

# Mouse embryonic stem cells efficiently lipofected with nuclear localization peptide result in a high yield of chimeric mice and retain germline transmission potency

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## Abstract

Embryonic stem (ES) cells are an important tool in developmental biology, genomics, and transgenic methods, as well as in potential clinical applications such as gene therapy or tissue engineering. Electroporation is the standard transfection method for mouse ES (mES) cells because lipofection is quite inefficient. It is also unclear if mES cells treated with cationic lipids maintain pluripotency. We have developed a simple lipofection method for high efficiency transfection and stable transgene expression by employing the nonclassical nuclear localization signal M9 derived from the heterogeneous nuclear ribonucleoprotein A1. In contrast to using 20 µg DNA for  $10 \times 10^6$  cells via electroporation which resulted in 10–20 positive cells/mm<sup>2</sup>, M9-assisted lipofection of  $2 \times 10^5$  cells with 2 µg DNA resulted in > 150 positive cells/mm<sup>2</sup>. Electroporation produced only 0.16% EGFP positive cells with fluorescence intensity (FI) > 1000 by FACS assay, while M9-lipofection produced 36-fold more highly EGFP positive cells (5.75%) with FI > 1000. Using  $2.5 \times 10^6$  ES cells and 6 µg linearized DNA followed by selection with G418, electroporation yielded 17 EGFP expressing colonies, while M9-assisted lipofection yielded 72 EGFP expressing colonies. The mES cells that stably expressed EGFP following M9-assisted lipofection yielded > 66% chimeric mice (8 of 12) and contributed efficiently to the germline. In an example of gene targeting, a knock-in mouse was produced from an ES clone screened from 200 G418-resistant colonies generated via M9-assisted lipofection. To our knowledge, this is the first report of generation of transgenic or knock-in mice obtained from lipofected mES cells and this method may facilitate large scale genomic studies of ES developmental biology or large scale generation of mouse models of human disease.

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## 1. Introduction

Pluripotent embryonic stem (ES) cells, derived from the embryo blastocyst inner cell mass, can be propagated in an undifferentiated state in vitro. Mouse ES (mES) cells were first isolated in the early 1980s [1,2] while human ES cells and human embryonic germ cells (EG cells) were isolated in 1998 [3,4]. ES cells are a major tool in knock-out mouse technologies, tissue engineering applications, developmental biology, and differentiation studies. ES cells also serve as a potential resource for gene therapy and cell therapy. Genetically

engineered mice have become invaluable biological tools for investigating gene function and disease pathogenesis. With the human and mouse genome projects completed or near completion, the demand for large numbers of mouse models bearing predetermined genetic alterations obtained via ES cell technology is higher than ever.

ES cells retain their pluripotency for a limited time in culture and are extremely difficult to lipofect. Most gene targeting methods utilize inefficient, expensive, and time consuming electroporation approaches to introduce foreign genes into ES cells [5–7]. Electroporation is typically used to transfect mES cells, although the rate of mES cell survival following electroporation can be as low as 10% [5]. Electroporation may be particularly problematic for human ES cell applications where

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survivability of rare human stem cells, such as those from autologous cord blood, is critical. Electroporation is also labor intensive since it requires millions of cells and large quantities of plasmid (a maxiprep) [5]. A simple and efficient lipofection approach for mES transfection would be valuable for ES cell techniques and developmental studies.

Lipofection has low efficiency in certain cell types due to intracellular barriers, such as poor endocytosis, poor endosome escape, and/or poor nuclear localization of the transfected DNA [8]. ES cells are notoriously difficult to lipofect, and most commercially available lipid-based transfection reagents have either low transfection efficiency or high toxicity when used with mES cells [9]. We sought to address this problem by testing a non-classical nuclear localization (NLS) peptide to assist in lipofection.

Nuclear import is believed to be a rate-limiting step during gene delivery. In an early micro-injection study, Capecchi [10] showed that less than 1% of cells expressed transgenes when plasmid was injected into cytoplasm, while up to 50% cells expressed transgenes when plasmid was injected into the nucleus directly. Classical nuclear localization signals (cNLS) have been tested, but result in only a modest or negligible increase in transfection efficiency in commonly used cell lines, such as HeLa and 3T3 cells [11–13]. To overcome these problems, we turned to a nonclassical NLS termed M9, which is derived from the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 [14,15]. By conjugating M9 with a cationic peptide sequence for DNA binding, transgene expression increased 63-fold in confluent bovine aortic endothelium cells [16].

In this study, we tested the use of the M9 sequence for lipofection of mES cells. Our results demonstrated that M9-assisted lipofection dramatically increased transgene expression in mES cells. In addition, mES transfected with M9-lipofection retain their pluripotency and can contribute to the germline. This simple transfection method provides an alternative to electroporation for introducing DNA into ES cells. To our knowledge, this is the first report of transgenic or knock-in mouse generation from lipofected cells. Such a method may facilitate efforts to create large numbers of mouse models for genomic investigations of gene function and disease pathogenesis.

## 2. Results

### 2.1. Transfection efficiency of electroporation and lipofection

To compare the transfection efficiency of conventional electroporation and liposome-based transfection, we transfected AB2.2 mES cells with the pcDNA3-

EGFP plasmid. Electroporation was performed as described in Section 4, using  $10 \times 10^6$  cells and  $20 \mu\text{g}$  plasmid DNA. Fluorescence microscopy performed 48 h after electroporation demonstrated less than 10–20 weakly fluorescent EGFP positive cells/ $\text{mm}^2$  (Fig. 1A). FACS analysis of the same mES cells showed that 4.8% of electroporated mES cells had fluorescence signals stronger than background but only 0.16% of the electroporated cells had fluorescence signals with fluorescence intensity (FI) > 1000, indicating the majority of transfected cells had very low EGFP expression (Fig. 2B, Table 1). With optimized conditions of  $2 \mu\text{g}$  plasmid and  $2.5 \times 10^5$  cells, transfection with lipofectamine alone also generated approximately 10–20 weakly fluorescent cells/ $\text{mm}^2$  (Fig. 1B). Transfection with lipofectamine alone produced 0.93% of cells with FI > 1000, 5.8-fold more than detected following electroporation (Fig. 2C, Table 1). To test the effect of M9-assisted lipofection on the efficiency of mES transfection,  $2 \times 10^5$  mES cells were transfected with  $2 \mu\text{g}$  pcDNA3-EGFP DNA using M9 peptide and lipofectamine. Fluorescence microscopy 48 h after transfection showed more than 150 intensely EGFP positive cells/ $\text{mm}^2$  (Fig. 1C). FACS analysis of the same cells demonstrated that almost 10% of mES cells expressed EGFP above background fluorescence. A total of 5.75% of cells were highly fluorescent (M3, FI > 1000), which was 36-fold greater than the level detected after electroporation. The average FI of the most positive cells (M3) after M9-assisted lipofection was 6011 compared with FI = 1807 for electroporated mES cells (Fig. 2). As seen in Table 1, M9-assisted lipofection provided more cells with higher EGFP expression while using less plasmid and cell reagents in comparison to electroporation.

To optimize lipofection conditions for mES cells, we tested different ratios of lipofectamine:DNA and M9 peptide:DNA using  $2 \times 10^5$  cells and  $2 \mu\text{g}$  pcDNA3-EGFP plasmid DNA. Cells were examined by fluorescence microscopy and subjected to FACS analysis 48 h following transfection. As shown in Fig. 3A, the percentage of highest EGFP expressing mES cells (M3) with FI > 1000 was dramatically improved by use of M9 peptide. A ratio of 2–2.5  $\mu\text{g}$  lipofectamine per  $2 \mu\text{g}$  plasmid provided optimal transfection efficiency with minimized cationic lipid utilization. For comparison, the results with electroporation are also shown in Fig. 3 using  $20 \times 10^6$  cells and  $20 \mu\text{g}$  DNA.

Based on these observations, the 1:50:2.5 (wt:wt:wt) ratio of plasmid:M9:lipofectamine transfection formulation was the optimal choice used in the transient lipofection experiments shown in Figs. 1 and 2. Also, mean triplex plasmid/M9/lipid particle size generated under this formulation was 61% of the mean size of plasmid/lipid complexes, and these triplex particles were more monodisperse than the lipid/DNA complex, as seen by a dynamic light scattering assay (Fig. 4).

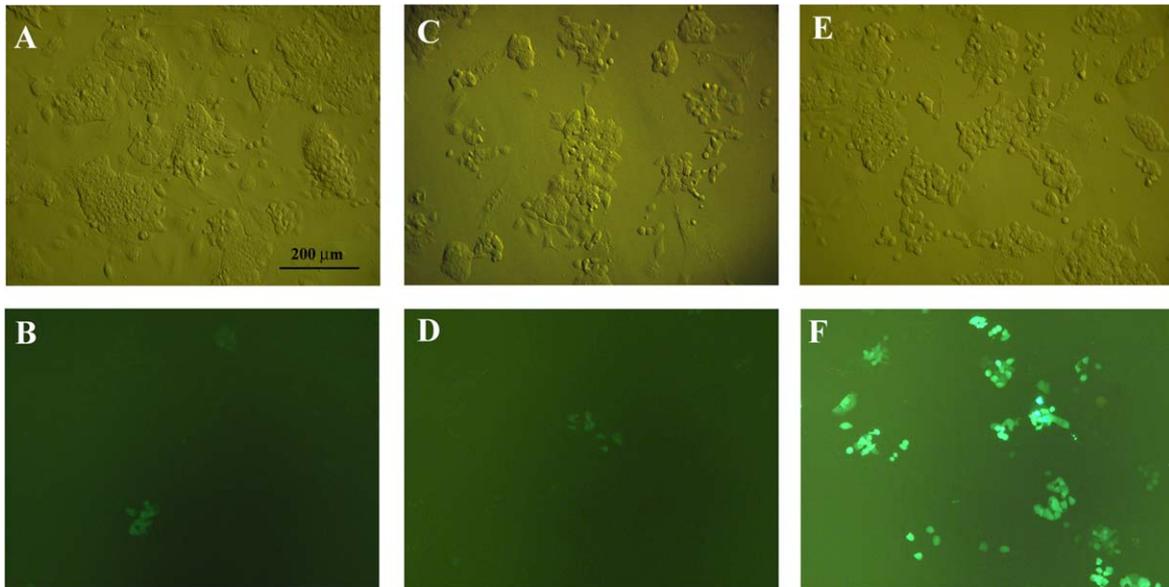


Fig. 1. ES cell transfections. Comparison of mES cell transfection by electroporation (A and B), lipofectamine (C and D), and M9-assisted lipofection (E and F). Upper panels (A, C, and E) are micrographs of cells viewed with phase-contrast; lower panels (B, D, and F) show the same field viewed by fluorescence microscopy to detect cells expressing EGFP.

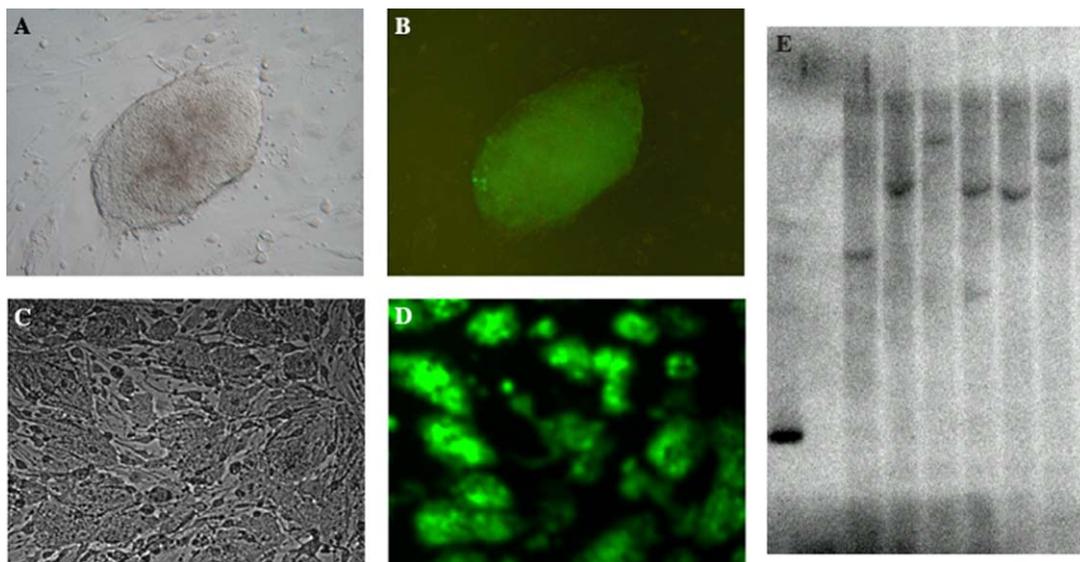


Fig. 5. Selection of stable ES-EGFP cell lines: An EGFP expressing colony during G418 selection is shown under light (A) and fluorescence microscopy (B). A stabilized EGFP expressing ES cell line is shown under light (C) and fluorescence microscopy (D). Southern blot analysis (E) of six clonal lines demonstrated single copy genomic integration of the EGFP sequence in five of the six clones (lane 4 shows 2 bands). Plasmid control shown on left side of blot.

## 2.2. DNA integration efficiency in electroporation and M9-lipofection

To determine if M9-lipofection is also more efficient than electroporation for establishing stable ES cell lines under identical cell and DNA conditions, we transfected  $2.5 \times 10^6$  mES cells with  $6 \mu\text{g}$  linearized pcDNA3-EGFP by either electroporation or M9-lipofection. Following transfection by both methods, the mES cells were sub-

jected to selection with G418. After 10 days of selection the total number of G418 resistant colonies and fluorescent colonies was counted. As shown in Table 2, M9-lipofection produced more than  $4 \times$  as many stable EGFP-expressing colonies than electroporation (72 vs 17). To verify integration of the pcDNA3-EGFP plasmid into the genomic DNA of the fluorescent ES cells after M9-assisted lipofection, 24 EGFP individual EGFP expressing colonies from the M9-lipofection were

Table 1  
Transient transfection of ES cells with electroporation and M9-assisted lipofection

Plasmid transfection	Electroporation	Lipofection	M9-assisted
DNA used	20 $\mu$ g	2 $\mu$ g	2 $\mu$ g
Total cells used	$10 \times 10^6$	$2 \times 10^5$	$2 \times 10^5$
EGFP positive cells/mm <sup>2</sup>	< 20	< 20	> 150
Average EGFP signal of Positive cells (M2 + M3, FI > 100)	300–400	500–600	4000–4500

picked and expanded (Figs. 5A and B). These EGFP-ES cells remained healthy in culture for up to 10 additional passages, with stable EGFP expression (Figs. 5C and D). Southern blot analysis of DNA from these lines indicated that half of these clones had one copy of pcDNA3-EGFP and remainder had two or three integrated copies of EGFP (Fig. 5E).

### 2.3. Generation of transgenic mice using lipofected ES cells

To determine if mES cells transfected with M9-lipofection retain their ability to produce chimeric mice and contribute to the germline, we selected a single clone of EGFP-ES cells for blastocyst injections. Injection of blastocysts, with six successful embryo transfers, resulted in the birth of 12 pups, 8 of which were chimeric. The extent of chimerism ranged between 99 and 50%; 6

pups were greater than 90% chimeric. The organs of one chimeric mouse were examined for EGFP expression. As can be seen in Fig. 6, cells in the heart, retina, and kidney demonstrated green fluorescence, confirming the presence of the EGFP transgene in those tissues.

To test the ability of M9-lipofected mES cells to contribute to the germline, 2 chimeric mice were mated with C57BL/6 mice. A total of 4F1 lines were generated, two from each of the chimeric mice. PCR analysis demonstrated transmission of the EGFP gene to 50% of pups. EGFP expression was found in many organs of the PCR positive progeny, including the retina, kidney, lung, and spleen (Fig. 7), confirming germline transmission of the transgene.

We studied the efficiency of homologous recombination in lipofected ES cells. We generated mice with targeted disruption of the Rpl gene using lipofection. For this experiment, three 100 mm dishes each containing  $2.5 \times 10^{-6}$  TL-1 ES cells were transfected with 6  $\mu$ g linearized targeting vector and 300  $\mu$ g M9 peptide per dish. Of the 200 G418-resistant colonies screened, one displayed the correct recombination at the Rpl locus. This clone was used successfully to generate highly chimeric mice. Germline transmission of the mutant Rpl allele was achieved from 7 out of 8 tested chimeras. This result demonstrated that lipofection can produce correctly targeted alleles at a rate comparable to or better than that obtained via electroporation (typically, 1 in 200 or worse). This is the first demonstration, to our knowledge, of a knock-in mouse generated by lipofection methods.

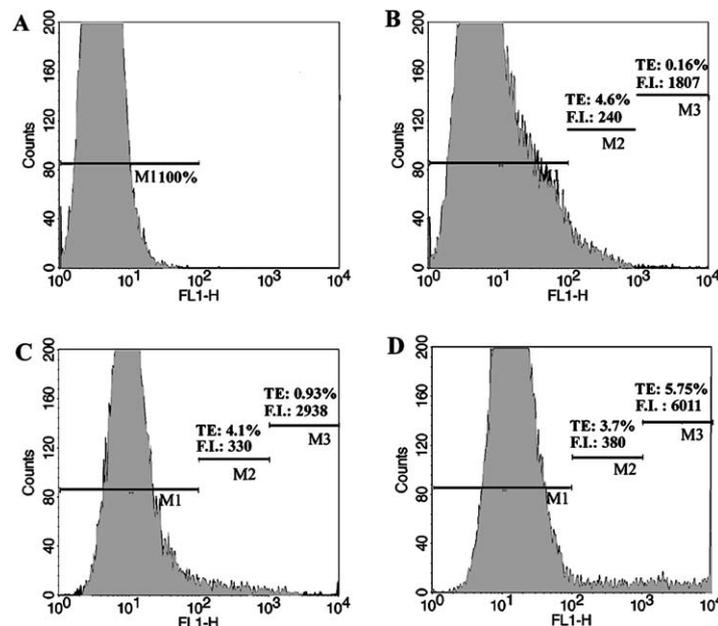


Fig. 2. Transfection efficiency. The efficiency of transfecting mES cells by electroporation (B), lipofectamine (C), and M9-assisted lipofection (D) was evaluated by FACS. The panels are histograms of the FACS analysis, showing the log of fluorescence intensity on the x-axis, and cell number on the y-axis. The FACS profile of untransfected control mES cells is shown in (A). The histograms are dissected into three categories to facilitate comparison. M1 is the background fluorescence with FI > 100 units. M2 is low fluorescence, with FI between 100 and 1000; M3 is high fluorescence, with FI > 1000 units. The transfection efficiency (TE) is indicated for M2 and M3 for each of the transfections.

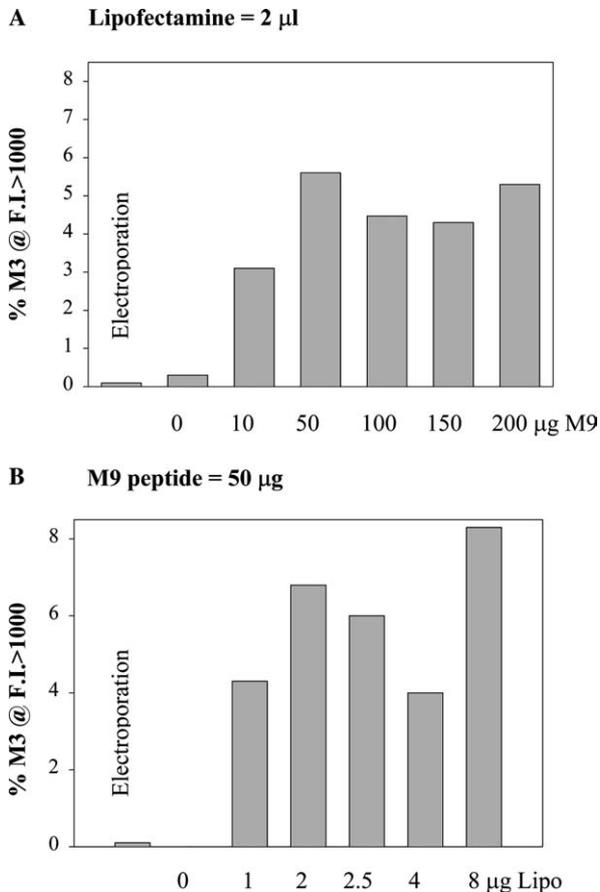


Fig. 3. Titration of M9 and lipofectamine for mES transfection. The percent of positive cells with FI > 1000 is plotted for each transfection condition. (A) M9 dose-dependent transfection with 2.5 µl lipofectamine. (B) Lipofectamine dose-dependent transfection with 50 µg M9. Twenty microgram plasmid and  $10 \times 10^6$  cells were used in electroporations and 2 µg plasmid and  $2 \times 10^5$  cells were used in all lipofections.

### 3. Discussion

ES cells are important resources for both fundamental research and potential therapeutic applications. The introduction of genes into ES cells is an important tool for analysis of DNA function, cell differentiation, cell/gene therapy, tissue engineering, and knock-out production [17,18]. We have demonstrated that M9-assisted lipofection of mES cells has higher transfection efficiency than electroporation and that this simple technique saves time and materials when used for gene targeting of ES cells. Most importantly, ES cells derived from this approach retained their ability to contribute to the germline.

Traditionally, transgenes are introduced into ES cells by electroporation. More than  $10 \times 10^6$  cells and 20–40 µg DNA are used in typical transfections [5,19]. During electroporation, about 90% ES cells are killed, and the final gene-targeting efficiency is less than two colonies per  $10^6$  treated cells [5]. We found similar

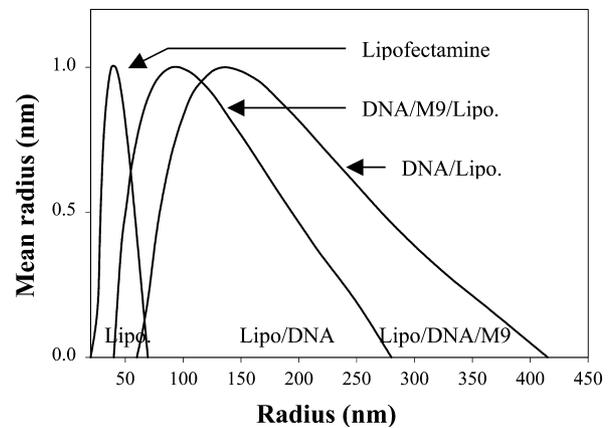


Fig. 4. Size distributions of the lipofection particles with and without addition of M9 peptide.

Table 2

Efficiencies for establishing ES-EGFP expressing cell lines and producing chimeric cells

Gene integration	Electroporation	M9-assisted
DNA used	6 µg	6 µg
Total cells used	$2.5 \times 10^6$	$2.5 \times 10^6$
G418 resistant colonies	38	198
EGFP positive colonies	17	72

transfection efficiency with electroporation, with 6–7 positive clones per  $10^6$  treated ES cells (Table 2). To find an alternative transfection method, many investigators have tried lipofection using different lipids. In one recent study, Watanabe et al. [9] tested eight different lipids for transfecting mouse primordial germ cells (PGCs) and found that less than 0.4% transfected cells expressed the marker gene.

We demonstrate an order of magnitude improvement both in transfection efficiency and level of gene expression per cell. To improve the efficiency of transfecting mES cells, we sought to use a new approach by employing the nonclassical nuclear localization signal, M9 [14,15] of the hnRNP A1 protein, which binds karyopherin-β2 (Transportin-1). M9-assisted lipofection has been tested and proved able to increase transfection efficiency and transgene expression level in nondividing cell lines such as confluent endothelium [8,16] as well as neurons [20]. M9-assisted lipofection of mES cells not only increased transfection efficiency, but also increased the transgene expression level. The fluorescence signal of the highest expressing mES cells (FI > 1000) following M9-assisted lipofection ES was 36-fold higher than that achieved by electroporation.

One important application of mES cells is to generate ES cells with targeted disruption of genes to use for producing mouse models of human diseases. Selecting and identifying correctly targeted ES clones is a rate-limiting step, since homologous recombination of the targeting vector with the endogenous gene is a very rare

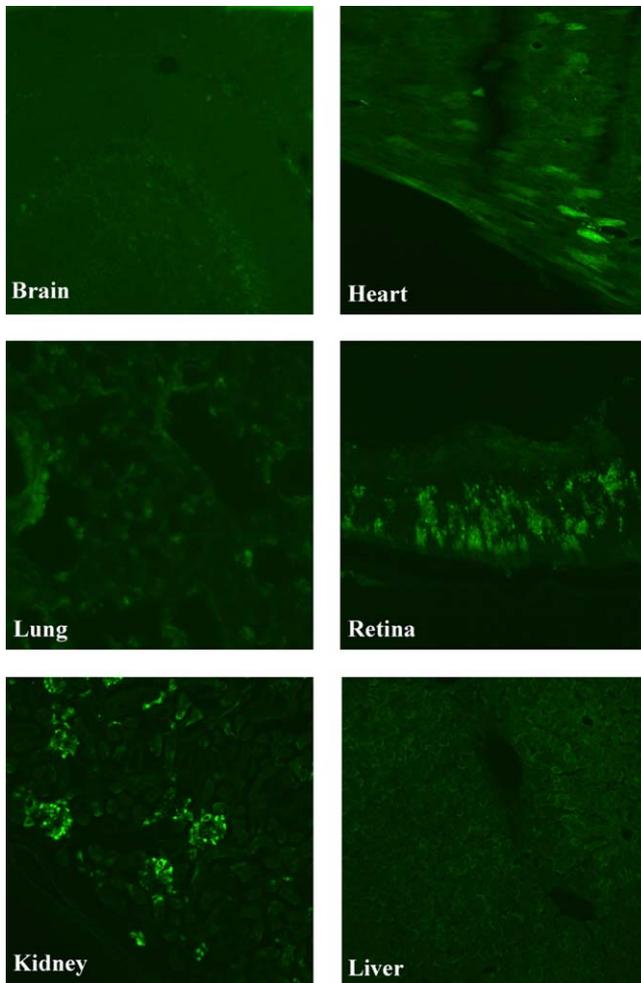


Fig. 6. The EGFP protein expression pattern in one of the chimeric mice. Heart, retina, and kidney had higher EGFP expression level than the lung, brain, and muscle (not shown), and no EGFP signal was detected in the liver.

event. Low nuclear importing of targeting vectors would further decrease the possibility of correct recombination. Our experiments demonstrated that M9-assisted lipofection generated higher transgene expression than electroporation, presumably due to delivery of higher levels of plasmid DNA to the nucleus. Consistent with this, M9-assisted lipofection generated  $4 \times$  more stable EGFP expressing clones than electroporation (Table 1). Improved delivery of vectors to the nucleus by M9-assisted lipofection could improve gene targeting efficiency. Increased transgene expression could also be beneficial for investigations of ES cell differentiation and for potential therapeutic uses of ES cells.

Experimental and therapeutic use of ES cells depends on their pluripotency. Our results indicate that M9-lipofected mES cells retain their pluripotency, as evidenced by their ability to efficiently produce chimeric mice capable of germline transmission of the EGFP transgene. M9-assisted lipofection has several advantages over electroporation that make it an attractive

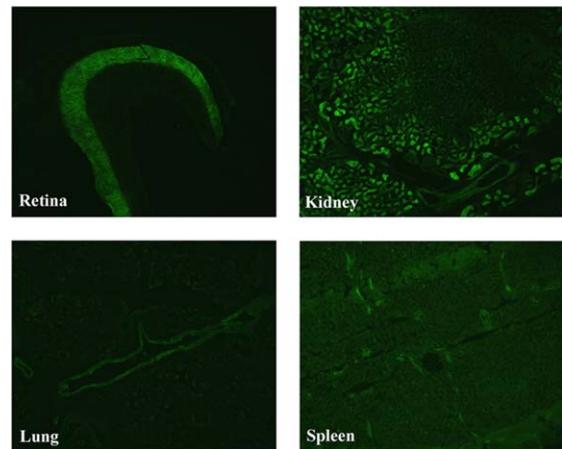


Fig. 7. The EGFP expression pattern in one F1 mouse. EGFP expression in retina, kidney, lung, and spleen confirms germline transmission of the transgene.

method for transfecting mES cells. It is easy to perform and requires fewer ES cells and less DNA than electroporation. Transfections can be performed in complete medium, with 15% serum, to diminish the risk of causing differentiation of the mES cells. Since fewer cells are needed for M9-assisted transfection, it may be possible to use lower passage number mES cells, and thus improve the success rate of gene-targeting experiments.

The simple lipofection technique described here could be useful for investigation of ES cell differentiation. The pluripotency of ES cells makes them attractive for therapeutic use, in that they could theoretically be used to replace any tissue. The factors which control ES cell differentiation, however, have not been completely defined [21]. For example, Schuldiner et al. [21] tested human ES cells with eight different kinds of growth factors and demonstrated that each factor has many effects on ES differentiation and a single type cell is extremely challenging to produce. M9-assisted lipofection could be used to transfect ES cells with genes that may be involved in differentiation and development, and thus facilitate these investigations.

In summary, M9-assisted transfection of mES cells provides a simple and efficient method for mES cell transfection. Since cells transfected by M9-lipofection retain their pluripotency, this technique should be useful for many types of experiments that utilize mES cells, including gene targeting techniques. It is also possible that M9-assisted lipofection could be used for transfection of human ES cells or stem cells derived from other tissues.

## 4. Materials and methods

### 4.1. Cell culture

AB2.2 mouse embryonic stem cells and ESQ feeder cells were purchased from Stratagene. STO feeder cells

were obtained from ATCC. Feeder cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) with 7–10% fetal bovine serum (FBS) (Hyclone). ES cells were cultured on Mitomycin C (10 µg/ml) inactivated feeders in ES medium (DMEM with 15% FBS, 1% nonessential amino acid (Gibco-BRL), 0.1 mM β-mercaptoethanol (Sigma), and 1250 U/ml leukemia inhibitory factor (LIF) (Chemicon)). For use in transfection experiments, mES cells were "panned" to remove feeder cells by plating trypsinized mES cells on gelatin-coated culture dishes for 30–45 min. The feeder-depleted mES cells were then collected and counted. For selection experiments, ES media were supplemented with 275 µg/ml G418 and 1% penicillin/streptomycin.

#### 4.2. Vector

The plasmid pcDNA3-EGFP was produced by inserting an *Xba*I fragment containing the EGFP coding sequence from pEGFP (Clontech) into pcDNA3.1 (Invitrogen). For establishing ES-EGFP stable cell lines, the vector was linearized with *Bgl*II or *Mse*I and then purified by phenol–chloroform extraction followed by ethanol precipitation.

#### 4.3. Electroporation

For electroporation experiments,  $10 \times 10^6$  mES cells in log phase were collected by centrifugation and resuspended in 0.8 ml DMEM. Plasmid (20 µg) was then added to the ES cells and the mixture was incubated for 5 min. mES cells were electroporated using a GenePulserII (BioRad) set at 250 V and 500 µF. In some experiments,  $2.5 \times 10^6$  mES cells were electroporated with 6 µg of linearized plasmid.

#### 4.4. Lipofection

Plasmid (2 µg) was complexed for 15 min with the cationic M9 peptide at a ratio of 1:50 (wt:wt) in 100 µl Optimem medium (Life Technologies) as previously described using G<sub>264</sub>–Y<sub>395</sub> of hnRNP A1 linked to a cationic peptide derived from a scrambled variant of the SV 40 NLS [16]. In some experiments, higher amounts of DNA were used as noted. The plasmid/peptide complex was mixed with lipofectamine (Life Technologies) in equal volume of Optimem for 45 min. At the end of this incubation,  $2 \times 10^5$  freshly panned mES cells/well were plated on inactivated feeder cells in a 6-well plate with 0.8 ml ES medium. The transfection mixtures were then added to the appropriate wells and the plate was centrifuged at 100g for 5 min. For some experiments,  $1 \times 10^5$  mES cells/well were seeded onto feeder-coated 6-well plates 18 h prior to transfection. M9-assisted lipofection was then performed using the same conditions

described above, except that the transfection mixtures were added to the adherent ES cells.

#### 4.5. Light scattering

Particle size distributions of DNA/lipofectamine, DNA/peptide/lipofectamine were analyzed with software DYNAMICS version 5.24.02 on a DynaPro 99 (Protein Solutions, Charlottesville, VA) at 26 °C. These samples were prepared using the same conditions used for mES cell transfections.

#### 4.6. EGFP expression

Following transfection, fluorescent mES cells were detected by fluorescence microscopy using a Nikon Diaphot inverted microscope. For quantitative determinations of transfection efficiency, fluorescent mES cells were detected by FACS using a FACScan flow cytometer (Becton–Dickinson) and CellQuest software. Instrument settings were kept constant for all samples within each experiment.

#### 4.7. Stable ES-EGFP cell lines

To produce mES cells that stably express EGFP,  $2.5 \times 10^6$  AB2.2 cells were transfected with 6 µg linearized pcDNA3-EGFP either by electroporation or M9-assisted lipofection. G418 (275 µg/ml) selection was started 24 h after electroporation or lipofection. After 10 days of selection with G418, the surviving ES cell colonies were examined by fluorescence microscopy. Fluorescent colonies were picked and expanded. The number of integrated copies of pcDNA3-EGFP was determined by Southern blot analysis using the EGFP coding region (*Xba*I fragment of pcDNA3-EGFP) as a probe.

#### 4.8. EGFP chimeric mice production and germline transmission

The mES cells from a single stable EGFP selected from lipofected ES clone were injected into blastocysts to create chimeric mice. These manipulations were performed in the Transgenic and Chimeric Mouse Facility at the University of Pennsylvania School of Medicine (Philadelphia, PA). The extent of chimerism of the resulting mice was estimated by examining coat color. The presence of the EGFP gene in chimeric mice was confirmed by PCR using genomic DNA isolated from tail or toe biopsies. The PCR primers used were as follows: EGFP2 5'-ACCTACGGCAAGCTGACCCTGAA-3'; EGFP3 5'-CGCTGCCGTCCTCGATGTTGTG-3'.

The EGFP expression in different organs of a chimeric mouse was also evaluated. The chimeric mouse was anesthetized with diethyl ether and sodium

pentobarbital and perfused via the heart with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The organs were removed and immersed in fixative for 6 h at 4 °C. After rinsing in PBS, the samples were immersed in 30% sucrose overnight at 4 °C, embedded in tissue freezing medium (Triangle Biomedical Science) and sectioned at 12 µm thickness for fluorescence microscopy. The germline transmission capability of the chimeric mice was tested by breeding two of the chimeric mice with C57BL/6 mice. The resulting litters were tested for EGFP DNA and expression by PCR and fluorescence microscopy of different organ tissue sections.

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