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**SHORT COMMUNICATION**

# An efficient transfection method for mouse embryonic stem cells

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*Embryonic stem (ES) cells are considered to have potentials for tissue regeneration and treatment of diverse human diseases. ES cells are capable of indefinite renewal and proliferation, which can be induced to differentiate into tissues of all three germ lines. Despite these exciting potential, it remains unclear as to how the renewal and differentiation programs are operated and regulated at the genetic level. Genetic manipulation such as delivery of exogenous gene expression or knockdown with small interfering RNA (siRNA) is commonly used in most of cancer or transformed cells but relatively rare in ES cells. In this study, we compare the transfection efficacies of several liposome-based transfection methods by introduction of a plasmid encoding enhanced green fluorescent protein (EGFP) into mouse ES (mES) cells. Our results show that transfection by Effectene achieves the efficiency of >98% in CCE and >80% in D3 cells. The optimal ratio of DNA:Effectene for EGFP transfection is between 1:4 and 1:8. Transient-expressed EGFP or endogenous protein kinase A (PKA) were significantly knocked down by Effectene transfection of specific siRNA. High EGFP level expression and accumulation in mES cells induces minor cytotoxicity but can be reduced by introducing siRNA of EGFP. Further, this transfection method did not significantly affect mES properties of proliferation or differentiation. Our results provide an optimal protocol to achieve an efficient transfection for mES cells.*

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Mouse embryonic stem (mES) cells derived from blastocyst-stage embryos are capable to proliferate with unlimited cell renewal and differentiate to all three germ layers including endoderm, ectoderm and mesoderm.[1,2] Due to its pluripotency, mES cells can provide the unlimited cell source for the study of regenerative medicine, developmental biology and cell-based gene therapy. Several nonviral gene delivery methods for mES cells such as liposome, electroporation and nucleofection have been developed to prevent the safety concern of viral vector based gene transfer. For example, a previous report of transfection efficiency for 20–70% using Effectene for mES cells has been described.[3] Further, it has been reported that 50–80% transfection efficiency could be achieved by lipofectamine in mES cells.[4] Recently, a reported study shows that nucleofection achieved an average of transfection efficiency at 63.66% whereas the percentage of electroporation is 6.41%.[5] The RNA interference approach has been developed as a powerful gene knockdown method to study the molecular and cell biology of eukaryotic system. Recently, it has been applied to the mES cells.[6–8] In this report, we describe a detail transfection protocol and DNA:Effectene ratio to show an extremely high transfection efficiency of mES cells. This transfection protocol achieves >98% of enhanced green fluorescent protein (EGFP) positive embryoid bodies (EB) and >90% of protein knockdown by introducing small interfering RNA (siRNA). We reported here an optimal liposome-based transfection method for delivery of genes or siRNA constructs into mES cells. To evaluate the gene delivery efficiency of liposome-based transfection, several liposomal reagents were compared for their transient transfection efficiency of mES cells. Among these transfection reagents, Effectene achieves higher transfection efficiency than the other liposomal reagents (Figure 1a). This significant result was confirmed by the western blot of EGFP protein (Figure 1b). We further determined the transfection efficiency of these reagents in D3 mES cells, which were maintained with the mouse embryonic fibroblasts as feeder cells. Both D3 and CCE cells were transfected with Effectene, Fugene 6 or Lipofectin, and transfection efficiency were examined by the percentage of EGFP positive EB. The transfection efficiency of Effectene for CCE and D3 cells were observed as >98 and 80%, respectively (Figure 1c). Relative low percentages of EGFP positive EB for Fugene 6 and Lipofectin were observed in both mES cells (Figure 1c). We next compared the transfection efficiency of Effectene with other gene transfer methods including electroporation, adenovirus (Ad) or adenovirus-associated virus (AAV).

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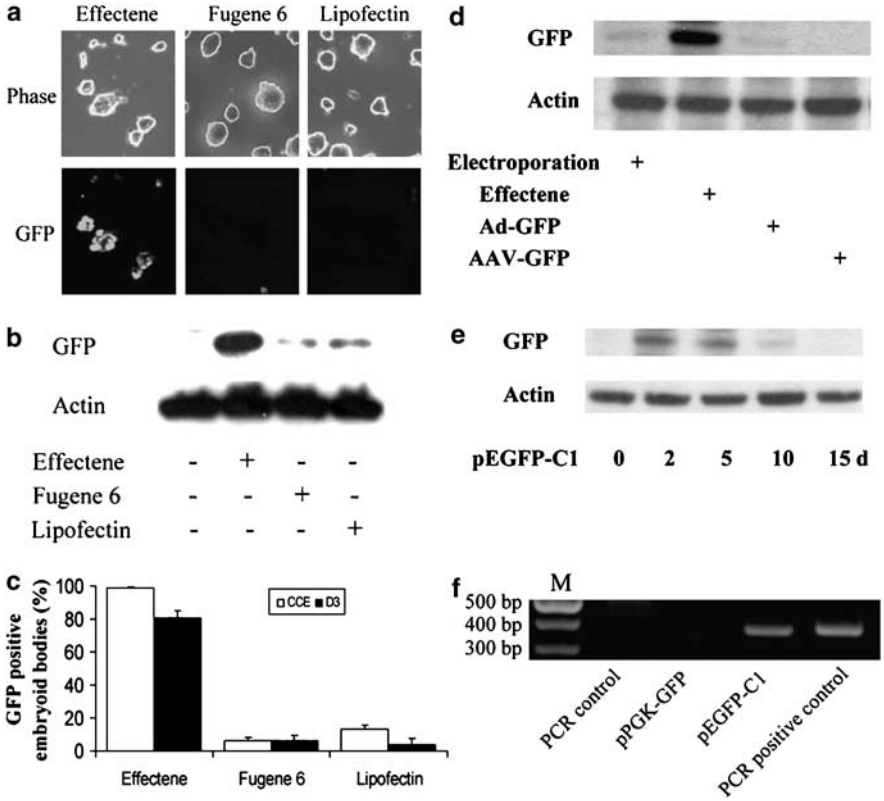


**Figure 1** Efficiency of liposome-based transfection methods. CCE mouse embryonic stem (mES) cells were obtained from StemCell Technologies (Vancouver, BC, Canada) with permission from Dr Robertson and Dr Keller.[9,10] Plastic tissue culture dishes were pretreated with 0.2% gelatin. Undifferentiated CCE mES cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing glutamine, sodium pyruvate, nonessential amino acids, β-mercaptoethanol, leukemia inhibitory factor (LIF, 1500 U/ml), 15% fetal bovine serum (FBS) and penicillin/streptomycin. CCE mES cells were transfected with a cytomegalovirus (CMV) promoter-driven enhanced green fluorescent protein (EGFP) expression vector pEGFP-C1 by Effectene, Fugene 6 and Lipofectin. For the transfection of Effectene, 1 μg pEGFP-C1 vector was suspended in 100 μl EC buffer followed by adding 3.2 μl enhancer and incubated at room temperature for 10 min. 8 μl of Effectene reagent was then added and incubated at room temperature for another 15 min. For Fugene 6 and Lipofectin transfection, 1 μg DNA of pEGFP-C1 was mixed with 3 μl of Fugene 6 or Lipofectin reagents in serum-free medium and incubated at room temperature for 15 min. CCE cells were washed with phosphate-buffered saline (PBS), trypsinized and centrifuged at 1000 r.p.m. for 5 min. Cell pellets were resuspended in complete medium and $2 \times 10^5$ CCE cells were incubated with each transfection mixture of pEGFP-C1 with Effectene, Fugene 6 or Lipofectin reagents and moved the a six-well culture plate. CCE cells formed embryoid bodies (EB) at 48 h and the transfection efficiency was determined by the percentage of EGFP positive EB via fluorescent microscope. (**a**) CCE cells transfected with pEGFP-C1 by Effectene, Fugene 6 or Lipofectin for 48 h. EGFP expression was examined by fluorescent microscope. (**b**) Protein level of EGFP expression was determined by western blotting. (**c**) CCE and D3 cells transfected with pEGFP-C1 by Effectene for 48 h. Transfection efficiency was determined by the percentage of EGFP positive EB. Each bar represents mean ± s.d. ($n = 3$). (**d**) CCE cells were transfected with pEGFP-C1 by electroporation, Effectene, Ad-GFP (20 multiplicity of infection) or AAV-GFP (20 multiplicity of infection) for 48 h. EGFP expression was determined by western blotting. (**e**) CCE cells transfected with pEGFP-C1 by Effectene and harvested at indicated time. EGFP expression was determined by western blotting. These western blots were representative of three independent experiments. (**f**) CCE cells were transfected with a transient vector (pPGK-GFP) or pEGFP-C1 by Effectene for 48 h. Cells were washed with PBS and incubated overnight in digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS and 0.1 mg/ml proteinase K). Genomic DNA was further extracted with phenol/chloroform/isoamylalcohol (25:24:1) followed by precipitation with 100% alcohol and resuspended in TE buffer. The extracted genomic DNA was served as templates for PCR. A forward primer (CATGGTCCTGCTGGAGTTCGTG) on CMV promoter region and a reverse primer on EGFP (TTTGTAACCATTATAAGCTGC) were employed for PCR reaction. An expected size of 382 bp PCR product was separated with 2% agarose gel. 20 ng of pPGK-GFP plasmid was used as template for PCR positive control. M denotes DNA marker.

CCE cells were electroporated with pEGFP-C1 or transfected with Ad-GFP and AAV-GFP at 20 multiplicity of infection for 48 h. Transfection efficiency were examined by western blotting and the result indicated that GFP expression levels are Effectene > electroporation > Ad-GFP > AAV-GFP (Figure 1d). We have further observed the expression of EGFP in CCE cells maximally at 48 h, and this could be detectable at 5–10 days but gradually lose its signal after 15 days (Figure 1e). Moreover, we have checked whether this transfection protocol enhanced DNA integration of the delivered gene. CCE cells transfected with a transient vector carrying EGFP (pPGK-GFP) or pEGFP-C1 stable vector and whole genomic DNA were extracted. Extracted genomic DNA (100 ng) was served as templates of PCR. A pair of specific primers for GFP was employed for PCR amplification. As show in Figure 1f, we did not observe any integration at the time point of 48 h when transfected with a transient vector pPGK-GFP but transfection of pEGFP-C1 show significant integration of genome. This result of genomic integration may due to pEGFP-C1 is a transposon (Tn5)-based stable expression vector which carrying a cassette of Neomycin resistant gene, therefore induced genomic integration of EGFP. To

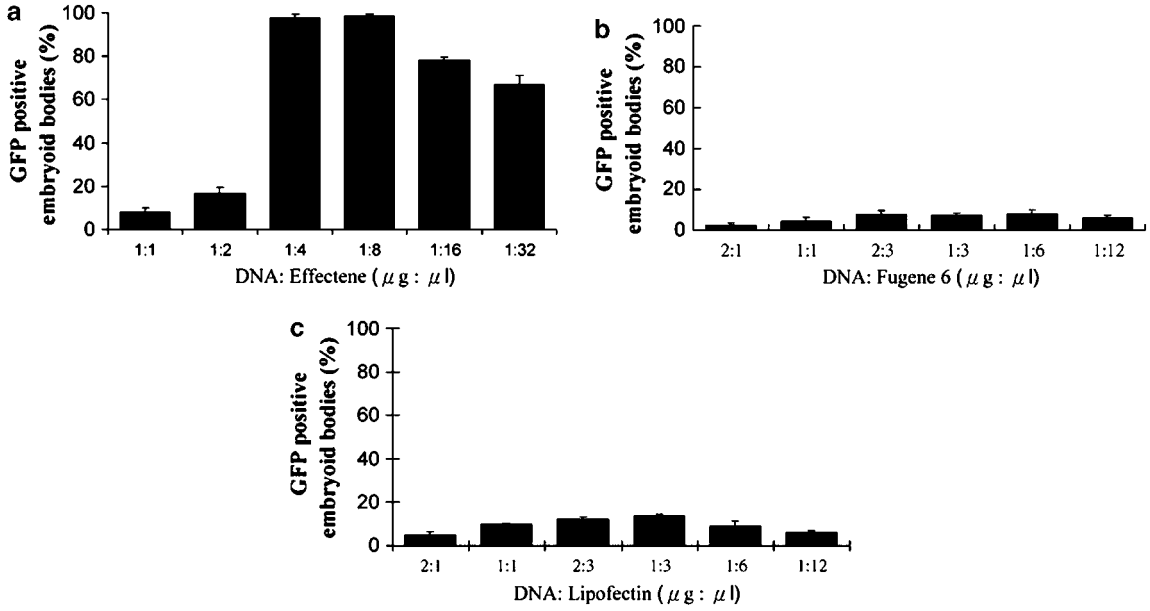


**Figure 2** The optimal ratio of DNA/liposomal reagents for transfection. CCE cells were transfected with different ratios of DNA with transfection reagents (**a**, Effectene; **b**, Fugene 6; **c**, Lipofectin) as indicated for 48 h and the transfection efficiency was examined by the percentage of enhanced green fluorescent protein (EGFP) positive embryoid bodies (EB). Each bar represents mean ± s.d. ($n = 3$).

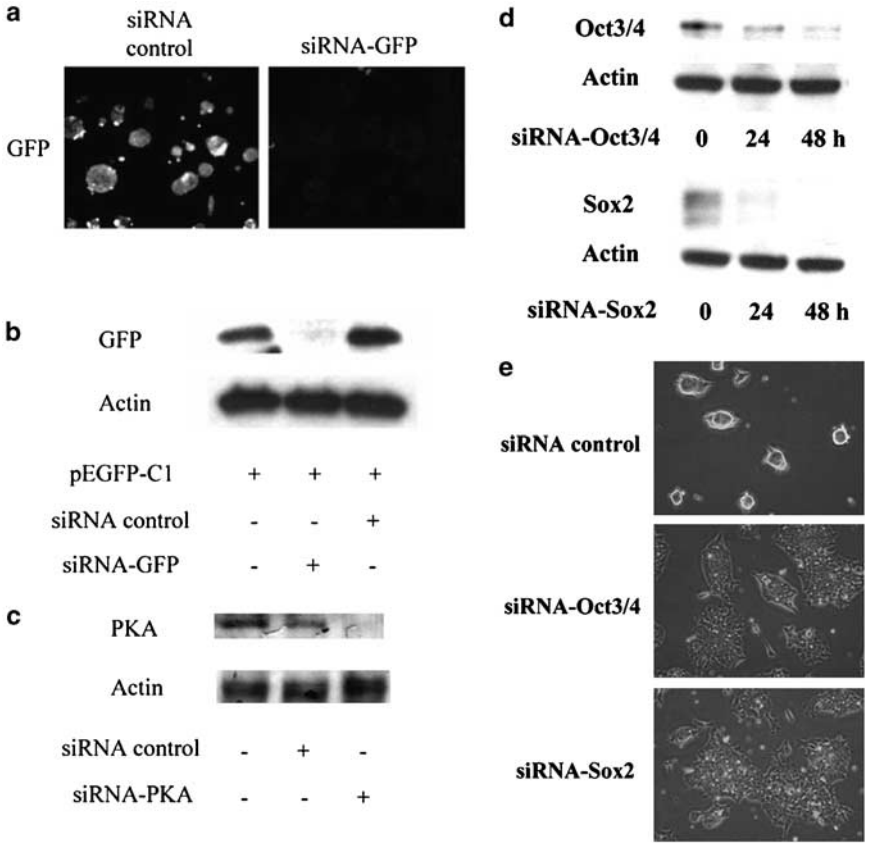


**Figure 3** Efficient knockdown gene expression by introducing small interfering RNA (siRNA). (**a**, **b**) Enhanced green fluorescent protein (EGFP) siRNA (targeted sequences: 5′-GGAGCGCACCATCTTCTTC-3′) was constructed to a pSuper-base base vector. CCE cells were cotransfected by Effectene with pEGFP-C1 plus EGFP siRNA or its control pSuper vector and incubated for 48 h. The EGFP expression level was examined by fluorescent microscope (**a**) and western blot analysis (**b**). (**c**) Protein kinase A (PKA) siRNA (SureSilencing shRNA plasmids, SuperArray, targeted sequences of 5′-CGTCCTGACCTTTGAGTATCT-3′), (**d**, **e**) Oct3/4 and Sox2 siRNA (purchased from Santa Cruz, Santa Cruz, CA, USA) were transfected into CCE cells by Effectene as described above. After 48 h, the transfected cells were harvested and the protein levels of PKA, Oct3/4 and Sox2 were determined by western blotting analysis. These western blots were representative of three independent experiments. (**e**) Representative CCE cell differentiation was demonstrated by phase microscopy.

investigate the optimal transfection condition, we compared the transfection efficiency of various ratios of DNA/transfection reagents. The best transfection efficiency was achieved at the ratio of DNA:Effectene at 1:4 and 1:8 with which >95% EGFP positive EB was observed. The transfection efficiency was decreased to about 78 and 70% when the ratio was increased up to 1:16 and 1:32, respectively (Figure 2a). Very low percentage of EGFP positive EB was observed when the ratio of DNA:Effectene was down to 1:1 and 1:2 (Figure 2a). In contrast, low percentage of EGFP positive EB was measured in both Fugene 6 and Lipofectin transfected CCE cells (Figure 2b and c). To confirm this optimal condition for gene delivery efficiency, we employed this optimal protocol to knockdown gene expression by introducing siRNA into mES cells. As shown in Figure 3a, EGFP expression level and the percentage of EGFP positive EB was markedly reduced in EGFP-siRNA transfected CCE cells. This result was further confirmed by the western blot analysis (Figure 3b). We next determined the suppressing effect of the endogenous protein kinase A (PKA) by introducing its siRNA vector. Endogenous PKA expression level was significantly suppressed to less than 10% of control cells in PKA-siRNA transfected CCE cells (Figure 3c). These results indicate that this optimized protocol is effective to knockdown genes expression by transfection of siRNA for both exogenous and endogenous protein expression. To further demonstrate the importance of this application, we transfected siRNA of Oct3/4 and Sox-2, which were reported to be important for maintaining cell renewal and preventing cell differentiation in mES cells. Our results indicated that introducing these siRNA reduced Oct3/4 and Sox-2 expressions (Figure 3d) and significantly induced CCE cell differentiation (Figure 3e). As mES cells possess the properties and potential to develop to different type of cells, it is important to maintain the pluripotent properties during manipulations for gene delivery. Therefore, we examined the cytotoxicity of these different liposomal transfection methods. CCE cells were transfected with EGFP, PKA-siRNA or control vector and cytotoxicity was determined by trypan blue stain. Neither blank nor transfection of siRNA-PKA showed significant cytotoxicity but high expression of EGFP increased the percentage of cell death (Figure 4a). Although EGFP was suggested to have low cytotoxicity for mammalian cells, a number of literatures reported that high expression of EGFP induced cell damage both *in vitro* and *in vivo*.[11–13] To investigate whether the cytotoxicity is due to the transfection procedure or the accumulation of EGFP, CCE cells were cotransfected with pEGFP-C1 and EGFP-siRNA. Cytotoxicity was obviously reduced by cotransfection of pEGFP-C1 with EGFP-siRNA, which suggests high level of EGFP accumulation but not transfection protocol induces mES cell damage (Figure 4b). Moreover, it is important to evaluate whether this transfection protocol affects mES cell differentiation and proliferation. To investigate these issues, CCE cells were transfected and percentage of EB formation or cell numbers were determined by phase microscopy. Transfection with Effectene, Lipofectin or Fugene 6 did not significantly affect percentage of EB formation (Figure 5a). Furthermore, cell numbers were not changed by transfection by Effectene protocol (Figure 5b), implying this liposomal base transfection reagents do not alter mES cell differentiation and proliferation. Taken together, we conclude that DNA:Effectene ratio of 1:4 or 1:8 is the most optimal condition for transient transfection of mES cells.

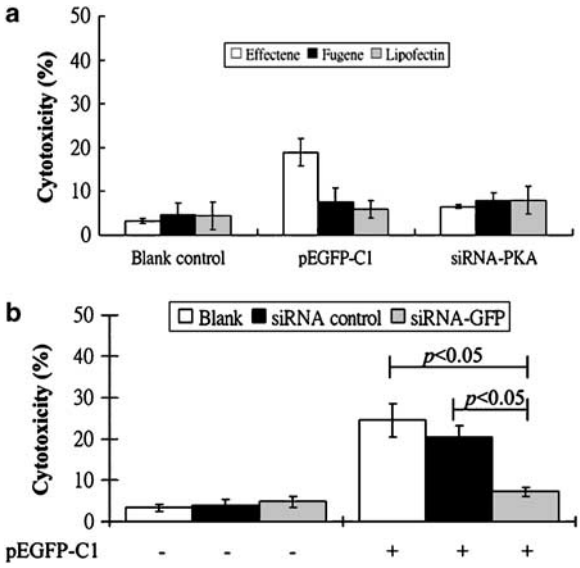


**Figure 4** The cytotoxicity analysis of transfection. (**a**) CCE cells transfected with blank control, pEGFP-C1 and siRNA-PKA by different transfection methods for 48 h. (**b**) CCE cells were cotransfected with pEGFP-C1 plus siRNA-EGFP or its control vector for 48 h. Percentage of cytotoxicity was determined by trypan blue staining. Each bar represents mean ± s.d. ($n = 3$). Analysis of variance was used to determine statistical differences of cytotoxicity between groups. A $P < 0.05$ was considered to be statistically significant.

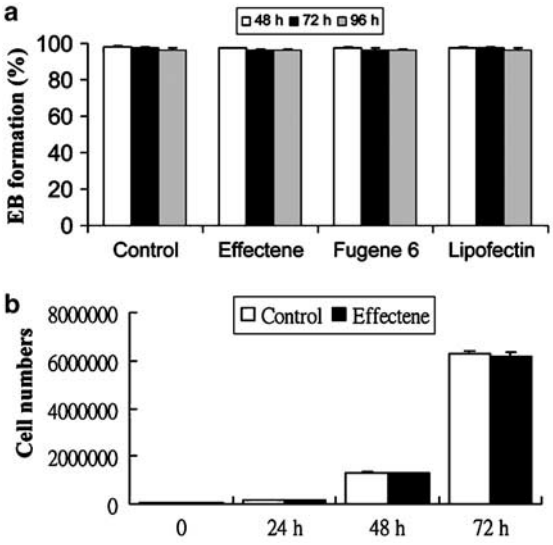


**Figure 5** The differentiation and proliferation analysis of transfection. (**a**) CCE cells were transfected with Effectene, Fugene 6 or Lipofectin for 48 h and percentage of embryoid bodies (EB) formation was examined by phase microscopy. (**b**) Cell numbers of transfected CCE cells were determined by phase microscopy from 0–72 h. Each bar represents mean ± s.d. ($n = 3$).

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