Chapter 29

Genetic Modification of Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSC) are currently considered the most promising type of adult stem cells for therapeutic applications, because they can be easily isolated from the bone marrow and other tissues, and manipulated for different applications. The genetic transformation of MSC using genes that enhance their homing ability, as well as their proliferation and survival capacities when transplanted to sites of injury, is an important alternative to improve MSC function, especially for tissue regeneration. This chapter describes protocols for the transformation of MSC using plasmid vectors by lipofection and electroporation, as well as retroviral vectors representing viral transformations.

Key words: Mesenchymal stem cells, Transduction, Transfection, Lipofection, Electroporation, Tissue regeneration, Gene therapy, Cell therapy

1. Introduction

Mesenchymal stem cells (MSC) were first described in the bone marrow, and today are known to be present in virtually every type of tissues (1). Protocols for the isolation of MSC from different sources and in different organisms have been well described (2). By definition, MSC are able to differentiate into adipocytes, chondrocytes, and osteocytes, but they have shown a potential to differentiate into other cell types as well (3–6). MSC also have a natural capacity to immune-protect-injured tissues and inhibit immunosurveillance, thus, allow for tissue regeneration through the production and release of immunomodulatory molecules (7). It has also been shown that MSC home to sites of injury (8). All of these properties account for MSC being currently considered the most promising adult cell type for therapeutic applications, especially in regenerative medicine.

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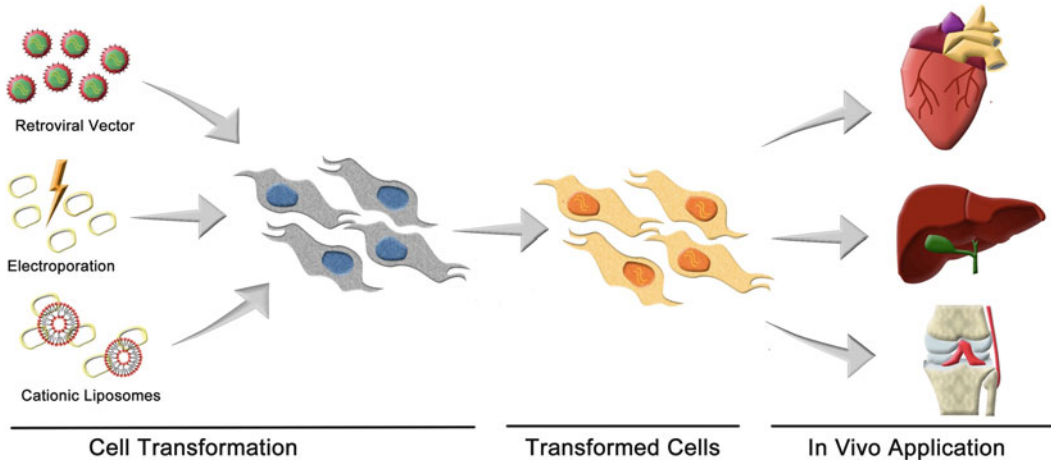

Fig. 1. Mesenchymal stem cells (MSC) may be transformed for the expression of genes that increase their therapeutic potential. For viral transduction, cells are incubated with a medium containing the viral vectors. For non-viral vectors modification, the main strategies include the use of electroporation or cationic liposomes, which facilitate intracellular delivery. Transformed cells may then be used to treat many different types of diseases affecting organs and tissues such as the heart, liver, or cartilage.

In fact, MSC are already being employed in clinical trials for pathologies such as severe acute graft-versus-host disease, Crohn's disease, ischemic stroke, multiple and amyotrophic lateral sclerosis, and cardiovascular diseases (reviewed in ref. (1)). Even though pre-clinical and clinical trials indicate promising results, reports show that the number and the functional activity of patient-derived cells are lower when compared to cells derived from healthy individuals, and that chronic diseased tissue produce lower levels of chemokines and growth factors when compared to healthy tissue (9, 10). These factors could impair the homing and engraftment of transplanted cells. Using viral and non-viral vectors, it is possible to introduce, or control the expression of, genes that could improve MSC properties. In this way, the genetic engineering of MSC can be a useful tool to enhance cell survival and deliver soluble factors to sites of injury (Fig. 1).

Viral and non-viral vectors have been extensively used in gene therapy protocols with MSC: plasmid (11), retrovirus (12), adenovirus (13), and adeno-associated virus vectors (14) have proven to be good options to genetically modify MSC.

Non-viral systems are generally based on plasmids, and their main advantages are large gene capacity, safe handling, and low immunogenicity. However, they have drawbacks such as low efficiency and difficulty in transfecting target cells. Despite their low efficiency, they are used in over 25.1% of the clinical trials using gene therapy (http://www.wiley.com/legacy/wileychi/genmed/clinical/). Different methods are used to maximize their efficacy, with most of them based on mechanical or electrical force, or chemical compounds. MSC can be transfected with plasmid vectors

by a number of methods, including cationic liposomes or lipid-rich reagents (11, 15).

Electroporation is a method that uses electric pulses to create transient pores in the plasma cell membrane that allows the passage of nucleic acids (reviewed in ref. (16)). Electroporation was originally designed for prokaryotic cells, and eukaryotic cells such as MSC; a square-wave electroporator should be used to allow control of parameters such as voltage, amperage, resistance, and time pulse (17). Nucleoporation is a variation of the basic method, and is based on a combination of parameters that enables entry of nucleic acids in the cell nucleus (18). Other techniques such as sonoporation and micro-electroporation are under development with encouraging results in the field of non-viral gene transfer (19, 20).

A wide variety of viral vectors have been used in clinical trials (reviewed in refs. (21–23)). Retrovectors comprise lentivectors (HIV, FIV, EIAV) and oncovectors (MLV). Lentivectors are capable of transducing even quiescent cells, whereas oncovectors need cell division to access the cell nucleus. Both of them can provide stable transgene integration. Adenoassociated vectors may also be considered integrative vectors and nowadays they are used as an alternative to retrovectors, based on biosafety questions and feasibility in viral production. Adenovectors may be employed when transient transgene expression is desired, but its immunogenicity is still an issue.

Conditions of target cell transduction must consider vector titer, proportion of viral vector related to target cell, and cell biology (which may hamper vector transduction). Examples of undesired consequences include variegation of transgene expression (24), transgene silencing due to chromosomal positioning/repressive chromatin (25), or even genotoxicity due to insertional mutagenesis, which are especially complicated when working with stem cells (26). This reinforces that each step of genetic modification through viral vectors must be carefully analyzed, whether in vivo or in vitro, for progenitors or nonprogenitor cells. Reports of MSC transduction are available (27, 28), but a strong background on viral vectorology is mandatory to develop critical thinking about how to change MSC without changing its progenitor characteristics.

In this chapter, we describe protocols for the transformation of MSC using plasmid vectors by lipofection and electroporation (in three modalities: exponentially decaying, square wave, or nucleoporation), and retroviral vectors representing viral transformations.

2. Materials

All reagents are prepared with sterile, ultrapure water. Solutions and reagents are kept at 4°C. Fetal calf serum (FCS), streptomycin–penicillin, trypsin–EDTA, and DNA samples are kept at –20°C.

Basic equipment and items will not be specified. All reagents used are from Sigma Chemical Co., unless otherwise stated. Plasticware is from TPP (Trasadingen). All waste should be disposed according to regulations. Biosafety regulations and guidelines may vary between different countries and should be followed accordingly.

2.1. MSC Culture

1. Procedures for cultivation of rodent or human MSC are described elsewhere (29, 30). Cultures should be healthy and in log phase of growth, which can be achieved using pre-confluent cultures (see Note 1).
2. Cells should be used in early passages (around 3–4), after cultivation in low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS (Cultilab) and 5.96 g/L HEPES buffer.
3. Antibiotics may be used. Our experience indicates that removing FCS from the culture medium reduces the recovery of viable cells after gene transfer with viral or non-viral vectors.

2.2. Plasmid Vectors

1. Plasmid vectors containing the gene of interest may be produced by standard methods or purchased from many different suppliers.
2. Their quality may be checked by agarose gel electrophoresis.
3. The samples should be quantified by spectrophotometry, aliquoted in microtubes and stored at –20°C until manipulation.
4. Plasmid DNA vectors with concentration higher than 1 μg/μL should be used.

2.3. Viral Vectors

Retroviral vectors may also be produced or purchased. Fresh stocks, already titred (see Note 2), should be used.

2.4. Components for Lipofection of MSC

1. Serum-free DMEM, pre-warmed at 37°C.
2. DMEM containing 10% FCS, pre-warmed at 37°C.
3. Solution of trypsin (0.25%) with 1 mM ethylenediamine (trypsin/EDTA).
4. Hanks' Balanced Salt Solution (HBSS).
5. 24-, 12- or 6-well culture plates.
6. Plus Reagent (Invitrogen Corp.).
7. Lipofectamine LTX (Invitrogen).

2.5. Components for Transfection of MSC by Exponential Decay Electroporation

1. Electroporation medium: RPMI-1640 medium without serum or antibiotics.
2. Recovery medium: low-glucose DMEM supplemented with 20% FCS and antibiotic (if used).
3. Solution of trypsin (0.25%) with 1 mM ethylenediamine (trypsin/EDTA).

4. 0.4-mm Electroporation cuvettes.
5. Exponential decay electroporator.

2.6. Components for Transfection of MSC via Electroporation Square Wave

1. Pulsing buffer (pH 5.5): 125 mM KCl, 15 mM NaCl, 3 mM HEPES, 25 mM glucose, 1.2 mM $MgCl_2$ (plus 10% FCS or 0.6% BSA).
2. Culture medium: DMEM supplemented with 20% FCS and antibiotics (if used).
3. 0.4-mm electroporation cuvettes.
4. Square wave electroporator.

2.7. Components for Transfection of MSC by Nucleoporation

1. Human MSC Nucleofector® Kit (cat. Nº PLV-100) (Lonza/Amaxa Biosystems).
2. Post-electroporation culture medium, DMEM supplemented with 20% FCS.
3. Electroporation cuvettes supplied with the kit.
4. Amaxa nucleoporator.

2.8. Components for Transduction of MSC with Retrovectors

1. DMEM containing 10% FCS, pre-warmed at 37°C.
2. Trypsin/EDTA.
3. HBSS.
4. 24-, 12- or 6-well culture plates.
5. Protamine sulfate (see Note 3).

3. Methods

All procedures should be carried out under sterile conditions.

3.1. Lipofection of MSC

1. On day prior to lipofection, plate cells at a density between 10^4 and 2×10^4 cells/well, in 24-well plates, so that wells will be 60–80% confluent when lipofected. Add 500 µL of complete medium per well.
2. Before starting the preparation of lipofection reagents, check the plates to make sure that the wells contain a similar cell density (see Note 4).
3. Start by diluting the plasmid vector in order to obtain a sample of 500 ng/µL to use for lipofection. Dilution can be made using HBSS.
4. Add 1 µL plasmid vector solution (500 ng/µL) to 100 µL serum-free DMEM/10 mM HEPES (see Note 5).
5. Add 0.5 µL Plus Reagent to the plasmid-medium mix obtained on step 4. Incubate 5 min at room temperature (RT) (see Note 6).

6. Add 1.25 μL Lipofectamine LTX to the mixture and incubate 30 min at RT.
7. Add 100 μL plasmid-lipofectamine mix to the well, dropwise. Mix well by rocking the plate.
8. Incubate the cells in 5% CO_2, at 37°C.
9. Add 500 μL fresh complete medium to every well 24 h after the lipofection.
10. Change the medium completely 48 h after lipofection. Add 1 mL fresh complete medium per well.
11. Check for reporter gene expression between 24 and 72 h after lipofection (see Note 7).

3.2. Transfection of MSC by Exponential Decay Electroporation

Four works can be found in the literature regarding the use of electroporation with exponential decay to transfect MSC (31–34). The protocols differ in the dose of DNA plasmids used (10–25 μg), voltage values (150–350 V), resistance (infinite), and capacitance (500–1,000 μF), as well as in the media used to resuspend the cells during application of the electric pulse (RPMI, DMEM, and MEM without FCS or antibiotics). The efficiency ranged from 30 to 58% and the viability of transfected cells, from 40 to 69% (when specified), always inversely proportional to the voltage used and transfection efficiency obtained. The protocol which has shown greater success (electroporation efficiency × viability) is described further in detail (32). All the procedures are at RT, without any pre-incubation step.

1. Detach MSC at 50–60% confluence using trypsin/EDTA, and resuspend in RPMI-1640 medium (without serum or antibiotics) at 1×10^6 cells/mL.
2. In a 0.4-mm electroporation cuvettes, add a total of 400 μL of the cell suspension mixed with 20 μg of plasmid DNA, at RT.
3. Set up the electroporator at 300 V, 500 μF, and ∞ Ohm.
4. Immediately following the electroporation, add 400 μL of recovery medium and transfer the suspension into a culture plate.
5. Analyze transfection efficiency and viability 24 h after electroporation.

3.3. Transfection of MSC via Electroporation Square Wave

Square wave electroporation has not been widely used with MSC, and is generally used for electroporation of RNA, small antisense oligonucleotides, or morpholino (35–38). All the procedures are conducted at RT, without any pre-incubation step.

1. Detach MSC at 50–60% confluence using trypsin/EDTA, and resuspend in pulsing buffer at 1×10^6 cells/mL.
2. Mix a total of 100 μL of the cell suspension with 20 μg of plasmid DNA in 0.4-mm cuvettes at room temperature.

3. Set up the electroporator at 150 V and 75 ms.
4. Immediately following electroporation, add 1 mL of culture medium to the cuvette to resuspend the cells and transfer the cell suspension into a 12-well culture plate.
5. Analyze transfection efficiency and viability 24 h after electroporation.

3.4. Transfection of MSC by Nucleoporation

A few reports have described the protocol to transfect MSC with the Amaxa nucleoporator, examining both the efficiency of transfection and cell viability. Two protocols may be used, program U-23 (high-transfection efficiency) or C-17 (high cell survival), with minor technical differences between them that may explain the differences in transfection efficiency and viability (39–43). Procedures are conducted at RT, without any pre-incubation step.

1. Detach MSC at 50–60% confluence using trypsin/EDTA, resuspend in DMEM/F12 medium supplemented with 10% FCS, and determine cell concentration.
2. Resuspend the cells again in 100 μL of Amaxa Nucleofector Solution specific for MSC at 2×10^6 cells/mL final concentration.
3. Add 10 μg of plasmid DNA.
4. Set up the nucleoporator at program U-23 if you desire high-transfection efficiency or C-17 if you prefer high cell survival.
5. Execute the nucleoporation step.
6. Immediately following the electroporation add 500 μL of DMEM supplemented with 20% FCS and transfer the suspension into a culture plate.
7. Analyze transfection efficiency and viability 24 h after electroporation.

3.5. Transduction of MSC with Retrovectors

The methods described below are designed for a standard MSC transduction based on retroviral vectors (lenti- or oncovectors). Adaptations may be needed considering the applications of transduced cells, for example for in vivo injection. In that case, we suggest that the vectors should be carefully purified to reduce cell mortality after the procedure (especially if working with lentivectors), which will improve cell culture recovery after transduction. It is highly recommended that biosafety guidelines are respected, because lentiviral vectors pseudotyped with VSV-G envelope are considered biosafety level 2. You may find a good biosafety background at the Centers for Disease Control (CDC) website. To fulfill the biosafety issue, a very interesting report considering different envelopes for HIV vectors may be helpful to evaluate alternative pseudotypes that may be manipulated in biosafety level 1 (44). However, altering the envelope may alter transduction efficiency. Thus, this alternative must be carefully evaluated and we recommend testing transduction conditions to determine a final protocol.

1. One day before transduction, plate 5×10^4 cells on 6-cm-diameter cultures dishes, with 4 mL of cell culture medium (see Note 8).
2. On the day of transduction, feed the culture with fresh medium with protamine sulfate (10 μg/mL final concentration) (see Note 3).
3. Apply the desired amount of vector to the culture (see Note 8).
4. After 20 h, stop the transduction by changing the cell culture medium (see Note 8).
5. Expand the culture or split the cells according to your selection agent (see Note 9).

4. Notes

1. Density adjustments may be necessary according to the tissue of origin and passage of MSC, which have implications for population doubling time. Make sure to test which cell concentration is best for your conditions.
2. For oncovector production, we suggest the protocol described by Pear et al. (45). For lentivector production, we highly recommend a technical report rich in details (46). The concentration of harvested retroviral stocks is not just about quantity, but also about quality: it helps to eliminate toxic proteins from the vector production step. We adapted the concentration of viral stocks from that reference: for each 4 mL of 20% sucrose (in ultrapure water), add 26 mL of harvested vector. Batches are concentrated in a Sorvall centrifuge (rotor SS34), at 16,000 rpm for 2 h. Round bottom tubes are employed in a swinging bucket rotor and we make a sign at the point where the pellet will be probably localized. The supernatant is drained off carefully to avoid disturbing the pellet. The tubes may be inverted on paper towels, to drain off all liquid. Alternatively, a short spin of 2 min at 5,000 × *g* may be used to determine the residual volume of the medium. Adjust this volume (matching the type of medium that will be employed in cell culture) and vortex the tube. After a spin at 5,000 × *g* for 10 min, allow the tubes to rest for 6 h at 4–8°C. Once again, repeat the cycle of vortexing and centrifugation at 5,000 × *g* for 10 min. Pool all tubes together. Aliquots should be immediately frozen at −80°C. Please note that the VSV-G envelopes allow such manipulation of viral stocks. If using other pseudotypes or other retrovectors (such as MLV), you may need to consider the volume input of vector in concentration and vector recovery, after a functional transduction assay (based on reporter gene expression or antibiotic resistence gene, for example) to

evaluate the amount of vector recovery. Save some unconcentrated stocks to compare with the concentrated ones. Do that on your target cell also because it may be easier to quantify and detect transgene expression in established cell lines employed in the titration procedure. You can refer to your titer as unit of vector/unit of volume in that specific cell line.

3. Protamine sulfate may be a better choice as adjuvant for MSC transduction than polybrene. Toxic effects of polybrene for the ex vivo expansion and selection of human CD34+ stem cells have been reported (47). In fact, we had better culture recovery and good expansion rate when using protamine. You will find examples of protocols employing polybrene with MSC (27). Thus, we recommend evaluation of this aspect in the transduction protocol, exposing cells to different concentrations of the reagent, during the same period used for the transduction process. Keep in mind that high MOI (multiplicity of infection) may also add toxicity to the experiment. In our experience, transduction efficiency will be greatly reduced if performed in free polybrene or protamine medium.
4. Cell counting and plating are not absolutely accurate procedures, unless you are using automated resources. Select wells that have a similar cell density to lipofect and maintain as controls.
5. All volumes expressed here are per well. When lipofecting multiple wells, adjust the reagents accordingly. For instance, to lipofect 5 wells, add 5 μL plasmid vector DNA to 500 μL serum-free medium; then add 2.5 μL Plus Reagent and incubate 5 min at RT; add 6.25 μL Lipofectamine LTX to the mix and incubate 30 min at RT; add 100 μL DNA-lipofectamine mix per well.
6. Lipofectamine LTX may be combined with Plus Reagent to enhance lipofection efficiency on primary cells.
7. When selecting transformants with geneticin, add the appropriate concentration of antibiotics to the transformed cells between 72 and 120 h after lipofection. We suggest that you test the appropriate concentration of antibiotics for your culture conditions. In our conditions, the recommended concentration of geneticin for MSC is between 350 and 400 μg/mL. We also recommend that you keep positive control wells (non-lipofected cells treated with geneticin) when using this selection method.
8. Define the layout of your experiment: cell plate density is very important for calculating the amount of virus that will be required. We suggest these conditions because MSC are large cells that should be comfortable in monolayer condition until transduction. This culture condition may work for lenti- and

oncovectors. You may start evaluating different MOI and setting a deadline to evaluate transgene expression. If changing the cell line, you may retest the protocol before the collection of data. The type of cell, lineage or primary culture, animal or human, MOI, single or double transduction, promoters employed in vector design, or transgene silencing are some examples of factors that may interfere with transduction efficiency. We use 20 h because of cell culture doubling time (extremely relevant for MLV vector). If working with lentivectors, you may reduce the time of transduction. If working with high MOI, time of exposure must be tested first for cell mortality due to toxicity.

9. It is possible to maintain individual cultures until they can be pooled together. Please note that it is important to evaluate if the selection agent should be maintained to prevent silencing of the transgene. We suggest replicates of the experiment that will be analyzed at the same time, but altering culture conditions (with or without selection agent).

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