ORIGINAL RESEARCH

# Comparison of plasmid DNA versus PCR amplified gene of insert DNA for nucleofection in Kasumi-1 cells

Kang Wu · Xu-jie Zhao · Ka-wing Wong · Xiao-yong Fan

**Abstract** Plasmid electroporation, or its optimized version nucleofection, is an important technique for gene transfection of cells in suspension. However, substantial cell death and/or low transfection efficiency are still common for some cell lines. By using enhanced green fluorescent protein (EGFP) as a reporter, we compared the use of PCR amplified EGFP (PaEGFP) and its parental plasmid (pEGFP-N2) for nucleofection in Kasumi-1 cells. We found that PaEGFP induced significantly lower cell death but had similar transfection efficiency compared to its parent plasmid (pEGFP-N2). Most importantly, contrary to the pEGFP-N2-nucleofected cells, the PaEGFP-nucleofected cells subsequently grew properly. Tests in other cell lines also implied that PaEGFP indeed induced consistently less cell death, but transfection efficiencies varied, being good in suspension cell lines but lower in adhesive cell lines. We suggest that direct transfection with PCR amplified genes can be a simple and useful approach for optimization of electropulse-based transfection not only of Kasumi-1 cells, but also may be useful for other cell lines that are difficult to transfect in suspension.

**Keywords** PCR amplified gene · Cell viability · Nucleofection · Plasmid · Transfection efficiency

Kang Wu and Xu-jie Zhao contributed equally to this work.

K. Wu · K. Wong · X. Fan (✉)
Key Laboratory of Medical Molecular Virology of MOE/MOH, Shanghai Public Health Clinical Center, Fudan University, 2901 Caolang Road, Shanghai 201508, China
e-mail: xyfan008@fudan.edu.cn

X. Zhao
State Key Laboratory of Medical Genomics, Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, 197 Ruijin Road II, Shanghai 200025, China

K. Wong · X. Fan
Shanghai Medical College, Fudan University, 138 Yixueyuan Road, Shanghai 200032, China

## Introduction

Transfection of nucleic acids (small non-coding RNA, mRNA, or plasmid) into cells is a valuable and widely utilized method in functional genomics (Kumar et al. 2010), vaccine development (Lowrie et al. 1999; Lowrie 2006), transcriptional delineation (Linggi et al. 2002), stem cell research (Kim et al. 2009), and inflammatory therapy (Zimmermann et al. 2012), to name just a few examples. Techniques for cell transfection, whether for cell lines or primary cells, can be broadly divided into two categories: viral-based and nonviral-based methods. For the nonviral-based methods, electroporation is one of the most important

approaches, especially for cells in suspension. In electroporation, an electrical pulse is applied to the cells, which induces transient permeability of the cell plasma membranes and results in the internalization of nucleic acids (Neumann et al. 1982). As an optimized method of electroporation, nucleofection encompasses not only electrical pulse, but also cell type-specific solutions, resulting in superior transfection efficiency and cell viability, especially for difficult-to-transfect cells (Maasho et al. 2004; Bertram et al. 2012).

The Kasumi-1 cell line, which is a leukemic cell line derived from peripheral blood of a Japanese boy with acute myeloid leukemia subtype M2 (Asou et al. 1991), holds a central position in the dissection of leukemogenesis by displaying plasticity at various levels (e.g., morphology, molecular composition, and immunophenotype) (Larizza et al. 2005). Our routine application of nucleofection in Kasumi-1 cells, with plasmids and small-interfering RNA (siRNA), revealed that plasmids always induced severe cell death and had lower transfection efficiency as compared to siRNA (unpublished data), which hindered the objective interpretation of relevant biological phenomenon. To help resolve this nucleofection dilemma, we compared the performance of the gene alone, PCR amplified from the plasmid, against the parent plasmid containing the gene. The PCR amplified gene (PaGene) harbored the gene promoter, protein coding region, and transcription terminator from the plasmid. The PaGene induced significantly lower cell death and had similar transfection efficiency as compared with its parent plasmid in Kasumi-1 cells. Preliminary study of nucleofection in several other cell lines indicated that PaEGFP consistently induced less cell death. However, the transfection efficiencies varied, with similar transfection efficiency in suspension cell lines, and slightly lower transfection efficiency in adhesive cell lines.

## Materials and methods

### Cell lines and *Escherichia coli* (*E. coli*) strain

Kasumi-1, NB4, and THP-1 cell lines, which were obtained from the cell bank of Chinese Academy of Sciences (CAS, Shanghai, China), were maintained in RPMI 1640 (GIBCO, Grand Island, NY, USA)

**Table 1** Primers used for amplification of PaEGFP and PaLuc

| Primers | Sequences |
| --- | --- |
| PaEGFP-F | 5′-GTATTACCGCCATGCATTAG-3′ |
| PaEGFP-R | 5′-GGACAAACCACAACTAGAAT-3′ |
| PaLuc-F | 5′-GGTACCGAGCTCTTACGCGT-3′ |
| PaLuc-R | 5′-TTCAGATCCTTATCGATTTT-3′ |

containing 15 % (for Kasumi-1) or 10 % (for NB4 and THP-1) fetal bovine serum (FBS) (Gibco) at 37 °C in 5 % $CO_2$. Cell lines 293FT and RAW264.7, which were also obtained from cell bank of CAS, were maintained in DMEM (GIBCO) containing 10 % FBS (Gibco) at 37 °C in 5 % $CO_2$. *E. coli* strain (Top10; Invitrogen, Carlsbad, CA, USA) was used for plasmid maintenance and propagation.

### Preparation of plasmids and PCR amplified genes (PaGenes)

Plasmids pEGFP-N2 (BD Biosciences, San Jose, USA) and pGL3-Control (Promega, Madison, WI, USA) were extracted using NucleoBond Xtra Midi Kit (Macherey–Nagel, Düren, Germany). Null eluent from empty *E. coli* (Top 10) was also prepared using AxyPrep™ Plasmid Miniprep kit (Axygen, Hangzhou, China). The extracted plasmids were subsequently used for PCR amplification of *EGFP* and firefly *luciferase* genes to give PCR products named PaEGFP and PaLuc respectively. In detail, these two genes were amplified using proofreading DNA polymerase KOD Plus (Toyobo, Osaka, Japan) and their cognate primer pairs (see primer sequences in Table 1). The forward primers were located upstream of the gene promoter ($P_{CMV\ IE}$ for *EGFP* in pEGF-N2 and the SV40 promoter for *luciferase* in pGL3-Control) and the reverse primers were located downstream of the transcription terminator (SV40 early poly(A) signal for *EGFP* in pEGFP-N2 and SV40 late poly(A) signal for *luciferase* in pGL3-Control) (Fig. 1). The primer pair locations were selected to permit the transcription and subsequent translation of PaEGFP and PaLuc intracellularly. PaGenes were purified with AxyPrep™ PCR Clean-up kit (Axygen), and used directly for nucleofection. DNA concentrations and the A260/A280 and A260/A230 ratios (both were higher than 1.8) were determined using a NanoDrop ND1000 spectrophotometer (Thermo

**Fig. 1** Schematic of gene amplification from plasmid and experimental design. Primer pair (Primer-F and Primer-R) was designed, with forward primer located upstream of the promoter and reverse primer downstream of the terminator. The primer pair was used to amplify the gene of interest by a proofreading DNA polymerase. The amplified gene (PaGene) was purified and used to transfect cells in parallel with its parent plasmid. Two different amounts of parent plasmid were used for transfection, one contained the same amount of nucleic acid (ng) as used with the PaGene, the other contained the same number of molecules (moles) as the PaGene
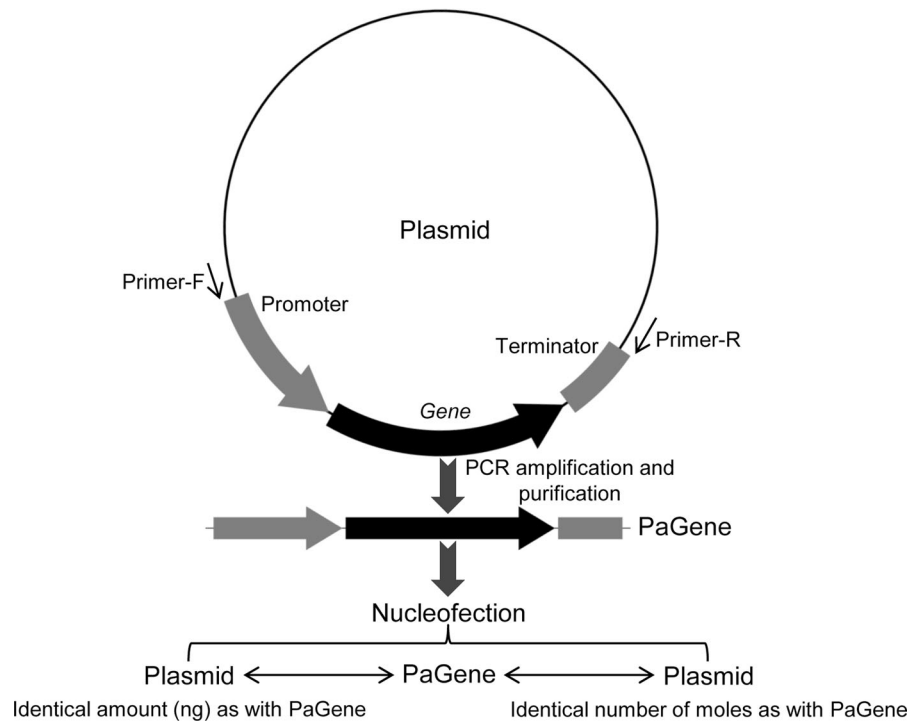


Scientific, Wilmington, MA, USA) to ensure integrity and purity. High quality super-coiled plasmid DNAs and PaGenes were also visually checked by agarose gel electrophoresis (Fig. S1).

Labeling of Kasumi-1 cell with cell proliferation dye eFluor 670

Cell Proliferation Dye eFluor 670 (eBioscience, San Diego, USA) is a fluorescent dye that can bind to any cellular protein containing primary amines. With successive divisions of the stained cells, the fluorescence intensity of eFluor 670 in the stained cells successively halves. Thus, flow cytometry of nucleofected cells pre-labeled with eFluor 670 can reveal cell proliferation. In brief, resuspended Kasumi-1 cells were mixed with an equal volume of 10 µM eFluor 670 and then incubated for 10 min at 37 °C in the dark. Then 4–5 volumes of cold complete RPMI 1640 were added and incubated on ice for 5 min to stop the staining. Finally, the labeled cells were washed three times with complete RPMI 1640 and used for nucleofection.

Nucleofection

$2 \times 10^6$ Kasumi-1 cells, either pre-labeled with eFluor 670 or not, were nucleofected using nucleofector$^{TM}$ with buffer V and program P-19 (Amaxa Biosystems, Cologne, Germany) then seeded in 2 mL pre-warmed medium. No optimized parameters for nucleofection of Kasumi-1 cells had been published, so we utilized the nucleofection protocol of Corsello et al. (2009). The protocol induced considerable cell death, and thus left scope for improvement. In comparing the use of plasmid DNA and PaGene for nucleofection, different amounts of nucleic acids were used. For the PaEGFP and pEGFP-N2 pair, 680 ng of PaEGFP and 2000 ng of pEGFP-N2 were used per electroporation cuvette (identical number of moles). In addition, null eluent from empty *E. coli* was used to control for possible lethal effects of any residual endotoxin. For the PaLuc and pGL3-Control pair, we used 830 ng of PaLuc and 2000 ng of pGL3-Control for equal moles. Comparisons between PaGene and parent plasmid were also done using the same amount of each (ng per cuvette); 680 ng was used for pEGFP-N2 and 830 ng for pGL3-Control. Four other cell lines

(NB4, THP-1, 293FT, and RAW264.7) were nucleofected under the same conditions as the Kasumi-1 cells, with the exception that the pulse programs were X-001 for NB4 cells, V-001 for THP-1 cells, A-023 for 293FT cells, and D-032 for RAW264.7 cells.

Flow cytometry analysis

Kasumi-1 cells were prepared for flow cytometry (FACSAria, BD Biosciences) analysis 24 h, 48 h, or 72 h post nucleofection with PaEGFP or pEGFP-N2. The cells were first gated based on parameters of forward-scatter (FSC) and side-scatter (SSC). From this dot plot, proportions of viable cells were calculated and then the viable cell populations were further analyzed for EGFP expression. Data were analyzed with WinMDI Version 2.9.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay

Equal volumes of Kasumi-1 cells (equivalent to 10,000 cells per well) that had been nucleofected with either PaEGFP or pEGFP-N2 were seeded into 96-well flat bottom plates. Supernatant was discarded 24, 48, or 72 h post nucleofection, MTT reagent (Beyotime, Shanghai, China) was added and the cells were incubated for a further 4 h, during which time MTT was reduced to purple formazan crystals by the mitochondria in living cells. DMSO was then added to solubilize the reduced crystals and the absorbance was measured at 570 nm by spectrophotometry.

Luciferase assay

72 h post nucleofection with PaLuc or pGL3-Control, Kasumi-1 cells were lysed and tested for firefly luciferase following the instruction manual of Dual-Luciferase Reporter Assay System (Promega). *Renilla* luciferase vector was not added as internal control because of its lethal effect to Kasumi-1 cells.

Western blot

72 h post nucleofection of Kasumi-1 cells with either PaEGFP or pEGFP-N2, an equal volume of cells was harvested and boiled in $2 \times$ loading buffer (20 mM Tris–HCl, pH 8.0, 100 mM DTT, 2 % SDS, 20 % glycerol, 0.016 % bromophenol blue) for the

subsequent Western blot to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and EGFP. Blotting for EGFP reflected the total amount of EGFP in live and dead cells (cell debris). Blotting of constitutively expressed GAPDH indicated the differential in cell viability and proliferation, since more GAPDH should be observed in more live cells.

Statistical analysis

Student's *t* test was applied for group comparisons. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

# Results

PaEGFP induces less cell death
during nucleofection in Kasumi-1 cells

As shown by flow cytometry analysis (left panels of Fig. 2a, b), PaEGFP induced a slightly greater degree of cell death as compared to the negative control (nucleofection without DNA) that was statistically insignificant at both 24 h and 72 h post nucleofection. However, nucleofection with the parental plasmid (pEGFP-N2) resulted in a prominent time-dependent and dose-independent cell death. At 72 h post nucleofection, nearly 50 % of Kasumi-1 cells from the pEGFP-N2 groups (both 680 and 2,000 ng) were dead. By contrast, less than 20 % of the cells from the PaEGFP group (left panel of Fig. 2b) were dead. In addition, cells nucleofected with null eluent from empty *E. coli* were killed comparable to the negative control and PaEGFP groups (left panel of Fig. 2b). When the cells were examined by light microscopy after nucleofection, the cell density in the PaEGFP group was much higher, and almost all cells showed good morphology (Fig. 3). However, cell densities from the pEGFP-N2 groups were lower, and cell debris could be easily seen (Fig. 3).

We confirmed this difference in degree of damage by using the MTT assay and Western blotting. The MTT assay showed that Kasumi-1 cells in the PaEGFP group underwent proper growth across the time points tested (24, 48, and 72 h post nucleofection). By contrast, cells in the pEGFP-N2 groups underwent no growth (Fig. 4a). Western blotting of the constitutively expressed GAPDH, as a marker for cell mass, showed a thicker band of stain with the PaEGFP group compared to the pEGFP-N2 groups (Fig. 4b).
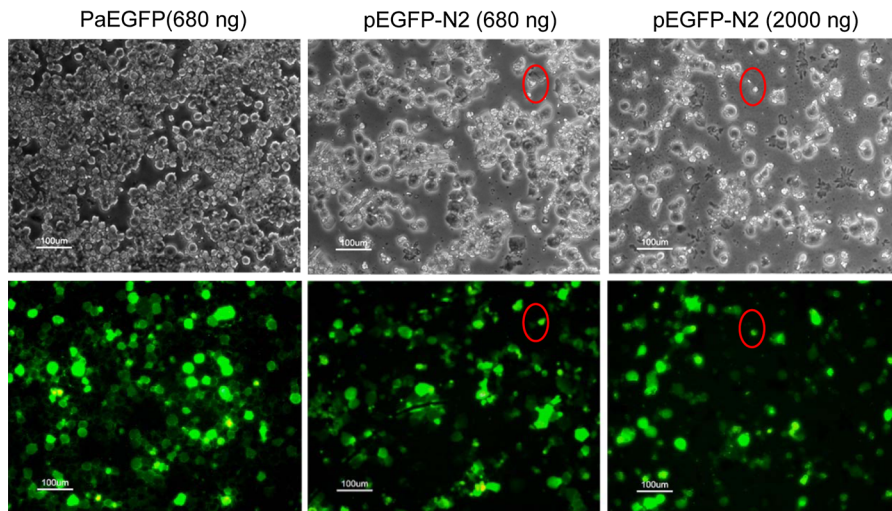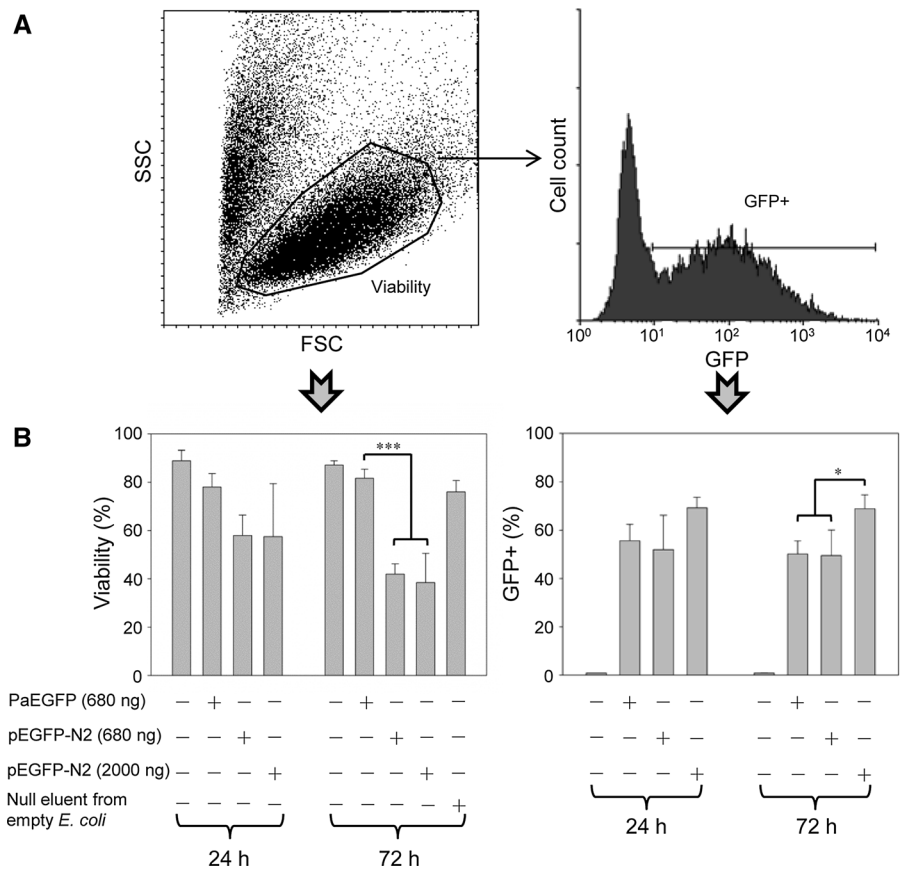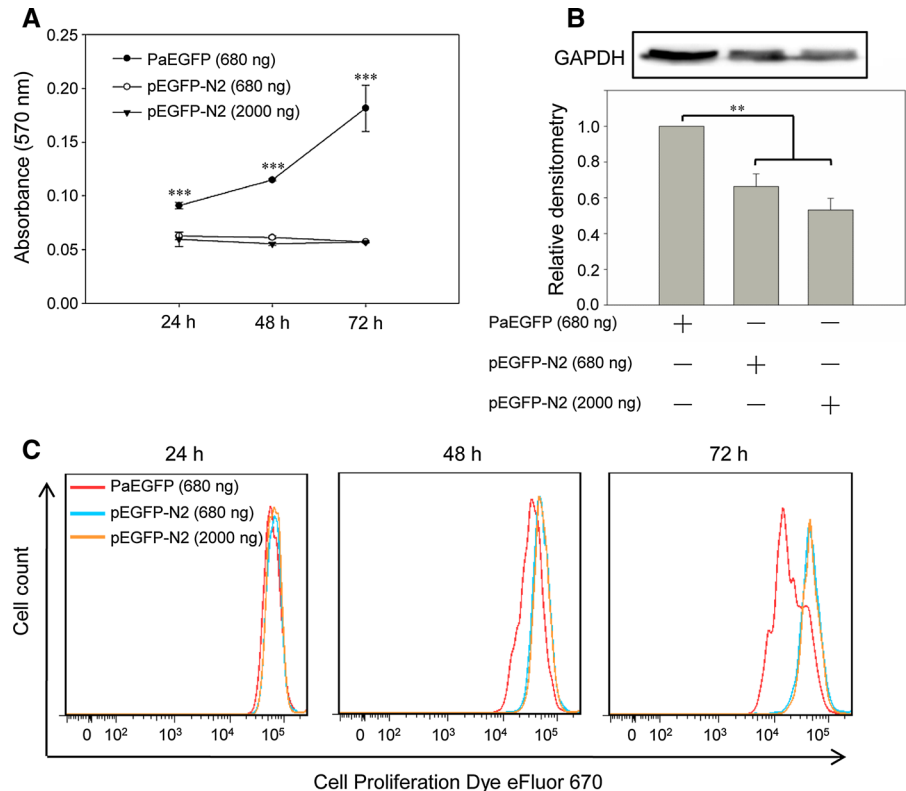
**Fig. 3** Nucleofection of Kasumi-1 cells with PaEGFP and pEGFP-N2. Cells were nucleofected with 680 ng of PaEGFP, or either 680 ng (*equal weight*) or 2,000 ng (*equal moles*) of pEGFP-N2. Cells in the volume of 100 µL were deposited by cytocentrifugation on glass slides 72 h post nucleofection, and examined by microscopy. Upper panels, light images. Lower panels, fluorescence images. *Scale bars*, 100 µM. *Red circles* indicate cell debris which retained the expressed EGFP

**Fig. 4** Viability of the nucleofected Kasumi-1 cells as revealed by other approaches. **a** MTT assay: equal volumes of cells (representing the same number of cells prior to nucleofection) from each group were accessed by MTT approach at the indicated hours. **b** Western blot of the internal control GAPDH. Equal volumes of cells from each group at 72 h post nucleofection were used for Western blot. The amount of GAPDH reflected the different degrees of cell death as well as cell proliferation among different groups of Kasumi-1 cells. **c** FACS assay of the nucleofected Kasumi-1 cells pre-labeled with cell proliferation dye eFluor 670. Error bars show mean ± SD of three independent assays. **p < 0.01; ***p < 0.001

FACS assay of nucleofected Kasumi-1 cells that had been pre-labeled with cell proliferation dye eFluor 670 validated the result of MTT assay by showing proper proliferation of Kasumi-1 cells in the PaEGFP group and weak proliferation in the pEGFP-N2 groups (Fig. 4c).

### PaEGFP induces proper EGFP expression after nucleofection in Kasumi-1 cells

Since PaEGFP induced less cell death as compared to its parental plasmid pEGFP-N2, we then determined the expression levels of EGFP in the different groups. The proportion of live cells expressing EGFP was similar in the cells induced with PaEGFP and those induced with 680 ng of pEGFP-N2 at both 24 h and 72 h post nucleofection. The highest proportion of EGFP-expressing cells observed was in the group induced with 2,000 ng of pEGFP-N2, although the differences were not statistically significant (right panels of Fig. 2a, b also in lower panel of Fig. 3). Overall expression of EGFP was also assayed by Western blotting against EGFP. In line with the result from FACS analysis (right panel of Fig. 2b), the group

nucleofected with 2,000 ng of pEGFP-N2 expressed the highest amount of EGFP protein, the 680 ng PaEGFP group expressed less EGFP, and the 680 ng pEGFP-N2 group expressed the lowest level of EGFP (Fig. 5a). When the expression levels of EGFP were normalized against GAPDH expression (Fig. 4b), there was a comparable EGFP expression level in PaEGFP (680 ng) and pEGFP-N2 (680 ng), whereas pEGFP-N2 (2,000 ng) had the highest EGFP expression level (Fig. 5b). This pattern of protein expression was confirmed when firefly luciferase was used as a reporter, with the highest signal in the group nucleofected with 2,000 ng of pGL3-Control, a lower signal in the group with 830 ng PaLuc, and the lowest signal in the group with 830 ng pGL3-Control (Fig. 5c). Taken all together, these results indicated that nucleofection with PaEGFP induced good EGFP expression in Kasumi-1 cells.

### Comparison of PaEGFP and pEGFP-N2 in nucleofection of other cell lines

Since PaEGFP induced less cell death but had similar transfection efficiency as compared to pEGFP-N2 in
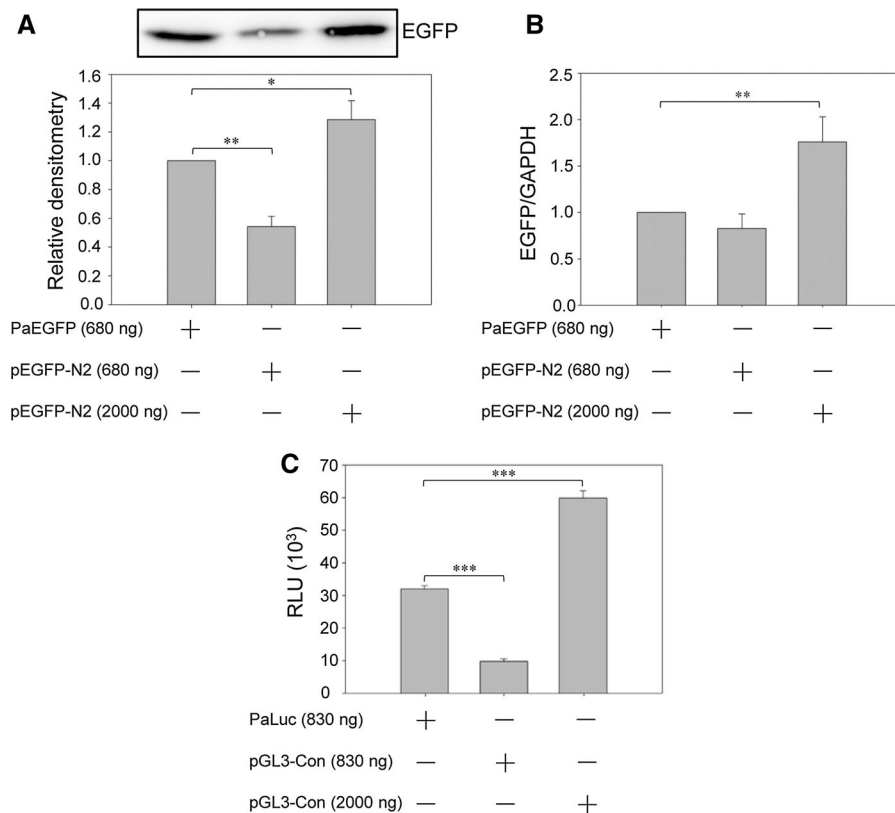
Fig. 5 Quantification of proteins in Kasumi-1 cells after nucleofection with PaGenes or their parent plasmids. **a** EGFP 72 h post nucleofection: upper panel, Western blot of EGFP; lower panel, digitization of bands with image J software. Band intensities were divided by the intensity of the PaEGFP-nucleofected cells. **b** Relative EGFP expression normalized by GAPDH 72 h post nucleofection. Digital value of EGFP band was normalized against cognate digital value of GAPDH band from a same protein sample (EGFP/GAPDH), then the relative ratios were divided by the relative ratio of the PaEGFP-nucleofected cells. **c** Relative luciferase units (RLU) of cells nucleofected with PaLuc or pGL3-Control 72 h post nucleofection. *Error bars* show mean ± SD of three independent assays. $*p < 0.05$; $**p < 0.01$

Kasumi-1 cells, we investigated whether the phenomenon would generalize to other cell lines. We selected four commonly used cell lines to test; two suspension cell lines (NB4 and THP-1) and two adhesive cell lines (293FT and RAW264.7). The results were similar to those in Kasumi-1 cells. For example, PaEGFP induced less cell death, and had even higher transfection efficiency compared to pEGFP-N2 in NB4 cells at 72 h post nucleofection (Fig. 6a). In THP-1 cells, PaEGFP also induced less cell death, but had similar transfection efficiency compared to pEGFP-N2 at 72 h post nucleofection (Fig. 6b). However, in adhesive cell lines (293FT and RAW264.7), PaEGFP induced lower transfection efficiencies, even though it allowed normal cell growth, when compared to pEGFP-N2 (Fig. 6c, d). These results implied that PaEGFP always induced consistently less cell death (in NB4 and THP-1) and permitted normal cell growth (in 293FT and RAW264.7). However, although there was at least comparable transfection efficiency in suspension cell lines, there was lower transfection efficiency in adhesive cell lines.

## Discussion

Fluorescent proteins (EGFP in most cases) are easily assayed, even in vivo, and thus have wide and diverse utilization in research. In this study, we systematically compared the use of PCR amplified gene (i.e. PaEGFP) and its parental plasmid (i.e. pEGFP-N2) for nucleofection in Kasumi-1 cells. To the best of our

**Fig. 6** Comparison of
nucleofection with PaEGFP
and pEGFP-N2 in diverse
cell lines. NB4 (**a**), THP-1
(**b**), 293FT (**c**), and
RAW264.7 (**d**) cells were
nucleofected with PaEGFP
and pEGFP-N2, then cell
viability (for NB4 and THP-
1) or proliferation (for
293FT and RAW264.7), as
well as transfection
efficiency were accessed
72 h post nucleofection.
*Error bars* show
mean ± SD of three
independent assays.
*p < 0.05; **p < 0.01;
***p < 0.001
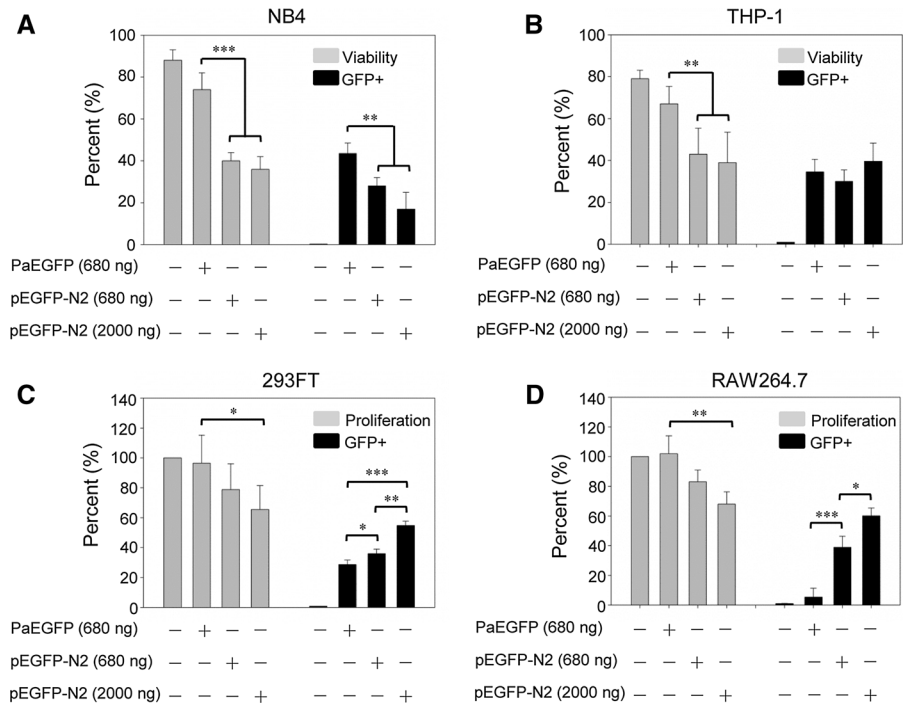


knowledge, we are not aware of other similar studies
in published literature. By using EGFP as a reporter of
gene expression, we illustrated that vector-less Pa-
Gene, when delivered into Kasumi-1 cells through
nucleofection, induced less cell death but had similar
transfection efficiency as compared to its parental
plasmid. When Kasumi-1 cells were nucleofected with
PaEGFP and pEGFP-N2 at identical amount (680 ng),
similar proportions of cells expressed EGFP. When
cells were nucleofected with PaEGFP and pEGFP-N2
at same number of moles (680 ng of PaEGFP *vs.*
2,000 ng of pEGFP-N2), slightly lower but compara-
ble proportion of cells expressed EGFP in the PaEGFP
group (Fig. 2). Nevertheless, either at the same
amount or at the same number of moles, significantly
lower proportions of viable cells were observed when
Kasumi-1 cells were nucleofected with pEGFP-N2
than when nucleofected with PaEGFP (Figs. 2, 3).
Furthermore, Kasumi-1 cells nucleofected with PaE-
GFP underwent proper cell proliferation (Fig. 4) and
displayed no loss of EGFP-expressing cells from 24 to
72 h post nucleofection (Fig. 2). These results clearly
indicated that PaEGFP induced less cell damage as
compared to pEGFP-N2 during Kasumi-1 cells nu-
cleofection. Since control *E. coli* null eluent had no
harmful effect, the toxicity of plasmid pEGFP-N2 was

probably attributable to the plasmid itself rather than
to endotoxin.

The Kasumi-1 cells nucleofected with pEGFP-N2
(2,000 ng) or with pGL3-Control (2,000 ng)
expressed the greatest amounts of protein as detected
by Western blot or luciferase assay (Fig. 5). It was
obvious that absolute amounts of protein were partly
from live cells and partly from ruptured cells. As we
observed in Fig. 3, the cell debris (as indicated by red
circles) from groups nucleofected with pEGFP-N2
also contained EGFP. If we only consider live cells
exclusively (Fig. 2a), the amount of protein from the
pEGFP-N2 groups is dwarfed since cells nucleofected
with plasmids underwent severe cell death. In other
words, on a live-cell basis, cells nucleofected with
PaEGFP or PaLuc expressed at least similar total
amounts of protein in the population of live cells when
compared to the cells nucleofected with pEGFP-N2
(2,000 ng) or pGL3-Control (2,000 ng).

So far, no recommended pulse condition or relevant
solution has been recommended by Amaxa for nucle-
ofection of Kasumi-1 cells. In one study, the authors
used program P-19 and solution V to nucleofect
Kasumi-1 cells (Corsello et al. 2009). Obviously, the
nucleofection parameters could be optimized further
in terms of cell viability and transfection efficiency

(Fig. 2). However, for the specific experiment in this study, the published nucleofection parameters were suitable for comparing performances between PaGene and its parental plasmid in terms of cell viability and transfection efficiency. The relatively low cell viability and adequate transfection efficiency guaranteed enough space improvement to allow monitoring the superior capacity of PaGene.

The basis for the differential between PaGene and plasmid nucleofection observed in this report might be attributed to, but not limited to, the bacterial plasmid backbone. The backbone itself, with its expressed antibiotic-resistance gene, the supercoiled DNA conformation, the larger size, or the DNA methylation of the bacterial plasmids etc. might be a lethal burden for cells made fragile by an electropulse. Ongoing work is needed for deciphering the exact mechanisms for the substantial cell death in plasmid nucleofection.

Besides Kasumi-1 cells, several other cells lines were also used for evaluation of PaEFP against pEGFP-N2. The cells nucleofected with PaEGFP instead of the plasmid always showed lower rates of cell death. However, superior transfection efficiencies were only seen with suspension cell lines rather than adhesive cell lines. For Kasumi-1 cells and probably other difficult-to-transfect suspension cells, after optimization of pulse conditions and solutions, utilization of PaGene might be a valuable new practical approach to nucleofection.

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