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Efficient gene expression in megakaryocytic cell line using nucleofection

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Abstract

To clarify the mechanism of platelet production from megakaryocytes, expression of target proteins by gene transfection was examined using various gene delivery techniques. Transfection into hematopoietic cells, including megakaryocytes, by conventional gene delivery techniques such as electroporation and lipofection are known to be difficult. In this study, in addition to electroporation and lipofection, we tested other gene-transfer methods (nucleofection, transfection using inactivated virus envelope, and transferrin-linked cationic polymer) with the green fluorescent protein (GFP) gene into the human megakaryocytic cell line MEG-01. We found that nucleofection, which uses a combination of special electrical parameters and specific solutions, was the best, judging from the expression ratio of GFP-positive cells (approximately 70% of cells) and low toxicity. The efficiency of GFP expression was not related to the amount of pDNA delivered into the MEG-01 cells. To verify the utility of nucleofection, the thrombopoietin (TPO) receptor c-mpl was transfected into MEG-01 cells. Transfected cells showed a higher responsiveness to TPO than mock-transfected MEG-01 cells. We propose that nucleofection is a useful method for transfecting target genes to megakaryocytic cells when addressing the mechanism of platelet production.

Keywords: Transfection; Megakaryocytes; MEG-01; Nucleofection

1. Introduction

Megakaryocytes, which are estimated to constitute approximately 0.4% of the total bone marrow cells (Levine, 1980), are differentiated from hematopoietic stem cells into platelets via many stages. Since various hemorrhagic and thrombotic disorders are strongly attributed to abnormalities in platelet number or function, understanding the mechanism of platelet production is important for establishing new pharmacologic strategies to regulate disordered or inappropriate platelet production. To clarify the molecular mechanisms of megakaryocyte differentiation and platelet production, approaches using gene expression analysis such as cDNA microarray, serial analysis of gene expression, differential display, and cDNA subtraction are available. On the basis of these analyses, candidate genes involved in the regulation of cellular differentiation have been identified. Knockout or transgenic technology is a powerful tool for validating the functions of candidate genes. Indeed, knockout mice have been established for several genes. Mice deficient in thrombopoietin (TPO) (Bartley et al., 1994), c-mpl (TPO receptor) (Carver-Moore et al., 1996), GATA-1 (Shivdasani et al., 1997), or nuclear factor erythrocyte 2 (Shivdasani et al., 1995) have been shown to have decreased numbers of platelets in the blood, suggesting that these proteins participate in platelet production. However, establishing transgenic or knockout mice generally takes a long time, and the number of candidate genes is sometimes too large to knockout all of them at once. Therefore, a simple gene validation system is required to clarify the mechanism of platelet production.

One powerful method is transfection of target genes relating to the target proteins into megakaryocytes. However, transfection into megakaryocyte and megakaryocytic cell lines by conventional methods is notoriously difficult and the efficiency is very low (less than 5%) (Wang et al., 1999). To overcome the

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low transfection efficiency, transgene-expressing cells have to be enriched to validate the function of the transgenes, and this is a time- and labor-consuming process. To overcome this problem, Burstein et al. (1999) used a retrovirus vector and showed that 41–82% of megakaryocytes were positive. In addition, Gillitzer et al. (2005) showed successful gene-transfer into $CD34^+$ stem cells using a retrovirus vector, and that the transfected cells differentiated into megakaryocytes in response to stimulation by TPO. However, the utility of viral vectors is limited by the time required, facilities, the overall expense, and safety considerations. A simple method of transfecting genes to megakaryocytes should be a great advantage.

In studies of megakaryocyte differentiation, K562 cells are among those most commonly used (Drexler et al., 2004). However, this cell line has both pro-erythroidic and megakaryocytic properties. In contrast, MEG-01 cells (a human megakaryocytic leukemia cell line) (Ogura et al., 1985) are committed to the megakaryocytic lineage and can produce platelet-like particles (Takeuchi et al., 1998). In addition, this cell line is frequently used in megakaryocyte differentiation studies and in gene function validation studies in megakaryocytopoiesis, platelet-like particle production, ploidy, and in vivo tumorigenesis (Zunino et al., 2001). In this study, we examined various gene-transfer methods with MEG-01 cells.

2. Materials and methods

2.1. Cell line

MEG-01 cells were purchased from American Type Culture Collection (VA, USA) and cultured in RPMI-1640 medium (Sigma, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH, KS, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, CA, USA) at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Plasmid DNA (pDNA)

pEGFP-C1, encoding green fluorescent protein (GFP), was purchased from Clontech (CA, USA). pGL3-Control, encoding luciferase, was purchased from Promega (WI, USA). pCMV-Script, a negative control plasmid, was purchased from Stratagene (CA, USA). pcDNA3 was purchased from Invitrogen. All plasmids were purified using an EndoFree Plasmid DNA Purification Kit (QIAGEN, CA, USA). A TPO receptor (c-mpl)-expressing plasmid was constructed as follows. Briefly, human c-mpl cDNA was amplified by reverse-transcriptase polymerase chain reaction (RT-PCR) using M-MLV Reverse Transcriptase (Invitrogen), KOD-plus DNA polymerase (Toyobo, Osaka, Japan), and a specific primer set (sense, 5′-CGCCACCATGCCCTCCTGGGCCCTCTTCAT-3′; antisense, 5′-TCAAGGCTGCTGCCAATAGCTTAGTGGTAG-3′). In the sense primer, the kozak consensus sequence (underlined) was included as the start codon. Full-length cDNA was subcloned into a pCR-Blunt vector (Invitrogen) and it was cloned into the EcoRI site of the expression vector pcDNA3. The orientation of the insert was determined by restriction mapping. The sequence of c-mpl was confirmed as accession number NM_005373 using a CEQ2000 DNA sequencer (Beckman Coulter, CA, USA).

2.3. Transfection

2.3.1. Electroporation

Electroporation was performed with an Electro Square Porator T820 electroporation system (BTX Inc., CA, USA) according to the manufacturer's recommended method. In brief, 1×10^6 cells suspended in 0.1 ml Dulbecco phosphate-buffered saline (PBS) were mixed with 15 μg of pGL3-Control plasmid. The mixture was transferred into a 2 mm gap electroporation cuvette and incubated on ice for 10 min. Then, electroporation was performed with various field strengths, pulse lengths, and pulse numbers. After electroporation, the cells were incubated on ice for 10 min and transferred into RPMI-1640 supplemented with 10% FBS. Ten minute or 24 h after electroporation, cell viability was determined by trypan blue dye exclusion assay. Twenty-four hour after electroporation, expression was determined by luciferase assay.

2.3.2. Hemagglutinating virus of Japan envelope (HVJ-E) vector

HVJ-E vector is an unique transfection tool that employs the cell fusion ability of the envelope of Sendai virus (HVJ) originally described by Kaneda (2003) (see review). Transfection with HVJ-E was performed according to the manufacturer's recommendations. Briefly, HVJ-E vector (25 μl) (GenomONE or GenomONE-Neo; Ishihara Sangyo, Osaka, Japan) was mixed with DNA (5 μg) and reagent B supplied in the kit (1 μl). The mixture was centrifuged at 10,000 × *g* for 5 min at 4 °C. The pellet was suspended with the buffer supplied in the kit (30 μl). Then, the supplied reagent C (5 μl) was added. An aliquot of the mixture (1–4 μl) was added to cells (2.5×10^5 cells/0.5 ml) in a microcentrifuge tube, and the cells were centrifuged at 10,000 × *g* for 30 min at 35 °C. The cell pellet was re-suspended with RPMI-1640 supplemented with 10% FBS and cultured in a 24-well plate. Twenty-four hour after transfection, GFP expression was determined using a flowcytometer as described below.

2.3.3. Lipid or cationic polymer

MEG-01 cells were transfected using Lipofectamine2000 (LFA2000; Invitrogen), FuGENE6 (Roche Diagnostics, Basel, Switzerland), GenePORTER2 (Gene Therapy Systems, CA, USA), Effectene (Qiagen), SuperFect (Qiagen), jetPEI (Q-Biogene, CA, USA), or TransFast (Promega). All transfections were performed according to the manufacturers' guidelines. Representative conditions for each reagent are shown in Table 1. Briefly, the reagents and pDNA were mixed to form the DNA–reagent complex. Then, the mixture was added to the cell suspension in the culture plate and cultured for 24 h. According to the manufacturers' recommendations, for all reagents, withdrawal of the transfection reagents from the culture was not necessary. Twenty-four hour after transfection, GFP expression was determined using a flowcytometer as described below.

Table 1
Transfection efficiency of liposome- and cationic polymer-based reagents with manufacturers' recommended conditions

Reagent (μl)		DNA (μg)	Cell number ($\times 10^5$)	Viability (%)	Positivity (%)	Efficiency (%)
LFA2000	4.0	1.0	2.5	84.9	1.7	1.4
FuGENE6	1.2	0.2	5	92.2	0.1	0.1
SuperFect	4.0	2.0	5	86.2	0.0	0.0
Effectene	10	0.4	5	88.4	0.0	0.0
TransFast	4.5	0.5	2	76.8	0.8	0.6
jetPEI	2.0	1.0	2.5	81.0	0.2	0.2
GenePORTER2	7.0	2.0	5	87.9	0.4	0.4
DuoFect	4.5	10	5	78.5	0.1	0.1

MEG-01 cells were transfected with commercially available liposome- or cationic polymer-based reagents complexed or mixed with pEGFP-C1 pDNA. Viability of the cells and GFP-positive cells were analyzed by flowcytometer 24 h after transfection.

2.3.4. Transferrin-linked cationic polymer

A transfection system (Duofect; Q-Biogene) using the interaction between transferrin (TF)-containing polyethyleneimine and TF receptors on the cell surface was tested. Transfection was performed according to the manufacturer's guidelines. In brief, 20 h before transfection, the cells were collected and re-suspended in RPMI-1640 supplemented with 10% FBS containing deferrioxamine. Deferrioxamine can increase the density of TF receptors on the cell surface, thus further enhancing gene delivery to the cell via TF receptor-mediated endocytosis. DNA/Duofect complex was added to the cells (5×10^5) and they were incubated at 37 °C for 4 h. The complex was then removed by aspiration, washed twice with RPMI-1640 supplemented with 10% FBS, and further cultured with RPMI-1640 supplemented with 10% FBS at 37 °C for 24 h. Twenty-four hour after transfection, GFP expression was determined using a flowcytometor as described below.

2.3.5. Nucleofection

MEG-01 cells were transfected using a Nucleofector Device from Amaxa Biosystems GmbH (Cologne, Germany). MEG-01 cells (1×10^6 cells) were collected by centrifugation ($300 \times g$, 5 min, 4 °C), re-suspended in 100 μl of Nucleofector Solution R, T, or V. Following addition of pEGFP-C1 pDNA (5 μg), the mixture was transferred into an electroporation cuvette. The cuvette was placed in the Nucleofector Device (Amaxa). Initially, eight programs with different intensities and lengths of electric pulse (A-23, A-27, T-20, T-27, T-16, T-01, G-16, and O-17) were used to obtain an optimal transfection condition. Control experiments were performed by processing MEG-01 cells in the same way, but without adding pDNA to the cells. Immediately after nucleofection, the MEG-01 cell suspension was transferred into a well of a 12-well plate containing 1 ml prewarmed RPMI-1640 supplemented with 10% FBS. After 24 h, the MEG-01 cells were collected by centrifugation ($300 \times g$, 5 min, 4 °C) and re-suspended in PBS containing 0.4% bovine serum albumin (BSA) and 5 mM EDTA. The ratio of transfected cells was determined by analyzing the expression of GFP by flowcytometry. Following the initial optimization study, another seven programs with higher transfection efficiencies (T-01, T-16, U-08, U-16, U-01, T-17, T-19, T-21, and T-09) are recommended by Amaxa Biosystems. Further details of the programs are proprietary information of Amaxa Biosystems. The transfection efficiencies were again measured by flowcytometry as described above.

2.4. Determination of transfection efficiency

2.4.1. Measurement for GFP

GFP-expression in MEG-01 cells was determined by flowcytometric analysis at 24 h post-transfection. Harvested cells were re-suspended in PBS containing 0.4% BSA and 5 mM EDTA, filtered through a 40-μm nylon cell strainer (BD Falcon, MA, USA), and analyzed using EPICS ELITE ESP equipped with a 488 nm argon laser (Beckman Coulter). The results are shown as positivity, viability, and efficiency. For each sample, 1×10^4 cells were analyzed and the live cell number was counted by the live gate (determined by forward scatter versus side scatter). Viability was calculated from the number of live and dead cells. To determine the GFP expression gate setting, mock (pCMV-Script)-transfected cells were used as a negative control. Positivity was the percentage of GFP-positive cells among the viable cells. The transfection efficiency was calculated as follows:

$$\text{Efficiency (\%)} = \frac{[\text{Positivity (\%)}] \times [\text{Viability (\%)}]}{100\ (\%)}$$

2.4.2. Determination of luciferase activity

Twenty-four hour after transfection, the cells were collected by centrifugation ($300 \times g$, 5 min, 4 °C) and lysed in 100 μl of Glo Lysis Buffer (Promega) for 5 min at room temperature. The cellular debris was spun down at $10{,}000 \times g$ for 10 min at 4 °C in a microcentrifuge and the supernatant was transferred into a new microtube and kept −20 °C until measurement of luciferase activity. This was done by mixing 50 μl of the supernatant with 50 μl of substrate solution (Bright Glo Luciferase Assay System; Promega), followed by measurement of light emission using ArvoSX (Perkin-Elmer, MA, USA). All luciferase activities were calibrated for viable cell numbers determined by trypan blue dye exclusion assay.

2.5. Determination of cell-associated pDNA by PCR

The pDNA delivered to cells following transfection was determined using the method of Tachibana et al. (2002a) with

minor modifications. Briefly, the cells were transfected by LFA2000 or nucleofection. After 4 h of transfection, the cells were collected by centrifugation ($300 \times g$, 5 min, 4 °C) and washed twice with cold PBS. The collected cells were suspended in 0.5 ml of DNA extraction buffer (10 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 10 mM NaCl). Then, 10 μl of 0.5 M EDTA, 10 μl of 10% SDS, and 5 μl of proteinase K (20 mg/ml) were added to the cell suspension. After incubation at 37 °C overnight, proteins were eliminated by phenol/chloroform treatment and the DNA was precipitated by the addition of ethanol. The precipitate obtained by centrifugation ($10{,}000 \times g$ for 10 min) was dissolved in TE buffer (1 mM EDTA, 10 mM Tris–HCl; pH 8.0) and used as a DNA sample. DNA concentrations were determined by measurement of the absorbance at 260 nm with an Ultrospec3000 spectrophotometer (Amersham Pharmacia, Uppsala, Sweden). A part of the GFP region of pEGFP-C1 in the DNA samples was then amplified by PCR using TaKaRa Ex Taq DNA polymerase with the primers 5′-GACGTAAACGGCCACAAGTTCAGCG-3′ and 5′-CTGCAGAATTCGAAGCTTGAGCTCG-3′. The PCR comprised denaturation at 94 °C for 2 min, followed by 26 cycles at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. After PCR, 10 μl of the reaction mixture was subjected to agarose gel electrophoresis, and the signal intensities of the amplified DNA bands were quantified using a FluorChem image analyzer (Alpha Innotech, CA, USA).

2.6. Evaluation of transgene function

2.6.1. Detection of phosphorylated STAT5 by Western blot

A c-mpl-encoding pDNA was transfected by nucleofection. To check whether the transgene was successfully expressed, Western blot analysis for phosphorylated STAT5, which is known to appear as a signal of TPO through the TPO receptor, c-mpl, was performed. MEG-01 cells transfected with the c-mpl expression plasmid or the control plasmid (pcDNA3) were cultured in RPMI 1640 containing 0.5% BSA for 16 h. The cells were stimulated with 100 ng/ml recombinant human TPO (Peprotech, NJ, USA) for 0, 10, and 30 min, and then directly lysed by SDS sample buffer (100 μl per 1×10^6 cells). The samples were separated on a 10% denaturing polyacrylamide gel (SDS-PAGE) and transferred to a 0.45-μm nitrocellulose membrane (Hybond ECL; Amersham, Uppsala, Sweden). The membranes were blocked with blocking buffer (Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% skim milk) for 1 h at room temperature. Subsequently, the membranes were incubated for 16 h at 4 °C with anti-STAT5 and phospho-STAT5 (Tyr 694) antibodies (Cell Signaling Technology, MA, USA) using 1:2000 and 1:1000 dilutions with primary antibody dilution buffer (TBS containing 0.1% Tween-20 and 5% BSA), respectively. After washing three times with TBS containing 0.1% Tween-20 (TBS-T), the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, CA, USA) using a 1:5000 dilution. After washing three times with TBS-T, immunocomplexes were visualized with a SuperSignal West Pico Kit (Pierce, IL, USA).

2.6.2. Cell proliferation assay

MEG-01 cells transfected with c-mpl-encoding pDNA or control pDNA (pcDNA3) were suspended with RPMI 1640 (serum free), seeded at 10,000 cells/well in a 96-well plate, and pre-cultured at 37 °C in 5% CO_2. After 16 h, recombinant human TPO (1, 10, and 100 ng/ml) was added to the wells and culture was continued for a further 2 days. A WST-8 assay was performed according to the manufacturer's recommendations (Dojindo, Kumamoto, Japan). Briefly, 10 μl of WST-8 reagent was added to each well following all treatments and the plates were then further incubated for 4 h at 37 °C in 5% CO_2. Subsequently, the color development was read at 450 nm using a plate-reader (E-Max; Molecular Devices, CA, USA).

2.7. Statistics

Data were analyzed using Student's *t*-test for unpaired samples. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Electroporation

An initial transfection experiment was performed using an Electro Square Porator T820 electroporation system (BTX). According to the manufacturer's guidelines, MEG-01 cells were electroporated using a broad range of different electrical conditions to check the effects of pulse field strength and pulse length. Fig. 1A shows cell viability as a function of pulse length and field strength. Cell viability declined steadily with increasing pulse length and field strength. It appears that cell are affected by both field strength and pulse length.

pGL3-Control pDNA, which can express luciferase, was transfected under various conditions, with viabilities above 30%. The higher the luciferase activity, the lower the cell viability (Fig. 1B). Luciferase activities increased with increasing pulse length and field strength. However, the activities were relatively lower (<300, cf. 27,000 in the case of LFA2000). Use of the lowest field strength (1.25 kV/cm), which showed higher viability (Fig. 1A), led to no luciferase activity at any pulse length or field strength (data not shown).

3.2. HVJ-E vector GenomONE and GenomONE-Neo

The inactivated HVJ-E vector systems GenomONE and GenomONE-Neo were tested. Unfortunately, GenomONE was very highly cytotoxic to MEG-01 cells. Thus, it was impossible to detect luciferase activity under the manufacturer's recommended conditions (data not shown). We examined another HVJ-E vector, GenomONE-Neo, with the pEGFP-C1 plasmid, which expresses GFP. Fig. 2 shows the relation between GFP positivity and viability following transfection. GFP-positive cells increased in response to the amount of complex added,

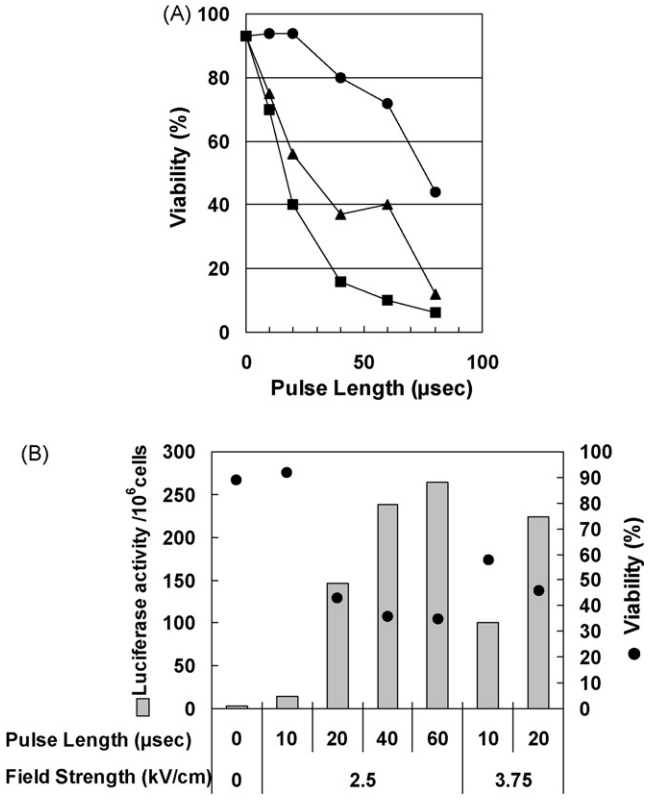

Fig. 1. Effect of electroporation and transfection efficiency with electroporation in MEG-01 cells. (A) MEG-01 cells (1×10^6) were treated with various field strengths and pulse lengths. Three pulses were applied at field strengths of 1.25 (●), 2.5 (▲), and 3.75 kV/cm (■) with various pulse lengths (0–80 μs). (B) MEG-01 cells (1×10^6) were transfected with pGL3-Control (15 μg) plasmid. Cell viability and luciferase activity were determined 20 h after transfection. The pulse number was kept at three in all electroporations.

while cell viability was inversely related to this amount. Dilution factors largely affected GFP expression and cell viability, but did not affect the positivity of GFP expression. To improve both the efficiency of GFP expression and cell viability, we tried to optimize the transfection conditions by modification of the preparation conditions, adding the volume and dilution ratio

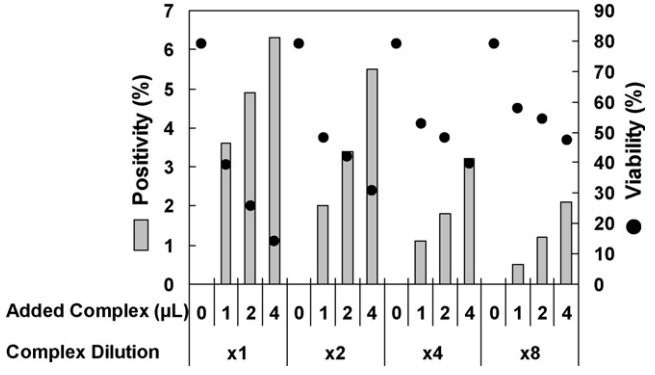

Fig. 2. Transfection efficiency of HVJ-envelope vector, GenomONE-Neo, in MEG-01 cells. MEG-01 cells (2.5×10^5) were treated with various amounts of GenomONE-Neo (25 μl)-pEGFP-C1 (5 μg) complex. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection.

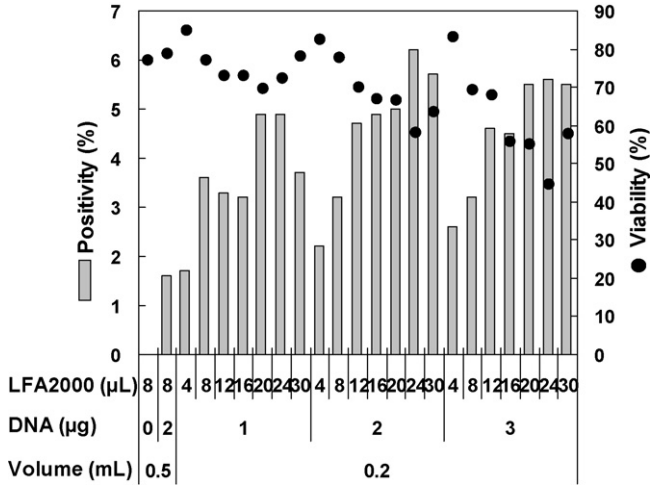

Fig. 3. Transfection efficiency of LFA2000 in MEG-01 cells. MEG-01 cells (5×10^5) were transfected with 1, 2, or 3 μg of pEGFP-C1 with various amounts of LFA2000. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection.

of the HVJ-E–DNA complex. However, no improvement was achieved (data not shown).

3.3. Liposome or cationic polymer-based reagents

LFA2000, FuGENE6, SuperFect, Effectene, TransFast, jet-PEI, GenePORTER2, and DuoFect were tested with pEGFP-C1 pDNA. Representative results based on the manufacturers' recommended protocols are given in Table 1. Among these reagents, LFA2000 showed the highest transgene positivity. This finding is consistent with the results of Chuang and Schleef (2001), who showed that the transfection efficiency with FuGENE6, SuperFect, Effectene, or GenePORTER2 was very low for MEG-01 cells (<0.001%). To obtain a higher positivity with LFA2000, parameters such as the amount of pDNA, the transfection volume, and the culture time for transfection or after transfection were changed. As shown in Fig. 3, increasing the amount of LFA2000 used to prepare the lipoplex with pDNA improved the positivity without an increase in cytotoxicity. Positivity was also improved by decreasing the incubation volume. Finally, we obtained conditions that achieved a transfection positivity of around 6% (Fig. 3).

3.4. Nucleofection

Nucleofection was tested with pEGFP-C1 pDNA. The results of preliminary experiments to optimize the transfection conditions are summarized in Table 2. Although there were some combinations of solution and program that gave a high positivity (up to 90%), the viabilities were lower. In terms of viability, solution R showed slight lower cytotoxicity than solutions T and V. We then tested the combination of solution R with another eight programs recommended by Amaxa Biosystems: T-01, T-16, U-08, U-16, U-01, T-17, T-19, T-21, and T-09. The results are shown in Table 3. Consequently, program U-01 was selected

Table 2
Transfection efficiency of nucleofection (I)

Program	DNA	Solution R			Solution T			Solution V		
		Viability (%)	Positivity (%)	Efficiency (%)	Viability (%)	Positivity (%)	Efficiency (%)	Viability (%)	Positivity (%)	Efficiency (%)
A-23	+	73.2	38.1	27.9	52.1	60.4	31.5	59.3	49.7	29.5
A-27	+	81.5	6.7	5.5	77.4	15.3	11.8	79.4	10.4	8.3
T-20	+	38.1	61.2	23.3	32.3	95.0	30.7	37.1	94.8	35.2
T-27	+	53.1	90.7	48.2	35.1	92.6	32.5	49.1	93.2	45.8
T-16	+	63.8	90.6	57.8	48.1	92.5	44.5	57.6	92.5	53.3
T-01	+	82.1	74.6	60.6	76.8	75.0	57.6	78.8	77.2	60.8
G-16	+	67.3	57.0	38.4	54.9	69.5	38.2	49.9	68.9	34.4
O-17	+	63.1	75.5	47.6	51.2	81.3	41.6	65.2	91.3	59.5
–	+	88.1	0.0	0.0	88.6	0.0	0.0	86.5	0.0	0.0
T-16	–	78.4	0.0	0.0	77.6	0.0	0.0	79.0	0.0	0.0

For each condition, MEG-01 cells (1×10^6) were suspended with Nucleofector Solution R, T, or V, together with 5 μg of pEGFP-C1 plasmid, and processed using various programs. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection.

for further experiments because of the higher efficiency and viability.

3.5. Amount of pDNA delivered to cells by nucleofection

To examine the reason for the high efficiency using nucleofection program U-01, we determined the amount of pDNA delivered by nucleofection. Program U-01 was compared with program A-27, which showed high viability and low transgene efficiency (Table 2). As a negative control, transfection with LFA2000 was selected. At 4 h after transfection, cell-associated pDNA was determined according the method described in Section 2. Interestingly, the amount of delivered pDNA by nucleofection was lower than that by LFA2000 irrespective of the use of program U-01 or A-27 (Fig. 4).

3.6. Nucleofection of c-mpl-encoding pDNA

To verify the utility of nucleofection, MEG-01 cells were transiently transfected with c-mpl-encoding pDNA. The effect of treatment with the ligand TPO on the transfected cells was then evaluated by Western blotting of phospholylated STAT5, which is activated by the TPO-mpl signal cascade, and by cell proliferation assay. As shown in Fig. 5B, STAT5 in c-mpl-transfected cells was more phospholylated (activated) than that in mock-transfected cells (arrowheads) following incubation with TPO. STAT5 phospholylation in c-mpl-transfected cells was enhanced 10 and 30 min after TPO treatment. The prolif-

Table 3
Transfection efficiency of nucleofection (II)

Program	Viability (%)	Positivity (%)	Efficiency (%)
T-01	72 ± 2	44 ± 9	32 ± 7
T-16	46 ± 7	82 ± 4	38 ± 7
U-08	51 ± 4	81 ± 5	41 ± 4
U-16	43 ± 4	87 ± 5	37 ± 5
U-01	68 ± 2	70 ± 7	48 ± 6
T-17	42 ± 4	87 ± 3	37 ± 3
T-19	41 ± 2	85 ± 6	35 ± 4
T-21	41 ± 3	83 ± 5	34 ± 4
T-09	47 ± 4	83 ± 4	39 ± 5
–	80 ± 4	0 ± 0	0 ± 0

For each condition, MEG-01 cells (1×10^6) were suspended with Nucleofector Solution R together with 5 μg of pEGFP-C1 plasmid, and processed using various programs. Cell viability and GFP positivity were analyzed by flowcytometer 24 h after transfection. Data are mean ± standard error of three independent experiments.

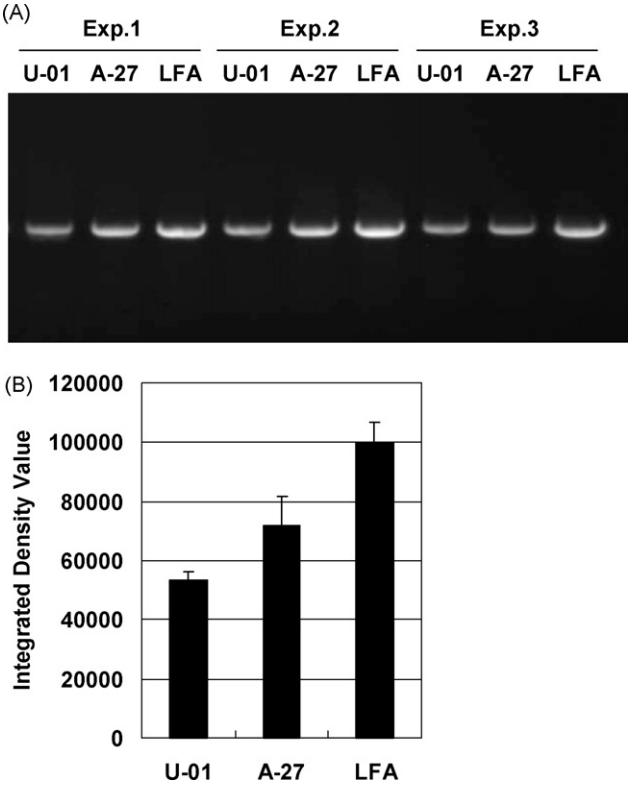

Fig. 4. Amount of cell-associated pDNA following nucleofection and lipofection. MEG-01 cells (1×10^6) were transfected with pEGFP-C1 plasmid using nucleofection (solution R, program U-01 or A-27) and LFA2000. At 4 h after transfection, pDNA was isolated from the cells and the amount was determined by PCR. (A) Agarose gel image of PCR products. (B) Integrated density value (IDV) of DNA bands. Data are averages of three independent experiments.

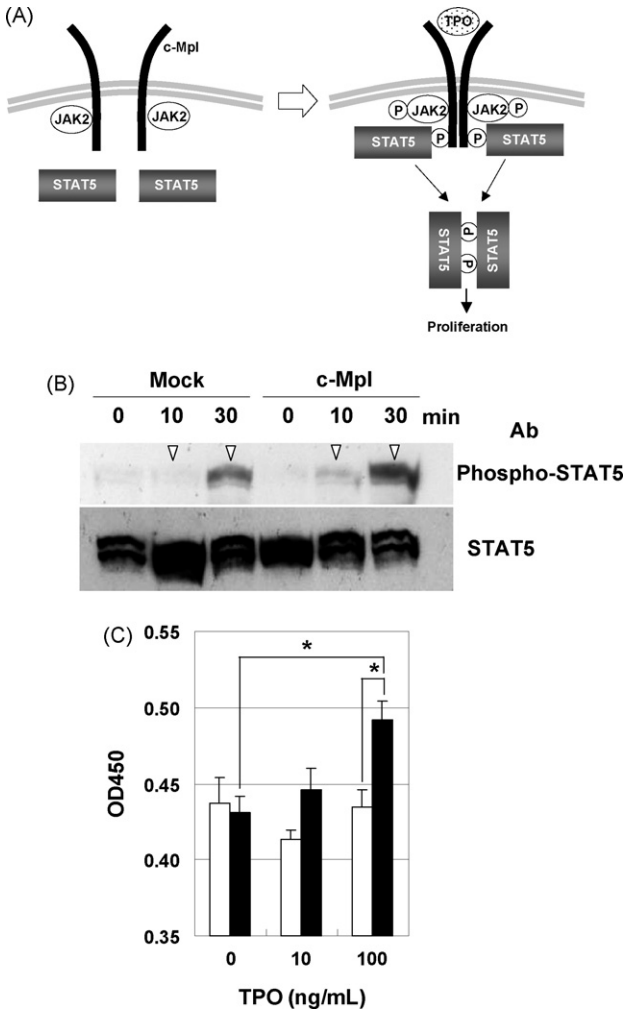

Fig. 5. TPO response in c-mpl-transfected cells. (A) Signal transduction cascades of TPO-induced STAT5 activation and proliferation of megakaryocytes. (B) Western blot of phospho-STAT5 and STAT5. MEG-01 cells (3×10^6) were transfected with c-mpl-encoding pDNA (5 μg) by nucleofection. Transfected cells were stimulated with TPO (100 ng/ml) for 0, 10, or 30 min. (C) Cell proliferation of c-mpl- (■) or mock (□)-transfected cells following incubation in the presence of TPO (0, 10, or 100 ng/ml) for 2 days.

eration assay indicated that c-mpl-transfected cells proliferated extensively after treatment with TPO (100 ng/ml) for 2 days (Fig. 5C). These results show the high utility of nucleofection in validating the function of genes in MEG-01 related to differentiation.

4. Discussion

Efficient transfection of megakaryocytes is required to study the mechanism of platelet production from megakaryocytes. However, conventional transfection methods are generally insufficient. In this study, we investigated several transfection methods (electroporation, commercially available liposomal reagents, transferrin-linked cationic polymer, inactivated virus envelope vectors, and nucleofection) to obtain efficient transgene expression in MEG-01 cells. The results indicate that, among the methods we tested, nucleofection was the only one to achieve efficient transgene expression in MEG-01 cells.

To clarify part of mechanism of efficient transgene expression by nucleofection, we determined the amount of pDNA delivered by transfection using two different nucleofection programs (U-01, most efficient; A-27, less efficient) and LFA2000 (negative control). The amount of pDNA delivered by nucleofection with U-01 was lower than that by nucleofection with A-27 and by LFA2000 (Fig. 4). It appears that there is not necessarily a good correlation between the amount of pDNA delivered by nucleofection and transgene expression. This observation is supported by the recent report of Hama et al. (2006). Even when transfected pDNA is incorporated into cells, there are many hurdles to clear, such as lysosomal traps and nuclear entry, before significant amounts of the gene product are expressed (Elouahabi and Ruysschaert, 2005). In nucleofection, electroporation based gene delivery technique, pDNA is assumed to be introduced directly into the cytoplasm and efficiently used for gene expression. In contrast, with LFA2000, lipofection based gene delivery reagent, pDNA forms complexes with the LFA2000, and the genes seem to be introduced into cells via endocytosis. This means that the genes are more likely to be trapped in endosomes and lysosomes, and cannot easily enter the nucleus. Tachibana et al. (2002b) reported that liposomes themselves inhibit transcription using a cell-free transcription and translation system. Hence, it is assumed that, though the amount of pDNA delivered to the cells by LFA2000 was more than that by nucleofection (U-01 or A-27), a relatively lower transfection efficiency was observed. Further investigation is needed to clarify the exact mechanism and achieve efficient gene expression with the relatively small amount of pDNA delivered by nucleofection.

To demonstrate the utility of nucleofection, we selected the c-mpl gene because c-mpl expression is very low (Graf et al., 1996) and the TPO signaling cascade is well investigated in megakaryocytes (Kaushansky, 2003). As shown in Fig. 5, there was a significant difference between c-mpl- and control pDNA-transfected cells in their responsiveness to TPO. This result strongly suggests that nucleofection is practical for validating gene function in MEG-01 cells.

Among the various gene-transfer methods, it has been reported that only methods using viral vectors show high transgene expression in megakaryocytes. Burstein et al. (1999) achieved 41–82% transgene-expressing megakaryocytes using a retroviral vector. Recently, Gillitzer et al. (2005) reported the use of a GFP-expressing retrovirus with $CD34^+$ cells; the infected cells were cultured in the presence of TPO and differentiated into megakaryocytes. After differentiation, GFP expression on the culture-derived platelets was 40%. This method may be suitable for transgene expression in megakaryocytes. However, viral methods are not suitable for large numbers of validations of transgene function. This is because, for each target gene, the procedure requires many steps: viral vector construction, transfection to package cells, virus production, virus purification, preparation, and titer check for efficient infection. Moreover, there is a safety problem when using the virus, which can infect human cells and must be handled with appropriate facilities. In contrast, nucleofection is a relatively fast and easy method.

Nucleofection has been reported as an efficient technique of pDNA delivery to primary cultured cells and non-adhered hematopoietic cells (Hamm et al., 2002). For example, primary keratinocytes was successfully transfected without any change in their cellular properties (Distler et al., 2005). Natural killer cells, a kind of hematopoietic cell, and cell lines were also successfully transfected with high efficiency (Trompeter et al., 2003; Maasho et al., 2004). This is the first study to achieve efficient transgene expression in a megakaryocytic cell line, MEG-01, by nucleofection.

In conclusion, we have obtained an efficient transfection method for a megakaryocytic cell line, MEG-01. This low cytotoxicity, higher efficiency, non-viral method may be useful for clarifying the kinds of genes that are involved in the mechanism of platelet production from megakaryocytes. Moreover, nucleofection may be a useful technique, not only for megakaryocytes but also for other hematopoietic cells. This method may help in clarifying the mechanisms of the functions of hematopoietic cells.

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