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Application of Magnet-based Nanofection in Embryonic Stem Cell Research

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1. Introduction

Embryonic stem (ES) cells are derived from the epiblast of the inner cell mass (ICM) of a blastocyst. These are pluripotent cells and can give rise to all derivatives of the three primary germ layers - ectoderm, endoderm, and mesoderm. ES cells require specific signals for specific lineage of differentiation - if injected directly into another body, ES cells will differentiate into many different types of cells, causing a teratoma (Wu et al., 2007).

The techniques for culturing mouse embryonic stem (ES) cells from the inner cell mass of the preimplantation blastocyst were first done in 1981 (Martin, 1981), and versions of these standard procedures are used today in laboratories throughout the world. The first successful derivation of human ES (hES) cells was reported by Thomson et al. (Thomson et al., 1998). They isolated and plated the cells onto mitotically inactivated MEF (mouse embryonic fibroblast) cells. In 2000, Reubinoff et al. confirmed that hES cells could be efficiently derived from surplus embryos and possess the differentiation potential under *in vitro* conditions. Since then, there has been rapid progress made and numerous studies have described the derivation of new hES cell lines including methods of growing both undifferentiated hES cells and their differentiated progeny. In last 6 years, there has been exponential increase in methods to improve culture conditions, differentiation patterns to produce human cells for transplantation and drug testing (Trounson, 2006, Gepstein, 2002) and genetic manipulation (Draper et al., 2004, Zwaka and Thomson, 2003).

Generating cultures of mouse or human ES cells that remain in a proliferating and undifferentiated state is multistep process. Typically, the inner cell mass of a preimplantation blastocyst is removed from the trophectoderm that surrounds it and cultured in the small plastic culture dishes containing growth medium supplemented with fetal calf serum. The culture dishes are sometimes coated with a "feeder" layer of non-dividing cells, which are often MEF cells that have been chemically inactivated so they will not divide. Mouse ES cells can be grown *in vitro* without feeder layers if the cytokine leukemia inhibitory factor (LIF) is added to the culture medium, but human ES cells do not respond to LIF.

The process of generating an embryonic stem cell line is somewhat difficult, so lines are not produced every time from the preimplantation-stage embryo maintained in a culture dish. However, if the plated cells survive, divide and multiply enough to crowd the dish, they are removed, and plated into several fresh culture dishes. The process of re-plating or

subculturing of the cells is repeated many times and maintained for many months. Once the cell line is established, the original cells yield millions of embryonic stem cells. Embryonic stem cells that have proliferated in cell culture for six or more months without differentiating, are pluripotent, and appear genetically normal and then referred to as an embryonic stem cell line.

As long as the embryonic stem cells in culture are grown under appropriate conditions, they can remain undifferentiated condition. But, if cells are allowed to clump together, known as embryoid bodies and they start to differentiate spontaneously. They can form any types of cells like muscle cells, nerve cells, and many other cell types. Although spontaneous differentiation is a good indication that a culture of embryonic stem cells is healthy, it is not a proficient way to produce cultures of specific cell types. To generate cultures of particular types of differentiated cells like heart muscle cells, blood cells, or nerve cells, researchers tried to control the differentiation of embryonic stem cells. They changed the chemical composition of the culture medium, altered the surface of the culture dish, or modified the cells by inserting specific genes. Through years of experimentation, scientists have established some basic protocols or "recipes" for the directed differentiation of embryonic stem cells into some targeted cell types.

- Derived from the inner cell mass/epiblast of the blastocyst.
- Capable of undergoing an unlimited number of symmetrical divisions without differentiating (long- term self-renewal).
- Exhibit and maintain a stable, full (diploid), normal complement of chromosomes (karyotype).
- Give rise to differentiated cell types that are derived from all three primary germ layers of the embryo (endoderm, mesoderm, and ectoderm).
- Capable of integrating into all fetal tissues during development (Mouse ES cells maintained in culture for long periods can still generate any tissue when they are reintroduced into an embryo to generate a chimeric animal).
- Capable of colonizing the germ line and giving rise to egg or sperm cells.
- Clonogenic, which is a single ES cell can give rise to a colony of genetically identical cells, or clones, which have the same properties as the original cell.
- Express the transcription factor Oct-4, which then activates or inhibits a host of target genes and maintains ES cells in a proliferative, undifferentiating state.
- Can be induced to continue proliferating or to differentiate.
- Lacks the G1 checkpoint in the cell cycle. ES cells spend most of their time in the S phase of the cell cycle, during which they synthesize DNA.
- Unlike differentiated somatic cells, ES cells do not require any external stimulus to initiate DNA replication.
- Do not show X chromosome inactivation. In every somatic cell of a female mammal, one of the two X chromosomes becomes permanently inactivated. X chromosome inactivation does not occur in undifferentiated ES cells.

Table 1. Characteristics of embryonic stem cells

The ability of ES cells to develop into all cell types of the body has fascinated many scientists in recent years. The transcription factor Oct4 has been used as a key marker for ES cells and for the pluripotent cells of the intact embryo and its expression must be maintained at a high

level for ES cells to remain undifferentiated (Mallon et al., 2006). However, the Oct4 protein itself is insufficient to maintain ES cells in the undifferentiated state (Matsuda et al., 1999). Two groups also identified another transcription factor, Nanog, which is essential for the maintenance of the undifferentiated state of mouse ES cells (Sato et al., 2004, Mitsui et al., 2003). By comparing gene expression patterns between different ES cell lines, other cell types such as adult stem cells and differentiated cells, genes that are enriched in the ES cells have been identified.

To understand the mechanism behind the undifferentiated property of ES cells and genes that play a vital role in the maintenance of the pluripotency of ES cells, development of efficient gene delivery system is necessary for embryonic stem cell research.

1.1 Significance of Gene delivery systems in embryonic stem cells

ES cells have generated great hope as a potential resource for cell and transplantion therapy, due to their ability to differentiate into many cell types (Odorico et al.,2001). However, such potential depends on efficient and reliable methods for specific tissue differentiation using several strategies, such as manipulation of the microenvironment by use of different cell culture conditions, and the use of growth factors or small molecules that can efficiently regulate cell differentiation at specific cell stages (Boheler et al., 2002, Thomson et al., 1998). Matrices designed for formation of specific tissues and genetic manipulation of stem cells include transient expression from exogenous constructs, expression of episomal vectors, stable expression from randomly integrated exogenous constructs, or site specific targeting by homologous recombination (Bethke and Sauer1997). The use of genetic manipulation in ES cells has provided a valuable tool in the research for gene function study (Dhara and Benvenisty, 2004).

For analyzing the molecular mechanism of the specific genes during proliferation and differentiation of ES cells, gene delivery into ES cells is very important technique. There are arrays of transfection reagents and methods which aim to achieve high efficiency in specific gene delivery. Determination of the best gene delivery approach for any particular experiment requires consideration of a number of different factors. Even with all the newest and currently available technologies, ES cells have very low efficiency in transduction during conventional transfection procedures, with their undifferentiated phenotype often being compromised. Many gene delivery systems are reported till today, like viral transfection, lipofection, and nucleofection, but all the methods have some or the other disadvantages. Here, we tried to explain several gene delivery systems used in ES cell research. Especially, we focus on magnet-based nanofection.

2. Gene delivery systems

In this chapter, we will mainly discuss about magnet-based nanofection and its benefits compared to the other viral and non viral gene delivery systems, but here we briefly explain several gene delivery techniques, such as calcium phosphate- (Jarcho, 1981), DEAE–dextran- (Gauss and Lieber, 1992), or liposome–mediated transfection (Schenborn and Oler, 2000), electroporation (Chu et al.,1987), nucleofection (Siemen et al., 2005), or viral vector-mediated transduction (Kaplitt et al., 1994). Under standard conditions, mammalian cells take up and express the externally applied DNA with very low efficiency. Various methods have been proposed to overcome this problem.

- Calcium phosphate-mediated transfection; Calcium phosphate relies on precipitates of plasmid DNA formed by its interaction with calcium ions. It is cheap and has been routinely used for both transient and stable transfection in a variety of cell types and easy technique to perform. However, this method is prone to high variability and is not suited for *in vivo* gene transfer.
- DEAE-dextran-mediated transfection; Diethylaminoethyl-dextran (DEAE–dextran) is a cationic polymer that tightly associates with negatively charged nucleic acids. This method successfully delivers nucleic acids into cells for transient gene expression, all of which seek to maximize the uptake of DNA and to minimize the cytotoxic effects. In this method, cells are exposed briefly to a high concentration of DEAE-dextran-DNA and then to chloroquine diphosphate, which acts as a facilitator for transfection. However, this technique is not generally useful for long-term transfection studies (stable transfection) that rely upon integration of transferred DNA into the chromosome.
- Liposome–mediated transfection; Liposomes are lipid bilayers entrapping a fraction of aqueous fluid. DNA is spontaneously associated to the external surface of cationic liposomes (by virtue of its negative charge) and these liposomes will interact with the cell membrane. However the disadvantages of this technique are poor target selectivity and decreased efficiency compared viral vectors and transient gene expression.
- Electroporation; Electroporation (or electropermeabilization) is a mechanical method which is used to introduce polar molecules into a host cell through the cell membrane. In this technique there is a significant increase in the electrical conductivity and permeability of the plasma membrane caused by an externally applied electrical field. It is generally used in molecular biology for introducing any new molecule into a cell, such as loading it with a molecular probe, a drug that can change the cell's function, or a piece of coding DNA.
- Nucleofection; Nucleofection is a gene delivery system, in which, there is a combination of electrical parameters, generated by a device called nucleofector, with cell-type specific reagents. The substrate is directly transferred into the cell nucleus and to the cytoplasm. The major concern of this technique involves safety risks, lack of reliability, and high costs.
- Viral vector-mediated transduction; Viruses are obligate intra-cellular parasites, designed through the course of evolution to infect cells, often with great specificity to a particular cell type. They tend to be very efficient at transfecting their own DNA into the host cell, which is expressed to produced new viral particles. By replacing genes that are needed for the replication phase of their life cycle (the non-essential genes) with foreign genes of interest, the recombinant viral vectors can transduce the specific cell type. Although a number of viruses have been developed, interest has centred on four types; retroviruses (including lentiviruses), adenoviruses, adeno -associated viruses, and herpes simplex virus type 1. However, the major disadvantages of viral vectors are that all viral vectors induce an immunological response to some degree and may have safety risks (such as insertional mutagenesis), and toxicity problems.

3. Magnet-based nanofection

In recent years, nanotechnology has reached such a level that allows nanoparticles with magnetic properties to be functionalized with specific ligands for many applications, such as site specific drug or gene delivery, magnetic resonance imaging, hyperthermic treatment for

cancer cells and tumour targeting (Latorre and Rinaldi, 2009). The term nanoparticle refers to particles less than 1 mm in size, typically less than 200 nm. Thus they are of a similar length scale to biomolecules, making nanoparticles ideal for combining with biomolecules for medical applications (Jain, 2007, Freitas, 2006).

Several research groups have independently developed magnet-based nanofection methods. This method of transfection was inspired by the concept of magnetic drug targeting (Plank, Anton et al., 2003), which dates back to the late 1970s (Hafeli, 2004), where drugs are bound covalently or noncovalently to magnetic particles and suitable magnetic gradient fields are used to accumulate such 'magnetic' drugs at target sites and/or to retain them there.

Magnet-based nanofection is a gene delivery system that utilizes nanotechnology. It is achieved by the application of a magnetic field to superparamagnetic iron peroxide particles which are associated with gene vectors (Lee et al., 2008). In this technique, the cationic polymer polyethyleneimine (PEI) is coated with superparamagnetic nanoparticles (tsMAG-PEI) are complexed to plasmid DNA. Apart from suitable magnetic nanoparticles, magnet-based nanofection also requires suitable magnetic fields, which are provided by the Magnetofactor plate, especially designed for magnet-based nanofection. This plate will produce a pattern of higher and lower densities of transfected cells according to the geometry of the magnetic field lines. As a reporter gene, enhanced GFP (green fluorescent protein) was transfected in D3 embryonic stem cells by magnet-based nanofection and gave better result (45 %), when compared to Fugene 6- and liposome-mediated transfection (15 %) (Plank, Schillinger et al., 2003).

There are several different types of commercially available magnet-based nanofection systems, which can be purchased from the OZ biosciences (France) or chemicell (Germany). PolyMag is a universally applicable magnetic particle preparation for high efficiency gene delivery. It can be used to deliver DNA into cells with a single step procedure and has been used successfully with plasmid DNA, antisense oligonucleotides, and siRNAs. While, CombiMag can be combined with any commercially available transfection reagent like poly cations and lipids which can be associated with plasmids DNA or siRNA. Recently, PolyMag, a type of magnet-based nanofection, has been reported to provide highly efficient transfection in numerous cell types (Gersting et al., 2004).

The coupling of magnetic nanoparticles to gene vectors in presence of static magnetic feild has been shown to result in dramatic increase in transfection efficiency of reporter genes when compared with other gene delivery methods (Plank, Anton et al., 2003, Plank, Schillinger et al., 2003). Magnet-based nanofection has an improved dose dependent response and enhances transfection efficiency in both permanent and primary airway epithelial cells and also it leads to significant transgene expression at very short incubation times in an ex vivo airway epithelium organ model (Gersting et al., 2004). Magnet-based nanofection in human umbilical vein endothelial cells (HUVEC) had an increased transfection efficiency of a luciferase reporter gene up to 360-fold in comparison with various conventional transfection systems. Apart from the efficicency, there was only an up to 1.6-fold increase in toxicity caused by magnet-based nanofection, suggesting the advantages of this method (Krotz et al.,2003).

Here, we briefly explain six types of commercially available magnet-based nanofection reagents.

1. PolyMag has been optimized to be used with all types of nucleic acids and can be applied to any magnetic particle preparation for increased efficiency of nucleic acid

delivery. This reagent has been used successfully with plasmid DNA, antisense oligonucleotides, and siRNA (Kamau et al., 2006, Namiki et al., 2009).

2. PolyMag Neo represents the latest development in magnet-based nanofection reagent and can be applied to wide variety of nucleic acid delivery, especially in the primary cell lines, where it is hard to transfect. Its universality and strong formulation were reported to allow in achieving higher gene expression level (Kim et al., 2009).
3. CombiMag has been used successfully with plasmid DNA, antisense oligonucleotides, mRNA, siRNA, and viruses. This reagent is designed that it can combine with any commercially available transfection reagent such as cationic polymers and lipids (Kadota et al.,2005).
4. SilenceMag provides more efficient method for delivery of siRNA even at low doses. This formulation was reported to provide reliable high gene silencing efficiencies in primary cell lines and introduce large quantities of siRNA duplexes into cells leading to exceptional gene knockdown effects (Li et al.,2008).
5. ViroMag is reported to be applicable on a various cell lines and primary cells (hard to transfect cell lines) and can be combined with any viruses (specifically designed for retrovirus and lentivirus). It is reported that combination with adenovirus allows up to 500-fold improvement of gene expression compared with other commercial available method (Kamau et al., 2006).
6. NeuroMag is dedicated to be a magnet-based nanofection reagent for neurons. It is perfect for primary neurons and can also be used for glial cells. This reagent is also called magnetoencephalogram (MEG), an analogoue of electroencephalogram (EEG). However, in MEG, the magnetic fields are produced by ion flow and are associated with neuronal activity rather than the electric potentials measured in the EEG. By this system magnetic feilds can be recorded by neuronal activity occurring in the brain (Jensen and Tesche, 2002).

Overall, magnet-based nanofection is easy to use and unique solution for transfection in various cell lines. It has many advantages than that of other gene delivery systems, as follows.

1. High transgene expression.
2. Lead to transfect non-permissive cells (ability of transfect in primary cells which are usually hard to transfect).
3. Rapidly accumulate all virus doses on the cells/ culture medium.
4. Synchronize cell adsorption/infection without alteration to the virus or its genome.
5. Significantly progress virus infectivity.
6. Confined to target specific area of transduction.
7. Can be used for various types of nucleic acids including plasmid DNA, siRNA, oligonucleotides, linearized DNA, double stranded RNA, mRNA, shRNA, virus, and protein.
8. Simple, easy to handle, and non-toxic.
9. Compatible with and without serum-containing medium.

4. Methodology of magnet-based nanofection

4.1 Optimization of Magnet-based nanofection

4.1.1 Magnet-based nanofection reagents

Magnet-based nanofection reagents are universally applicable magnetic particle preparation for high efficiency gene delivery. The gene of interest to be transfected is mixed with

magnetic particles in a single step. Magnet-based nanofection reagents have been used successfully with plasmid DNA, antisense oligonucleotides, and siRNA.

4.1.2 Magnetofactor plate

Apart from suitable magnetic nanoparticles, magnet-based nanofection reagent requires appropriate magnetic fields. A magnetofactor plate (Chemicell, Germany) is specially designed for magnet-based nanofection reagent.

Due to its special geometry, it produces powerful magnetic fields under different plate formats (T-75 flasks, 60 -100 -mm dishes, 6, 12, 24, and 96-well plates). The magnetofactor plate is designed in such way that it exerts a heterogeneous magnetic field that magnetizes the nanoparticles in solution, forms a very strong gradient, and covers all the surface of the plate.

4.1.3 Generalized protocol of magnet-based nanofection

For the better efficiency of transfection, it is always recommended to seed the cells on the previous day of the transfection. The suitable cell density will depend on the growth rate and the cells conditions. Cells should be 60-90 % confluent during magnet-based nanofection. Immediately preceeding transfection, the medium can be replaced with fresh medium (optionally without serum) if necessary.

It is always better to incubate at least 24 hours before exposing the transfected cells to selection media. Vectors are prepared in medium without serum or in PBS. Along with the standard magnet-based nanofection protocol, the serum- and supplement-free vector cocktail is added to the cells that are cultured in medium. Therefore, the addition of the transfection reagent will result in the further dilution of standard culture medium. For most cell types, change of media is not required after magnet-based nanofection. However, it may be necessary for cell lines that are sensitive to serum/supplement concentration. Alternatively, the cells may be kept in serum-free medium during magnet-based nanofection. In this case, a changing of media may be required after magnet-based nanofection.

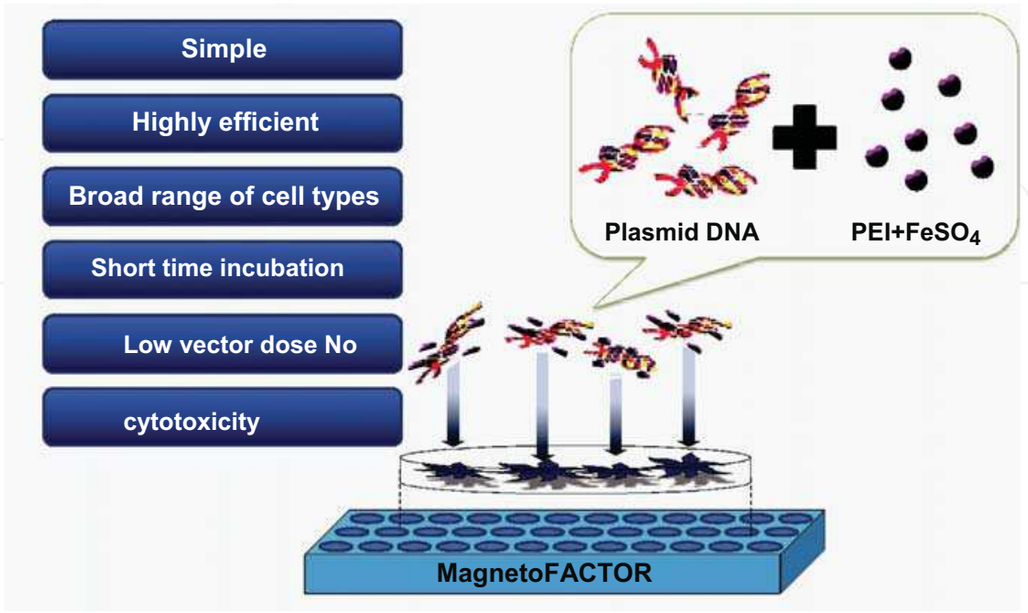

Fig. 1. Generalized view of magnet-based nanofection

4.1.4 Time

Usually, transfection reagents must be in contact with cells for a period of time, then additional medium is added or the medium is replaced to assist decrease toxic effects of the reagent. The optimal transfection time depends on the cell lines, transfection reagent, and nucleic acid used. These reagents usually require significantly shorter time when compared to other transfection reagents and can be decreased to as little as 30 minutes with certain cell lines. This shortened transfection time may help in considerably decreased risk of cell death.

4.1.5 Serum condition

Transfections by magnet-based nanofection protocols often require serum-free conditions for optimal performance, because serum can interfere with many commercially available transfection reagents. It has to be noted that the best results are obtained when variability is minimized among lot of serum.

4.1.6 DNA delivery mechanism of magnet-based nanofection

The magnetic nanoparticles are majorly made up of iron oxide particles, which are fully biodegradable, coated with specific cationic property of the molecules depending upon the applications. Their association with the gene vectors (DNA, siRNA, ODN, virus, etc.) is achieved by salt-induced colloidal aggregation or electrostatic interaction. The magnetic particles are then concentrated towards the target cells by the force of an external magnetic field generated by magnets. The cellular uptake of the genetic material is accomplished by endocytosis and pinocytosis, two biologically natural processes. Consequently, membrane architecture and structure are reported to stay intact, in contrast to other physical transfection methods that damage the cell membrane (Kamau et al., 2006). The nucleic acids are then released into the cytoplasm. However, the releasing of nucleic acid may be by different mechanisms depending upon the formulation used:

1. The proton sponge effect caused by cationic polymers coated on the nanoparticles promotes endosome osmotic swelling, disruption of the endosome membrane, and intracellular release of DNA.
2. The destabilization of endosome by cationic lipids coated on the particles that release the nucleic acid into cells by flip-flop of cell negative lipids and charge neutralization
3. The usual viral infection mechanism when virus is used in magnet-based nanofection works for primary cells and the cells which are hard to transfect, that are not dividing or slowly dividing, meaning that the genetic materials can go to the cell nucleus without cell division. Coupling magnetic nanoparticles to gene vectors results in a dramatic increase of the uptake of these vectors and consequently high transfection efficiency.

The biodegradable cationic magnetic nanoparticles are reported to be non toxic at the recommended doses and also even at higher doses (Mykhaylyk et al., 2010). Complexes of gene vectors and magnetic nanoparticles are seen in cells after 10–15 minutes which is much faster than any other transfection method. After 24, 48, or 72 hours, most of the particles are localized in the cytoplasm, vacuoles (membranes surrounded structure into cells) and occasionally inside the nucleus.

Thus, magnet-based nanofection uses a simple protocol for high efficient transfection in most of cell lines, up to several thousand fold increased levels of transgene expression upon short-term incubation (about 15~20 minutes). Moreover, great transfection rates and transgene expression levels are achievable with extremely low vector concentrations and without intense cytotoxicity.

5. Application of Magnet-based nanofection

5.1 Cell lines

The introduction of nucleic acids into cells is relevant for therapeutic (gene therapy) purposes. Many scientists have reported that high gene delivery can be obtained by magnet-based nanofection in various cell lines. Magnetic drug targeting was used to enhance nonviral transgene expression in airway epithelial cells and tracheal airway epithelium ex vivo (Gersting et al., 2004). They also found that efficiency of transfection by magnet-based nanofection increased compared to other non viral gene delivery methods like polyfection and lipofection by 2–3 orders of magnitude.

Transfection efficiency of a luciferase reporter gene was increased up to 360-fold by magnet-based nanofection in umbilical vein endothelial cells (HUVEC) compared to various conventional methods (Ido et al.,2002). In contrast, there was only an up to 1.6-fold increase in toxicity caused by magnet-based nanofection, signifying that the advantages of magnet-based nanofection outbalanced the increase in toxicity. It is quite simple, effective, and cheap improvement of plasmid gene delivery that results in hundred fold increased efficiency in gene expression in target cells (Plank, Anton et al., 2003). In this respect, they established a powerful tool in molecular physiological research and at the same time demonstrated its potential in gene therapies for cardiovascular diseases. As part of this study, they also showed the potency of magnet-based nanofection for the delivery of siRNA (small interfering RNA).

Among the many strategies that have been proposed, immuno gene therapy has been one of the most of the common approaches. The authors have introduced cytokine genes into tumor cells which when re-administered after irradiation to a patient, are supposed to elicit an immune response against tumor antigens by the immunostimulatory effect of the expressed cytokine in general.

In another study, the gene coding for human GM-CSF (granulocyte macrophage colony stimulating factor) under the influence of the CMV promoter in magnetic formulation was administered twice in a one week interval prior to surgery into the biologically active margins of the fibrosarcoma. Equal volume of plasmid DNA and magnetic particles (trans-MAG-PEI, chemicell, Berlin, Germany) was mixed in physiological saline. A neodymium–iron–boron permanent magnet was fixed on the tumor adjacent to the injection site during vector injection. They found that expression of the magnetofected cytokine gene was increased in tumor (Gersting et al., 2004).

5.2 Embryonic stem cells and magnet-based nanofection

5.2.1 The Magnet-based nanofection method: non-viral gene transfection technologies for mammalian cells

Although magnet-based nanofection was studied in various cell lines, Lee et al. originally used this technology for gene delivery in embryonic stem cells in 2008 (Lee et al., 2008). In this method, superparamagnetic nanoparticles (tsMAG) coated with cationic polymer polyethyleneimine (tsMAG-PEI) were combined with plasmid DNA. The tsMAG-PEI and DNA complex was generated by salt-induced aggregation and used for magnet-based nanofection.

In this study, we compared the transfection efficiency of different DNA delivery methods such as PolyMag-mediated magnet-based nanofection, FuGENE 6-mediated transfection, liposome–mediated transfection, nucleofection, and calcium phosphate-mediated transfection both in NIH3T3 and embryonic stem cells.

		Magnet-based nanofection	FuGENE 6-mediated transfection	Liposome–mediated transfection	Nucleofection (6well)	Calcium phosphate-mediated transfection
PlasmidDNA µg per 24-well plate		0.5-2	0.2-0.4	0.8 -1.2	1-5	1.1
Reagent µl per 24-well plate		0.5-2	0.6-1.8	2	100	1X HBS = 18, 2.5M CaCl2 =1.1
Cells per well (24-well plate)		70-80 %	70-80 %	70-80 %	70-80 %	70-80 %
Transfection efficiency	NIH3T3	> 90 %	> 90 %	> 80~90 %	> 80~90 %	20 ~100 %
	ES cells	> 70~80 %	> 40~50 %	> 40~50 %	-	-

Table 2. Comparison of transfection efficiency of different DNA delivery systems

Under standard conditions, mammalian cells take up and express externally applied DNA with very low efficiency. This is commonly due to the lipid bilayer of the eukaryotic cell membrane, which oppose the entry of charged molecules into a cell. All transfection methods cannot be applied to all types of cells or experiments. The different methods vary greatly with respect to the level of gene expression that can be achieved.

Liposome–mediated transfection is a cationic liposome based reagent that provides high transfection efficiency and increased levels of the transgene expression in a range of mammalian cell lines *in vitro* using simple protocol (Dalby et al., 2004). However, this transfection reagent had highest efficiency and also highest toxicity. These results indicate that liposome–mediated transfection cannot be a widely accepted gene delivery method in ES Cells.

FuGene 6-mediated transfection is one of the non liposomal transfection methods and has been used since 1997. Since that time, its popularity has increased due to its ease of use, minimal to no cytotoxicity, and the high level of transfection in various cell lines. This reagent can be easily used to transfect cells, very efficient and little numbers of cells are required for quantifiable response by following the standard protocol. High levels of transfection can be obtained by this method, compared to the traditional reagents (Mykhaylyk et al.,2007, Namiki et al., 2009, Plank, Schillinger et al., 2003). It was also reported that the efficiency of transfection by Fugene 6 in stem cells was very low (15 %) (Siemen et al.,2005). These results indicate that FuGENE 6 cannot be feasible method for ES cells.

Recently, it has been reported that magnet-based nanofection gave a significantly higher efficiency (45 %) of gene delivery in stem cells than did the FuGENE 6-mediated transfection method (15 %) and found that these cells maintained their usual un-differentiated characteristics of self-renewal and pluripotency for a long time (> 50 passages) (Lee et al., 2008).

5.2.2 Application of magnet-based nanofection in ES cell research

Many non viral systems have come up recently for gene delivery in embryonic stem cells. But due to low efficiency levels, scientists started focusing on other alternative methods. Considering that these non viral gene delivery methods do not significantly affect basic properties of ES cells.

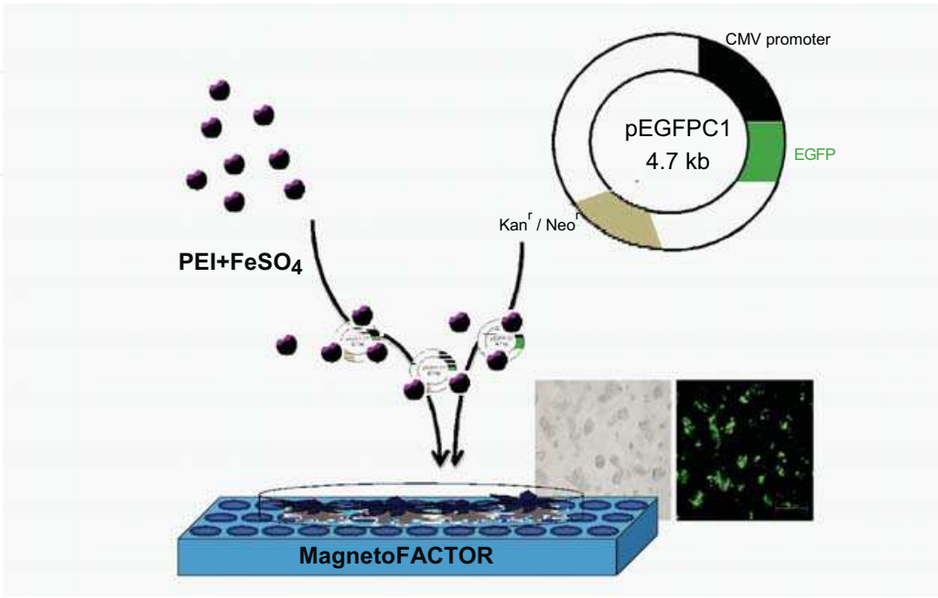

Fig. 2. Applicaton of the magnet-based nanofection method in ES cells

In this chapter, we discuss about magnet-based nanofection in detail. In this technique, plastic tissue culture dishes were pre-treated with 0.1 % gelatin in 24-well plate on previous day of transfection. Undifferentiated mouse embryonic stem cells (D3) were cultured in Dulbecco's modified eagle's medium (DMEM) and transfected with a CMV promoter-driven enhanced green fluorescent protein (pEGFP) expression vector through magnet-based nanofection. For the transfection method, 1 µg pEGFP vector was suspended in 98 µl serum free buffer and 1 µg PolyMag was added to the mixture and incubated at room temperature for 15~20 minutes. The complex was added to the serum-free DMEM medium in each of the 24 wells. The plates were placed on the magnetofactor plate device for 15~20 minutes at 37^0 C in 5 % CO_2 incubator. After selection by using G418 antibiotic for 2 weeks, the positive colonies were subcultured separately in a new cell culture dishes which were pretreated with 0.1 % gelatin. To confirm that the cells are in undifferentiated state, some of the basic confirmatory tests were performed.

1. AP staining protocol:

- Cells were cultured on sterile glass cover slips or slides overnight at 37 ^{o}C.
- Add fixative solution for 30 seconds.
- Rinse gently in deionised water for 45 seconds.
- Add to alkaline-dye mixture and incubate at 18^o C ~ 26^o C for 15 minutes in dark.
- Wash the excess dye with distilled water and observe under microscope.

2. Immunocytochemistry protocol:

- Fix the cells in 4 % PFA (paraformaldehyde) for 20~30 minutes at 37^o C.
- Permeabilization of cells using 0.1 % triton X

- Incubate the cells in 10 % goat serum for 1 hour at room temperature.
- Incubate the cells with the primary antibody at 4° C overnight.
- Rinse the cells in 1 % goat serum for 10 minutes.
- Incubate the cells with secondary antibody.
- Mount coverslips and visualize under microscope.

3. FACS (*Fluorescence-activated cell sorting*) analysis protocol:

- Harvest the cells using 0.25 % trypsin and resuspend the cells in PBS (Phosphate buffer saline).
- Fix the cells in 0.5 ~ 1 ml of cold 70 % ethanol and incubate for 20~30 minutes at 4° C.
- Add 0.5 ~ 1 ml of cold PI (propidium iodide) solution to the cell pellet.
- Suspend the cells immediately and acquire using flow cytometry.

4. EB (Embryonic body) formation protocol:

- Harvest the cells using 0.25 % tryPLE.
- Decant the supernatant and resuspend the cells in ES medium.
- Gently mix the cells to form a single cell suspension.
- Spin down the cells and remove the supernatant and resuspend the cells in ES medium.
- Count the cells and add ES medium without LIF (Leukemia inhibitory factor).
- Incubate the cells for 24 hours at 37 ^{o} C incubator.

5. Spontaneous differentiation protocol:

- EB (Embryonic body) formation by the 8 day induction method ($4^{-}/4^{+}$).
- EB's were plated onto 0.1 % gelatin-coated plates.
- Cultured for 15 days on N2 medium with 20 ng/ml bFGF (basic fibroblast growth factor).

6. Teratoma formation protocol:

- Embryonic stem cells are harvested.
- Centrifuge the cells for 5 minutes at 1, 200 rpm.
- Inject cells into the leg muscle of 4-week-old SCID-beige mice (male).
- After 10 weeks, examine for the teratoma formation.

7. Methylation analysis protocol:

- Isolation of genomic DNA and restriction enzyme digestion.
- Alkaline lysis and denaturation of DNA at 37° C for 15 minutes.
- Add 2 x volumes of 2 % low melting agarose to the DNA solution and mix thoroughly.
- Prepare the agarose beads by pipetting 10 µl aliquots of DNA/agarose mixture in cold mineral oil.
- Transfer beads in the tube containing 1 ml of modifying solution.
- Incubate the tubes for 4 hours at 50°C in dark.
- Wash the beads 6 times for 15 minutes with tris EDTA (ethylene diamine tetraacetic acid).
- Incubate for 15 minutes in 0.2N NaOH (sodium hydroxide) and wash the beads for 15 minutes with double distilled water.
- Methylation levels are checked by PCR (Polymerase chain reaction).

8. Chimera & germline transmission:

- Construct the homologous recombinant gene and transfect in cultured mouse embryonic stem cells by electroporation.
- Positive cells are selected by antibiotic mediated resistance marker provided in insertion cassette.
- Targeted gene is inserted into diploid mouse blastocyst.

- The injected blastocysts were implanted into pregnant female surrogate mouse, which directs the embryos to full term development and give birth to a mouse whose germline is derived from the donor mouse's ES cells.

So, the magnet-based nanofection is considered as extremely easy, less time- consuming, non cytotoxic and did not affect properties of ES cells like proliferation or differentiation. Magnet-based nanofection system may be the tool of choice for gene delivery and expression in ES cells.

5.3 Induced pluripotent stem (iPS) cells and magnet-based nanofection

5.3.1 Introduction of iPS cells

Most of the research on ES cells was on pluripotent stem cells, which are derived from the inner cell mass (ICM) of embryos in the blastocyst stage of development (Martin, 1981). Self-renewal and pluripotency are the two most essential characteristics of ES cells (Martin, 1981, Brimble et al., 2007) These cells owe the potential to differentiate into any cell type and provides a promising application in regenerative medicine (Mimeault et al.,2007, Keller, 2005). ES cells are the acknowledgment as a cell culture model for researching developmental mechanisms and their therapeutic modulations. The proliferative and developmental capacity of ES cells promises an essentially unlimited supply of specific cell type for basic research and transplantation therapies. However, one of the major concerns in ES cell research has been the ethical implications in using stem cells which are derived from embryo and also the therapeutic applications of stem cells depends on the availability that are limited by technical, ethical, or immunological considerations (Lowry et al., 2008, Wernig et al., 2007). Somatic cells reprogramming can be achieved by viral-mediated transduction using defined transcription factors (Feng et al.,2009). The iPS cells showed ES cell-like properties, such as morphology, gene expression profiles, differentiation into three germ layer lineages, formation of teratomas, and epigenetic status of several pluripotency markers (Lee et al., 2008). Their therapeutic potential is thought to be similar to that of ES cells in several disease models (Martin, 1980). Various reports from many labs gave contributed to a growing list of reprogramming factors used for iPS cells generation, such as Oct4, Sox2, Klf4, c-Myc, Lin28, Tcl1, Esrrb, Sall4, miRNA, SV40LT antigen, and telomerase reverse transcriptase (hTERT). Several small molecules also have been reported that enhances ES cell-like colony formimg efficiency such as supplementation with AZA (Azacytidine), VPA (Valproic acid), TSA (Tricholoro acetic acid), SAHA (suberoylanilide hydroxamic acid), BIX (Histone methyl transferase inhibitor), BayK (Calcium channel agonist), RG108 (DNA methyl transferase inhibitor), hypoxia condition, protein extract treatment, and knockdown of p53, p21, mdm2, and p16 (Plank, Anton et al., 2003, Plank, Schillinger et al., 2003).

5.3.2 Current trend of iPS cells research

Many researchers have discussed different methods for generating iPS cells. In this section, we will discuss the current techniques that are employed to introduce the reprogramming factors required for iPS cells generation (Zhao et al., 2009). Retroviral vectors were used for the initial derivation of iPS cells by Takahashi et al. But retroviral vectors may lead to many obstacles like oncogenesis and teratoma development in therapeutic applications. To avoid or reduce the potential oncogenic effect of genomic integration of viral vectors, non viral gene delivery methods was introduced (Wiles and Johansson, 1999, Lakshmipathy et al., 2004, Zeng et al., 2003, Plank, Schillinger et al., 2003). These non-viral gene delivery systems appear to have numerous advantages, including ease to manipulate, increased stability, low

cost, safety, and high flexibility regarding the size of transgene delivery (Kircheis, Wightman et al.,2001, Nimesh et al., 2006). Several researchers reported non-viral gene delivery methods, such as adenoviral vectors (D. Kim et al., 2009), non-integrating plasmids (Papapetrou et al., 2009), Cre recombinase/loxP system (Wang et al., 2007), piggyBac transposon-based system (Yusa et al., 2009), RNA transfection (Fusaki et al.,2009), and reprogramming by recombinant proteins (D. Kim et al., 2009). However, the efficiency of non-viral gene delivery systems was less compared to that of viral gene delivery methods.

Sl.no.	Method of Gene delivery	Factors for Pluripotency	Cell lines	Efficiency	Reference
1	Virus mediated Transfection	Oct4, Sox2, Klf4, c-Myc	Fibroblast	0.0001 %	Takahashi and Yamanaka, et al.,2006
2	Virus mediated transfection	Oct4, Sox2, Nanog and Lin28	fibroblast	> 0.01 %	Yu et al., 2007.
3	Lentiviral vectors	Pax5 shRNA, C/EBPa	Mouse B cell	> 0.001 %	Hanna et al., 2008
4	Retroviral vectors	Oct4, Sox2, Klf4	Neural stem cells	0.1-1.0 %	Kim et al., 2008
5	Retroviral vectors	Oct4, Sox2, Klf4	hepatocytes	0.5-1 %	Aoi et al., 2008
6	Retroviral vectors	Oct4, Sox2, Klf4	MEF	0.001-0.010 %.	Nakagawa et al., 2008
7	Lentiviral vectors using Cre recombinase /loxP system	Oct4, Sox2, Klf4, c-Myc	human fibroblast	0.001-0.002 %	Soldner et al., 2009
8	Piggy BAC transposons	Oct4, Sox2, Klf4, c-Myc	fibroblast	2.5 %	Yusa K et al., 2009
9	Episomal Vectors	OCT4, SOX2, NANOG, LIN28, c-Myc, KLF4, and SV40LT	Human foreskin fibroblasts	~ 0.1%	Yu et al., 2009
10	Lipofection	OCT4, SOX2, NANOG, LIN28, c-Myc, and KLF4	Mouse fibroblasts	> 0.01 %	Okita et al., 2008

Table 3. Different methods and sources for generating iPS cells

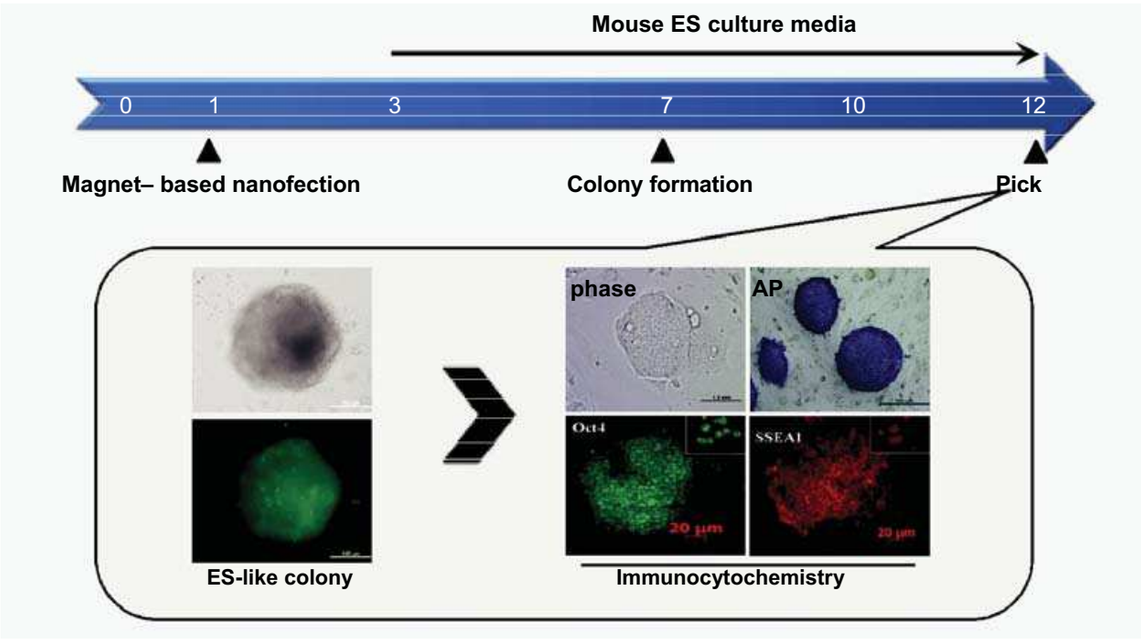

Fig. 3. Schematic representation of generation of iPS cells from MEF and characterization of ES-like colony with analysis of pluripotent marker expression

5.3.3 Generation of iPS cells using magnet-based nanofection

Since the initial derivation of iPS cells by Shinya Yamanaka's group, the field has advanced at a rapid pace. Main progress has been made in identifying new strategies to enhance the reprogramming efficiency and methods to improve clinical safety (Plank, Schillinger et al., 2003). In a recent study, generation of iPS cells using magnet-based nanofection was proved to be a very simple, highly efficient, cost -effective, and minimal to no cytotoxicity, implying that this system may be beneficial for generating safe iPS cells, compared to the other methods described previously.

Induced pluripotent stem cells were first generated by transfecting four factors in MEF cells (Takahashi and Yamanaka, 2006). For transfection, MEF cells were seeded (70~80 %) into each well of 96-well plates on the previous day of transfection and then four factors (Oct4, Sox2, Klf4, and c-Myc) was transfected into MEF by the magnet-based nanofection and ES cell media was changed alternative day. Some colonies of ES-like morphology were observed on seven days after transfection, and the iPS cell colonies generated by transduction of four factors were picked on day 12. The iPS cells were cultured in ES cell media on STO feeder cells, using standard embryonic stem cell culture protocol (Thomson et al., 1998, Xiao et al.,2006).

Magnet-based nanofection-derived iPS cells showed ES cell-like characteristics, such as expression of high levels of akaline phosphatase, expression of undifferentiated ES cell-specific cell surface markers (such as SSEA-1 and Oct4), and expression of endogenous pluripotency genes (Oct4, Nanog, Sox2, Klf4, and c-Myc). These cell lines also differentiated into various cell types through the embryid body (EB) formation. The three germ layer cell differentiation markers was observed through immunocytochemistry; endoderm (Tie2 and AFP), mesoderm (Brachury and Tn1) and ectoderm (Tuj1 and A2B5). Of particular interest, transcriptome, and proteome expression profiles of iPS cells confirmed that the iPS cells

more similar to ES cells, but different from MEF. Magnet-based nanofection-derived iPS cells identified 2,102 differentially expressed genes (DEGs) between MEF and ES cells (J1 and R1). Clustering analysis was performed for the expression of DEGs for MEF, ES cells, and magnet-based nanofection-derived iPS cells. Gene expression profiles for the magnet-based nanofection derived iPS cells was highly similar to that of ES cells (J1 and R1) and clearly distinct from that of MEF. As expected, 80-90 % of proteins found in magnet-based nanofection derived iPS cell lines were similar to those in ES cell line (D3). When transcriptome was compared to proteomic analysis, it showed a more significant difference in protein expression patterns between iPS cells and MEF. According to gene ontology analysis, functional classification appeared to be similar between cell lines with stemness (magnet-based nanofection derived iPS cells and ES cells) and MEF in most biological processes, such as protein metabolism and modification, cell cycle, transport, immunity, cell proliferation, differentiation, and apoptosis etc.

Recent study showed that primate ES cells can be derived by magnet-based nanofection from mouse embryonic fibroblast cells. In near future, this novel gene delivery system may open the door to the possibility of generating mouse /human cells for "patient-specific" iPS cells.

4. Summary

Mouse embryonic stem cells, which are derived from the epiblast of inner cell mass of the blastocyst have the ablity to self-renewate and differentiate into any cell types. The therapeutic use of these cells depends on the availability of pluripotent cells that are limited by technical, ethical, or immunological considerations.One of major technical drawback of gene delivery in ES cells is method of viral gene delivery systems, which may cause insertional mutagenesis, and also may induce cytotoxicity problems. Apart from the viral gene delivery systems, there are many non viral methods of gene delivery systems like calcium phosphate-, DEAE -dextran-, Fugene 6-, or liposome-mediated transfection and nucleofection. However, due to low efficiency, high toxicity, and economical problems, these methods are not widely accepted.

In this review, we have described application of magnet-based nanofection in various cell lines including ES and iPS cells. Among all the non viral gene delivery methods, magnet-based nanofection is an appropriate tool to overcome the strong barriers like low efficiency, slow vector accumulation, low vector concentration at target tissues, and toxicity problems. It is very simple, less time-consuming, cost-effective, and highly efficient even with low doses of nucleic acids. In this methodology magnetic field is applied to superparamagnetic iron peroxide particles which are associated with gene vectors. Gene vectors and magnetic nanoparticles complexes are seen inside the cells after 15-20 minutes, which is much faster than any other transfection method. After 24, 48, or 72 hours, most of the particles are localized in the cytoplasm, vacuoles, and frequently in the nucleus. Importantly, this type of design is universally applicable to all types of gene vectors. The concept of magnet-based nanofection greatly benefits from the fact that the individual modules of the system can be optimized independently and variants can be assembled in a combinatorial manner, thus facilitating optimization towards specific applications. The size and surface chemistry of magnetic particles can be tailored to meet specific demands on physical and biological characteristics, and the linkage between vector and magnetic particle can be designed accordingly.

Magnet-based nanofection is used as gene delivery tool in several cell lines and also has been used in ES cells. Recently, iPS cells were generated by using magnet-based nanofection technique and there are several perspectives to the future use of magnet-based nanofection. The three important features of magnet-based nanofection are:

1. The drastically lowered vector dose.
2. The reduced incubation time required to achieve high efficiency.
3. The possibility of gene delivery in non dividing cells.

In conclusion, the magnet-based nanofection will be an ideal research tool where the available vector dose, the required process time, and the sustainable costs of the procedure are limiting factors. In near future, magnet–based nanofection may become a strong choice for the clinical applications. Obvious target diseases are cancer, cardiovascular, or neurological diseases, and potentially also genetic diseases. Combined with existing advanced concepts of gene delivery, magnet–based nanofection may provide additional specificity and efficiency which are required in many gene therapy approaches.

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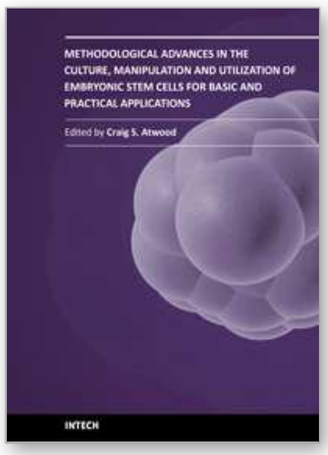

Methodological Advances in the Culture, Manipulation and Utilization of Embryonic Stem Cells for Basic and Practical Applications

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes methodological advances in the culture and manipulation of embryonic stem cells that will serve to bring this promise to practice.

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