

Influence of Seeding Density and Extracellular Matrix on Bile Acid Transport and Mrp4 Expression in Sandwich-Cultured Mouse Hepatocytes

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Abstract: This study was undertaken to examine the influence of seeding density, extracellular matrix and days in culture on bile acid transport proteins and hepatobiliary disposition of the model bile acid taurocholate. Mouse hepatocytes were cultured in a sandwich configuration on six-well Biocoat plates with an overlay of Matrigel (BC/MG) or gelled-collagen (BC/GC) for 3 or 4 days at seeding densities of 1.0, 1.25, or 1.5×10^6 cells/well. The lower seeding densities of 1.0 and 1.25×10^6 cells/well resulted in good hepatocyte morphology and bile canaliculi network formation, as visualized by 5-(and 6)-carboxy-2',7'-dichlorofluorescein accumulation. In general, taurocholate cellular accumulation tended to increase as a function of seeding density in BC/GC; cellular accumulation was significantly increased in hepatocytes cultured in BC/MG compared to BC/GC at the same seeding density on both days 3 and 4 of culture. In general, *in vitro* intrinsic biliary clearance of taurocholate was increased at higher seeding densities. Levels of bile acid transport proteins on days 3 and 4 were not markedly influenced by seeding density or extracellular matrix except for multidrug resistance protein 4 (Mrp4), which was inversely related to seeding density. Mrp4 levels decreased ~2- to 3-fold between seeding densities of 1.0×10^6 and 1.25×10^6 cells/well regardless of extracellular matrix; an additional ~3- to 5-fold decrease in Mrp4 protein was noted in BC/GC between seeding densities of 1.25×10^6 and 1.5×10^6 cells/well. Results suggest that seeding density, extracellular matrix and days in culture profoundly influence Mrp4 expression in sandwich-cultured mouse hepatocytes. Primary mouse hepatocytes seeded in a BC/MG configuration at densities of 1.25×10^6 cells/well and 1.0×10^6 , and cultured for 3 days, yielded optimal transport based on the probes studied. This work demonstrates the applicability of the sandwich-cultured model to mouse hepatocytes.

Keywords: Sandwich-cultured mouse hepatocytes; Mrp4; taurocholate; BEI; *in vitro* Cl_{biliary} , carboxydichlorofluorescein diacetate

Introduction

Primary hepatocytes are a widely accepted *in vitro* tool to evaluate hepatic drug uptake, metabolism and cytochrome P450 induction. Maintaining hepatocytes between two layers

of gelled collagen (sandwich-culture configuration) facilitates the development of intact canaliculi networks, reestablishment of polarized excretory function and maintenance of hepatic transport protein expression and function.¹⁻⁵ Dunn et al. first demonstrated that the sandwich configuration enhanced the morphology and viability of hepatocytes, and helped maintain normal secretion of many liver-specific proteins and organic compounds.⁶⁻⁸ Subsequent studies have demonstrated that the sandwich configuration facilitates the formation of gap junctions and functional bile canaliculi

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networks during culture.^{2,3} Several studies also have shown that sandwich-cultured hepatocytes are capable of performing a wide variety of cellular functions normally attributed to hepatocytes *in vivo* including albumin secretion, bile acid synthesis and excretion, and P450 enzyme induction, especially when compared to hepatocytes cultured under conventional conditions.^{2,3,8–11}

Sandwich-cultured hepatocytes have been used to investigate hepatic accumulation and excretion of a wide variety

of substrates.^{12–14} In fact, the *in vitro* intrinsic biliary clearance generated from the sandwich-cultured hepatocyte model has been shown to correlate with *in vivo* biliary clearance data in rats^{15,16} and humans.^{17,18} Thus, the sandwich-cultured hepatocyte model is very promising for characterizing biliary excretion and the hepatic disposition of drug candidates. With all relevant hepatic transport proteins and xenobiotic metabolizing enzymes expressed, substrate uptake, metabolism, and parent/metabolite efflux can be assessed. Recent work has demonstrated that sandwich-cultured primary mouse hepatocytes maintain metabolic competence longer than rat hepatocytes based on gene expression profiles and cytochrome P450 activity.¹⁹ Furthermore, the application of this method to hepatocytes from gene-disrupted mice in which specific transport proteins have been knocked out may provide new insight into the many roles of hepatic transport proteins. Numerous investigators have examined the influence of culture conditions on hepatic transport protein expression and function in sandwich-cultured rat and human hepatocytes.^{9,20–23} However, culture conditions have not been optimized for transport protein

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expression and function in sandwich-cultured mouse hepatocytes. As interest grows in utilizing the phenotypic diversity of various mouse strains (*e.g.*, the Collaborative Cross²⁴) to predict drug-induced hepatotoxicity, higher throughput screening systems, such as sandwich-cultured mouse hepatocytes, may prove to be particularly useful.

Hepatic transport proteins play an important role in the vectorial transport of bile acids from sinusoidal blood into the bile canaliculi. Sodium taurocholate cotransporting polypeptide (Ntcp) is expressed exclusively in the liver and mediates sodium-dependent uptake of bile acids.^{25,26} The organic anion-transporting polypeptides (Oatps), also localized on the basolateral membrane, translocate bile acids in a sodium-independent manner.²⁶ The multidrug resistance proteins 3 and 4 (Mrp3 and Mrp4) are involved in the basolateral excretion of organic anions and bile acids from the hepatocyte across the sinusoidal membrane into blood.^{27–29} In addition, data generated in mice deficient in the organic solute transporter (Ost)- α subunit have suggested that Ost α -Ost β , a heteromeric organic solute and steroid transporter, also is involved in the basolateral efflux of bile acids.³⁰ The apical (canalicular) transport proteins involved in biliary excretion of bile acids include the bile salt export pump (Bsep),³¹ the multidrug resistance protein 2 (Mrp2)^{32,33} and more recently, multidrug resistance P-glycoprotein (Mdr1a/

1b).³⁴ Interestingly, triple knockout mice deficient in Mdr1a/1b and Bsep exhibited a significantly more severe phenotype than Bsep^{-/-} and wild-type mice, including impaired bile formation, jaundice, flaccid gallbladder and hepatic inflammation.³⁴

The purpose of the present studies was to examine the influence of seeding density, extracellular matrix and days in culture on hepatic bile acid transport protein expression and taurocholate hepatobiliary disposition. The criteria used for the selection of optimal conditions were hepatocyte morphology, transport protein expression, substrate accumulation, as well as the biliary excretion index (BEI) and biliary clearance (*in vitro* Cl_{biliary}) of taurocholate.

Experimental Details

Materials. Dulbecco's modified Eagle's medium (DMEM), MEM nonessential amino acids solution (100 \times), L-glutamine, insulin, penicillin G–streptomycin solution, and 5-(and 6)-carboxy-2',7' dichlorofluorescein (CDF) diacetate (CDFDA) were purchased from Invitrogen (Carlsbad, CA). Collagenase (type 4), fetal bovine serum (FBS), sodium taurocholate, Triton X-100, dexamethasone, and Hanks' balanced salt solution (HBSS) modified with (H-1387) or without (H-4891) calcium chloride were obtained from Sigma-Aldrich (St. Louis, MO). BioCoat collagen I plates, Matrigel basement membrane matrix, rat tail collagen (type I), and ITS+ (insulin/transferrin/selenium) culture supplement were purchased from BD Biosciences Discovery Labware (Bedford, MA). [³H]Taurocholate (5 Ci/mmol, >97% purity) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Bio-Safe II liquid scintillation cocktail was obtained from Research Products International (Mt. Prospect, IL). Bicinchoninic acid (BCA) protein assay reagents and BSA for the protein assay standard were purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals and reagents were of analytical grade and available from commercial sources.

Isolation and *in Vitro* Culture of Primary Mouse Hepatocytes. Male C57BL/6 mice (27.7–30.3 g) were purchased from Charles River Laboratories, Inc. (Raleigh, NC). Mice had free access to water and food prior to surgery. All animal procedures complied with the guidelines of the Institutional Animal Care and Use Committee (University of North Carolina, Chapel Hill, NC). Hepatocytes were isolated by a two-step collagenase perfusion, as described previously,³⁵ with modifications. The inferior vena cava was cannulated and buffers were perfused retrograde to blood

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flow. Cell viability, determined by trypan blue exclusion, was 89–93%. In pilot studies, hepatocytes were seeded on six-well plates with gelled collagen solution, as described previously,²¹ or six-well Biocoat plates. In subsequent studies, hepatocytes were seeded at 1.0, 1.25, or 1.5 × 10⁶ cells/well in 6-well BioCoat plates in DMEM without phenol red supplemented with 2 mM L-glutamine, 1% (v/v) MEM nonessential amino acids, 100 units penicillin G sodium, 100 μg streptomycin sulfate, 1 μM dexamethasone, 5% (v/v) FBS, and 10 μM insulin (day 0 of culture), and allowed to attach for 2–6 h in a humidified incubator (95% O₂, 5% CO₂) at 37°C. After cell attachment, culture plates were swirled gently and the culture medium was replaced with the same medium. Cells were overlaid 16–24 h (day 1 of culture) after seeding with ice-cold Matrigel basement membrane matrix (BC/MG) or gelled collagen (BC/GC). Matrigel was overlaid at a concentration of 0.25 mg/mL in 2 mL/well cold serum-free DMEM containing 2 mM L-glutamine, 1% (v/v) MEM nonessential amino acids, 100 units penicillin G sodium, 100 μg streptomycin sulfate, 0.1 μM dexamethasone, and 1% (v/v) ITS+. Gelled collagen solution (~1.5 mg/mL) was prepared by adding 4 mL of rat tail type I collagen, 4 mL of sterile deionized water, and 1 mL of 10× DMEM, and the pH was adjusted to 7.4 with 2 N NaOH. Hepatocyte cultures were aspirated, and the monolayer was overlaid with 0.1 mL/well of ice-cold neutralized type I gelled collagen and placed at 37°C in a humidified incubator for ~1 h to allow the matrix to gel, followed by the addition of 1.5 mL/well of warm DMEM with the same supplements as the cells overlaid with matrigel. The culture medium was changed every 24 h until experiments were performed on day 3–4 of culture.

Accumulation Experiments. The method to determine substrate accumulation in sandwich-cultured hepatocytes has been described previously.¹⁶ Briefly, hepatocytes were rinsed twice with 2 mL of warm HBSS containing Ca²⁺ (standard buffer) or Ca²⁺-free HBSS, and incubated with 2 mL of the same buffer for 10 min at 37°C to maintain tight junction integrity and bile canaliculi networks (cells + bile), or disrupt tight junctions and open bile canaliculi networks (cells), respectively. The buffer was removed, and the cells were incubated for 10 min at 37°C with 1.5 mL of [³H]taurocholate (1 μM) or CDFDA (2 μM) in standard buffer. Hepatocytes were rinsed vigorously three times with 2 mL ice-cold standard buffer following the incubation. CDFDA-treated hepatocytes were viewed immediately and digital images captured with a Zeiss Axiovert 200TV inverted phase contrast microscope. Taurocholate-treated hepatocytes were lysed with 1 mL of 0.5% (v/v) Triton X-100 in phosphate-buffered saline by placing plates on an orbital shaker for a minimum of 20 min at room temperature. Taurocholate uptake was corrected for nonspecific binding by subtracting uptake on blank six-well Biocoat plates overlaid with Matrigel or gelled collagen. Data were normalized to protein concentration in each well, determined in duplicate aliquots using BCA protein assay reagent kit (Pierce) as instructed by the manufacturer. BSA, as supplied

by the manufacturer, was used as a standard (0.2–2 mg/mL). The [³H]taurocholate samples were analyzed by liquid scintillation spectroscopy in a Packard Tri-Carb scintillation counter (PerkinElmer Life and Analytical Sciences).

Cytotoxicity Assay. The activity of lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis, was measured in the medium of sandwich-cultured hepatocytes using a cytotoxicity kit according to the manufacturer's instructions (Roche, Indianapolis, IN). The degree of LDH release was expressed as a percentage of the maximum cellular LDH release, measured by adding 0.5% Triton X-100 to sandwich-cultured mouse hepatocytes at each seeding density.

Immunoblots. Cells were washed once with HBSS and resuspended in lysis buffer consisting of 1% SDS, 1 mM EDTA and Complete protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by the BCA assay. Whole-cell lysates (30–40 μg) were resolved on NuPAGE 4 to 20% Bis-Tris gel (Invitrogen Corp, Carlsbad, CA), and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in 5% milk-Tris-buffered saline with Tween 20 (TBST) for 30 min, blots were incubated overnight at 4 °C with the following antibodies: Mrp2 (*Abcc2*; M2III-6), Mrp3 (*Abcc3*; M3II-21), Mrp4 (*Abcc4*; M4I-10), Mdr1a/1b (*Abcb1*; C-219), and breast cancer resistance protein [Bcrp (*Abcg2*); BXP-53], which were supplied by Alexis Biochemicals, San Diego, CA; Bsep (*Abcb11*; K44) and Ntcp (*Slc10a1*; K4), which were a kind gift from Drs. Bruno Stieger and Peter Meier; and β-actin (C4, Chemicon, San Francisco, CA). After incubation with HRP-conjugated secondary antibody, signals were detected by chemiluminescent substrate Supersignal West Dura (Pierce, Rockford, IL) with a Bio-Rad VersaDoc imaging system; densitometry analysis was performed using Quantity One v4.1 software (Bio-Rad Laboratories, Hercules, CA).

Data Analysis. For accumulation studies, the biliary excretion index (BEI, %) and *in vitro* intrinsic biliary clearance (Cl_{biliary}, mL/min/kg) were calculated using B-CLEAR technology (Qualyst, Inc., Raleigh, NC):¹⁶

$$\text{BEI} = \frac{\text{accumulation}_{\text{cells+bile}} - \text{accumulation}_{\text{cells}}}{\text{accumulation}_{\text{cells+bile}}} \times 100 \quad (1)$$

where substrate accumulation in the cells+bile compartments was determined in hepatocytes preincubated in standard buffer; cellular accumulation of substrate was determined in hepatocytes preincubated with Ca²⁺-free HBSS.

$$\text{in vitro Cl}_{\text{biliary}} = \frac{\text{accumulation}_{\text{cells+bile}} - \text{accumulation}_{\text{cells}}}{\text{AUC}_{0-T}} \quad (2)$$

where AUC_{0–T} represents the product of the incubation time (T) and the initial taurocholate concentration in the medium. *In vitro* Cl_{biliary} values were scaled per kilogram body weight using 0.63, 0.72, and 0.78 mg of protein per 1.0, 1.25, and 1.5 × 10⁶ cells, respectively (the average value obtained in

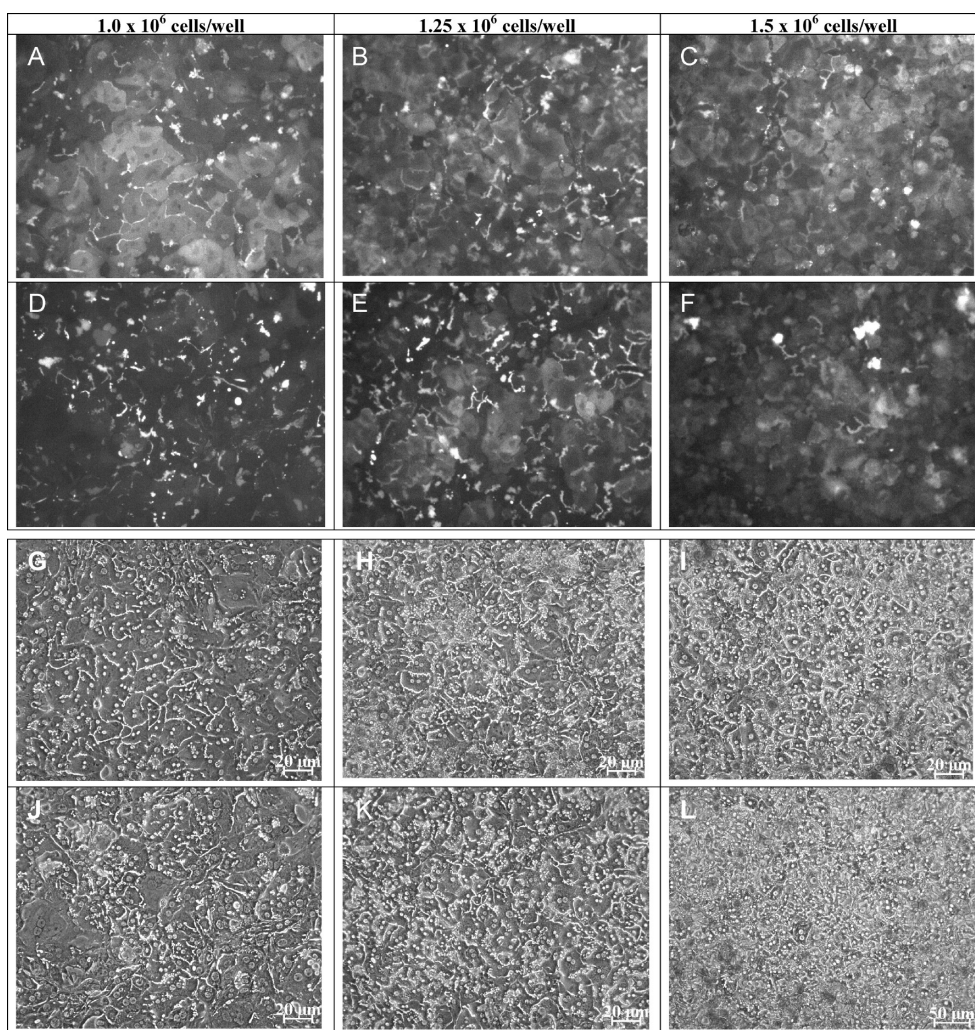


Figure 1. Effect of seeding density and extracellular matrix on cell morphology and bile canicular network formation in day 3 sandwich-cultured mouse hepatocytes. Representative ($n = 3$) CDF fluorescence (A–F) and light microscopy (G–L) images of mouse hepatocytes cultured in a Biocoat/Matrigel (BC/MG; A–C and G–I) or Biocoat/gelled-collagen (BC/GC; D–F and J–L) sandwich configuration in six-well plates (maintained with DMEM for 3 days); seeding densities (1.0 , 1.25 , or 1.5×10^6 cells/well) are noted at the top of each column. Sandwich-cultured mouse hepatocytes were incubated with $2 \mu\text{M}$ CDFDA for 10 min.

all preparations), 135×10^6 hepatocytes/g of mouse liver tissue,³⁶ and 87.5 g of liver tissue per kg of body weight ($1.75 \text{ g}/0.02 \text{ kg mouse}$).³⁷

Statistical analysis of the [^3H]taurocholate cellular accumulation and *in vitro* intrinsic biliary clearance data was performed using a three-way analysis of variance for the factors of day in culture, seeding density and extracellular matrix, with Bonferroni's post hoc test (SAS version 9.1, Cary, NC).

Results

Sandwich-Cultured Mouse Hepatocyte Viability and Bile Canicular Network Formation. Initial experiments plating mouse hepatocytes on gelled collagen plates resulted in decreased attachment and adherence after overlay compared to Biocoat plates. Therefore, subsequent experiments utilized Biocoat plates only, on which hepatocytes flattened to form confluent monolayers. Hepatocyte morphology was evaluated by light microscopy, and canicular network

formation was determined by CDF imaging. Mouse hepatocytes cultured in sandwich configuration and maintained in DMEM for up to 4 days were cuboidal in shape and formed extensive canicular networks (Figure 1 and 2). The intensity of CDF accumulation in the bile canaliculi appeared to be more extensive in mouse hepatocytes cultured in the BC/GC configuration compared to the BC/MG configuration based on visual inspection; the BC/MG matrix appeared to have greater intracellular accumulation of CDF compared to BC/GC. Based on the light microscopy images, the higher seeding density of 1.5×10^6 cells/well resulted in greater accumulation of dead cells and debris compared to the lower seeding densities (Figure 1 and 2). Based on LDH leakage, cell death primarily occurred in the first 24 h before overlay; medium LDH levels during the first 24 h after plating reached $\sim 25\%$ of Triton X-100 control. Cumulative LDH leakage after overlay through day 4 in culture was negligible ($<12\%$

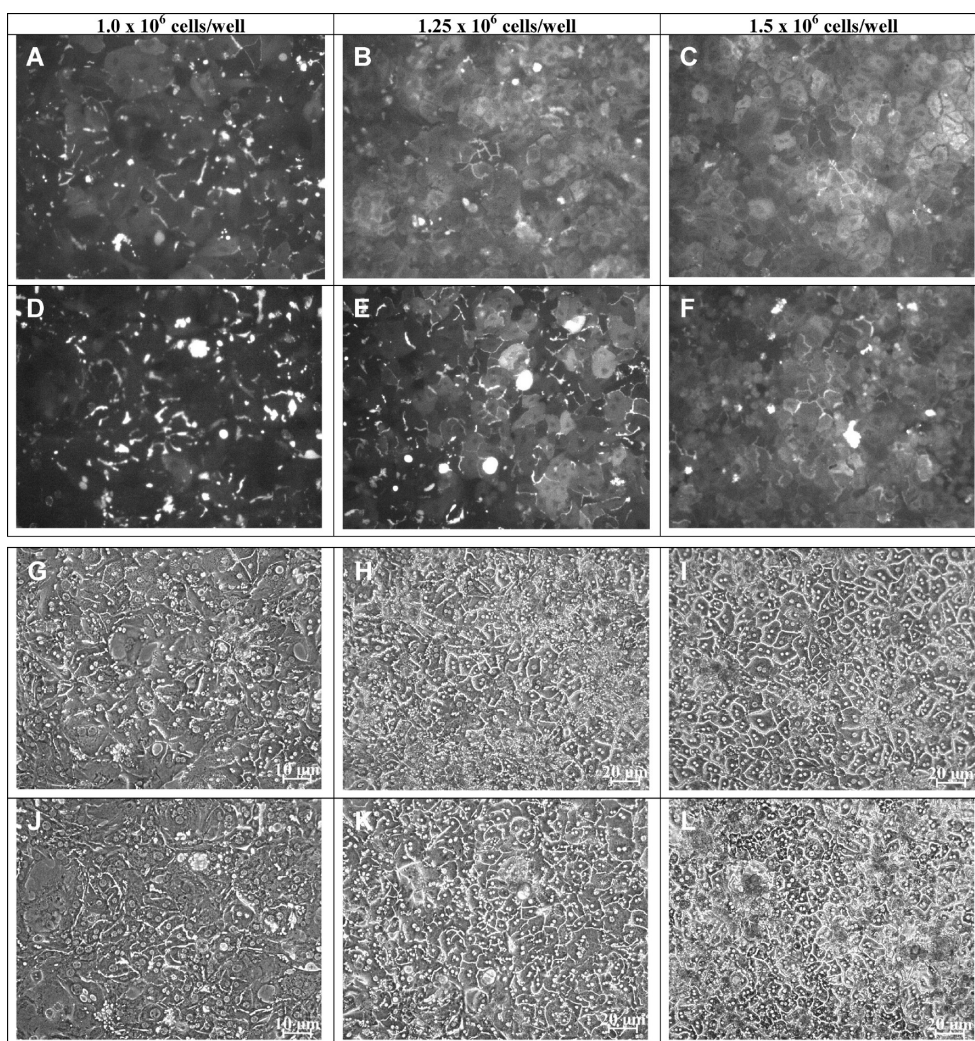


Figure 2. Effect of seeding density and extracellular matrix on cell morphology and bile canaliculi network formation in day 4 sandwich-cultured mouse hepatocytes. Representative ($n = 3$) CDF fluorescence (A–F) and light microscopy (G–L) images of mouse hepatocytes cultured in a Biocoat/Matrigel (BC/MG; A–C and G–I) or Biocoat/gelled-collagen (BC/GC; D–F and J–L) sandwich configuration in six-well plates (maintained with DMEM for 4 days); seeding densities (1.0 , 1.25 , or 1.5×10^6 cells/well) are noted at the top of each column. Sandwich-cultured mouse hepatocytes were incubated with $2 \mu\text{M}$ CDFDA for 10 min.

of Triton X-100 control), regardless of seeding density or extracellular matrix (data not shown).

Transport Protein Expression. Representative immunoblots of Ntcp, Mrp4, Mrp3, Mrp2, Bsep, Bcrp, and Mdr1a/1b in sandwich-cultured mouse hepatocytes seeded at different densities on BC/MG and BC/GC are shown in Figure 3. Relevant bile acid transport proteins were expressed in sandwich-cultured mouse hepatocytes by day 3; culture configuration and seeding density had modest effects on Mrp3, Mrp2, Bcrp, Ntcp, Mdr1a/1b and Bsep protein. Interestingly, Mrp4 protein increased (~ 2 -fold or greater) as seeding density decreased, regardless of extracellular matrix on both days 3 and 4 (Figure 3 and 4).

Hepatobiliary Disposition of the Model Bile Acid Taurocholate. The influence of seeding density, overlay matrix composition and days in culture on the accumulation and biliary excretion of [^3H]taurocholate was assessed in

sandwich-cultured mouse hepatocytes. Cellular accumulation of [^3H]taurocholate was significantly lower on day 4 compared to day 3 (Figure 5). [^3H]Taurocholate cellular accumulation tended to increase as a function of seeding density for BC/GC; [^3H]taurocholate cellular accumulation was significantly greater in mouse hepatocytes cultured in the BC/MG configuration compared to the BC/GC configuration at each seeding density on both days 3 and 4 of culture (Figure 5). In general, *in vitro* intrinsic biliary clearance of taurocholate was increased at higher seeding

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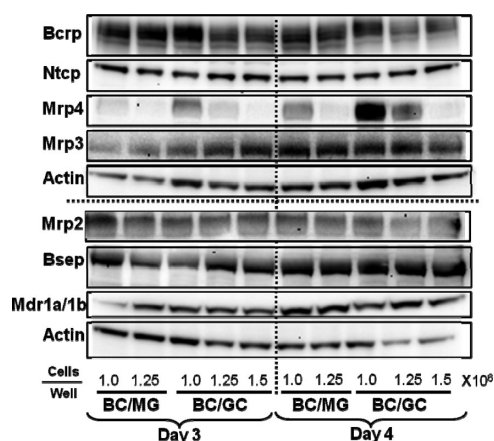


Figure 3. Influence of seeding density, extracellular matrix and days in culture on transport protein levels in sandwich-cultured mouse hepatocytes. Representative immunoblots of Bcrp, Ntcp, Mrp4, Mrp3, Mrp2, Bsep, and Mdr1a/1b in mouse hepatocytes cultured in Biocoat/Matrigel (BC/MG) or Biocoat/gelled-collagen (BC/GC) sandwich configuration in six-well plates and maintained with DMEM for 3 or 4 days ($n = 2$). β -Actin was used as a loading control.

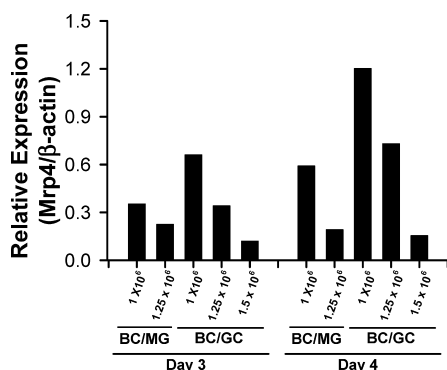


Figure 4. Relative expression of Mrp4 protein compared with β -actin in sandwich-cultured mouse hepatocytes cultured in Biocoat/Matrigel (BC/MG) or Biocoat/gelled-collagen (BC/GC) sandwich configuration in six-well plates and maintained with DMEM for 3–4 days. Densitometry was performed with Quantity One software (version 4.1; Bio-Rad, Hercules, CA).

densities; taurocholate BEI values were not significantly different across seeding density (Table 1).

Discussion

Sandwich-cultured primary hepatocytes have been used extensively to study hepatic uptake, metabolism and efflux. To date, limited data have been published utilizing sandwich-cultured mouse hepatocytes to study hepatic transport mechanisms and biliary excretion. Previous reports have demonstrated the influence of culture conditions including the type of culture media, media supplements, extracellular matrix and confluency on cell morphology, bile canaliculi network formation, substrate accumulation and BEI in both rat and cryopreserved human hepatocytes.^{20–22} In the present study, the influence of seeding density, extracellular matrix and days

in culture on protein levels of bile acid transporters, and the hepatobiliary disposition of taurocholate in sandwich-cultured mouse hepatocytes were assessed.

Both extracellular matrix overlay and seeding density had profound effects on the health, morphology, and bile canaliculi network formation of sandwich-cultured mouse hepatocytes. The cell morphology of mouse hepatocytes cultured on Biocoat plates was similar to prior observations with rat hepatocytes; cells spread out to form a confluent monolayer resulting in a flattened appearance on this rigid collagen when compared to hepatocytes cultured on gelled collagen.^{2,22} Mouse hepatocytes overlaid with Matrigel (BC/MG) exhibited a similar attachment efficiency and cell morphology compared to those overlaid with gelled collagen (BC/GC). However, Matrigel provided a more uniform, reproducible overlay. CDFDA, which is hydrolyzed to fluorescent CDF inside hepatocytes, is a good Mrp2 probe substrate³⁸ that has been used extensively to visualize the bile canaliculi.^{2,20,21} CDF was excreted into bile canaliculi in all culture conditions. However, more CDF accumulation in bile canaliculi networks was observed by fluorescence microscopy in BC/GC compared to BC/MG at lower seeding densities, which may be attributed to more extensive formation of bile canaliculi networks (Figures 1 and 2). Although levels of Mrp2 protein were similar across extracellular matrix and days in culture (Figure 3), differences in the amount of functional Mrp2 protein on the apical membrane also may contribute to the increased accumulation of CDF in bile canaliculi networks observed at the lower seeding densities. Furthermore, CDF intracellular accumulation was greater in mouse hepatocytes cultured on BC/MG compared to BC/GC based on visual inspection of the fluorescent images (Figures 1 and 2); similar findings were noted for taurocholate cellular accumulation (Figure 5).

Mouse hepatocytes seeded at 1.5×10^6 cells/well resulted in greater accumulation of dead cells and decreased network formation, as visualized by CDF. Cell death occurred primarily during the first day of culture, shortly after hepatocytes were seeded, based on LDH measurements. Most of the unattached and dead cells were washed away when the medium was changed at 24 h, prior to overlay, but some cell debris presumably was stuck to the collagen, as visualized in Figures 1 and 2. The cell death observed in this study may be attributed primarily to collagenase digestion used during hepatocyte isolation, which has been shown by many investigators to influence cell viability,^{39–42} and cannot be explained by complete consumption of nutrients or growth factors in the cell medium. Greater cell death at higher seeding densities was

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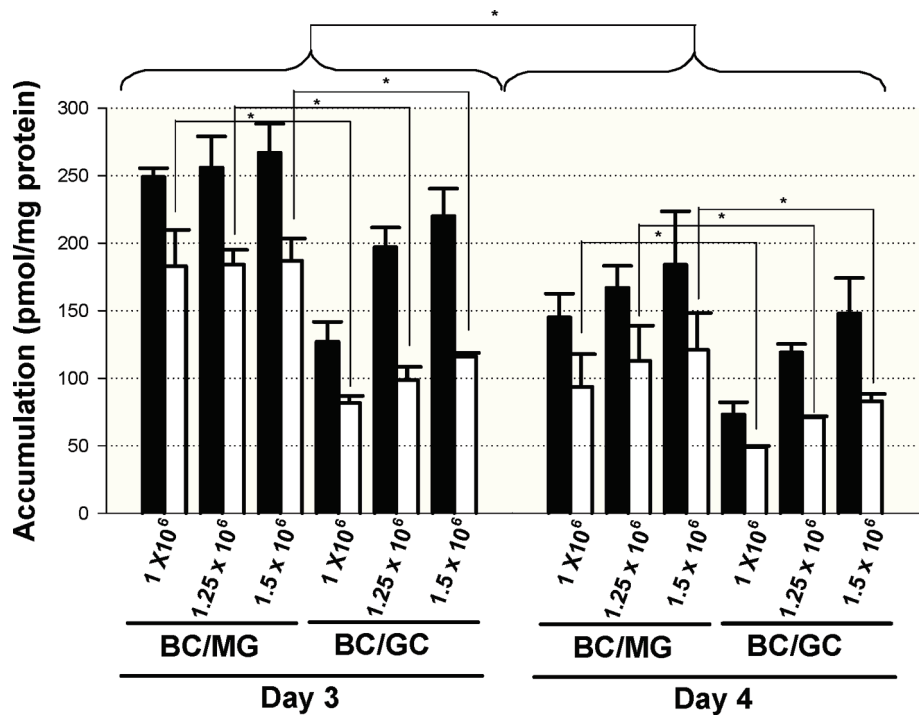


Figure 5. Accumulation in cells + bile (black bars) and cells (white bars) after a 10-min incubation with 1 μ M [3 H]taurocholate in mouse hepatocytes cultured in Biocoat/Matrigel (BC/MG) or Biocoat/gelled-collagen (BC/GC) sandwich configuration in six-well plates and maintained with DMEM for 3 or 4 days (mean \pm SEM; $n = 3$). Cellular accumulation (white bars) of [3 H]taurocholate was significantly increased on day 3 compared to day 4, and significantly greater when cultured in BC/MG configuration compared to the BC/GC configuration on both days 3 and 4 of culture. * $p < 0.05$.

Table 1. Mean Biliary Excretion Index (BEI) and *in vitro* Biliary Clearance (*in vitro* Cl_{biliary}) of [3 H]Taurocholate after a 10-min Incubation with 1 μ M [3 H]Taurocholate in Sandwich-Cultured Mouse Hepatocytes^a

		BC/MG			BC/GC		
day in culture		1.0 $\times 10^6$ cells/well	1.25 $\times 10^6$ cells/well	1.5 $\times 10^6$ cells/well	1.0 $\times 10^6$ cells/well	1.25 $\times 10^6$ cells/well	1.5 $\times 10^6$ cells/well
3	BEI (%)	26.3 \pm 10.8	27.6 \pm 5.5	29.6 \pm 8.2	35.4 \pm 3.9	49.9 \pm 6.3	46.9 \pm 6.4
	<i>in vitro</i> Cl _{biliary} (mL/min/kg)	48.7 \pm 20.5	60.4 \pm 15.8	73.7 \pm 24.6	33.6 \pm 7.6†	84.0 \pm 15.6†	96.0 \pm 21.1†
4	BEI (%)	36.2 \pm 9.0	32.5 \pm 10.3	34.2 \pm 5.0	32.4 \pm 7.7	39.9 \pm 3.5	43.5 \pm 6.4
	<i>in vitro</i> Cl _{biliary} (mL/min/kg)	38.3 \pm 5.0**	45.4 \pm 10.6	57.8 \pm 14.7**	18.0 \pm 6.4*	40.4 \pm 5.7*	60.3 \pm 19.3*

^a Mean \pm SEM; $n = 3$ per group. Primary mouse hepatocytes were seeded at designated densities and cultured in Biocoat/Matrigel (BC/MG) or Biocoat/gelled-collagen (BC/GC) sandwich configuration in six-well plates and maintained with DMEM for 3 or 4 days. *In vitro* intrinsic biliary clearance of [3 H]taurocholate was significantly increased as a function of seeding density († $p < 0.05$, 1.0 vs. 1.25 and 1.0 vs. 1.5 $\times 10^6$ cells/well on day 3; * $p < 0.05$, 1.0 vs. 1.25 and 1.0 vs. 1.5 $\times 10^6$ cells/well on day 3; ** $p < 0.05$, 1.0 vs. 1.5 $\times 10^6$ cells/well on day 4).

similar to findings observed in mouse hepatocytes cultured at an overconfluent stage, which resulted in cell death by apoptosis.⁴³ The observation that mouse hepatocytes perform better at a lower seeding density compared to other species (rat and human hepatocytes typically are seeded at $\sim 1.75 \times 10^6$ cells/well on 6-well plates^{44,45})

may be attributed to the increased oxygen demand *in vivo* in mice (liver blood flow in mice is ~ 90 mL/min/kg compared to ~ 55.2 and ~ 20.7 mL/min/kg in rats and humans, respectively).³⁷

Overlay matrix composition (BC/MG or BC/GC) and days in culture (day 3 or 4) appeared to have little effect on protein levels of Bsep, Bcrp, Ntcp, Mrp3, Mrp2 and Mdr1a/1b in sandwich-cultured mouse hepatocytes. Surprisingly, protein levels of Ntcp were well-maintained

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compared to sandwich-cultured rat hepatocytes, where significant downregulation of Ntcp protein³ (~80% decrease⁴⁶) and a ~75–80% decrease in mRNA⁴⁷ have been reported over days in culture.

Another profound observation noted in the present studies was that Mrp4 protein levels were higher at lower seeding densities (Figure 3 and 4). Mrp4 is localized on the basolateral membrane of hepatocytes and has been shown to efflux bile acids in a glutathione-dependent manner.²⁸ Mrp4 plays an important role in detoxifying the liver; bile-duct ligated *Mrp4*^{-/-} mice exhibited increased liver toxicity based on histological analysis, with a 4-fold decrease in serum bile acid concentrations and increased serum liver enzymes, compared to bile duct-ligated wild-type mice.⁴⁸ Although levels of the basolateral efflux transport protein Mrp3 were increased modestly in common bile duct-ligated mice compared to sham-operated controls,⁴⁹ in bile duct-ligated mice lacking Mrp3, serum and liver bile acid concentrations were similar compared to sham-operated wild-type controls.^{50,51} Furthermore, Mrp4 protein was increased more than 2-fold in bile duct-ligated wild-type mice while

Mrp3 protein was unchanged compared to sham-operated wild-type controls.⁴⁸ As an adaptive response to cholestasis, Mrp3 and Mrp4 are upregulated to efflux bile acids across the basolateral membrane, thereby reducing intracellular accumulation of bile acids in hepatocytes and subsequent toxicity due to the detergent effects on the cell membrane and resulting mitochondrial dysfunction.^{52,53} At lower seeding densities in sandwich-cultured mouse hepatocytes, Mrp4 may be upregulated as a compensatory mechanism to increase efflux of toxic bile acids; no change in Mrp3 protein was observed in sandwich-cultured mouse hepatocytes across extracellular matrix and days in culture (Figure 3).

Taurocholate accumulation in mouse hepatocytes cultured on both BC/MG and BC/GC supported the observation that Mrp4 protein decreased as a function of increased seeding density (Figure 4 and 5). Clearly, on day 4 of culture, cellular accumulation (white bars) of [³H]taurocholate increased from 93.6 to 121 pmol/mg protein in BC/MG, and from 49.2 to 83.0 pmol/mg protein in BC/GC in mouse hepatocytes seeded at 1.0×10^6 and 1.5×10^6 cells/well, respectively (Figure 5). This could not be attributed to differences in the primary bile acid transport proteins because total protein levels of Ntcp, Mdr1a/1b and Bsep were similar across seeding density and collagen matrix (Figure 4). However, Mrp4 was decreased ~3-fold when mouse hepatocytes were seeded at a density of 1.25×10^6 cells/well compared to 1.0×10^6 cells/well on BC/MG, and decreased ~8-fold when mouse hepatocytes were seeded at a density of 1.5×10^6 cells/well compared to 1.0×10^6 cells/well on BC/GC (Figure 4). Perhaps the increased Mrp4 expression at lower seeding densities resulted in redirection of some taurocholate excretion across the basolateral rather than the canalicular membrane, thus reducing the cellular accumulation of taurocholate compared to the higher seeding density in which Mrp4 expression was ~3- to 8-fold lower and cellular accumulation was higher (Table 1, Figures 3–5). Furthermore, the cellular accumulation of [³H]taurocholate in BC/MG was higher than BC/GC for all seeding densities, whereas Mrp4 protein was lower in BC/MG compared to BC/GC at a given seeding singular density.

The hepatocyte accumulation of [³H]taurocholate in sandwich-cultured mouse hepatocytes was increased when compared to sandwich-cultured rat and human hepatocytes. The cellular accumulation of taurocholate on day 3 following a 10-min incubation with 1 μ M [³H]taurocholate in sandwich-cultured mouse hepatocytes seeded at 1.5×10^6 cells/well was 187 ± 16 and 116 ± 3 pmol/mg protein on BC/MG

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and BC/GC, respectively (Figure 5). This was much greater than the reported cellular accumulation of taurocholate on day 4 following a 10-min incubation with 1 μM [^3H]taurocholate in sandwich-cultured rat hepatocytes on BC/MG and BC/GC ($<10^{44,54}$ and $\sim 50^{16}$ pmol/mg protein, respectively), or day 7 sandwich-cultured human hepatocytes (67.0 ± 25.0^{18} and 65.8 ± 11.3^{55} pmol/mg protein, both cultured on BC/MG). The *in vitro* intrinsic biliary clearance also was greater in mouse sandwich-cultured hepatocytes relative to rat and human sandwich-cultured hepatocytes. On day 3, the *in vitro* $\text{Cl}_{\text{biliary}}$ in mouse sandwich-cultured hepatocytes seeded at 1.5×10^6 cells/well on BC/MG and BC/GC, was 73.7 ± 24.6 and 96 ± 21.1 mL/min/kg, respectively, compared to 34.9 ± 12.0^{44} and $\sim 55^{16}$ mL/min/kg, respectively, in day 4 rat sandwich-cultured hepatocytes, and 15.8 ± 4.5^{18} and 15.2 ± 5.0^{55} mL/min/kg (both cultured on BC/MG only) in day 7 human sandwich-cultured hepatocytes. The differences in taurocholate accumulation and *in vitro* intrinsic biliary clearance are controlled by the transport proteins responsible for uptake, biliary excretion and basolateral efflux of bile acids. The observed differences are not due to the affinity of these hepatic transport proteins for taurocholate in mouse compared to rat and human hepatocytes because the K_m values for NTCP/Ntcp, OATPs/Oatps and BSEP/Bsep are similar between species.^{48,56–60} K_m values have been reported for human MRP4 (25.8 μM).²⁷ Therefore, the reason for greater taurocholate accumulation

and *in vitro* intrinsic biliary clearance in sandwich-cultured mouse hepatocytes relative to sandwich-cultured rat and human hepatocytes may be attributed, in part, to maintenance of Ntcp and decreased Mrp4 (Figure 4) protein when seeded at 1.5×10^6 cells/well. Alternatively, species-dependent differences in the taurocholate K_m for Mrp4 may exist.

Conclusion

In summary, extracellular matrix, seeding density and days in culture impact Mrp4 protein expression, bile canalicular network formation and taurocholate hepatobiliary disposition in sandwich-cultured primary mouse hepatocytes. Mouse hepatocytes seeded at densities of 1.25×10^6 cells/well or 1.0×10^6 cells/well, resulted in improved attachment, more extensive bile canalicular network formation and overall healthier hepatocytes. Total protein levels of Mrp3, Mrp2, Ntcp, Bcrp, Mdr1a/1b and Bsep were similar across seeding density and collagen matrix. Taurocholate accumulation was greater in BC/MG compared to BC/GC, and tended to increase as a function of seeding density for BC/GC. *In vitro* intrinsic biliary clearance of taurocholate was greatest at 1.5×10^6 cells/well. Hepatocellular accumulation and *in vitro* intrinsic biliary clearance of taurocholate were greater on day 3 compared to day 4. Primary mouse hepatocytes seeded in a BC/MG configuration at densities of 1.25×10^6 or 1.0×10^6 cells/well, and cultured for 3 days, yielded optimal transport based on the probes utilized in this study. This higher-throughput *in vitro* system could maximize the use of gene-disrupted mice from various strains to study hepatic function and to investigate the influence of gene disruption on hepatobiliary drug/metabolite disposition and hepatotoxicity.

Abbreviations Used

Ntcp, sodium taurocholate cotransporting polypeptide; Mrp 4, multidrug resistance protein 4; Mrp3, multidrug resistance protein 3; Ost, organic solute transporter; Bsep, bile salt export pump; Mrp2, multidrug resistance protein 2; Bcrp, breast cancer resistance protein; Mdr1a/1b, multidrug resistance P-glycoprotein; BEI, biliary excretion index; *in vitro* $\text{Cl}_{\text{biliary}}$, biliary clearance; DMEM, Dulbecco's modified Eagle's medium; CDFDA, 5-(and 6)-carboxy-2',7'-dichlorofluorescein diacetate; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; BCA, bicinchoninic acid; MG, Matrigel basement membrane matrix; GC, gelled rat tail collagen type I; PVDF, polyvinylidene difluoride; TBST, Tris-buffered saline with Tween 20.

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