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A rapid two-step method for isolation of functional primary mouse hepatocytes: cell characterization and asialoglycoprotein receptor based assay development

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Abstract Primary mouse hepatocytes are an important tool in the biomedical research field for the assessment of hepatocyte function. Several methods for hepatocyte isolation have been published; however, many of these methods require extensive handling and can therefore compromise the viability and function of the isolated cells. Since one advantage of utilizing freshly isolated cells is to maintain an environment in which the cells are more comparable to their in vivo state, it is important to have robust methods that produce cells with high viability, good purity and that function in a similar manner to that in their in vivo state. Here we describe a modified two-step method for the rapid isolation and characterization of mouse primary hepatocytes that results in high yields of viable cells. The asialoglycoprotein receptor (ASGPR), which is one of the most abundant cell surface receptors on hepatocytes, was used to monitor the function of the isolated hepatocytes by demonstrating specific binding of its ligand using a newly developed flow cytometry based ligand-receptor binding assay. Also, an in vitro screening method for siRNA drug candidates was successfully developed utilizing freshly isolated hepatocytes with minimum culture time.

Keywords Hepatocytes · Two-step isolation method · Asialoglycoprotein receptor (ASGPR) · GalNAc

Introduction

Hepatocytes are involved in many biological processes including protein synthesis, sugar metabolism, detoxification, as well as synthesis and secretion of cholesterol, bile acids, and phospholipids to name a few. Because of their central role in liver function, the use of freshly isolated primary hepatocytes has become an important tool to investigate various aspects of liver biology including metabolic disease, toxicology, oncology, and parasitology (Goncalves et al. 2007; Qian et al. 2007; Wallace et al. 2010; Warner et al. 2003; Wu et al. 2002). Hepatocytes isolated using the standard two step perfusion method have been used in culture as well as in cell suspension assays for many years (Klaunig et al. 1981; Li et al. 2010; Warner et al. 2003). In these ex vivo assays, cells are used immediately after isolation to avoid changes in gene regulation, polarization and de-differentiation (Bhandari et al. 2001; Godoy et al. 2010; Talamini et al. 1997; Wallace et al. 2010). The main downside of the current protocols is the requirement for cell purification through gradient

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centrifugation which extends the time that the cells are manipulated and can result in reduced cell number and viability. Here we describe an isolation method that removes the need for the gradient centrifugation purification step and results in high yields of viable cells that retain their function as measured by the activity of the highly expressed asialoglycoprotein receptor (ASGPR).

A well-established target for specific drug delivery to hepatocytes is the hepatic C-type lectin asialoglycoprotein receptor (ASGPR). ASGPR is expressed at high levels on hepatocytes (approximately 500,000 copies/cell) and plays an important role in the clearance of desialylated glycoproteins from serum via receptor mediated endocytosis (Braun et al. 1996; Ishibashi et al. 1994: Mu et al. 1993). The ASGPR has a high affinity for terminal β-linked galactose (Gal) or N-acetylgalactosamine (GalNAc) residues and binding of its ligands results in rapid internalization (Ashwell and Harford 1982; Park et al. 2005; Spiess 1990). The binding affinity of GalNAc ligands to ASGPR depends on the number of GalNAc clusters present in the molecule with the tetraantennary (GalNAc$_4$) showing higher affinity followed by triantennary (GalNAc$_3$) and biantennary (GalNAc$_2$) ligands. This ligand-receptor binding characteristic was observed in primary hepatocytes, but not when the isolated receptor was used in cell-free based assays (Connolly et al. 1982; Rensen et al. 2001). We demonstrate that the ASGPR is present, active, and functional after using the isolation protocol described in this article.

The following describes a rapid two-step perfusion method for the isolation of murine hepatocytes, a culturing technique using commercially available media additives, ex vivo binding assay of GalNAc compounds and a high throughput system for screening of siRNA-GalNAc conjugates. The protocol for murine hepatocyte isolation has also been applied to rat hepatocyte isolation with minimum changes in flow rates and perfusion time with similar outcomes as for the mouse primary hepatocytes (results not shown).

Materials and methods

Equipment

40 °C water bath, 22G feeding needle round tip (Fine Science Tools), monofilament nylon (suture), 75 µm cell strainer (Millipore), surgical instruments, heated operating table (Harvard Apparatus), lamp, pump (Watson-Marlow Sci-Q 401U/D Series), 50 mL conical tubes (Corning), syringes, hemocytometer and trypan blue (Sigma-Aldrich).

Reagents for hepatocyte isolation and culture

Solution 1: Hank's Balanced Salt Solution (HBSS, Gibco), EDTA 0.5 mM, pH = 8. *Solution 2*: Dulbecco's Modified Eagle's Medium (DMEM, Gibco), Collagenase Type I 0.8 mg/mL (Worthington Biochemical Group). *Solution 3*: Dulbecco's Modified Eagle's Medium. Ketamine and Xylazine, bovine serum albumin (BSA, Sigma-Aldrich), hepatocyte Plating/Thawing and Maintenance Media (Gibco), rat tail collagen (Sigma-Aldrich), tissue culture plates (Falcon).

Isolation procedure

1. 8–10 weeks old C57/Bl6 females are utilized.
2. All solutions are prepared fresh (just before isolation).
3. Pre-warm Solution 1 and 2 to 40 °C, set up tubing and perfusion pump.
4. Set up operating table at 40 °C.
5. Anesthetize the animals by injecting a mixture of Ketamine (80 mg/kg) and Xylazine (5 mg/kg) in 200 µL of saline intraperitoneally.
6. Prepare the animal for surgery (clean/sanitize using 70% ethanol).
7. Immobilize animal with tape. Dissect the abdominal cavity, move the gastric system to the right and expose the visceral vena cava. Remove the rib cage taking care not to rupture any blood vessels.
8. While the heart is still beating, insert the catheter (22G feeding needle/round tip, connected to Solution 1) into the vena cava through the right atrium. Keep the catheter in place by applying a surgical knot (to stabilize the catheter to prevent rupture/puncture of the vessel).
9. Start perfusion, and as the flow begins and the buffer reaches the liver make a small cut in the visceral vena cava. The flow rate should be 5 mL/min.
10. If inserted correctly, all of the blood will drain out in 1–2 min. Create back pressure by blocking

the visceral vena cava with a finger (every 2 min) for a few seconds (liver should inflate). Perfuse for 5–7 min.

11. Position a lamp a few inches above the animal to help maintain the abdominal cavity at ∼37 °C Continue to monitor the cavity temperature throughout the procedure. Do not overheat.

12. Switch the tubing from Solution 1 to Solution 2 and continue perfusion for 7–8 min (flow rate 5 mL/min).

13. Collect the liver (sever the portal vein and the connective tissue and separate from the body) into tube containing 15 mL of Solution 2.

14. Cut the liver sac to release the hepatocytes.

15. Add 35 mL of Solution 3 and pass the liver suspension through a cell strainer (use spatula to gentle stir tissue on strainer).

16. Spin cells at 50 g for 1 min.

17. Wash the pellet twice with Solution 3 (45 g, 1 min). The supernatant should appear clear after the last wash.

18. Count cells with a hemocytometer, calculate viability based on Trypan Blue staining and re-suspend pelleted cells in DMEM/2% BSA at 1 million cells/mL. Spin once again at 50 g and re-suspend the cells in plating media for cell culture.

Assessment of hepatocyte purity

Approximately 100,000 cells were lysed in 1 mL of Tissue and Cell Lysis Solution (Epicenter) containing 10 µL of 20 mg/mL Proteinase K (Ambion). Lysates were incubated in a tube shaker for 1.5 h at 65 °C and spun at 10,000 RPM for 5 min. The capture plate was loaded according to the branched DNA (bDNA)

Table 1 Hepatocyte culture volumes and cell number according to surface area (plate type)

Plate type	Coating volume (µL)	Cell number/ well	Medium volume (µL)
96 well	50	12,000	100
24 well	250	40,000	500
12 well	500	100,000	1,000
6 well	1,000	330,000	2,000

Collagen volume (plate pre-coating), cell density, and tissue culture medium volume for plating of freshly isolated mouse hepatocytes

protocol from Affymetrix. Mouse specific probes for *Transthyretin* (*TTR*), *Collagen IaI*, *CD45*, *Tie2* and *GAPDH* (loading control) were used to quantitate mRNA levels. Cells were also run through a flow cytometer (BD LSRII, BDFACSDiva Software, BD Bioscience.) where hepatocytes and non-hepatocytes were separated based on cell size using forward scatter (FSC) and side scatter (SSC). Data were analyzed with FlowJo Software (Tree Star, Inc.).

Hepatocyte culture and microscopy

Cell culture plates were coated with 0.1% rat tail collagen (Sigma) 24 h before plating cells. After isolation, cell number and viability was determined. The cell pellet was resuspended by gently pipetting up and down in the appropriate volume of Plating medium (at 37 °C) according to the plate size (see Table 1 for details). Cells were seeded gently and incubated in a tissue culture incubator set at 37 °C, 5% CO_2. After 4.5–5 h, cells were washed once with DPBS and the appropriate volume of Maintenance medium was added to each well (see Table 1 for details). Phase-contrast pictures were taken with an Axiovert 40 CFL (Zeiss) microscope.

ASGR1-2 and CYP2A13 regulation in culture

Hepatocyte mRNA levels of *ASGR1* and *ASGR2* (mouse specific probes from Affymetrix) were measured by bDNA after isolation at 5, 24, 48, 72 and 96 h in culture. Western blot analysis utilizing specific antibodies was performed to estimate CYP2A13 and ASGPR1 (Abcam) levels. *β*-Actin (Sigma-Aldrich) was used as a loading control. The western blot was scanned using the Odyssey System (Li-cor).

Biotin-GalNAc binding assay by flow cytometry

Hepatocytes were aliquoted into Stripwell Microplates (Corning) at a density of 200,000 cells/tube/1 mL medium (DMEM/2% BSA) at 4 °C. Biotin-GalNAc was added to the cells to final concentrations of 30, 15, 7.5, 3.8, 1.9 and 0.9 nM and incubated at 4 °C for 2 h in an orbital shaker set at 150 rpm. Cells were centrifuged at 50 g/4 °C for 2 min. 750 µL of supernatant was removed and washed with 750 µL of cold DPBS/2% BSA. The washing step was repeated twice. After the 2nd wash, 750 µL of DMEM/2% BSA

containing streptavidin-Alexa647 (Invitrogen) was added at a dilution of 1:200. Cells were incubated for 20 min at 4 °C and washed once. Hepatocytes were re-suspended in 250 μL of cold DPBS/2% BSA. Cells were put on ice until Median Fluorescent Intensity (MFI) was measured by flow cytometry. Data was analyzed by FlowJo Software (Tree Star, Inc) and PRISM 4.0 Software (GraphPad).

In vitro activity of GalNAc-siRNA conjugates

Either a bi- or tri-antennary GalNAc ligand conjugated to small interfering RNA duplexes targeting *Apolipoprotein B* (siApoB) were generated to test in vitro silencing of *ApoB* mRNA in primary mouse hepatocytes. Mouse hepatocytes were plated as previously described in this article in 96 well plates containing GalNAc-siApoB conjugates. Final concentrations of siRNA-conjugates were 5, 0.83, 0.14, 0.02, 0.003, 0.0006 and 0.0001 μM with a cell density of 12,000 cells/well/100 μL of media. After 4 h of incubation cells were washed once with PBS and lysed according to the bDNA protocol from Affymetrix. Mouse specific probes for *Apolipoprotein B* (ApoB) and *GAPDH* were used to detect mRNA levels.

Results and discussion

Cell number, viability and purity after isolation

Hepatocyte isolation, by the method described herein, was highly reproducible as demonstrated by the generation on average of 18–20 million cells per mouse having cell viabilities between 93 and 96% as calculated by the Trypan Blue exclusion assay ($n \geq 120$ isolations). For assessment of cell purity, mRNA levels of genetic markers for both hepatocytes and non-hepatocytes were measured using the branched DNA (bDNA) method for detection. Figure 1a shows that mRNA levels for three non-hepatocyte genetic markers, *CD45* (immune cell marker), *Collagen IaI* (stellate cell marker) and *Tie2* (endothelial cell marker) are much lower than the hepatocyte-specific gene, Transthyretin (*TTR*) (1.6% CD45, 5.7% Collagen IaI and 5.7% Tie2, respectively). These results indicate that an enriched cellular suspension of $\geq 80\%$ hepatocytes relative to other cell types can be achieved using this streamlined isolation protocol. A

similar purity was determined by a separate analysis using flow cytometry to separate cells based on size where $\sim 20\%$ of cells were shown to be non-hepatocytes and 80% hepatocytes (Fig. 1b).

Hepatocyte culture and cellular changes

The proper cell density and medium conditions to achieve an approximately 85% confluent culture after 24 h were determined empirically. Table 1 summarizes these conditions for various plate configurations. It is important not to seed hepatocytes at very high density because both attachment and subsequent viability decreases rapidly at high cell density (typically observed as early as 5 h in culture). It is also important to keep the culture plates undisturbed for at least 4 h after plating to allow proper cell adherence. Figure 2 shows hepatocytes in culture as early as 5 h and out to 120 h. As seen by phase contrast microscopy, the morphology of the hepatocytes changes over time in culture consistent with documented morphological changes (Bhandari et al. 2001; Godoy et al. 2010; Li et al. 2010; Talamini et al. 1997: Wallace et al. 2010). These cellular changes include cell polarization, de-differentiation as well as down regulation of gene expression (Bhandari et al. 2001; Godoy et al. 2010; Li et al. 2010; Talamini et al. 1997: Wallace et al. 2010). Consistent with the literature, changes in two widely known hepatocellular markers, *ASGR* and the Cytochrome P450 family member CYP2A13, were observed over time in culture. Figure 3 shows approximately 50% reduction in mRNA expression levels of *ASGR1* and *ASGR2* as early as 5 h after cell plating with a concomitant decrease in ASGPR protein levels by 48 h in culture (Fig. 4). A similar reduction was also observed for CYP2A13 protein as early as 24 h after plating (Fig. 5). These data are consistent with previous research demonstrating that hepatocytes in culture show rapid changes in regulation of gene expression. These cellular changes are important to keep in mind when developing and interpreting in vitro assays using cultured hepatocytes.

GalNAc binding as functional read-out

To demonstrate that the hepatocytes are functional after isolation, we evaluated the cells ability to bind *N*-acetylgalactosamine (GalNAc), a well-documented

Fig. 1 **a** mRNA levels for different cellular markers from liver resident cells. *Transthyretin* (*TTR*), *CD45*, *Collagen IaI* and *Tie2* mRNA levels were measured in freshly isolated hepatocytes by a branched DNA method (bDNA). Percent mRNA for non-hepatocytes cell markers relative to the housekeeping gene GAPDH were 1.6% for *CD45*, 5.7% for *Collagen IaI* and 5.7% for *Tie2*. *Error bars* represent the standard deviation. **b** Determination of hepatocyte population by flow cytometry. Forward scatter (FSC) and side scatter (SSC) were used to separate hepatocytes and non-hepatocytes based on cell size. The *dot plot* shown was gated on the live cell population
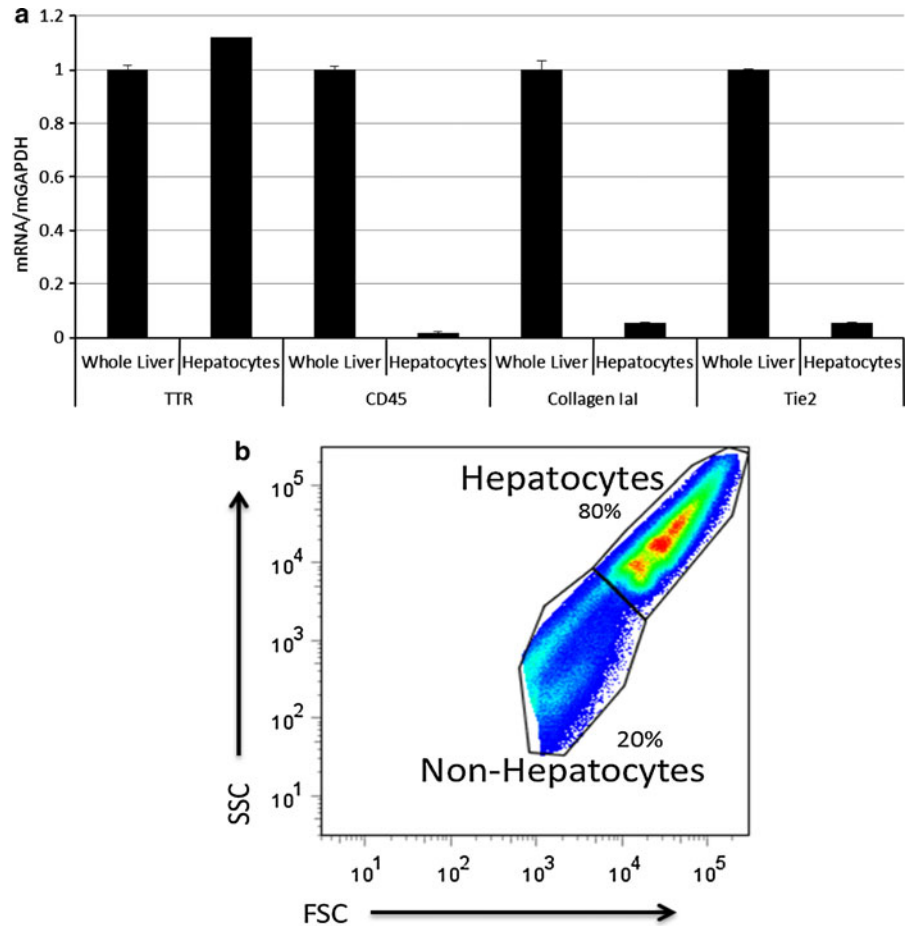
ligand for the asialoglycoprotein receptor (ASGPR). Ligand binding was assessed using flow cytometry to monitor the fraction of cells bound to biotinylated-GalNAc as a function of ligand concentration The calculated Kd values for the bi- and triantennary GalNAc were 28.3 and 2.3 nM respectively, consistent with binding affinities obtained for radiolabeled ligand-receptor assays previously reported where calculated Kd values were 4.3 nM for $GalNAc_3$ and 25 nM for a bi-antennary ligand ($GalNAc_2$) (Connolly et al. 1982; Connolly et al. 1983; Biessen et al. 1995; Lee et al. 1983; Rensen et al. 2001) (Fig. 6).

In vitro activity of GalNAc-siRNA

We have developed a method to screen the activity of GalNAc-siRNA conjugates in vitro using freshly isolated mouse hepatocytes in suspension and with minimal culture time (4 h). Figure 7 shows the concentration dependent uptake of GalNAc-conjugated siRNA and resulting silencing of the target ApoB mRNA in primary hepatocytes using bi- and tri-antennary GalNAc conjugated ApoB siRNA. IC50 values determined from the dose response curves were 1 and 80 nM for the $GalNAc_3$-siApoB and $GalNAc_2$-siApoB, respectively. The efficacy results for the siRNA-GalNAc conjugates correlates with the binding observed for the bi- and tri-antennary GalNAc ligand in that the ApoB siRNA attached with the tri-antennary ligand ($GalNAc_3$-siApoB) had a lower IC50 for target mRNA knockdown than the siRNA with the biantennary GalNAc ligand ($GalNAc_2$-siApoB). This correlation indicates that both assays can be done in cells in suspension or with minimum culture time (4 h incubation for siRNA activity) at times when the ASGPR is still present (Fig. 3).

Fig. 2 Hepatocyte morphology after plating. Representative phase-contrast microscopy pictures (40× magnification) of plated mouse hepatocytes taken at 5, 24, 48, 72, 96 and 120 h after plating. *Scale bars* represent 35 μm
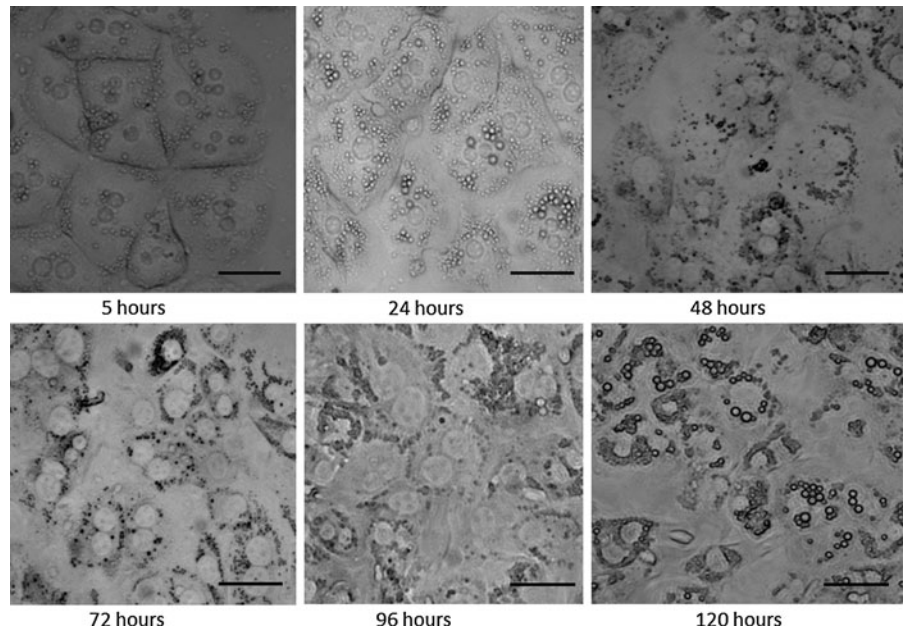

Fig. 3 *ASGR1-2* mRNA down regulation in plated hepatocytes. *ASGR1and ASGR2* mRNA levels were quantitated relative to the housekeeping gene GAPDH using the branched DNA method and probes specific to mouse ASGR1 and ASGR2. Cell lysates were collected at 5, 24, 48, 72 and 96 h after cell plating and compared to lysates generated from freshly isolated hepatocytes. *Error bars* represent the standard deviation
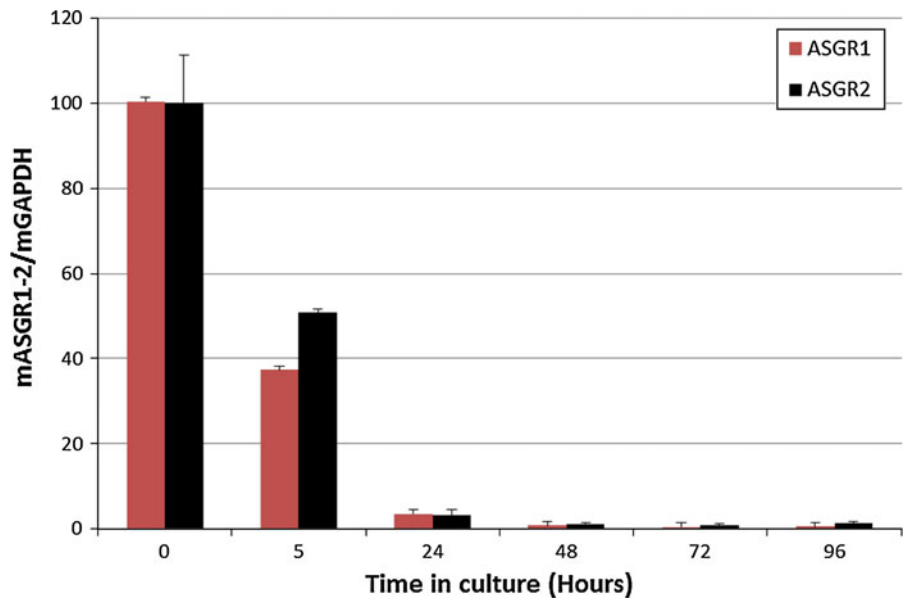

Conclusions

Here we described a modified two-step protocol for rapid isolation of hepatocytes to obtain cells with both high viability and purity. Table 2 summarizes several parameters (viability, cell number, etc.) for hepatocyte isolation comparing the method described here to two previously published isolation protocols (Goncalves et al. 2007; Li et al. 2010). We show that we can obtain a cell suspension enriched with hepatocytes having a
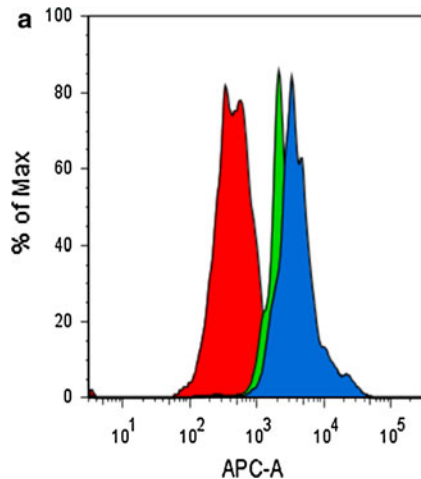
cell viability >90% in a short period of time. Most of the protocols for murine hepatocyte isolation are performed in a period of time of around 20 min or more. The time difference can be appreciated when isolation needs to be performed from numerous animals (e.g. drug pre-treated, genetically modified, etc.) when even a 5 min time difference per animal could negatively impact the quality of isolated cells. In addition, we developed a receptor-ligand binding assay to assess hepatocyte function. This assay
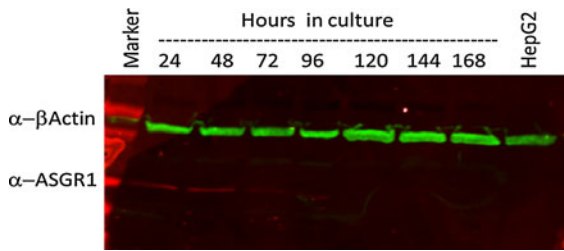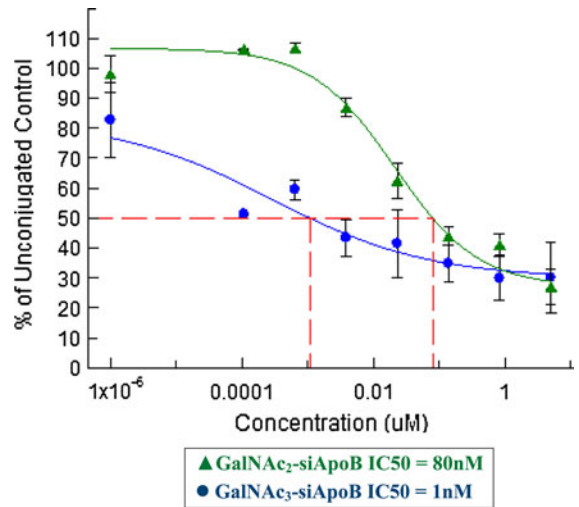
Fig. 4 Protein levels of the major subunit of the Asialoglyco-protein receptor (ASGR1) in cultured hepatocytes. Western blot showing ASGR1 levels in hepatocyte lysates collected after 24, 48, 72, 96, 120, 144 and 168 h after plating. β-Actin was used as a loading control and HepG2 cells were used as a negative control for ASGR1 expression
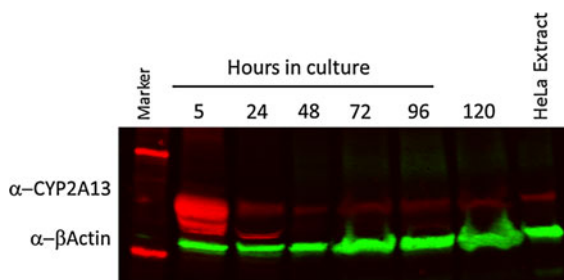
Fig. 5 CYP2A13 protein levels in cultured hepatocytes. Western blot showing Cytochrome P450 levels from lysates collected after 5, 24, 48, 72, 96 and 120 h of culture. β-Actin was used as loading control and HeLa cell extract was used as a control for low CYP2A13 expression
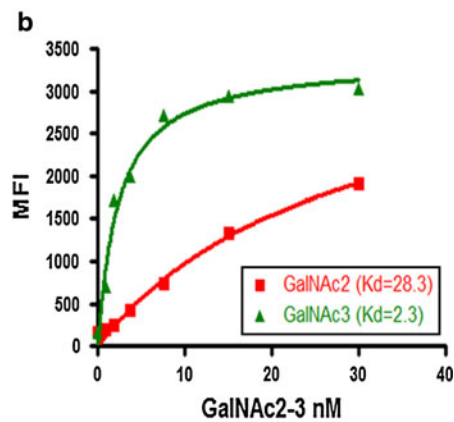

Fig. 7 *Apolipoprotein B* mRNA silencing by GalNAc-siApoB conjugates in primary mouse hepatocytes. Cells in suspension were incubated for 4 h with 5, 0.83, 0.14, 0.02, 0.003, 0.0006 and 0.0001 μM of GalNAc-siApoB conjugates with either a bi or tri-antennary GalNAc ligand. b DNA analysis was performed to monitor *ApoB* mRNA relative to *GAPDH* mRNA as a function of the concentration of the siRNA conjugate. IC50 curves were determined by a dose response one site fit model (Microsoft Excel Fit). IC50 values are expressed in nM

monitors the ability of the isolated cells to bind the well characterized asialoglycoprotein receptor ligand, GalNAc. Utilizing flow cytometry, we demonstrated

Fig. 6 a Histogram showing fluorescence intensity from hepatocytes incubated with 30 nM of GalNAc$_2$ (*green*), 30 nM of GalNAc$_3$ (*blue*) and blank (*red*). **b** GalNAc ligand binding assay. Freshly isolated hepatocytes in suspension were incubated for 2 h with 30, 15, 7.5, 3.8, 1.9 and 0.9 nM of

biotinylated GalNAc$_2$ and GalNAc$_3$. Median Fluorescent Intensity (MFI) was measured by flow cytometry after incubation with streptavidin-Alexa647. Kd values (nM) were calculated using a model of non-linear regression curve fit (Prism 4.0 Software)

Table 2 Comparison of different mouse hepatocyte isolation protocols

Parameters/protocol	This study	Goncalves	Li
Perfusion time (min)	15	+30	+20
Viability (%)	93–96%	80–90	N/D
Cell number (million)	18–20	1–3	100–400
Gradient Centrifugation	No	Yes	No
Cell purity markers	*CD45, Tie2, Collagen IaI*, Cell size	CD45, CD95	N/D

Parameters such as perfusion time, cell viability, cell number and cell purity are compared between isolation methods previously published by Goncalves et al. (2007) and Li et al. (2010) and the isolation method described here

binding of biotin labeled ligands with nM binding affinities consistent with previously published Kd values for similar ligand designs (Connolly et al. 1982: Connolly et al. 1983; Biessen et al. 1995; Lee et al. 1983; Rensen et al. 2001). In addition, we demonstrated that the binding of the GalNAc ligand led to cellular uptake and gene silencing when attached to an ApoB-targeting siRNA. The siRNA-conjugate results were consistent with the binding affinities measured for the ligand showing a lower IC50 value for GalNAc$_3$-siApoB compared to that for the GalNAc$_2$-siApoB conjugate. Hepatocytes isolated and cultured by the method described here can be used in either cell suspension assays such as the flow cytometry assay described or with assays using short culture times as an important tool in the areas of biological and medical research.

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