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Transient Gene Delivery for Functional Enrichment of Differentiating Embryonic Stem Cells

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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.

Keywords

stem cell differentiation; gene delivery; transient transfection; cell sorting; Cyp450 detoxification activity

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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., 2002). 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, 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.

Materials and Methods

Cell Culture

The ES cell line D3 (ATCC, Manassas, VA) was maintained in an undifferentiated state in T-75 gelatin-coated flasks (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), 1,000 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 1×10^6 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), 100 U/mL penicillin, 100 U/mL streptomycin (Gibco), 10 μg/mL gentamicin (Gibco). Embryoid bodies were formed and cultured for 3 days using the hanging drop method (1,000 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 5×10^4 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.

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°C in an 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 an 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 (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). 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.

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 (1×) 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). 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.

Sandwich ELISA for Detection of Albumin Secretion

Media samples were collected directly from cell cultures at the specified time points and stored at −20°C 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 37°C followed by three washes. This was followed by the addition of 200 μL of blocking solution and 30 min incubation at 37°C. 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 37°C 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 37°C 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 to 10,000 ng/mL and a linear fit of the standards was used to determine the albumin concentration in each sample.

Urea Secretion

Media samples were collected directly from cell cultures at the specified time points and stored at −20°C 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 37°C 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 with a 585 nm emission filter. A standard curve was generated by creating serial dilutions of a urea standard from 0 to 300 μg/mL and a linear fit of the standards was used to determine the urea concentration in each sample.

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. 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.

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 downstream of both insertion sites. The two vectors are hereby referred to as pAlb-DsRedExpress1 and pCyp7A1-DsRed-Express1. 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.

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™ 2000 (Invitrogen) into stem cells differentiated for 17 days post EB formation. Four hours 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.

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 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.

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.

Results

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™ 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. 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 fivefold 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™ 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 threefold, thus creating a total of nine DNA-to-reagent ratios in which to measure transfection efficiency (Fig. 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 threefold, 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.

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. 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. 3B).

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 2 days before phenotypic assays were performed (Fig. 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. 3D).

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™ 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 ($P < 0.05$) (Fig. 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 ($P < 0.01$) in albumin secretion as compared to the day 17 albumin-based sorted population (Fig. 5). The Cyp7A1-based sorted cells exhibited albumin secretion values at approximately threefold that of Hepa 1–6 cells and the albumin-based sorted cells, and sixfold 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 fourfold 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. 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 ($P < 0.001$). 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. 7). Cyp7A1-based sorted cells showed a highly significant enrichment of Cyp1A2 functional activity. The increased function in these cells was over sevenfold greater than that measured in the albumin-based sorted, day 17 non-sorted and CMV-based sorted cells and over threefold 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.

Discussion

The purpose of this study was to develop a transient gene delivery system for targeting and purifying a sub-population 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 6 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 (Heo et al., 2006; Ishii et al., 2005; Lavon et al., 2004; 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 (Heo et al., 2006; Ishii et al., 2005; Lavon et al., 2004).

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 threefold 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, 1994; 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, 1993; 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 2 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-DsRed-Express1 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 auto-fluorescence or further stimulate the Cyp7A1 promoter (Lam et al., 2006; Lee et 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., in press). 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 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 paper 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.

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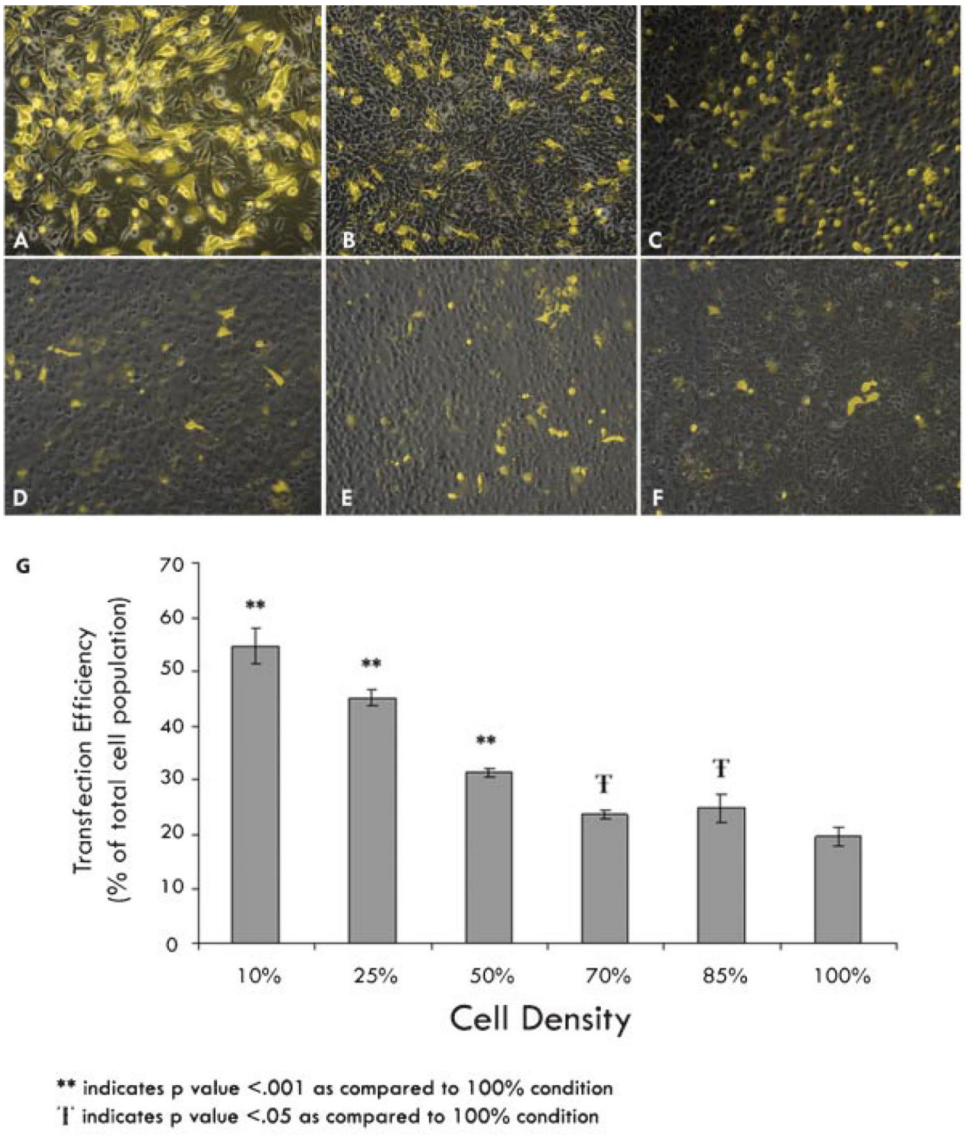

Figure 1.

Composite phase/fluorescent overlay images of day 17 differentiated ES cells transfected with the pDsRed2-C1 vector at varying cell-seeding densities. **A**: Ten percent cell-seeding density. **B**: Twenty-five percent cell-seeding density. **C**: Fifty percent cell-seeding density. **D**: Seventy percent cell-seeding density. **E**: Eighty-five percent cell-seeding density. **F**: One hundred percent 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. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

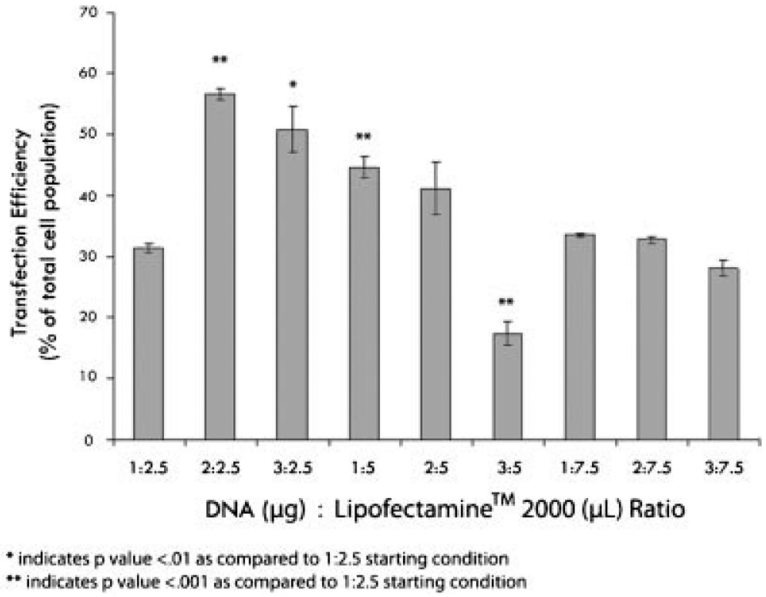

Figure 2.

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.

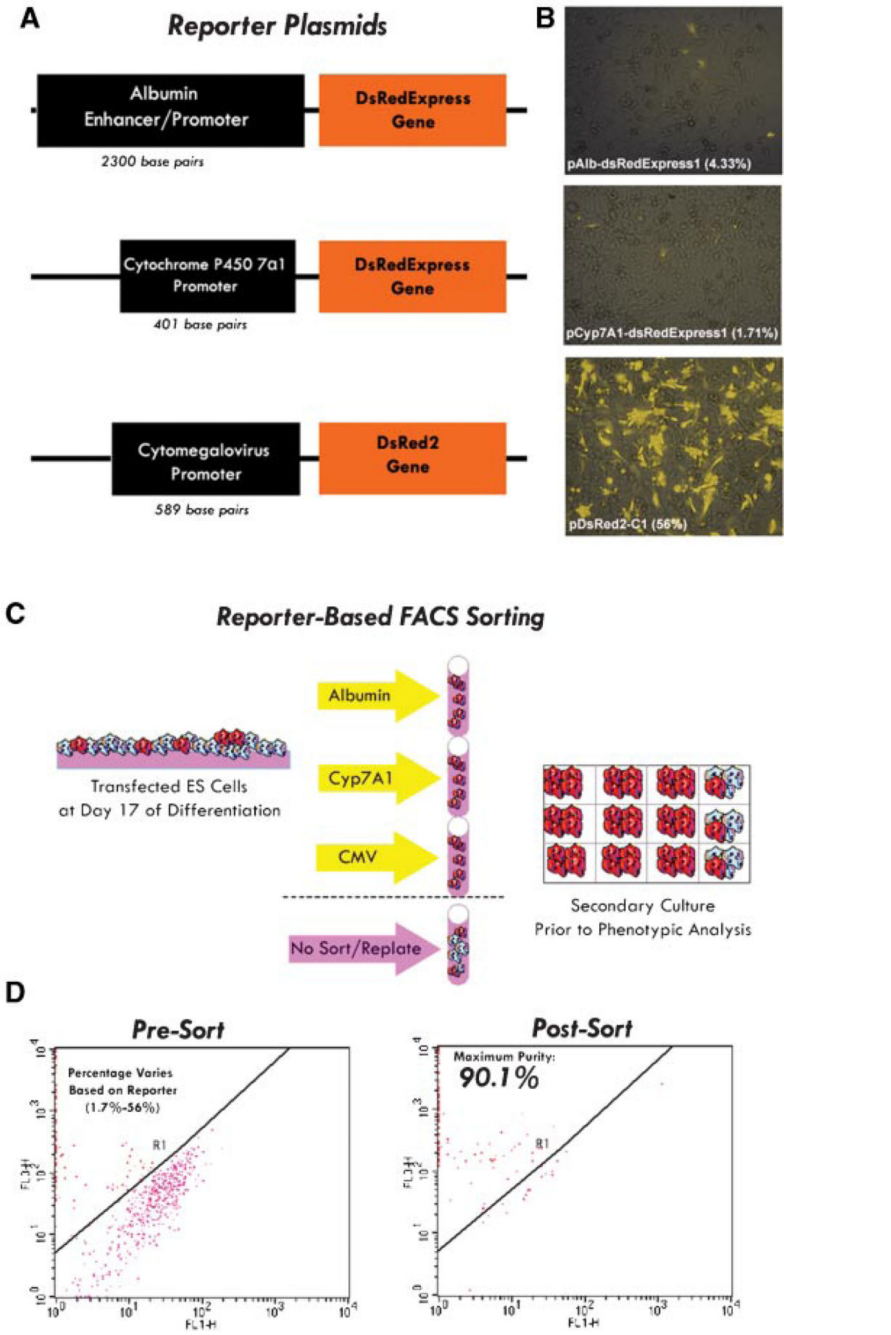

Figure 3.

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. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

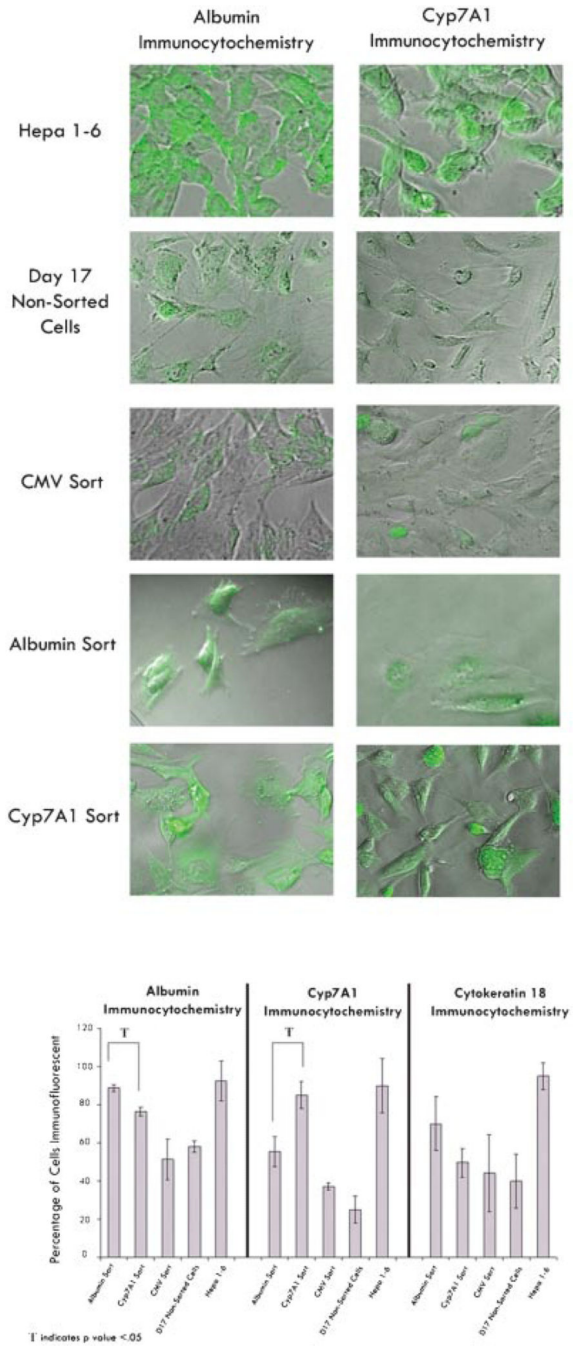

Figure 4.

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. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

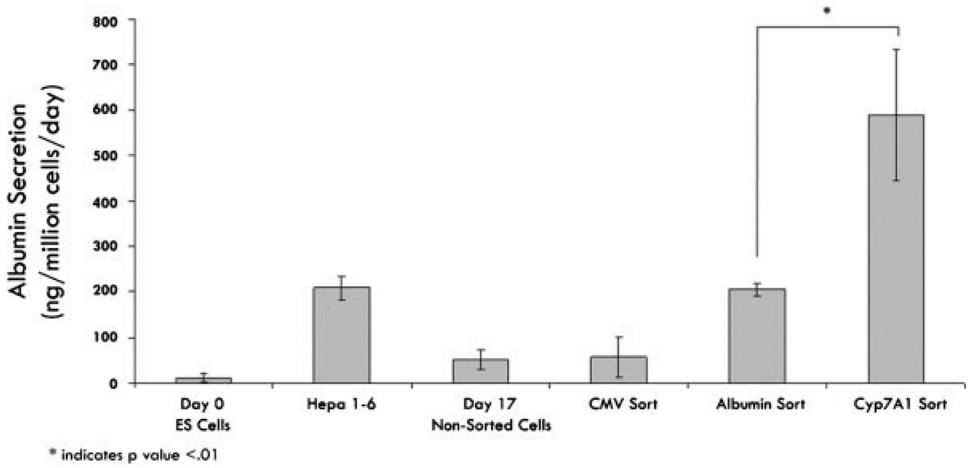

Figure 5.

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.

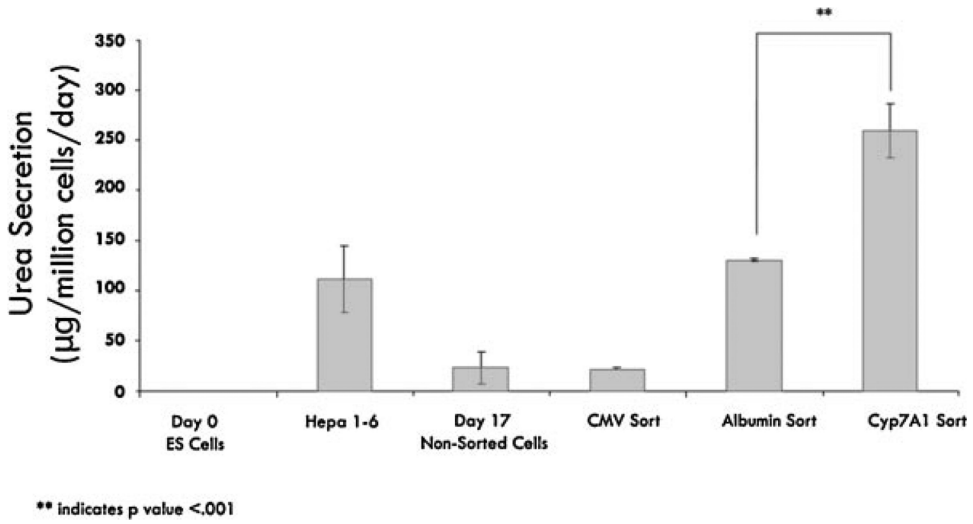

Figure 6.

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.

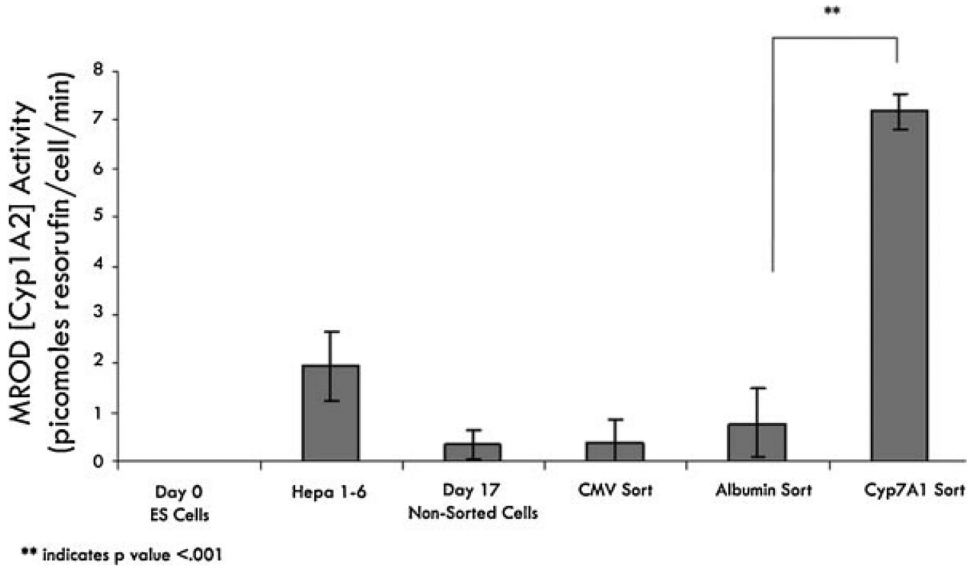

Figure 7.

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).