Published in final edited form as:

Ann Biomed Eng. 2012 September ; 40(9): 1851–1861. doi:10.1007/s10439-012-0555-y.

Ex Vivo Gene Delivery to Hepatocytes: Techniques, Challenges, and Underlying Mechanisms

Shan Gao¹, Erkin Seker², Monica Casali¹, Fangjing Wang¹, Shyam Sundhar Bale¹, Gavrielle M. Price¹, and Martin L. Yarmush^{1,3}

¹Center for Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School and Shriners Hospitals for Children, 51 Blossom Street, Boston, MA 02114, USA

²Department of Electrical and Computer Engineering, University of California Davis, Davis, CA 95616, USA

³Department of Biomedical Engineering, Rutgers University, Piscataway, NJ 08854, USA

Abstract

Gene delivery to primary hepatocytes is an important tool for a number of applications including the study of liver cell biology and pathology, drug screening, and gene therapy. Robust transfection of primary hepatocytes, however, is significantly more difficult to achieve than in cell lines or readily dividing primary cells. In this report, we investigated *in vitro* gene delivery to both primary rat hepatocytes and Huh7.5.1 cells (a hepatoma cell line) using a number of viral and non-viral methods, including Lipofectamine 2000, FuGene HD, Nucleofection, Magnetofection, and lentiviruses. Our results showed that Lipofectamine 2000 is the most efficient reagent for green fluorescent protein (GFP) gene delivery to primary rat hepatocytes ($33.3 \pm 1.8\%$ transfection efficiency) with minimal adverse effect on several hepatic functions, such as urea and albumin secretion. The lentiviral vectors used in this study exhibited undetectable gene delivery to primary rat hepatocytes but significant delivery to Huh7.5.1 cells ($>80\%$ transfection efficiency). In addition, we demonstrated lentiviral-based and spatially defined delivery of the GFP gene to Huh7.5.1 cells for use in biological microelectromechanical systems.

Keywords

Non-viral transfection; Lentiviruses; Primary rat hepatocytes; Huh7.5.1; Transfection efficiency; Hepatic function

INTRODUCTION

The liver is a central organ where essential metabolic processes take place in the human body, including metabolism of carbohydrates, lipids, amino acids, alcohol, and drugs. Genetically engineered hepatocyte cultures have played an important role in studying liver biology and pathology, as well as in screening for therapeutic drug candidates.^{2,13,23} To this end, gene delivery to hepatocytes has been extensively used for decades from basic

© 2012 Biomedical Engineering Society

Address correspondence to Martin L. Yarmush, Center for Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School and Shriners Hospitals for Children, 51 Blossom Street, Boston, MA 02114, USA. ireis@sbi.org.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (doi: 10.1007/s10439-012-0555-y) contains supplementary material, which is available to authorized users.

research²⁹ to clinical trials.¹⁴ Both non-viral vectors and viral vectors have been employed for delivering plasmid DNA and siRNA to hepatocytes *in vitro* and *in vivo* in order to either initiate or inhibit the expression of specific proteins for pathway studies and for therapeutic applications. Similar to the methods used in other types of cells or tissues, non-viral gene delivery to hepatocytes includes commercially available transfection agents,^{6,36} liposomes,³⁷ electroporation,^{2,36} carbohydrate-based gene delivery vectors,³⁰ and hydrodynamic injection¹²; while viral gene delivery vectors include retroviruses^{36,41} (especially lentiviruses²⁴), adenoviruses³ and adeno-associated viruses.²⁰ It has often been more challenging, however, to transfect primary hepatocytes compared to cell lines or to readily dividing primary cells. In addition, primary hepatocytes have a limited life-span varying from 48 h to a maximum of 2 weeks depending on the quality of isolation, culture conditions, and the effect of transfection on cell functions. Therefore, it is important to identify the most efficient gene delivery methods to hepatocytes by systematically comparing common and commercially available methods in terms of their transfection efficiency and their adverse effects on cellular functions.

Reporter plasmid vectors have frequently been used to study cellular pathways and transcription factor activities in cells, including in primary hepatocyte cultures.^{8,29} Numerous studies have focused on hepatic processes at cellular and molecular levels by introducing exogenous transgenes or reporters into primary hepatocytes *via* non-viral² or viral²⁹ gene delivery methods. Among these reporters, fluorescent reporters are superior to traditional enzymatic reporters because they enable the study of the temporal dynamics of response elements. For instance, previous studies from our group have demonstrated real-time monitoring of transcription factor dynamics using a fluorescent hepatoma reporter cell line (H35)—this was prepared by stably transfecting H35 cells with a series of reporter vectors containing a destabilized green fluorescent protein (d2GFP) gene controlled by various response elements.^{31,40} By incorporating these fluorescent reporter cell lines into a microfluidic platform that enables parallel exposure to certain soluble stimuli, we acquired massive amounts of data that revealed the temporal profile of transcription factor dynamics in a high-throughput fashion.¹⁶ While stably transfected reporter cell lines provide important insight into the genetic machinery of the cell, hepatoma cell lines do not fully represent the functions of primary hepatocytes, such as complex metabolic processes. It is important, therefore, to systematically develop protocols that maximize transfection efficiency for both cell lines and primary hepatocytes.

In this study, we evaluated *ex vivo* gene delivery to primary rat hepatocytes (cells that exhibit *in vivo*-like metabolic response) and Huh7.5.1 cells (a cell line used extensively in hepatitis C viral infection studies) using a number of viral and commercially available non-viral methods (Fig. 1). We examined the gene delivery efficiency, as well as the specific hepatic functions of transfected primary rat hepatocytes, such as urea and albumin secretion. We also investigated potential mechanisms of low levels of lentiviral-mediated transgene expression in primary rat hepatocytes. To our knowledge, this is the first study to uniformly evaluate and compare a number of frequently used non-viral and viral vectors, including Lipofectamine 2000, FuGene HD, Amaxa Nucleofection, Magnetofection, and lentiviruses, to deliver fluorescent transgenes to primary rat hepatocyte cultures and to a human hepatoma cell line (Huh7.5.1). In addition, we demonstrated that, using cell patterning, efficient *ex vivo* gene delivery methods enabled spatially defined reporter gene delivery for studying *ex vivo* hepatic gene regulation upon stimulation. We expect these integrated techniques yield an *in vitro* liver model, using primary hepatocyte reporters for studying hepatic pathways or drug screening.

METHODS

Cell Culture

Freshly isolated primary hepatocytes from female Lewis rats were seeded directly into tissue culture wells coated with either collagen gel (1.11 mg/mL), collagen (50 µg/mL), or fibronectin (20 µg/mL), at a density of 105,000 cells/cm². Primary rat hepatocytes were maintained in DMEM medium, supplemented with 10% heat inactivated FBS, hydrocortisone, glucagon, insulin, EGF, penicillin, and streptomycin. The medium was replenished daily.

Huh7.5.1 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL). The cells were harvested from tissue culture flasks using trypsin/ EDTA, pelleted by centrifugation, and re-suspended to 1×10^6 cells/mL in culture medium. Cells were then seeded in a 12-well tissue culture plate at a density of 60,000 cells/cm². The medium was replenished every other day.

All cells were cultured at 37 °C in a fully humidified atmosphere of 10% CO₂.

Non-viral Transfection

Lipofectamine 2000 and FuGene HD—Lipofectamine 2000 and FuGene HD were used to transfect primary rat hepatocytes and Huh7.5.1 cells with pmaxGFP (Lonza) following the manufacturer's protocols. Fluorescent and phase images were captured at 3, 24, 48, and 96 h post-transfection. The transfection efficiency was evaluated by taking the ratio of cells expressing GFP to total cell number. At least four images were counted for each sample with each image containing about 300 cells.

Amaya Nucleofection—Freshly isolated primary rat hepatocytes were transfected with pmaxGFP following the manufacturer's protocols and then seeded into a 6-well culture plate coated with collagen gel (1.11 mg/mL) that contained 1 mL of medium per well. The medium was replaced 3 h after cell seeding. Fluorescent images of GFP expression were captured at 3, 24, 48, and 96 h post-transfection. The transfection efficiency was calculated as previously described in "Lipofectamine 2000 and FuGene HD".

Magnetofection—Freshly isolated primary rat hepatocytes and Huh7.5.1 cells (seeded the day before) were transfected with pmaxGFP using PolyMag Neo nucleic acid delivery reagent following the manufacturer's protocol. Fluorescent images of GFP expression were captured at 3, 24, 48, and 96 h post-transfection.

Viral Gene Delivery Systems

Lentiviral Constructs and Production—The reported data in this study was obtained using pLVmnd14lucRFPttk (pLVR) vector containing luciferase and red fluorescent protein (RFP) fusion gene under the control of a MND promoter (modified myeloproliferative sarcoma virus promoter), followed by a woodchuck post-transcriptional regulatory element (WPRE). The constructs of three other lentiviral vectors used in this study are described in Supplemental Materials.

We used the second generation of a lentiviral vector production system to produce LentiRFP lentiviruses. HEK-293T cells were co-transfected with pLVR, pVSV-G, and pΔ using FuGene HD following the optimized protocol developed in our laboratory. Specifically, HEK-293T cells were maintained in culture medium (DMEM containing 4500 mg/L glucose and 4 mM glutamine supplemented with 10% FBS and 1 mM sodium pyruvate) and were plated at approximately 50% confluence in T-175 flasks coated with poly-lysine (0.1 mg/

mL). After 20–24 h, the medium was replaced with 20 mL of Opti-MEM supplemented with 25 μ M chloroquine. Vector mixture solution was prepared by adding 4.7 μ g of pLVR, 4.7 μ g of p Δ , and 3.1 μ g of pVSV-G into 47 μ L TE buffer. In a separate tube, 31.1 μ g of FuGene HD reagent was added to 627 μ L Opti-MEM. The vector mixture was then added to the FuGene HD in Opti-MEM and incubated at room temperature for 15 min. The transfection mixture was added to the HEK-293T cells in the flask while the flask was gently agitated. After 7 h, sodium butyrate was added to the culture medium to the final concentration of 10 μ M. After 24 h, the medium was discarded and replaced with 20 mL of culture medium. After another 24 h after medium change, the medium was collected and filtered through a 0.45 μ m-filter and 20 mL of fresh culture medium was added to the flask. The filtered medium was centrifuged at 3000 $\times$ g for 10 min and aliquots of supernatant were stored at -80°C . Virion-containing medium was collected and filtered again 24 h later. The viral titer was obtained by infecting HEK-293T cells with serial dilutions of viral suspension. Three days post-infection, the cells were harvested and analyzed by flow cytometry (FACS-Calibur; Becton Dickinson, San Jose).

Lentiviral Transduction—Primary rat hepatocytes or Huh7.5.1 cells were seeded in a 12-well plate the day before infection. Thawed lentiviral stocks were added to the cell culture at a Multiplicity of Infection (MOI) of 1, 5, and 20, in the presence of 8 μ g/mL of polybrene. Growth medium was added to the final volume of 1.5 mL/well for primary hepatocytes, while DMEM growth medium was added to a final volume of 1 mL/well for Huh7.5.1 cells. Cells were incubated with the virion-containing medium overnight. The growth medium was replaced with fresh medium the next day.

Urea and Albumin Secretion Assays

Conditioned medium from hepatocyte cultures was collected daily and stored at -80°C . Urea concentration was measured using BUN test kit from Stanbio Laboratory. Albumin concentration was measured using competitive ELISA developed in our lab. Urea and albumin secretion is given as $\mu\text{g}/1 \times 10^6$ cells/24 h.

Data Analysis

Images Analysis—All fluorescence images were captured with a Zeiss 200 Axiovert microscope. The average fluorescence intensity of the LentiRFP-infected cells was quantified using ImageJ (NIH shareware, <http://rsb.info.nih.gov/ij/index.html>) and an image processing macro that was written in-house. Briefly, the macro smoothed each image *via* a median filter, subtracted the background, and enhanced the image contrast using built-in ImageJ functions. The image was segmented at a threshold value that corresponded to the sum of average pixel intensity and standard deviation for each image. The average cell fluorescence was taken as signal intensities above a selected threshold. Average intensity was further normalized by the exposure time for each image; the intensity varied linearly with exposure time within the dynamic intensity range used in our experiments.

Flow Cytometry Analysis—Huh7.5.1 cells transfected with pmaxGFP were analyzed using Quanta SC flow cytometer (Beckman Coulter). Viable cells were gated in forward and side-scatter plots. The cells exhibiting green fluorescence were detected through the FL1 channel (excitation: 488 nm, emission: 520 ± 10 nm) on the gate threshold that was set based on measurements made with untreated cells. The GFP gene delivery efficiency was evaluated as the percentage of green cells within total viable cells.

Lentiviral Infection Efficiency by Quantitative RT-PCR Analysis—Cells transfected with LentiRFP viruses were lysed at set time points and the lysate was stored at -80°C . Genomic DNA and total RNA were purified using an AllPrep DNA/RNA mini kit

(Qiagen). The qRT-PCRs were performed using the SuperScript[®] III Platinum[®] SYBR[®] Green One-Step qRT-PCR Kit (Invitrogen) and a Stratagene Mx3000P machine. A standard curve was obtained using serial dilutions of pLVR as described in the Supplemental Materials. The normalized copy number (ng^{-1}) of each DNA or RNA sample was obtained by dividing the copy number (derived from the standard curve) by the amount of total DNA or total RNA in each reaction.

Statistical Analysis—The Student's *t* test was used for all statistical comparisons, with *p* < 0.05 considered significant.

RESULTS AND DISCUSSION

Transfection Efficiency and Specific Hepatic Functions of Transfected Primary Rat Hepatocytes

In this study, we evaluated several transfection methods for delivering fluorescent transgenes to primary rat hepatocytes: (1) two widely used non-viral transfection agents (Lipofectamine 2000 and FuGene HD), (2) two non-viral physical force-mediated transfection methods (Amaxa Nucleofection and Magnetofection), and (3) four lentiviral vectors. We chose human hepatoma Huh7.5.1 cells⁴³ as a control.

Non-viral Transfection Agents/Methods for Gene Delivery to Hepatocytes

The transfection efficiencies in primary rat hepatocytes and Huh 7.5.1 cells using different methods are listed in Table 1. Lipofectamine 2000 and Amaxa Nucleofection are the most efficient among the non-viral transfection methods in this study, with a transfection efficiency of $33.3 \pm 1.8\%$ and $39.3 \pm 8.7\%$, respectively, compared to FuGene HD and Magnetofection ($1.31 \pm 0.62\%$ and $4.14 \pm 0.55\%$, respectively). Our data agrees with previous studies that show Lipofectamine 2000 is a preferred transfection agent for primary hepatocyte culture.^{6,11} A major challenge in *ex vivo* gene delivery to primary cells is to not only produce successful transgene expression but also maintain the specific tissue functions of the transfected cells. Therefore, we examined the hepatic functions of the primary hepatocytes transfected with Lipofectamine 2000 and Nucleofection by measuring the urea and albumin secretion (Fig. 2). Although the Nucleofection protocol has been optimized for primary hepatocytes by the manufacturer, it caused dramatic reduction in urea and albumin secretion (Figs. 2a and 2b). We also tested two other plasmids containing DsRed or red fluorescent protein transgene, which showed a similar trend (data not shown). It is well known that collagen is the preferred extra cellular matrix (ECM) substrate for primary hepatocyte cultures and the application of a top layer of collagen further improves hepatic functions *in vitro*.^{4,5} The application of the top layer of collagen on the cells, however, did not restore the urea and albumin secretion (Supplemental Materials) when subjected to Nucleofection. Interestingly, the albumin secretion dramatically decreased as early as 24 h post-transfection (Fig. 2b) while the urea secretion (Fig. 2a) gradually decreased until it was nearly undetectable by day 6, suggesting that the cells quickly lost their hepatic functions after Nucleofection. In contrast, when Lipofectamine 2000 was used, transfected cells exhibited comparable urea and albumin secretion to untreated cells (Figs. 2c and 2d), indicating that hepatic functions were minimally altered by transfection.

Fibronectin is reported to modulate the efficiency of gene transfer to cells.^{9,19} We did not observe a significant difference between fibronectin- and collagen-treated surfaces on transfection efficiency for primary rat hepatocytes using Lipofectamine 2000 (Table 1 and Supplemental Materials). Hepatocytes cultured on fibronectin, however, exhibited significantly reduced urea and albumin secretion (Supplemental Materials) compared to the cells cultured on collagen gels, which is consistent with previous studies.¹⁸

For Huh7.5.1 cells, FuGene HD exhibited the least toxicity among the tested methods (Table 1). Although Lipofectamine 2000 could achieve high transfection efficiency (>50%), the transfected Huh7.5.1 cells did not proliferate as well as the untreated cells and few cells survived for 3 days post-transfection. In summary, Lipofectamine 2000 is preferred for *ex vivo* transgene delivery to primary rat hepatocytes cultured on collagen gel while FuGene HD is the preferred transfection agent for Huh7.5.1 cells due to its low toxicity.

Viral Vectors for Gene Delivery to Hepatocytes

During the last 20 years, lentiviruses have gained considerable attention due to their lower immunogenicity compared to adenoviruses, as well as their ability to transduce non-dividing cells and maintain gene expression over the long term.²⁷ Lentiviral vectors are reported to be efficient for transgene delivery to various cell lines,⁴² primary cells and stem cells,^{26,38} including neurons,^{1,7} T cells³⁵ and hepatocytes.^{21,24,28,34,36} In this study, we examined four different lentiviral constructs containing RFP or GFP transgenes under a CMV, MND, EF-1 α , or PGK promoter and with or without the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) downstream of the transgene (Figure S1, Supplemental Materials). To our surprise, none of the lentiviral constructs produced detectable fluorescent protein expression in primary hepatocytes at MOI = 5–20, while all of them showed high transgene delivery efficiency (>80%) to Huh7.5.1 cells at MOI = 1. The result of using MND-RFP lentiviral (lentiRFP) delivery to primary rat hepatocytes and Huh7.5.1 cells at MOI = 1–5 is shown in Table 1. The representative result of using EF-1 α -EGFP lentiviral (lentiGFP) delivery to primary rat hepatocytes and Huh7.5.1 cells at MOI = 20 is shown in Fig. 3S, Supplemental Materials. Previous studies have shown similar results in quiescent rodent hepatocyte cultures. For instance, Nguyen *et al.* demonstrated that rodent hepatocytes exhibit a significant degree of resistance to human immunodeficiency virus (HIV) vector-mediated transduction while human hepatocyte cultures are highly sensitive to the same vectors.²¹ Park *et al.* also documented inefficient transduction of quiescent hepatocytes from mice and rats using lentiviral gene transfer.²² Seppen *et al.* reported low transduction efficiency ($5 \pm 1\%$) in primary rat hepatocytes using unmodified lentiviral vectors.²⁸ Our results agree with the findings in these studies. In addition, several groups have engineered lentiviral constructs for better trans-gene delivery to (1) mouse liver cells *in vivo* by incorporating HIV central polypurine and termination tract and WPRE,³⁴ (2) *ex vivo* non-human primate hepatocytes by using hepatic apolipoprotein A-II promoter,²⁴ (3) primary rat hepatocytes by incorporating HIV central polypurine tract (PPT) and HBV fragment containing enhancers and post-transcriptional regulatory element,²⁸ (4) rat hepatocytes *ex vivo* by using albumin promoter enhancer and WPRE.³⁶ It was demonstrated that primary mice hepatocytes infected with lentiviral vectors, but not human hepatocytes, lacked late viral DNA from 1 to 24 h post-infection, which indicated inefficient reverse transcription of viral RNA after virion internalization in murine hepatocytes.²¹ The four lentiviral vectors in this study exhibited high transgene delivery efficiency (>80%) to Huh7.5.1 cells at MOI = 1. These results are comparable to the results obtained by Nguyen *et al.*²¹ that a third-generation HIV-derived vector system expressing the enhanced GFP cDNA from the CMV promoter transduced more than 80% freshly isolated and cultured human hepatocytes. The discrepancy between lentiviral delivery to primary rat hepatocytes and human hepatoma Huh7.5.1 cells may be attributed to the species-dependent reverse transcription of lentiviral RNA. Our data suggests that (1) specific modifications of lentiviral vectors are necessary for significant gene delivery to rodent hepatocytes; (2) human hepatocytes Huh7.5.1 are readily susceptible to lentiviral transduction, even with the most common construct comprising VSV-G and the HIV-1 *gag*, *pol*, and *rev* genes.

DNA and RNA Levels of RFP Transgene in LentiRFP-Infected Cells

To better determine the underlying mechanisms of inefficient transgene expression in rat hepatocytes when using lentiviral vectors, we monitored the copy number of the RFP gene in genomic DNA and total RNA of lentiRFP-infected rat hepatocytes (Figure 3). First, we obtained the RFP copy number in genomic DNA and total RNA in infected Huh7.5.1 cells up to 19 days post-infection (Fig. 3a). The copy number of the RFP transgene reached a peak level in the host genome the day after infection, gradually decreased until day 7, then stabilized up to 19 days. The mRNA level of RFP followed a similar trend except for reaching its peak level on day 5 followed by a minor peak on day 7 before reaching a stable level. The average fluorescence intensity of the lentiviral-infected cells reached its highest level on day 6 and gradually decreased until day 19 before stabilizing for up to day 32. This infection kinetics is similar to a previous report describing the transfection of cardiomyocytes, *in vitro*, using a lentiviral vector containing an EGFP transgene under a CMV promoter.¹⁰

Although no significant RFP fluorescence was detected in the lentiviral-infected primary hepatocytes (Fig. 3b) compared to untreated cells at any time up to 5 days post-infection, we observed a significant amount of RFP gene present in genomic DNA and total RNA in the infected cells up to day 2; but these levels dropped dramatically afterwards. Compared to the dividing Huh7.5.1 cells in culture, primary rat hepatocytes are quiescent in culture. Because of the limited primary hepatocyte culture life-span on the dilute collagen-treated surface, we did not detect a stable level of RFP transgene level in genomic DNA and total RNA of the lentiviral-infected hepatocytes. Nevertheless, the presence of RFP in DNA and RNA indicated efficient integration of transgene into the host genome up to day 2 and significant transgene mRNA presence in cytoplasm. This infection kinetics may indicate a different mechanism for low transgene expression level from aforementioned inefficient unspliced mRNA²⁸ from the nucleus. It is notable that the integration of the RFP gene into the Huh7.5.1 genome peaked at 24 h post-infection, while this peak occurred in rat hepatocytes at 48 h post-infection; this indicates a much slower reverse transcription and integration of the viral transgene, which may explain the previously discussed lack of elongated transgene reverse transcripts up to 24 h post-infection for rat hepatocytes.²¹ In addition, the MND-RFP lentiviral construct contains WPRE element, which did not appear to increase the RFP gene delivery efficiency to rat hepatocytes. Apoptosis and necrosis of hepatocytes in culture may also be responsible for the decrease of the transgene DNA and mRNA. It is also possible that a hepatocyte-specific promoter, rather than the ubiquitous promoter used in this study, could enhance the transgene expression in rat hepatocyte culture. Nevertheless, the high transduction efficiency of all lentiviral constructs to Huh7.5.1 cells demonstrated the advantage of the lentiviral vector for gene delivery.

In summary, Lipofectamine 2000 is the superior transfection reagent for gene delivery to primary rat hepatocytes (20–30%) with minimal adverse effects on specific hepatic functions (i.e., urea and albumin secretion). Although similar transfection efficiencies can be obtained by Nucleofection of primary rat hepatocytes, urea and albumin secretion is significantly impaired in the transfected cells, which cannot be restored by culturing cells on collagen gels or in a collagen sandwich. FuGene HD and Magnetofection exhibit much lower transfection efficiency (1–2%) for primary cells but much higher efficiency for Huh7.5.1 cells. FuGene HD is the preferred non-viral transfection reagent for Huh7.5.1 cells because it is less toxic to the cells than Lipofectamine 2000. The lentiviral constructs used in this study exhibit no detectable gene delivery to primary rat hepatocytes but significant delivery to Huh7.5.1 cells (>80%). RFP gene transfer kinetics in primary rat hepatocytes and Huh7.5.1 cells may indicate species-related specificity of successful lentiviral transfection. Human hepatocytes are susceptible to lentiviral vectors with common construct consisting

VSV-G, the HIV-1 *gag*, *pol*, and *rev* genes, while successful gene delivery to rodent hepatocytes may require specific modifications of lentiviral vectors, including specific promoters^{24,36} and post-transcriptional regulatory elements.^{28,36}

FUTURE DIRECTIONS: INTEGRATION OF *EX VIVO* GENE DELIVERY, REPORTERS AND BioMEMS

Efficient *ex vivo* gene delivery to cell cultures enables manipulation of cells at the gene-level in biological microelectromechanical systems (BioMEMS), enabling high-throughput and/or real-time study of cellular functions at the molecular level.³⁹ For example, as shown in Fig. 4, we transduced Huh7.5.1 cells with the lentiviruses containing dual fluorescent genes^{32,33} (kindly provided by Dr. Stelios Andreadis at University at Buffalo). The lentiviral vector contains a transgene consisting of a constitutive DsRed gene under a PGK promoter, and a destabilized ZsGreen gene under a CMV_{min} promoter controlled by a GAS response element. On the third day after lentiviral transduction, >80% of the cells showed significant DsRed fluorescence (Figs. 4a and 4c). After addition of interferon- γ (50 ng/mL), more than 50% of the cells showed significant ZsGreen fluorescence within 24 h (Fig. 4b), while few cells showed ZsGreen fluorescence without the interferon- γ treatment (Fig. 4d); this indicated effective lentiviral transduction and GAS response to interferon- γ . Similar lentiviral reporters³² can be used to study transcription factor dynamics in various cell types, including primary human hepatocytes. For high-throughput studies, either cells of interest or vectors of interest can be patterned for spatially defined *ex vivo* or *in vitro* gene delivery. In addition, we patterned lentiviruses within collagen for reverse infection of Huh7.5.1 cells (Fig. 5). Co-culture of patterned primary cells and other stromal cells may enhance the gene delivery and/or physiological functions of primary cells. For example, Ploss *et al.* demonstrated that micropatterned cocultures (MPCCs) of primary human hepatocytes and supportive stromal cells promotes the polarization of hepatocytes, resembling *in vivo* physiological hepatic properties,¹⁵ which enabled sustained hepatitis C viral replication *in vitro*.²⁵ A powerful platform can be established for *ex vivo* manipulation of cells of interest by integrating efficient *ex vivo* gene delivery system, reporter vectors, and well defined patterning in BioMEMS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Stelios Andreadis and Feng Zhang for providing us dual fluorescent lentiviruses (GAS-RE), pVSV-G and p Δ vectors, respectively. Authors gratefully acknowledge support by NIH/NBIB (P41) EB002503 and Shriners Hospitals for Children in Boston. ES acknowledges support by Massachusetts General Hospital Fund for Medical Discovery award 217035.

REFERENCES

1. Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci.* 2005; 8:1263–1268. [PubMed: 16116447]
2. Chen NK, Sivalingam J, Tan SY, Kon OL. Plasmid-electroporated primary hepatocytes acquire quasiphenological secretion of human insulin and restore euglycemia in diabetic mice. *Gene Ther.* 2005; 12:655–667. [PubMed: 15703765]
3. Cristiano RJ, Smith LC, Woo SL. Hepatic gene therapy: adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. *Proc. Natl. Acad. Sci. USA.* 1993; 90:2122–2126. [PubMed: 8384712]

4. Dunn JC, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnol. Prog.* 1991; 7:237–245. [PubMed: 1367596]
5. Dunn JC, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. *FASEB J.* 1989; 3:174–177. [PubMed: 2914628]
6. Edwards M, et al. Transfection of primary cultures of rat hepatocytes. *Methods Mol. Biol.* 2006; 320:273–282. [PubMed: 16719399]
7. Ehrenguber MU, et al. Gene transfer into neurons from hippocampal slices: comparison of recombinant Semliki Forest Virus, adenovirus, adeno-associated virus, lentivirus, and measles virus. *Mol. Cell. Neurosci.* 2001; 17:855–871. [PubMed: 11358483]
8. Enoru-Eta J, et al. Development of a UGT1A1 reporter gene assay for induction studies: correlation between reporter gene data and regulation of UGT1A1 in human hepatocytes. *Drug Metab. Lett.* 2010; 4:31–38. [PubMed: 20201778]
9. Esendagli G, et al. Transfection of myeloid leukaemia cell lines is distinctively regulated by fibronectin substratum. *Cytotechnology.* 2009; 61:45–53. [PubMed: 19967401]
10. Fleury S, et al. Multiply attenuated, self-inactivating lentiviral vectors efficiently deliver and express genes for extended periods of time in adult rat cardiomyocytes in vivo. *Circulation.* 2003; 107:2375–2382. [PubMed: 12695294]
11. Gardmo C, Kotokorpi P, Helander H, Mode A. Transfection of adult primary rat hepatocytes in culture. *Biochem. Pharmacol.* 2005; 69:1805–1813. [PubMed: 15885657]
12. Herrero MJ, et al. Analysis of metabolic and gene expression changes after hydrodynamic DNA injection into mouse liver. *Biol. Pharm. Bull.* 2011; 34:167–172. [PubMed: 21212539]
13. Janorkar AV, King KR, Megeed Z, Yarmush ML. Development of an in vitro cell culture model of hepatic steatosis using hepatocyte-derived reporter cells. *Biotechnol. Bioeng.* 2009; 102:1466–1474. [PubMed: 19061238]
14. Kay MA. State-of-the-art gene-based therapies: the road ahead. *Nat. Rev. Genet.* 2011; 12:316–328. [PubMed: 21468099]
15. Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat. Biotechnol.* 2008; 26:120–126. [PubMed: 18026090]
16. King KR, et al. A high-throughput microfluidic realtime gene expression living cell array. *Lab Chip.* 2007; 7:77–85. [PubMed: 17180208]
17. Love Z, et al. Imaging of mesenchymal stem cell transplant by bioluminescence and PET. *J. Nucl. Med.* 2007; 48:2011–2020. [PubMed: 18006616]
18. Mooney D, et al. Switching from differentiation to growth in hepatocytes: control by extracellular matrix. *J. Cell. Physiol.* 1992; 151:497–505. [PubMed: 1295898]
19. Moritz T, et al. Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymo-tryptic carboxy-terminal fragments. *Blood.* 1996; 88:855–862. [PubMed: 8704241]
20. Nakai H, et al. Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. *J. Virol.* 2005; 79:214–224. [PubMed: 15596817]
21. Nguyen TH, et al. Highly efficient lentiviral vector-mediated transduction of nondividing, fully reimplantable primary hepatocytes. *Mol. Ther.* 2002; 6:199–209. [PubMed: 12161186]
22. Park F, Ohashi K, Chiu W, Naldini L, Kay MA. Efficient lentiviral transduction of liver requires cell cycling in vivo. *Nat. Genet.* 2000; 24:49–52. [PubMed: 10615126]
23. Park JS, Surendran S, Kamendulis LM, Morral N. Comparative nucleic acid transfection efficacy in primary hepatocytes for gene silencing and functional studies. *BMC Res Notes.* 2011; 4:8. [PubMed: 21244687]
24. Parouchev A, et al. Efficient ex vivo gene transfer into non-human primate hepatocytes using HIV-1 derived lentiviral vectors. *J. Hepatol.* 2006; 45:99–107. [PubMed: 16723167]
25. Ploss A, et al. Persistent hepatitis C virus infection in microscale primary human hepatocyte cultures. *Proc. Natl. Acad. Sci. USA.* 2010; 107:3141–3145. [PubMed: 20133632]

26. Robinson DA, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* 2003; 33:401–406. [PubMed: 12590264]
27. Sakoda T, Kasahara N, Kedes L, Ohyanagi M. Lentiviral vector-mediated gene transfer to endothelial cells compared with adenoviral and retroviral vectors. *Prep. Biochem. Biotechnol.* 2007; 37:1–11. [PubMed: 17134978]
28. Seppen J, Rijnberg M, Cooreman MP, Oude Elferink RP. Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes. *J. Hepatol.* 2002; 36:459–465. [PubMed: 11943415]
29. Sheahan S, Bellamy CO, Treanor L, Harrison DJ, Prost S. Additive effect of p53, p21 and Rb deletion in triple knockout primary hepatocytes. *Oncogene.* 2004; 23:1489–1497. [PubMed: 14647424]
30. Sizovs A, McLendon PM, Srinivasachari S, Reineke TM. Carbohydrate polymers for nonviral nucleic acid delivery. *Top. Curr. Chem.* 2010; 296:131–190. [PubMed: 21504102]
31. Thompson DM, et al. Dynamic gene expression profiling using a microfabricated living cell array. *Anal. Chem.* 2004; 76:4098–4103. [PubMed: 15253648]
32. Tian J, Alimperti S, Lei P, Andreadis ST. Lentiviral microarrays for real-time monitoring of gene expression dynamics. *Lab Chip.* 2010; 10:1967–1975. [PubMed: 20520864]
33. Tian J, Andreadis ST. Independent and high-level dual-gene expression in adult stem-progenitor cells from a single lentiviral vector. *Gene Ther.* 2009; 16:874–884. [PubMed: 19440229]
34. VandenDriessche T, et al. Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. *Blood.* 2002; 100:813–822. [PubMed: 12130491]
35. Verhoeven E, Costa C, Cosset FL. Lentiviral vector gene transfer into human T cells. *Methods Mol. Biol.* 2009; 506:97–114. [PubMed: 19110622]
36. Wang X, Mani P, Sarkar DP, Roy-Chowdhury N, Roy-Chowdhury J. Ex vivo gene transfer into hepatocytes. *Methods Mol. Biol.* 2009; 481:117–140. [PubMed: 19096805]
37. Wang Z, Yuan Z, Jin L. Gene delivery into hepatocytes with the preS/liposome/DNA system. *Biotechnol. J.* 2008; 3:1286–1295. [PubMed: 18830969]
38. Wang F, et al. Transcriptional profiling of human mesenchymal stem cells transduced with reporter genes for imaging. *Physiol. Genomics.* 2009; 37:23–34. [PubMed: 19116247]
39. Wheeler DB, et al. RNAi living-cell microarrays for loss-of-function screens in *Drosophila melanogaster* cells. *Nat. Methods.* 2004; 1:127–132. [PubMed: 15782175]
40. Wieder KJ, et al. Optimization of reporter cells for expression profiling in a microfluidic device. *Biomed. Microdevices.* 2005; 7:213–222. [PubMed: 16133809]
41. Yee JK, et al. A general method for the generation of high-titer, pantropic retroviral vectors: highly efficient infection of primary hepatocytes. *Proc. Natl. Acad. Sci. USA.* 1994; 91:9564–9568. [PubMed: 7937806]
42. Zhang B, et al. The significance of controlled conditions in lentiviral vector titration and in the use of multiplicity of infection (MOI) for predicting gene transfer events. *Genet. Vaccines Ther.* 2004; 2:6. [PubMed: 15291957]
43. Zhong J, et al. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA.* 2005; 102:9294–9299. [PubMed: 15939869]

FIGURE 1.

Survey of conventional gene delivery schemes. Cationic polymer-mediated delivery: Negatively charged nucleic acids self-assemble with cationic polymers or lipids and produce nucleic acid-polymer nanoparticles or liposomes. The net positive charge on the complex of nucleic acid-polycations or lipids attracts the complexes to the negatively charged cell membrane, thereby increasing the probability of endocytosis. The complex is then internalized by the cells within an endosome. If the complex or nucleic acid successfully escapes into the cytosol from the endosome before it develops into a lysosome (where the content is degraded), the escaped nucleic acids can be transported into nucleus where the transgene is transcribed into mRNA using the cellular genetic machinery and then translated

into the protein of interest in the cytosol. Magnetofection: Polycation-coated magnetic beads and nucleic acids form complexes for gene delivery. An external magnetic field was applied during the transfection in order to promote interaction of the cell membrane with the nucleic acid-magnetic bead complex, thereby enhancing endocytosis. The internalization and intracellular trafficking of the complex follow a similar pathway to that of the nucleic acids delivered *via* cationic polymers/lipids. Electroporation: An external electric field is applied to the suspension of highly concentrated cells and nucleic acids in order to transiently increase the permeability of the cellular and nuclear membranes by creating pores large enough to accommodate translocation of neat nucleic acids directly into the nucleus. These nucleic acids are subsequently transcribed into mRNA and translated into proteins. Viral delivery: Viral particles that carry the nucleic acid of interest attach to specific receptors on the cell membrane. For lentiviruses, the viral RNA is delivered into the cells, reverse-transcribed into DNA and translocated into the nucleus. For adenoviruses, the viral DNA is delivered into the cell and then into the nucleus. The nucleic acids then progress through the processes that lead to translation into proteins.

FIGURE 2.

Urea and albumin secretion of transfected primary rat hepatocytes. (a) Urea secretion by transfected primary rat hepatocytes using Nucleofector. (b) Albumin secretion by transfected primary rat hepatocytes using Nucleofector. (c) Urea secretion by transfected primary rat hepatocytes using Lipofectamine 2000. (d) Albumin secretion by transfected primary rat hepatocytes using Lipofectamine 2000.

FIGURE 3. DNA and RNA level of RFP transgene in lentiRFP-infected Huh7.5.1 cells and primary rat hepatocytes. (a) Huh7.5.1 cells, (b) Primary rat hepatocytes.

FIGURE 4.

Lenti-GAS-RE infected Huh7.5.1 cells. (a) and (b): DsRed channel and ZsGreen channel of Huh7.5.1 cells 92 h post lentiviral infection and 44 h post IFN- γ removal; (c) and (d): DsRed channel and ZsGreen channel of Huh7.5.1 cells 92 h post lentiviral infection.

FIGURE 5.

Patterned lentiviral delivery (EGFP) to Huh7.5.1 cells. (a) Schematic of patterned lentiviral delivery to Huh7.5.1 cells; (b) and (c) Green fluorescent channel and bright field of Huh7.5.1 cells cultured on collagen containing lentiviruses 72 h post-seeding.

TABLE 1

Ex vivo gene delivery efficiency in primary rat hepatocytes and Huh7.5.1 cells.

Gene delivery method/agent	Vector	Culture surface treatment for hepatocytes	Transfection efficiency (# viable fluorescent cells/# viable cells)	
			Hepatocytes	Huh7.5.1
FuGene HD	pmaxGFP	50 µg/mL collagen	1.3 ± 0.6%	12.5 ± 3.2%
Lipofectamine 2000	pmaxGFP	50 µg/mL collagen; 1.11 mg/mL collagen gel	33.3 ± 1.8% ^a	48.6 ± 6.7% ^b
Nucleofection	pmaxGFP	1.11 mg/mL collagen gel	39.3 ± 8.7% ^a	—
Magnetofection	pmaxGFP	1.11 mg/mL collagen gel	4.1 ± 0.5%	7.6 ± 1.8%
Lentiviruses	pLVR	50 µg/mL collagen	Undetectable	63.2 ± 7.8% (MOI = 1)

For non-viral transfection, data was obtained 24–48 h post-transfection. For lentiviral gene delivery, data was obtained 72–96 h post-transfection. Primary rat hepatocytes were cultured up to 5 days while Huh7.5.1 cells were cultured up to 30 days with necessary passaging unless otherwise indicated.

^aThe specific hepatic functions, i.e., urea and albumin secretion, were analyzed for these samples and listed in Fig. 2.

^bData obtained 24 h post-transfection because majority of the cells under-went apoptosis 48 h post-transfection and the cells were cultured for 4 days when few viable cells left in the culture wells.