TRANSIENT GENE DELIVERY FOR FUNCTIONAL ENRICHMENT OF DIFFERENTIATING EMBRYONIC STEM CELLS

by

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ABSTRACT OF THE DISSERTATION

Transient Gene Delivery for Functional Enrichment of Differentiating Embryonic Stem Cells

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There is a critical need for new sources of hepatocytes, both clinically to provide support for patients with liver failure and in drug discovery for toxicity, metabolic and pharmacokinetic screening of new drug entities. One major challenge in the field of differentiating embryonic stem (ES) cells is the limitation to selectively purify and enrich these cells from a heterogeneous population. We developed a transient gene delivery system that uses fluorescent gene reporters for purification of the cells. Following a transient transfection, the cells were purified through a fluorescence-activated cell sorter (FACS), re-plated in secondary culture and subsequent phenotypic analysis performed. We engineered two non-viral plasmid reporters, the first driven by the mouse albumin enhancer/promoter and the second by the mouse cytochrome P450 7A1 (Cyp7A1) promoter. We optimized the transfection of these genes into spontaneously differentiated ES cells and sorted independent fractions positive for each reporter 17 days after inducing differentiation. We found that cells sorted based on the Cyp7A1 promoter showed significant enrichment in terms of albumin secretion, urea secretion and cytochrome P450 1A2 detoxification activity as compared to enrichment garnered by the albumin promoter-based cell sort. In a second study, we explored improving the efficiency of a transient gene delivery system to differentiating ES cells by serum starving the cells for three days. We found that under serum starvation, expression of a constitutively-controlled plasmid increases from ~50% to ~83% of the population. When

probed with the Cyp7A1 liver-specific reporter vector, the expression increases from ~1.4% to ~3.7% of the population. These trends were assessed using a Cy3-tagged oligonucleotide, which enabled rapid quantification of DNA uptake and was a valid predictor of ultimate cell transfection efficiency. These results suggest that modifications in media components prior to transfection of cells can have a profound effect on improving non-viral gene delivery. Efficiently genetically engineering cellular systems while circumventing the need to induce permanent gene changes will be critical for the generation of clinically-acceptable cellular material in the future.

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Chapter 1: Introduction

1.1 Thesis Overview and Specific Aims

Gene and stem cell therapies have the potential to treat a variety of inherited and acquired human diseases. Significant progress has been made in recent years in uncovering new information regarding gene architecture and their links to diseases. Similarly, novel materials have been developed to more efficiently delivery nucleic acids into cells and tissues. Embryonic stem (ES) cells have been sought after because of their ability to differentiate into cell types of all three body germ layers. Differentiation strategies have been developed to direct cells toward a specific lineage, with the hope of generating large volumes of mature, single cell-type populations that could be used clinically for cell therapies, tissue engineered devices or *in vitro* for research-based pharmacologic and toxicology studies. Furthermore, ES cells serve as an *ex vivo* model system of organogenesis, tissue-layer development and disease progression. A variety of differentiation protocols for generating single end-point mature populations of cells have begun to be explored over the past decade. Many of these protocols for controlling differentiation produce heterogeneous end-populations. Due to noise in the system as a result of the interaction of many types of cells, research has been hindered in terms of fully assessing the true phenotype of a particular cell of interest. Recently, investigators have been able to induce pluripotency in adult somatic cells by introducing three or four stem cell-specific genes, using retroviral or lentiviral vectors.

While sophisticated techniques may exist for performing functional assessment of cells and tissues, these are generally applied to a pure population of cells or tissues, in many instances requiring chemical fixation and permeabilization. Visual techniques for monitoring gene expression patterns *in situ* in live cells, especially in a mixed cell population, are of

paramount utility in understanding the dynamic patterns of ES cell differentiation. The first aim is geared to developing a set of liver-specific non-viral gene reporter vectors to dynamically assess hepatocyte-like activity in the differentiating ES cells. Once this characterization is complete, the second aim is to utilize the gene reporters to enrich specific subpopulations of hepatocyte-like cells and to assess the enhancement by the reporters in terms of phenotypic functions. In cases of nuclear transfer into a cell, whether it involves delivering a synthetic oligonucleotide, a RNA molecule or a large DNA molecule, careful consideration is needed to develop a vector that can support the cargo and efficiently and safely target the given cell population. These considerations apply both for delivery of genes to a patient *in vivo* or for modifying cells or tissues *in vitro.* In our case, since we want to target as large a fraction of the given cell populations using the reporter genes, an efficient method for non-viral plasmid vector delivery to the ES cell system is critical. Once an optimal reporter vector is chosen, the third aim is to increase the number of cells expressing the vector. This is completed in tandem with the use of a constitutive plasmid that measures overall transfection efficiency. As a new method is identified for improving overall DNA uptake and delivery in the differentiating ES cells, we begin probing the mechanism that leads to an increase in transfection efficiency.

To address the issues outlined above, the specific aims of this dissertation are as follows:

Specific Aim 1: To develop a set of fluorescent gene reporters controlled by the regulatory elements associated with liver-specific gene activity; and to deliver these genes in order to characterize cells in a differentiating ES cell environment.

Specific Aim 2: To utilize the gene reporters to enrich hepatocyte-like subpopulations.

Specific Aim 3: To enhance transfection efficiency of the reporter vectors in the differentiating ES cell system.

Chapter 2 will deal with Specific Aims 1 and 2, and Chapter 3 will deal with Specific Aim 3.

1.2 Significance and Motivation

Cirrhosis and other liver diseases rank as the eighth leading cause of death in the United States. Over 25 million Americans are affected; approximately 30,000 patients die each year, and one of every 10 persons dies while waiting for a liver transplant (American Liver Foundation URL). A functional alternative that can sustain those with diseased organs (e.g., liver failure) is needed. Functional mature hepatocytes used in an extracorporeal bio-artificial liver device may bridge transplant waiting time and provide mediating therapy. However, large volumes (i.e., 10-100 billion cells) of pure hepatocytes are required to make treatment a clinical reality (Chan et al., 2004; Tilles et al., 2002). With limited culture potential of hepatocytes *in vitro* and the dearth of organ donors, ES cells have emerged as a possible solution to this cell sourcing problem. Theoretically, methods for generating pure populations of hepatocytes as well as other differentiated cell types (e.g., pancreatic β cells, oligodendrocytes, cardiomyocytes and endothelial cells) could provide utility in a variety of cell therapy applications (Chan et al., 2004).

Hepatotoxicity is a critical issue in the drug development industry (Prestwich, 2008). Of the estimated 90% of lead candidates that fail to become drugs, hepatotoxicity accounts for 22% of this failure rate. While great strides have been made to establish models of human hepatotoxicity (e.g., primary hepatocytes, cell lines, animal studies) that are successful in

predicting acute hepatotoxicity, it has been difficult to predict *in vivo* hepatotoxicity using cytotoxicity screens (Dambach et al., 2005; DiMasi et al., 2003; Imming et al., 2006; Li, 2004). This is in contrast to comparable cell-based assays that have been successful predictors in non-liver systems (e.g., cardiovascular, hematologic and gastrointestinal) (Collings and Vaidya, 2008). Idiosyncratic reactions to drugs generally are not detected until they reach the clinic, if they are noted at all before a drug reaches the general market (Gómez-Lechón et al., 2007). Even with animal models (which are the only *in vivo* model of bioactivation), ~80% of the drugs that pass animal testing still fail due to interspecies differences in hepatic metabolism (Prestwich, 2008). An understanding of the mechanisms governing drug-induced hepatotoxicity has led to the development of a variety of *in vitro* cytotoxicity assays using primary human hepatocytes (e.g., mitochondrial functional inhibition, enzyme or transporter inhibition, calcium signaling, reactive metabolities) (Gómez-Lechón et al, 2007). Despite the ability of the liver to regenerate *in vivo,* attempts to apply implicated growth factors crucial to the process (i.e., hepatocyte growth factor, epidermal growth factor, interleukin-6, fibroblast growth factor, insulin, thyroid hormones and vascular endothelial growth factor) for *in vitro* expansion of hepatocytes have not been successful (LeClyuse, 2001; Roymans et al., 2004; Roymans et al., 2005). As a result, metabolism and hepatotoxicology research has been limited, making the use of primary hepatocytes costly and transitory for testing purposes. Furthermore, these assays have low sensitivity because they often cannot incorporate preincubation of cells for days prior to the test, evaluate one static endpoint, or cannot assess events at the single-cell level (samples are derived from bulk liver microsome extracts). The development of a new source of hepatocytes from stem cells may perhaps lead to an improved model to study drug metabolism and enzyme induction, where specific functions can be defined in the engineered hepatocyte, thereby alleviating the limiting cell sourcing issue.

The utility of endoderm-derived ES cells to differentiate into hepatocytes is explored using a murine ES cell development model. While a mouse ES differentiation system exhibits distinctive differentiation kinetics from human ES cells, the use of mouse cells for scientific study is beneficial as mouse reagents are in plentiful supply, tissue culture maintenance does not involve the use of a feeder layer and ethical issues of using human ES cells are avoided. This system is sufficient to generate useful information about hepatic differentiation. Ultimately, however, the lessons learned here will need to be transferred to human ES cell models if the generation of functional cells for human use is to be implemented.

The continual changing states exhibited by differentiating ES cells, in terms of morphology, proliferation versus differentiation dynamics, cell membrane biology and protein secretion, present a complex model for studying mechanisms of gene delivery to a variety of cell types. Delivering genes into mammalian cells faces many challenges, whether the target cell is deep within a living organ or among a monolayer of cells *in vitro.* This is due to the complexities involved with gene targeting, including the need for a delivery vector to be produced in high purity free of cellular and bacterial contaminants, to efficiently and stably enter non-proliferating cells, to induce long-term transgene expression, and, in some cases, to target specific tissues, all while not generating cytotoxic, immunogenic or inflammatory responses. Non-viral plasmids, despite their low transfection efficiency, are useful in that they can accommodate large inserts and do not have the same risks as the HIV-based adeno-associated virus or lentivirus family (Bessis et al., 2004; Buning et al., 2004; Tenenbaum et al., 2003). Commercial reagents have been developed for the non-viral gene delivery field in the form of charge-neutralizing liposome materials that complex with the DNA molecules. Nuclear-

localization signaling peptides can also be built in to these reagents to increase efficiency of DNA transport to the cell membrane.

Related to the cell therapy goal, it is crucial to develop the appropriate technology to be able to manipulate and modify specific gene behavior in all types and states of stem cells. By using our gene reporter system as an example of cell sub-type targeting, we demonstrate the need for high efficiency of gene transfer and purity of cell function. The application of these studies is to generate a pure end-population of hepatocyte-like cells. The lessons related to gene delivery for cell recovery lay the groundwork for application to a broader scope of cell types as well as different forms of functional manipulation, which can repair or replace a diseased organ or tissue. The focus of this dissertation will be to develop strategies for efficiently isolating hepatocyte-like cells with the initial goal of using these cells as models of liver metabolism and enzyme activity in mind. Thus, we will assess these cells in terms of their hepatic functional phenotype. It is not within the scope of this dissertation, however, to experimentally address the use of these cells as a clinical tool for use in a device such as a bioartificial liver or an extracorporeal liver system.

1.3 Overview of Approach, Prior Work in the Field and Selection of Methods

1.3.1 Stem Cells as a Source for Hepatocytes

The basis for ES cell biology dates back to development studies of mouse embryogenesis. A fertilized egg, or zygote, can generate an entire organism and this capacity, known as totipotency, remains through the eight-cell morula stage (Loebel et al., 2003). Here, a blastocyst is formed, composed of an outer trophoectoderm and an undifferentiated inner mass (Fig. 1.1). The cells of the inner mass are no longer totipotent but retain the ability to develop into all cell types of the embryo and thus are pluripotent. It is the cells of the inner

cell mass that are the source of ES cells. For mouse systems, ES cells can be maintained in culture with leukemia inhibitor factor (LIF) supplementation and retain an undifferentiated state. Upon removal of LIF, the cells can be cultivated into cells of all three germ layers by varying substrate conditions, ES cell formation and growth factor/cytokine supplementation. Formation of ES cells into an embryoid-body (EB) for four days followed by plating in a tissue-culture environment can spontaneously differentiate into a variety of cell types without the addition of specialized growth factors. A large portion of these cells are of the endoderm origin and are believed to express hepatocyte-like function. Table 1.1 summarizes selected ES cell-based studies that have reported hepatocyte-like activity. Most of the techniques for generating a particular cell types of the three germ layers involve inducing cellular aggregation or three-dimensional hanging drops and by varying media supplementation with soluble factors, such as growth factors or cytokines (Hamazaki et al., 2001; Loebel et al., 2003). Some groups have sorted differentiated ES cells with fluorescent reporters driven by the albumin gene (Heo et al, 2006; Lavon et al., 2004). This is done using stable transfection approaches. While preferentially expressed in the liver, the hepato-specificity of the albumin gene is not clear, as it may be expressed in both the liver and visceral endoderm of the yolk sac during development (Asahina et al., 2004). In addition, instances of albumin expression in mature adult cells have been found in bovine mammary gland, bone, lymph, skeletal and intestinal tissues (Shamay et al., 2005; Yamaguchi et al., 2003). A study probed the fetal yolk sac and the fetal liver for what is believed to be a more liver-specific gene, cytochrome P450 7α1 (Cyp7A1), and found expression of Cyp7A1 only in the fetal liver while albumin was expressed in both the fetal yolk sac and fetal liver. Using stably-transfected ES cells for Cyp7A1, the group found activation of the reporter in <0.06% of cells differentiated in EBs for 23 days, which made cell sorting impractical (Asahina et al., 2004).

In order to test the reporters on a reproducible system for differentiating ES cells, our approach begins with a natural, spontaneous differentiation scheme that has been developed in our laboratory (Novik et al., 2007). We chose a spontaneous differentiation system because it resembles natural embryogenesis in terms of producing cells of all three germ layers. While the system produces a heterogeneous mixture of differentiated cells, a strong presence of endoderm function and hepatocyte-linked activity has been indicated (Hamazaki et al., 2003; Novik et al, 2007). By evaluating the spontaneous route, we hope that the tools developed will enable the conception of future methods that increase the yields of hepatocyte-like cells gained from stem cell cultures.

1.3.2 Liver-Specific Gene Development During Embryogenesis

The selection of liver-specific gene reporters was guided by hepatic gene expression during development. The first sign of liver gene expression in the mouse is 8.5 days postcoitum (ED) and day 9.5 for the rat. The ventral floor of the foregut endoderm thickens to form the liver diverticulum, from which hepatocytes migrate into the septum transversum and form the definitive endoderm. Transcription factors *Gata-4 and HNF-3β* bind to distinct sites in the albumin enhancer in the undifferentiated foregut endoderm. The hepatocyte nuclear factor (HNF) family activates liver-specific genes and cross-regulates each other's promoters (Dabeva et al., 2000; Jochheim et al., 2004; Jones et al., 2002; Odom et al., 2004). Fetal liver progenitor cells initiate expression of alpha fetoprotein (AFP) at ED9.5 in the rat (Dabeva et al., 2000). This is shortly followed by expression of albumin, alkaline phosphatase and intermediate filament CK-14. The early endoderm has the potential to differentiate into liver, extra-hepatic bile duct or pancreas. The endoderm-to-hepatoblast pathway is strongly influenced by several tissue-cytokine networks, including the production of bone morphogenic protein by the septum transversum mesenchyme, the production of fibroblast

growth factor by the cardiac mesoderm and, in the case of *Xenopus* embryos, combined action of Wnt/β-catenin and TGF-β induces endoderm. A comparison of the three endoderm specific lineages reveals a strong presence of pancreatic duodenal homeobox (PDX) transcription factor in the pancreas, low expression of PDX in bile duct cells and an absence of PDX in the liver. By ED12, when the liver buds from the endoderm, a bipotential hepatoblast is formed that must make a cell fate decision between becoming an hepatocyte or bile duct epithelium. This decision is strongly influenced by the Notch pathway. At ED15-16, immature hepatocytes have formed and the number of bipotential cells gradually decreases (Petkov et al., 2000). Comparative studies have used complementary DNA microarrays in parallel with quantitative PCR to plot $\log_2$ expression of albumin (where values are taken as fold difference compared to newborn tissue) (Jones et al., 2002). Quantitative PCR plots from one study showed steady expression at a relative value of -4 from ED13-ED18, rising at birth and peaking to +4 at adulthood, while the arrays show -0.75 - -0.50 expression between ED13-ED16, a rise to +0.2 at ED18, a drop to normalized 0 value at birth and a rise to +2 at adulthood (Odom et al., 2004).

Studies of embryonic, fetal and adult liver tissues compared with gene expression levels of differentiated mouse ES cells and cultured adult hepatocytes reveal the following:

1. Mechanisms underlying differentiation into hepatic cells under *in vitro* cell culture conditions are poorly understood;
2. ES cell differentiation is driven by transcription events similar to those seen in liver organogenesis, but the kinetics of activation may be dissimilar;
3. The induction of liver development *in vivo* is particularly highlighted by a distinct point of initiation of HNF-6, HNF-4α, HNF-1α at ED7.5 and HNF-3α between ED9.5-11.5, most of which drop between ED11.5-13.5. C/EBPα rises steadily at

ED11.5 and C/EBPβ is already high in the early embryonic tissue but rises higher at ED13.5 (Odom et al., 2004);

4. AFP becomes detectable *in vivo* at ED9.5 and increases 1000-fold between ED9.5 and 15.5, before dropping dramatically in the adult liver (Petkov et al., 2000; Petkov et al., 2004);
5. Albumin expression becomes detectable at ED10.5 and increases steadily until it peaks at adulthood (Odom et al., 2004; Petkov et al., 2000; Petkov et al., 2004);
6. Groups report immunofluorescent staining of AFP and albumin in *in vitro* differentiation schemes ranging from 15-24% of ES cells that have been differentiated for 15-35 days. In these same tissues, however, albumin mRNA expression is detectable in day 25 differentiated ES cells but is over two orders of magnitude lower than primary hepatocytes. In contrast, AFP mRNA levels were reported to be 3-4 fold higher than adult primary hepatocytes, a major indicator of lack of mature function (Petkov et al., 2004);
7. Cyp7A1, a liver-specific and rate-limiting enzyme in the conversion of cholesterol to bile acids, appears between days 12-14 in the developing mouse fetal liver, is sustained through birth and adulthood and is noted in differentiating mouse ES cell cultures by day 15 (Asahina et al., 2004; Mataki et al., 2007).

We ultimately chose to utilize reporters driven by the regulatory elements of the albumin and Cyp7A1 genes, as these are both mature markers of hepatocytes. Chapter 2 will discuss their development and compare their functions in enriching hepatocyte-like cells. Chapter 3 will address methods for improving the number of cells expressing the Cyp7A1 (and a constitutive CMV) reporter in the differentiating ES cell environment.

1.3.3 Dynamic Gene Expression Profiling

Many advanced technologies exist for monitoring gene expression in cells and tissues. These include Northern blots, cDNA microarrays and reverse-transcription polymerase chain reaction. The technologies enable quantitative analysis of gene expression in single cells as well as systemic analysis at the tissue, organ or organism level. While improvements have been made in increasing the accuracy of these assays, certain technical aspects (e.g., template quality, subjectivity in analysis and operator variability) and experimental requirements (e.g., large cell mass) as well as their general tendency to be based upon multiple measurements at discrete time points limit their application in (1) understanding time-dependent behavior associated with ES cell differentiation without culture termination and (2) accurately quantifying gene expression activity per cell in a mixed population of cell types. A need exists for a nondestructive technique to monitor these continuous gene changes. The use of reporter technology in the form of green fluorescent protein (GFP) or *Discosoma sp.* red fluorescent protein (DsRed) (and associated fluorescent proteins), firefly luciferase, ß-galactosidase gene (lacZ), chloramphenicol transferase and secreted alkaline phosphatase are powerful assays of gene expression that correlate to the production of a neutral internal by-product (i.e., fluorescence, luminescence) (Armone et al., 2004; Maggi et al., 2004; Spergel et al., 2001). Enzymatic assays (e.g., CAT and lacZ) are limited to fixed (and hence, destructive) samples. LacZ are useful for looking at spatially distributed gene patterns but are limited to the manual nature of the staining technique (Spergel et al., 2001). Non-destructive luciferase and fluorescent protein assays permit visualization within living cells, tissues and organs. Luciferase activity is typically activated by a substrate D-luciferin and is significantly more sensitive than the CAT assay (Armone et al., 2004; Rosochacki et al., 2002). For fluorescent protein reporter activity, sensitivity may be limited by weak promoter activity. The expression of a particular fluorophore in a live cell enables cell sorting/purification via

fluorescence-activated cell sorting (FACS). The challenge presented by a mixed ES cell population necessitates the use of a dynamic gene reporter to assess and quantity gene expression and to enable cell selection of a given subpopulation of interest. These non-invasive techniques are also advantageous for studying cell function and dynamic response to a range of stimulants and culture environments.

1.3.4 Labeling of Live Cells for Cell Sorting: Selection of a Gene Reporter System

Aside from plasmid-driven gene reporters, alternative approaches do exist for studying live cells. It is possible to label cells with antibodies that target cell-specific surface antigens. Cells could be labeled in suspension with a primary antigen and then conjugated to a fluorescent secondary antibody. There is a severe limitation in cell surface markers that are specific for hepatocytes (Crosby et al., 2002; Watt and Forrester, 2006). A novel GCTM-5 monoclonal antibody specific to human embryonic hepatoblasts has been identified (Stamp et al., 2005). However, no commercial antibodies specific for mouse embryonic lineages nor that have been tested for targeting live hepatocytes were available for these studies. Thus, the development of a dynamic gene reporter that could hone in on live cells expressing liver-specific genes was chosen instead.

Another alternative to using gene reporters would be the use of a molecular beacon. These are single-stranded short hairpin loop oligonucleotides that contain a probe sequence specific to a target DNA or mRNA sequence. These beacons contain a fluorophore on one arm and a quencher on the other that, due their close proximity when unbound, keep the probe dark. When bound to a target probe, the conformation of the beacon reorganizes so that the loop dissociates, thereby restoring fluorescence. While these probes can be designed to be very sensitive to mRNA levels and may have applications in identifying stem cell subpopulations,

their utility is currently limited as they do not naturally crossover the cell membrane and are typically microinjected into cells (Mhlanga and Tyagi, 2006; Perlette and Tan, 2001). Complexing of these probes with lipid-based delivery reagents has improved the amount introduced to the cell, but this approach is further limited as these reagents shuttle the probes to the nucleus (as opposed to releasing in the cytoplasm) and, when bound to the target probe, can lead to an amplified, blurry signal due to the residual liposome (Marras et al., 2006; Mhlanga and Tyagi, 2006; Santangelo et al., 2006). We felt that the use of a plasmid gene reporter could obviate these issues for a variety of reasons, including that the production of a fluorescence protein signal from a reporter would have a longer half-life than the availability of mRNA for binding, the production of plasmid DNA could be scaled up in bacterial cultures and that a variety of delivery reagents currently exist to support plasmid transfection.

1.3.5 Molecular Biology of Plasmid Vectors

Plasmid vectors are circular molecules of double-stranded DNA derived from bacterial-based natural plasmids. A plasmid can be cut at specific restriction endonuclease sites, and the foreign DNA fragment can be inserted into this region and ligated to re-circularize and complete the modified plasmid. In the event the host DNA and foreign DNA do not contain compatible restriction sites, a blunt digestion can be performed to bypass the need for a sticky end. Once a plasmid is created, it can be cloned by transforming the DNA material into *E. coli* cells, growing stocks of bacteria and purifying the circular plasmid. Successful integration of the insert can be confirmed by identification of the band length on an electrophoretic gel and by DNA sequencing before and after the insert site (Sambrook and Russell, 1989).

In the case of a fluorescent reporter plasmid, the cDNA encoding the fluorescent protein is downstream but in-frame from a multiple cloning site (MCS). A promoter sequence or a combinatorial promoter/enhancer/repressor regulatory package can be inserted into the MCS, thus acting as a switch for the fluorescent protein. Additional components of a plasmid vector typically include a selection site for neomycin/G418, enabling knock-out of non-transfected cells by treatment with a selection agent. Additional plasmids can be modified to incorporate a fusion protein attached to the N- or C-terminus region of the fluorescent reporter molecule, thus permitting visualization of an internal protein as it is processed and localized in its cellular milieu. Cloning strategies, vector and insert preparation, transformation and DNA isolation methods are described in detail in the Methods and Materials sections of Chapters 2 and 3.

1.3.6 Gene Delivery to Mammalian Cells

Delivery of foreign DNA to mammalian cells poses many challenges, both at the intracellular and extracellular level. In order for gene transfer systems to be efficacious, they must be able to target a select cell or cells of interest, be transcriptionally active for the desired length of time, be prepared in highly concentrated, high purity form and be immunologically inactive.

Many options exist for gene delivery in either a viral or non-viral format. Amongst the vector options, the adeno-associated virus (AAV) exhibits the highest natural efficiency of DNA delivery into cells (McCarty et al., 2004). The virus genomic DNA, in particular the REP and CAP genes, is replaced with the therapeutic gene, so that no viral genes are contained within. Compared to other viral vectors that are large, complex and may lead to an immune response, the AAV is composed of three virus coating proteins and a single-stranded piece of DNA with self-complementary regulatory elements. While the transduction efficiency of AAV is

high in both dividing and non-dividing cells, scale-up purification can be limited when producing an end-product (Gao et al., 2000; McCarty et al., 2004). A retrovirus efficiently integrates into a host genome and provides stable gene expression, but random integration can lead to insertion mutagenesis. A lentivirus can infect both dividing and non-dividing cells efficiently without causing significant cytotoxicity, but it is derived from HIV-1 viral components and additional steps would be required to remove genes that encode viral accessory proteins (Braun, 2006; Salmon and Trono, 2006). To circumvent these safety issues, we utilized a non-viral plasmid vector integrated with a commercially-available liposome-based reagent as a delivery approach that would be manageable and scalable, despite the inherently low efficiency rates (Audou et al., 2002; Fraley et al., 1980; Kumar et al., 2003; Trachant et al., 2004; Zhdanov et al., 2002). The challenge is to effectively target large numbers of cells in order to identify a strong signal in cells expressing the reporter and utilize this fluorescent property to select for cells of interest. Lipofectamine^{TM} 2000 is a cationic lipid that has been optimized in many mammalian systems to achieve reasonable levels of transfection efficiency and was chosen as the reagent for transfection in our experiments.

1.3.7 Transient versus Stable Non-Viral Gene Delivery

With non-viral vectors, several additional options exist for transfecting the plasmid DNA (Fig. 1.2). A transfection can be either transient or stable. A transient transfection is the initial expression of the inserted plasmid DNA. During a transient transfection, the plasmid DNA is expressed as an episome within the nucleus but is generally not taken up into the chromosomal DNA. If the gene is to be integrated permanently into the chromosome, however, a stable transfection must take place. A stable transfection begins with a transient transfection. In very few cells (<1/10,000), the plasmid DNA will integrate randomly into the

genome (Merrihew et al., 1996). These cells can be selected if the plasmid DNA expresses a resistance gene to an antibiotic. After 24-48 h of a transient transfection, an antibiotic is added to the culture medium, and this selection pressure over a period of time will enable the cells with a stable transfection to proliferate while killing the remaining cells. These stably-transfected cells can be further propagated. Another form of stable transfection is what is known as a gene knock-in that enables a genetic sequence to be inserted into a specific locus in the chromosome. A gene is designed with two 34-base pair flanking DNA sequences termed loxP, which, when transfected in a linearized format and upon addition of the Cre recombinase enzyme, can homologously recombine at the specific locus (Araki et al., 2002). This enables site-specific control of gene expression.

Despite advances in the use of stable transfections for inserting exogenous genes into cells, we opted to use a transient approach for a variety of reasons. First, we wanted to generate a robust system where reporters could be rapidly developed and inserted into the differentiating ES cells without the need to create multiple stable cell lines. This could take 6-8 weeks and would require additional confirmatory testing to validate that the initial pluripotency was not lost or that additional stress was not induced in the cells during this process. Second, despite the extra costs of transfecting at each time of analysis, we wanted to evaluate whether a transient approach could be useful for manipulating stem cells. We explore one instance where transient transfection is appropriate (i.e., cell sorting), but this can be applied to many other applications, including the temporal activation of pluripotency genes to generate induced pluripotent stem (iPS) cells. Third, it is not clear that stable transfection of genes provides the same signal of expression as a transient transfection. For instance, it is reported that the albumin gene promoter is appropriately regulated in transient but not in stable transfections (Berland and Chasin, 1988). With a stable transfection, the regulation of a

reporter gene is influenced by the site into which it integrates. Clone-to-clone variation can vary tremendously, making it necessary to characterize multiple cloned cell lines to normalize the reporter signal. While we do not preclude the advantages of using a stable transfection in cases where a reporter gene more accurately mimics its natural chromatic configuration and function, we reduce some of the limitations ascribed to transient transfection by improving transfection efficiency to the differentiating ES cells and by determining early on that these reporters can be readily detected by fluorescence microscopy and via flow cytometry. Thus, we aim to develop a transient gene delivery system that can be used for the functional enrichment of differentiating ES cells.

Chapter 2: Transient Gene Delivery for Functional Enrichment of Differentiating Embryonic Stem Cells

2.1 Abstract

There is a critical need for new sources of hepatocytes, both clinically to provide support for patients with liver failure and in drug discovery for toxicity, metabolic and pharmacokinetic screening of new drug entities. We have reported previously a variety of methods for differentiating murine embryonic stem (ES) cells into hepatocyte-like cells. One major challenge of our work and others in the field has been the ability to selectively purify and enrich these cells from a heterogeneous population. Traditional approaches for inserting new genes (e.g., stable transfection, knock-in, retroviral transduction) involve permanent alterations in the genome. These approaches can lead to mutations and involve the extra costs and time of developing, validating and maintaining new cell lines. We have developed a transient gene delivery system that uses fluorescent gene reporters for purification of the cells. Following a transient transfection, the cells are purified through a fluorescence-activated cell sorter (FACS), re-plated in secondary culture and subsequent phenotypic analysis is performed. In an effort to test the ability of the reporters to work in a transient environment for our differentiation system, we engineered two non-viral plasmid reporters, the first driven by the mouse albumin enhancer/promoter and the second by the mouse cytochrome P450 7A1 (Cyp7A1) promoter. We optimized the transfection efficiency of delivering these genes into spontaneously differentiated ES cells and sorted independent fractions positive for each reporter 17 days after inducing differentiation. We found that cells sorted based on the Cyp7A1 promoter showed significant enrichment in terms of albumin secretion, urea secretion and cytochrome P450 1A2 detoxification activity as compared to enrichment garnered by the albumin promoter-based cell sort. Development of gene reporter

systems that allow us to identify, purify and assess homogeneous populations of cells is important in better understanding stem cell differentiation pathways. And engineering cellular systems without making permanent gene changes will be critical for the generation of clinically-acceptable cellular material in the future.

2.2 Introduction

Embryonic stem (ES) cells are a pluripotent and highly proliferative starting population for the differentiation of cells of all three adult body germ layers. ES cells also serve as an *ex vivo* model of organogenesis and tissue-layer development. Defined differentiation protocols for generating single end-point mature populations of cells have been explored extensively over the past decade (Loebel et al., 2003). Differentiation strategies have been developed to direct cells specifically toward a hepatocyte lineage, with the hope of generating mature, functional populations that could be used clinically for cell therapy or tissue engineered devices and industrially for drug toxicology screens. Use of mature hepatocyte-like functional cells in an extracorporeal bio-artificial liver device may help to bridge transplant waiting time and provide mediating therapy for those with cirrhosis and other liver diseases (Chan et al., 2004; Tilles et al., 2005). However, large numbers of pure hepatocytes (approximately 10 billion cells) are required to make treatment a clinical reality (Chan et al., 2004).

Techniques for generating particular cell types of the endoderm germ layer range from inducing cellular aggregation, forming three-dimensional hanging drops, introducing ECM proteins, and supplementing media with soluble factors such as growth factors or cytokines (Hamazaki et al., 2001; Heo et al., 2006; Loebel et al., 2003). In prior studies, our laboratory has explored the differentiation of mouse ES cells into hepatocyte-like cells using a

combination of these induction strategies (Maguire et al., 2006; Maguire et al., 2007; Novik et al., 2006; Sharma et al., 2006). While these systems and those of others display markers of hepatocyte differentiation, they are inherently heterogeneous and produce limited yields of functional hepatocytes (Hamazaki et al., 2001; Rambhatla et al., 2003; Shirahashi et al., 2004). This issue could either be resolved by developing well-defined differentiation protocols for generating pure populations of cells or by physically sorting the cells of interest using a molecular tool known as a gene reporter.

The use of gene reporters has emerged as a non-destructive and real-time method of assessing cells expressing a gene of interest (Schenborn and Groskreutz, 1999). This is accomplished by inserting the gene's regulatory elements (i.e., promoter, enhancer/promoter, enhancer/repressor/promoter, etc.) upstream from a gene that encodes a marker (i.e., fluorescent or colorimetric). Traditional approaches have developed reporter cell lines where the genes are delivered through a virus or as a result of creating a stably-transfected cell line using a non-viral plasmid. Despite the high DNA delivery rates achievable in certain cell populations and with viral systems, these approaches have inherent challenges and limitations. The use of retroviruses, knock-in constructs and stable transfections for molecular reprogramming can lead to insertional mutagenesis over time (Goessler et al., 2006; Hanna et al., 2007). There are significant costs, equipment and time involved in creating stable cell lines, along with potential harmful effects of the continuous antibiotic selection pressure (Nagarajan and Sinha, 2008; Rosser et al., 2005). Furthermore, there is a need for validation testing of the cloned cell lines to ensure batch homogeneity and minimal phenotypic changes from the starter passage (Soliman et al., 2007). Our gene delivery system used a transient epigenetic transfection approach where non-viral DNA material was inserted into the cells but not permanently taken up into the genome. This eliminated many of the

steps involved in stable cell clone selection while allowing us to effectively target cells for the purpose of activating reporter fluorescence and enriching specific subpopulations.

Previous work by other investigators differentiating hepatocyte-like cells from stem cells used stably-transfected albumin promoter-based fluorescent reporters as a means to purify and enrich this population. These sorted populations have shown functional enhancement of albumin secretion and ammonia detoxification and gene transcript enrichment of liver-specific markers assessed via RT-PCR (Heo et al., 2006; Lavon et al., 2004; Soto-Gutiérrez et al., 2006). However, the question remains as to the hepato-specificity of the albumin gene, especially in the case of selecting hepatic progenitor cells that have not yet reached a terminal differentiation state. There is evidence that the albumin gene may be expressed in other non-hepatic differentiated stem cell populations and, when observed in healthy adult animal tissue, in bone, lymph, skeletal, intestinal and mammary gland tissues (Novik et al., 2006; Shamay et al., 2005; Yamaguchi et al., 2003). We thus developed a gene reporter driven by a known liver-specific gene, cytochrome P450 7α1 (Cyp7A1), and compared it with a reporter driven by the albumin enhancer/promoter. The reporters targeted the hepatocyte-like differentiated cells within a spontaneously differentiating EB system (i.e., no growth factors and no specialized substrates). The reporters were delivered transiently into the live differentiating stem cell cultures. We previously demonstrated in the alginate encapsulation stem cell culture system that the Cyp7A1 promoter-driven DsRed fluorescent reporter can be used as a marker of hepatocyte-specific activity and correlates well with other phenotypic assays (Maguire et al., 2007). To overcome the inherent low efficiency of transient transfection, we optimized cell-seeding densities and DNA-to-transfection reagent ratios. Cells were sorted at 17 days following the start of differentiation using the albumin or Cyp7A1 reporters and assessed for a range of hepatocyte functions and detoxification

activity. This study demonstrates the application and utility of a transient gene delivery approach for evaluating hepatocyte-specific functional activity of differentiated stem cells enriched by two liver-specific gene reporters.

2.3 Methods and Materials

2.3.1 Cell Culture

The ES cell line D3 (ATCC, Manassas, VA) was maintained in an undifferentiated state in T-75 gelatin-coated flasks (Biocoat, 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 μg/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 embryonic stem cell differentiation. Every 2 days, media was aspirated and replaced with fresh media. Cultures were split and passaged every 5-6 days, following media aspiration and washing with 6 mL of phosphate buffered solution (PBS) (Gibco). Cells were detached following incubation with 3 mL of trypsin (0.25%)-EDTA (Gibco) for three min, resulting in a single cell suspension, followed by the addition of 12 mL of Knockout DMEM. Cells were then re-plated in gelatin-coated T-75 flasks at a density of 10^6 cells/mL.

In order to induce differentiation, cells were suspended in Iscove's Modified Dulbecco's Medium (Gibco) containing 20% fetal bovine serum (Gibco), 4mM L-glutamine (Gibco), 100U/mL penicillin, 100 U/mL streptomycin (Gibco), 10 μg/mL gentamicin (Gibco). Embryoid bodies were formed and cultured for three days using the hanging drop method

(1000 ES cells per 30 µl droplet). Hanging drops where 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. EB cells were detached following incubation with 0.5 mL of trypsin-EDTA (Gibco) for three min, resulting in a single cell suspension, and neutralized by the addition of IMDM media. Cells were then re-plated in 12-well tissue culture treated polystyrene plates (BD-Biosciences) at an initial seeding density of $5x10^4$ day 17 cells per well for further analysis. These cells are hereby referred to as day 17 differentiated ES cells. Culture medium was changed every 2-3 days.

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). Hepa 1-6 cells were used as positive control for the hepatocyte-specific assays.

All cell cultures were incubated in a humidified 37°C, 5% CO_2 environment

2.3.2 Intracellular Albumin and Cytochrome P450 7A1 Immunofluorescent Analysis

Cells were washed for 10 min in cold PBS and fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at room temperature. The cells were washed twice for 10 min in cold PBS and then twice for 10 min in cold saponine/PBS (SAP) membrane permeabilization buffer containing 1% bovine serum albumin (BSA) (Sigma-Aldrich), 0.5% saponine (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich). To detect intracellular albumin, the cells were subsequently incubated for 30 min at 4^oC in a SAP solution containing rabbit anti-

mouse albumin antibody (150 μg/mL) (MP Biomedicals, Irvine, CA), or normal whole rabbit serum (150 μg/mL) (MP Biomedicals) as an isotype control, washed twice for 10 min in cold SAP buffer, and then treated for 30 min at 4°C with the secondary antibody, FITC-conjugated goat anti-rabbit IgG, diluted 1:200 (Jackson Immuno Labs, Westgrove, PA). To detect the presence of cytochrome P450 7A1 enzyme, the cells were incubated for 30 min at 4°C in a SAP solution containing rabbit anti-mouse Cyp7A1 antibody (IgG) (1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), or the IgG fraction of normal rabbit serum serum (1:50 dilution) (MP Biomedicals) as an isotype control, washed twice for 10 min in cold SAP buffer, and then treated for 30 min at 4°C with the secondary antibody, FITC-conjugated goat anti-rabbit IgG, diluted 1:200 (Jackson Immuno Labs, Westgrove, PA). For both stains, cells were then washed once with cold SAP buffer and once with cold PBS. Fluorescent images were acquired using a computer-interfaced inverted Olympus IX70 microscope. Specimens were excited using a 515 nm filter. Fluorescent intensity values were determined for each cell using Olympus Microsuite™ software. Experimental intensity values for each cell were calculated after subtracting the average intensity of the isotype control. An alternate method for quantification was performed using flow cytometry where the isotype control was used to determine the negative-gated region.

2.3.3 *In Situ* Indirect Immunofluorescent Cytokeratin 18 Analysis

Cells were washed for 10 min in cold PBS and fixed in 1% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at room temperature. The cells were washed twice for 10 min in cold PBS and then with Triton X-100 (1X) diluted in PBS (Sigma-Aldrich). To detect cytokeratin 18, cells were incubated for 30 min at 4°C in a PBS solution containing rabbit anti-mouse cytokeratin 18 antibody (IgG) (1:50 dilution) (Santa Cruz Biotechnology) or the IgG fraction of normal rabbit serum (1:50 dilution) (Santa Cruz Biotechnology) as an isotype

control, and then treated for 30 min at 4°C with the secondary antibody, FITC-conjugated goat anti-rabbit IgG, diluted 1:200 (Jackson Immuno Labs, Westgrove, PA). Cells were washed once with cold PBS. Fluorescent images were acquired using a computer-interfaced inverted Olympus IX70 microscope. Specimens were excited using a 515 nm filter. Fluorescent intensity values were determined for each cell using Olympus Microsuite™ software. Experimental intensity values for each cell were calculated after subtracting the average intensity of the isotype control. An alternate method for quantification was performed using flow cytometry where the isotype control was used to determine the negative-gated region.

2.3.4 Sandwich ELISA for Detection of Albumin Secretion

Media samples were collected directly from cell cultures at the specified time points and stored at -20ºC for subsequent analysis. Albumin secretion was detected using a commercially available kit (Bethyl Laboratories, Montgomery, TX). Anti-albumin capture antibody was diluted 1:100 in coating buffer and 100 µL was added to each well of a 96-well Nunc-Immuno MaxiSorp plate (NUNC, Denmark). The plates were incubated for 1 h at 37ºC followed by three washes. This was followed by the addition of 200 µL of blocking solution and 30 min incubation at 37ºC. The plate was washed three times and 100 µL of standards and samples were added to their respective wells. The plate was incubated for 1 h at 37ºC and washed three times. A horseradish peroxidase conjugated anti-mouse albumin antibody was diluted 1:10,000 and 100 µL was added to each well, incubated for 1 h at 37ºC and washed five times. An *o*-phenylene-diamine (OPD) (Sigma-Aldrich) substrate solution was prepared, 100 µL was added to each well and incubated for 15 min at room temperature. The reaction was stopped by the addition of 100 µL 2M H_2SO_4. Absorbance readings were obtained using a Bio-Rad Model 680 plate reader (Hercules, CA) with a 490 nm emission filter. A standard

curve was generated by creating serial dilutions of albumin standard from 7.8 ng/mL to 10,000 ng/mL and a linear fit of the standards was used to determine the albumin concentration in each sample.

2.3.5 Urea Secretion

Media samples were collected directly from cell cultures at the specified time points and stored at -20ºC for subsequent analysis for urea content. Urea secretion was assayed using a commercially available kit (StanBio, Boerne, TX). Urea enzyme reagent (100 µL) was added to each well of a 96-well plate followed by addition of 10 µL of standards/samples to the enzyme reagent. The plates were centrifuged at 1,000 RPM for 1 min and then placed in a water bath at 37ºC for 5 min. Urea color reagent (100 µL) was then added to each well followed by centrifugation and water-bath incubation. Absorbance readings were obtained using a Bio-Rad Model 680 plate reader (Hercules, CA) with a 585 nm emission filter. A standard curve was generated by creating serial dilutions of a urea standard from 0 µg/mL to 300 µg/mL and a linear fit of the standards was used to determine the urea concentration in each sample.

2.3.6 Measurement of Cytochrome P450 Activity

Cytochrome P450 activity was induced by treatment with 3-methylcholanthrene (2 µM) (Sigma–Aldrich) for 48 h prior to the activity assay. Cytochrome P450-dependent resorufin *o*-dealkylase activity was measured using resorufin substrates methoxyresorufin (MROD) from a Resorufin Sampler Kit (Invitrogen, Carlsbad, CA). The incubation mixture contained the methoxyresorufin substrate (5 mM) and dicumarol (80 mM) in phenol red free Earle's Balanced Salt Solution (EBSS) (Gibco). The prepared solutions were preheated to 37°C prior to incubation with cells. The 12-well plates were washed with 2 mL of EBSS (37°C) and

further incubated with 2 mL of EBSS at 37°C for 5–7 min, to remove the residual medium. Following removal of EBSS, the incubation mixture was added (2 mL per well), and the dishes were incubated at 37°C in a 5% CO_2 incubator. At various time points (10, 20, 30, 40 min) following incubation, 100 μL of the mixture was transferred into a 96-well plate. The fluorescence of the plate was measured using a DTX880 fluorescence plate reader (Beckman Coulter, Fullerton, CA) with an excitation of 530 nm and emission of 590 nm at the end of 25 min incubation. A standard curve of resorufin fluorescence was constructed at each time point using concentrations ranging from 1 to 1,000 nmol in EBSS. Linear curves were obtained with an $r^2 \geq 0.98$. The constructed standard curves were used to convert the fluorescence values obtained from the plate reader to nanomoles of resorufin. Rate of formation of resorufin, as calculated from the early linear increase in the fluorescence curve, was defined as cytochrome P450 activity and expressed as pmol/cell/min.

2.3.7 Cloning of Albumin Enhancer/Promoter and Cytochrome P450 7α1 (Cyp7A1) Promoter into pDsRedExpress1 Vector

The pDsRedExpress1 plasmid vector was attained from BD Biosciences Clontech (Mountain View, CA). The murine albumin enhancer/promoter was attained from a liver specific expression vector in a pBluescript plasmid donated from Dr. Joseph Dougherty (UMDNJ-RWJMS, Piscataway, NJ). The cytochrome P450 7α1 (Cyp7A1) vector was donated in the form of a PGL3-Promoter vector from Dr. Gregorio Gil (Virginia Commonwealth University, Richmond, VA). The promoter regulatory elements were each excised at a blunt and a sticky end and inserted via ligation into respective blunt and sticky sites in the parent pDsRedExpress1 vector. Correct insertion of the regulatory elements into the pDsRedExpress1 vector was confirmed by screening bacterial clones via test transfections in mouse Hepa 1-6 cells and by DNA sequencing up- and down-stream of both insertion sites.

The two vectors are hereby referred to as pAlb-DsRedExpress1 and pCyp7A1-DsRedExpress1. An additional vector, pDsRed2-C1, driven by the constitutive cytomegalovirus immediate early promoter, was used as a control for positive transfection and sorting of the differentiated ES cells.

2.3.8 Transient Transfection of Liver-Specific Vectors into Differentiated Stem Cells

The liver-specific expression vectors, pAlb-DsRedExpress1 and pCyp7A1-DsRedExpress1, along with the constitutive pDsRed2-C1 plasmid, were transiently transfected using Lipofectamine^{TM} 2000 (Invitrogen, Carlbad, CA) into stem cells differentiated for 17 days post EB formation. Four h prior to transfection, the EBs were trypsinized and re-plated to attain uniform and monolayer cell distribution. Following 24 h, red fluorescent activity was detected via flow cytometry and imaged using a computer-interfaced inverted Olympus IX70 microscope.

2.3.9 Flow Cytometry and Cell Sorting

The BD FACSCalibur^{TM} (San Jose, CA) system is a four-color, dual-laser, benchtop system capable of both cell analysis and sorting. To quantify DsRed expression, cell medium was aspirated, cells were washed with PBS and trypsinized for 1 min and resuspended in PBS. Instrument settings were calibrated using mock transfected and non-transfected cells. Cells were then analyzed using dot plots measuring forward versus side scatter and FL-3 (red fluorescence) versus FL-1 (green fluorescence), as well as histogram plots measuring count values of FL-1 and FL-3. Using the flow cytometry values as described above, the region of interest was then selected for the cell sort gating threshold. Sorted lines were cleansed with 70% ethanol and buffered with sterile PBS. Positive-gated cells were collected in tubes that were pre-incubated in cold FBS. Tubes were centrifuged at 950 RPM for 7 min, and cells

were re-plated in IMDM differentiation medium. The yield of cells recovered in each sort was approximately 80% of the number of cells gated and counted by the flow cytometer. Media was replenished after approximately 4-5 h after the sort to remove any additional contaminants or debris remaining once the sorted cells adhered to the cell culture plates. The cells to be assessed for cytochrome P450 detoxification function were treated with 3-methylcholanthrene for 48 h.

2.3.10 Statistical Analysis of Functional Assays

Each data point represents the mean of three or greater experiments (each with biological triplicates), and the error bars represent the standard deviation of the mean. Statistical significance was determined using the Student's *t*-test for unpaired data. Differences were considered significant if the *P*-value was less than or equal to 0.05.

2.4 Results

2.4.1 Optimization of Transient Transfection Conditions

In order to overcome the inherent low transfectability of the day 17 differentiated ES cell cultures, we optimized several parameters of the transfection process. Using the Lipofectamine^{TM} 2000 transfection reagent complexed with the CMV-controlled pDsRed2-C1 constitutive reporter DNA, we first examined the effect of varying the cell seeding density of the targeted differentiated ES cells. We chose the pDsRed2-C1 vector because of its commercial availability and ease of assessing expression in many mammalian cell types. Day 17 of the differentiation process was chosen based on previous indicators of hepatocyte-like expression at this time point (Novik et al., 2006). At day 17, we disassociated the EBs and re-plated the cells at 10%, 25%, 50%, 70%, 85% and 100% coverage of the surface area of single wells of a 12-well plate. The cells were allowed to attach for 4 h prior to

transfection. After 24 h, we found via flow cytometry and fluorescent microscopy the greatest transfection efficiency in the least confluent wells and the lowest efficiency in the most confluent wells (Fig. 2.1a-g). The range of transfection efficiency varied from 20% in the lowest case to 55% in the highest case. We note that where cell mass in the 50% confluency condition was half that of the 100% confluency condition, the efficiency only increased one-half fold, and where the cell mass was five-fold less in the 10% confluency condition versus the 50% confluency condition, the efficiency nearly doubled, both cases indicating a non-linear trend between cell surface coverage (i.e., cell number) and transfection efficiency.

We then chose to utilize the 50% confluency condition and the pDsRed2-C1 plasmid vector for further transfection optimization. We began with the vendor recommended DNA : Lipofectamine^{TM} 2000 reagent ratio of 1 μg DNA : 2.5 μL reagent (for a single well of a 24-well plate) and increased either the DNA mass or reagent volume or both two- or three-fold, thus creating a total of nine DNA-to-reagent ratios in which to measure transfection efficiency (Fig. 2.2). We attained the greatest transfection efficiency by using a 2 μg DNA : 2.5 μL reagent ratio, which yielded nearly 60% transfection efficiency, a level which surpassed the maximum efficiency in the previous cell-seeding density variation experiment. We note here that in cases where the 1 μg DNA : 2.5 μL mathematical ratio was conserved (i.e., 2:5; 3:7.5) but the overall DNA-lipoplex availability was increased two- or three-fold, the transfection efficiency increased only in the 2:5 ratio case and declined slightly in the 3:7.5 case. The fluorescence activity was lost in virtually the entire cell population after 6 days in culture post-transfection.

2.4.2 Analysis of Reporter Expression

We chose the 50% confluency condition and the 2 μg DNA : 2.5 μL reagent ratio for subsequent transfections using the three reporter plasmids (Fig. 2.3a). These optimized conditions yielded the greatest increase in expression for the albumin and Cyp7A1 reporters as well. Assessing the real values of expression of the three gene reporters 24 h after transfection, we found 1.71% of the day 17 differentiated ES cells expressed the Cyp7A1 reporter, 4.33% expressed the albumin reporter and 56% expressed the CMV-driven pDsRed2-C1 reporter plasmid (Fig. 2.3b).

2.4.3 Reporter-Based Cell Sorting

The protocol for sorting the transiently-transfected day 17 differentiated ES cells consisted of three separate sorts using the albumin, Cyp7A1 and CMV-based reporters, respectively. These sorted cells along with non-sorted pDsRed2-C1-transfected cells were re-plated into a 96-well plate and fed with IMDM media for an additional two days before phenotypic assays were performed (Fig. 2.3c). The maximum cell sorting purity attained, as determined by the number of post-sorted fluorescent cells reanalyzed through the flow cytometer, was 90.1% (Fig. 2.3d).

2.4.4 Enrichment of Purified Cell Populations

The three sorted populations were probed with albumin, Cyp7A1 and cytokeratin 18 protein antibodies, imaged and quantified via Olympus MicroSuite^{TM} software and compared to mouse Hepa 1-6 cells (positive control), re-plated/non-sorted day 17 differentiated ES cells (negative control) and day 0 non-differentiated ES cells (negative control). The differential enrichments among the two liver-specific reporter sorted populations and enrichment over the CMV-sorted and day 17 re-plated/non-sorted cells as measured via albumin and Cyp7A1 immunocytochemistry staining of cells were visually apparent and numerically significant

when fluorescence was quantified ($P<.05$) (Fig. 2.4). However, the differential enrichments in the albumin and Cyp7A1-based sorts varied with the reporter used; the albumin-based sorted cells yielded a population with greater number of cells positive for the albumin protein, and the Cyp7A1-based sorted cells yielded a greater percentage of cells positive for Cyp7A1. The percentage of cells staining positive for albumin was 51.4% in the re-plated day 17 differentiated ES cells and 58% in the CMV-based sorted cells. The percentage of cells expressing albumin increased to ~89% in the albumin-based sorted cells and ~77% in the Cyp7A1-based sorted cells. In terms of Cyp7A1 staining, 85% of the Cyp7A1-based sorted cells were positive, compared to ~56% of the albumin-based sorted cells, ~37% of the CMV-based sorted cells and 25% of the re-plated day 17 differentiated cells. In terms of cytokeratin 18 expression, the increases in percentages of cells positive exhibited by the albumin-based and Cyp7A1-based sorted cells were not statistically different from that of the CMV-based sorted and day 17 re-plated cell population, nor was there statistical differential enrichment between the two liver-specific sorted populations. Albumin, Cyp7A1 and cytokeratin 18 expression were not detected in the day 0 non-differentiated ES cells.

Albumin secretion was assessed via a sandwich ELISA in the sorted cells as a test of hepatocyte-specific function. The day 17 Cyp7A1-based sorted cells showed a significant enhancement ($P<.01$) in albumin secretion as compared to the day 17 albumin-based sorted population (Fig. 2.5). The Cyp7A1-based sorted cells exhibited albumin secretion values at approximately three-fold that of Hepa 1-6 cells and the albumin-based sorted cells, and six-fold that of the day 17 non-sorted and CMV-based sorted cells. The albumin secretion rate in the albumin-based cell population was nearly identical to that of Hepa 1-6 cells, which was close to four-fold greater than that of the day 17 non-sorted and CMV-based sorted cells. Day 0 non-differentiated ES cells exhibited negligible levels of albumin secretion.

Urea secretion was measured using a colorimetric assay as an additional marker of hepatocyte function. Urea secretion levels were enriched in both the Cyp7A1- and albumin-based sorted populations, as compared to the day 17 non-sorted and CMV-based sorted cells (Fig. 2.6). This enrichment was greatest in the Cyp7A1-based sort (nearly 10-fold), while the albumin-based sorted cells secreted urea at levels similar to that of Hepa 1-6 cells, both of which were approximately five times that of the day 17 non-sorted and CMV-based sorted cells. The greater functional enrichment for urea secretion in the Cyp7A1-based sort versus the albumin-based sort was highly significant ($P<.001$). Day 0 non-differentiated ES cells showed negligible urea secretion.

To further examine the hepatocyte-like function of the sorted populations, the mature cytochrome P450 detoxification enzymatic functional assay for Cyp1A2 was selected on the basis of its enriched presence and activity in the liver (Edwards et al., 1994; Guengerich and Turvy, 1991; Schweikl et al., 1993; Shmueli et al., 2003; Yanai et al., 2005). Following a 48-h induction with 3-methylcholanthrene, the sorted cells were assessed for their ability to convert methoxyresorufin (MROD) substrate into the fluorescent molecule resorufin. The production of fluorescence at several time points over a 40-min time period was correlated to the activity of the isoenzyme (Fig. 2.7). Cyp7A1-based sorted cells showed a highly significant enrichment of Cyp1A2 functional activity. The increased function in these cells was over seven-fold greater than that measured in the albumin-based sorted, day 17 non-sorted and CMV-based sorted cells and over three-fold greater than that of the Hepa 1-6 cells. The albumin-based sorted cells demonstrated MROD activity in the same range as that of the day 17 non-sorted and CMV-based sorted cells. Day 0 non-differentiated ES cells had no detectible MROD activity.

2.5 Discussion

The purpose of this study was to develop a transient gene delivery system for targeting and purifying a subpopulation of differentiated embryonic stem cells and assess for enriched hepatocyte-like activity. Transient transfection of the gene reporters in our case allowed us to overcome the need to create stable transgenic cell lines that permanently integrate plasmid DNA and are maintained under antibiotic selection pressure. Stable transfection necessitates validation testing of cloned cell lines and running several experimental lines in parallel in order to account for variation in fluorescent signal due to inconsistent genomic integration (Eiges et al., 2001; Kim et al., 2005). With our approach, we could select ES cells at any desired passage number, at which time the genes could be delivered. This rapid generation of plasmids and delivery into cells was accomplished without a major investment of the resources and bioreactor equipment normally required to generate single clones (Rosser et al., 2005). The fluorescent reporter activity lasted up to six days, which was ample time to perform the fluorescence-based cell sorting. Furthermore, it has been reported for the albumin gene that its regulatory elements behave differently in transient versus stable transfections and is appropriately regulated in the transient case (Berland and Chasin, 1988). This is despite the transient gene lacking the chromatin and chromosome context of transcription associated with a stable integration. Industrially, stable cell line generation would be even more burdensome given the challenge of testing thousands of newly-discovered genes that may be relevant in drug discovery. In response, the use of transient transfections has already been implemented industrially in large-scale cell culture operations for generating recombinant proteins (Geisse and Henke, 2005).

Addressing the extra reagents needed per experiment and the lower efficiency associated with transient transfection, we optimized two parameters: cell-seeding density and DNA :

Lipofectamine™ 2000 reagent ratio. This allowed us to increase our efficiency of DNA delivery to near 60% of the targeted cells. We aim in future work to use models of particle adsorption and cellular uptake to better understand the phenomena of increased transfection efficiency at lower cell-seeding densities. Higher transfection efficiencies will be needed to maximize the number of cells can be targeted for cell enrichment, thereby increasing the sorting yield and overall purity of the FACS-sorted cells, which was at a maximum of 90.1% in these studies. While ideally we would like the purity of cells to be greater than 95%, the width of our detection thresholds were widened to be sensitive to the greatest numbers of fluorescent cells due to the low percentages of cells positive for the liver-specific reporters. This most likely accounted for the fraction of non-fluorescent cells mixing into the sorted population.

We tested our gene delivery system by assessing the ability of two liver-specific gene reporters to produce purified hepatocyte-like populations of differentiated murine ES cells. This was measured by a panel of hepatocyte-specific markers of protein expression, albumin secretion, urea secretion and cytochrome P450 detoxification. While primary hepatocytes represent the most authentic model for *in vitro* drug metabolism and toxicity studies, the expense in isolating these cells and their lack of long-term stability has led many investigators to explore alternative sources of hepatocyte-like cells. Embryonic stem cells may represent a renewable source of hepatocyte-like cells, and many laboratories including ours have explored a variety of strategies and systems for directing differentiation toward a hepatocyte lineage. Previous groups have implemented fluorescent gene reporter systems into ES differentiation cultures and examined and sorted for the presence of endoderm-specific alpha-fetoprotein (AFP) and albumin genes (Benvenisty et al., 2004; Heo et al., 2006; Ishii et al., 2005; Soto-Gutiérrez et al., 2006; Teratani et al., 2005; Yamamoto et al., 2003). One

group reported the expression of Cyp7A1 but did not sort these cells (Asahina et al., 2004). Cells enriched based on AFP and albumin reporters in other studies exhibited differentially-expressed hepatocyte-specific genes but were limited in performing hepatocyte-like functions (Benvenisty et al., 2004; Heo et al., 2006; Ishii et al., 2005).

Cyp1A2 is one of the most abundant cytochrome P450 enzymes in the human liver (Edwards et al., 1994; Guengerich and Turvy, 1991; Schweikl et al., 1993). A recent human ES cell study identified the presence of Cyp1A2 and Cyp3A4/7 at the mRNA and protein level but not enzymatically (Ek et al., 2007). In our Cyp7A1-enriched day 17 differentiated ES cell population, we found Cyp1A2 activity to be six-times greater than the day 17 non-sorted and CMV-based controls and three-times greater than the albumin-based sorted cells and the Hepa 1-6 positive control cell line. In a murine hepatic differentiation ES cell study, Cyp2B, Cyp2D, Cyp2C29 and Cyp3A P450 enzyme activities were detected by measuring testosterone metabolite products (Tsutsui et al., 2006). In a human direct differentiation study, only Cyp3A4 activity was detected by the production of a testosterone metabolite (Hay et al., 2007).

We showed that the MROD (Cyp1A2) activity was nearly three-fold that of the Hepa 1-6 cell line control. This may be explained by the inability of the Hepa 1-6 cell line to retain mature expression of cytochrome P450 activity, the lack of which has been reported to be common in hepatoma cell lines (Bock, 1993; Gonzalez, 1990). Li et al. (1998) reported Cyp1A2 activity upon induction in the human colon carcinoma cell line LS180, while others were only able to detect Cyp1A2 activity at the mRNA level in hepatoma cell lines (Chung and Bresnick, 1994; Fukuda et al., 1992). Only recently, the human hepatocarcinoma-derived

HepaRG cell line was found to express P450 ezymatic activity (Cyp1A1/2, Cyp2C2, Cyp2E9, Cyp3A4) when seeded at high density for two weeks (Aninat et al., 2006).

Enrichment of cytokeratin 18 expression in both the albumin- and Cyp7A1-based sorted populations was found to be statistically insignificant both amongst each other and when compared to the CMV-based sorted and day 17 re-plated cells. This may be due to the inherent heterogeneity and expression pattern of the cytokeratin 18 gene (Toietta et al., 2003). Several genome-wide human tissue expression studies found mRNA levels for the cytokeratin 18 protein present in kidney, lung, prostate and pancreatic tissues within close range or greater than that of liver tissue. These same studies found Cyp1A2 expression levels in the liver to be at least 10-fold greater than all other tissue groups sampled, making it a suitable choice for measuring hepatocyte-specific cytochrome P450 detoxification enzymatic activity (Shmueli et al., 2003; Yanai et al., 2005).

The differences in functional enhancement between the two liver-specific sorted populations demonstrate the concern for specificity of gene promoter choice for sorting hepatocyte-like cells even at later stages of differentiation. Albumin is a key plasma protein produced by the liver that binds toxins and drugs and plays a role in transporting fatty acids and steroid hormones. In earlier work by our laboratory where we performed cDNA microarray analysis on day 17 EB-mediated differentiated cells, albumin expression was detected by immunocytochemistry but was not one of the limited mature hepatocyte gene expression markers found to be upregulated via cDNA microarray analysis (Novik et al., 2006). Asahina et al. (2004) notes that albumin may be a well-characterized product of the liver, but it is expressed in both the liver and the visceral endoderm of the yolk sac, making it unclear whether differentiated ES cells expressing albumin are definitive endoderm or visceral

endoderm, or both. Albumin expression and secretion is also found, for example, in bovine mammary gland tissue and is believed to be part of the mammary gland innate immune system (Shamay et al., 2005). There is additional evidence that the albumin gene is also expressed in bone, lymph, skeletal and intestinal tissue (Yamaguchi et al., 2003). This may explain why the pAlb-DsRedExpress1 expression rate was 2.5-fold that of pCyp7A1-DsRedExpress1 vector expression in the day 17 differentiated ES cells. Similarly, nearly half of the non-sorted heterogeneous population of day 17 differentiated ES cells stained positive for albumin immunocytochemistry, whereas the expression of Cyp7A1 was limited to about a quarter of the population.

The disparity between the percentages of cells staining for albumin and Cyp7A1 versus the low expression of the reporter plasmids in these cells may have several possible explanations. The lack of correlation between mRNA expression/promoter activation and protein abundance is well known, with differences reported to be as great as 20-fold in either direction (Greenbaum et al., 2003; Nie et al., 2006). One single mRNA molecule may yield many protein molecules, leaving it up to the cell's cytoplasmic machinery to determine where and when proteins are expressed (Tyagi, 2007). Sensitivity of detecting the reporters may be limited, and thus it may be necessary to apply more sophisticated imaging techniques (e.g., selective filters, spectral analysis) to overcome background autofluorescence or further stimulate the Cyp7A1 promoter (Lam et al., 2006; Lee at al., 1994; Serganova and Blasberg, 2005). Potentially slow degradation and turnover rate of the albumin protein and the Cyp7A1 isoenzyme may explain why levels detected via immunostaining in the cells are higher than otherwise indicated by the real-time reporter vectors (Cupp and Tracy, 1997; Døssing et al., 1983; Lai et al., 1978; Michalets, 1998; Schreiber et al., 1971; Sterling, 1951; Urban et al., 1972).

The paucity of specific surface protein markers for hepatocytes has promoted the use of gene targeting and gene reporters for enriching hepatocyte-like cells in stem cell culture systems (Watt and Forrester, 2006). Asahina et al. (2004) identified Cyp7A1 as a gene expressed in the liver but suppressed in yolk-sac tissues, thus making it a very suitable marker for identifying hepatocytes. Cyp7A1 catalyzes the conversion of cholesterol, NADPH and oxygen to 7α-hydroxycholesterol, NADP(+) and water. In addition to regulating cholesterol homeostasis, Cyp7A1 is the rate-limiting enzyme in bile acid biosynthesis. Its predominant expression in the liver may be attributed to the narrow substrate specificity between the P450 active site and cholesterol (Pikuleva, 2006). The hepatic-specific regulatory elements in the Cyp7A1 promoter region have been well-characterized, and agonists which suppress the availability of these transcription factors have been found to inhibit gene expression (Cooper et al., 1997; Marrapodi and Chiang, 2000). Cyp7A1 expression in fetal liver is restricted to perivenous hepatocytes, and *in vitro* expression of Cyp7A1 is greater in attached culture than in suspension culture, possibly due to the EB environment being more conducive to gastrulation and thus hepatic differentiation (Asahina et al., 2004). A major concern when identifying hepatocyte-like cells in stem cell differentiation systems is to properly distinguish visceral endoderm (which has similar morphology, physiology and function to hepatocytes) from true definitive endoderm (Asahina et al., 2004; Jollie, 1990; McGrath and Palis, 1997). We feel that cell sorting of differentiated ES cells based on Cyp7A1 expression overcomes this challenge as we demonstrated greater cases of hepatocyte-specific enrichment when using the Cyp7A1 reporter as compared to the albumin reporter. The cells sorted from our day 17 EB-mediated differentiation system represented a small fraction of the overall population. It is apparent that the cells sorted with both the albumin and Cyp7A1 reporters did not resemble the classical morphology of mature hepatocytes. Recent work in our

laboratory has shown that EB-differentiated stem cells can attain hepatocyte morphology when re-plated for 10 days in a collagen gel sandwich secondary culture environment with media containing 250 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Novik et al., 2008). In this present study, in order to assess the sorted cells as soon as possible after being removed from the EB differentiation environment, we chose a time-point of 48 h after the sort to perform our analysis. We aim in future work to further validate the phenotypic profile of the sorted cells (e.g., via RT-PCR) and to increase the percentages of cells that express Cyp7A1 by combining these multiple differentiation approaches of our laboratory with gene reporter-based cell sorting.

We believe that the approaches explored in these studies will not only improve the development of hepatocyte differentiation systems but can be applied broadly to the stem cell field as well. The scale-up of this system for *in vitro* pharmaceutical applications (estimated to be about 132-fold) could be feasible both in cost and in equipment to generate cells, but additional systems would need to be developed to reach clinical scales. Recently, there have been reports of generating induced pluripotent stem cells from autologous skin. These systems have utilized retroviral vectors for gene delivery and rapid generation of stable clones, and the authors point out in these pilot studies a potential cancer hazard due to insertional mutagenesis (Hanna et al., 2007; Park et al., 2008). Alternative strategies for molecular reprogramming, such as a transient epigenetic gene expression system as developed in this chapter along with further methods for screening isolated stable transfection events, may overcome the critical challenge of generating clinically-acceptable cellular material from any potential cell source in the future.

Chapter 3: Serum Starvation Media Conditioning Improves DNA Uptake and Transfection Efficiency in Differentiating Embryonic Stem Cells

3.1 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. Despite scientific advances in the field and in order for these cells to have therapeutic clinical potential, techniques for controlling gene expression need to be developed that minimize or eliminate the risk of oncogenesis and mutagenesis. Possible routes for achieving this outcome could come in the form of a transient non-viral gene delivery system. In this study, we explored improving the efficiency of a transient gene delivery system to differentiating murine embryonic stem (ES) cells by serum starving the targeted cells for three days prior to transfection. We found that under serum starvation, expression of a constitutively-controlled plasmid increases from ~50% to ~83% of the population. When probed for a liver-specific reporter vector, the expression increases from ~1.4% to ~3.7% of the population with serum starvation. These trends were assessed using a Cy3-tagged oligonucleotide, which enabled rapid quantification of DNA uptake and was a valid predictor of ultimate cell transfection efficiency. These results suggest that modifications in media components prior to transfection of cells can have a profound effect on improving non-viral gene delivery.

3.2 Introduction

The efficient delivery of DNA using non-viral plasmid vectors has been a major challenge in the fields of gene therapy, stem cell research, cellular therapeutics and RNAi/oncology (Bleiziffer et al., 2007; Clements et al., 2007; Dalby et al., 2004; Goessler et al., 2006; Šarić

and Hescheler, 2008). 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 (Clements et al., 2007; Douglas et al., 2006; Douglas, 2008; Douglas et al., 2008; Tsai et al., 2002). 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 (Wallenstein et al., 2008). 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 transfection efficiency (maximum≈56%) of plasmid expression. 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 (Hanna et al., 2007; Meissner et al., 2007; Stadtfeld et al., 2008). Despite the success of these techniques and in order 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 (Liu, 2008; Pera and Hasegawa, 2008).

The goal of this work was to explore the role of serum starvation in improving non-viral 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 (Coquelle et al., 2006; Golzio et al., 2002; Yu et al., 2006). Different cell types respond differently, if at all, to the degree and length of serum starvation (Oya et al., 2003). 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 (Gustincich and Schneider, 1993; Gustincich et al., 1999). 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 7α1 (Cyp7A1) promoter.

In earlier work, we found that purifying differentiating ES cells expressing Cyp7A1-driven reporter yields greater hepato-specific functional enrichment than cells sorted with a more ubiquitous reporter driven by the albumin enhancer/promoter (Wallenstein et al., 2008). In this study, we first assessed transfection efficiency using a general constitutive CMV-driven plasmid. We then specifically targeted liver-specific cells using the Cyp7A1 reporter in an effort to expand the number of cells in this subpopulation expressing the reporter. The Cy3 oligonucleotides proved to be a useful tool that was accurate in predicting long-term transfection efficiency. Through several functional assays, we determined that serum starvation did not disrupt the integrity of the cells. 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 non-viral gene delivery solutions in the future.

3.3 Methods and Materials

3.3.1 Cell Culture

The ES cell line D3 (ATCC, Manassas, VA) was maintained in an undifferentiated state in T-75 gelatin-coated flasks (Biocoat, 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 μg/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 embryonic stem cell differentiation. Every 2 days, media was aspirated and replaced with fresh media. Cultures were split and passaged every 5-6 days, following media aspiration and washing with 6 mL of phosphate buffered solution (PBS) (Gibco). Cells were detached following incubation with 3 mL of trypsin (0.25%)-EDTA (Gibco) for 3 min, resulting in a single cell suspension, followed by the addition of 12 mL of Knockout DMEM. Cells were then replated in gelatin-coated T-75 flasks at a density of $1x10^6$ cells/mL.

In order to induce differentiation, cells were suspended in Iscove's Modified Dulbecco's Medium (Gibco) containing 20% fetal bovine serum (Gibco), 4mM L-glutamine (Gibco), 100U/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 where 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 3 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 $5x10^4$ day 17 cells per well for further analysis. 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% CO_2 environment

3.3.2 Cloning of Cytochrome P450 7α1 (Cyp7A1) Promoter into pDsRedExpress1 Vector

The pDsRedExpress1 plasmid vector was attained from Clontech (Mountain View, CA). The cytochrome P450 7α1 (Cyp7A1) vector was donated in the form of a PGL3-Promoter vector from Dr. Gregorio Gil (Virginia Commonwealth University, Richmond, VA). The promoter region was excised at a blunt and a sticky end and inserted via ligation into a respective blunt and sticky site in the parent pDsRedExpress1 vector. Correct insertion of the regulatory segment into the pDsRedExpress1 vector was confirmed by screening bacterial clones via test transfections in mouse Hepa 1-6 cells and by DNA sequencing up- and down-stream of both insertion sites. This vector is hereby referred to as pCyp7A1-DsRedExpress1. An additional vector, pDsRed2-C1 (Clontech, Mountain View, CA), driven by the constitutive cytomegalovirus immediate early promoter, was used as a control for positive transfection and sorting of the differentiating ES cells.

3.3.3 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%.

3.3.4 Particle Size and Zeta Potential Measurements

In order 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^{TM} 2000 complex and the plasmid DNA/Lipofectamine^{TM} 2000 complex after 20 min of particle complex formation. The solutions were diluted in Opti-MEM reduced serum media (Invitrogen) 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 : Lipofectamine^{TM} 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.

3.3.5 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 Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) 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 : Lipofectamine™ 2000 ratio of 0.8 μg DNA : 1.0 μL reagent (optimized based on prior work) was used in a 48-well plate (Wallenstein et al., 2008). 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 serum-free Opti-MEM Reduced-Serum media (Invitrogen, Carlsbad, CA) 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 (Table 3.1).

3.3.6 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 detected via flow cytometry and imaged using a computer-interfaced inverted Olympus IX70 microscope.

3.3.7 Flow Cytometry and Cell Sorting

The BD FACSCalibur™ (San Jose, CA) system is a four-color, dual-laser, benchtop system capable of both cell analysis and sorting. To quantify DsRed expression, cell medium was aspirated, cells were washed with PBS and trypsinized for 1 min and resuspended in PBS. Instrument settings were calibrated using mock transfected and non-transfected cells. Cells were then analyzed using dot plots measuring forward versus side scatter and FL-3 (red fluorescence) versus FL-1 (green fluorescence), as well as histogram plots measuring count values of FL-1 and FL-3. Using the flow cytometry values as described above, the region of interest was then selected for the cell sort gating threshold. Sorted lines were cleansed with 70% ethanol and buffered with sterile PBS. Positive-gated cells were collected in tubes that were pre-incubated in cold FBS. Tubes were centrifuged at 950 RPM for 7 min, and cells were replated in IMDM differentiation medium. The yield of cells recovered in each sort was approximately 80% of the number of cells gated and counted by the flow cytometer. Media was replenished after approximately 4 h after the sort to remove any additional contaminants or debris remaining once the sorted cells adhered to the cell culture plates.

3.3.8 F-Actin Visualization, Phase-Contrast Imaging and Determination of Cell Area

A rhodamine-phalloidin actin filament (F-actin) staining kit was obtained from Cytoskeleton, Inc. (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 (Pellegrin and Moore, 2007). Phase-contrast images were acquired using an Olympus IX70 inverted microscrope. To determine two-dimensional cell area, at least 10 cells were selected from three distinct images of each respective condition using the Olympus Microsuite^{TM} 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 yield cell area values in pixel^2 units.

3.3.9 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 -20ºC 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 non-random fragmentation ladder pattern of DNA on the gel. Necrotic samples would be indicated by a randomly-digested DNA smear. Non-necrotic and non-apoptotic cells would be indicated by a single band comprising the entire genomic DNA.

3.3.10 Urea Secretion

Media samples were collected directly from cell cultures 2 days after replating from the cell sort and stored at -20ºC for subsequent analysis for urea content. Urea secretion was assayed using a commercially available kit (StanBio, Boerne, TX). Urea enzyme reagent (100 µL) was added to each well of a 96-well plate followed by addition of 10 µL of standards/samples to the enzyme reagent. The plates were centrifuged at 1000 RPM for 1 min and then placed in a water bath at 37ºC for 5 min. Urea color reagent (100 µL) was then added to each well followed by centrifugation and water-bath incubation. Absorbance readings were obtained using a Bio-Rad Model 680 plate reader (Hercules, CA) with a 585 nm emission filter. A standard curve was generated by creating serial dilutions of a urea standard from 0 µg/mL to 300 µg/mL and a linear fit of the standards was used to determine the urea concentration in each sample.

3.3.11 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 -20ºC for subsequent analysis. Albumin secretion was detected using a commercially available kit (Bethyl Laboratories, Montgomery, TX). Anti-albumin capture antibody was diluted 1:100 in coating buffer and 100 µL was added to each well of a 96-well Nunc-Immuno MaxiSorp plate (NUNC, Denmark). The plates were incubated for 1 h at 37ºC followed by three washes. This was followed by the addition of 200 µL of blocking solution and 30 min incubation at 37ºC. The plate was washed three times and 100 µL of standards and samples were added to their respective wells. The plate was incubated for 1 h at 37ºC and washed three times. A horseradish peroxidase conjugated anti-mouse albumin antibody was diluted 1:10,000 and 100 µL was added to each well, incubated for 1 h at 37ºC and washed five times. An *o*-phenylene-diamine (OPD) (Sigma-Aldrich) substrate solution was prepared, 100 µL was added to each well and incubated for 15 min at room temperature. The reaction was stopped by the addition of 100 µL 2M H_2SO_4. Absorbance readings were obtained using a Bio-Rad Model 680 plate reader (Hercules, CA) with a 490 nm emission filter. A standard curve was generated by creating serial dilutions of albumin standard from 7.8 ng/mL to 10,000 ng/mL and a linear fit of the standards was used to determine the albumin concentration in each sample.

3.3.12 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 was determined using the Student's *t*-test for unpaired data. Differences were considered significant if the *P*-value was less than or equal to 0.05.

3.4 Results

3.4.1 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 overall 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 serum-free media. Cells were sampled 12 min after the addition of the Cy3 oligonucleotide lipoplexes (Figure 3.1a). 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 ($P<0.01$) 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 ($P<0.01$ for 1 and 3 days; $P<0.05$ for 2 days), where less than ~15% of the cells transfected at each of these days contained particles 12 min after transfection. After 60 min of transfection, all conditions exhibited Cy3 uptake in at least 90% of the cells; and after 4 h, >99% of the cells indicated Cy3 uptake (data not shown). 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).

3.4.2 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 3.1b). Compared to the control replated ES cell group, which had an efficiency of ~50% of the total cell population, the transfection efficiency of the high serum populations did not decrease significantly after 1 or 2 days. However, with 3 days of incubation in high serum-containing media, transfection efficiency dropped to ~28% (P<0.01). In contrast, the transfection efficiency of the serum-starved populations increased after 2 and 3 days of conditioning, when compared to the replated population, with the greatest efficiency noted in the 3-day serum-starved group (~83%) (P<0.01). 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).

3.4.3 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 3.1c). We found that with respect to the replated ES cell population, which showed expression in ~1.5% of all cells, the serum-starved groups increased in the 1- and 3-day treatment case but not in the 2-day case, with the greatest expression noted at 3 days of serum starvation (~3.7%) (P<0.05). The cells treated with high serum for 2 or 3 days exhibited decreased levels of Cyp7A1 expression, with the lowest noted after 2 days (~0.49%) (P<0.05). We did not test Cyp7A1 reporter expression on cells beyond the 3-day serum starvation point due to loss of basal function.

3.4.4 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 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) (Figure 3.1d). In parallel, the transfection efficiencies of the Hepa 1-6 cells were lower in both the serum-starved (P<0.01) and high serum-treated (P<0.05) conditions as compared to the replated Hepa 1-6 control cells (Figure 3.1e).

3.4.5 Microscopic Evaluation of Media-Conditioned Cells

In an effort to understand the intracellular changes that accompany serum starvation, we examined the overall morphology as well as the 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 3.2a) revealed flat and uniformly-sized cells, as compared to the serum-starved differentiating ES cells (Figure 3.2b), 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 3.2c) and after treatment with high serum for 3 days (Figure 3.2d). However, minimal F-actin staining was noted for the serum-starved differentiating ES cells (Figure 3.2e). 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 3.2f). F-actin staining of the high serum-treated Hepa 1-6 cells (Figure 3.2g) and serum-starved Hepa 1-6 cells (Figure 3.2h) showed similar distribution patterns of F-actin 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 3.2i). We found that the differentiating ES cells serum-starved for 3 days showed greater average area ($pixel^2$) than the cells treated with high serum ($P<0.01$). 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 appear to occur concomitantly with the effect of serum starvation on cell transfectability.

3.4.6 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 3.3a). 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 non serum-starved 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 3.3b), 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 control plasmid 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 3.3c) and albumin secretion (Figure 3.3d). For both assays, the media conditioning showed no significant functional difference between the serum-starved and high serum-treated groups. The Cyp7A1-sorted cells were significantly enriched ($P<0.05$) from their CMV-sorted counterparts in both functional assays.

3.5 Discussion

The purpose of this study was to explore the effect that serum starvation has on improving gene delivery efficiency to 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^{TM} 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 (Luo and Saltzman, 2000). 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 after 3 days). This trend paralleled the plasmid transfection efficiencies for expression of both the CMV- and Cyp7A1-driven plasmids, thus demonstrating the utility of the Cy3 oligonucleotides as an early predictive measure of plasmid DNA transfection expression (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 and the activation of the serum deprivation response gene (Gustinich and Schneider, 1993; Kim et al., 2002; Park et al., 2004; Schratt et al., 2001).

Both the CMV- and Cyp7A1-transfected cells were sorted based on fluorescent reporter expression, and it was found that the serum starvation media did not alter the functional capacity of either group, when 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 DNA uptake and transfection efficiency may help us to develop improved cellular pre-transfection 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 (Lenne et al., 2006; Raucher and Sheetz, 1999B; Raucher and Sheetz, 2000; Titushkin and Cho, 2006; Titushkin and Cho, 2007). This expanded membrane may have lead to an increased endocytosis rate (Raucher and Sheetz, 1999A). The presence and formation 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 another group's study on serum-starved Swiss 3T3 cells to occur as early as two minutes after serum-containing media was added (Ridley and Hall, 1992). The actin cytoskeleton is believed to play an important role in clathrin-mediated endocytosis, the main internalization mechanism believed to be employed by Lipofectamine^{TM} 2000 (Colin et al., 2000; Douglas, 2008; Hoekstra et al., 2007; Yarar et al., 2005). Proteins involved in the nucleation step of actin filament polymerization are implicated in the formation of endocytic vesicles (Engqvist-Goldstein and Drubin, 2003; McPherson, 2002; Merrifield 2004; Munn, 2001). Thus, extended actin filaments formed as cells recover from the serum starvation may help draw endocytic vesicles from the plasma membrane. An additional endocytic mechanism by which serum starvation may have increased DNA uptake and transfection efficiency 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 (Jones, 2007). 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 5 minutes (Amyere et al., 2002; Nakase et

al., 2007). A decrease in membrane-cytoskeleton adhesive forces may occur in parallel with increased rates of fluid phase endocytosis (Raucher and Sheetz, 2001).

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 (Vecchi et al., 2001). 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 the 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. Delivering genetic vectors through non-viral 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.

Chapter 4: Conclusions and Future Applications

4.1 Summary of Studies

These studies demonstrate that non-viral liver-specific reporter vectors can be delivered transiently to differentiating ES cells to identify and enrich a subpopulation of cells that exhibit enhanced phenotypic function. We were able to assess the utility of the reporters using fluorescent reporters driven by the albumin and Cyp7A1 regulatory elements. Initially, transfection efficiency was optimized by altering cell-seeding densities and DNA : Lipofectamine™ 2000 reagent ratios. We later were able to improve transfection efficiency of both a constitutive CMV and the liver-specific Cyp7A1 plasmids dramatically by media conditioning the differentiating ES cells under serum starvation conditions for three days.

4.2 Future Work

4.2.1 Hepatocytes for Drug Toxicity Models

The hepatocyte-like cells developed in this dissertation may have the potential to serve as models of drug metabolism, enzyme induction and toxicity. The use of stem cells has the potential to provide a long-term solution to the supply issue of attaining cells. Translating the technology to human ES cell-based systems pose additional challenges, including the need to maintain a feeder culture layer, more complex induction pathways and longer time of differentiation to generate functional end-point hepatocyte-like cells. While it may be conducive to use rat ES cells to validate industrial rat hepatocyte/animal models, the propagation of rat ES cells has proved difficult and only recent reports have been generated showing feasibility of differentiation (Schulze et al., 2006). There also exist adult tissues that have been known to transdifferentiate into hepatocytes, as well as alternative progenitor

populations, including placental or fetal stem cells (Chen et al., 2008; Chien et al., 2006). In evaluating the potential of engineering stem cells, it is important to identify what parameters are critical in assessing the maturing of a cell into a hepatocyte. Essentially, hepatocytes derived from non-primary sources such as stem cells will be required to perform liver-specific protein synthesis (i.e., albumin, urea, bile, lipids, lipoproteins and fibrinogen), store glycogen and fat soluble vitamins (A, D, E, K), and undergo phase I metabolism (Cyp1A2, 2A6, 2B6, 3A4) and phase II metabolism (UDP-glucuronosyltranferase, glutathione-*S*-transferase, sulfotransferase) at levels comparable to primary hepatocytes (Hengstler et al., 2005). A modification to our purification approach may be to adapt it to a two-stage strategy to select for potential hepatocyte-like cells by sorting progenitor populations of cells (AFP+), further differentiating these cells on fibronectin-coated collagen gels supplemented with follistatin (an activin inhibitor), and then selecting and sorting for mature markers (e.g., Cyp7A1 activity, Cyp1A2 activity). The addition of a vector controlled by the regulatory elements of the Cyp1A2 promoter, or another mature phase I metabolite cytochrome P450 gene, can be constructed using the techniques described in these studies to ensure hepato-maturity of the differentiated stem cell population.

Using molecular gene reporters, cell reporter assays have been developed in the past few years as a screening tool for Cyp450 gene activity (e.g., Cyp3A4) (Vermeir et al., 2005). The assays use either transient or stable transfection of a gene into a cell line that is then dosed with a compound, which activates a reporter molecule. The reporter gene is constructed in a manner identical to that described in these studies, where a plasmid containing the regulatory elements of the gene of interest upstream from a reporter cDNA coding gene generates a detectable product (e.g., luciferase,

fluorescent protein, alkaline phosphatase, β-galactosidase). Cyp3A4 gene reporter assays have been shown to accurately rank the potency of xenobiotics (El-Sankary et al., 2001). This can be extended to developing reporters of inducers of oxidative stress and reactive metabolites. Thus, a panel of gene reporters can be adapted to dynamically monitor cells for fluorescence and assess response to stimuli without sacrificing cells. This is amenable to a high-throughput environment where multiple wells can be scanned under a fluorescent microscope or samples can be drawn into a flow cytometer. The engineering of functional, hepatocyte-like cells that are validated in pharmacological settings may spur the further development of these cells for clinical applications. However, the constraints of scale-up, purity, long-term function and immunologic tolerance will first need to be resolved.

4.2.2 Improving Methods for Non-Viral Gene Delivery

We demonstrated that 3-day serum starvation could enhance the transfection efficiency of both a constitutive CMV plasmid and a liver-specific Cyp7A1 plasmid in the differentiating ES cells. It is not clear the precise mechanism for this increase. We suspect the involvement of F-actin in altering cell shape and permitting macropinocytosis/membrane ruffling, but we would like to further probe these phenomena. For the Hepa 1-6 cells, where we did not see any improvement in transfection efficiency after three days, we may need to reduce the amount of serum in the medium to less than 0.5% or alter the length of the conditioning. An additional understanding of the mechanisms involved in improving efficiency may help to adapt and improve protocols that improve efficiency even greater or shorten the length of time required for media conditioning.

4.2.3 Transient Non-Viral Gene Delivery for Broader Applications

The major thrust of this work demonstrated that transient transfection, despite a lower efficiency than viral-mediated approaches, can be applicable to studies where short-term activation of genes is appropriate. This approach worked well where the differentiating ES cells were activated by a fluorescent reporter within 24 h and could be sorted up to 2-3 days after transfection. A future extension of this work could be to test a transient non-viral gene delivery approach in generating induced pluripotent stem (iPS) cells. Currently, groups use retro- or lentiviral vectors to insert key stem cell genes (e.g., Oct3/4, Sox2, Klf4, c-Myc, Nanog, Lin28) into adult somatic cells but point out the long-term clinical safety barriers of using viral-based components and oncogenes in this process (Lowry et al., 2008; Mali et al., 2008). Thus, it may be possible to accomplish two goals with a non-viral transient transfection system: identifying a safer means for introducing genetic material and avoiding permanent genetic modifications in the newly-formed iPS cells. A major biological question will be whether iPS cells can maintain pluripotency for sufficient duration with a transient transfection approach. Engineering solutions will need to address the complexity and feasibility of delivering multiple plasmids to be expressed simultaneously in each cell (i.e., delivery of genes in series, together as one complex, or in parallel as individual complexes).

4.3 Conclusions

Using non-viral fluorescent plasmids integrated with the regulatory elements of liver-specific genes and delivered via transient transfection, we were able to dynamically probe their activity at any desired time point in the differentiation of murine ES cells. This enabled us to identify day 17 as a key point for the purification and enrichment of the fluorescent cells. We were able to distinguish between the liver-specific functional enrichment augmented by the Cyp7A1 reporter over the albumin reporter. Using dynamic reporters can

improve the development of hepatocyte differentiation systems and can be applied broadly to the stem cell field or other systems where mixed populations are presented.

By serum starving the differentiating ES cells for three days, we were able to enhance DNA uptake and increase the transfection efficiency of a constitutive CMV-driven plasmid and the percentage of cells expressing the Cyp7A1 plasmid. This increased the number of recoverable Cyp7A1 (+) cells increased by an order of magnitude. A deeper understanding of the mechanisms by which serum starvation increase DNA uptake and transfection efficiency may help develop improved pre-transfection techniques that can be applied immediately before transfection.

Table 1.1 Hepatic differentiation protocols using embryoid bodies.

Group	Year	Source	Extracellular Matrix Proteins	Growth Factors	Analysis Methods
Hamazaki et al.	2001	Mouse	Collagen	FGFm HGF, [OSM, DEX, ITS]	RT-PCR: TTR, ALB, AFP, AAT, G6P, TAT
Yamamoto et al.	2003	Mouse	In vivo transplant	None	RT-PCR: ALB, AAT, TTR, CK19, tryptophan 2,3-dioxygenase
Asahina et al.	2004	Mouse	Gelatin or suspension	None	Cyp7A1-GFP reporter; RT-PCR: Cyp1A2, Leap2, Sth2, TAT
Lavon et al.	2004	Human	None	None	pALB-GFP sorting; Microarray and RT-PCR: Apoliliproteins, ALB, fibrinogen, alcohol dehydrogenase 1C
Heo et al.	2006	Mouse	Collagen	None	pALB-GFP sorting; PCR: ALB, AAT, G6P, TO, transplantation
Novik et al.	2006	Mouse	+/- Collagen	+/- aFGF & HGF	cDNA microarray analysis; intracellular albumin expression
Soto-Gutiérrez et al.	2006	Mouse	Co-culture	Activin A, FGF-2, HGF, Dex	pALB-GFP sorting, albumin secretion, ammonia detoxification, lidocaine, diazepam
Novik et al.	2008	Mouse	Collagen gel	OSM, SNAP	Intracellular albumin expression; Cyp1A2, Cyp2B2 detoxification

Table 3.1 Cell proliferation of media-conditioned differentiating ES cells at varying cell-seeding densities. Bold values indicate transfected conditions.

Condition	Replated (Control)	1 Day	2 Day	3 Day
Very Low Density Differentiating ES 0.5%	2000	4000	**8000**	**9000**
Very Low Density Differentiating ES 20%	2000	6000	**10000**	**22000**
Low Density Differentiating ES 0.5%	6000	**8000**	**12000**	**16000**
Low Density Differentiating ES 20%	6000	**12000**	**16000**	**24000**
Medium Density Differentiating ES 0.5%	**13000**	**14000**	14000	18000
Medium Density Differentiating ES 20%	**13000**	**18000**	18000	N/A
High Density Differentiating ES 0.5%	**16000**	**16000**	18000	N/A
High Density Differentiating ES 20%	**16000**	**18000**	N/A	N/A

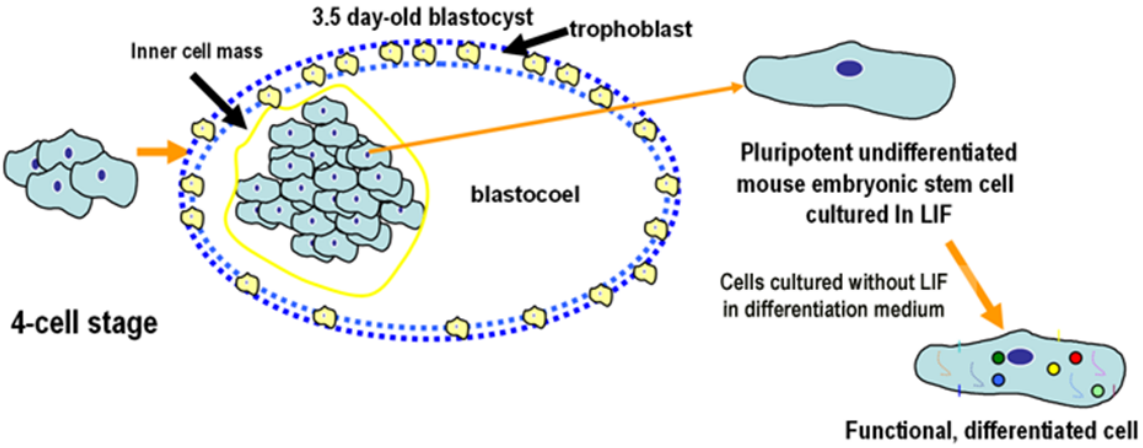

Figure 1.1 Schematic of murine ES cell derivation. A pluripotent undifferentiated mouse ES cell is derived form the inner cell mass of a 3.5 day-old blastocyst. The cell is maintained in a proliferative mode in culture with LIF and can be differentiated with removal of LIF.

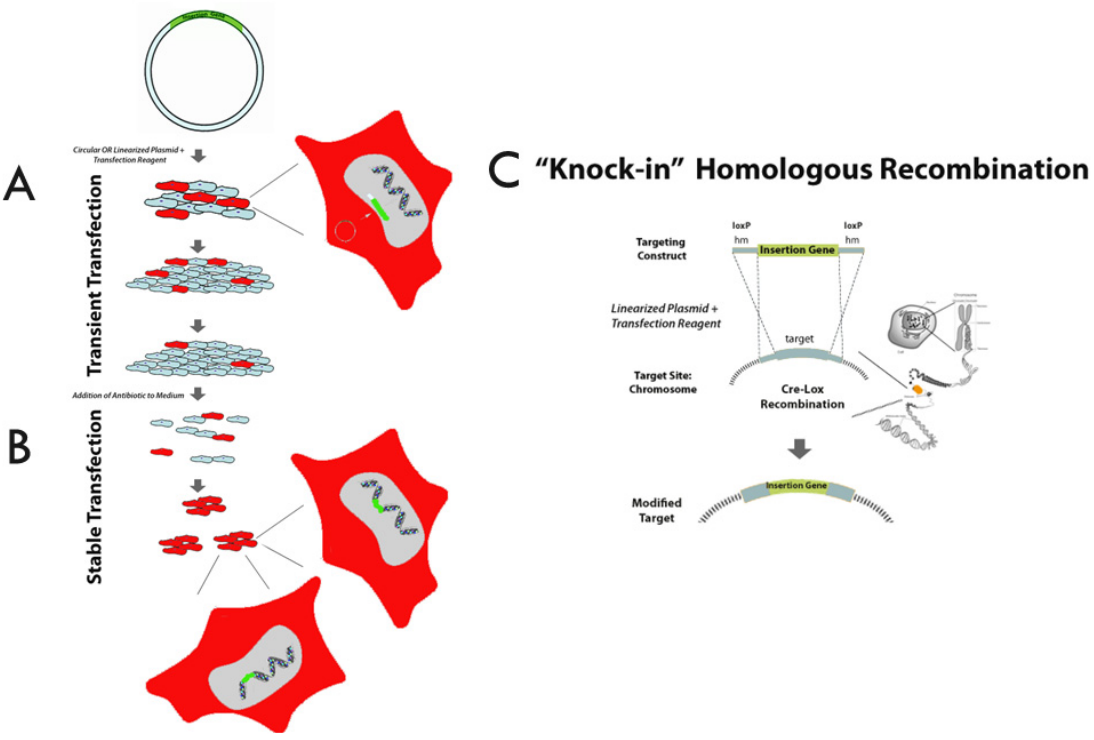

Figure 1.2 Types of transfection approaches. (**a**) Plasmid DNA is transiently transfected where a plasmid is expressed exogenously from the chromosomal DNA. (**b**) Plasmid DNA is stably integrated into random loci of the chromosomes. (**c**) A knock-in construct is designed with two flanking loxP sites to target a specific locus in the genome.

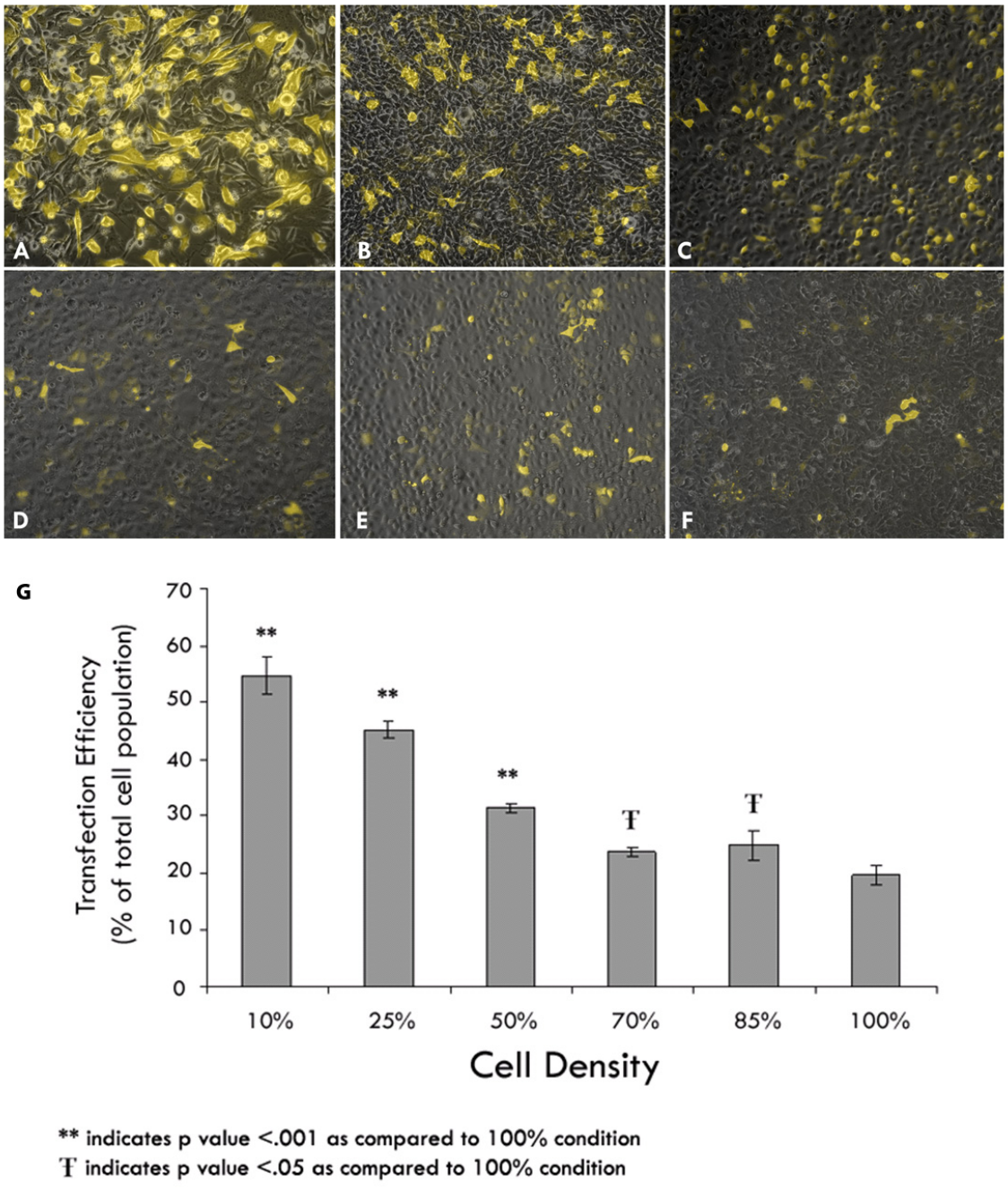

Figure 2.1. Composite phase/fluorescent overlay images of day 17 differentiated ES cells transfected with the pDsRed2-C1 vector at varying cell-seeding densities. (**a**) 10% cell-seeding density. (**b**) 25% cell-seeding density. (**c**) 50% cell-seeding density. (**d**) 70% cell-seeding density. (**e**) 85% cell-seeding density. (**f**) 100% cell-seeding density. (**g**) Transfection efficiency of day 17 differentiated ES cells at varying cell-seeding densities. Efficiency was measured as the percentage of cells fluorescently expressing the constitutive CMV-based pDsRed2-C1 plasmid.

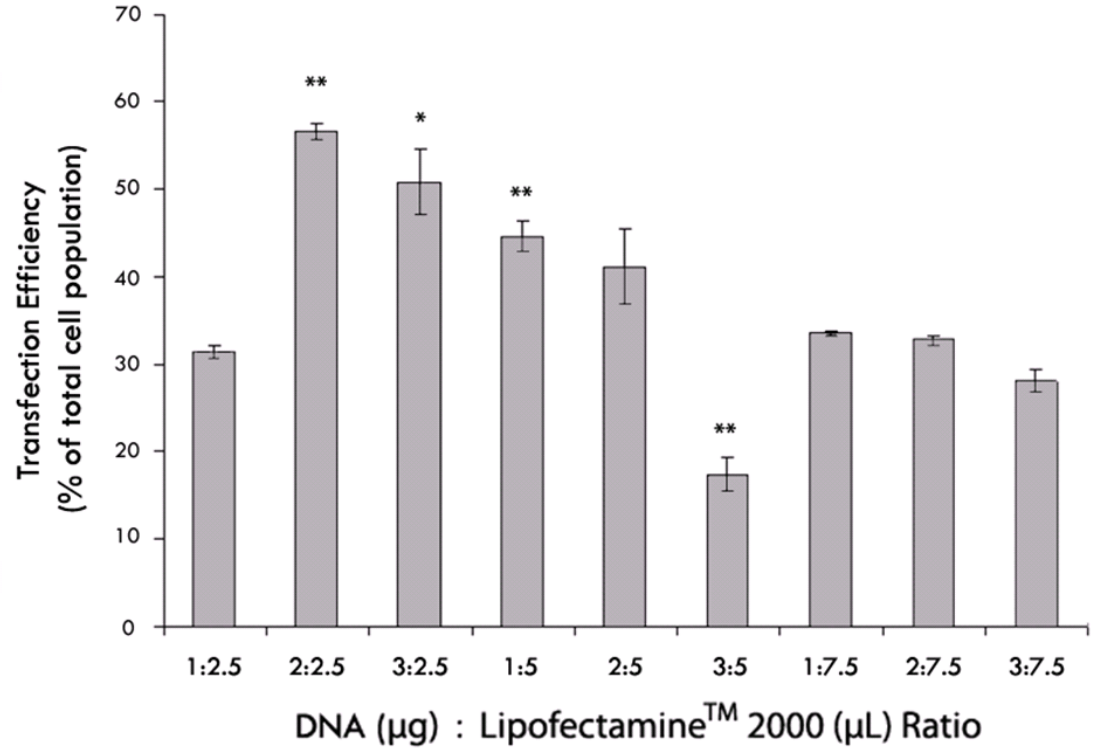

Figure 2.2. Variation in transfection efficiency of day 17 differentiated ES cells as a function of DNA : Lipofectamine™ 2000 ratios. Efficiency was measured as the percentage of cells fluorescently expressing the constitutive CMV-based pDsRed2-C1 vector. Cells were seeded at 50% confluency 4 h prior to transfection.

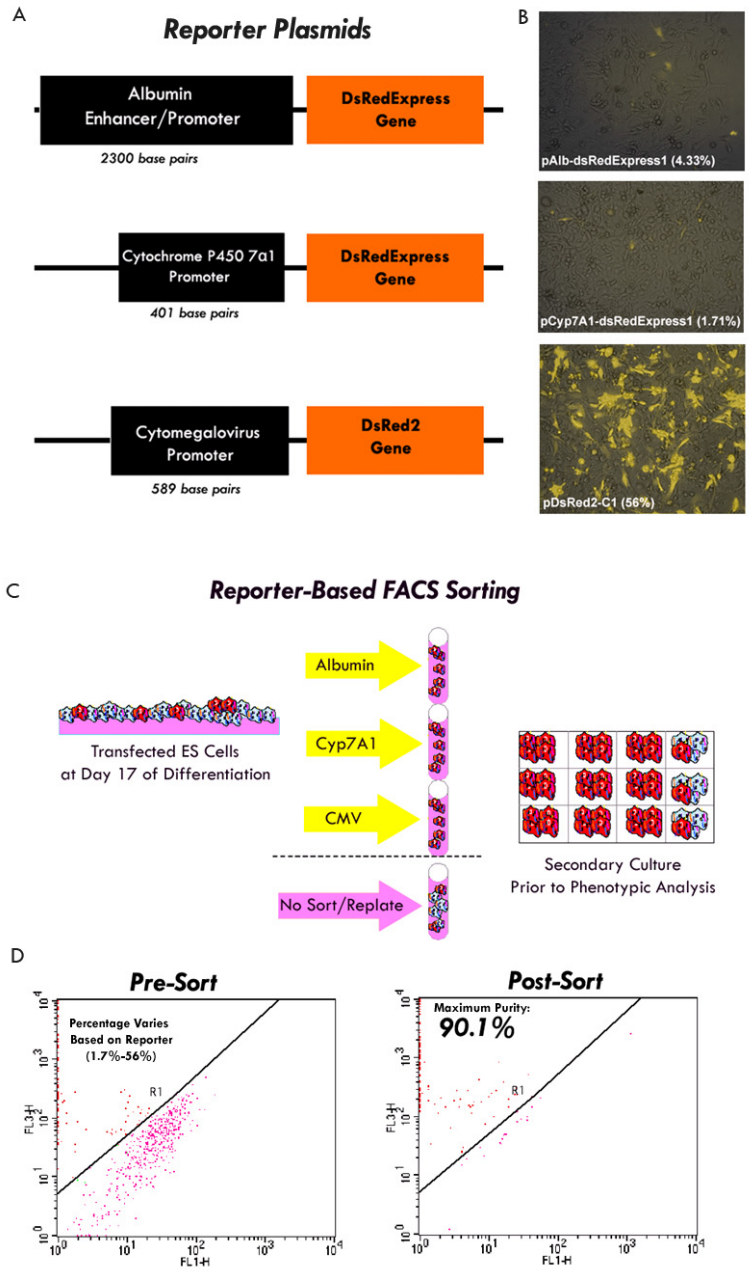

Figure 2.3. Schematic of gene reporters and cell sorting. (**a**) Reporter library of the three non-viral plasmid vectors used in the sorting study. DsRed fluorescent protein controlled by the mouse albumin enhancer/promoter, the mouse cytochrome P450 7A1 promoter and the constitutive human CMV immediate early gene promoter. (**b**) Composite phase/fluorescent overlay images of day 17 differentiated ES cells transiently expressing the reporter plasmids pCyp7A1-DsRedExpress1, pAlb-DsRedExpress1 and pDsRed2-C1, respectively. Percentages indicated were measured via flow cytometry. (**c**) Cell enrichment and analysis protocol. Three separate populations of day 17 differentiated and transfected ES cells with the albumin, Cyp7A1 and CMV plasmids are sorted 24 h post-transfection and re-plated in a 96-well plate for subsequent analysis. A non-sorted population of cells is also re-plated as a negative control. (**d**) Flow cytometry analysis of a representative transfected population pre- and post-FACS sorting.

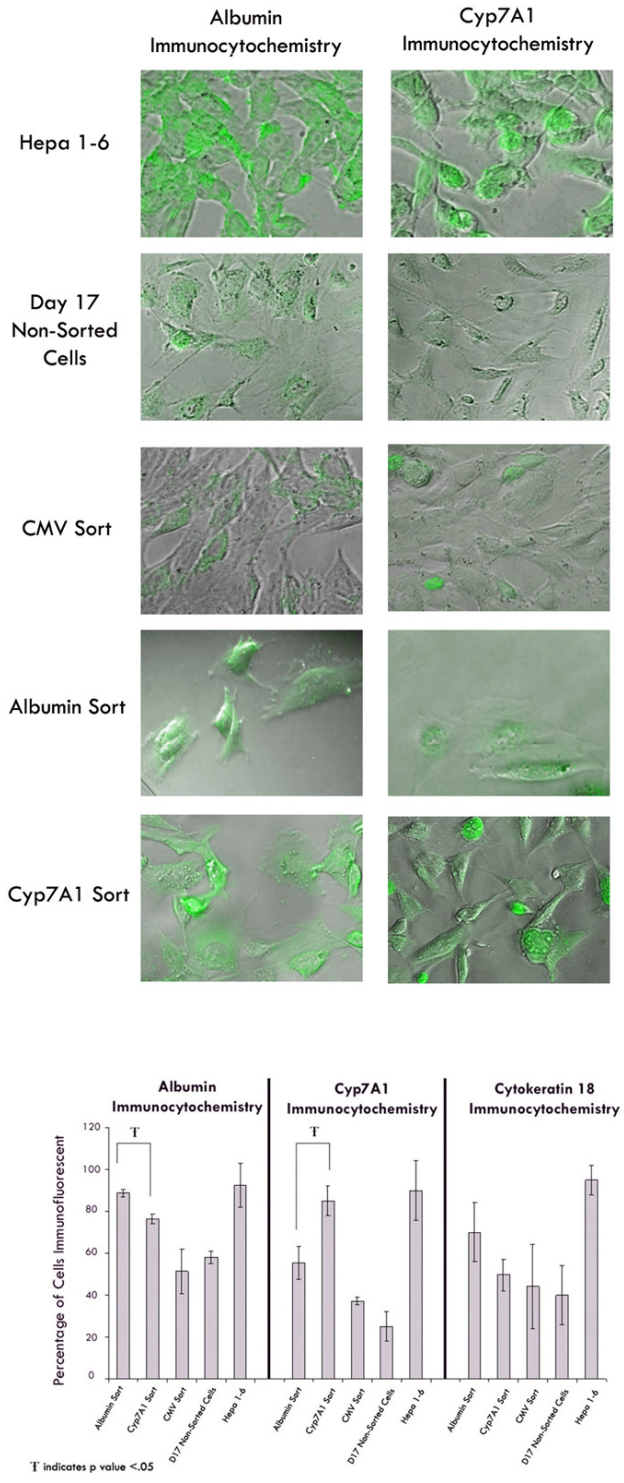

Figure 2.4. Immunocytochemistry staining and analysis of sorted populations 48 h post-sort. The cells sorted for albumin, Cyp7A1 and CMV activity in addition to a non-sorted control population and Hepa 1-6 cells were fixed and immunofluorescently stained for albumin, Cyp7A1 and cytokeratin 18. Immunofluorescence was quantified by subtracting intensity of isotype controls.

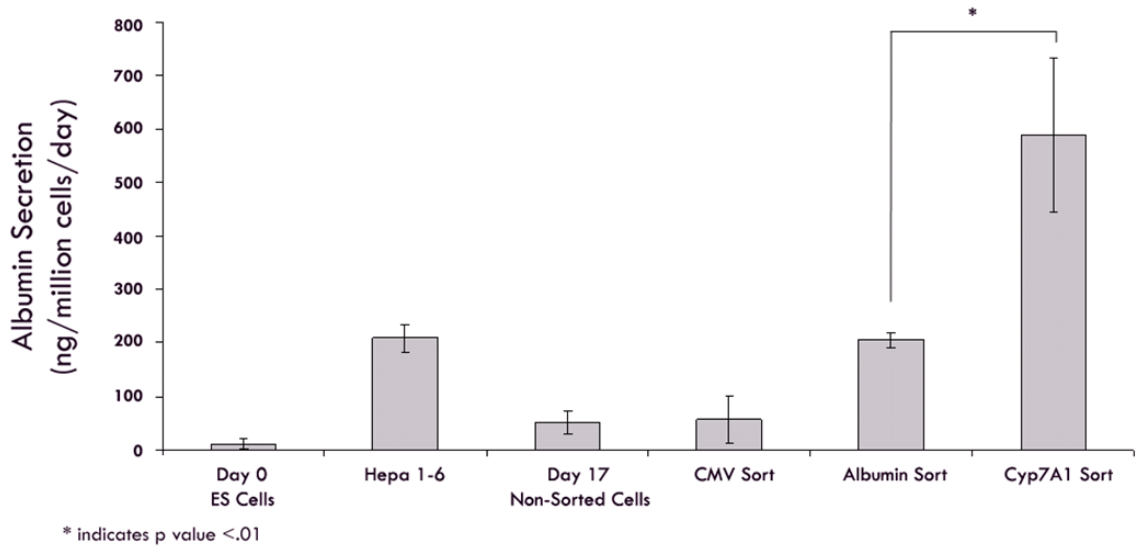

Figure 2.5. Albumin secretion of sorted day 17 differentiated ES cells, day 17 re-plated cells, Hepa 1-6 cells and day 0 non-differentiated ES cells. Albumin secretion was detected using a sandwich ELISA.

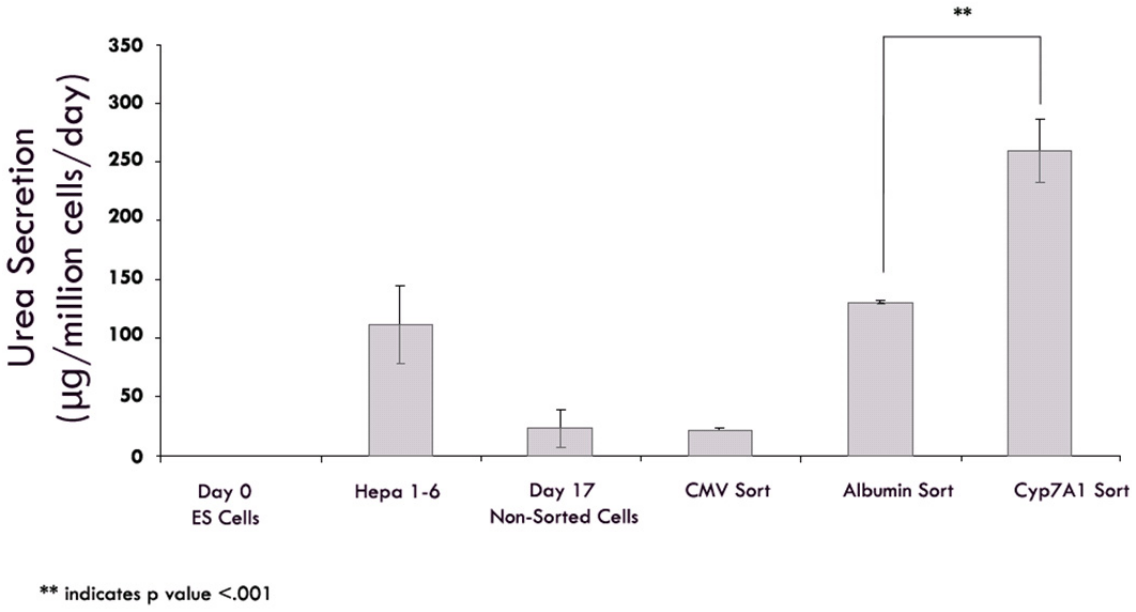

Figure 2.6. Urea secretion of sorted day 17 differentiated ES cells, day 17 re-plated cells, Hepa 1-6 cells and day 0 non-differentiated ES cells. Urea secretion was detected using a colorimetric assay.

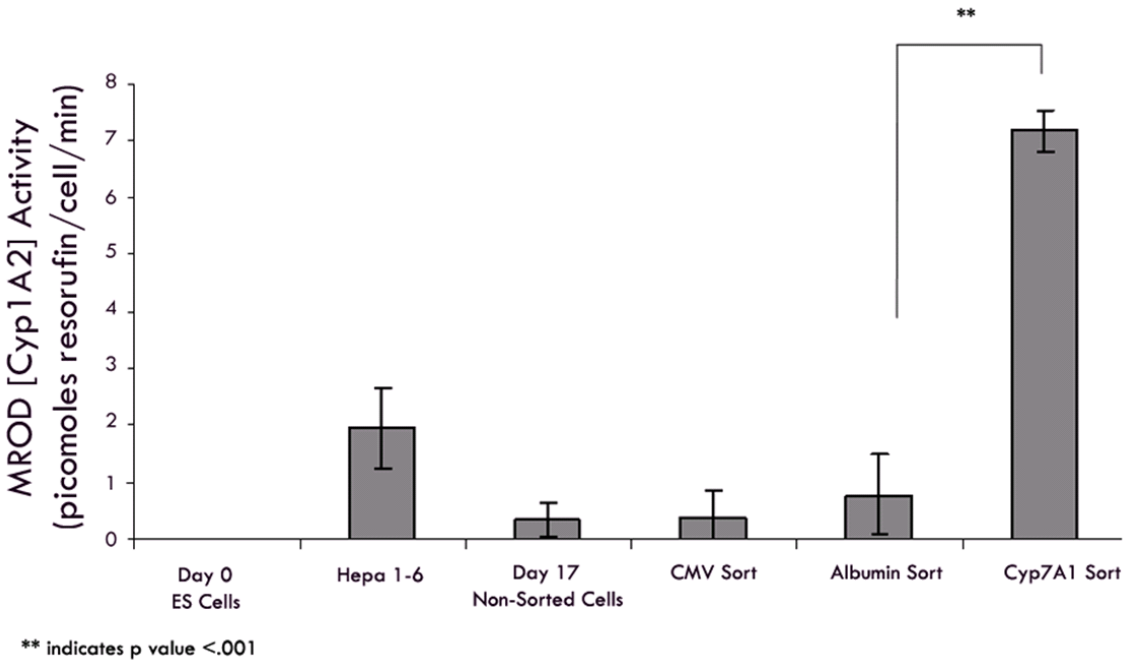

Figure 2.7. Cytochrome P450 1A2 detoxification activity of sorted day 17 differentiated ES cells versus day 17 re-plated cells and Hepa 1-6 cells. Cytochrome P450 activity was determined by measuring the formation of resorufin due to the activity of the isoenzyme methoxyresorufin-O-dealkylase (MROD, Cytochrome P450 1A2).

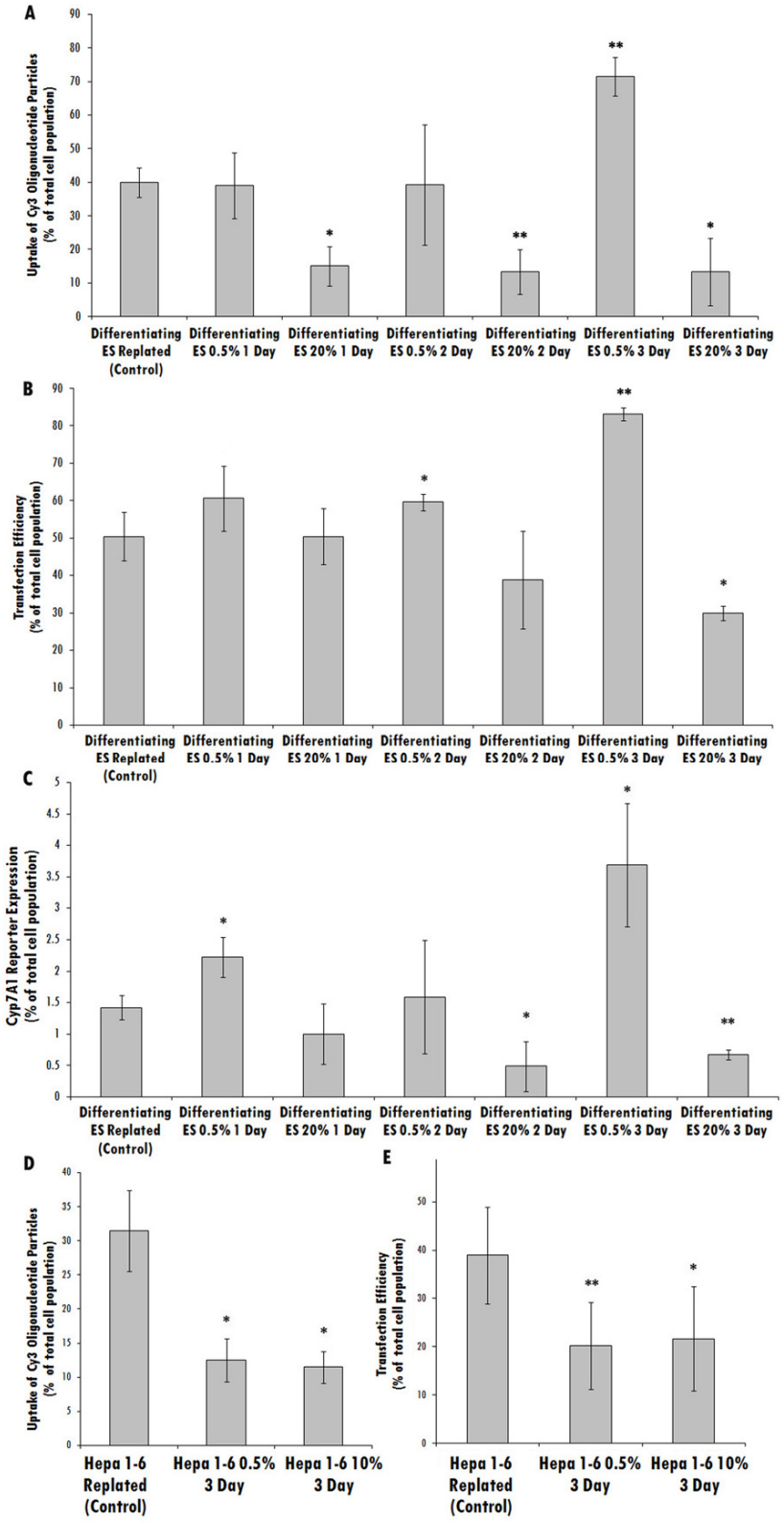

Figure 3.1. Cy3 oligonucleotide lipoplex uptake predicts plasmid transfection efficiency. (**a**) Uptake of Cy3 oligonucleotides in media-conditioned differentiating ES cells was determined

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. [* indicates *P*-value <0.05 as compared to Control group; ** indicates *P*-value <0.01 as compared to Control group]

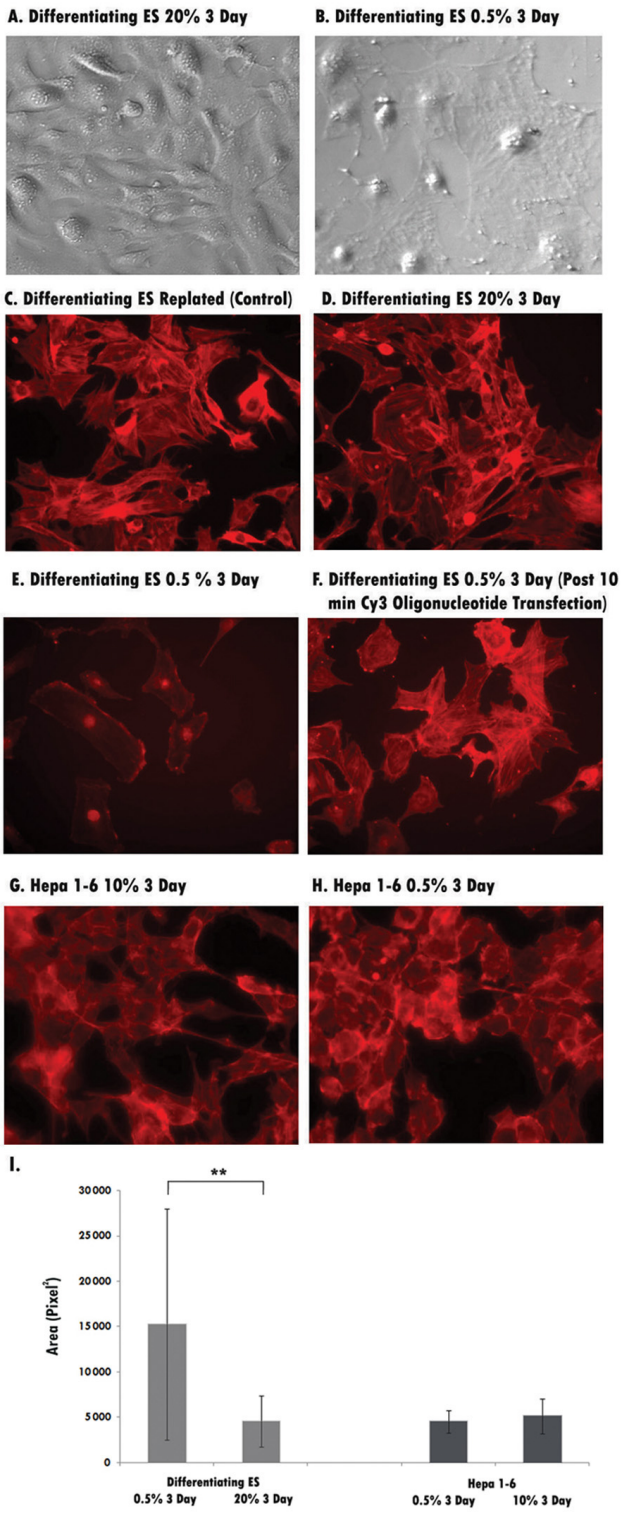

Figure 3.2. Microscopic evaluation of media-conditioned cells. Phase-contrast images were acquired using an Olympus IX70 inverted microscrope. F-actin staining was performed using a rhodamine-phalloidin visualization kit on fixed cells. (**a**) Phase-contrast image of differentiating ES cells treated 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 and then fixed and stained. (**d**) F-actin staining of differentiating ES cells treated with high serum media for 3 days prior to fixation and staining. (**e**) F-actin staining of differentiating ES cells serum-starved for 3 days prior to fixation and staining. (**f**) F-actin staining of differentiating ES cells serum-starved for 3 day and then transfected with Cy3 oligonucleotides for 10 min prior to fixation and staining. (**g**) F-actin staining of Hepa 1-6 treated with high serum media for 3 days prior to fixation and staining. (**h**) F-actin staining of Hepa 1-6 serum-starved for 3 days prior to fixation and staining. (**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 pixel^2 using Olympus Microsuite^{TM} software. [n=30 per bar for Differentiating ES; n=10 per bar for Hepa 1-6; ** indicates P-value <0.01]

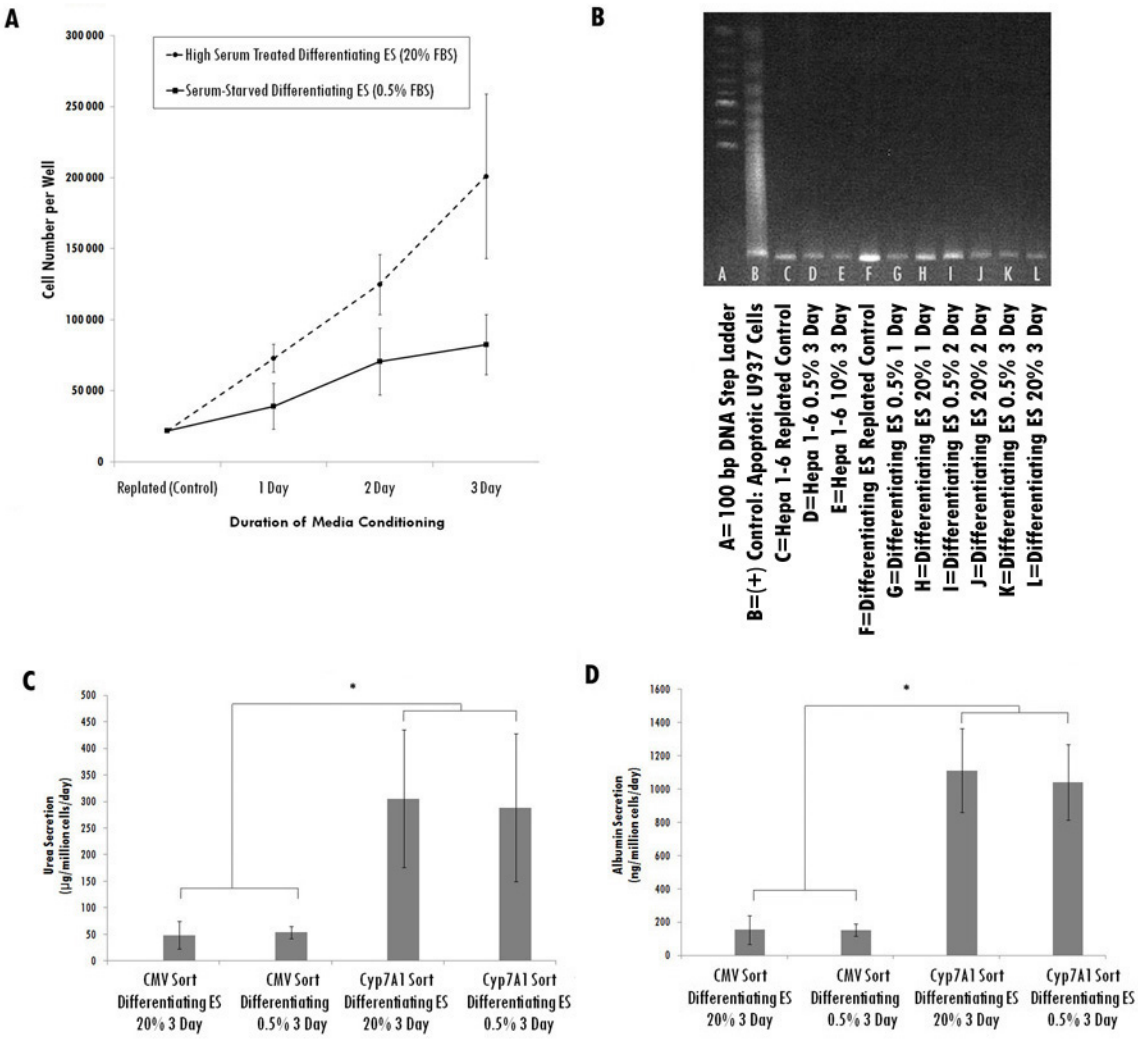

Figure 3.3. Proliferative and functional integrity of media–conditioned cells. (**a**) Cell proliferation of media-conditioned differentiating ES cells. Day 17 differentiating ES cells were uniformly plated into a 12-well plate. After 6 h, media was replenished with either 0.5% FBS-containing media or 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. [* indicates *P*-value <0.01 when comparing each of the CMV-based sorted samples to each of the Cyp7A1-based sorted samples]

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1998-2002 B.S., Biomedical Engineering, May 2002
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Academic Research Experience

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Summer 2003 Student Research Fellow, Center for Engineering in Medicine, Shriners Burns Hospital, Harvard Medical School, Boston, Massachusetts

2000-2002 Student Researcher, Drug Delivery Laboratory, NJ Center for Biomaterials, UMDNJ, Newark, New Jersey

Summer 1996 Student Researcher, Genetics Research Department, Technion University, Haifa, Israel

Life Sciences Industrial Experience

Summer 2001 Intern, Department of Neurochemistry, Aventis Pharmaceuticals, Inc., Bridgewater, New Jersey

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Publications

Wallenstein, Eric J., Jeffrey Barminko. Rene S. Schloss, Martin L. Yarmush. 2008. Transient gene delivery for functional enrichment of differentiating embryonic stem cells. Biotechnol Bioeng 101:859-872.

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Wallenstein, Eric J. and Daniel Fife. 2001. Temporal patterns of NSAID spontaneous adverse event reports: the Weber effect revisited. Drug Saf 24:233-237.

Instructional Experience and Course Development

College Level:

"Tissue Engineering I", Department of Biomedical Engineering, Rutgers University, Fall 2005, Fall 2006, Fall 2007

"Measurements & Analysis Laboratory", Department of Biomedical Engineering, Rutgers University - Fall 2003, Fall 2004

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High School:

New Jersey Governor's School at Rutgers University, July-August 2004

Community:

Instructor in basic life support, first aid and defibrillation operation, February 1998-December 2006