Research Highlights

Highlights from the latest articles in regenerative medicine

Stable transduction or transient transfection?

Evaluation of: Cao F, Xie X, Gollan T *et al.*: Comparison of gene-transfer efficiency in human embryonic stem cells. *Mol. Imaging Biol.* (2009) (Epub ahead of print).

The preclinical identification of mediators responsible for key events in tissue repair offers the potential for cell-based therapy to advance to cell-based gene therapy, which can enhance the effects of cell transplantation by temporally and spatially regulating the release of the gene product by carrier stem cells. The clinical application of stem cell-mediated gene therapy approaches is based on the development of safe and efficient methods to deliver genes to the cells. An ideal transgene-delivery vector should be safe, nonimmunogenic, easy to produce, capable of targeting specific tissue, controllable, and result in effective transfection and long-lasting expression [1,2].

Vehicles for gene transfer include both viral and nonviral vector systems. Using a double-fusion reporter gene with a ubiquitin promoter driving an enhanced green fluorescent protein reporter and firefly luciferase along with a neomycin selection marker, Cao *et al.* have compared gene-transfer efficiency using various nonviral gene delivery, such as lipofection, electroporation and nucleofection, with viral (e.g., lentiviral) transduction of two human embryonic stem cell (hESC) lines (male H1 and female H9) to identify the most efficient, reliable and simple gene-transfer system for *in vitro* and *in vivo* applications. Lentiviral transduction demonstrated the highest level of *Fluc* gene expression (H1: $25.3 \pm 4.8\%$ and H9: $22.4 \pm 6.5\%$). Nucleofection resulted in lower transfection efficiency of $16.1 \pm 3.6\%$ (H1) and $5.8 \pm 3.2\%$

(H9). Minimal transfection efficiency was observed with electroporation (H1: $2.1 \pm 0.4\%$ and H9: $1.9 \pm 0.3\%$) and lipofection (H1: $1.5 \pm 0.5\%$ and H9: $1.3 \pm 0.2\%$). Lentiviral transduction generated a greater number of hESC lines stably expressing the double-fusion reporter gene compared with other transfection techniques. Interestingly, the H1 cell line had higher transfection efficiency than the H9 hESC line for all protocols tested, suggesting varying susceptibility to gene transfer in different hESC lines. Lentiviral transduction demonstrated not only the highest level of gene transfer (22–25%) but also the best cell viability following treatment (>95%). A mildly decreased level of cell viability (70–75%) was observed with nucleofection, with poor viability following treatment with electroporation (20–25%).

Cao *et al.* performed serial bioluminescence imaging on days 2, 7, 14, 35, 42 and 56. H9 cells containing the double-fusion reporters (via stable lentiviral transduction) demonstrated persistent engraftment and survival. Following imaging at day 56, animals were sacrificed and the developed teratoma explanted. Histologic analysis of control hESCs and hESC double-fusion reporter teratomas demonstrates mixed regions of endodermal, mesodermal and ectodermal differentiation, suggesting that lentiviral transduction does not affect the differentiation of hESC into the three germ layer cell types *in vivo*.

Furthermore, stable cell lines with lentiviral transduction and plasmid nucleofection protocols were constructed using the double-fusion reporter genes and their growth and differentiation *in vivo* over several months monitored. Immunofluorescence and histological analysis demonstrated that the stable hESC lines consisted of undifferentiated cells that

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expressed numerous stem cell markers. After several weeks, these undifferentiated cells developed into subcutaneous teratomas.

This study demonstrates that virus-based vectors are more efficient at introducing gene constructs into cells. Both lentiviral transduction and plasmid nucleofection protocols successfully generated stable hESC clones that stably expressed the reporter gene. Although lentivirus generated higher numbers of stable clones, nucleofection has the advantage of being a simple, reproducible and efficient technique for transient as well as stable transfection.

In vivo viral gene delivery integrating the gene sequence into the host cell genome has demonstrated the highest levels and most durable gene expression that can be achieved. The clinical use of viral vectors is limited by their immunogenicity, nonspecific delivery of viral particles to the tissue and the risk of random integration at undesirable chromosomal locations, which can lead to adverse side effects, such as insertional mutagenesis. Although viral vectors are designed to be replication defective, the possibility of introducing replication-competent viruses exists [3].

By contrast, nonviral vectors are easier to engineer and manufacture, have greater ease of handling, lower cost of preparation, fewer side effects from toxicity and immunogenicity, and more compatibility with clinical protocols than viruses.

However, it is more difficult to deliver genes efficiently into cells with nonviral vectors, and lack of chromosomal integration ability precludes long-term therapeutic effects when delivered to living tissues. The ultimate benefit of nonviral gene delivery led to continuous improvement of gene-delivery vectors and transfection methods.

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Stable gene expression using nonviral transfection technology I

Evaluation of: Dieterlen MT, Wegner F, Schwarz SC *et al.*: Non-viral gene transfer by nucleofection allows stable gene expression in human neural progenitor cells. *J. Neurosci. Methods* 178, 15–23 (2009).

It has recently been shown that adult mouse neural progenitor cells (NPCs) can be reprogrammed to induced pluripotent stem cells using retroviral transfer of transcription factors Klf4 and Oct4 [1]. Human NPCs (hNPCs) also seem to be an attractive tool for the stable transfer of foreign genes or application of growth factors within the CNS. The potential applications are to use such cells for reprogramming to induce pluripotent stem cells or

for secretion of proteins into the brain or to cure degenerative neurologic disorders [2]. Two viral systems for gene transfer were previously tested in hNPCs: the adenoviral transduction of hNPCs was ineffective with transduction rates of less than 5%. The lentiviral-mediated gene transfer achieved transduction rates of less than 30% but resulted in a markedly reduced proliferation capacity of hNPCs.

In the study, Dieterlen *et al.* investigated whether nucleofection can also provide large quantities of stably transfected hNPCs without loss of their proliferation and differentiation capacity. Nucleofected cells were cultivated for 6–8 weeks until the first clones were formed. According to their proliferation potential, stably transfected clones were transferred to six-well plates, then passaged regularly after reaching more

than 95% confluency. Cell counts, vitality and transfection efficiency was documented every week using a fluorescence microscope.

The nucleofector technology offered a time-saving method to combine acceptable viability ($44 \pm 3\%$) and high transfection rates of hNPCs. The plasmid C1-enhanced green fluorescent protein (EGFP) contained a neomycin-resistance cassette that allowed selection of stably nucleofected hNPCs using neomycin or G418. The highest initial nucleofection efficiency was obtained 24 h after nucleofection with the programs A-33 ($20.5 \pm 1.1\%$) and G-13 ($20.2 \pm 1.0\%$). Both programs proved to be suitable for short-term nucleofection experiments on human NPCs.

To determine an appropriate promoter system for long-term gene expression in hNPCs, the research group tested two

eukaryotic promoter systems: the cytomegalovirus immediate early promoter and the cytomegalovirus (CMV) enhancer/chicken β -actin (CBA) promoter. To compare both promoter systems, transcriptional activities of EGFP were detected by flow cytometry over a period of 42 days. Both promoters demonstrated comparable initial transgene expression that was increased via selection to $91.3 \pm 0.2\%$ with CBA promoter-driven constructs and to $46.3 \pm 0.4\%$ with CMV promoter-driven constructs 14 days after nucleofection. The enhanced activity of CBA promoter-driven EGFP expression compared with the CMV promoter was documented for a period of 6 weeks. Nucleofection efficiency decreased over that period, suggesting that ongoing drug selection is necessary for producing long-term cultures of stably nucleofected hNPCs. A total of 6–8 weeks after nucleofection, stably transfected colonies in cultures nucleofected with CBA promoter-driven constructs were detected. These results suggest that CBA promoter-driven gene expression enables long-term expression of nucleofected genes in hNPCs *in vitro*.

Human neural progenitor cells were stereotactically implanted into the striatum of two anesthetized female Sprague–Dawley

rats. After differentiation for 3 weeks *in vitro*, the transfected hNPCs transplanted into the the striatum expressed voltage-gated sodium channels, suggesting the development of functional properties during neuronal maturation.

Thus, nucleofected hNPCs demonstrating stable EGFP expression maintain the capacity to differentiate *in vitro* and *in vivo* after transplantation, making them an interesting tool for morphological and functional analyses of neuronal maturation. These data provide a basis for future studies using hNPCs to investigate the neuronal differentiation *in vivo* after transplantation, the feasibility as a vector for gene (protein) therapy and the induction of pluripotent stem cells.

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Stable gene expression using nonviral transfection technology II

Evaluation of: Magg T, Hartrampf S, Albert MH: Stable nonviral gene transfer into primary human T cells. *Hum. Gene Ther.* (2009) (Epub ahead of print).

Application of genetically modified T cells is a promising tool for treating malignancies, viral infections, genetic disorders and autoimmune disease [1,2]. Magg *et al.* describes an easy and reliable nonviral transfection strategy for generation and purification of stably transfected primary human CD4⁺ T cells exhibiting genomic integration and homogenous long-term expression of a gene of interest.

Magg and colleagues created a dual-expression plasmid, which allowed the

coexpression of a gene of interest and a selection marker for immunomagnetic purification. Transfection of the C-terminal truncated version of the low-affinity NGF receptor/green fluorescence protein (pLNGFR/GFP) plasmid with the Amaxa nucleofector device yielded $58.0 \pm 2.0\%$ LNGFR-positive cells, $26.4 \pm 1.7\%$ GFP-positive cells and $25.6 \pm 1.6\%$ LNGFR/GFP double-positive cells. Simultaneous expression of two transgenes could be demonstrated in primary human T cells with good efficiency using this nonviral, dual-promoter plasmid transfection strategy. The highest percentage of LNGFR and GFP double-positive cells was obtained when expressing GFP under the control of the cytomegalovirus promoter and LNGFR expression driven by the elongation factor-1 α promoter.

Within 7 days of nucleofection of 10^7 cells, the expression of both reporter proteins declined to background signal except for $0.5 \pm 0.1\%$ of the cells that remained double positive for LNGFR and GFP. LNGFR/GFP double-positive cells were enriched using magnetic cell sorting. After a third enrichment, a total of $6.4 \times 10^5 \pm 1.2 \times 10^5$ cells, with a purity of $98.9 \pm 0.6\%$, could be harvested. These T cells were cultured for 8 weeks after nucleofection and analyzed for LNGFR and GFP expression. Both marker genes remained at constantly high levels during the observation period. To confirm that these cells were indeed stable transfectants, genomic PCR analysis was performed with plasmid-specific oligonucleotides 35 days post-transfection. Western blot analysis

and PCR confirmed that the transgene was in fact integrated into the genome at multiple sites. These findings implicate that nucleofection is suitable for generation and purification of stably transfected primary human T cells.

Phenotypic characterization of transiently and stably transfected primary human T cells revealed that nucleofected T cells exhibited a significant reduction in ^3H thymidine incorporation upon T-cell stimulation as compared with nontransfected control cells or stably transfected T cells, suggesting impaired DNA synthesis of freshly transfected T cells immediately after nucleofection. Freshly transfected T cells also exhibited significantly decreased secretion of IL-5, -10 and -13, IFN- γ and TNF- α , but not IL-4 and -12 compared with nontransfected control cells, whereas stably transfected T cells demonstrated a similar cytokine secretion pattern as nontransfected T cells cultured under identical conditions. The freshly transfected T cells displayed a slightly reduced proliferation capacity compared with control T cells ($84.4 \pm 3.0\%$ proliferating cells vs $95.0 \pm 0.5\%$, $p = 0.004$), whereas stably transfected T cells displayed a normal proliferative response compared with untransfected cells cultured under identical conditions ($81.7 \pm 2.2\%$ vs $79.9 \pm 2.7\%$). There was a significantly higher proportion of apoptotic cells upon nucleofection during the first 16 h of the culture period but not thereafter.

In this way, the authors demonstrated that nucleofection is a suitable method for transient and also stable transfection

of resting primary human T cells. This provides an efficient method, not only for studying gene functions in primary human T cells, but also potentially for therapeutic application of genetically modified T cells.

In vivo viral gene delivery integrating the gene sequence to the host cell genome has demonstrated the highest levels and most durable gene expression that can be achieved. However, currently, the primary consideration of the cell-based gene transfer is the safety. Independent from the gene-transfer method used, integration of plasmid DNA into the chromosome bears a risk for insertional mutagenesis. Indeed, in a clinical trial, with genetically modified hematopoietic stem cells, the induction of leukemia in patients has been reported [3]. Nucleofection has demonstrated higher levels of transfection efficiency with the possibility of low-level stable transfection, with significantly improved levels of cell viability over other nonviral transfection methods.

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