available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

# Effect of culture conditions on the expression and function of Bsep, Mrp2, and Mdr1a/b in sandwich-cultured rat hepatocytes

*Ryan Z. Turncliff* [1], *Xianbin Tian*, *Kim L.R. Brouwer* *

*School of Pharmacy, University of North Carolina at Chapel Hill, Unites States*

ARTICLE INFO

*Article history:*
Received 21 November 2005
Accepted 3 February 2006

*Classification code:*
(9) Pulmonary
Renal and Hepatic Pharmacology

*Keywords:*
Sandwich-cultured
Hepatocytes
Transport proteins
Biliary excretion
In vitro models

ABSTRACT

Rat hepatocytes cultured in a sandwich configuration form functional canalicular networks. The influence of extracellular matrix configuration, medium composition, and confluency on the expression and function of Bsep, Mrp2, and Mdr1a/b in sandwich-cultured (SC) rat hepatocytes was examined. Primary rat hepatocytes were: (1) maintained in various extracellular matrix sandwich configurations, (2) cultured in Dulbecco's modified Eagle's medium (DMEM), Modified Chee's medium (MCM) or Williams' E medium (WME), and/or (3) plated at decreasing cell density. Bsep, Mrp2, and Mrdr1a/b expression in day 4 SC rat hepatocytes was assessed by Western blot; function was measured by accumulation of taurocholate, 5(and 6)-carboxy-2′,7′-dichlorofluorescein, and rhodamine 123, respectively, in canalicular networks. In general, the extracellular matrix conditions examined resulted in similar protein expression and function. Function of Bsep, Mrp2, and Mdr1a/b was higher in SC rat hepatocytes maintained in DMEM or WME. Mrp2 and Mdr1a/b expression, representative of total cellular content, did not always correlate directly with function, which should be reflective of canalicular membrane expression. Mrp2 expression decreased significantly as cell density decreased in SC hepatocytes. Low plating density in Biocoat™ plates resulted in poor canalicular network formation and reduced function of Mrp2 and Mdr1a/b. Expression and/or function of Mrp2 and Mdr1a/b in rat hepatocytes cultured in a sandwich configuration may be influenced by plating density and media type.



Recently, primary hepatocytes have been used in vitro to investigate hepatic transport processes responsible for the accumulation and excretion of a wide variety of drugs. An in vitro model of biliary excretion, the sandwich-cultured (SC) rat hepatocyte model, has been used to predict the in vivo biliary clearance of some drugs [1]. The influence of extracellular matrix and medium composition on metabolic enzymes [2] including glutathione S-transferase [3], sulfotransferase and sulfatase expression [4], and regulation of transporter gene expression [5] in primary rat hepatocytes has been explored. However, the influence of culture conditions on hepatic transport protein expression and function has not been investigated. As canalicular efflux may be the rate-limiting step in the biliary excretion of xenobiotics, the influence of culture conditions on the expression and function of the bile salt export pump (Bsep; Abcb11), the multidrug resistance protein (Mrp2; Abcc2), and multidrug resistance protein (Mdr1a/b; Abcb1; P-glycoprotein) should be defined for the SC rat hepatocyte model.

* *Corresponding author at*: School of Pharmacy, C.B. #7360, Kerr Hall, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7360, United States. Tel.: +1 919 962 7030; fax: +1 919 962 0644.
E-mail address: kbrouwer@unc.edu (Kim L.R. Brouwer).
[1] Alkermes Inc., Cambridge, MA, United States.

0006-2952/$ – see front matter 
doi:10.1016/j.bcp.2006.02.004

Bsep represents the rate-limiting step in the excretion of bile acids such as taurocholic acid [6]. Bsep is expressed in primary rat hepatocytes [7] and localization at the canalicular domain in hepatocyte couplets was confirmed by confocal microscopy [8]. In addition to Bsep, sulfate and glucuronide conjugates of bile acids also are transported actively by Mrp2 [9]. Mrp2, however, is primarily responsible for the active transport of organic anions, glucuronide and sulfate conjugates (other than bile acids), and glutathione conjugates [10]. One such organic anion, 5(and 6)-carboxy-2′,7′-dichlorofluoresein, accumulated in the canalicular networks of SC rat hepatocytes isolated from transport competent [11,12], but not Mrp2-deficient ($TR^-$) Wistar rats [13]. Mdr1 appears to have some substrate overlap with Mrp2 [14,15]. In the rodent Mdr1a/b, has been localized to the canalicular domain of SC rat hepatocytes [16] and is primarily responsible for the biliary excretion of organic cations [17], including rhodamine123 [18]. A review of hormonal and xenobiotic regulation of Bsep, Mrp2 and Mdr1a/b (in vitro and in vivo) was provided by Fardel et al. [19].

SC rat hepatocytes re-polarize during culture and many of the structural and functional attributes found in normal liver, including canalicular networks, are apparent by day 4 in culture [20,21]. A thorough review of hepatocyte culture conditions that allow for extended time in culture was published by LeCluyse et al. [22]. To date, a range of culture media choices [e.g., Dulbecco's modified Eagle medium (DMEM), modified Chee's medium (MCM), Williams' medium E (WME)], and supplements [i.e., dexamethasone (DEX), insulin] have been used successfully to culture primary rat hepatocytes that exhibit canalicular networks [21,23,24]. Furthermore, many dish types (e.g., polystyrene, Permanox™), and extra-cellular matrix configurations (e.g., gelled collagen, Biocoat™, Matrigel™) have been used to culture primary hepatocytes. Early investigations by Schuetz et al. [25], demonstrated that primary hepatocytes cultured on Matrigel™ were capable of in vivo-like induction of cytochrome P450's compared to hepatocytes cultured on type I collagen, while Clemet and Yamada [26] utilized freshly isolated hepatocytes to reveal the importance of hepatocyte cell surface proteins which recognize basement membrane proteins such as collagen IV, heparan sulfate proteoglycan, and laminin. Extra-cellular matrix configurations affect hepatocyte morphology [27], which may directly influence the formation of canalicular networks and the expression and function of Bsep, Mrp2 and Mdr1a/b in primary rat hepatocytes. The use of either gelled-collagen or Matrigel™ to overlay hepatocytes has resulted in in vivo-like morphology [2].

The effects of collagen volume, time of collagen overlay, culture medium and DEX concentration on the function of Bsep and Mdr1a/b recently have been examined in our laboratory [28,29]. The influence of isolation procedure, extracellular matrix configuration, and medium supplementation with DEX on the gene expression of membrane transporters also has been reported [5]. Additional microenvironment conditions which may influence the formation of canalicular networks and directly or indirectly modulate the expression and/or function of canalicular transport proteins (e.g., dish type, extracellular matrix configuration, hepatocyte confluency) have yet to be examined. These experiments were undertaken to explore the potential modulation of expression and function of the canalicular proteins Bsep, Mrp2 and Mdr1a/b in SC rat hepatocytes maintained in a variety of culture conditions.

## 1. Methods

### 1.1. Chemicals

[$^3$H] Taurocholate (2 Ci/mmol, purity > 95%; Perkin-Elmer Life Sciences, Boston, MA), Collagenase (type 1, class 1; Worthington Biochemical Corporation, Freehold, NJ), Dulbecco's modified Eagle's medium (DMEM), modified Chee's medium (MCM), Williams' medium E (WME), insulin (Invitrogen/GIBCO, Carlsbad, CA), ITS$^{+TM}$, rat tail collagen (type I), Matrigel™ Matrix (BD Biosciences, Bedford, MA), DMEM (10×), penicillin–streptomycin solution, fetal bovine serum (FBS), taurocholic acid (TC), dexamethasone (DEX), Triton X-100, and soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), 5(and 6)-Carboxy-2′,7′-dichlorofluorescein (CDF) diacetate (CDF-DA), and rhodamine 123 (Rh123) (Molecular Probes, Eugene, OR) were purchased. All other chemicals and reagents were of analytical grade.

### 1.2. Isolation and in vitro culture of primary rat hepatocytes

Hepatocytes were isolated from male Wistar rats (270–325 g; Charles River Laboratories, Raleigh, NC) using a collagenase perfusion, as described previously [11]. All animal procedures were compliant with the guidelines of the Institutional Animal Care and Use Committee (University of North Carolina at Chapel Hill). Hepatocyte viability was >85% as determined by trypan blue exclusion. Hepatocytes were resuspended in DMEM (5% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 4 mg/l insulin, and 1 μM DEX) and diluted to a final concentration of $1.0 \times 10^6$ cells/ml. Lower plating densities were achieved by serial dilution.

### 1.3. Preparation of culture dishes and sandwich-cultured hepatocytes

Polystyrene 60 mm dishes (Corning, Corning NY), 60 mm Permanox™ dishes (Nunclon; Nalge Nunc International, Rochester, NY) and six-well Polystyrene plates (Corning, Corning NY) were coated with a collagen solution [gelled-collagen, (GC)] as previously described [11]. Alternatively, BD BioCoat™ (BC) six-well plates (BD Biosciences, Bedford MA) were used. All dishes and plates were placed overnight in a 37 °C incubator. Hepatocytes were plated in 60 mm dishes (~$3.0 \times 10^6$ cells/dish) or in six-well plates (~$2.0 \times 10^6$ cells/well [100% plating density]). The plating medium was replaced with DMEM (5% FBS, 4 mg/l insulin, 0.1 μM DEX) after 1–3 h. After 24 h, medium was aspirated and cells were overlaid with rat tail gelled-collagen type I solution (GC/GC or BC/GC). One hour later, DMEM + ITS™ (1% ITS™, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1 μM DEX) was added to hepatocytes overlaid with gelled collagen. Alternatively,

Matrigel™ (M; 0.25 mg/ml), added to ice-cold culture medium (DMEM + ITS), formed a gelled overlay as the medium returned to 37 °C in an incubator (GC/M or BC/M). Medium was replaced every 24 for 96 h (Day 4). The effect of media composition was evaluated in hepatocytes plated in 60 mm polystyrene dishes and maintained in GC/GC configuration. Hepatocytes were maintained with DMEM, MCM or WME medium, each supplemented with 1% ITS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1 μM DEX, from day 1 through 4.

### 1.4. Light microscopy

Light microscopy was used to confirm the integrity of the canalicular networks and to evaluate the effect of culture medium on hepatocyte morphology. Light microscopy images were taken with a Nikon TMS light microscope at 200× magnification.

### 1.5. Accumulation and biliary excretion experiments (Day 4)

Methods to determine the accumulation and the biliary excretion index (BEI) of substrates in SC rat hepatocytes have been described previously [30]. Briefly, hepatocytes were rinsed twice with 3 ml warm standard Hanks balanced salts solution (HBSS) to maintain tight junctions and bile canalicular (BC) networks [Cells + BC], or $Ca^{2+}$-free HBSS to disrupt tight junctions [Cells], and subsequently incubated with the same buffer for 10 min at 37 °C. Hepatocytes were incubated with 1 μM (TC) or 10 μM (TC, CDF-DA, Rh123) substrate for 10 min (TC, CDF-DA) or 30 min (Rh123) in standard HBSS, and subsequently were rinsed vigorously three times with 3 ml ice-cold HBSS. Hepatocytes were lysed with 2 ml (60 mm dishes) or 1 ml (six-well plates) 0.5% Triton X-100 by orbital shaking.

### 1.6. Sample analysis

TC was analyzed by liquid scintillation spectroscopy (Packard Tricarb scintillation counter; Meriden, CT). CDF-DA (measured as CDF) and Rh123 were analyzed by fluorescence spectroscopy (BioTek FL600; BioTek, Winooski, VT). The protein content of each dish was measured with the BioRad bicinchoninic acid assay kit (BioRad, Hercules, CA) using BSA as the standard. All data were corrected for nonspecific binding to GC/GC, BC/GC, GC/M, or BC/M hepatocyte-free culture dishes.

### 1.7. Western blot analysis of canalicular transport proteins (Day 4)

Lysing buffer (1 mM EDTA, 1% SDS, pH 8) was added to each dish, cells were scraped using a cell lifter, and homogenization was achieved by repeated pipetting (stored at −80 °C until analysis). Samples ($n = 3$) were separated by SDS-polyacrylamide gel electrophoresis and were electrotransferred to PVDF membranes. Blots were blocked overnight (tris-buffered saline, 5% nonfat dry milk, 0.3% Tween 20) and subsequently probed with the following antibodies: Mrp2 ($M_2$III-6; Alexis Biochemicals, Carlsbad CA), Bsep (Kamiya Biomedical, Seattle, WA), Mdr1a/b (Ab-1; Oncogene Research Products, Cambridge, MA), β-Actin (Chemicon, Temecula, CA). Molecular mass standards (SeeBlue®Plus2; Invitrogen, Carlsbad, CA) were loaded with samples on each gel. Immunoreactive protein bands were detected by chemiluminescence on film or using a BioRad VersaDoc® (BioRad, Hercules, CA) imaging system.

### 1.8. Data analysis

Accumulation data (determined in triplicate from three individual livers) are expressed as mean ± S.E.M. The BEI (%) was determined using B-CLEAR technology (Qualyst, Inc., Research Triangle Park, NC) by dividing the difference in substrate accumulation between Cells + BC and Cells by the accumulation in Cells + BC, multiplied by 100 [11]. BEI was calculated as a mean of BEI values from the individual livers and is expressed as mean ± S.E.M. In vitro biliary clearance ($Cl_{b\ in\ vitro}$; ml/min/mg protein) was calculated by dividing the difference in substrate accumulation between Cells + BC and Cells by the product of the incubation concentration and the time of the incubation.

### 1.9. Statistical analysis of data

A one-way analysis of variance (ANOVA) table was constructed to assess significant differences in accumulation data and BEI values among groups. A two-way ANOVA table was constructed to examine the effects of two factors (plating density and substratum) on $Cl_{B\ in\ vitro}$ values. The criterion for statistical significance was $P < 0.05$.

## 2. Results

### 2.1. Influence of dish type on rat hepatocytes cultured in sandwich configuration

#### 2.1.1. Morphology

Rat hepatocytes cultured between two layers of gelled collagen and maintained in DMEM for 4 days exhibited chord-like arrays, were cuboidal in shape, and formed extensive canalicular networks. Rat hepatocytes cultured in BC/GC sandwich configuration flattened to form confluent monolayers and exhibited extensive canalicular networks (Fig. 1). Canalicular network formation appeared slightly more extensive in BC/GC compared to rat hepatocytes cultured in GC/GC configuration.

#### 2.1.2. Accumulation and BEI of probe substrates

Accumulation of TC and CDF (administered as CDF-DA) in Cells + BC was similar in both polystyrene and Permanox™ dishes for a given substrate; likewise, accumulation in Cells was similar for a given substrate in both dish types (Fig. 2). There were no significant differences in BEI values as a function of dish type for either TC or CDF in SC rat hepatocytes cultured in DMEM, however, the BEI of Rh123 was significantly influenced by dish type (Table 1). The non-specific binding of TC was lowest in BC/GC hepatocyte-free dishes, while the non-specific binding of Rh123 was lowest in GC/GC hepatocyte-free coated Permanox™ dishes (Table 2).

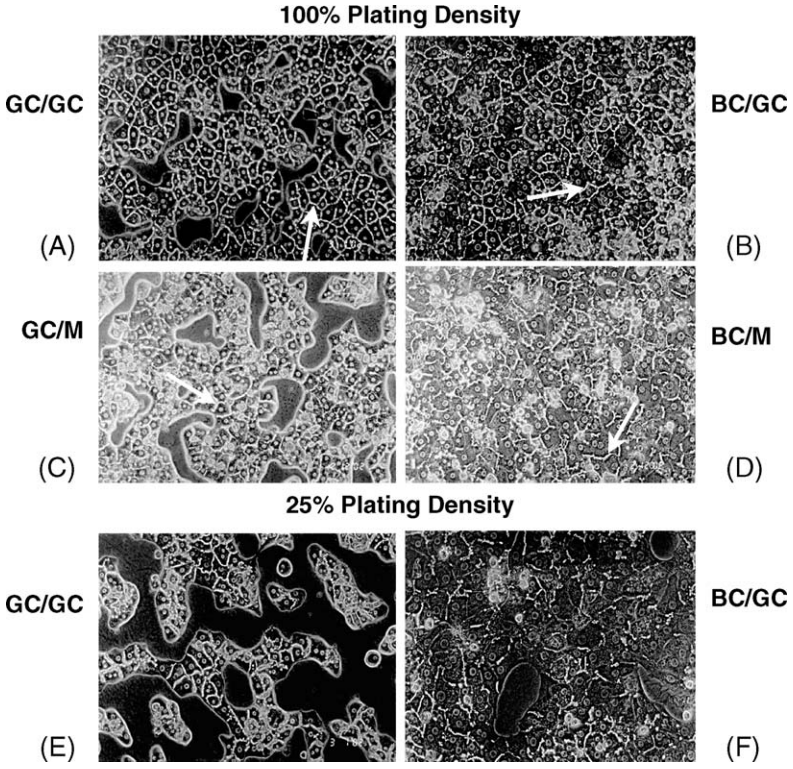


**Fig. 1 – Influence of sandwich configuration and plating density on Day 4 hepatocyte morphology and canalicular network formation. Light microscopy images of SC rat hepatocytes maintained in DMEM for 4 days in six-well polystyrene plates. Hepatocytes cultured on a gelled-collagen (GC) substratum (A, C, and E) and overlaid with either gelled-collagen (GC/GC; A and E) or Matrigel™ (GC/M; C) formed cord-like arrays. Hepatocytes cultured on a Biocoat™ (BC) matrix (B, D, and F) and overlaid with either gelled-collagen (BC/GC; B and F) or Matrigel™ (BC/M; D), flattened to form confluent monolayers. Canalicular networks (arrows) were extensive in sandwich-cultured rat hepatocytes cultured at 100% plating density ($1.25 \times 10^5$ cells/cm$^2$) (A–D). Canalicular network formation appeared to be proportional to plating density in SC rat hepatocytes maintained on gelled-collagen (E) but not Biocoat™ substratum (F) at 25% cell density.**

### 2.2. *Influence of extracellular matrix on rat hepatocytes cultured in sandwich configuration*

#### 2.2.1. *Protein expression of transporters*

Representative Western blots of Bsep, Mrp2 and Mdr1a/b expression in rat hepatocytes cultured in GC/GC, BC/GC, GC/M, and BC/M sandwich configurations are shown in Fig. 3. Bsep expression was similar in all sandwich configurations. The expression of Mrp2 and Mdr1a/b was more variable in SC rat hepatocytes plated on Biocoat™ substratum.

#### 2.2.2. *Accumulation and biliary excretion of probe substrates*

The influence of substratum and overlay matrix composition on the accumulation of probe substrates was determined in hepatocytes cultured in GC/GC, BC/GC, GC/M, and BC/M sandwich configurations (Fig. 4). No significant differences were noted in the accumulation of TC as a function of sandwich configuration. The BEI values of TC in rat hepatocytes cultured in BC/GC (57 ± 5%), GC/M (70 ± 13%), and BC/M (85 ± 6%) sandwich configurations were not significantly different from GC/GC (63 ± 8%) (mean ± S.E.M.; $n = 3$). CDF accumulation in Cells + BC was significantly lower in rat hepatocytes maintained in BC/M compared to GC/GC sandwich configuration (Fig. 4). CDF BEI values were not significantly different in rat hepatocytes maintained in GC/GC, BC/GC, GC/M and BC/M (31 ± 5%, 18 ± 8%, 40 ± 3% and 30 ± 4%, respectively; mean ± S.E.M.; $n = 3$). No significant differences were noted in the accumulation of Rh123 as a function of sandwich configuration. BEI values of Rh123 determined in SC rat hepatocytes maintained in GC/GC (10 ± 2%), BC/GC (8 ± 2%) and BC/M (13 ± 4%) sandwich configurations were not significantly different. The BEI value of Rh123 in SC rat hepatocytes maintained in GC/M configuration could not be calculated because a difference between accumulation in Cells + BC and Cells was not observed.

$Cl_{B\ in\ vitro}$ (ml/min/kg) values of TC in rat hepatocytes cultured in GC/GC (15 ± 4%), BC/GC (20 ± 1%), GC/M (21 ± 6%) and BC/M (21 ± 3%) sandwich configurations were not significantly different (mean ± S.E.M.; $n = 3$). Similarly, CDF $Cl_{B\ in\ vitro}$ (ml/min/kg) values in rat hepatocytes maintained in GC/GC, BC/GC, GC/M and BC/M sandwich configurations (6 ± 2%, 3 ± 1%, 3 ± 1%, 5 ± 2%, respectively) were not significantly different. $Cl_{B\ in\ vitro}$ (ml/min/kg) values of Rh123 determined in SC rat hepatocytes maintained in GC/GC (3 ± 0.5%), BC/GC (2 ± 0.2%) and BC/M (4 ± 0.5%) sandwich configurations were not significantly different.

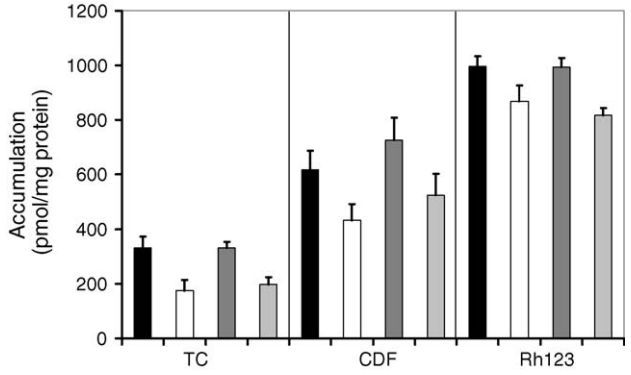


**Fig. 2 – Day 4 accumulation of prototypical substrates in polystyrene and Permanox™ dishes. Primary rat hepatocytes were cultured in 60 mm polystyrene dishes (Black, White) or 60 mm Permanox™ dishes (dark grey, light grey) in sandwich configuration and were maintained with DMEM. Accumulation of TC (10 μM, 10 min), CDF (administered as CDF-DA, 10 μM, 10 min), and Rh123 (10 μM, 30 min) was similar in polystyrene and Permanox™ dishes in day 4 SC rat hepatocytes pre-incubated in standard HBSS (Black, Dark Grey) for a given substrate. Likewise, accumulation was similar in $Ca^{2+}$-free HBSS (White, Light Grey) for a given substrate. Data represent mean ± S.E.M. from three experiments performed in triplicate.**

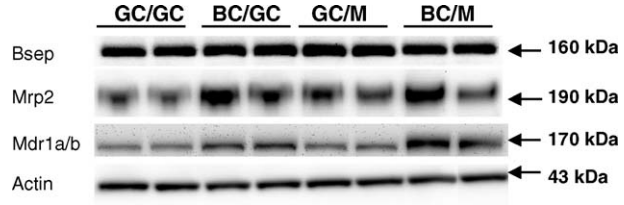


**Fig. 3 – Influence of extracellular matrix configuration on expression of canalicular transport proteins in Day 4 SC rat hepatocytes. Representative Western blots of Bsep, Mrp2 and Mdr1a/b expression primary rat hepatocytes cultured in gelled-collagen/gelled-collagen (GC/GC), Biocoat™/gelled-collagen (BC/GC), gelled-collagen/Matrigel™ (GC/M), or Biocoat™/Matrigel™ (BC/M) sandwich configurations in six-well plates and maintained with DMEM for 4 days. Each lane represents a separate rat hepatocyte isolation.**

### 2.3. *Influence of media composition on canalicular protein expression and function*

#### 2.3.1. *Morphology*

The morphology of primary rat hepatocytes maintained in sandwich configuration in DMEM, MCM or WME (data not shown) was consistent with a previous report from our laboratory [28]. SC rat hepatocytes maintained in MCM had increased vacuole formation and less extensive canalicular network formation compared to SC rat hepatocytes maintained in DMEM.

#### 2.3.2. *Protein expression*

Representative Western blots of Bsep, Mrp2, and Mdr1a/b expression as a function of media type are shown in Fig. 5. Expression of Bsep appeared to be lower in SC rat hepatocytes maintained in MCM and higher in SC rat hepatocytes maintained in DMEM. Mrp2 expression appeared to be similar in SC rat hepatocytes maintained in all media types. Mdr1a/b expression appeared to be highest in SC rat hepatocytes maintained in DMEM.

#### 2.3.3. *Accumulation and biliary excretion of probe substrates*

The accumulation of TC, CDF and Rh123 in Cells + BC and Cells only was lowest in SC rat hepatocytes maintained in MCM compared to DMEM and WME (data not shown). The BEI of CDF and Rh123 were significantly lower in SC rat hepatocytes maintained in MCM compared to DMEM (Table 1).

### 2.4. *Influence of plating density on substrate accumulation and canalicular transport protein expression*

The significance of cell–cell contacts and hepatocyte morphology on the formation of functional canalicular networks was examined in SC rat hepatocytes plated in six-well polystyrene plates on either GC or BC substratum. Based on the data shown in Figs. 3 and 4, hepatocytes were overlaid with GC rather than Matrigel™ to achieve sandwich configuration, and were maintained in DMEM for 4 days prior to protein sample collection and biliary excretion studies.

**Table 1 – Influence of media type and dish type on the biliary excretion index (BEI) of TC, CDF, and Rh123 in Day 4 SC rat hepatocytes**

| | DMEM | | | MCM | WME |
|---|---|---|---|---|---|
| | Polystyrene | Permanox™ | Biocoat™ | Polystyrene | Polystyrene |
| TC | 61 ± 11 | 48 ± 12 | 60 ± 3 | 43 ± 3 | 58 ± 10 |
| CDF | 43 ± 5 | 35 ± 7 | 28 ± 8 | 15 ± 4* | 31 ± 6 |
| Rh123 | 13 ± 1 | 18 ± 2* | 8 ± 2* | 0* | 12 ± 3 |

Rat hepatocytes were cultured between two layers of gelled-collagen (polystyrene, Permanox™ dishes) or between rigid-collagen and gelled-collagen (Biocoat™ dishes) and were maintained in Dulbecco's modified Eagle's medium (DMEM), modified Chee's medium (MCM) or Williams' medium E (WME) from days 1 to 4. Taurocholate (TC; 10 μM, 10 min), carboxyfluorescein (CDF; administered as CDF-DA; 10 μM, 10 min) and rhodamine 123 (Rh123; 10 μM, 30 min) BEI values are expressed as mean ± S.E.M. ($n = 3$).

* $P < 0.05$ vs. DMEM-Polystyrene; single-factor ANOVA.

**Table 2 – Non-specific binding of TC, CDF and Rh123 in gelled-collagen coated polystyrene, Permanox™ and Biocoat™ culture dishes**

| | Polystyrene[a] | | Permanox™ [a] | | Biocoat™ [b] | |
|---|---|---|---|---|---|---|
| | Cells + BC | Cells | Cells + BC | Cells | Cells + BC | Cells |
| TC | 0.7 ± 0.3 | 0.4 ± 0.3 | 0.3 ± 0.2 | 0.2 ± 0.2 | 0.13 ± 0.11 | 0.10 ± 0.05 |
| CDF | 0.3 ± 0.3 | 0.6 ± 0.5 | N.D. | N.D. | N.D. | N.D. |
| Rh123 | 1.0 ± 0.4 | 1.0 ± 0.6 | 0.5 ± 0.4 | 0.4 ± 0.3 | 1.0 ± 0.0 | 0.9 ± 0.4 |

Collagen coated blank dishes (hepatocyte-free) were pre-incubated in Cells + BC or Cells and subsequently were incubated with substrate in standard HBSS. Following incubation, dishes were rinsed three times with ice-cold standard HBSS; residual substrate was recovered in 0.5% Triton X-100. Data represent the percentage of initial substrate recovered in Triton X-100 solution following rinsing (mean ± S.E.M.; $n = 3$–6). N.D.: not detectable.

[a] Polystyrene and Permanox™ dishes were coated with gelled-collagen.

[b] Biocoat™ dishes were overlaid with gelled-collagen.

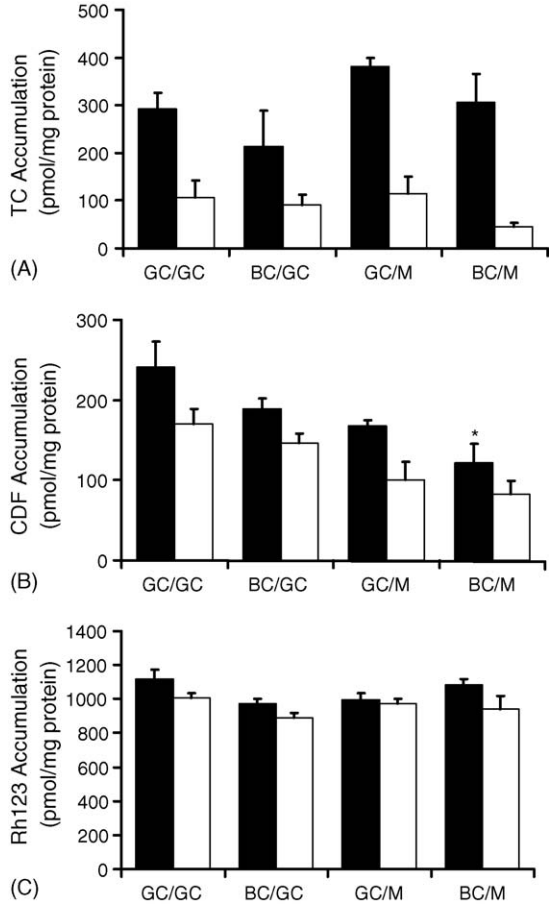


**Fig. 4 – Influence of sandwich configuration on the accumulation of TC, CDF, and Rh123 in Day 4 SC rat hepatocytes. Primary rat hepatocytes were cultured in gelled-collagen/gelled-collagen (GC/GC), Biocoat™/gelled-collagen (BC/GC), gelled-collagen/Matrigel™ (GC/M), or Biocoat™/Matrigel™ (BC/M) sandwich configuration in six-well plates and maintained with DMEM for 4 days. Accumulation in standard (solid bars) and $Ca^{2+}$-free (open bars) HBSS (mean ± S.E.M.; $n = 3$) was determined for (A) TC (10 μM, 10 min), (B) CDF (administered as CDF-DA; 10 μM, 10 min) and (C) Rh123 (10 μM, 30 min). $^{*}P < 0.05$ vs. GC/GC in the same $Ca^{2+}$ condition.**

#### 2.4.1. *Morphology*

SC rat hepatocytes maintained on GC substratum formed chord-like arrays at all plating densities; however, on BC substratum, hepatocytes spread to form confluent monolayers at 100% (Fig. 1). A cell plating density of 25% on BC substratum resulted in extensive flattening and less extensive canalicular network formation.

#### 2.4.2. *Protein expression*

Representative Western blots of canalicular protein expression in rat hepatocytes maintained in GC/GC and BC/GC sandwich configurations at decreasing cell densities are shown in Fig. 6. Expression of Mdr1a/b and Bsep was similar on both substratum matrix conditions at all plating densities. Mrp2 expression appeared to be lower in SC rat hepatocytes plated at 25% density compared to 100% density on both GC and BC substratum (Fig. 6).

#### 2.4.3. *Accumulation and biliary excretion of probe substrates*

The BEI values for TC in rat hepatocytes plated at 100% and 50% density in GC/GC sandwich configuration were 57 ± 3% and 60 ± 4%, respectively. Slightly higher TC BEI values were observed in rat hepatocytes cultured in BC/GC sandwich configuration at 100% and 50% cell plating densities (87 ± 5% and 78 ± 3%, respectively). However, at a plating density of 25% the TC BEI was significantly lower in both GC/GC and BC/GC sandwich configurations (36 ± 3% and 42 ± 2%, respectively). The BEI value of CDF was not significantly different as a function of sandwich configuration. However, the BEI value of CDF in rat hepatocytes cultured in GC/GC and BC/GC sandwich configurations at 25% cell density (13 ± 2% and 26 ± 7%, respectively) was significantly lower than at 100% cell density (24 ± 4% and 45 ± 9%, respectively). There was not a significant difference in the Rh123 BEI value as a function of plating density or sandwich configuration. The $Cl_{B\ in\ vitro}$ values of TC, CDF and Rh123 are listed in Table 3. The in vitro biliary clearance of TC was significantly lower in SC rat hepatocytes maintained in BC/GC sandwich configuration relative to GC/GC as plating density decreased. CDF $Cl_{B\ in\ vitro}$ was significantly lower in rat hepatocytes cultured at 25% cell density compared to 100% or 50%, regardless of sandwich configuration (Table 3). When different densities were evaluated in the GC/GC and BC/GC configurations, both density and the substratum influenced Rh123 $Cl_{B\ in\ vitro}$.

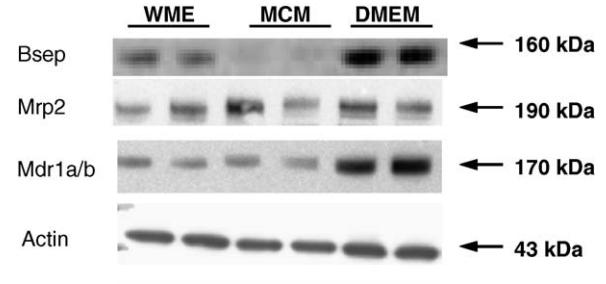


**Fig. 5 – Influence of media type on expression of canalicular transport proteins in Day 4 SC rat hepatocytes. Representative Western blots of Bsep, Mrp2 and Mdr1a/b expression in primary rat hepatocytes cultured in gelled-collagen/gelled-collagen sandwich configuration in 60 mm polystyrene dishes and maintained in Williams' medium E (WME), modified Chee's medium (MCM) or Dulbecco's modified Eagle's medium (DMEM) for 4 days. Each lane represents a different rat hepatocyte preparation.**

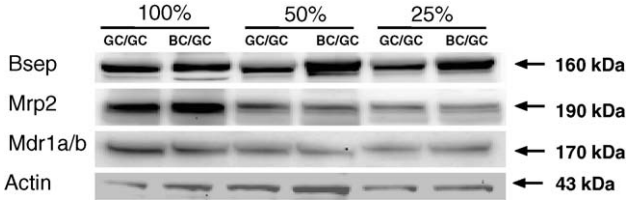


**Fig. 6 – Influence of cell density on canalicular transport protein expression in Day 4 SC rat hepatocytes. Representative Western blots of Bsep, Mrp2, and Mdr1a/b expression in primary rat hepatocytes cultured in gelled-collagen/gelled-collagen (GC/GC) or Biocoat™/gelled-collagen (BC/GC) sandwich configuration at 100%, 50% or 25% cell density in six-well plates and maintained in DMEM for 4 days.**

## 3. Discussion

The influence of culture conditions on primary hepatocyte morphology, longevity, and function has been described in great detail [27,31–36]. However, emphasis to date has been placed on the modification of conventional culture conditions to optimize the metabolic capacity of primary hepatocytes [2,23,37–39]. More recently, the importance of hepatic transport mechanisms responsible for the accumulation and excretion of xenobiotics has been realized. Formation of bile canalicular networks in primary rat hepatocytes is dependent on the thickness of the cell monolayer, the presence of medium supplements, and the extracellular matrix overlay [22]. In the present study, a series of experiments was conducted systematically to evaluate the influence of culture conditions on the expression and function of the transport proteins Bsep, Mrp2 and Mdr1a/b.

The type of plastic (Permanox™ versus polystyrene) did not affect hepatocyte morphology or canalicular network formation, nor the BEI of TC or CDF. However, the BEI of Rh123 was significantly greater in Permanox™ dishes. In accordance with a recently published perspective [40], the non-specific binding of new chemical entities should be determined to assess the validity of in vitro experiments. The non-specific binding of TC, CDF, and Rh123 in Permanox™ dishes was lower than in polystyrene dishes, confirming our earlier results regarding the non-specific binding of Rh123 [29]. Decreased non-specific binding may result in slight, but significant, changes in BEI noted above for Rh123. Permanox™ is an oxygen permeable polymer that is chemically resistant to acids, alkalis and some hydrocarbons (Tech Note, vol. 3, no. 15; Nalge Nunc International), which may explain the apparent reduced non-specific binding of probe substrates. Importantly though, in polystyrene dishes and six-well plates, non-specific binding was decreased to 1% or less of the initial concentration following vigorous rinsing. Increasing trends toward higher-throughput models that utilize multi-well plates to both optimize efficiency and maximize resources may directly impact the choice of culture dish or multi-well plate; at present, the Permanox™ polymer is available in limited formats.

Culture medium influenced the formation of canalicular networks and expression of canalicular transport proteins. SC rat hepatocytes maintained in DMEM appeared to express the highest levels of canalicular transport proteins Bsep and Mdr1a/b. In agreement with previous reports [28], SC rat hepatocytes maintained in WME exhibited more extensive canalicular network formation compared to DMEM. Nonetheless, BEI values of probe substrates were similar between SC rat hepatocytes maintained in DMEM or WME. MCM has been shown to support the induction of P450 enzymes and maintain

**Table 3 – Influence of plating density on in vitro biliary clearance ($Cl_{B\ in\ vitro}$) values of TC, CDF and Rh123 in Day 4 SC rat hepatocytes cultured on gelled-collagen (GC) and Biocoat™ (BC) substratum**

| | $Cl_{B\ in\ vitro}$ (ml/min/kg) | | | | | |
|---|---|---|---|---|---|---|
| | 100% | | 50% | | 25% | |
| | GC/GC | BC/GC | GC/GC | BC/GC | GC/GC | BC/GC |
| TC* | 25 ± 6 | 18 ± 4 | 31 ± 14 | 10 ± 3 | 23 ± 12 | 10 ± 1 |
| CDF† | 4.0 ± 0.5 | 6.6 ± 0.8 | 4.0 ± 0.8 | 3.5 ± 0.8 | 2.7 ± 0.4 | 3.5 ± 0.1 |
| Rh123*† | 4.5 ± 0.4 | 1.8 ± 1.0 | 6.1 ± 2.0 | 1.4 ± 0.8 | 8.7 ± 0.3 | 3.2 ± 0.6 |

Hepatocytes cultured in GC/GC or BC/GC sandwich configuration at 100% (~$1.25 \times 10^5$ cell/cm$^2$), 50% and 25% cell densities were incubated with the probe substrates TC (1 μM, 10 min), CDF (administered as CDF-DA, 10 μM, 10 min) and Rh123 (10 μM, 30 min). Data represent mean ± S.E.M. from three independent experiments performed in triplicate.

* $P < 0.05$ sandwich configuration.

† $P < 0.05$ plating density

normal cell morphology [22]. However, in the current experiments, the accumulation and BEI values of TC, CDF and Rh123 were lower in SC rat hepatocytes maintained in MCM compared to DMEM; these results were consistent with previously reported data for TC and Rh123 [28,29]. The expression of Bsep and Mdr1a/b appeared to be lower in SC rat hepatocytes maintained in MCM compared to DMEM, which may explain the decreased BEI values of TC and Rh123. The BEI of CDF was significantly lower in SC rat hepatocytes maintained in MCM compared to DMEM. However, Mrp2 expression was similar in SC rat hepatocytes maintained in all media types. It is possible that the level of expression of Mrp2 on the canalicular domain may be lower than the specific protein reflected in an immunoblot of total cell homogenate because it has been shown that Mrp2 is stored in intracellular vesicles that can be recruited to the canalicular domain when necessary [41]. MCM, originally developed for the maintenance of tumor cell lines [42], is an enriched medium that contains amino acid concentrations that are 5–10 times higher than DMEM. WME contains high levels of amino acids such as L-alanine to support hepatocyte replication in vitro [31,43]. Increased amino acid concentrations have been shown to reduce lysosomal protein degradation and preserve cellular protein levels [22,34]. The induction potential of glutathione S-transferase enzymes was greater in primary rat hepatocytes maintained in DMEM compared to MCM [3]. Sidhu et al. [39] reported that WME was superior to MCM in the maintenance of cytochrome P450 2B and 3A induction response. These results emphasize the importance of appropriate media selection a priori in the culture of primary rat hepatocytes for metabolic or biliary excretion experiments. The results described herein suggest that DMEM or WME would be suitable to culture SC rat hepatocytes for the purpose of biliary excretion experiments.

The substratum matrix is the primary determinant of cell attachment and survival [44]. Morphologic differences were evident between primary rat hepatocytes cultured on gelled collagen (GC) and Biocoat™ (BC) substratum. These results were in agreement with prior observations of hepatocyte flattening on rigid collagen and chord-like formation of hepatocytes plated on gelled collagen type I [21]. In addition to allowing for cell attachment to fibronectin, collagens and laminin, the interactions of hepatocytes with an extracellular matrix regulates other cellular functions such as cell proliferation, differentiation and migration [45]. Matrigel™ is composed of 60% laminin, 30% type IV collagen and 3% heparin sulfate proteoglycan [46]. In the present experiments, rat hepatocytes maintained in BC/M sandwich configuration flattened to a confluent monolayer and formed extensive canalicular networks. The expression of Bsep, Mrp2 and Mdr1a/b in SC rat hepatocytes maintained in BC/M sandwich configuration was similar to GC/GC sandwich configuration. Consistent with these findings, the BEI and $Cl_{B\ in\ vitro}$ values of TC, CDF, and Rh123 were similar in rat hepatocytes maintained in either BC/GC or BC/M sandwich configurations. The flattened (i.e., non-cuboidal) morphology of rat hepatocytes maintained in BC/M sandwich configuration was similar to hepatocytes cultured on dried rat tail collagen which expressed high levels of cytoskeletal mRNA and proteins [47]. LeClusye et al. [21] reported that actin filaments are integral in the maintenance of the hepatocyte architecture and canalicular network contraction, but were not critical for the formation of the biliary pole in SC rat hepatocytes. The preparation of gelled-collagen coated culture dishes and the subsequent overlay of gelled-collagen are time consuming procedures that are not readily amenable to higher-throughput assays. Results of the present studies suggest that GC/GC and BC/M sandwich configurations yield similar expression and functional data for the canalicular transport proteins examined, and support the use of Biocoat™ multi-well plates with a Matrigel™ overlay for higher-throughput assays to quantitate biliary excretion.

Hepatocytes plated on hydrated rat tail collagen at high density form compact trabecular aggregates that maintain a pattern of liver-specific gene expression, whereas at low density and on rigid rat tail collagen gene expression is predominately cytoskeletal related [47]. Plating density also has been shown to influence the attachment efficiency of hepatocytes cultured on simple collagen substratum [33,34]. In the current experiments, decreased cell–cell contacts resulted in an apparent decrease in canalicular network formation in hepatocytes plated at 25% density compared to 100% density. In rat hepatocytes plated between two layers of gelled collagen at 25% plating density, the formation of canalicular networks was evident between hepatocytes in direct contact with another hepatocyte following the initial plating. In recent experiments utilizing human hepatocytes, Hamilton et al. [48] observed that despite exhibition of a more physiologic morphology when plated on Matrigel™, decreased cell density resulted in a loss of cellular induction response to xenobiotics due to the decreased cell–cell contact. While the expression of Bsep was similar at all plating densities in the current experiments, the BEI of TC was significantly lower at 25% cell density compared to 100%. These results suggest that the capacity of the canalicular network may limit the calculation of BEI and $Cl_{B\ in\ vitro}$ for compounds that are extensively excreted in bile such as TC [1].

In conclusion, these studies have shown that the function and expression of the canalicular transport proteins Bsep, Mrp2 and Mdr1a/b in primary rat hepatocytes are influenced by culture conditions. SC rat hepatocytes represent a unique and robust model to study hepatobiliary transport processes of xenobiotics. The use of multi-well plates may serve to increase throughput of such experiments, as well as to maximize precious resources such as human hepatocytes. Primary rat hepatocytes cultured in a gelled-collagen/gelled-collagen or Biocoat™/Matrigel™ sandwich configuration and maintained in DMEM resulted in optimal canalicular network formation and transport protein expression and function.

## Acknowledgments

This research was supported by Pfizer Global Research and Development and The National Institutes of Health, Grant GM41395.

## REFERENCES

[1] Liu X, Chism JP, LeCluyse EL, Brouwer KR, Brouwer KL. Correlation of biliary excretion in sandwich-cultured rat hepatocytes and in vivo in rats. Drug Metab Dispos 1999;27:637–44.

[2] LeCluyse E, Bullock P, Madan A, Carroll K, Parkinson A. Influence of extracellular matrix overlay and medium formulation on the induction of cytochrome P-450 2B enzymes in primary cultures of rat hepatocytes. Drug Metab Dispos 1999;27:909–15.
[3] LeCluyse EL, Ahlgren-Beckendorf JA, Carroll K, Parkinson A, Johnson J. Regulation of glutathione S-transferase enzymes in primary cultures of rat hepatocytes maintained under various matrix configurations. Toxicol In Vitro 2000;14:101–15.
[4] Slaus K, Coughtrie MW, Sharp S, Vanhaecke T, Vercruysse A, Rogiers V. Influence of culture system and medium enrichment on sulfotransferase and sulfatase expression in male rat hepatocyte cultures. Biochem Pharmacol 2001;61:1107–17.
[5] Luttringer O, Theil FP, Lave T, Wernli-Kuratli K, Guentert TW, de Saizieu A. Influence of isolation procedure, extracellular matrix and dexamethasone on the regulation of membrane transporters gene expression in rat hepatocytes. Biochem Pharmacol 2002;64:1637–50.
[6] Schmitt M, Kubitz R, Lizun S, Wettstein M, Haussinger D. Regulation of the dynamic localization of the rat Bsep gene-encoded bile salt export pump by anisoosmolarity. Hepatology 2001;33:509–18.
[7] Rippin SJ, Hagenbuch B, Meier PJ, Stieger B. Cholestatic expression pattern of sinusoidal and canalicular organic anion transport systems in primary cultured rat hepatocytes. Hepatology 2001;33:776–82.
[8] Crocenzi FA, Mottino AD, Cao J, Veggi LM, Pozzi EJ, Vore M, et al. Estradiol-17{beta}-D-glucuronide induces endocytic internalization of Bsep in rats. Am J Physiol Gastrointest Liver Physiol 2003;285:G449–59.
[9] Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H, et al. Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. Biochim Biophys Acta 2001;1511:7–16.
[10] Suzuki H, Sugiyama Y. Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition. Adv Drug Del Rev 2002;54:1311–31.
[11] Liu X, LeCluyse EL, Brouwer KR, Gan LS, Lemasters JJ, Stieger B, et al. Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. Am J Physiol 1999;277:G12–21.
[12] Zamek-Gliszczynski MJ, Xiong H, Patel NJ, Turncliff RZ, Pollack GM, Brouwer KL. Pharmacokinetics of 5(and 6)-carboxy-2′,7′-dichlorofluorescein and its diacetate promoiety in the liver. J Pharmacol Exp Ther 2003;304:801–9.
[13] Turncliff, RZ. Expression and function of transport proteins and CYP3A in sandwich-cultured hepatocytes: application to the hepatobiliary disposition of drugs and generated metabolites. PhD Dissertation, University of North Carolina at Chapel Hill, 2004. UMI Microform 3129827, Proquest Information and Learning Co., Ann Arbor, MI.
[14] Borst P, Evers R, Kool M, Wijnholds J. The multidrug resistance protein family. Biochim Biophys Acta 1999;1461:347–57.
[15] Kruh GD, Zeng H, Rea PA, Liu G, Chen ZS, Lee K, et al. MRP subfamily transporters and resistance to anticancer agents. J Bioenerg Biomembr 2001;33:493–501.
[16] Hoffmaster KA, Turncliff RZ, LeCluyse EL, Kim RB, Meier PJ, Brouwer KLR. P-glycoprotein expression, localization, and function in sandwich-cultured primary rat and human hepatocytes: relevance to the hepatobiliary disposition of a model opioid peptide. Pharm Res 2004;21(7):1294–302.
[17] Kusuhara H, Suzuki H, Sugiyama Y. The role of P-glycoprotein and canalicular multispecific organic anion transporter in the hepatobiliary excretion of drugs. J Pharm Sci 1998;87:1025–40.
[18] Shapiro AB, Ling V. Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. Eur J Biochem 1997;250:130–7.
[19] Fardel O, Payen L, Courtois A, Vernhet L, Lecureur V. Regulation of biliary drug efflux pump expression by hormones and xenobiotics. Toxicology 2001;167:37–46.
[20] LeCluyse EL, Audus KL, Hochman JH. Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. Am J Physiol 1994;266:C1764–7.
[21] LeCluyse EL, Fix JA, Audus KL, Hochman JH. Regeneration and maintenance of bile canalicular networks in collagen-sandwiched hepatocytes. Toxicol In Vitro 2000;14:117–32.
[22] LeCluyse EL, Bullock PL, Parkinson A, Hochman JH. Cultured rat hepatocytes. Pharm Biotechnol 1996;8:121–59.
[23] Richert L, Binda D, Hamilton G, Viollon-Abadie C, Alexandre E, Bigot-Lasserre D, et al. Evaluation of the effect of culture configuration on morphology, survival time, antioxidant status and metabolic capacities of cultured rat hepatocytes. Toxicol In Vitro 2002;16:89–99.
[24] De Smet K, Cavin C, Vercruysse A, Rogiers V. Collagen type I gel cultures of adult rat hepatocytes as a screening induction model for cytochrome P450-dependent enzymes. Altern Lab Anim 2001;29:179–92.
[25] Schuetz EG, Li D, Omiecinski CJ, Muller-Eberhard U, Kleinman HK, Elswick B, et al. Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix. J Cell Physiol 1988;134:309–23.
[26] Clement B, Yamada Y. A Mr 80K hepatocyte surface protein(s) interacts with basement membrane components. Exp Cell Res 1990;187:320–3.
[27] Dunn JC, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. FASEB J 1989;3:174–7.
[28] Chandra P, Lecluyse EL, Brouwer KL. Optimization of culture conditions for determining hepatobiliary disposition of taurocholate in sandwich-cultured rat hepatocytes. In Vitro Cell Dev Biol Anim 2001;37:380–5.
[29] Annaert PP, Turncliff RZ, Booth CL, Thakker DR, Brouwer KL. P-glycoprotein-mediated in vitro biliary excretion in sandwich-cultured rat hepatocytes. Drug Metab Dispos 2001;29:1277–83.
[30] Liu X, LeCluyse EL, Brouwer KR, Lightfoot RM, Lee JI, Brouwer KL. Use of Ca2+ modulation to evaluate biliary excretion in sandwich-cultured rat hepatocytes. J Pharmacol Exp Ther 1999;289:1592–9.
[31] Williams GM, Gunn JM. Long-term cell culture of adult rat liver epithelial cells. Exp Cell Res 1974;89:139–42.
[32] Laishes BA, Williams GM. Conditions affecting primary cell cultures of functional adult rat hepatocytes. II. Dexamethasone enhanced longevity and maintenance of morphology. In Vitro 1976;12:821–32.
[33] Williams GM, Bermudez E, Scaramuzzino D. Rat hepatocyte primary cell cultures. III. Improved dissociation and attachment techniques and the enhancement of survival by culture medium. In Vitro 1977;13:809–17.
[34] Jauregui HO, McMillan PN, Driscoll J, Naik S. Attachment and long term survival of adult rat hepatocytes in primary monolayer cultures: comparison of different substrata and tissue culture media formulations. In Vitro Cell Dev Biol 1986;22:13–22.
[35] Reid LM, Narita M, Fujita M, Murray Z, Liverpoool C, Rosenberg L. Matrix and hormonal regulation of differentiation in liver cultures. In: Research in Isolated and Cultured Hepatocytes. Inserm, London: John Libbey Eurotext Ltd.; 1986. p. 225–58.

[36] Dunn JC, Tompkins RG, Yarmush ML. Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. Biotechnol Prog 1991;7:237–45.
[37] Schuetz EG, Schuetz JD, May B, Guzelian PS. Regulation of cytochrome P-450b/e and P-450p gene expression by growth hormone in adult rat hepatocytes cultured on a reconstituted basement membrane. J Biol Chem 1990;265:1188–92.
[38] Akrawi M, Rogiers V, Vandenberghe Y, Palmer CN, Vercruysse A, Shephard EA, et al. Maintenance and induction in co-cultured rat hepatocytes of components of the cytochrome P450-mediated mono-oxygenase. Biochem Pharmacol 1993;45:1583–91.
[39] Sidhu JS, Farin FM, Omiecinski CJ. Influence of extracellular matrix overlay on phenobarbital-mediated induction of CYP2B1, 2B2, and 3A1 genes in primary adult rat hepatocyte culture. Arch Biochem Biophys 1993;301:103–13.
[40] Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, et al. The conduct of in vitro and in vivo drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. Drug Metab Dispos 2003;31:815–32.
[41] Kipp H, Arias IM. Trafficking of canalicular ABC transporters in hepatocytes. Annu Rev Physiol 2002;64:595–608.
[42] Chee DO, Boddie AW, Roth JA, Holmes EC, Morton DL. Production of melanoma-associated antigen(s) by a defined malignant melanoma cell strain grown in chemically defined medium. Cancer Res 1976;36:1503–9.
[43] Williams GM, Weisburger EK, Weisburger JH. Isolation and long-term cell culture of epithelial-like cells from rat liver. Exp Cell Res 1971;69:106–12.
[44] Macdonald JM, Xu ASL, Hiroshi K, LeCluyse E, Hamilton G, Liu H, et al. Ex vivo maintenance of cell from the liver lineage. In: Lanza WL, Langer R, Vacanti J, editors. Methods of tissue engineering. San Diego, CA: Academic Press; 2001.
[45] Ruoslahti E, Vaheri A. Cell-to-cell contact and extracellular matrix. Curr Opin Cell Biol 1997;9:605–7.
[46] Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 1982;21:6188–93.
[47] Ben-Ze'ev A, Robinson GS, Bucher NL, Farmer SR. Cell–cell and cell–matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. Proc Natl Acad Sci USA 1988;85:2161–5.
[48] Hamilton GA, Jolley SL, Gilbert D, Coon DJ, Barros S, LeCluyse EL. Regulation of cell morphology and cytochrome P450 expression in human hepatocytes by extracellular matrix and cell–cell interactions. Cell Tissue Res 2001;306:85–99.