STEM CELLS®

Original Article

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Efficient Transfection of Embryonic and Adult Stem Cells

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ABSTRACT

The ability of embryonic stem cells and adult stem cells to differentiate into specific cell types holds immense potential for therapeutic use in cell and gene therapy. Realization of this potential depends on efficient and optimized protocols for genetic manipulation of stem cells. In the study reported here, we demonstrate the use of nucleofection as a method to introduce plasmid DNA into embryonic and adult stem cells with significantly greater efficiency than electroporation or lipid-based transfection methods have. Using enhanced green fluorescent protein (eGFP) as a reporter gene, mouse embryonic stem cells were transfected both transiently and stably at a rate nearly 10-fold higher than conventional methods. The transfected cells retained their stem cell properties, including continued expression of the stem cell markers SSEA1, Oct4, and Rex1; formation of embryoid bodies; differentiation into cardiomyocytes in the presence of appropriate inducers; and, when injected into developing blastocysts, contribution to chimeras. Higher levels of transfection were also obtained with human embryonic carcinoma and human embryonic stem cells. Particularly hard-to-transfect adult stem cells, including bone marrow and multipotent adult progenitor cells, were also transfected efficiently by the method of nucleofection. Based on our results, we conclude that nucleofection is superior to currently available methods for introducing plasmid DNA into a variety of embryonic and adult stem cells. The high levels of transfection achieved by nucleofection will enable its use as a rapid screening tool to evaluate the effect of ectopically expressed transcription factors on tissue-specific differentiation of stem cells. ***Stem Cells*** *2004;22:531–543*

INTRODUCTION

Mouse embryonic stem (ES) cells have been successfully used to study the developmental milestones from a single cell to a mouse. Generation of knockout mouse models by targeted disruption of essential genes provides useful insights into genes that regulate development, allowing investigators to dissect molecular developmental mechanisms. Gene targeting has also been used to create mouse models for many human genetic diseases, facilitating development of therapeutic strategies [1–4]. ES cells are also useful as an in vitro

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model system for the study of cell differentiation. Several transcription factors have been demonstrated to regulate differentiation of stem cells to specific cell types such as heart, pancreas, liver, and neuron [5–8]. Ectopic overexpression of such factors stimulates ES cells to differentiate to certain cell types [9–12].

Besides mouse ES cells, several other types of stem cells have been well characterized. Human embryonic carcinoma (hEC) cells isolated from germ line tumors resemble ES cells and have been extensively used to study embryogenesis [13]. Recently, human embryonic stem (hES) cells have been isolated from human blastocysts [14, 15]. Several types of adult stem cells have been isolated from various sources that display varying differentiation potential [16–20]. Such a repertoire of stem cells and their ability to be coaxed to differentiate into specific cell types provides new opportunities in regenerative medicine.

Several methods are available to achieve introduction of foreign DNA that carries a gene of interest for ectopic expression in cells. The most commonly used method for generation of transiently and stably transfected mouse ES cells has been electroporation. Several commercial liposome-based methods have also been used, with Lipofectamine (Life Technologies; Invitrogen, Carlsbad, CA) being the most widely used method [12, 21]. Human ES cells have been transfected by Exgen [22], a liposome-based method, and by electroporation [23]. Viral vectors such as adenovirus, adeno-associated virus, and lentivirus can also be used to overexpress cDNA in mouse and human ES cells [24, 25]. However, the use of these vectors requires each cDNA to be cloned into specific vectors, thereby hindering rapid screening of potential transcription factors involved in differentiation of ES cells into definitive cell types.

Here we describe a nonviral, high-efficiency method of transfecting a variety of mouse and human stem cells using the Amaxa Nucleofector (Amaxa Biosystems, Cologne, Germany). The Amaxa Nucleofector technology is an electroporation-based method in which a combination of a specific nucleofector solution and specific electric parameters achieves delivery of plasmid DNA into the cell nucleus, thereby resulting in enhanced gene expression. This method is effective in transfecting hard-to-transfect cells such as T cells [26] and dendritic cells [27]. Using enhanced green fluorescent protein (eGFP) as the reporter, we demonstrate that nucleofection achieves greater than 60% transient transfection efficiency in mouse ES cells and increases stable transfection of mouse ES cells by 10-fold compared with electroporation. Further, stable transfected cells maintain their ES cell properties. Nucleofection can be used for rapid screening to study the effect of ectopic expression of various transcription factors.

We further show that nucleofection transfects the human EC cell line, Ntera2, and hES, as well as multipotent adult progenitor cells (MAPCs) and hematopoietic progenitors from fresh mouse bone marrow cells. Based on these studies, we conclude that nucleofection is an efficient method for gene transfer into stem cells from human and mouse.

Materials and Methods

Materials

Mouse ES cells (line R1) were obtained from Dr. Janet Rossant, Mount Sinai Hospital, Toronto, Canada. Normal mouse embryonic fibroblasts (MEFs) were generated in the lab from 13 days postcoitum mouse embryos according to established procedure [28]. Human ES cells (H1) were obtained from WiCell (Madison, WI). MEFs resistant to neomycin were obtained from Specialty Media (Phillipsburg, NJ). Dulbecco's Modified Eagle's Media (DMEM) high glucose, DMEM low glucose, DMEM/F12, knockout serum replacement (SR), Stem Pro–34 serum-free medium (SFM), fetal calf serum (FCS), β-mercaptoethanol, L-glutamine, streptomycin-penicillin, nonessential amino acids, sodium pyruvate, trypsin, and Matrigel were obtained from Gibco-BRL (Grand Island, NY). FCS for MAPC culture was obtained from HyClone (Logan, UT); epithelial growth factor (EGF), dexamethasone, ascorbic acid 2-phosphate, linoleic acid-bovine serum albumin (LA-BSA), insulin, transferrin, and selenium (ITS), MCDB, gelatin, fibronectin (FN), and 3,3' diamino benzidine (DAB) tablets were from Sigma Chemical Corp. (St. Louis, MO); platelet-derived growth factor-BB (PDGF-BB), recombinant mouse stem cell factor (SCF), mouse interleukin-3 (IL-3), and thrombopoietin were from R&D Systems (Minneapolis, MN); and leukemia inhibitory factor (LIF) was from Chemicon Inc. (Temecula, CA). Nucleofector solutions (hMSC kit, mES kit, hCD34$^+$ kit) were obtained from Amaxa Inc. (Gaithersburg, MD). The calcium phosphate transfection kit and the expression vector pEGFP-N1 were purchased from Clontech (Palo Alto, CA). The Neurogenin3-green fluorescent protein (Ngn3-GFP) construct was kindly provided by Dr. Gerald Gradwohl (INSERM, Strasbourg, France). The transfection reagents, Superfect and Effectine, and DNA and RNA isolation kits were from Qiagen Inc. (Valencia, CA). Dimyristoyl Rosenthal inhibitor ether-C (DMRIE-C) and basic fibroblast growth factor (bFGF) were purchased from Invitrogen, and the Adenovirus Enhanced Transferrinfection (AVET) kit was purchased from Bender Med Systems (Vienna, Austria). The DNA-free RNA kit was obtained from Ambion Inc. (Austin, TX). Reverse transcription (RT) reagents and Taqman SYBR green universal mix polymerase chain reaction

(PCR) buffers were from Perkin Elmer Applied Biosystems (Boston, MA). Tissue-Tek OCT compound was from Sakura Finetek (Torrance, CA). Antibodies against goat antihuman cardiac troponin C were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY) goat antimouse Nkx2.5 from Santa Cruz Biotech (Santa Cruz, CA), and antigoat secondary antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC) from Jackson Immuno Research Laboratories (West Grove, PA). Rabbit anti-GFP was purchased from Abcam Inc. (Cambridge, MA), and biotin-labeled donkey antirabbit secondary antibody from Amersham Biosciences (Piscataway, NJ). Avidin-biotin complexed to horseradish peroxidase (HRP) was from Dako Cytomation (Carpinteria, CA). SSEA1 and SSEA4 antibodies were from the Developmental Studies Hybridoma Bank (Iowa City, IA), and secondary antibodies conjugated to phycoerythrin (PE) were obtained from Caltag Lab (Burlingame, CA). Biotin-labeled mixtures of lineage-specific antibodies, including B220, CD4, CD8, Ter119, Gr1, and Mac1, and streptavidin-labeled secondary antibody conjugated to allophycocyanin (APC) were from BD Biosciences Pharmingen (San Diego, CA).

Cell Culture

Mouse ES Cells

The mouse R1 ES cells (mES-R1) were cultured on irradiated MEFs on 0.1% gelatin-coated tissue culture dishes in DMEM supplemented with 15% FCS, 2 mM L-glutamine, 50 μM β-mercaptoethanol, streptomycin-penicillin, sodium pyruvate, nonessential amino acids, and 10 ng/ml recombinant LIF. Since mES cells can develop cytogenetic abnormalities in culture, leading to decreased capacity to contribute to germ line when injected into blastocyst [29], chromosome counting was carried out prior to injection on at least 20 spreads; more than 70% of the cells contained euploid cells.

Human EC and ES Cells

Human ES cells (H1) were grown on irradiated MEFs or on Matrigel-coated plates in 80% DMEM/F12, 20% knockout SR, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acids, and 4 ng/ml bFGF. The human embryonic carcinoma cell line Ntera2 was grown on gelatin-coated plates in DMEM (high glucose), with 10% FCS, 2 mM L-glutamine, 1% nonessential amino acids, and 1% penicillin-streptomycin. Like mouse ES cells, it was recently shown that human ES cells cultured for over 35 passages develop karyotypic abnormalities such as trisomy of chromosome 17 and 12 [30]. The H1 cells used in our studies were similarly 100% trisomy 12.

Total Bone Marrow Cells

Freshly isolated total bone marrow cells were depleted of red blood cells with ammonium chloride treatment. The remaining cells were either maintained as such or transfected and seeded in Stem Pro–34 SFM (Gibco), supplemented with 10 μg/ml mouse stem cell factor, 5 μg/ml thrombopoietin, and glutamine.

Multipotent Adult Progenitor Cells (MAPCs)

MAPCs from the bone marrow of either human, mouse, or rat were isolated as described earlier [18, 19]. Cells were maintained on 10 ng/ml FN-coated flasks in 60% DMEM (low glucose), 40% MCDB 201 with 1× ITS, 1× LA-BSA, 0.5 μM dexamethasone, 0.1 mM ascorbic acid 2-phosphate, 100 U penicillin-streptomycin, 10 ng/ml PDGF-BB, 10 ng/ml EGF, and 10^3 units/ml LIF (for mouse and rat MAPCs), with 2% FCS. Recent unpublished studies from our lab have shown that, like ES cells, a number of mouse and, to a lesser extent, rat MAPC populations maintained for more than 50 passages contain cells with a non-euploid number of chromosomes. Some of the murine MAPCs used after more than 60 passages for studies described here were 65% aneuploid, whereas others, at earlier passages, were more than 65% euploid. No differences in nucleofection efficiency were seen among the different populations.

Transfection Methods

Cells were harvested by trypsinization and then pelleted via centrifugation. Excess media was removed to obtain a tight cell pellet, which was resuspended in either the normal culture medium for electroporation or in the appropriate nucleofector solution for the nucleofection procedure. Several nucleofection programs were tested to choose the optimal program for each cell type. Some of the nucleofector programs used for optimization were A23, A27, A29, T20, T27, T16, T01, G16, O17, and U08. Electroporations were carried out using parameters earlier described for the particular cell type. For nucleofection, mES-R1 cells were resuspended in nucleofector solution, and program A23 was used to transfect the cells. For mES-R1 cells, the electroporation parameters used were 330 V and 250 μF; for human NTERA2 cells, the parameters used were 300 V and 500 μF, and 320 V and 200 μF. Human ES cells were grown on Matrigel to eliminate MEFs for one to two generations before nucleofection. Cells were harvested from the dishes by collagenase treatment, and after centrifugation, the cells were nucleoporated as clumps in nucleofector solution (mouse ES kit; Amaxa). Human H1 ES cells were electroporated using two different parameters: 220 V and 960 μF, and 320 V and 200 μF. Total mouse bone marrow cells (30–40 million cells) depleted of red blood cells were resuspended in nucleofector solution

(kit for human CD34+ cells; Amaxa) and following transfection were resuspended in Stem Pro media supplemented with SCF, thrombopoietin, and glutamine. MAPCs (human, rat, or mouse) were trypsinized, and 0.2–0.5 million cells were resuspended in nucleofector solution (human MSC kit; Amaxa); after nucleofection, the cells were plated on FN-coated dishes in MAPC culture media.

Differentiation of mES Cells into Cardiomyocytes

The mES-R1 cells were differentiated into cardiomyocytes using methods described by Maltsev et al. [31], with modifications. For formation of embryoid bodies, 4 million mES cells were plated in 60-mm low-binding culture dishes for 2 days in the presence of enterococcosel broth (EB) media (DMEM and 10% FCS in the absence of LIF) and an additional 5 days in bacteriological petri dishes for maintaining the cells in suspension. For cardiac differentiation, 7-day-old embryoid bodies were plated on gelatin-coated plates and maintained in the presence of 1% dimethylsulfoxide (DMSO) for 6 days [31]. Presence of beating cells was detected around the third day of DMSO treatment. At day 7, beating cells were collected in RLT buffer (Qiagen) for RNA isolation and RT-PCR analysis using the primers listed in Table 1.

Overexpression of Ngn3-GFP

The mES-R1 cells were seeded in DMEM media with 10% FCS in the absence of LIF, and cells were induced to differentiate by incubating the cells in the presence of 0.1 µM retinoic acid for 4 days. Cells were then harvested by trypsinization and nucleoporated with either pEGFP-N1 plasmid as a control or the Ngn3-GFP construct. After 72 hours, cells were harvested in RLT buffer for RNA isolation and RT-PCR analysis using the primers listed in Table 1.

RNA Isolation, Reverse Transcription, and Quantitative PCR

Total RNA was harvested from cells using the Qiagen RNeasy Kit. The resulting total RNA was subjected to DNase treatment using the DNA-*free* kit for 1 hour at 37°C to remove any contaminating genomic DNA. RNA was then quantified by absorbance at A260 nm and 2 µg of total RNA used for cDNA synthesis using Taqman reverse transcription reagents. Of the transcribed cDNA, 20 ng were used for quantitative PCR (QPCR), which was carried out in Taqman SYBR green universal mix PCR reaction buffer using an ABI PRISM 7700 (Perkin Elmer Applied Biosystems, Boston, MA).

Fluorescence-Activated Cell Sorter (FACS) Analysis

The mES-R1 cells transfected with pEGFP–N1 were seeded on gelatin-coated plates and harvested 24, 48, or 72 hours post-transfection. Transfected hES cells seeded on Matrigel or irradiated MEFs were treated with a mixture of trypsin and EDTA and washed with PBS. Following trypsinization and centrifugation of cells, the cell pellet was resuspended in FACS staining media containing 0.1% BSA in 0.01% sodium azide in PBS. For SSEA1 staining, cells were stained with the biotin-labeled anti-SSEA1 antibody or isotype control for 30 minutes on ice. The cells were then washed with staining media, and PE-conjugated streptavidin secondary antibody was added to both the isotype control and the SSEA1-stained cells and incubated on ice for 30 minutes. For SSEA4 staining for hEC and hES cells, cells were incubated with 1:50 diluted SSEA4 antibody and incubated on ice for 30 minutes. Cells were then washed, and secondary antibody conjugated to PE was added and incubated for 30 minutes on ice. Total mouse bone marrow cells were labeled with a cock-

Table 1. List of primers used

Gene	Forward primers (5'-3')	Reverse primers (5'-3')
GAPDH	CATGGCCTTCCGTGTTCCTA	CTGGTCCTCAGTGTAGCCCAA
Oct4	GAAGCCGACAACAATGAGAAC	ACAGAACCATACTCGAACCACA
Rex1	AAGCGTTTCTCCCTGGATTTC	TTTGCGTGGGTTAGGATGTG
eGFP	AGAACGGCATCAAGGTGAAC	TGCTCAGGTAGTGGTTGTCG
Ngn3	CCCCAGAGACACAACAACCT	TCTTCGCTGTTTGCTGAGTG
Pax6	AACAACCTGCCTATGCAACC	ACTTGGACGGGAACTGACAC
NeuroD	CAAAGCCACGGATCAATCTT	CCCGGGAATAGTGAAACTGA
Is11	TCATCCGAGTGTGGTTTCAA	CCATCATGTCTCTCCGGACT
GATA4	CTGTCATCTCACTATGGGCA	CCAAGTCCGAGCAGGAATTT
Nkx2.5	CAGTGGAGCTGGACAAAGCC	TAGCGACGGTTCTGGAACCA
A C Act.	CTGAGATGTCTCTCTCTCTCTTAG	ACAATGACTGATGAGAGATG

tail of lineage markers conjugated to biotin. Following incubation on ice for 30 minutes, streptavidin-linked secondary antibody conjugated to PE was added and further incubated for 30 minutes on ice. Labeled cells were washed, and cells were resuspended in 0.5–1 ml of staining media containing propidium iodide. Dead cells and MEFs (in the case of human ES cells) were excluded from analysis by propidium iodide staining and by forward- and side-scatter gating. Data were analyzed using the software Cell Quest Pro (BD Biosciences Pharmingen).

Immunocytochemistry

Cells differentiated into cardiomyocytes were fixed with 4% paraformaldehyde. Blocking was carried out in 1% BSA and 5% donkey serum in PBS. Antibodies against cardiac troponin C and Nkx2.5 were diluted to 1:100. Incubation with primary antibody was carried out overnight at 4°C. Slides were washed three times with 1% BSA in PBS. Secondary antibody conjugated to TRITC was added and incubated at room temperature for 45 minutes. The samples were washed with 1% BSA in PBS and mounted using mounting media for fluorescence. Cells were examined by fluorescence microscopy using a Zeiss Axiovert scope (Carl Zeiss Inc., Thronwood, NY).

Immunohistochemistry

Tissues were harvested from chimeras and control mice immediately after euthanization and then embedded in Tissue-Tek OCT embedding medium and frozen. The 10-μ sections were collected on glass slides using a Leica Cryotome. Sections were fixed in 4% paraformaldehyde and blocked with hydrogen peroxide to neutralize endogenous peroxidase activity. Sections were incubated with anti-GFP antibody overnight at 4°C, washed three times with 0.1% BSA in PBS, and incubated with biotinylated secondary antibody. Following incubation, the secondary antibody was washed, and Avidin-Biotin complex coupled to HRP (Dako Cytomation) was added to the section and color developed using DAB (Sigma). Images were collected using a phase-contrast microscope.

Generation of Chimeras

Stable mES-R1 clones were generated following nucleofection with pEGFP-N1 plasmid and 2-week selection in Geneticin on irradiated, nonresistant MEFs. Clones that had high levels of GFP expression by fluorescence microscopy were chosen for blastocyst injection into C57Bl/6 embryos. The embryos were then implanted into pseudopregnant mice, and the progeny were assayed for chimerism based on coat color. The coat color of C57B1/6 is black, while the mES-R1 cells yield agouti coat color. The percentage of chimerism was also assayed by presence of the eGFP cDNA by QPCR analysis of genomic DNA and expression of the GFP protein by FACS analysis of tissues.

Genomic DNA Isolation

Tail snips of chimeras were suspended in buffer, and genomic DNA was isolated using the Qiagen DNeasy kit according to manufacturer's recommendation. Isolated DNA was used as a template to quantify GFP by QPCR analysis.

Statistical Analysis

All in vitro experiments were carried out at least three times with three independent samples. The mean value and the standard deviation were calculated using Microsoft Excel.

Results

Nucleofection Achieves High Transient Transfection Efficiency Compared with Electroporation

The mouse ES cell line R1 (mES-R1) was used to study the potential of nucleofection as a transfection method in these cells. The transfection efficiency by nucleofection was compared to electroporation as both methods achieve transfection in solution and the treatment of cells is similar in both, eliminating differences in transfection efficiencies arising from cell handling. The mES-R1 cells were harvested, and 1 million cells were either electroporated at 330 V 250 μF, nucleoporated using the mES cell nucleofection solution (program A23), or left untreated. Figure 1A shows the brightfield and green fluorescence images of cells electroporated (top two panels) and cells nucleoporated (bottom two panels), 24 hours after transfection. Green fluorescent cells were visible in the nucleoporated sample as early as 8–10 hours post-transfection, and fluorescence intensity increased by 24 hours after transfection. In contrast, the number of green fluorescent cells and their intensity did not significantly increase, even after 48 and 72 hours in the case of electroporation (data not shown). Figure 1B shows the average GFP fluorescence 48 hours post-transfection. The percentage of GFP^+ cells was determined by FACS analysis. While the percentage of GFP^+ cells is 6.41% (+4.59%; $n = 3$) in the case of electroporation, nucleofection achieved a transfection efficiency of 63.66% (+9.36%; $n = 8$) under the conditions tested.

Increased Stable Transfection via Nucleofection

The mES-R1 cells transfected with pEGFP-N1 plasmid were subjected to drug selection for 2 weeks in the presence of 400 mg/ml Geneticin (G418) to select for ES cells with stably integrated plasmid. Figure 1C shows the percentage of G418-resistant cells relative to untransfected mES-R1 cells main-

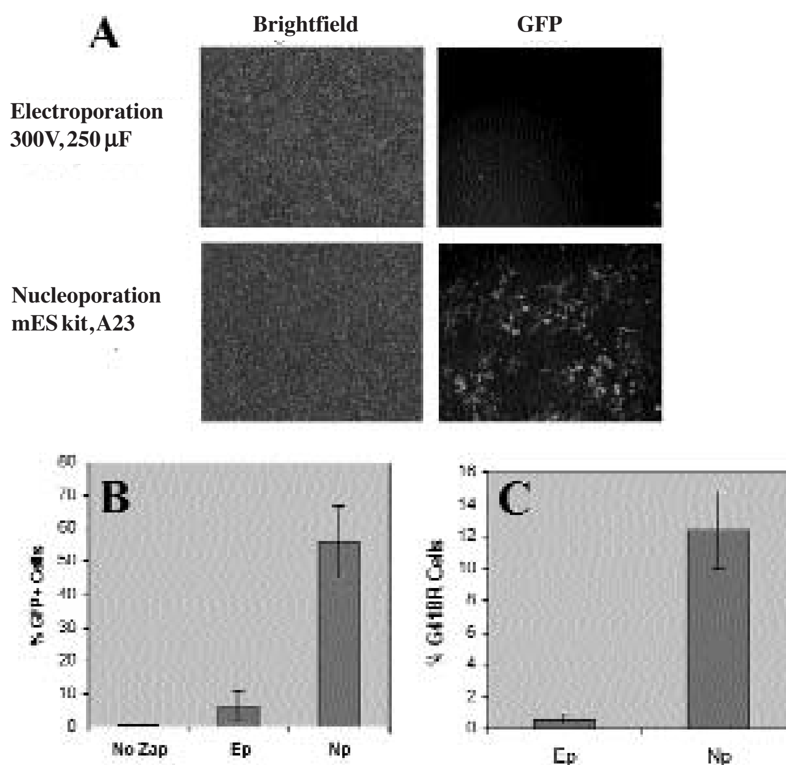


Figure 1. Transfection efficiency of mouse embryonic stem cells (mES-R1) with enhanced green fluorescent protein (eGFP). (**A**): Approximately 1 million mES-R1 cells grown on gelatin were transfected with the plasmid pEGFP-N1, either by electroporation at 330 V and 250 μF or by nucleofection using the program A23. Cells were visualized for GFP 24 hours later using fluorescence microscopy. **(B)**: Transient transfection efficiency: fluorescence-activated cell sorter (FACS) analysis was carried out 24 hours post-transfection to monitor the percentage of GFP$^+$ cells with no transfection (No Zap, Avg. $n = 8$), following electroporation (Ep; Avg. $n = 3$), or following nucleofection (Np; Avg. $n = 8$). Standard deviation is represented as error bars. **(C)**: Stable transfection efficiency: Cells transfected with pEGFP-N1 either via electroporation (Ep) or by nucleofection (Np) were treated with 400 μg/ml Geneticin 48 hours post-transfection, and the number of G418-resistant cells were counted following 2 weeks of selection. The average of three independent experiments is represented as a bar graph. Standard deviation is represented as error bars.

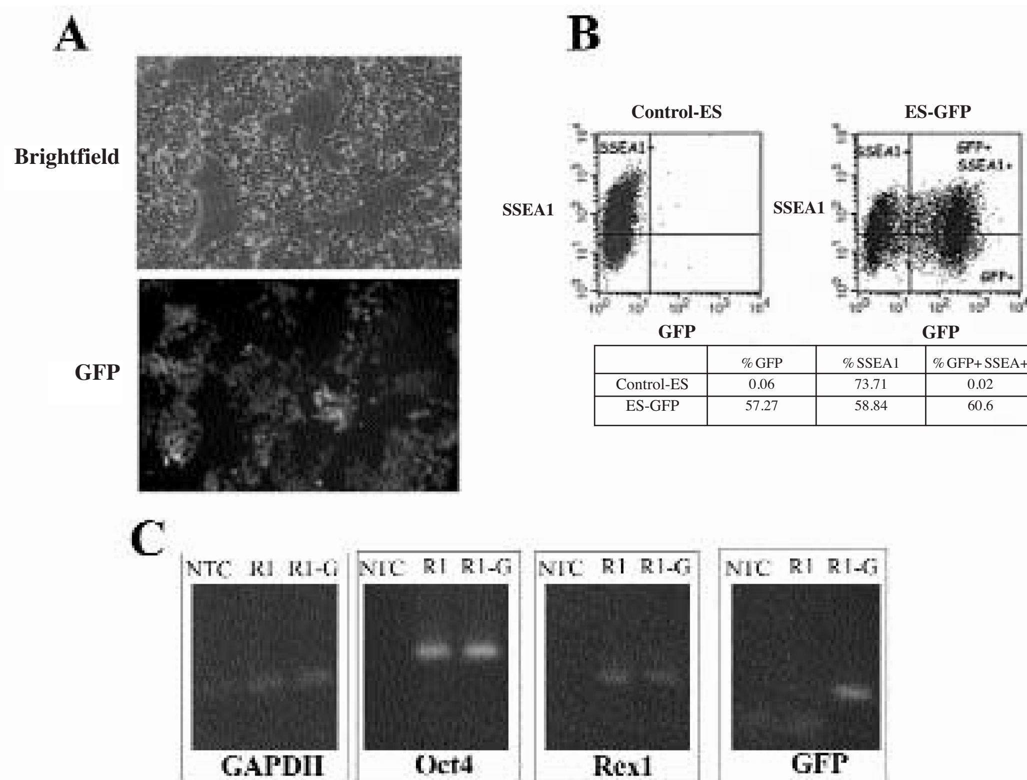
tained in parallel in the absence of G418. Results are represented as an average of three independent sets of experiments. The percentage of stable transfectants arising from electroporation was 0.59% (+0.33%; $n = 3$), while the rate of stable transfection via nucleofection was 12.34% (+2.43%; $n = 3$).

Pooled G418-Resistant Clones

Nucleoporated G418-resistant mES-R1 clones (over 150 clones on a 10-cm dish) were pooled following 2 weeks of selection and cultured for an additional 2 weeks. The cells were then analyzed for expression of ES cell markers SSEA1, Oct4, and Rex1. Figure 2A shows brightfield and fluorescence images of the pooled stable cells showing that the majority of cells still express GFP. In the control unmanipulated mES-R1 cells, 74% were SSEA1$^+$ by FACS analysis; 57% of G418-resistant mES-R1 were GFP$^+$ cells, and 61% of the GFP$^+$ cells were SSEA1$^+$ (Fig. 2B). QRT-PCR analysis revealed identical levels of Oct4 and Rex1 expression in unmanipulated ES cells and pooled G418-resistant clones (Fig. 2C). These results demonstrate that several key stem cell characteristics are maintained in mES-R1 cells following nucleofection.

Differentiation of an EGFP mES-R1 Clone into Cardiac Cells

Cardiac differentiation was evoked by culturing the GFP$^+$ mES-R1 cells as embryoid bodies using a slight modification of the method described by Maltsev et al. [31]. Following 7 days of differentiation, beating cells were either harvested in RLT buffer for RNA isolation and QRT-PCR analysis for cardiac markers (Gata4, Nkx2.5, and α cardiac actinin) or seeded for immunocytochemistry. Figure 3 shows staining patterns of cardiac markers in the mES-R1 cells expressing GFP after differentiation. The top panels show staining of GFP$^+$ cells with the early cardiac marker Nkx2.5, and the bottom panels are cells stained with the late cardiac-specific marker, cardiac troponin T. As seen in the merged panel of GFP and TRITC–labeled cardiac-specific antibodies, stable



	% GFP	% SSEA1	% GFP+ SSEA+
Control-ES	0.06	73.71	0.02
ES-GFP	57.27	58.84	60.6

Figure 2. Pooled mouse embryonic stem cells (mES-R1) stable clones expressing enhanced green fluorescent protein (eGFP). **(A)**: Brightfield (top panel) and green fluorescence (bottom panel) of GFP-mES-R1 stable cells following 6 weeks in culture. **(B)**: G418-resistant colonies were subjected to fluorescence-activated cell sorter (FACS) analysis to monitor the percentage of cells positive for both GFP and the stem cell marker SSEA1. The left panel shows SSEA1 staining of untransfected mES-R1 cells, and the right panel shows SSEA1 staining of eGFP-transfected, G418-resistant mES-R1 cells. **(C)**: Polymerase chain reaction (PCR) analysis of stem cell markers Oct4 and Rex1 in G418-resistant cells and untransfected mES cells. Abbreviations: NTC, no template control; R1, untransfected mES-R1 cells; R1-G, eGFP-transfected mES-R1 cells.

GFP expressing mES-R1 cells induced to the cardiac lineage express early or late cardiac markers. Further analysis was carried out by QRT-PCR for expression of the cardiac markers Nkx2.5, Gata4, and α cardiac actinin. The results obtained are summarized in Table 2. Relative to levels in undifferentiated cells, expression of Gata4 was increased by 79-fold, while Nkx2.5 and α cardiac actinin increased by 8- and 24-fold, respectively.

Contribution of EGFP-mES-R1 Cells to Chimeras

The mES-R1 cells nucleoporated with the pEGFP-N1 construct were selected in G418 for 4 weeks, and the resulting colonies were replated into 24-well plates; each clone was expanded separately. The expanded clones with highest fluorescence intensity as determined by fluorescence microscopy and greater than 95% GFP$^+$ cells determined by FACS analysis were chosen for blastocyst injection. From 10–12 cells were injected into each blastocyst. The injected blastocysts were implanted into pseudopregnant female mice, and pups born were monitored for chimerism based on coat color. Figure 4 shows the pups obtained from one such litter.

QPCR analysis for GFP was performed on genomic DNA isolated from tail clips of chimeric animals. The GFP$^+$ mES-R1 cells used for blastocyst injection were used as a positive control, and the amount of PCR product obtained from these cells was set as 100%. Based on the amount of GFP PCR product relative to the positive control, animals were classified as 0%, <10%, and >20% chimeric. We used FACS analysis of cells isolated from various organs to detect GFP+ cells in animals with more than 20% chimerism. In one chimera analyzed in detail, 2%–3% GFP$^+$ cells were detected in hematopoietic tissue such as peripheral blood, spleen, and thymus, while nearly 16% GFP$^+$ cells were detected in kidney. Presence of GFP$^+$ cells was further confirmed by immunohistochemical staining on frozen sections of kidney using anti-GFP antibodies (Table 3). In animals with no contribution (as assessed by QRT-PCR of genomic DNA) FACS and immunohistochemistry failed to detect any GFP$^+$ cells.

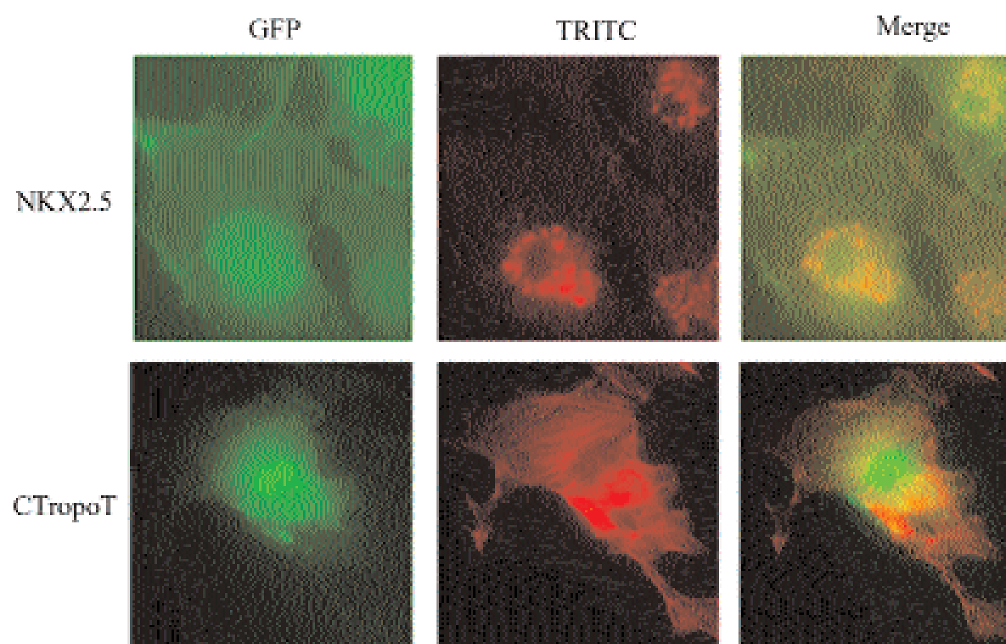


Figure 3. Differentiation of green fluorescent protein (GFP)–positive mouse embryonic stem cells (mES-R1) into cardiomyocytes. Immunostaining of GFP-mES-R1 cell–derived cardiomyocytes for Nkx2.5 and cardiac troponin T after 7 days of dimethylsulfoxide (DMSO) treatment.

Ectopic Expression of Ngn3 in mES-R1 Cells

Enhanced transfection efficiencies obtained in mES cells via nucleofection are particularly valuable for screens in which stem cells are induced to differentiate into specific cell types by ectopic expression of regulatory genes such as transcription factors. One such gene that has been well characterized is Ngn3, known to be required early during pancreas development [32]. Overexpression of *Ngn3* in mouse ES cells activates downstream genes that specify pancreatic development such as *Pax6*, *Is11*, and *NeuroD* [12]. In those studies, Lipofectamine (Invitrogen), which generally achieves 20%–25% transfection efficiency in mES cells, was used as the method of transfection. We hypothesized that nucleofection of mES cells with *Ngn3* would yield greater numbers of transfected cells and result in a more homogenous population of differentiating ES cells. The mES-R1 cells were grown in the presence of RA to induce *Gata4* expression. Cells were then trypsinized and nucleoporated with either a GFP or an Ngn3-GFP construct and plated on gelatin-coated dishes. After 72 hours, cells were harvested in RLT buffer, and QRT-PCR was performed to monitor for expression of *Pax6*, *Is11*, and *NeuroD*. Figure 5 shows the image of an agarose gel with the QRT-PCR products of various genes tested. Quantitation of the QRT-PCR results are summarized in Table 4. Cells transfected with GFP plasmid alone show levels of Ngn3 mRNA that are 0.3% of that obtained using fresh mouse pancreas. The relative level of Ngn3 (compared with mouse pancreas) increases to 515% in cells transfected with Ngn3-GFP, a 1,618-fold increase over the control GFP-transfected ES cells. This demonstrates that Ngn3 is introduced into the cells

Table 2. Quantitative polymerase chain reaction (QPCR) analysis of Nkx2.5, Gata4, and α cardiac actin in differentiated and undifferentiated GFP-mES-R1 cells (see Fig. 3)

	GFP-ES cells		GFP-ES cells diff.		
Gene	**Cycle no.**	**Rel. to mHeart**	**Cycle no.**	**Rel to mHeart**	**Fold increase**
Nkx2.5	38.02	1.8	27.43	14.5	8
Gata4	38.9	20.4	31.61	1605.6	79
α C Act.	33.55	3	24.65	72.2	24

Values are normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are relative to mouse heart gene expression.
Abbreviations: ES, embryonic stem; GFP, green fluorescent protein; Rel to mHeart, relative to mouse heart.

Figure 4. Contribution of stable green fluorescent protein (GFP) expressing mouse embryonic stem cells (mES-R1) to chimeras. Chimeras obtained from one representative experiment showed one pup with wild-type coat color (black), two pups with less than 20% chimerism, and one pup with more than 20% chimerism as estimated by coat color.

and is being transcribed efficiently. To assess functional NGN3 PROTEIN activity, we assayed for levels of genes known to be activated by Ngn3 during pancreas development, including *Pax6*, *Is11*, and *NeuroD*. Expression levels of these genes were found to increase by 32-fold, 32-fold, and 15-fold, respectively, relative to GFP transfected cells (Table 4).

Transfection of hEC and hES Cells

The plasmid pEGFP-N1 was transfected into the hEC cell line, Ntera2, either by electroporation or by nucleofection. FACS analysis of GFP expression 48 hours post-transfection was used to quantitate transfection efficiency. Ntera2 cells were either electroporated or nucleoporated using two different conditions (Fig. 6A). Electroporation yielded 50% transient transfection efficiency, whereas we achieved nearly 95% efficiency using nucleofection. Average transient transfection efficiencies obtained with Ntera 2 cells ($n = 3$) are twofold higher via nucleofection than by electroporation (Table 5). Recently, Zwaka and Thompson [23] described electroporation conditions of 320 V and 200 μF as being optimal for obtaining high-efficiency transfection of hES cells. The transient transfection of human H1 ES cells as clumps via this method was compared with nucleofection methods. Figure 6B gives the results of a representative FACS analysis, showing percentage of GFP$^+$ cells under various electroporation and nucleofection conditions, 72 hours post-transfection. While electroporation at two different electric parameters yielded 5%–6% transfectants, nucleofection achieved 20%–22% transfection (Table 5).

Adult Stem Cell and Progenitor Cells

MAPCs are a rare population of cells derived from adult bone marrow [18, 19]. These cells must be maintained at low density to maintain multipotency (200–1,000 cells/cm^2). Consequently, various transfection methods that require high-density cell cultures such as calcium phosphate (Clontech), AVET (Bender MedSystems), Effectine (Qiagen), and DMRIE-C (Invitrogen), are largely ineffective in transfecting these cells. However, modest transfection efficiency (<5% by FACS analysis) was obtained with the liposome-based Superfect system (Qiagen). Electroporation yielded 10% GFP$^+$ cells 24 hours post-transfection, but this number drastically decreased with time, indicating that this method is associated with significant toxicity to the cells (UL and CMV, unpublished data). Nucleofection yielded 11%, 25%, and 35% transfection in human, mouse, and rat MAPCs, respectively (Table 6). GFP expression was seen as early as 10 hours post-transfection, and the intensity continued to

Table 3. Percentage of chimerism in three representative mice, as determined by genomic DNA analysis of tail clips and FACS analysis of isolated kidney cells and peripheral blood (PB) (see Fig. 4)

	% GFP Cells		
	FACS		Immunohistochemistry
Genomic PCR	PB	Kidney	Kidney
0%	0	0	Negative
<10%	1.16	1.79	Negative
>20%	2.61	15.55	Positive

Presence or absence of GFP was also determined by immunohistochemistry.
Abbreviations: FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; PCR, polymerase chain reaction.

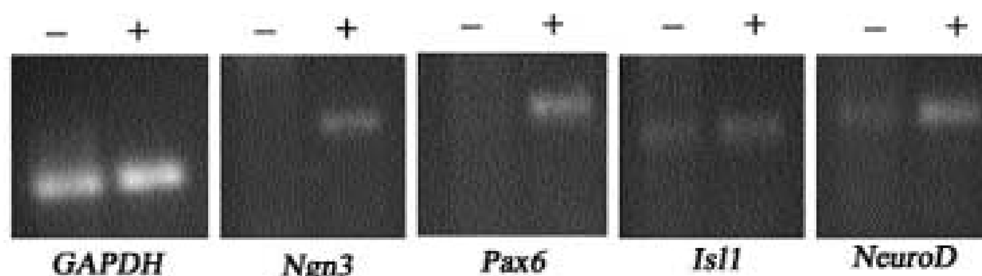


Figure 5. Overexpression of *Neurogenin3* (*Ngn3*) in mouse embryonic stem cells (mES-R1). Quantitative reverse transcription polymerase chain reaction (QRT-PCR) analysis of pancreatic markers in mES-R1 cells transfected with *GFP* (-) or *ngn3-GFP* (+).

increase, with maximal intensity around 24–48 hours post-transfection. Unlike electroporation, stable cell lines could be successfully created by nucleofection using a plasmid containing the hygromycin resistance gene (U.L. and C.M.V., unpublished data).

Total mouse bone marrow cells were also efficiently transfected via nucleofection. However, this method resulted in nearly 90% cell death following nucleofection under optimum conditions. Total bone marrow cells depleted for lineage-positive cells (Lin^- fraction) were also transfected at similar frequencies as total bone marrow cells (25% versus 18%; Table 6). Colony-forming assays of the transfected bone marrow cells on methylcellulose for 2 weeks yielded green fluorescent CFU-M (macrophage) and CFU-GM (granulocyte/macrophage) colonies, indicating that hematopoietic progenitors are indeed stably transfected via nucleofection (data not shown).

Discussion

Electroporation and lipofection (liposome-mediated) are the most commonly used methods to transfect mouse ES cells. However, their transient transfection efficiency is typically limited to 20%–25% [12]. A modified suspension method of Lipofectamine transfection under optimal conditions has been reported in which 50% transient transfection efficiency has been achieved in mouse ES cells [21]. However, for successful gene targeting, the best transfection method is via electroporation, which has been shown to be more effective than liposome-based methods for this purpose [33].

In this study, we show that nucleofection achieves nearly 10-fold higher transient and stable transfection efficiency than electroporation. Importantly, the transfected mouse ES cells retain their stem cell properties, as determined by three independent assays. First, expression of the stem cell markers Oct4, Rex1, and SSEA1 in the transfected ES cells was similar to that in the untransfected controls. Second, transfected cells could form embryoid bodies and, upon exposure to DMSO, give rise to cardiomyocytes. Third, when transfected mouse ES cells were injected into blastocysts, they were able to contribute to chimera formation.

The high transfection efficiency obtained with nucleofection allows its use as a screening tool to study the effect of specific transcription factors on differentiation. Since nucleofection achieves higher transfection efficiency than do elec-

Table 4. Quantitative reverse transcription polymerase chain reaction (QRT-PCR) data showing the expression profiles of pancreatic markers in GFP- and Ngn3-GFP–transfected mES-R1 cells (see Fig. 5)

	GFP		Ngn3-GFP		
Gene	Cycle no.	Rel. to mPanc.	Cycle no.	Rel. to mPanc.	Fold increase
Ngn3	40	0.3	26.52	515	1618.00
Pax6	40	0.02	32.17	0.7	32.22
Is11	39.1	0.04	31.27	1.2	32.33
NeuroD	38.22	0.08	31.52	1.2	14.67

Cycle number indicates the cycle at which the PCR product was first detected. Values obtained for each gene are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels and represented as gene expression relative to mouse pancreas. The difference in the expression of pancreatic markers between GFP and ngn3-GFP transfected cells is represented as fold-increase.

Abbreviations: GFP, green fluorescent protein; Ngn3, Neurogenin3; Rel. to mPanc., relative to mouse pancreas.

Table 5. Average percentage of GFP+ cells as measured by FACS analysis (see Fig. 6)

Ntera2	GFP+ cells (%)	hES-H1	GFP+ cells (%)
Control	0.655 ± 0.01	Control	0.99 ± 0.014
A23	86.915 ± 11.56	A23	22.33 ± 1.287
A27	81.67 ± 1.58	A29	19.92 ± 0.594
Ep300V	44.56 ± 10.85	Ep220V	5.33 ± 0.049
Ep320 V	32.2 ± 10.62	Ep320 V	6.17 ± 0.248

Values represent mean + standard deviation.
Abbreviations: FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein.

troporation or lipid-based transfection methods, a more homogeneous differentiation should be achieved upon overexpression of such factors by nucleoporation. This was found to be correct in the case of Ngn3 overexpression in mouse ES cells. As predicted, the increase in expression of pancreatic markers following nucleofection with Ngn3 was twofold to threefold greater than that reported using Lipofectamine [12].

Human ES cells are known to be hard to transfect. Nucleofection of human H1 ES cells yielded a threefold greater number of transfectants than did electroporation. The hEC cells, Ntera2, were also transfected to significantly higher transfection efficiency than with electroporation. Besides ES cells, certain types of adult stem cells are particularly hard to transfect, one such example being MAPCs. Several commercially available transfection methods failed to transfect MAPCs. The requirement that MAPCs be grown at densities between 200 and 1,000 cells/cm^2 precludes most of the liposome-based methods where high cell density is essential. As culture at high density results in differentiation of MAPCs, an alternate transfection method is required. Electroporation resulted in 10% transient transfection levels in MAPCs, but the process was highly toxic to the cells, resulting in gradual

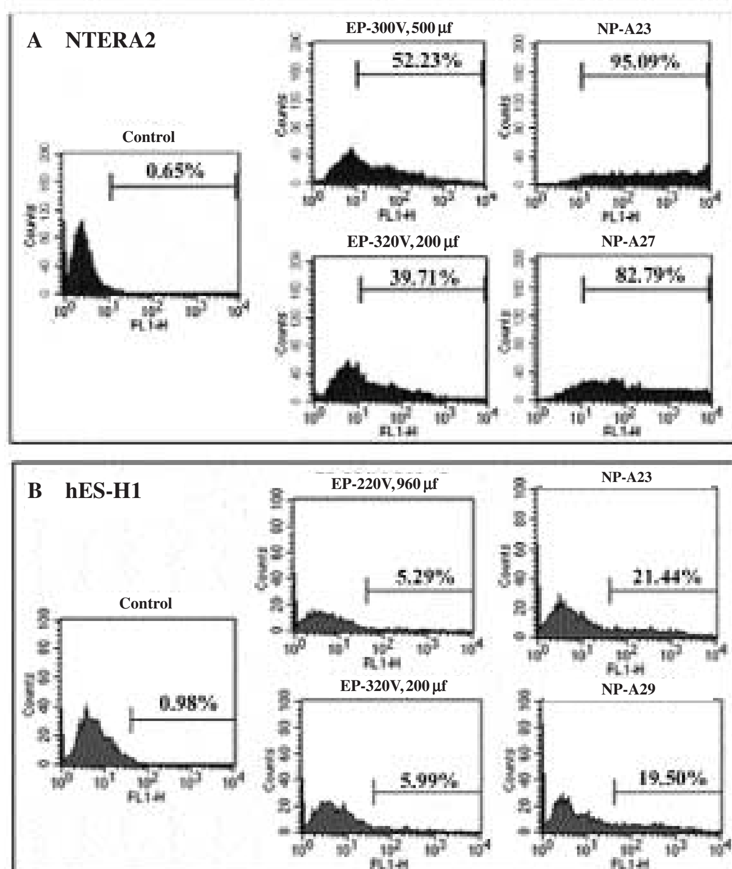


Figure 6. Transfection efficiency in human embryonic carcinoma (hEC) and human embryonic stem (hES) cells. Cells were transfected with the plasmid pEGFP-N1 and the presence of green fluorescent protein (GFP) was monitored in transfected cells by fluorescence microscopy and fluorescence-activated cell sorter (FACS) analysis. Data were collected from at least three independent experiments to calculate mean values and standard deviation. **(A)**: Representative FACS analysis of NTERA2 cells. Approximately 10^6 cells were either subjected to electroporation at the electric parameters of 330 Vand 500 µF or 320 V and 200 µF or were nucleoporated using programs A23 or A27. After 58 hours, cells were trypsinized and subjected to FACS analysis for GFP. **(B)**: 1–2 × 10^6 human H1 ES cells were either electroporated or nucleoporated as clumps. The parameters used for electroporation were 220 V and 960 µF or 320 V and 300 µF. Cells were nucleoporated using programs A23 or A27. Cells were trypsinized 72 hours post-transfection and subjected to FACS analysis for GFP.

Table 6. Transfection efficiency of progenitor cells from adult tissue

Cell type	Kit	Program	Transfection efficiency (%)
mTBM	$hCD34^+$	U08	17.5 + 14.49
$mLin^-$	$hCD34^+$	U08	24.51 + 13.95
mMAPC	hMSC	A23	25.37 ± 6.01
hMAPC	hMSC	A23	11.84 ± 0.88
rMAPC	hMSC	A23	34.5 ± 9.25

Abbreviations: mTBM, mouse total bone marrow; $mLin^-$, mouse bone marrow lineage negative cells; mMAPC, hMAPC, and rMAPC, mulitpotent adult progenitor cells from mouse, human, and rat, respectively.

loss of transfected cells with time. Consequently, stable transfectants could not be isolated by this method. In contrast, nucleofection was successfully used to derive drug-resistant stable mouse MAPC clones that could be grown in culture for extended periods of time. Nucleofection therefore provides an ideal method for transfecting MAPCs, allowing culture of cells at low density but yielding transfection to high efficiency. Finally, total mouse bone marrow cells, which have been traditionally transfected via electroporation, were also efficiently transfected via nucleofection (0.5%–0.8% versus 17%–25%). However, nearly 90% of the cells died during the process of nucleofection. Electroporation causes relatively lower levels of cell death in total mouse bone marrow cells (approximately 50%) but also achieves lower transient transfection efficiency (0.5%–0.8%).

Based on our results, we conclude that nucleofection is an efficient method for transfecting ES cells and other hard-to-transfect stem cells, and it appears to be superior to current available methods. Although liposome-based methods can produce high transfection efficiencies, this method requires that the cells be exposed to serum-free conditions, which may lead to differentiation. Further, the optimal conditions for efficient transfection via the liposome method is based on a critical cell-to-lipid ratio that may vary from experiment to experiment. This, coupled with the risk of spontaneous differentiation of stem cells under serum-free conditions, could lead to heterogeneity within an ES or other stem cell population, complicating studies. Nucleofection avoids this problem and further achieves greater transfection efficiency. While electroporation is not associated with complications arising from exposure to serum-free conditions, the efficiency of transfection is low. Nucleofection offers an ideal method to achieve high levels of transfection while retaining the quality of stem cells.

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