Human Foetal Hepatocytes: Isolation, Characterization, and Transplantation

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Abstract

Hepatocyte transplantation has become an alternative to orthotopic liver transplantation for the treatment of liver metabolic diseases. However, there is an increasing lack of donor organs and isolated mature hepatocytes are difficult to manipulate and cannot be expanded in vitro. It is therefore necessary to find alternative sources of hepatocytes, and different approaches to evaluate the therapeutic potential of stem cells of different origins are being developed. Hepatic progenitors (hepatoblasts) and/or foetal hepatocytes isolated from foetal livers may be one potential source to generate fully differentiated hepatocytes. We have reported that human foetal liver cells can be isolated and cultured. These cells also engraft and differentiate into mature hepatocytes in situ after transplantation into immunodeficient mice. Foetal cell populations could also be used as targets for gene therapy since efficient gene transfer is achieved with retroviral vectors. Use of such experimental approaches will help design strategies for clinical applications of liver cell therapy with hepatic progenitors.

Key words: Foetal hepatocytes, lentiviral vector, transduction, transplantation.

1. Introduction

Hepatocyte transplantation as an alternative to whole-organ transplantation has become a reality and to date more than 20 patients with liver metabolic diseases have been transplanted (1, 2). However, this approach is hampered not only by the shortage of organs but also by the poor engraftment efficiency of adult hepatocytes, which also do not proliferate in a quiescent liver (3). One possibility to generate hepatocytes for transplantation is the
use of foetal cells. Development of foetal liver begins when the ventral foregut endoderm buds off and gives rise to the early hepatic epithelium through signals from both the cardiac mesoderm and the septum transversum (4, 5). The cells within the liver arise from a multipotent stem cell that will give rise to the liver, pancreas, intestine, and stomach and are recognized as hepatoblasts. These progenitors are bipotent and give rise to hepatocytes and bile duct epithelial cells (or cholangiocytes).

Foetal hepatic cells display two specific characteristics common to stem cells, a spontaneous ability to proliferate, which, however, decreases with the developmental stage, and a size half that of adult cells (10–15 vs 20–30 μm), which should allow them to migrate and engraft in the parenchymal plates after transplantation better than their adult counterparts. In vitro studies with rodent and human foetal liver tissue have shown that multipotent progenitor cells, which have features of mesenchymal–epithelial transition and retain capability to differentiate into fat, cartilage bone, and endothelial cells as well as into hepatocytes and bile duct cells, can be isolated (6, 7). Recently hepatic stem cells were also isolated from foetal and post-natal human donors and have been shown to give rise to hepatoblasts in vitro and more mature hepatocytes in vivo after transplantation into the liver of NOD/SCID mice (8). We have isolated a cell population composed mostly of bipotent progenitor cells from human livers at an early stage of development (9).

In vivo studies have shown that these hepatic progenitors (hepatoblasts) after transplantation into the liver of non-conditioned immunodeficient mice were able to partially repopulate (up to 7%) transplanted liver by contrast to adult fully differentiated hepatocytes.

In this chapter we described methods for isolating from human foetal liver tissues and to characterize the cells in culture. We also describe methods for cell transplantation and in situ detection after lentiviral gene marking.

2. Materials

Foetal livers were obtained after the termination of pregnancy performed at 10–13 weeks of gestation and with the informed consent of mothers as recommended by the French Agency of Biomedicine and the local Ethics Committee of Paris XI University (Paris, France).

2.1. Human Foetal Hepatic Cell Isolation

1. Collagenase solution: 0.1 M HEPES (free Acid, ULTROL Grade, Merck), 0.002 M KCl (Sigma), 0.013 M fructose (Sigma), 0.12 M NaCl (Sigma), 2.8 mM Na₂HPO₄
12H₂O₂ (Sigma) supplemented with 10 mM CaCl₂ (Sigma) and collagenase: Worthington type 1 CLS-1 (129 U/ml).

2. Wash and plating medium: Dulbecco’s modified Eagle’s medium DMEM/HAM F12/WILLIAMS E (1:1:2) (Eurobio) supplemented with 10% heat-inactivated foetal calf serum (FCS, PAA Laboratories GmbH, Austria), 0.1% bovine serum albumin, 2 mM glutamine, and 1% antibiotics (penicillin/streptomycin, 50,000 UI, Eurobio).

2.2. Foetal Hepatic Cell Culture

1. Primaria culture dish (9.6 cm²) (BD Bioscience).
2. DMEM/HAM F12/WILLIAMS E supplemented with 5% foetal bovine serum and with:
   - 0.1% linoleic acid–albumin (Sigma Chemicals Co.); store the solution at +4°C.
   - 10⁻⁸M insulin (Novo Nordisk, Denmark); store at +4°C.
   - 10⁻⁶M hydrocortisone (Merck Sharp & Dohme, Germany); dissolve 100 mg in ethanol and dilute with PBS. Store aliquots at −20°C.
   - 10⁻⁷M 3,3',5-triodo-L-thyronine (Sigma). Dissolve in distilled water to a concentration of 25 mM and neutralize by NaOH 10 M.
   - 100 μg/ml ascorbic acid (Roche).
   - 2 mM glutamine and 1% antibiotics (Eurobio).

2.3. Reverse Transcription-Polymerase Chain Reaction Analysis

1. TRIzol reagent (Invitrogen).
2. Superscript II reverse transcriptase (Invitrogen).
3. GoTaq Flexi DNA polymerase (Promega).

2.4. Double Immunostaining

1. Collagen I pre-coated glass coverslips (BD Biocoat Cells Environment, BD).
2. Phosphate-buffered saline (PBS) (Invitrogen) solution 1× at pH 7.4.
3. Fixation solution: Formaldehyde (Sigma): Prepare a 4% (v/v) solution fresh for each experiment.
4. Quench solution: 50 mM NH₄Cl in PBS.
5. Permeabilization solution: 0.1% (v/v) Triton X-100 in PBS.
6. Blocking solution: 1% gelatin (Sigma) in PBS (Sigma).
7. Antibody dilution buffer: 1% gelatin in PBS.
8. Primary antibody: monoclonal mouse anti-human CK19 antibody (Dako, Glostrup, Denmark).
10. FITC-conjugated goat anti-human albumin antibody (Bethyl Laboratories).

2.5. Immunocytochemistry for Green Fluorescent Protein Expression

1. PBS solution 1× at pH 7.4.
2. Formaldehyde (Sigma): Prepare a 4% (v/v) solution fresh for each experiment.
3. Inhibition of endogenous peroxidase solution: 3% H₂O₂ in distilled water.
4. Quench solution: 50 mM NH₄Cl in PBS.
5. Permeabilization solution: 0.1% (v/v) Triton X-100 in PBS.
6. Blocking solution: 3% (w/v) bovine serum albumin (BSA) in PBS.
7. Primary antibody: Anti-GFP antibody, BD Living Colors A.v (Clontech, BD Biosciences, CA, USA).
8. Antibody dilution: 0.1% Tween 20 + 3% BSA in PBS.
10. Covalent conjugate between avidin and an enzyme: peroxidase-conjugated avidin (Vector Laboratories).
11. Peroxidase substrate solution: Diaminobenzidine (DAB) chromogene (Dako).

2.6. Western Blot Analysis for ERK Expression

2.6.1. Cell Lysis

1. PBS solution 1×.
2. Protease inhibitor tabs (Complete – Roche).
3. Cell lysis buffer (1X): 20 mM Tris (pH 8), 150 mM NaCl, 50 mM EDTA, 1% Triton X-100 (v/v), 2% BSA (w/v), and Complete (1X).
4. Teflon cell scrapers (Fisher).

2.6.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Mini-Protean 3 Cell (Biorad Laboratories Inc.).
2. Separating buffer (1X): 1.5 M Tris-Cl (pH 8.8), 0.4% SDS.
3. Stacking buffer (1X): 0.5 M Tris-Cl (pH 6.8), 0.4% SDS.
4. Acrylamide/bis (30% A, 2.67% bis) (Biorad Laboratories Inc.).
5. N,N,N,N′-tetramethyl-ethylenediamine (Temed – Biorad Laboratories Inc.).
6. Ammonium persulphate (APS) (Biorad Laboratories Inc.): prepare 10% solution in water and immediately freeze in single use (200 μl aliquots) at –20°C.

7. Running buffer (10X): 0.25 M Tris, 1.92 M Glycine, 10% SDS.

8. Sample buffer (3X): Tris-Cl 0.5 M, 10% SDS (w/v), 0.3 M DTT, 15% glycerol (v/v), and 0.5% (w/v) bromophenol blue.

9. Pre-stained molecular weight markers (kaleidoscope markers; Biorad Laboratories).

2.6.3. Western Blotting

1. PVDF transfer membrane Hybond-P (Amersham Biosciences).

2. Transblot SD Semi-dry Electrophoretic Transfer Cell (Biorad laboratories Inc.).

3. Bio-Dot SF filter paper (Biorad Laboratories Inc.).

4. Transfer buffer: 48 mM Tris (do not adjust pH), 39 mM Glycine.

5. PBS with Tween (T-PBS) (1X): 0.001% Tween 20 in PBS.

6. Blocking buffer: 5% (w/v) non-fat dry milk in T-PBS.

7. Primary antibody: rabbit anti-ERK1 (SC94) and mouse monoclonal anti-p-ERK (E4) (Santa Cruz Biotechnology, CA, USA).

8. Appropriated secondary antibodies conjugated to horseradish peroxidase (HRP) (Amersham Biosciences).

9. ECL western blotting detection system and Hyperfilm ECL (Amersham Biosciences).

2.7. Lentiviral Vectors

Lentiviral plasmid: the GFP gene is under regulatory sequences of apolipoprotein A-II gene (APOA-II). It has been constructed in the laboratory (10). Recombinant lentiviruses are produced by Vectalys (Toulouse, France).

2.8. Transplantation

1. PBS solution 1X.

2. Trypsin EDTA 0.25% (InVitrogen).

3. Hoechst 33258 (Sigma).

4. NOD/SCID mice (Charles River Laboratories).


6. Insulin syringe with a 30-gauge needle (BD® Micro lance 3).
2.9. Immunohistochemistry for Albumin Expression

1. PBS solution 1X at pH 7.4.
2. Formaldehyde (Sigma): Prepare a 4% (v/v) solution fresh for each experiment.
3. Inhibition of endogenous peroxidase solution: 3% H₂O₂ in distilled water.
4. Quench solution: 50 mM NH₄Cl in PBS.
5. Permeabilization solution: 0.1% (v/v) Triton X-100 in PBS.
6. Blocking solution: 1% (w/v) gelatine from porcine in PBS.
7. Primary antibody: Anti-Alb antibody (Bethyl laboratories, France).
8. Antibody dilution: 0.1% Tween 20 + Antibody Diluent (Dako).
9. Secondary antibody: rabbit anti-goat Ig linked to HRP (Thermo Scientific).

3. Methods

3.1. Cell Isolation and Culture

1. Transfer the tissue on ice in medium. If necessary transfer into a 60 mm Petri dish and mince the residual tissue with a sterile forceps and a surgical scalpel to small pieces.
2. Transfer the tissue into a 15 ml centrifuge tube and wash it once in HEPES buffer and successive centrifugation at 50×g for 2 min.
3. Put the tissue in a sterile beaker containing the collagenase solution and a magnetic cross-barrel placed in the hood on a heating magnetic stirrer at 37°C (see Notes 1 and 2).
4. Incubate under slow agitation to gently mix the tissue with the collagenase solution for 1 h (usually 50 mg in 25 ml). Every 15 min dissociate mechanically by gently pipetting up and down (see Note 3).
5. Add a volume of plating medium and filter the cell suspension using a 70 μm cell strainer.
6. Transfer the cell solution into 15 ml centrifuge tubes and pellet the cells by centrifugation at 50×g for 5 min.
7. Wash the cells three times by addition of 10 ml plating medium and successive centrifugations at 50×g for 5 min.
8. Suspend the cells in plating medium by gently pipetting up and down several times.

9. Count the viable cells with trypan blue using a Malassez cell (see Note 4).

10. Seed the cells in plating medium at a density of 25,000/cm\(^2\) on 3-cm Primaria dishes (see Note 5).

11. Change the medium after 24 h for culture medium and then everyday. Depending on the experiments culture medium can be supplemented with 5% FCS and/or cells can be grown in the presence of various growth factors including 20 ng/ml hepatocyte growth factor (HGF) (kind gift of Genentech, San Francisco, USA) (Fig. 2.1).

### 3.2. Characterization

#### 3.2.1. RT-PCR Analysis

1. Total RNA is extracted by using TRIzol reagent.

2. RNA is reverse transcribed by using the Superscript II reverse transcriptase.

3. cDNA samples are subjected to PCR amplification with DNA primers (Table 2.1).

4. RT-PCR is performed using the GoTaq Flexi DNA polymerase and the following programme conditions: first step of 5 min at 94°C, 30 cycles for 30 s at 94°C, a 30 s annealing step at 55–60°C and 30 s at 72°C, and extension for 10 min at 72°C (Table 2.1).
### Table 2.1
**Primers and conditions used for RT-PCR and size of final products**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers sequences</th>
<th>Product length (pb)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
</table>
| HNF4alpha  | Sense: CTG CTC GGA GCC ACC AAG AGA TCC ATG  
               Antisense: ATC ATC TGC CAC GTG ATG CTC TGC A  | 370                 | 55                        |
| HNF6       | Sense: GGG CAG ATG GAA GAG ATC AA  
               Antisense: TGC GTT CAT GAA GAA GTT GC  | 449                 | 55                        |
| CEBPalpha  | Sense: CTC GAG GCT TGC CCA GAC CGT  
               Antisense: GCG GGC TTG TCG GGA TCT CAG  | 395                 | 58                        |
| AFP        | Sense: AGA ACC TGT CAC AAG CTG TG  
               Antisense: GAC AGC AAG CTG AGG ATG TC  | 675                 | 55                        |
| ALB        | Sense: CCT TTG GCA CAA TGA AGT GGG TAA CC  
               Antisense: CAG CAG TCA GCC AT TCA CCA TAG G  | 354                 | 55                        |
| AAT        | Sense: AGA CCC TTT GAA GTC AAG CGA CC  
               Antisense: CCA TTG CTG AAG ACC TTA GTG ATG C  | 558                 | 55                        |
| ApoA-II    | Sense: GGA GAA GGT CAA GAG CCC GAG  
               Antisense: AGC AAA GAG TGG GTA GGG ACA G  | 247                 | 60                        |
| Factor IX  | Sense: TGT TGG TGT CCC TTT GGA TT  
               Antisense: TCA CTC AAA GCA CCC AAT CA  | 312                 | 55                        |
| Cyp3A7     | Sense: AAG TCT GGG GTA TTT ATG ACT  
               Antisense: CGC TGG TGA ATG TTG GAG AC  | 220                 | 60                        |
3.2.2. Fluorescence Double Immunostaining

1. Culture the cells on collagen I pre-coated glass coverslips.
2. Wash the cells with PBS twice. Fix the cells with 4% paraformaldehyde (PFA) in PBS for 15 min at 4°C.
3. Wash the cells with PBS three times.
4. Pretreat the cells with 1% gelatin in PBS.
5. Incubate the cells for 1 h at room temperature with monoclonal mouse anti-human CK19 antibody (1/100).
6. Wash the cells three times.
7. Incubate the cells for 1 h at room temperature with Cy-3-conjugated goat anti-mouse antibodies (1:400).
8. Wash the cells three times with PBS.
9. Incubate the cells for 1 h at room temperature with FITC-conjugated goat anti-human albumin antibody.
10. Counterstain nuclei with DAPI.

3.2.3. Western Blot Analysis

By contrast to adult human hepatocytes, foetal cells express the phosphorylated form of ERK, which is induced after stimulation by HGF.

1. Rinse cells with PBS (4°C) and scrape the cells in ice cold lysis buffer (1 ml/10⁶ cells).
2. Sonicate samples for 10 s and centrifuge 5 min at 19,000×g.
3. Collect the supernatants and quantify proteins using BCA assay. Store aliquots at –20°C.
4. Prepare a 1.5 mm thick, 8% gel by mixing 1.35 ml of acrylamide/bis solution, 1.25 ml separating buffer, 2.37 ml water, 25 μl APS 10%, and 5 μl Temed. Pour the gel, leaving space for a stacking gel, and overlay with isopropanol. The gel should polymerize in about 30 min. Pour off isopropanol and rinse the top of the gel twice with water.
5. Prepare the stacking gel by mixing 0.325 ml of acrylamide/bis solution, 0.625 ml stacking buffer, 1.5 ml water, 25 μl APS 10%, and 5 μl Temed. Pour the stack and insert the comb. Wash the wells with running buffer (1X).
6. Mix sample (10 μg whole lysate) with one-third of sample buffer (3X) and boil for 5 min, cool on ice for 10 min, and spin a few seconds.
7. Load samples, include one well for molecular weight prestained marker.
8. Complete the assembly of the gel unit and carry out electrophoresis at 20 mA through the stacking gel and 40 mA through the separating gel (4 h).
9. Soak the gel in transfer buffer for 15 min.
10. Cut PVDF membrane and six pieces of filter paper to the dimension of the gel.
11. Soak the membrane briefly in 100% methanol, then in distilled water for 5 min, and in transfer buffer for 15 min.
12. Saturate filter papers and membrane in transfer buffer for 30 min.
13. Assemble the sandwich transfer onto the anode as follows: three filter papers, PVDF membrane, gel, and three filter papers.
14. Exclude all air bubbles by rolling a pipet over the surface of the paper. Transfer is accomplished within 1 h at 160 mA.
15. Incubate the membrane in blocking buffer overnight at 4°C on a rocking platform then wash it three times for 15 min each with T-PBS.
16. Add the primary antibody for 2 h at room temperature on a rocking platform (anti-ERK1, E4 at 1:500 or anti-p-ERK, SC-94 at 1:2,000) and wash the membrane four times for 15 min each with T-PBS.
17. Add the appropriate secondary antibody in blocking buffer (anti-rabbit 1:2,000 and anti-mouse 1:1,000) for 1 h at room temperature on a rocking platform, then wash five times for 10 min each with T-PBS.
18. Reveal immunoreactive bands by the enhanced chemiluminescence system.
19. Once a satisfactory exposure for the p-ERK has been obtained, the membrane is then stripped of that signal and then reprobed with antibody that recognizes unphosphorylated ERK.
20. The stripping is realized according to the conditions described by the manufacturer.
21. The membrane is then blocked as mentioned earlier and then ready to be reprobed anti-ERK as described above.

3.3. Cell Labelling with the Hoechst Fluorescent Dye

1. Remove the culture medium and wash the cells with Ca++/Mg++-free PBS.
2. Remove PBS and add 0.25% trypsin solution. Put the dish back to the incubator for a few minutes and monitor dissociation under an invert phase-contrast microscope.
3. When cells are released add several millilitres of plating medium to inhibit trypsin, centrifuge at 50×g for 5 min.
4. Wash once in PBS and adjust hepatocyte suspension at \(10^7\) cells/ml in serum-free medium per 12 ml conical tube.

5. Add 5 \(\mu\)l of Hoechst 33258 at 10 mg/ml to the cell suspension and incubate for 30 min at 37\(^\circ\)C with gentle agitation.

6. Stop the reaction by addition of 1 ml FCS then addition of 9 ml medium containing 10% FCS (plating medium).

7. Transfer the cells to new 12 ml conical tubes and wash the cells three times in plating medium and centrifugation at 50\(\times\)g for 5 min.

8. Suspend the cells in PBS at 4 \(\times\)10\(^5\) cells/10\(\mu\)l for transplantation.

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### 3.4. Retroviral Transduction

Transferring genes into foetal hepatic cells could enhance the scope of cell transplantation. Recombinant vectors derived from the onco-retroviruses (Moloney murine leukaemia virus) can be used to efficiently transduce foetal hepatic cells, by contrast to adult hepatocytes, since the cells divide extensively the first days after plating (9). However, since these vectors infect both dividing and non-dividing cells, and since the design of lentiviral vectors leads to safer recombinant lentiviruses, devoid of viral enhancer and promoter sequences, with high titre it is recommended to use lentiviral vectors rather than oncoretrovirus. These self-inactivating vectors express the gene of interest from internal promoters. An important consideration in designing vectors is promoter selection, especially if in situ gene marking of transplanted cells is to be performed. Viral promoters, including cytomegalovirus immediate-early promoter (CMV-IE), are silenced in situ and therefore ubiquitous promoter such as the eukaryotic initiation factor 1 alpha promoter (EF1\(\alpha\)) or hepatocyte-specific promoter such as alpha 1-antitrypsin have to be used. As most of the vectors are pseudotyped with the vesicular stomatitis G (VSV-G) envelope, they can be concentrated to yield high-titre viral particles.

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### 3.4.1. Cell Transduction

Foetal cells are isolated as described above.

They are suspended in plating medium without serum at a concentration of 1–10 \(\times\)10\(^6\) cells/ml in cryotubes and incubated with recombinant lentivirus at MOI=30 for 2–3 h at 37\(^\circ\)C. The tubes are left unscrewed to allow gas exchanges (see Notes 6 and 7).

After incubation the transduced cells are plated in plating medium.

After 24 h the medium is replaced by HDM supplemented with 5% FCS and after 4–5 days of culture (see Note 8).
3.4.2. Detection of Transduced Cells

1. Wash the transduced cells three times with PBS.
2. Fix with 4% PFA for 15 min at 4°C then wash three times for 5 min.
3. Incubate in blocking solution for 20 min at room temperature.
4. Incubate in PBS/0.1% Triton X-100 for 10 min at room temperature.
5. Incubate in PBS/1% bovine serum albumin for 1 h at room temperature.
6. Incubate the cells with the anti-GFP antibody for 1 h at room temperature then wash three times for 5 min each in 0.1% Tween/PBS.
7. Apply the secondary biotinylated antibody according to the M.O.M kit staining procedure, then wash three times for 5 min each in 0.1% Tween/PBS.
8. Reveal by incubation with a solution of amino-ethylcarbazol for 10 min at room temperature and wash three times for 5 min each. Mount in glycergel (Fig. 2.2).

Fig. 2.2. Transduction of foetal hepatocytes using a GFP-expressing lentiviral vector. Phase-contrast micrograph (magnification ×200).

3.5. Cell Transplantation

1. 4- to 5-week-old NOD/SCID mice.
2. Anaesthetize with suitable medication (ketamine, 50 mg/kg +xylazine 20 mg/kg), place in right decubitus position, and clean the abdominal wall with iodine.
3. Make 0.5–1 cm incision below the left subcostal abdominal wall with sharp scissors.
4. Inject $0.8–1 \times 10^6$ cells through a 1 ml insulin syringe with a 30-gauge needle (BD® Microlance 3) into the spleen.
5. Close the abdominal incision with 4-0 nylon sutures.
6. Return the animal into its cage, keep warm under heating lamp until recovery from anaesthesia, and administer analgesia.

3.6. Identification of Engrafted Hepatocytes by Albumin Histochemical Staining

1. Fix the samples in formaldehyde solution for 10 min at room temperature.
2. Wash the samples three times for 5 min each with PBS.
3. Inhibit endogenous peroxidases with 3% H$_2$O$_2$ in PBS for 30 min at room temperature and wash twice with PBS.
4. Incubate in NH$_4$Cl for 15 min at room temperature to quench residual formaldehyde and then wash three times with PBS.
5. Incubate in PBS/0.1% Triton X-100 for 10 min at room temperature and then rinse three times with PBS.
6. Incubate in blocking buffer for 1 h at room temperature.
7. Incubate the sections with the anti-Alb antibody for 1 h at room temperature in a humid chamber and wash three times with PBS.
8. Apply the secondary antibody linked to HRP, then wash the sections twice with PBS.

Fig. 2.3. Detection of transplanted hepatocytes. Transplanted cells are visualized in recipient mouse liver using an anti-human albumin antibody (magnification ×400).
9. Apply DAB solution on the sections and control development times under a microscope (between 2 and 10 min in the dark), then wash with distilled water three times for 2 min each.

10. Mount the samples in glycergel or glycerol (90% in PBS) if counterstaining is necessary (Fig. 2.3).

4. Notes

1. Filter HEPES solution after adjusting pH to 7.4. Prepare collagenase solution immediately before dissociation and filter it.

2. The batch of collagenase is critical for cell viability. Batches are first tested for their ability to produce high yields, maximum viability, and membrane recovery of rat hepatocytes. Currently collagenase type 3 (PAA Laboratories, France) or type 1 CLS-1 Worthington is used.

3. Agitation of the collagenase solution for liver tissue dissociation must be controlled and slow. The choice of the magnetic stirrer is therefore important. We purchased the stirrer from Fisher Bioblock Scientific.

4. The number of cells plated is difficult to estimate as the cells are isolated in clusters.

5. Unattached cells mainly haematopoietic cells wash easily from the surface of monolayer cultures during medium changes after 48 h. Additional washes can be performed.

6. We use aliquots of the virus stocks to prevent decrease of virus titre after freeze–thaw cycle. Virus can be thawed and frozen once if rapidly frozen in liquid nitrogen prior to storage in –80°C.

7. Polybrene is generally used at 8 μg/ml. We rather use 3 μg/ml for foetal and adult hepatocytes for retroviral transduction.

8. We wait at least 5 days after transduction, so that all virus particles are integrated but also to avoid any possible pseudotransduction, i.e. passive incorporation of GFP protein into the viral particle, or phagocytosis of plasmid DNA interfering with the results (11). As control, cells can also be pre-incubated for 1 h prior to transduction with different concentrations (from 1 to 10 μM) of 5′-azido thymidine (AZT, GlaxoSmithKline), an inhibitor of reverse transcriptase, which is also added every 24 h to the culture medium.
until cell harvest. In this sample transduction rate is expected to be close to 0%.

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References

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