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Serum Starvation Improves Transient Transfection Efficiency in Differentiating Embryonic Stem Cells

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Abstract

Control of genetic expression is a critical issue in the field of stem cell biology, where determining a cell fate or reprogramming adult somatic cells into pluripotent cells has become a common experimental practice. In turn, for these cells to have therapeutic clinical potential, techniques for controlling gene expression are needed that minimizes or eliminates the risk of oncogenesis and mutagenesis. Possible routes for achieving this outcome could come in the form of a transient nonviral gene delivery system. In this study, we improved the efficiency of transient gene delivery to differentiating murine embryonic stem (ES) cells via serum starvation for 3 days before transfection. The transient expression of a constitutively-controlled plasmid increased from ~50% (replated control) to ~83% when transfected after 3 days of serum starvation but decreased to ~28% when transfected after 3 days in normal high serum-containing media. When probed with a liver-specific reporter, Cyp7A1, expression increased from ~1.4% (replated control) to ~3.7% when transfected after 3 days of serum starvation but decreased to ~0.7% when transfected after 3 days in high serum-containing media. Cy3-tagged oligonucleotides were used to rapidly quantify DNA uptake and predict ultimate transfection efficiency. This study suggests that modifications in media serum levels before transfection can have a profound effect on improving nonviral gene delivery.

Keywords

serum deprivation; stem cells; gene delivery; Cyp7A1; hepatocyte

Introduction

The efficient delivery of DNA using nonviral plasmid vectors has been a major challenge in the fields of gene therapy, stem cell research, cellular therapeutics, and RNAi/oncology.^{1–5} Over the past decade, novel engineered materials have been developed along with quantitative physical characterization assays in an attempt to meet the highly efficient transduction capacity of viral vectors, such as retroviral and lentiviral systems, while maintaining a high level of safety, minimal toxicity, robustness for scale-up and the ability to carry large cargo.^{2,6–9} Recently, we developed a transient gene delivery system to deliver two fluorescent liver-specific reporter plasmids into differentiating, semi-mature murine embryonic stem (ES) cells for enriching a subpopulation of hepatocyte-like cells.¹⁰ The

benefit of the transient expression of the plasmids fulfilled the system's needs, as the activation of the fluorescent reporters was only necessary up to and through the completion of the cell sorting. Nonetheless, the scale-up of this system is limited by inherent low plasmid transfection efficiency. Improving the transfection efficiency to this population could dramatically improve the targeting of smaller subpopulations of cells to attain better sensitivity of expression values and to improve the recovery fraction. Recently, many groups have targeted adult somatic cells to generate induced pluripotent stem (iPS) cells by using retroviral vectors to express genes associated with pluripotency.^{11–13} Despite the success of these techniques and to realize the therapeutic potential of these cells in the future, the studies highlight the need to develop alternate delivery methods that would minimize the risk of oncogenesis due to the random insertion of genes.^{14,15}

The aim of this work was to explore the role of serum starvation in improving nonviral gene delivery (i.e., transfection) to mammalian cells, with a particular focus on differentiating ES cells. Serum starvation, the dramatic deprivation of the portion of serum in media, is a cell culture technique used in a variety of applications, including cell cycle synchronization at the G_0/G_1 phase, reduction of cellular activities to basal levels by inactivating growth factor-stimulated kinases and the induction of quiescence and/or apoptosis.^{16–18} Different cell types respond differently, if at all, to the degree and length of serum starvation.¹⁹ The serum deprivation response gene (SDR or SDPR) is expressed in serum-starved cells and is believed to be activated in a pathway distinct from cell–cell contact inhibition.^{20,21} In this study, we assessed the effect of serum starvation on the uptake of Cy3-tagged 20-mer oligonucleotides and the transfection efficiencies of reporters driven by either the constitutively-expressed cytomegalovirus (CMV) promoter or the liver-specific cytochrome P450 7a1 (Cyp7A1) promoter.

In earlier work, we found that purifying differentiating ES cells expressing the Cyp7A1-driven reporter yields greater hepato-specific functional enrichment than cells sorted with a more ubiquitous reporter driven by the albumin enhancer/promoter.¹⁰ In the current study, we first assessed the role of serum starvation in modulating transfection efficiency using a general constitutive CMV-driven plasmid. We then specifically targeted liver-specific cells using the Cyp7A1 reporter in an effort to purify the number of cells in this subpopulation expressing the reporter. We determined that serum starvation did not disrupt the genomic nor functional integrity of the cells. In addition, Cy3 oligonucleotides proved to be a useful tool in accurately predicting long-term transfection efficiency. A better understanding of the mechanisms of DNA uptake and cell-specific gene transfer idiosyncrasies may lead to the development of novel, safe, and efficient nonviral gene delivery solutions in the future.

Materials and Methods

Cell culture

The ES cell line D3 (ATCC, Manassas, VA) was maintained in an undifferentiated state in T-75 gelatin-coated flasks (Bio-coat, BD-Biosciences, Bedford, MA) in Knockout Dulbecco's Modified Eagles Medium (Gibco, Grand Island, NY) containing 15% knockout serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), 10 μ g/mL gentamicin (Gibco), 1000 U/mL ESGRO™ (Chemicon,

Temecula, CA), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). ESGRO™ contains leukemia inhibitory factor (LIF), which prevents ES cell differentiation. Every 2 days, media was aspirated and replaced with fresh media. Cultures were split and passaged every 5–6 days. Cells were detached following incubation with trypsin (0.25%)-EDTA (Gibco) for 2 min, resulting in a single cell suspension, followed by the addition of Knockout DMEM. Cells were then replated in gelatin-coated T-75 flasks at a density of 10⁶ cells/mL.

To induce differentiation, cells were suspended in Iscove's Modified Dulbecco's Medium (Gibco) containing 20% fetal bovine serum (Gibco), 4 mM L-glutamine (Gibco), 100 U/mL penicillin, 100 U/mL streptomycin (Gibco), 10 µg/mL gentamicin (Gibco). Embryoid bodies were formed and cultured for 3 days using the hanging drop method (1000 ES cells per 30 µL droplet). Hanging drops were transferred to suspension culture in 100 mm Petri dishes and cultured for an additional day. The EBs were then plated, one EB per well, in 12-well tissue culture polystyrene plates (BD-Biosciences) for an additional 13 days. Culture medium was changed every 3 days. EB cells were detached following incubation with 0.5 mL of trypsin-EDTA (Gibco) for 2 min, resulting in a single cell suspension, and neutralized by the addition of IMDM media. Cells were then replated in 12-well tissue culture treated polystyrene plates (BD-Biosciences) at an initial seeding density of 5 × 10⁴ day 17 cells per well for further manipulation. These cells are hereby referred to as day 17 differentiating ES cells.

The Hepa 1–6 cell line (ATCC, Manassas, VA) was maintained in Dulbecco's Modified Eagles Medium (Gibco) containing 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), and 4 mM L-glutamine (Gibco). Hepa 1–6 cells were grown on tissue culture treated T-75 flasks (Falcon, BD Biosciences, San Jose, CA).

All cell cultures were incubated in a humidified 37°C, 5% CO₂ environment.

Cloning of cytochrome p450 7α1 (Cyp7A1) promoter into pDsRedExpress1 vector

The pCyp7A1-DsRedExpress1 vector was prepared as previously described.¹⁰ An additional vector, pDsRed2-C1 (Clontech), driven by the constitutive CMV immediate early promoter, was used as a positive control for transfection and as a negative control for sorting of the differentiating ES cells.

Selection of Cy3 oligonucleotide

The Cy3 fluorescently-tagged oligonucleotide was obtained from Integrated DNA Technologies (Coralville, IA). The 20-base sequence modified at the 5' end with a Cy3 fluorescent dye consisted of random oligonucleotides selected for each base. The GC content of the oligonucleotides as reported by the manufacturer was 51.6%.

Particle size and zeta potential measurements

To evaluate the physicoelectrical properties of the DNA lipoplexes, we characterized both the particle size and zeta potential of the two DNA lipoplexes: the Cy3 oligonucleotide/Lipofectamine™ 2000 complex and the plasmid DNA/Lipofectamine™ 2000 complex after

20 min of particle complex formation. The solutions were diluted in Opti-MEM Reduced-Serum Medium (Invitrogen, Carlsbad, CA) at an equivalent volume that would be added to a cell culture well and analyzed using a Brookhaven Particle Size and ZetaPALS Analyzer (Holtsville, NY). The ratio of 3.2 μg DNA: 4.0 μL reagent ratio (for 1 mL final solution volume) was identical in both complexes. For the plasmid DNA, we used the pDsRed2-C1 plasmid, as this vector would be used as a measure of overall transfection efficiency in all cell types tested. We found that the Cy3 oligonucleotide lipoplex particles had a diameter of 749.7 ± 100.3 nm, while the plasmid DNA lipoplex particles had a diameter of 766.0 ± 87.8 nm. The zeta potential of the Cy3 oligonucleotide lipoplex particles was 13.8 ± 8.5 mV, and that of the plasmid DNA lipoplex particles was 13.5 ± 9.8 mV. By altering the ratio of the DNA: LipofectamineTM 2000, we could adjust the sizes and zeta potentials of the two lipoplexes and bring them within close range of each other. The size and zeta potential differences between the Cy3 oligonucleotide lipoplex and the plasmid DNA lipoplex did not hamper its function as a predictor of DNA uptake.

Transient transfection of differentiating stem cells and hepatoma cells

The liver-specific expression vector, pCyp7A1-DsRedExpress1, along with the constitutive pDsRed2-C1 plasmid, were transiently transfected using LipofectamineTM 2000 (Invitrogen) into the replated and media-conditioned differentiating stem cells. Only the pDsRed2-C1 vector was transfected into the replated or media-conditioned Hepa 1–6 hepatoma cells. A DNA: LipofectamineTM 2000 ratio of 0.8 μg DNA: 1.0 μL reagent (optimized based on prior work) was used in a 48-well plate.¹⁰ This ratio was conserved while volumes were adjusted as per the manufacturer's protocol for different sized well plates. The transfection complexes were prepared and delivered in Opti-MEM reduced-serum medium and replenished with serum-containing media after 4 h. Visual cell confluency of cells was maintained so that cells in the 40–70% surface coverage range were chosen for transfection.

Serum starvation and sample collection

The EBs were trypsinized and replated in IMDM media containing 20% FBS. Cells were initially seeded at different densities in parallel wells to compensate for varying proliferative rates. The control population of differentiating ES cells consisted of cells transfected with the Cy3 oligonucleotides or the reporter plasmids within 6 h of replating. Media conditioning was initiated by replating cells for 4–6 h in 20% FBS-containing IMDM media, removing the media and replenishing these cells with IMDM media containing 0.5% FBS (serum-starved condition) or 20% FBS (high serum) for 1, 2, and 3 days, after which they were immediately transfected with the Cy3 oligonucleotides or the reporter plasmids. Hepa 1–6 cells were plated in DMEM media containing 10% FBS. The Hepa 1–6 control population consisted of cells transfected with the Cy3 oligonucleotides or the reporter plasmids within 6 h of replating. Media-conditioned Hepa 1–6 cells were replated for approximately 4–6 h, at which point the media was removed and replenished with DMEM media containing 0.5% FBS (serum-starved condition) or 10% FBS (high serum) for an additional 3 days. After 3 days, they were immediately transfected with the Cy3 oligonucleotides or the reporter plasmids.

Cells transfected with the Cy3 oligonucleotides were trypsinized 12 min after addition of the complexes, and fluorescent activity/particle uptake was determined via flow cytometry. Cells transfected with the reporter plasmids were trypsinized after 48 h, and red fluorescent activity/transfection efficiency was confirmed using a computer-interfaced inverted Olympus IX70 microscope and detected via flow cytometry.

Flow cytometry and cell sorting

Cell preparation, flow cytometry and cell sorting using the BD FACSCalibur™ (San Jose, CA) was conducted as previously described.¹⁰ The yield of cells recovered in each sort was ~80% of the number of cells gated and counted by the flow cytometer. Once the sorted cells adhered to the cell culture plates (~4 h after the sort), media was replenished after to remove any remaining contaminants or debris.

F-actin visualization, phase-contrast imaging and determination of cell area

A rhodamine-phalloidin actin filament (F-actin) staining kit was obtained from Cytoskeleton (Denver, CO). Cells were washed with buffer for 30 s, fixed with a formaldehyde-based fixative solution for 10 min, washed for 30 s, permeabilized for 5 min and washed for another 30 s, all at room temperature. Samples were stained with the rhodamine-phalloidin dye for 30 min at room temperature in the dark. Cells were washed three times with buffer and with PBS. Actin filaments were then visualized via fluorescence microscopy (excitation filter 525 nm; emission filter 585 nm) using an inverted Olympus IX70 microscope and a confocal Olympus IX81 microscope. We were specifically interested in overall F-actin presence and the formation of bundles of filaments, which we will refer to as stress fibers.²² Phase-contrast images were acquired using an Olympus IX70 inverted microscope. To determine two-dimensional cell area, at least 10 cells were selected from three distinct images of each respective condition using the Olympus Microsuite™ software. Cell regions of interest (ROIs) were defined by manually encircling cell membranes. The ROIs were then detected using the software, and the particle results yielded cell area values in pixel² units.

Apoptotic DNA detection

An apoptotic DNA ladder kit was obtained from Roche Diagnostics (Indianapolis, IN) to detect the presence of apoptotic or necrotic cell death. At each collection time point, cells were trypsinized, diluted in PBS, centrifuged and frozen at -20°C until analysis. At the time of analysis, binding/lysis buffer was added to the cell pellet and a positive control from the kit (lyophilized apoptotic U937 cells), vortexed for 5–10 s and incubated for 10 min at room temperature. Isopropanol was added to this solution, vortexed briefly, and the solution was placed in a collection column. The column was centrifuged for 1 min at 8000 RPM on table top centrifuge to bind the nucleic acids to the filter. The filter was washed and the sample eluted. The media-conditioned samples, U937 positive control cells and a 100 bp DNA step ladder (New England Biolabs, Beverly, MA) were run through a 1% agarose tris-borate EDTA (1X) gel containing ethidium bromide for 60 min at 100 V. The gel was then visualized and a digital image printed using a UV Transilluminator (UVP, Upland, CA). Apoptotic samples would be indicated by a nonrandom fragmentation ladder pattern of DNA on the gel. Necrotic samples would be indicated by a randomly-digested DNA smear. Non-

necrotic and nonapoptotic cells would be indicated by a single band comprising the entire genomic DNA.

Urea secretion

Media samples were collected directly from cell cultures 2 days after replating from the cell sort and stored at -20°C for subsequent analysis for urea content. Urea secretion was assayed as previously described.¹⁰

Sandwich ELISA for detection of albumin secretion

Media samples were collected directly from cell cultures 2 days after replating from the cell sort and stored at -20°C for subsequent analysis. Albumin secretion was detected as previously described.¹⁰

Measurement of cytochrome p450 1A2 activity

Cytochrome P450 1A2 (Cyp1A2) activity was induced by treatment with 3-methylcholanthrene ($2\ \mu\text{M}$) (Sigma–Aldrich) for 48 h before the activity assay. Cytochrome P450-dependent resorufin *O*-dealkylase activity was measured using a methoxyresorufin (MROD) substrate from a Resorufin Sampler Kit (Invitrogen) as previously described.¹⁰

Statistical analysis of measurements

Each data point represents the mean of three or more experiments, and the error bars represent the standard deviation of the mean. Statistical significance between two treatment groups was determined using the Student's *t*-test for unpaired data. One-way ANOVA was used to compare the effect of day of treatment and media conditioning of the differentiating ES cells versus replated differentiating ES control in the particle uptake, transfection efficiency and reporter expression studies, and to compare the Hepa 1–6 replated control to media-conditioned Hepa 1–6 cells. Individual comparisons were then made using a post hoc Dunnett's multiple comparisons test. Two-way ANOVA was used to determine the interaction between the plasmids used to sort the differentiating ES cells versus type of media conditioning in the cell functional integrity studies, with individual comparisons made using post hoc Bonferroni testing. Differences were considered significant if the *P*-value was less than or equal to 0.05. GraphPad Prism 5 software was used for statistical analyses.

Results

Effect of serum starvation on uptake of Cy3 oligonucleotides

The initial steps of DNA transfection consist of the absorption and uptake of DNA across the plasma membrane. Therefore, experiments were designed to determine whether serum starvation could increase DNA uptake and whether this would ultimately predict transfection efficiency. Measuring the fluorescence of Cy3 oligonucleotide lipoplex particles inside cells shortly after transfection (i.e., after 12 min) was facilitated via flow cytometry and enabled rapid quantification of the percentages of cells that acquired the DNA. To assess the effect of serum changes at different time points, we explored seven different conditions. Day 17 differentiating ES cells were trypsinized from their culture environment, and a control group

consisted of cells that were replated and transfected within 6 h. To assess the effects of long-term media conditioning, six additional groups of cells consisted of cells that were replated after trypsinization and then replenished with either IMDM media containing 20% FBS (high serum) or IMDM media containing 0.5% FBS (serum-starved) for 1, 2 and 3 days. For each condition, Cy3 oligonucleotides were transfected into the cells in Opti-MEM media without serum. Cells were sampled 12 min after the addition of the Cy3 oligonucleotide lipoplexes (Figure 1A). The control cell population (transfected within 6 h of replating) had an uptake level of ~40% of the cell population. Serum starvation for 1 or 2 days did not lead to any significant changes in DNA uptake. The 3-day serum-starved condition, however, had the greatest DNA particle uptake ($P < 0.01$; one-way ANOVA for treatment condition followed by post hoc Dunnett's multiple comparisons tests) as compared to the control, with ~70% of the cells containing Cy3 oligonucleotides. The differentiating ES cells treated with high serum for 1, 2, and 3 days all had a decrease in uptake as compared to the control ($P < 0.05$ for 1, 2, and 3 days; one-way ANOVA for treatment condition followed by post hoc Dunnett's multiple comparisons tests), where less than ~15% of the cells transfected at each of these days contained particles 12 min after transfection. We also tested whether naked Cy3 oligonucleotides could be transfected without complexing with Lipofectamine™ 2000. We found that less than 2% of the cells in any condition take up the Cy3 oligonucleotides by 12 min (data not shown).

Correlation of Cy3 oligonucleotide uptake to CMV plasmid transfection efficiency with media conditioning

Next, we explored the correlation of Cy3 oligonucleotide uptake rates to the transfection efficiency of the constitutive CMV plasmid. The seven experimental groups described above (i.e., control, serum-starved for 1, 2 and 3 days, and high serum for 1, 2, and 3 days) were transfected with pDsRed2-C1 plasmid DNA complexed with Lipofectamine™ 2000 as described in the Materials and Methods section. After 48 h, the number of fluorescent cells was quantified via flow cytometry (Figure 1B). Compared to the control replated ES cell group, which had an efficiency of ~50% of the total cell population, the transfection efficiency of the serum-starved populations increased after 2 and 3 days of conditioning, with the greatest efficiency noted in the 3-day serum-starved group (~83%) ($P < 0.01$; one-way ANOVA for treatment condition followed by post hoc Dunnett's multiple comparisons tests). The transfection efficiency of the high serum populations did not change significantly after 1 or 2 days. However, with 3 days of incubation in high serum-containing media, transfection efficiency dropped to ~28% ($P < 0.05$; one-way ANOVA for treatment condition followed by post hoc Dunnett's multiple comparisons tests). Cells serum-starved for 4 or 5 days were not transfected as significant cell death (> 10%) and diminishment of hepato-specific functions became apparent beyond 3 days (data not shown).

Cyp7A1 expression rates with media conditioning

Once we established that serum starvation could improve transfection efficiency of a constitutively-expressed CMV promoter-driven plasmid, we sought to determine if these conditions would enhance the numbers of cells expressing a liver-specific Cyp7A1 promoter-driven plasmid. The control and media-conditioned differentiating ES cell groups were transfected with the pCyp7A1-DsRedExpress1 plasmid and assessed in a manner

identical to that of the pDsRed2-C1 plasmid transfection (Figure 1C). We found that with respect to the replated ES cell population, which showed expression in ~1.5% of all cells, the serum-starved groups exhibited the greatest enhancement of expression with 3 days of serum starvation (~3.7%) ($P < 0.05$; one-way ANOVA for treatment condition followed by post hoc Dunnett's multiple comparisons tests). The cells treated with high serum for 1–3 days showed no significant change in Cyp7A1 expression, as compared to the control replated ES cells. We did not test Cyp7A1 reporter expression on cells beyond the 3-day serum starvation point due to loss of basal function (data not shown).

Use of the Cy3 oligonucleotides to predict transfection efficiency in serum starvation of a non ES-cell type

To determine if the serum starvation media conditioning could be applied to another hepatocyte-like cell type and whether the Cy3 oligonucleotides could be used to predict such trends, we explored the media conditioning of Hepa 1–6 mouse hepatoma cells. Based on the Cy3 oligonucleotide assessment, both 3-day serum-starved (0.5% FBS) and high serum-treated (10% FBS) groups of Hepa 1–6 cells demonstrated less DNA uptake than that of the replated Hepa 1–6 control cells ($P < 0.05$; one-way ANOVA for treatment condition followed by post hoc Dunnett's multiple comparisons tests) (Figure 1D). In parallel, the transfection efficiencies of the Hepa 1–6 cells were lower in both the serum-starved and high serum-treated ($P < 0.05$; one-way ANOVA for treatment condition followed by post hoc Dunnett's multiple comparisons tests) conditions as compared to the replated Hepa 1–6 control cells (Figure 1E).

Microscopic evaluation of media-conditioned cells

In an effort to understand the intracellular changes that accompany serum starvation, we examined the overall morphology, presence and structure of F-actin in the media-conditioned differentiating ES cells and Hepa 1–6 cells. Phase-contrast images of the high serum-treated differentiating ES cells (Figure 2A) revealed flat and uniformly-sized cells, as compared to the serum-starved differentiating ES cells (Figure 2B), which appeared thinly spread out with the exception of an elevation around what is presumably the nucleus. We stained the differentiating ES cells and Hepa 1–6 cells with a rhodamine-phalloidin dye and compared the effect of media conditioning. F-actin fibers were strongly present in differentiating ES cells after replating (Figure 2C) and after treatment with high serum for 3 days (Figure 2D). However, minimal F-actin staining was noted for the serum-starved differentiating ES cells (Figure 2E). We then stained 3-day serum-starved cells that were fixed 10 min after transfection with Cy3 oligonucleotides and found that a greater number of F-actin stress fibers appeared to have formed (Figure 2F). F-actin staining of the high serum-treated Hepa 1–6 cells (Figure 2G) and serum-starved Hepa 1–6 cells (Figure 2H) showed similar distribution patterns of F-actin in both populations around the plasma membrane.

We used the F-actin staining images to quantify and compare the two-dimensional cell area of the media-conditioned differentiating ES and Hepa 1–6 cells (Figure 2I). We found that the differentiating ES cells serum-starved for 3 days showed greater average area (pixel²) than the cells treated with high serum ($P < 0.01$; Student's *t*-test). In the 3-day media-conditioned Hepa 1–6 cells, however, both groups showed similarly-sized cell areas.

Changes in cell morphology and two-dimensional area were consistent with F-actin ultrastructural changes, which appeared to occur concomitantly with the effect of serum starvation on cell transfectability.

Proliferative ability and functional evaluation of cells during and following media conditioning

To assess the proliferative ability of the media-conditioned cells, we plotted cell growth by uniformly plating cells into a multiwell plate and counting the number of cells 6 h after replating and after 1, 2, and 3 days of media conditioning (Figure 3A). Cells treated with high serum grew at a quicker rate than the serum-starved cells. At all test points of media conditioning, negligible number of cells appeared to be detached and less than 5% of the cells were dead as determined by Trypan blue exclusion staining. At 4 and 5 days of serum starvation, the number of dead cells in each sample exceeded 10% of the population (data not shown). Despite the fact that the growth rate of cells under serum starvation is lower than that of high serum-treated cells, the yield of transfected and recoverable Cyp7A1-expressing serum-starved cells was nearly 10-fold that of the replated control and ~2.25-fold that of the high serum-treated cells [i.e., 3046 Cyp7A1 (+) 3-day serum-starved cells (3.69% of 82,555) versus 1346 Cyp7A1 (+) 3-day high serum-treated cells (0.67% of 201,000) versus 337 Cyp7A1 (+) replated control cells (1.53% of 22,000)].

To ascertain whether media conditioning led to apoptosis or necrosis in the media-conditioned cells, we extracted genomic DNA from all treatment conditions and ran them via gel electrophoresis. In the event of apoptosis, the genomic DNA would produce step bands in its lane. In the event of necrosis, the genomic DNA would smear across the lane. All media-conditioned treated cells produced one solid band without smearing (Figure 3B), qualitatively indicating neither apoptosis nor necrosis occurred under any treatment condition. A positive control sample of apoptotic cells is shown in lane B producing step bands.

Finally, we evaluated the effect of media conditioning on the functional capacity of the differentiating ES cells. We sorted the cells after three days of media conditioning using both the CMV and the Cyp7A1 reporter plasmids. The CMV plasmid was used as a negative control for the cell sort. The Cyp7A1 plasmid was used as a liver-specific reporter to target the hepatocyte-like subpopulation of differentiating ES cells. Cells were replated after sorting for an additional two days and assessed for urea secretion (Figure 3C), albumin secretion (Figure 3D) and Cyp1A2 detoxification activity (Figure 3E). In all three assays, the media conditioning did not lead to any significant functional difference between the serum-starved and high serum-treated groups. The Cyp7A1-sorted cells were significantly enriched from their CMV-sorted counterparts in the three functional assays ($P < 0.01$ for urea secretion; $P < 0.001$ for albumin secretion; $P < 0.05$ for Cyp1A2 activity; two-way ANOVA for reporter plasmid followed by Bonferroni post hoc comparison tests).

Discussion

The purpose of this study was to explore the effect that serum starvation has on improving the gene delivery efficiency of differentiating ES cells. We demonstrated that serum

starvation increases the transfection efficiency of differentiating ES cells as well as the number of viable and functional cells ultimately recoverable. In an effort to quantify DNA delivery trends, we sought a reproducible, rapid and inexpensive uptake tool. This was accomplished using a Cy3-linked 20-mer oligonucleotide. The Cy3 oligonucleotides were transfected with Lipofectamine™ 2000, and the uptake of the lipoplex particles was quantified via flow cytometry after 12 min. This time point was chosen to permit the settling and concentration of the lipoplex particles closer to the cell surface.²³ We found that the serum-starved differentiating ES cells as a whole showed significantly higher Cy3 oligonucleotide uptake rates than their high serum-treated counterparts (with the highest rate occurring at 3 days). While many factors contribute to transfection efficiency (e.g., plasmid uptake, trafficking to the nucleus, mRNA translation efficiency and/or mRNA and protein stability), the oligonucleotide uptake trend paralleled the plasmid transfection efficiencies for expression of both the CMV- and Cyp7A1-driven plasmids after three days of media treatment. This demonstrates the utility of the Cy3 oligonucleotides as a useful predictive measure of plasmid DNA uptake and a potential tool for predicting transfection efficiency (which is typically assessed 1–2 days later).

By reducing the levels of serum from 20% to 0.5% in the media of EB-mediated day 17 differentiating ES cells for 3 days, we were able to achieve a significant increase in the percentage of cells that were transfected with a CMV-driven fluorescent reporter vector. The increase garnered by the serum-starved media also translated into a significant increase in the percentage of cells expressing a liver-specific Cyp7A1 fluorescent reporter vector. Both groups showed the greatest enhancement after 3 days of serum starvation. The specific timing of 3 days of conditioning that was applicable to the differentiating ES cells may be due to a variety of factors idiosyncratic to highly proliferative or transformed cells, including growth rate and metabolic activity, tempered resistance to serum starvation, demand and depletion of nutrients in the media, ability to respond to stress, phenotypic changes in the cell population and the activation of the SDR gene.^{20,24–26} While serum starvation could have directly altered the differentiation profile of the ES cell population by directly increasing the number of Cyp7A1 expressing cells, we believe that the concomitant increases in CMV transfection efficiencies as well as oligonucleotide uptake do not support this possibility.

Both the CMV- and Cyp7A1-transfected cells were sorted based on fluorescent reporter expression, and it was found that serum starvation did not alter the functional capacity of either group, as compared to cells treated with high levels of serum. Furthermore, there was negligible cell loss within the 3 days of media conditioning, and there was no indication of necrosis or apoptosis in any treatment group. As typical cell culture protocols call for the changing of media every 2–3 days, we do not find the 3-day serum starvation conditioning time period to be impractical. However, a better understanding of the mechanisms by which serum starvation increases oligonucleotide and plasmid DNA uptake may help us to develop improved cellular pretransfection methods that can be completed in a shorter period of time and achieve similar or greater transfection efficiency.

Staining the media-conditioned differentiating ES cells for F-actin revealed the presence of stress fibers in the replated and high serum-treated cells. In the serum-starved differentiating

ES cells, the cells were larger in cross-sectional area, which may have increased the probability of lipoplex/cell membrane interaction, and the presence of actin was localized around the nucleus and cell membrane, suggesting a disrupted F-actin network as a result of serum starvation. This may have reduced the tension of the plasma membrane, thus leading to an expanded plasma membrane.^{27–31} This expanded membrane may have led to an increased endocytosis rate.³² The presence and formation of F-actin fibers appeared to resume when serum-starved cells were transfected with Cy3 oligonucleotides and assessed after 10 minutes. A rapid stimulation of stress fibers was reported in a study on serum-starved Swiss 3T3 cells to occur as early as two minutes after serum-containing media was added.³³ The actin cytoskeleton is believed to play an important role in clathrin-mediated endocytosis, the main internalization mechanism believed to be employed by Lipofectamine™ 2000.^{7,34–36} Proteins involved in the nucleation step of actin filament polymerization are implicated in the formation of endocytic vesicles.^{37–40} Thus, extended actin filaments that formed as cells recovered from the serum starvation may have helped draw endocytic vesicles from the plasma membrane.

An additional endocytic mechanism by which serum starvation may have increased DNA uptake could be the induction of macropinocytosis. It has been shown that, via growth factor stimulation, macropinocytosis can take place in cells that do not naturally phagocytose.⁴¹ Macropinocytosis depends on signaling to the actin cytoskeleton and utilizes an actin-driven mechanism to protrude the plasma membrane and engulf large volumes of fluid in macropinosomes, which can be induced by growth factors to occur within five minutes.^{42,43} A decrease in membrane-cytoskeleton adhesive forces may occur in parallel with increased rates of fluid phase endocytosis.³² This signaling cascade may be triggered by trace elements, proteins, nucleosides and growth factors present in the Opti-MEM media in which the oligonucleotides and plasmid DNA complexes are delivered to the cells.

The original motivation for exploring improvements in transient gene delivery was to increase the yield of differentiating ES cells expressing the Cyp7A1 reporter vector. We found that not only did the cells continue to proliferate under serum-starvation conditions (albeit at a slower rate than high serum-treated cells), but that the number of cells expressing the Cyp7A1 reporter plasmid vector increased 2.4-fold, as compared to the replated control cells. This translated into a near 10-fold yield in cells available for recovery following sorting. While endocytosis and nucleocytoplasmic shuttling of endocytic proteins are believed to be independent processes, some endocytic proteins do play a role in transcriptional regulation.⁴⁴ An increased presence of endocytic proteins upon transfection of the serum-starved cells, irrespective of the type of endocytic pathway activated, may have led to an increased signal of the reporter in addition to the number of cells that were transfected, thus enabling detection of Cyp7A1 reporter-expressing cells in a larger fraction of the population.

When media conditioning the Hepa 1–6 cells, we found that transfection efficiencies after 3 days decreased as compared to the replated control cells. The Cy3 oligonucleotide uptake data was accurate in predicting this decline. The decrease may be due to Hepa 1–6 cells' status as a carcinoma cell line or that the depletion of serum from 10% to 0.5% in Hepa 1–6 cells may not be as significant as the drop from 20% to 0.5% in the differentiating ES cells.

We also noted that there was an insignificant difference in average cell areas between the two media-conditioned Hepa 1–6 cells, while the serum-starved differentiating ES cells were significantly larger than their high serum-treated counterparts. Furthermore, we did not optimize the length of media conditioning in Hepa 1–6 cells, as was the case in the differentiating ES cells, so we cannot conclusively exclude these cells from being subject to serum starvation-dependent transfection enhancement. Through additional optimization, it may be possible to increase DNA uptake and transfection efficiency in these cells as well.

The methods developed in these studies have the capacity to improve gene delivery to differentiating stem cells and, with further optimization, may be applicable to the genetic manipulation of other mature, somatic cells as well. The enhancement in transfection efficiency attained via serum starvation is comparable to or exceeds levels obtained through other methods when studying the ES D3 cell line in the proliferative state (e.g., 45% when using magnetic nanoparticles and > 80% efficiency using standard lipid-based techniques).^{45,46} Delivering genetic vectors through nonviral plasmids is amenable to safer and clinically-sighted manufacturing practices, and developing efficient systems for completing this task will make scale-up feasible and transferable to industrial cellular engineering systems.

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Literature Cited

1. Bleiziffer O, Eriksson E, Yao F, Horch RE, Kneser U. Gene transfer strategies in tissue engineering. *J Cell Mol Med.* 2007; 11:206–223. [PubMed: 17488473]
2. Clements BA, Incani V, Kucharski C, Lavasanifar A, Ritchie B, Uluda H. A comparative evaluation of poly-L-lysine-palmitic acid and lipofectamine 2000 for plasmid delivery to bone marrow stromal cells. *Biomaterials.* 2007; 28:4693–4704. [PubMed: 17686514]
3. Dalby B, Cates S, Harris A, Ohki EC, Tilkins ML, Price PJ, Ciccarone VC. Advanced transfection with lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. *Methods.* 2004; 33:95–103. [PubMed: 15121163]
4. Goessler UR, Riedel K, Hormann K, Riedel F. Perspectives of gene therapy in stem cell tissue engineering. *Cells Tissues Organs.* 2006; 183:169–179. [PubMed: 17159343]
5. Šri T, Hescheler J. Stem cells and nuclear reprogramming. *Minim Invasive Ther Allied Technol.* 2008; 17:64–78. [PubMed: 18465442]
6. Douglas KL, Piccirillo CA, Tabrizian M. Effects of alginate inclusion on the vector properties of chitosan-based nanoparticles. *J Control Release.* 2006; 115:354–361. [PubMed: 17045691]
7. Douglas KL. Toward development of artificial viruses for gene therapy: a comparative evaluation of viral and non-viral transfection. *Biotechnol Prog.* 2008; 24:871–883. [PubMed: 18335953]
8. Douglas KL, Piccirillo CA, Tabrizian M. Cell line-dependent internalization pathways and intracellular trafficking determine transfection efficiency of nanoparticle vectors. *Eur J Pharm Biopharm.* 2008; 68:676–687. [PubMed: 17945472]
9. Tsai JT, Furstoss KJ, Michnick T, Sloane DL, Paul RW. Quantitative physical characterization of lipid-polycation-DNA lipo-polyplexes. *Biotechnol Appl Biochem.* 2002; 36:13–20. [PubMed: 12149118]

10. Wallenstein EJ, Barminko J, Schloss RS, Yarmush ML. Transient gene delivery for functional enrichment of differentiating embryonic stem cells. *Biotechnol Bioeng*. 2008; 101:859–872. [PubMed: 18942772]
11. Hanna J, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science*. 2007; 318:1920–1923. [PubMed: 18063756]
12. Meissner A, Wernig M, Jaenisch R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat Biotechnol*. 2007; 25:1177–1181. [PubMed: 17724450]
13. Stadtfeld M, Brennand K, Hochedlinger K. Reprogramming of pancreatic beta cells into induced pluripotent stem cells. *Curr Biol*. 2008; 18:890–894. [PubMed: 18501604]
14. Liu SV. iPS cells: a more critical review. *Stem Cells Dev*. 2008; 17:391–397. [PubMed: 18426340]
15. Pera MF, Hasegawa K. Simpler and safer cell reprogramming. *Nat Biotechnol*. 2008; 26:59–60. [PubMed: 18183017]
16. Coquelle A, Mouhamad S, Pequignot MO, Braun T, Carvalho G, Vivet S, Métivier D, Castedo M, Kroemer G. Enrichment of non-synchronized cells in the G1, S and G2 phases of the cell cycle for the study of apoptosis. *Biochem Pharmacol*. 2006; 72:1396–1404. [PubMed: 16765323]
17. Golzio M, Teissié J, Rols MP. Cell synchronization effect on mammalian cell permeabilization and gene delivery by electric field. *Biochim Biophys Acta*. 2002; 1563:23–28. [PubMed: 12007621]
18. Yu JN, Ma SF, Miao DQ, Tan XW, Liu XY, Lu JH, Tan JH. Effects of cell cycle status on the efficiency of liposome-mediated gene transfection in mouse fetal fibroblasts. *J Reprod Dev*. 2006; 52:373–382. [PubMed: 16518081]
19. Oya N, Zölzer F, Werner F, Streffer C. Effects of serum starvation on radiosensitivity, proliferation and apoptosis in four human tumor cell lines with different p53 status. *Strahlenther Onkol*. 2003; 179:99–106. [PubMed: 12590320]
20. Gustincich S, Schneider C. Serum deprivation response gene is induced by serum starvation but not by contact inhibition. *Cell Growth Differ*. 1993; 4:753–760. [PubMed: 8241023]
21. Gustincich S, Vatta P, Goruppi S, Wolf M, Saccone S, Della Valle G, Baggiolini M, Schneider C. The human serum deprivation response gene (SDPR) maps to 2q32–q33 and codes for a phosphatidylserine-binding protein. *Genomics*. 1999; 57:120–129. [PubMed: 10191091]
22. Pellegrin S, Mellor H. Actin stress fibres. *J Cell Sci*. 2007; 120:3491–3499. [PubMed: 17928305]
23. Luo D, Saltzman WM. Enhancement of transfection by physical concentration of DNA at the cell surface. *Nat Biotechnol*. 2000; 18:893–895. [PubMed: 10932162]
24. Kim HR, Jeong JA, Park CH, Lee SK, Lee WK, Jang YS. A role for cell cycle proteins in the serum-starvation resistance of Epstein-Barr virus immortalized B lymphocytes. *Biochem Cell Biol*. 2002; 80:407–413. [PubMed: 12234093]
25. Park CH, Kim HR, Kim J, Jang SH, Lee KY, Chung GH, Jang YS. Latent membrane protein 1 of Epstein-Barr virus plays an important role in the serum starvation resistance of Epstein-Barr virus-immortalized B lymphocytes. *J Cell Biochem*. 2004; 91:777–785. [PubMed: 14991769]
26. Schratt G, Weinhold B, Lundberg AS, Schuck S, Berger J, Schwarz H, Weinberg RA, Rüther U, Nordheim A. Serum response factor is required for immediate-early gene activation yet is dispensable for proliferation of embryonic stem cells. *Mol Cell Biol*. 2001; 21:2933–2943. [PubMed: 11283270]
27. Lenne PF, Wawrezinieck L, Conchonaud F, Wurtz O, Boned A, Guo XJ, Rigneault H, He HT, Marguet D. Dynamic molecular confinement in the plasma membrane by microdomains and the cytoskeleton meshwork. *EMBO J*. 2006; 25:3245–3256. [PubMed: 16858413]
28. Raucher D, Sheetz MP. Characteristics of a membrane reservoir buffering membrane tension. *Biophys J*. 1999; 77:1992–2002. [PubMed: 10512819]
29. Raucher D, Sheetz MP. Cell spreading and lamellipodial extension rate is regulated by membrane tension. *J Cell Biol*. 2000; 148:127–136. [PubMed: 10629223]
30. Titushkin I, Cho M. Distinct membrane mechanical properties of human mesenchymal stem cells determined using laser optical tweezers. *Biophys J*. 2006; 90:2582–2591. [PubMed: 16399828]
31. Titushkin I, Cho M. Modulation of cellular mechanics during osteogenic differentiation of human mesenchymal stem cells. *Biophys J*. 2007; 93:3693–3702. [PubMed: 17675345]

32. Raucher D, Sheetz MP. Phospholipase C activation by anesthetics decreases membrane-cytoskeleton adhesion. *J Cell Sci.* 2001; 114:3759–3766. [PubMed: 11707527]
33. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* 1992; 70:389–399. [PubMed: 1643657]
34. Colin M, Maurice M, Trugnan G, Kornprobst M, Harbottle RP, Knight A, Cooper RG, Miller AD, Capeau J, Coutelle C, Brahimi-Horn MC. Cell delivery, intracellular trafficking and expression of an integrin-mediated gene transfer vector in tracheal epithelial cells. *Gene Ther.* 2000; 7:139–152. [PubMed: 10673719]
35. Hoekstra D, Rejman J, Wasungu L, Shi F, Zuhorn I. Gene delivery by cationic lipids: in and out of an endosome. *Biochem Soc Trans.* 2007; 35:68–71. [PubMed: 17233603]
36. Yarar D, Waterman-Storer CM, Schmid SL. A dynamic actin cytoskeleton functions at multiple stages of clathrin-mediated endocytosis. *Mol Biol Cell.* 2005; 16:964–975. [PubMed: 15601897]
37. Engqvist-Goldstein AE, Drubin DG. Actin assembly and endocytosis: from yeast to mammals. *Annu Rev Cell Dev Biol.* 2003; 19:287–332. [PubMed: 14570572]
38. McPherson PS. The endocytic machinery at an interface with the actin cytoskeleton: a dynamic, hip intersection. *Trends Cell Biol.* 2002; 12:312–315. [PubMed: 12185847]
39. Merrifield CJ. Seeing is believing: imaging actin dynamics at single sites of endocytosis. *Trends Cell Biol.* 2004; 14:352–358. [PubMed: 15246428]
40. Munn AL. Molecular requirements for the internalisation step of endocytosis: insights from yeast. *Biochim Biophys Acta.* 2001; 1535:236–257. [PubMed: 11278164]
41. Jones AT. Macropinocytosis: searching for an endocytic identity and role in the uptake of cell penetrating peptides. *J Cell Mol Med.* 2007; 11:670–684. [PubMed: 17760832]
42. Amyere M, Mettlen M, Van Der Smissen P, Platek A, Payraastre B, Veithen A, Courtoy PJ. Origin, originality, functions, subversions and molecular signalling of macropinocytosis. *Int J Med Microbiol.* 2002; 291:487–494. [PubMed: 11890548]
43. Nakase I, Tadokoro A, Kawabata N, Takeuchi T, Katoh H, Hiramoto K, Negishi M, Nomizu M, Sugiura Y, Futaki S. Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. *Biochemistry.* 2007; 46:492–501. [PubMed: 17209559]
44. Vecchi M, Polo S, Poupon V, Van De Loo JW, Benmerah A, Di Fiore PP. Nucleocytoplasmic shuttling of endocytic proteins. *J Cell Biol.* 2001; 153:1511–1517. [PubMed: 11425879]
45. Lee CH, Kim EY, Jeon K, Tae JC, Lee KS, Kim YO, Jeong MY, Yun CW, Jeong DK, Cho SK, Kim JH, Lee HY, Riu KZ, Cho SG, Park SP. Simple, efficient, and reproducible gene transfection of mouse embryonic stem cells by magnetofection. *Stem Cells Dev.* 2008; 17:133–141. [PubMed: 18271700]
46. Ko BS, Chang TC, Shyue SK, Chen YC, Liou JY. An efficient transfection method for mouse embryonic stem cells. *Gene Ther.* 2009; 16:154–158. [PubMed: 18668145]

Figure 1. Cy3 oligonucleotide lipoplex uptake predicts plasmid transfection efficiency

(A) Uptake of Cy3 oligonucleotides in media-conditioned differentiating ES cells was determined via flow cytometry as the percentage of positively fluorescent cells 12 min after addition. (B) Transfection efficiency was measured as the percentage of cells expressing the constitutively-expressed CMV fluorescent plasmid 48 h after transfection. (C) Expression of the liver-specific Cyp7A1 fluorescent plasmid for each treatment duration was measured via flow cytometry as the percentage of positively fluorescent cells 48 h after transfection. (D) Hepa 1–6 hepatoma cells were used to assess the effect of serum starvation on a non-ES cell type. Cy3 oligonucleotide uptake was measured 12 min after addition into Control and media-conditioned Hepa 1–6 cells. (E) Transfection efficiency was assessed by quantifying expression of the constitutively-expressed CMV fluorescent plasmid in Control and media-conditioned Hepa 1–6 cells. [One-way ANOVA for treatment condition followed by post hoc Dunnett's multiple comparisons tests; * indicates P -value <0.05 as compared to Control group; ** indicates P -value <0.01 as compared to Control group].

Figure 2. Microscopic evaluation of media-conditioned cells

Phase-contrast images were acquired using an Olympus IX70 inverted microscope. F-actin staining was performed using a rhodamine-phalloidin visualization kit on fixed cells. (A) Phase-contrast image of differentiating ES cells treated with high serum media for 3 days. (B) Phase-contrast image of differentiating ES cells serum-starved for 3 days. (C) F-actin staining of differentiating ES cells replated. (D) F-actin staining of differentiating ES cells treated with high serum media for 3 days. (E) F-actin staining of differentiating ES cells serum-starved for 3 days. (F) F-actin staining of differentiating ES cells serum-starved for 3 days and then transfected with Cy3 oligonucleotides for 10 min. (G) F-actin staining of Hepa 1–6 treated with high serum media for 3 days. (H) F-actin staining of Hepa 1–6 serum-starved for 3 days. (I) Quantification of cell area of serum-starved (0.5% FBS) and high serum-treated (20% FBS for differentiating ES and 10% FBS for Hepa 1–6) cells after 3 days. Cells were fixed and stained for F-actin to aid in identifying distinct cell regions. Cell areas were then quantified as pixel² using Olympus Microsuite™ software. [$n = 30$ per bar for Differentiating ES; $n = 10$ per bar for Hepa 1–6; ** indicates P -value < 0.01; Student's t -test].

Figure 3. Proliferative and functional integrity of media-conditioned cells

(A) Cell proliferation of media-conditioned differentiating ES cells. Day 17 differentiating ES cells were uniformly plated into a 12-well plate. After 6 h, media was replenished with either 0.5% FBS-containing media or 20% FBS-containing media for an additional 1–3 days. Cells were quantified after trypsinization via Trypan blue exclusion. The solid line represents the serum-starved cells. The dashed line represents the high serum-treated cells.

(B) Screening assay for fragmented DNA in apoptotic or necrotic cells. Genomic DNA from all media-conditioned and replated cells studied (lanes C–L) was extracted and run via gel electrophoresis to detect for evidence of apoptosis (step band) or necrosis (smeared band). A positive control (lane B) is indicative of apoptotic activity. Lane A is a 100 bp DNA step ladder.

(C) Urea secretion of CMV-based and Cyp7A1-based sorted differentiating ES cells after 3 days of serum starvation (0.5% FBS) or high serum media (20% FBS) treatment. Urea secretion was detected using a colorimetric assay 2 days after replating from the sort.

(D) Albumin secretion of CMV-based and Cyp7A1-based sorted cells after 3 days of serum starvation (0.5% FBS) or high serum media (20% FBS) treatment of differentiating ES cells. Albumin secretion was detected using a sandwich ELISA 2 days after replating from the sort.

(E) Cytochrome P450 1A2 detoxification activity of CMV-based and Cyp7A1-based sorted cells after 3 days of serum starvation (0.5% FBS) or high serum media (20% FBS)

treatment of differentiating ES cells. Cytochrome P450 activity was determined by measuring the formation of resorufin due to the activity of the isoenzyme methoxyresorufin-*O*-dealkylase (MROD) 2 days after replating from the sort. [Two-way ANOVA for reporter plasmid followed by post hoc Bonferroni comparison tests; * indicates P -value <0.05 , ** indicates P -value <0.01 , *** indicates P -value <0.001 when comparing each of the CMV-based sorted samples to each of the Cyp7A1-based sorted samples].