

Comparison of Ectopic Gene Expression Methods in Rat Neural Stem Cells

Woosuk Kim*, Ji Hyeon Kim*, Sun-Young Kong, Min-Hye Park, Uy Dong Sohn, and Hyun-Jung Kim

Laboratory of Stem Cell and Molecular Pharmacology, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea

Neural stem cells (NSCs) have the ability to proliferate and differentiate into various types of cells that compose the nervous system. To study functions of genes in stem cell biology, genes or siRNAs need to be transfected. However, it is difficult to transfect ectopic genes into NSCs. Thus to identify the suitable method to achieve high transfection efficiency, we compared lipid transfection, electroporation, nucleofection and retroviral transduction. Among the methods that we tested, we found that nucleofection and retroviral transduction showed significantly increased transfection efficiency. In addition, with retroviral transduction of Ngn2 that is known to induce neurogenesis in various types of cells, we observed facilitated final cell division in rat NSCs. These data suggest that nucleofection and retroviral transduction provide high efficiency of gene delivery system to study functions of genes in rat NSCs.

Key Words: Electroporation, Lipid-Mediated transfection, Neural stem cells, Nucleofection, Retrovirus

INTRODUCTION

Stem cells are promising sources for cell therapy and drug screening because of their regeneration potential and pluri- or multi-potency [1-5]. Due to the therapeutic potential of stem cells, many researchers are intensively studying stem cells. To manipulate stem cells to express foreign genes to study the role of genes in stem cell biology, it is necessary to achieve high gene transduction efficiencies. Up-regulation or down-regulation of genes provides information about their roles in stem cell fate determination and is important to seek for the essential functions of genes [6-8]. However, stem cells are hard to be transfected with ectopic genes [9-13]. Among many types of stem cells, neural stem cells (NSCs) are multipotent cells that can proliferate and can give rise to different types of cells consisting of the nervous system such as neurons, astrocytes and oligodendrocytes [14]. Since NSCs can only differentiate into cells that compose the nervous system, they have been considered safe to be transplanted into body [3,15]. In many cases, NSCs have been transplanted into neurodegenerative conditions to replace degenerated neurons [2,3,16,17].

In order to elucidate the molecular mechanisms controlling NSCs fate, it is critical to achieve efficient gene delivery [18].

It has been previously reported that basic helix-loop-helix (bHLH) protein family including Ngn and ASCL1 play important roles in the generation of neurons [19,20]. During the development of the nervous system, generation of various types of neurons is temporally and spatially controlled. Neuronal differentiation may occur through direct transcriptional regulation by bHLH protein family [21-23]. To identify the functions of bHLH protein in stem cell, efficient gene delivery method has to be determined. Because NSCs are one of the hard-to-transfect cell types, we compared lipid mediated transfection, electroporation, nucleofection and viral transduction in NSCs derived from fetal rat cortex by using plasmid that encode green fluorescence protein (GFP). Furthermore, we observed Ngn2 introduced stem cell behavior using real time microscope and showed that over-expression of Ngn2, a bHLH protein family, leads to an increased final cell division. The development of an efficient gene delivery method in NSCs may be useful in determining functions of genes in stem cells and used to develop stem cell based drug screening system.

Received October 16, 2012, Revised November 19, 2012,
Accepted November 29, 2012

Corresponding to: Hyun-Jung Kim, Laboratory of Stem Cell and Molecular Pharmacology, College of Pharmacy, Chung-Ang University, 221, Heukseok-dong, Dongjak-gu, Seoul 156-756, Korea. (Tel) 82-2-820-5619, (Fax) 82-2-815-5619, (E-mail) hyunjungkim@cau.ac.kr
*These authors contributed equally to this work.



This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

ABBREVIATIONS: NSC, neural stem cell; bHLH, basic helix-loop-helix; GFP, green fluorescent protein; SD, Sprague Dawley; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PDL, poly-d-lysine; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole; ESC, embryonic stem cell; HSC, hematopoietic stem cell; AAV, adeno-associated virus; SCF, stem cell factor.

METHODS

Rat NSC cultures

The cortex of E14 Sprague Dawley (SD) rat brain was isolated and dissociated with accutase (Millipore, USA). Starting from 200,000/ml density, cells were expanded as neurospheres in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Invitrogen, USA) containing 1% antibiotic-antimycotic (v/v) (Invitrogen, USA), 2% B27 (v/v) (Invitrogen, USA), 20 ng/ml epidermal growth factor (EGF, Millipore, USA) and 20 ng/ml basic fibroblast growth factor (bFGF, Millipore, USA). The medium was conditionally changed every other day by removing half the media and adding fresh media containing 2% B27 and 20 ng/ml EGF and bFGF. After 1 week, cells were dissociated with accutase and used for lipid-mediated transfection, electroporation, nucleofection, and retrovirus transduction.

Lipid-mediated transfection

Lipid-mediated transfection was performed using X-tremeGENE 9 DNA Transfection Reagent (Roche, Germany) according to the manufacturer's protocol. After plating 6×10^4 cells at the poly-d-lysine (PDL, Sigma-Aldrich, USA) and laminin (Invitrogen, USA) coated microscope cover glasses (Superior, Germany) in 24 well plates (Nunc, Germany), NSCs were cultured for 1 day to 50~60% confluency and washed twice with DMEM/F12 and then the media was changed to Opti-MEM (Invitrogen, USA). Each well was added with 250 ng retroviral GFP vector, 0.75 μ l X-tremeGENE 9 DNA Transfection Reagent and 25 μ l Opti-MEM mixture. After 8 hours incubation, cells were washed twice with DMEM/F12 and then medium was changed to proliferation media containing 2% B27 and 20 ng/ml EGF and bFGF. Two days after the transfection, cells were fixed for immunocytochemistry.

Electroporation

Electroporation was performed using NEPA21 electroporator (Nepagene, Japan) according to the manufacturer's recommendation. 1×10^6 dissociated rat NSCs were centrifuged at 90 g for 10 minutes at room temperature and resuspended in 100 μ l mixture of Opti-MEM and 10 μ g retroviral GFP vector. The mixture received two types of pulses, poring pulse and transfer pulse. Poring pulse condition was as below: 150 V, 5 milliseconds (ms) pulse length, total two pulses, 50 ms interval between the pulses, 10% decay (D). Rate with + polarity. The transfer pulse condition was as below: 20 V, 50 ms pulse length, total five pulses, 50 ms interval among the pulses, 40% D. Rate with +/- polarity. After the electroporation, cells were immediately seeded onto PDL and laminin coated microscope cover glasses in 24 well plates. Two days after the electroporation, cells were fixed for immunocytochemistry.

Nucleofection

Nucleofection was performed using Amaxa 4D nucleofector (Lonza, Switzerland) using P3 and P4 Primary Cell 4D-Nucleofector[®] X Kit L (Lonza, Switzerland) according to the manufacturer's protocol. 5×10^6 dissociated rat NSCs were centrifuged at 90 g for 10 minutes at room temperature and resuspended in 100 μ l nucleofector solution. 5

μ g of retroviral GFP vector was added and the mixture was transferred to 100 μ l Single Nucleocuvette. After the nucleofection, cells were immediately seeded onto PDL and laminin coated microscope cover glasses in 24 well plates. Two days after the nucleofection, cells were fixed for immunocytochemistry.

Retrovirus preparation and transduction

Retroviral GFP vector and retroviral Ngn2 vector were used to generate retroviruses. PLAT-E cells transduced with gag-pol and env were used for the packaging of viruses. Briefly, PLAT-E cells were transfected with retroviral GFP vector or Ngn2 vector using X-tremeGENE 9 following manufacturer's instruction. Supernatant containing viral particles was collected 2 days after transfection and centrifuged at 12,500 g for 12 hours to concentrate retroviruses. NSCs were plated at the PDL/laminin-coated microscope cover glasses in 24 well plates and cultured for 1 day to 50~60% confluency, and then NSCs were exposed to retroviruses for 6 hours in the presence of polybrene (4 μ g/ml, Sigma-Aldrich, USA). After washing twice with DMEM/ F12, cells were cultured in the presence of bFGF and EGF for 2 days. Then NSCs were fixed for immunocytochemistry.

Immunocytochemistry and cell counting

NSCs were fixed for 30 minutes with 4% paraformaldehyde (Affymetrix, USA) and rinsed twice with phosphate-buffered saline (PBS). Fixed cells were blocked for 30 minutes in PBS containing 5% normal goat serum (Millipore, USA) and 0.2% triton X-100 (Amresco, USA) and incubated 1 hour with anti-GFP primary antibody (1 : 500, Molecular Probes, USA). Following rinsing with PBS, cells were incubated with Alexa Fluor 488-conjugated secondary antibody (1 : 1,000, Molecular Probes, USA). Following rinsing with PBS, cell nuclei were stained by 4'-6-Diamidino-2-phenylindole (DAPI) (0.5 μ g/ml in PBS, Sigma-Aldrich, USA) to count the total cell number. The images were obtained with an inverse fluorescence microscopy (DMIL; Leica, Germany). Cell counting was performed in 3 randomly chosen microscopic fields. The ratio of GFP-positive cells to total cells was calculated to draw transfection or transduction efficiency.

Time-lapse analysis

Inverted Nikon microscope (Nikon, Japan) was used to capture images for GFP-positive cells. Fluorescence images were acquired every 4 minutes to trace behaviors of the GFP-positive cells. Cells were kept in humidified, 37°C temperature control chamber with 4~6% CO₂ during the image recording. Acquired images were assembled into movies using Metamorph imaging system (Molecular Devices, USA). The movies were used to evaluate the behavior and cell division of individual cells.

Statistics

Differences between groups were tested for significance using Student's *t*-test and considered to be statistically significant if $p < 0.05$. Quantitative data were expressed as mean \pm S.D.

RESULTS

Lipid-mediated transfection showed low transfection efficiency in NSCs

Cell therapy using stem cells and understanding the mechanisms how stem cells proliferate and differentiate require safe, efficient and stable delivery of foreign genes without making any harmful effect and changing essential properties. Lipid-mediated transfection is one of the most widely used gene delivery methods. To explore the efficiency of lipid mediated transfection in rat NSCs, we used retroviral vector encoding GFP. Cortical NSCs were cultured in the presence of EGF and bFGF for 1 week, then dissociated and plated onto PDL and laminin coated coverslips. One day after plating, NSCs were transfected with X-tremeGENE9 alone or with X-tremeGENE 9+retroviral GFP vector. Two days after transfection, NSCs were fixed and immunostained with anti-GFP antibody to determine the transfection efficiency. To count the total cell number, nuclei were stained with DAPI. As shown in Fig. 1, the efficiency of lipid-mediated transfection in rat NSCs was only around 4.48%. These data suggest that lipid mediated transfection is not effective in NSCs for ectopic expression of foreign genes.

Nucleofection and retrovirus transduction showed better transfection efficiency

Since lipid-mediated transfection efficiency was only around 3~4% (Fig. 1B, 1C and 2A), we next decided to compare

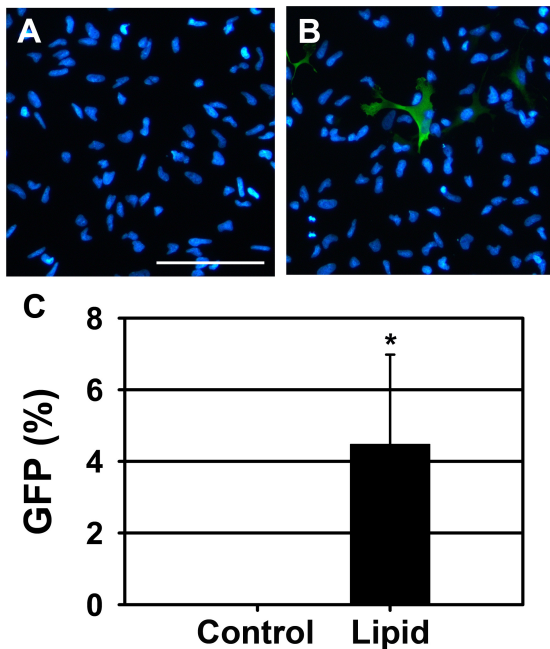


Fig. 1. Transfection efficiency of rat NSCs 48 hours after lipid-mediated transfection. (A, B) Representative merged images of NSCs transfected with (A) lipid alone and (B) lipid with GFP vector. GFP was visualized by immunostaining with anti-GFP primary antibody and Alexa Fluor 488-conjugated secondary antibody (green). Nuclei were visualized by DAPI staining (blue). Scale bar=100 μ m. (C) Quantification of the immunostaining data. The ratio of GFP-positive cells to the total cells was calculated and presented. The data are shown as mean \pm S.D. (* p <0.05 vs. negative control).

transfection efficiencies of electroporation, nucleofection and retrovirus transduction in NSCs. NSCs were expanded as neurospheres for a week in the presence of EGF and bFGF. Then NSCs were dissociated to make single cell suspension and directly used for electroporation or nucleofection. In the electroporation experiments using NEPA21 electroporator, 1×10^6 cells were mixed with 10 μ g of retroviral GFP vector in 100 μ l Opti-MEM and then electroporated using following conditions. NSCs and GFP vector were mixed and electroporated twice with 150 V pulse for 5 ms and between the pulses 50 ms interval was given (this is to make pores in the NSCs). Then, additional five times of 20 V pulse for

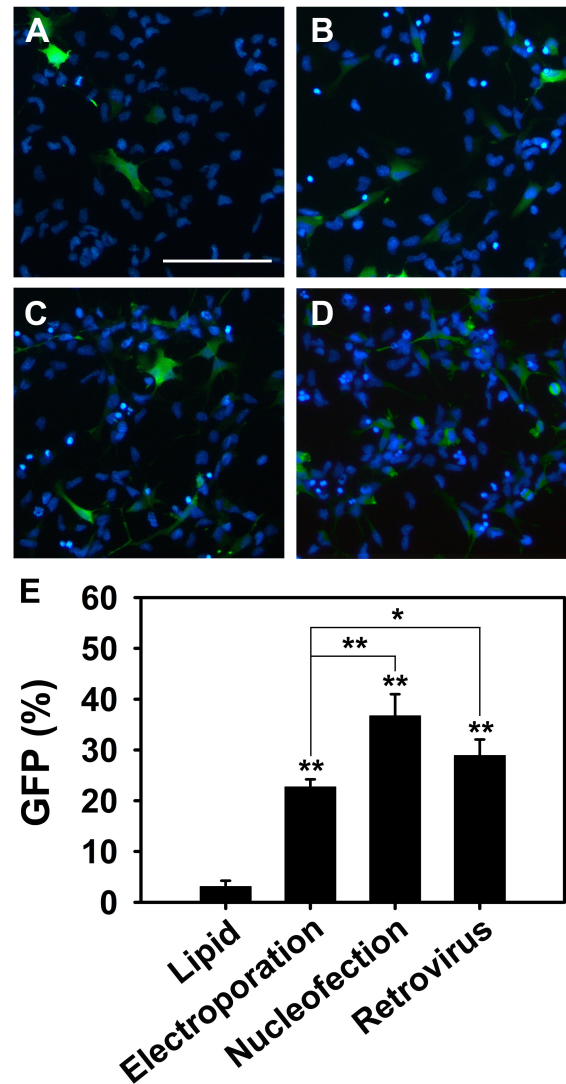


Fig. 2. Comparison of transfection efficiency of lipid-mediated transfection, electroporation, nucleofection and retroviral transduction. (A~D) Representative merged images of NSCs transfected with GFP vector for 48 hours using (A) X-tremeGENE9 transfection reagent, (B) NEPA21 electroporator, (C) Amaxa 4D Nucleofector, and (D) retroviral transduction. By immunostaining GFP positive cells are shown in green and nuclei are shown in blue. Scale bar=100 μ m. (E) Quantification data of the transfected cells. The ratio of GFP-positive cells to the total cells was calculated. Quantitative data are shown as mean \pm S.D. (** p <0.01, * p <0.05).

50 ms with 50 ms intervals among the pulses (this is to deliver GFP vector to pored NSCs). After the electroporation, cells were directly transferred to pre-warmed medium containing EGF, bFGF and 2 $\mu\text{g/ml}$ laminin and grown as monolayer for 2 days. Then NSCs were fixed and immunostained with anti GFP antibody. Using electroporation, the transfection efficiency was improved to $22.72\pm 1.48\%$ (Fig. 2B and 2E) which was seven times higher than lipid-mediated transfection.

Nucleofection is also an electroporation-based technique, however, it aims to transfer DNA or RNA directly into the cell nucleus. To transfect NSCs by nucleofection, 5×10^6 cells were resuspended in 100 μl mixture of solution provided by the manufacturer (P4 Primary Cell 4D-Nucleofector[®] X Kit) and 5 μg of retroviral GFP vector DNA was added. After the nucleofection using CA137, CL133, CU110, DC100, DC104, DR114 and EM110 programs, cells were plated into 24 well plates and grown as monolayer for additional 2 days in serum free media with EGF and bFGF. Among the 7 programs, CA137 showed the highest transfection efficiency ($36.71\pm 4.29\%$) in rat NSCs (Fig. 2C and 2E).

To explore gene delivery efficiency of retroviral transduction, NSCs were dissociated and 6×10^4 cells were plated onto PDL and laminin coated 24 well plates. One day after plating, NSCs were transduced with retroviruses (1 MOI) encoding the GFP and cultured in media with EGF and bFGF. Two days after retroviral transduction, NSCs were fixed and immunostained with anti-GFP antibody. Around 30% ($28.90\pm 3.16\%$) of total cells were expressing GFP (Fig. 2D). These data show that electroporation, nucleofection and retroviral transduction are more effective to introduce ectopic genes to NSCs than lipid-mediated transfection. In addition, among the conditions that we tested, nucleofection and retroviral transduction significantly increased the numbers of transfected NSCs compared to electroporation.

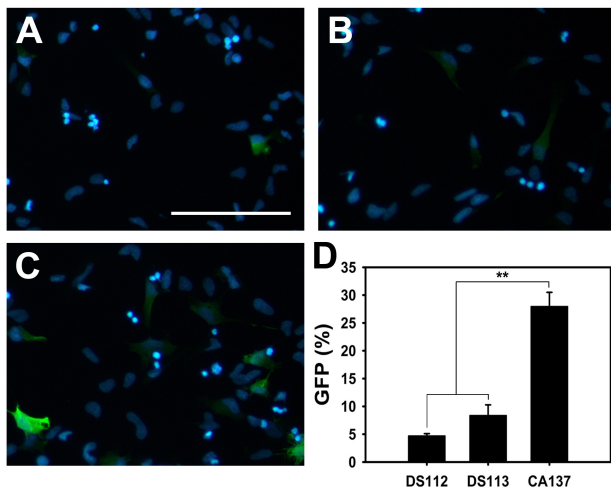


Fig. 3. Comparison of transfection efficiency of three programs -DS112, DS113 and CA137- of nucleofection. (A~C) Representative merged images of NSCs nucleofected using program (A), (B) DS113 and (C) CA137 for 48 hours. GFP positive cells are shown in green and DAPI stained nuclei are shown in blue. Scale bar=100 μm . (D) Quantification of the transfected NSCs. The ratio of GFP-positive cells to the total cells was calculated and presented. Quantitative data are shown as mean \pm S.D. (** $p<0.01$).

Recently, Bertram and colleagues reported that mouse NSCs showed more than 80% transfection efficiency when they used DS112 and DS113 nucleofection programs and P3 primary cell 4D-nucleofector[®] X kit solution [18]. Therefore, we compared transfection efficiency of DS112, DS113 using P3 primary cell 4D-nucleofector[®] X kit solution and CA137 using P4 primary cell 4D-nucleofector[®] X kit solution in rat NSCs. Surprisingly, DS112 ($4.71\pm 0.39\%$) and DS113 ($8.38\pm 1.91\%$) showed far lower transfection efficiency than CA137 ($27.96\pm 2.53\%$) (Fig. 3). These results indicate that for rat NSCs transfection, using CA137 program with P4 solution is more efficient than using DS112 and DS113 with P3 solution.

Ectopic expression of Ngn2 facilitates final cell division

Our data showed that electroporation, nucleofection and retroviral transduction provides above 20% gene delivery in NSCs. To explore transfected gene functions in NSCs, we next overexpressed Ngn2 that are known to be involved in neurogenesis [24]. After retroviral transduction of Ngn2 in NSCs, time-lapse analysis revealed enhanced cell division. About 8 hours after viral transduction, NSCs started to express ectopic genes that are visualized by GFP. Time-lapse microscopy data showed 1.3 times higher cell division in NSCs expressing Ngn2 than those expressing GFP alone (Fig. 4). These data suggest that Ngn2 promotes final cell division before differentiate into neuron. Our data prove the concept that ectopic genes function in NSCs using retroviral transduction.

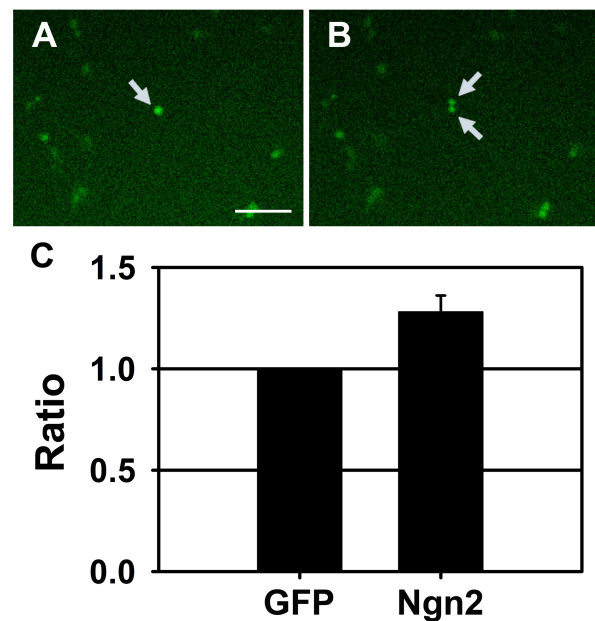


Fig. 4. Induction of final cell divisions by Ngn2. (A, B) Representative fluorescence time-lapse microscopic images of NSCs transduced with Ngn2 retrovirus. (A) Arrow-pointed cell rounded up and divided into (B) two daughter cells. Scale bar=100 μm (C) Quantification of cell division observed in NSCs transduced with control GFP retrovirus or Ngn2 retrovirus. Time-lapse video was recorded for about 24 hours. The ratio of dividing GFP-positive cells to the total GFP-positive cells was calculated and presented. Quantitative data are shown as mean \pm S.D.

DISCUSSION

Until recently, ectopic genes were introduced into various types of mammalian cells by the methods using calcium phosphate, polycations and liposome [25]. Among those, lipid-mediated transfection has been most frequently used for overexpression in various cell types [26,27]. However, some of cells including embryonic stem cells (ESCs) and NSCs are not effectively transfected by lipid mediated methods [9,11]. It is reported that with lipofection, about 10.7% of transfection efficiency has been achieved [10,12,13]. However, our study with lipid mediated transfection, we could only achieve around 3~4% of transfection efficiency. If we consider the fact that after NSCs differentiation, different types of cells including neurons, astrocytes, oligodendrocytes and undifferentiated NSCs exist in a culture well, with such low efficiency, it is hard to determine the effects of transgenes. Thus several alternative methods have been addressed to enhance ectopic gene expression. Viral transduction using retrovirus, lentivirus or adenovirus, electroporation and nucleofection provide better efficiencies [12, 18,28-33].

In this study, when compared with lipid-mediated transfection, electroporation or nucleofection significantly improved transfection efficiency. With both methods, we could observe around 20~30% of GFP positive cells. Electroporation is widely used for *in vitro* and *in vivo* transfection and utilizes high voltage to permeabilize cell membrane to transport DNA into the cells [29-31,33]. Nucleofection is an electroporation-based technology and has been shown to introduce ectopic genes in various types of cells including stem cells and primary cells [32,34-36]. Nucleofection is preferentially used to deliver ectopic genes into hard-to-transfect stem cells such as ESCs, mesenchymal stem cells, hematopoietic stem cells (HSCs) and NSCs due to high gene transfer efficiency [11,12,18,28,37-39]. Nucleofection showed better transfection efficiency over electroporation in human ESCs (hESCs) and rodent NSCs, probably because DNA was able to reach not only in the cytoplasm but also in the nucleus [12]. Similar to this, in our experiment, nucleofection showed higher transfection efficiency than electroporation. However, it is still possible that the optimal conditions for electroporation have not been determined yet. A recent study by Bertram and colleagues showed 88% transfection efficiency in mouse fetal cortical NSCs using amaxa 4D nucleofector program DS113 [18]. However, using the same protocol, we could only detect $4.71 \pm 0.39\%$ of transfected rat NSCs with DS112, and $8.38 \pm 1.91\%$ with DS113. In addition, the program CA137 was three times more efficient for the transfection of rat NSCs. The difference between our data and theirs could be from the different origin of NSCs (mouse or rat), or different GFP expression vectors that were used to trace transfected cells.

Viral transduction is another method to introduce foreign genes into cells [40-43]. In our experiments, gene delivery efficiency using retrovirus was comparable to that of nucleofection (around 30%). Although the transfection efficiency can be improved by using high titered virus, with 1MOI virus, we usually achieved around 30% transduction. With retrovirus transduction ectopic gene functions have been identified in NSCs [24,44]. We showed that the overexpression of bHLH proteins using retroviral transduction significantly induced neurogenesis in NSCs [24]. Lu and colleagues also showed that genetic modification with neurotrophin-3 using retroviruses promoted the survival, pro-

liferation, neuronal differentiation and elongation of neurites in human NSCs [44]. It has been previously reported higher efficiencies can be achieved using other types of virus in different types of cells [1,45-47]. With adeno-associated virus (AAV), it has been reported that up to 50% of hESCs can be transfected [47]. Similarly, adenovirus mediated transfection also showed about 50% of efficiency in adult rat subventricular zone-derived NSCs [46]. With lentivirus, the transfection efficiency goes up much higher, almost 80% of human and rat NSCs can express transgenes [1,45]. Because only cells that are replicating at the time of infection can be transduced by retrovirus, transduction using retrovirus shows lower transduction efficiency compared to AAV, adenovirus or lentivirus [48,49]. Retroviral transduction requires breakdown of the nuclear envelope that occurs during mitosis [49-51]. In contrast, AAV, adenovirus and lentivirus can infect terminally differentiated, non-dividing cells as well as dividing cells [52,53]. However, one of the advantage of using retrovirus is that since they only infect cells that are dividing at the time of transduction, terminally differentiated cells can be excluded and only multipotent or pluripotent stem cells can be marked by the transgene.

To improve retroviral transduction efficiency, supplement of growth factor has been used to boost up the mitosis. It is reported that the use of stem cell factor (SCF) in combination with interleukin-6 in murine HSCs improved retroviral transduction efficiency [54]. Granulocyte-colony stimulating factor/SCF or Flk-2/Flt3 ligand/interleukin-3 are also suggested to improve retroviral transduction efficiency in HSCs [55,56]. Even in the lentivirus mediated transduction, it has been known that EGF or hepatocyte growth factor markedly improved gene transfer [1,57]. Those factors may provide protective effects onto cells to improve transduction efficiency in viral transduction. In addition, to improve viral transduction efficiency, magnetically guided AAV delivery system was used and this significantly enhanced cellular transduction in human NSCs (hNSCs) and reduced the virus incubation time [58]. Elastin-like polypeptide-treated polystyrene surfaces are known to enhance AAV-mediated gene delivery efficiency in hNSCs [59]. It is also reported that ultrasound can enhance retrovirus-mediated gene transfer efficiency in various cell types [60,61]. It is suggested that exposure times of virus or timing of infection could affect viral transduction efficiency. However, the optimum time of virus exposure to cells may vary depending on cell types [20,46].

Cationic agents such as polybrene are generally used for retroviral-mediated gene transfer because they have been known to increase the efficiency of retroviral transduction [62-64]. Both the lipid membranes of the cell and viral particles have net negative charges. Cationic polymers are capable of overcoming this electrostatic repulsion via neutralization of negative cells and virus surface charges, and thus enhance retroviral transduction efficiency [64-66]. Since cationic polymers show their role in enhancement of transduction efficiency via charge neutralization, anionic polymers are considered to have the reverse effect on transduction efficiency and some studies showed that anionic polymers inhibit the transduction processes [66-68]. However, other studies showed opposite results that retroviral transduction efficiency is enhanced when an anionic polymer is added with equal or higher weight concentration of a cationic polymer [69,70]. Recently, several studies have demonstrated that some other cationic agents including

cationic liposomes can increase retroviral transduction efficiency compared to polybrene [62,63,71,72]. In our study, we have used polybrene to increase the transduction efficiency. Without polybrene, we could observe only around 3% transduction efficiency (data not shown). It is considered that enhancing effects of retroviral transduction efficiency by cationic polymers depends on both the type of target cells and envelope proteins used for pseudotyping [73]. Thus we can achieve better retroviral transduction efficiencies in NSCs by choosing an appropriate cationic agent.

Several earlier studies reported that ectopic expression of bHLH genes like Ngn2 can induce the generation of neurons from different types of cells [20,24,74-76]. In our current study, we observed facilitated cell division from NSCs that are retrovirally transduced with Ngn2. It has been known that neurons are born by both asymmetric and symmetric divisions [77]. The induction of cell divisions in Ngn2 expressing cells shown by time-lapse analysis in our study indicates that Ngn2 may facilitate final symmetric cell divisions and produce two daughter neurons. This observation is consistent with the previous findings that Ngn2 infected colonies of neural crest stem cells or rat NSCs were smaller and contained a higher percentage of neurons than ASCL1 infected colonies [20,24,78].

Recently, a new transfection technology has been introduced. Pickard and colleagues reported that they could efficiently transfect mouse NSCs with using magnetofection, a technique that utilizes magnetic nanoparticles for gene delivery [79]. Thus, it will be interesting to compare viral infection, nucleofection and magnetofection in terms of transfection efficiency, cell viability and toxicity.

In this study, we have compared the transfection efficiency of lipofection, electroporation, nucleofection and retroviral transduction using rat NSCs and demonstrated that the nucleofection and retroviral transduction were better than lipid mediated transfection and electroporation. Furthermore, we have demonstrated that ectopic expression of Ngn2 can induce final cell division for differentiation indicating using these techniques, the functions of genes can be explored in stem cells.

ACKNOWLEDGEMENTS

This research was supported by the Chung-Ang University excellent student scholarship and by the industrial core technology development program funded by the Ministry of Knowledge Economy (No.10041913, Development of stem cell culture system based on surface-nanostructure control (human adult cells: more than 12 passages, human embryonic cells: more than 1 complete differentiation of incomplete differentiable)] and by National Research foundation of Korea grant funded by the Korea Government (MEST) (No.2010-0012635).

REFERENCES

1. Capowski EE, Schneider BL, Ebert AD, Seehus CR, Szulc J, Zufferey R, Aebischer P, Svendsen CN. Lentiviral vector-mediated genetic modification of human neural progenitor cells for ex vivo gene therapy. *J Neurosci Methods*. 2007;163:338-349.
2. Kim HJ. Stem cell potential in Parkinson's disease and molecular factors for the generation of dopamine neurons. *Biochim Biophys Acta-Mol Basis Dis*. 2011;1812:1-11.
3. Müller FJ, Snyder EY, Loring JF. Gene therapy: can neural stem cells deliver? *Nat Rev Neurosci*. 2006;7:75-84.
4. Segers VF, Lee RT. Stem-cell therapy for cardiac disease. *Nature*. 2008;451:937-942.
5. Kim HJ, Jin CY. Stem cells in drug screening for neurodegenerative disease. *Korean J Physiol Pharmacol*. 2012;16:1-9.
6. Groszer M, Erickson R, Scripture-Adams DD, Lesche R, Trumpp A, Zack JA, Kornblum HI, Liu X, Wu H. Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene *in vivo*. *Science*. 2001;294:2186-2189.
7. Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*. 2000;287:1489-1493.
8. Van der Flier LG, van Gijn ME, Hatzis P, Kujala P, Haegebarth A, Stange DE, Begthel H, van den Born M, Guruyev V, Oving I, van Es JH, Barker N, Peters PJ, van de Wetering M, Clevers H. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell*. 2009;136:903-912.
9. Bauer M, Meyer M, Sautter J, Gasser T, Ueffing M, Widmer HR. Liposome-mediated gene transfer to fetal human ventral mesencephalic explant cultures. *Neurosci Lett*. 2001;308:169-172.
10. Kim YC, Shim JW, Oh YJ, Son H, Lee YS, Lee SH. Co-transfection with cDNA encoding the Bcl family of anti-apoptotic proteins improves the efficiency of transfection in primary fetal neural stem cells. *J Neurosci Methods*. 2002;117:153-158.
11. Lakshminpathy U, Pelacho B, Sudo K, Linehan JL, Coucouvanis E, Kaufman DS, Verfaillie CM. Efficient transfection of embryonic and adult stem cells. *Stem Cells*. 2004;22:531-543.
12. Cesnulevicius K, Timmer M, Wesemann M, Thomas T, Barkhausen T, Grothe C. Nucleofection is the most efficient nonviral transfection method for neuronal stem cells derived from ventral mesencephali with no changes in cell composition or dopaminergic fate. *Stem Cells*. 2006;24:2776-2791.
13. Tinsley RB, Fajerson J, Eriksson PS. Efficient non-viral transfection of adult neural stem/progenitor cells, without affecting viability, proliferation or differentiation. *J Gene Med*. 2006;8:72-81.
14. Gage FH. Mammalian neural stem cells. *Science*. 2000;287:1433-1438.
15. Brüstle O, McKay RD. Neuronal progenitors as tools for cell replacement in the nervous system. *Curr Opin Neurobiol*. 1996;6:688-695.
16. Amariglio N, Hirshberg A, Scheithauer BW, Cohen Y, Loewenthal R, Trakhtenbrot L, Paz N, Koren-Michowitz M, Waldman D, Leider-Trejo L, Toren A, Constantini S, Rechavi G. Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. *PLoS Med*. 2009;6:e1000029.
17. Rosser AE, Zietlow R, Dunnett SB. Stem cell transplantation for neurodegenerative diseases. *Curr Opin Neurol*. 2007;20:688-692.
18. Bertram B, Wiese S, von Holst A. High-efficiency transfection and survival rates of embryonic and adult mouse neural stem cells achieved by electroporation. *J Neurosci Methods*. 2012;209:420-427.
19. Osório J, Mueller T, Rétaux S, Vernier P, Wullmann MF. Phylogenetic expression of the bHLH genes Neurogenin2, Neurod, and Mash1 in the mouse embryonic forebrain. *J Comp Neurol*. 2010;518:851-871.
20. Kim HJ, McMillan E, Han F, Svendsen CN. Regionally specified human neural progenitor cells derived from the mesencephalon and forebrain undergo increased neurogenesis following overexpression of ASCL1. *Stem Cells*. 2009;27:390-398.
21. Helms AW, Battiste J, Henke RM, Nakada Y, Simplicio N, Guillemot F, Johnson JE. Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons. *Development*. 2005;132:2709-2719.

22. Sugimori M, Nagao M, Bertrand N, Parras CM, Guillemot F, Nakafuku M. Combinatorial actions of patterning and HLH transcription factors in the spatiotemporal control of neurogenesis and gliogenesis in the developing spinal cord. *Development*. 2007;134:1617-1629.
23. Bertrand N, Castro DS, Guillemot F. Proneural genes and the specification of neural cell types. *Nat Rev Neurosci*. 2002;3:517-530.
24. Kim HJ, Sugimori M, Nakafuku M, Svendsen CN. Control of neurogenesis and tyrosine hydroxylase expression in neural progenitor cells through bHLH proteins and Nurr1. *Exp Neurol*. 2007;203:394-405.
25. Thomas M, Klibanov AM. Non-viral gene therapy: polycation-mediated DNA delivery. *Appl Microbiol Biotechnol*. 2003;62:27-34.
26. Fraley R, Subramani S, Berg P, Papahadjopoulos D. Introduction of liposome-encapsulated SV40 DNA into cells. *J Biol Chem*. 1980;255:10431-10435.
27. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA*. 1987;84:7413-7417.
28. Aluigi M, Fogli M, Curti A, Isidori A, Gruppioni E, Chiodoni C, Colombo MP, Versura P, D'Errico-Grigioni A, Ferri E, Baccarani M, Lemoli RM. Nucleofection is an efficient nonviral transfection technique for human bone marrow-derived mesenchymal stem cells. *Stem Cells*. 2006;24:454-461.
29. Saito T, Nakatsuji N. Efficient gene transfer into the embryonic mouse brain using *in vivo* electroporation. *Dev Biol*. 2001;240:237-246.
30. Tabata H, Nakajima K. Efficient *in utero* gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. *Neuroscience*. 2001;103:865-872.
31. Mertz KD, Weisheit G, Schilling K, Lüers GH. Electroporation of primary neural cultures: a simple method for directed gene transfer *in vitro*. *Histochem Cell Biol*. 2002;118:501-506.
32. Richard I, Ader M, Sytnyk V, Dityatev A, Richard G, Schachner M, Bartsch U. Electroporation-based gene transfer for efficient transfection of neural precursor cells. *Brain Res Mol Brain Res*. 2005;138:182-190.
33. De Vry J, Martínez-Martínez P, Losen M, Temel Y, Steckler T, Steinbusch HW, De Baets MH, Prickaerts J. *In vivo* electroporation of the central nervous system: a non-viral approach for targeted gene delivery. *Prog Neurobiol*. 2010;92:227-244.
34. Dityateva G, Hammond M, Thiel C, Ruonala MO, Delling M, Siebenkotten G, Nix M, Dityatev A. Rapid and efficient electroporation-based gene transfer into primary dissociated neurons. *J Neurosci Methods*. 2003;130:65-73.
35. Hamm A, Krott N, Breibach I, Blindt R, Bosserhoff AK. Efficient transfection method for primary cells. *Tissue Eng*. 2002;8:235-245.
36. Gresch O, Engel FB, Nestic D, Tran TT, England HM, Hickman ES, Körner I, Gan L, Chen S, Castro-Obregon S, Hammermann R, Wolf J, Müller-Hartmann H, Nix M, Siebenkotten G, Kraus G, Lun K. New non-viral method for gene transfer into primary cells. *Methods*. 2004;33:151-163.
37. Siemen H, Nix M, Endl E, Koch P, Itskovitz-Eldor J, Brüstle O. Nucleofection of human embryonic stem cells. *Stem Cells Dev*. 2005;14:378-383.
38. Von Levetzow G, Spanholtz J, Beckmann J, Fischer J, Kögler G, Wernet P, Punzel M, Giebel B. Nucleofection, an efficient nonviral method to transfer genes into human hematopoietic stem and progenitor cells. *Stem Cells Dev*. 2006;15:278-285.
39. Dieterlen MT, Wegner F, Schwarz SC, Milosevic J, Schneider B, Busch M, Römuss U, Brandt A, Storch A, Schwarz J. Non-viral gene transfer by nucleofection allows stable gene expression in human neural progenitor cells. *J Neurosci Methods*. 2009;178:15-23.
40. Cone RD, Mulligan RC. High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. *Proc Natl Acad Sci USA*. 1984;81:6349-6353.
41. Eglitis MA, Kantoff P, Gilboa E, Anderson WF. Gene expression in mice after high efficiency retroviral-mediated gene transfer. *Science*. 1985;230:1395-1398.
42. Park SW, Won KJ, Lee YS, Kim HS, Kim YK, Lee HW, Kim B, Lee BH, Kim JH, Kim DK. Increased HoxB4 inhibits apoptotic cell death in Pro-B cells. *Korean J Physiol Pharmacol*. 2012;16:265-271.
43. Park KS, Wiederkehr A, Wollheim CB. Defective mitochondrial function and motility due to mitofusin 1 overexpression in insulin secreting cells. *Korean J Physiol Pharmacol*. 2012;16:71-77.
44. Lu H, Li M, Song T, Qian Y, Xiao X, Chen X, Zhang P, Feng X, Parker T, Liu Y. Retrovirus delivered neurotrophin-3 promotes survival, proliferation and neuronal differentiation of human fetal neural stem cells *in vitro*. *Brain Res Bull*. 2008;77:158-164.
45. Ostenfeld T, Tai YT, Martin P, Déglon N, Aebischer P, Svendsen CN. Neurospheres modified to produce glial cell line-derived neurotrophic factor increase the survival of transplanted dopamine neurons. *J Neurosci Res*. 2002;69:955-965.
46. Kameda M, Shingo T, Takahashi K, Muraoka K, Kurozumi K, Yasuhara T, Maruo T, Tsuboi T, Uozumi T, Matsui T, Miyoshi Y, Hamada H, Date I. Adult neural stem and progenitor cells modified to secrete GDNF can protect, migrate and integrate after intracerebral transplantation in rats with transient forebrain ischemia. *Eur J Neurosci*. 2007;26:1462-1478.
47. Hirsch ML, Fagan BM, Dumitru R, Bower JJ, Yadav S, Porteus MH, Pevny LH, Samulski RJ. Viral single-strand DNA induces p53-dependent apoptosis in human embryonic stem cells. *PLoS One*. 2011;6:e27520.
48. Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol*. 1990;10:4239-4242.
49. Roe T, Reynolds TC, Yu G, Brown PO. Integration of murine leukemia virus DNA depends on mitosis. *EMBO J*. 1993;12:2099-2108.
50. Kulkosky J, Skalka AM. Molecular mechanism of retroviral DNA integration. *Pharmacol Ther*. 1994;61:185-203.
51. Goff SP. Genetics of retroviral integration. *Annu Rev Genet*. 1992;26:527-544.
52. Naldini L. Lentiviruses as gene transfer agents for delivery to non-dividing cells. *Curr Opin Biotechnol*. 1998;9:457-463.
53. Lewis P, Hensel M, Emerman M. Human immunodeficiency virus infection of cells arrested in the cell cycle. *EMBO J*. 1992;11:3053-3058.
54. Luskey BD, Rosenblatt M, Zsebo K, Williams DA. Stem cell factor, interleukin-3, and interleukin-6 promote retroviral-mediated gene transfer into murine hematopoietic stem cells. *Blood*. 1992;80:396-402.
55. Elwood NJ, Zogos H, Willson T, Begley CG. Retroviral transduction of human progenitor cells: use of granulocyte colony-stimulating factor plus stem cell factor to mobilize progenitor cells *in vivo* and stimulation by Flt3/Flk-2 ligand *in vitro*. *Blood*. 1996;88:4452-4462.
56. Orlic D, Girard LJ, Anderson SM, Barrette S, Broxmeyer HE, Bodine DM. Amphotropic retrovirus transduction of hematopoietic stem cells. *Ann N Y Acad Sci*. 1999;872:115-123.
57. Rothe M, Rittelmeyer I, Iken M, Rüdlich U, Schambach A, Glage S, Manns MP, Baum C, Bock M, Ott M, Modlich U. Epidermal growth factor improves lentivirus vector gene transfer into primary mouse hepatocytes. *Gene Ther*. 2012;19:425-434.
58. Kim E, Oh JS, Ahn IS, Park KI, Jang JH. Magnetically enhanced adeno-associated viral vector delivery for human neural stem cell infection. *Biomaterials*. 2011;32:8654-8662.
59. Kim JS, Chu HS, Park KI, Won JI, Jang JH. Elastin-like polypeptide matrices for enhancing adeno-associated virus-mediated gene delivery to human neural stem cells. *Gene Ther*. 2012;19:329-337.
60. Lee YH, Peng CA. Enhanced retroviral gene delivery in

- ultrasonic standing wave fields. *Gene Ther.* 2005;12:625-633.
61. **Naka T, Sakoda T, Doi T, Tsujino T, Masuyama T, Kawashima S, Iwasaki T, Ohyanagi M.** Ultrasound enhances retrovirus-mediated gene transfer. *Prep Biochem Biotechnol.* 2007;37: 87-99.
 62. **Porter CD, Lukacs KV, Box G, Takeuchi Y, Collins MK.** Cationic liposomes enhance the rate of transduction by a recombinant retroviral vector *in vitro* and *in vivo*. *J Virol.* 1998;72:4832-4840.
 63. **Themis M, Forbes SJ, Chan L, Cooper RG, Etheridge CJ, Miller AD, Hodgson HJ, Coutelle C.** Enhanced *in vitro* and *in vivo* gene delivery using cationic agent complexed retrovirus vectors. *Gene Ther.* 1998;5:1180-1186.
 64. **Coelen RJ, Jose DG, May JT.** The effect of hexadimethrine bromide (polybrene) on the infection of the primate retroviruses SSV 1/SSAV 1 and BaEV. *Arch Virol.* 1983;75:307-311.
 65. **Coller BS.** Polybrene-induced platelet agglutination and reduction in electrophoretic mobility: enhancement by von Willebrand factor and inhibition by vancomycin. *Blood.* 1980;55: 276-281.
 66. **Davis HE, Rosinski M, Morgan JR, Yarmush ML.** Charged polymers modulate retrovirus transduction via membrane charge neutralization and virus aggregation. *Biophys J.* 2004; 86:1234-1242.
 67. **Baba M, Snoeck R, Pauwels R, de Clercq E.** Sulfated polysaccharides are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human immunodeficiency virus. *Antimicrob Agents Chemother.* 1988;32: 1742-1745.
 68. **Batra RK, Olsen JC, Hoganson DK, Caterson B, Boucher RC.** Retroviral gene transfer is inhibited by chondroitin sulfate proteoglycans/glycosaminoglycans in malignant pleural effusions. *J Biol Chem.* 1997;272:11736-11743.
 69. **Toyoshima K, Vogt PK.** Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions. *Virology.* 1969;38:414-426.
 70. **Le Doux JM, Landazuri N, Yarmush ML, Morgan JR.** Complexation of retrovirus with cationic and anionic polymers increases the efficiency of gene transfer. *Hum Gene Ther.* 2001;12:1611-1621.
 71. **Kaneko Y, Tsukamoto A.** Structural characteristics of cationic liposomes with potent enhancing effect on retroviral transduction into human hepatoma cells. *Cancer Lett.* 1996; 107:211-215.
 72. **Hodgson CP, Solaiman F.** Virosomes: cationic liposomes enhance retroviral transduction. *Nat Biotechnol.* 1996;14:339-342.
 73. **Jang J, Lee J, Kim ST, Lee KY, Cho JY, Kweon DH, Kwon ST, Koh YH, Kim S, Yoon K.** Polycation-mediated enhancement of retroviral transduction efficiency depends on target cell types and pseudotyped Env proteins: implication for gene transfer into neural stem cells. *Neurochem Int.* 2012;60:846-851.
 74. **Farah MH, Olson JM, Sucic HB, Hume RI, Tapscott SJ, Turner DL.** Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development.* 2000;127: 693-702.
 75. **Thoma EC, Wischmeyer E, Offen N, Maurus K, Sirén AL, Schartl M, Wagner TU.** Ectopic expression of neurogenin 2 alone is sufficient to induce differentiation of embryonic stem cells into mature neurons. *PLoS One.* 2012;7:e38651.
 76. **Ribes V, Stutzmann F, Bianchetti L, Guillemot F, Dollé P, Le Roux I.** Combinatorial signalling controls Neurogenin2 expression at the onset of spinal neurogenesis. *Dev Biol.* 2008; 321:470-481.
 77. **Huttner WB, Kosodo Y.** Symmetric versus asymmetric cell division during neurogenesis in the developing vertebrate central nervous system. *Curr Opin Cell Biol.* 2005;17:648-657.
 78. **Lo L, Dormand E, Greenwood A, Anderson DJ.** Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. *Development.* 2002;129:1553-1567.
 79. **Pickard MR, Barraud P, Chari DM.** The transfection of multipotent neural precursor/stem cell transplant populations with magnetic nanoparticles. *Biomaterials.* 2011;32:2274-2284.