
I. Place column in 1.5 ml microcentrifuge tube.
J. Add 50 µl of the DEPC H_2O in the center of the column, stand for 1 min, centrifuge at 12000g for 1 min.
K. Take 1 µl of DNA (plasmid), add 99 µl of TE buffer, pH 8.0, measure DNA concentration at OD260 nm and OD280 nm (OD260 nm/OD280 nm should be >1.7).
L. Redissolve the DNA in 50 µl of TE (pH 8.0) containing DNAase-free pancreatic RNAase (20 µg/ml). Vortex briefly. Store at -20°C.
M. Calculate the concentration of the plasmid DNA: 1 $OD_{260\ nm}$ = 50 µg of plasmid DNA/ml. Store the DNA in aliquots at -20°C.

3.2.3 Transfer human interleukin-2 gene into rat heart muscle cells:

(1) Transferred gene: Human interleukin-2 (IL-2) gene cloned in plasmid pBR322 inserted in *E. coli* can be bought from American Type Culture Collection (ATCC, Rockville, MD, USA).

(2) Transfection: ~2×10^7 of heart muscle cells suspended in 0.2 ml medium are seeded into a tissue culture chamber. 48-72 hours later, remove medium and add 0.2 ml fresh medium, then add 0.5 µg of plasmid in 0.05 ml calcium phosphate-HEPES-buffered saline, pH 7.0, at 37°C.

3.2.4 Detection of interleukin-2:

12~48 hours after the addition of plasmid and incubation, the amount of interleukin-2 is measured with the indirect enzyme-linked immunosorbent assay (ELISA) in medium. Antibody of anti-interleukin-2 (human) can be gotten from Sigma (Sigma Chemical Co., St Louis, MO, USA).

3.3 DEAE-dextran mediated

DEAE-dextran (diethylaminoethyloethyl-dextran) was used to introduce poliovirus RNA and SV40 and polyomavirus DNAs into cells in 1960s (Pagano, 1965; McCutchan, 1968; Warden, 1968).

There are three points that DEAE-dextran mediated transfection differs from calcium phosphate coprecipitation. (1) It is used for transient transfection. (2) It works more efficiently with cell lines of BSC-1, CV-1 and COS, etc. (3) It is more sensitive.

The DEAE-dextran mediated transfection could be done by the following steps:

(1) Harvest exponentially growing cells by trypsinization and transfer then into 60-mm tissue culture dished at a density of 105 cells/dish.

(2) Add 5 ml complete growth medium.

(3) Incubate 24 hours at 37^{o}C with 5% CO_2.

(4) Prepare DNA/DEAE-dextran/TBS-D solution by mixing 2 mg of superoiled plasmid DNA into 1 μg/ml DEAE-dextran in TBS-D.

(5) Remove medium and wash tree times with PBS and twice with TBS-D.

(6) Add DNA/DEAE-dextran/TBS-D solution 250 μl.

(7) Incubate 60 min at 37^{o}C with 5% CO_2.

(8) Remove DNA/DEAE-dextran/TBS-D solution.

(9) Wash with TBS-D three time and PBS twice.

(10) Add 5 ml medium supplemented with serum and chloroquine (0.1 mM).

(11) Incubate 4 hours at 37^{o}C with 5% CO_2.

(12) Remove medium.

(13)Wash with serum-free medium three times.

(14) Add to cells 5 ml of medium supplement with serum, and incubate 48 hours at 37^{o}C with 5% CO_2.

(15) Harvest the cells after the 48 hours transferction.

(16) Analyze RNA or DNA by hybridization, or analyze expressed protein by radiommunoassay, immunoblotting, immuniprecipitation, or by enxzymoatic activity in cell extract.

3.4 Electroporation

Pulse electrical fields can be used to introduce DNA into cells of animal, plant and bacteria. Factors that influence efficiency of transfection by electroporation: applied electric field strength, electric pulse length, temperature, DNA conformation, DNA concentration, and ionic composition of transfection medium, etc.

Steps of the electroporation transfection:

(1) Harvest cells in the mid- to late-logarithmic phase of growth.

(2) Centrifuge at 500 g (2000 rpm) for 5 min at 4^{o}C.

(3) Resuspend cells in growth medium at concentration of 1 X 10^7 cells/ml.

(4) Add 20 μg plasmid DNA in 40 μl cells.

(5) Electric transfect by 300 V / 1050 μF for 1-2 min.

(6) Transfer the electroporated cells to culture dish and culture the cells.

(7) Assay DNA, RNA or protein and continuously culture the cells to get positive cell lines. .

3.5 Polybrene

Several polycations, including polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide) (Chaney, 1986) and poly-L-ornithine (Nead, 1995), have been used in gene transfection with the DMSO enhancement. Normal steps are following:

(1) Harvest exponential cells by trypsinzationin and replate at a density of 5,000 cells/mm^2 in 10 ml MEM-α containing 10% fetal calf serum.

(2) Incubate 24 hours at 37^{o}C in 5% CO_2.

(3) Replace medium with 3 ml pre-warmed medium containing serum, 10 μg DNA and 30 μg polybrene (37^{o}C). Mix the medium before adding polybrene.

(4) Incubate 12 hours with a gent shake each hour.

(5) Remove medium and add 5 ml 30%^ DMSO in serum-containing medium.

(6) After 4 min incubation, aspirate the DMSO solution. Wash the cells twice with warmed (37^{o}C) serum-free medium, and add 10 ml complete medium containing 10% fetal calf serum.

(7) Incubate 48 hours at 37°C in 5% CO_2.

(8) Examine the cells everyday after the transfer-ction.

(9) For stable transfection, continue incubate 3 weeks with changing medium every 2 days.

3.6 Virus

Viruses are highly adapted to the process of gene transfer. Viral vectors have the ability to transfer DNA to a high fraction of cells, but using virus as the vector will be potentially arouse cancer leukaemia (Cavazzana-Calvo, 2004). Common vectors used for gene transfer in cell culture are derived from retroviruses. Adenovirus and other agents are used for the gene delivery.

3.7 Biolistics (Gene gun, or called microparticle bombardment)

Some cells, tissues and intracellular organelles are impermeable to foreign DNA, especially plant cells. biolistics, including particle bombardment, is a commonly used method for genetic transformation of plants and other organisms. To resolve this problem in gene transfer, the gene gun was made by Klein at Cornell University in 1987 (Klein, 1987; Kikkert, 2005). On the gene gun technique, Klein and Sanford et al published papers, obtained patents and formed a company called Biolistics (Klein, 1987).

The gene gun is part of the gene transfer method called the biolistic (also known as biobalistic or particle bombardment) method. In this method, DNA or RNA adhere to biological inert particles (such as gold or tungsten). By this method, DNA-particle complex is put on the top location of target tissue in a vacuum condition and accelerated by powerful shot to the tissue, then DNA will be effectively introduce into the target cells. Uncoated metal particles could also be shot through a solution containing DNA surrounding the cell thus picking up the genetic material and proceeding into the living cells. The efficiency of the gene gun transfer could be depended on the following factors: cell type, cell growth condition, culture medium, gene gun ammunition type, gene gun settings and the experimental experiences, etc.

Briefly for gene gun practice, the target cells or tissues on the polycarbonate membranes could be positioned in a Biolistic PDS-1000/HE Particle Delivery System (Bio-Rad Laboratories GmbH, München, Germany). Biolistic parameters are 15 in. Hg of chamber vacuum, target distance of 3 cm (stage 1), 900 psi to 1800 psi particle acceleration pressure, and 1.0 μm diameter gold microcarriers (Bio-Rad, USA). Gold microcarriers are prepared, and circular plasmid DNA is precipitated onto the gold using methods recommended by Bio-Rad with the following: 0.6 mg of gold particles carrying ~5 μg of plasmid DNA is used per bombardment.

The detail protocol for the gene gun transfection is described as follows:

(1) Prepare gold or tungsten particles: 60 mg gold or tungsten in 1 ml 70% ethanol, centrifuge at 10,000 rpm for 10 seconds and collect particles, and wash with H2O three times by centrifugation.

(2) Prepare DNA-coated particles: Mix 50 □l (about 3 mg) metal, 2.5 □l plasmid DNA (about 2.5 □g), CaCl2 50 □l (2.5 M), spermidine 20 □l (0.1M). Vortex and stand for 5 min. Centrifuge, remove supernatant, and add 140 □l 70% ethanol over the pelleted particles, and repeat the ethanol and centrifugation three times, then add 50 □l ethanol.

(3) Place a macrocarrier in the metal holder of gene gun and wash twice with ethanol.

(4) Vortex and spread 0.5 mg pellet slurry on the macrocarrier.

(5) Load the macrocarrier into the gene gun, and shoot it. Repeat the shoot until all the areas are shot. .

(6) For transient expression, examine cells 48 hours after the shooting, by immunology or other methods.

(7) For stable transfection, continue culture the transfected cells or tissues.

In our studies, we did gene transfection with the self-design CO_2 propelled gene gun (200 psi, distance 3 cm with 400 mesh nylon screen) using tungsten particle (600 nm diameter) coated with plasmid expressing anti-ampicillin gene, the plasmid with anti-ampicillin gene was transferred into E. Coli cells.

3.8 Laser transfection

As the example of our experiments, UV excimer laser (XeCl2, 308 nm) is used in the gene transfection (5 min by a 0.7 0.9, 1.4 or 2.0 mm diameter fiber with fluence of 45 and 60 mj/mm2 - real laser energy 2.3,

5.9, 13.1, 32.0 mj/pulse, 25 Hz) (CVX-300 Excimer Laser System, Spectranetics Corporation, Colorado Springs, CO, USA). Also, we used to make experiments with Nd:Yag, Ho:Yag in the gene transfection. All the methods of excimer, Nd:Yag and Ho:Yag laser transfection are effective.

3.9 Transfection enhanced by elevated temperature

Our studies show that high temperature enhances the gene transfection. In our experiments, rat heart muscle cells were cultured in medium 199 with 10% FBS and human aorta smooth muscle cells were cultured in F12K medium. Human interleukin-2 gene was transfected into rat heart cells and swine growth hormone gene was transfected into human aorta smooth muscle cells by calcium phosphate coprecipitation at various temperatures: 23ºC, 37ºC and 43ºC. Transfected interleukin-2 and swine growth hormone expressions were detected using an indirect ELISA. The heated cultured rat myocytes had a significantly higher expression of the transfected interleukin-2 gene. Ambient temperature rise to 43^{o}C for up to 30 min provided greater transient transfection of the interleukin-2 gene when compared to ambient temperatures at 37^{o}C and 23^{o}C ($p<0.01$). The greatest effects occurred within 10 min of incubation and persisted up to 30 min. These results suggest that even a few degrees of ambient temperature rise can significantly increase gene transfer into muscle cells. This may be of value when using gene therapy with transfection procedures (Ma, 2004b; Ma, 2004c).

3.10 Plant gene transfer

Agriculture and plant breeding relied solely on the accumulated experience of generations of farmers and breeders that is, on sexual transfer of genes between plant species. However, developments of plant molecular biology and genomics now give us access to knowledge and understanding of plant genomes and the possibility of modifying them. There are two most powerful technologies for transferring gene into plants: Agrobacterium-mediated transformation and biolistics. As plants have cell wall, the biolistics is very useful in the plant gene transfer (Rasco-Gaunt, 2001).

4 Discussion

The current century will bring tremendous changes to the science, technology, and the practice of medicine (Lushai, 2002). Gene therapy is part of a growing field in molecular medicine, which will gain importance in the treatment of human diseases (Gunther, 2005). As a critical topic, gene transfection gives people the hope to treat many diseases but it also could create dangerous species in the earth, so that it attracts plenty attention by the whole human society (Lanza, 2002). This simply means that the success of gene transfer technique will be benefit for the civilization, and also create the danger for the life in the earth either (Schiemann, 2003). Gene transfection procedures are used in the critic procedure animal clone (Chesne, 2002; Heyman, 2002), and the animal clone is challenged by the religious groups and ethnic extremists (Houdebine, 2003). As our personal views, no matter how big challenges from whatever aspects, the gene transfection and animal clone will develop quickly. The world is a complex place composed by different people. For the science and technology such as gene transfer and animal clone, no country can prevent other countries from the pursuing. We need to develop the technique even if the technique could be used in the danger action, and we need to consider the social effects of a technique when we develop it either.

Science development will be benefit to all the human society. As the gene therapy developing, many more desperate diseases could be cured and many human livings could be saved, such as the life of Pope John Paul II and Terri Schindler-Schiavo. Hope that the gene transfer techniques described in this article could be useful for the researches in the gene therapy field and help to advance the life science study.

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