Liver and Kidney on Chips: Microphysiological Models to Understand Transporter Function

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Because of complex cellular microenvironments of both the liver and kidneys, accurate modeling of transport function has remained a challenge, leaving a dire need for models that can faithfully recapitulate both the architecture and cell-cell interactions observed in vivo. The study of hepatic and renal transport function is a fundamental component of understanding the metabolic fate of drugs and xenobiotics; however, there are few in vitro systems conducive for these types of studies. For both the hepatic and renal systems, we provide an overview of the location and function of the most significant phase I/II/III (transporter) of enzymes, and then review current in vitro systems for the suitability of a transporter function study and provide details on microphysiological systems that lead the field in these investigations. Microphysiological modeling of the liver and kidneys using "organ-on-a-chip" technologies is rapidly advancing in transport function assessment and has emerged as a promising method to evaluate drug and xenobiotic metabolism. Future directions for the field are also discussed along with technical challenges encountered in complex multi-organ-on-chips development.

HEPATIC MICROPHYSIOLOGICAL SYSTEMS

Basic aspects of xenobiotic metabolism in the liver

The creation and maintenance of an in vitro microphysiological system that correctly mimics hepatic function to study drug and xenobiotic metabolism must include functional biotransformation enzymes and transporters. In this section, we review the primary hepatic phase I/II/III enzymes and transporters and survey hepatic in vitro systems with an emphasis on how well they have been used to study metabolism. Overall, there has been a trend for the development of more complex systems that utilize multiple cell types, physiologically relevant geometries, and microfluidics to capture the complex interactions within the liver. Together, these attributes create a favorable cellular microenvironment that allows for the expression and correct cellular orientation of the transporters.

The liver is the main organ in which xenobiotics and endogenous compounds are metabolized and excreted due to its physiological placement "downstream" from the gastrointestinal tract, high perfusion rate, large size, and high concentration of biotransformation enzymes.

To predict a compound’s metabolic fate, including detoxification or bioactivation to a toxic metabolite, it is very critical to understand both the biotransformation enzymes and transporters expressed in the liver. Because the liver receives nearly all of the blood perfusing the gastrointestinal tract, it is anatomically situated to be able to potentially remove xenobiotics (drugs and other chemicals foreign to the body) absorbed from the gut before reaching the systemic circulation—the hepatic first pass effect (Figure 1). To model a correctly functioning hepatic in vitro microphysiological system, it is imperative that the biotransformation enzymes are expressed and active in order to accurately study the parent compound and metabolite transporter dynamics.

Biotransformation enzymes are often called drug-metabolizing enzymes. These enzymes are involved in xenobiotic disposition via the processes of absorption, distribution, metabolism, and elimination and are classified as either phase I (generally oxidation, reduction, or hydrolysis reactions), phase II (generally conjugation or hydrolysis reactions), or phase III (transmembrane transporters), based on their metabolic function.

Generally, phase I enzymes are responsible for catalyzing the hydrolysis, reduction, and oxidation of xenobiotics. Among the phase I enzymes, cytochromes P450 (CYPs; detailed below) are the largest and most important superfamily: CYP3A4, CYP2D, and CYP2C subfamilies are responsible for 50%, 25%, and 20% of the biotransformation of all drugs, respectively.¹ Phase II enzymes can transfer a functional group to xenobiotics—which includes acetylation, methylation, glutathione conjugation, sulfate conjugation, and glucuronidation.

When xenobiotics are absorbed into hepatocytes from the portal vein and hepatic artery, transporters in the sinusoidal (basolateral) membrane of hepatocytes assist xenobiotics to enter hepatocytes for later biotransformation.

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The most abundant phase I/II enzymes and transporters expressed in the human liver are listed in Supplemental Table S1. One result of absorption, distribution, metabolism, and elimination is to convert toxic xenobiotics into nontoxic, water-soluble compounds that can easily be eliminated—a process called detoxification or inactivation. However, in certain cases, phase I and/or phase II enzymes can transform xenobiotics into more toxic chemicals—a process called bioactivation. The concept of bioactivation has been adapted to the pharmacological idea of the prodrug, in which the administered parent molecule has little or no pharmacological activity but one or more metabolites act as the major contributor to the desired pharmacological response. For example, codeine is a morphine prodrug that requires oxidative demethylation by CYP2D6 to form morphine to achieve its pharmacological activity.

PHASE I ENZYMES OF PHARMACOLOGICAL AND TOXICOLOGICAL SIGNIFICANCE

Hydrolysis
There are multiple gene products with a wide variety of potential substrates, including: carboxylesterase; alkaline phosphatase; dipeptidyl peptidase-4; epoxide hydrolases (microsomal epoxide hydrolase and cytosolic epoxide hydrolase); and paraoxonase (paraoxonase-1, paraoxonase-2, and paraoxonase-3).

Reduction
NAD(P)H-quinone oxidoreductases (NQO1 and NQO2), aldo-keto reductases, carbonyl reductase, CYP b5/NADH-cytochrome b5, nicotinamide adenine dinucleotide phosphate: P450 reductase, aldehyde oxidase.

Oxidation
Examples of enzymes involved in xenobiotic oxidation include: aldehyde dehydrogenases 1 and 2, alcohol dehydrogenases-1, aldehyde oxidase, xanthine oxidase, monoamine oxidase, peroxidase-glutathione peroxidase, flavin-dependent-monoxygenase 3, 4, and 5, and Cytochromes P450. CYPs are generally considered to be the most important group of oxidative enzymes involved in phase I biotransformation of xenobiotics. There are 56 different human CYP genes, but many are involved only in endogenous metabolic processes. Major human forms involved in xenobiotic biotransformation are: CYP1A1, IA2, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 3A4, 3A5, 3A7, and 4F3.

Phase II enzymes: conjugation
UDP-Glucuronosyltransferase. There are two major UDP-Glucuronosyltransferase (UGT) families involved in xenobiotic conjugation, UGT1 and UGT2. Within the UGT1 family are multiple gene variants that give rise to 10 (UGT1A1–1A10) distinct gene products. The UGT2 family has two subfamilies, UGT2A (3 members) and UGT2B (4 members). All UGT enzymes use uridine diphosphoglucuronic acid as the cofactor.

Sulfotransferases (SULTs) are too multigene families of enzymes, with SULT1A, 1B, 1E, and 2A1 involved in xenobiotic conjugation. All SULT enzymes use 3′ phosphoadenosine-5′ phosphosulfate as the cofactor. Glutathione S-transferases (GSTs) are also involved in xenobiotic conjugation, with approximately 15 different human genes, in five classes (GSTA1-5, GSTM1-5, GSTT1 and 2, GSTO1 GSTS1, and GSTZ1). In addition, GSTs use the tripeptide, glutathione, as a cofactor. Other important conjugation reactions include N-acetyltransferase (NATs; NAT1 and NAT2), with the cofactor, acetyl coenzyme A, and several methyltransferases with different substrate specificities (O-methyltransferase, N-methyltransferase, and S-methyltransferase).

Basolateral uptake transporters
These transporters mediate the hepatic uptake of xenobiotics and endogenous substances, such as bile acid and cholesterol. These uptake transporters belong to a multigene family of solute carriers (SLCs). In humans, the main uptake transporters are organic anion-transporting peptides (OATPs; OATP1A2, 1B1, 1B3, 2A1, and 2B1); the sodium-dependent taurocholate co-transporting protein (Na+-taurocholate co-transporting polypeptide [NTCP]); and the organic cation transporters (OCTs; OCT1 and OCT2). Many of these carrier proteins can transport...
substrates bidirectionally, depending on the substrate concentration gradient.

OATPs are integral membrane proteins with 12 transmembrane helices and are the main drug carrier proteins supporting the sodium-independent hepatic uptake of organic anions. Because of the prominent expression of OATPs on the basolateral membrane of hepatocytes, OATPs are responsible for a critical mechanism of chemical uptake into the liver, including a variety of substrates containing steroidal or peptide structural backbones and/or anionic or cationic chemicals. For example, OATP1A2 is associated with the uptake of sulfobromophthalein, BQ-123, [d-Pen², d-Pen⁵]-enkephalin, fexofenadine, levofloxacin, ouabain, and methotrexate. OATP1B1 and OATP1B3 are the OATP1B isoforms expressed in human livers and can transport bilirubin and its glucuronide conjugates. OATP1B1 not only transports various statin drugs, but can also carry thyroxine, tauroursodeoxycholate, glycochenodeoxycholate, and glycocholate. In addition, BSEP can transport pharmaceuticals, such as pravastatin (reviewed by ref. 7).

BCRP transports a highly diverse range of hydrophobic substrates, including chemotherapeutic agents, such as mitoxantrone, methotrexate, topotecan, and irinotecan. BCRP can also transport hydrophilic-conjugated organic anions, particularly sulfated conjugates with high affinity (reviewed by ref. 13).

Genetic defects or secondary consequences of hepatobiliary obstruction or destruction can cause cholestasis, which are often involved in impaired function or a sustained inhibition of these apical efflux transporters. Inherited mutations in the human MDR3 gene can cause progressive familial intrahepatic cholestasis type-3, a rare disease characterized by an early onset of cholestasis that leads to cirrhosis and liver failure before adulthood. In addition, mutations in BSEP are responsible for patients with progressive familial intrahepatic cholestasis type-2 with high serum bile acid concentrations and low biliary bile acid but normal serum γ-glutamyltranspeptidase activity and cholesterol.

Basolateral efflux transporters

Removal of endogenous and xenobiotic chemicals from hepatocytes to sinusoidal blood is mediated by transporters on the basolateral side, including MRP3, MRP4, and organic solute and steroid transporter, Ost alpha-Ost beta (OSTα/β).

MRP3 has a high affinity for glucuronide conjugates which are involved in detoxification and excretion of polar chemicals that have undergone the process of glucuronidation, including morphine-3-glucuronide, bilirubin-glucuronide, etoposide-glucuronide, and acetaminophen-glucuronide. It is suggested that MRP3 has a defense-related function and contributes to the excretion of toxic anions, as expression is upregulated during hepatic injury, such as cholestasis as it is associated with bile acid homeostasis in spite of low affinity for bile acids (reviewed in ref. 7).

MRP4 has a wide range of substrates, including antiviral agents (azidothymidin, adefovir, and ganciclovir), anticancer agents (methotrexate, 6-mercaptopurin, and camptothecins), and cardiovascular agents (loop diuretics, thiazides, and angiotensin II receptor antagonists), as well as endogenous chemicals (steroid hormones, prostaglandins, bile acids, and the cyclic nucleotides cAMP and cGMP). MRP4 has higher affinity for sulfate conjugates of bile acids and steroids. MRP4 is similar to MRP3, as both are upregulated in cholestasis, suggesting a protective role in preventing hepatotoxicity. Furthermore, MRP4-null mice developed cholestasis after bile duct ligation, implying that MRP4 is important in bile acid homeostasis (review by ref. 7).

OSTα/β proteins are present as heterodimers and/or hetero-multimers in the cell membrane. OSTα/β-mediated transport is bidirectional (uptake or efflux) and ATP-independent, depending on the electrochemical gradient. Although it is expressed in high levels in the liver, OSTα/β is also expressed widely in the small intestines, colon, kidneys, testes, ovaries, and adrenal gland, the latter of which is involved in steroid and bile acid homeostasis. The evidence from a study with Ost alpha null mice...
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ATP, adenosine triphosphate; BAL, bioartificial liver; BSEP, bile salt export pump; CMFDA, carboxy-2’, 7’-dichlorofluorescein diacetate; CYP, cytochrome; EC, endothelial cells; EH, epoxide hydrolase; ELISA, enzyme-linked immunosorbent assay; FMO, flavin-dependent monooxygenase; GM-CSF, granulocyte-macrophage colony-stimulating factor; GSH, glutathione; GST, glutathione S-transferase; HC, hepatocyte cells; HSC, hepatic stellate cell; IHC, immunohistochemical; II, interleukin; LC-MS/MS, liquid chromatography tandem mass spectrometry; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAO, monoamine oxidase; MDR, multidrug resistant; MRP, multidrug resistance-associated protein; MTT, methythiazol tetrazolium; NATs, N-acetyltransferases; NPC, nonparenchymal cell; NTCP, Na+-taurocholate co-transporting polypeptide; OCT, organic cation transporter; ROS, reactive oxygen species; SQL-SAL, sequentially layered, self-assembly liver; SULT, sulfotransferase; TNF, tumor necrosis factor; UGT, UDP-glucuronosyltransferase; XO, Xanthine oxidase (XO).
demonstrated OSTα/β as a target for interrupting the enterohepatic circulation of bile acids. OSTα/β substrates include steroid hormones and endogenous compounds, such as estrone sulfate and dehydroepiandrosterone sulfate, bile acids, and PGE2, as well as the cardiac glycoside digoxin (reviewed by ref. 16).

The study of hepatic transporter function has relied on (1) in vitro/ex vivo hepatocytes in suspension or two-dimensional (2D) plated monolayer cell formats for uptake and/or accumulation efflux assays using radioactive or fluorescent probe substrates, and (2) in vivo pharmacokinetic studies on mutant animals with deficiencies in specific transporter genes or transporter gene knock-out mice (reviewed by ref. 17). There are several in vitro methods used in assessing human drug metabolism and active transport of the drug, including the use of immortalized cell lines with transient or stable overexpression of transporters. In vitro transporter assays can help determine whether a compound is taken up at the sinusoidal surface by hepatocytes and/or whether its metabolites can be eliminated at the canalicular membrane for biliary excretion. Uptake and inhibition assays often involve OATP1B1, OATP2B1, and OATP1B3, and biliary efflux assays may include MDR1, MRP2, and BCRP, which can be used to predict compound disposition. The BSEP inhibition assay can be applied to screen whether the compound can lead to cholestasis or hyperbilirubinemia (reviewed by ref. 18).

In some instances, preclinical in vitro cell model systems poorly predict biotransformation and elimination in humans. First, extrapolation from in vitro findings to the in vivo situation remains complex with poor in vitro-to-in vivo correlation. Additionally expression levels of phase I/II enzymes and transporter in transformed cell lines, such as HepG2 (human hepatoma cells), are very low and variable (reviewed by ref. 19). OCT1 and OATP1B1 mRNA were abundantly expressed in human liver tissue, whereas these two transporters were expressed at low levels in HepG2 cells.20 Thus, transporter expression in HepG2 did not match the tissue expression pattern. Furthermore, due to overlapping substrate specificities and lack of selectivity with currently available inhibitors, it is challenging to fully understand the role of a given transporter in the disposition of specific drugs. Even though in vivo pharmacokinetic studies can provide integrated analysis of a drug’s disposition, the preclinical results from transgenic or mutant animal models sometimes fail to predict the clinical outcomes. Expression profiles of transporters in laboratory animals, such as rats and mice, are different from humans. For example, rodent mdr1a and mdr1b genes are correlated to the MDR1 gene in humans despite functional studies showing MDR1, mdr1a, and mdr1b expressing cells demonstrating substrates for rodent mdr1a and mdr1b are unlikely to be substrates for human MDR1, showing species-dependency in the spectrum of drug efflux activity.21

We review in vitro/ex vivo human hepatic cell systems used in biotransformation and transporter studies from traditional assays to the most recently advanced three-dimension (3D) cultures, including microsomes, cell lines cultured in 2D, primary hepatocyte suspensions, liver slices, sandwich cultures, 3D culture systems, and microphysiological system (MPS) culture systems (Figure 2 and Table 1).

Human liver subcellular fractions, microsome/supersomes, cytosol fractions, and S9 fractions
These subcellular fractions contain CYPs, UGTs or NATs, SULTs, and GSTs, which are useful for xenobiotic biotransformation research. These assays are traditionally used for in vitro-based predictions of metabolic clearance and drug-drug interactions. However, due to the loss of structural integrity of the cell, and the optimization of enzyme kinetic conditions by adding cofactors, such as nicotinamide adenine dinucleotide phosphate and 3’ phosphoadenosine-5’ phosphosulfate that are at concentrations irrelevant to physiological conditions, the results using these methods cannot be accurately used for transporter studies and quantitative estimations of in vivo human biotransformation.

Cell lines cultured in 2D
The HepG2 cell line is the most frequently used and best characterized immortalized human hepatoma cell line. However, compared with primary human hepatocytes, overall CYP activity remains low.22 Expression profile of transporters in HepG2 cells is not highly correlated to human liver tissue so the HepG2 cell line is not a suitable model for human transport assays. In general, 2D culture conditions cannot provide the optimal microenvironment for cells to establish polarization; thus, the use of 2D cell cultures has architectural limitations in transporter assays.

A new human liver cell line derived from a hepatocellular carcinoma (HepaRG) recently drew substantial attention in the field of pharmaceutics and toxicology. Differentiated HepaRG cells expressed high levels of phase I/II enzymes, including transporter levels comparable to freshly isolated human hepatocytes.22 HepaRG cells can maintain a proliferative state in undifferentiated culture medium for several weeks and can differentiate into hepatocytes and biliary epithelial cells by adding differentiation culture medium after reaching confluence.22

Primary human hepatocytes suspension and cultured in 2D
Primary human hepatocyte cultures are a preferred in vitro system for predicting in vivo drug biotransformation and clearance as they maintain critical metabolic features. After isolation by collagenase perfusion, primary human hepatocytes in suspension are viable for only a few hours thus they are immediately used as rodent models for kinetic characterization of transporter function. However, this rapid use and characterization is generally not possible for human hepatocytes. Thus, studies with human hepatocytes rely on establishing primary cultures. Once plated in a monolayer culture, human primary hepatocytes maintain suitable viability for several days. However, they usually lose cell-specific functions, such as albumin production and CYP expression, as both decline quickly over the first 24–48 hours of culture when the cells lose their differentiation status. Due to the scarcity of available human liver tissue and successful cryopreservation techniques, a good supply of human primary hepatocytes has become commercially available. In culture, previously cryopreserved hepatocytes can recover and maintain phase I/II enzyme activity after thawing for at least 7 days.23 Individual donor variation in metabolic enzyme activity due to genetic polymorphisms and other factors can be compensated for by the mixing of...
hepatocytes from multiple donors to generate homogeneous enzyme activities. Hepatocytes represent the majority of the hepatic cellular mass (about 80%), whereas other nonparenchymal cells (NPCs), including vascular and biliary epithelial cells (i.e., cholangiocytes), Kupffer cells, and hepatic stellate cells, provide key physiological functions. For example, cholangiocytes not only can contribute to bile secretion, but also can enable the absorption of ions, bile acids, amino acids, glucose, and other molecules, playing an important role in the modification of hepatic canalicular bile (reviewed by ref. 24).

In vivo, stellate and Kupffer cells have been shown to play an important role in the hepatoxicity of some compounds, and, thus, the absence NPCs in primary culture systems is a potentially serious limitation for toxicology studies.

**Precision-cut liver slices**

Precision-cut liver slices have several advantages for drug biotransformation and toxicology studies as they maintain the native liver structure with multiple cell types and zonation, and have good *in vitro-in vivo* correlations of drug biotransformation features. Cultured liver slices can retain phase II enzyme activity, albumin production, and gluconeogenesis for up to 20–96 hours, while regulating gene expression of the uptake transporters, NTCP, OATP, and efflux transporters, BSEP, MDR1, and MRP2.25

Despite the preservation of the overall hepatic architecture, drug biotransformation and intrinsic clearance rates are lower than isolated hepatocytes as necrosis can occur after 48–72 hours whereas CYP activities are greatly reduce within 6–72 hours.26

**Sandwich culture**

Sandwich cultures with primary human hepatocytes plated between two layers of extracellular matrix (ECM; collagen or Matrigel, derived from Engelbreth-Holm-Swarm sarcoma) were developed to maintain liver-specific functions over longer culture periods. The use of ECM overlays allows for a favorable cellular attachment environment and is thought to be one of the main reasons why cells polarize in this type of culture. Hepatocyte sandwich cultures with various medium constituents have been shown to maintain albumin secretion, viability, and cuboidal-shape morphology with phase I/II/transporter expression similar to that of liver tissue.27 Biliary excretion can also be evaluated in sandwich cultures with both basal and inducible biliary enzyme activities that allow assessments of hepatobiliary disposition. Taken together, sandwich cultures can provide a robust means to evaluate hepatic compound uptake, metabolism, efflux and biliary excretion, while closely mimicking *in vivo* characteristics. Compared with other 2D models, sandwich cultures have significant advantages and can be considered as a bridge between 2D and 3D cultures technologies (reviewed by ref. 28).

**Transwell culture for drug efflux**

Evaluation of the human hepatocyte uptake and efflux transporters MDR and MRP2 have been performed in transwell systems with transfected cell lines, including porcine kidney epithelial cells and Mad-Darby canine kidney (MDCK) epithelial cells. Transwell culture cells are grown on a permeable membrane filter that allows for the physical separation of the apical and basolateral domains and has been used to study drug uptake and efflux transporter activity.29

**Co-culture systems, 3D culture systems, and MPS culture systems**

Traditional *in vitro* methods, such as microsomes and suspension cultures, usually have too short of a time window to perform essential disposition assays, which can lead to imprecisions in the prediction of human biotransformation/clearance and toxicity. In order to improve the predictability of drug safety and efficacy in clinical development, and to have a clearer perspective of toxicity outcomes...
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BSA, bovine serum albumin; CdCl2, cadmium chloride; ciPTEC, conditionally immortalizing proximal tubule epithelial cell; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; GSH, glutathione; HFM, hollow fiber membrane; HK, human kidney; hKDC, human kidney-derived cell; HO, heme oxygenase; KIM, kidney injury molecule; LDH, lactate dehydrogenase; MDCK, Madine-Darby canine kidney; MDR, multidrug resistant; N/A, not applicable; OAT, organic anion transporter; OCT, organic cation transporter; OH, hydroxy; PDMS, polydimethyl siloxane; qPCR, quantitative polymerase chain reaction; SIS, small intestinal submucosa; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.
while reducing the use of animals for toxicity studies, recent research efforts have been focusing on development of advanced in vitro models based on the applications of co-culture systems, 3D cultures, and MPS cultures that utilize microfluidic flow, often are referred to as “organ-on-chips” or “organoid culture.”

The liver is a complex organ, consisting of hepatocytes, NPCs, and various ECMs. NPCs play an important role in hepatic physiological functions as well as hepatotoxicity. Many studies have demonstrated that co-cultures of hepatocytes with NPCs can sustain liver-specific function, morphology, and expression of liver-specific transcription factors via the activation of cell adhesion molecules and redistribution of cytoskeleton involved in cell-cell and cell-matrix interactions (reviewed in ref. 31). Several liver organoid cultures based on the application of co-culture have been reported, and some have been commercialized. These include advanced 3D culture systems based on cellular microenvironment dynamics between ECMs, microperfusion flow rates, and co-cultures of various cell types. The MPS model represents an interconnected set of cellular constructs designed to recapitulate the structure and function of human organs. Here, we review current advanced hepatic culture systems (Table 1).

HepatoPac is a co-culture system of human hepatocytes with mouse fibroblasts (3T3-J2 fibroblast), commercially available through Hepregen (Medford, MA). This system consists of micropatterned hepatocyte islands surrounded and stabilized by stromal cells in a 24-well plate format. This culture system can maintain liver-specific function for up to 6 weeks, including stable albumin secretion, urea synthesis, phase I/II drug biotransformation, and formation of bile canaliculi with efflux transporters (reviewed by ref. 32). Studies with the human hepatic HepatoPac platform have demonstrated an in vitro-in vivo correlation with hepatic uptake of faldaprevir by multiple transporters (including OATP1B1 and Na+/K+-dependent transporters) and biotransformation by CYP3A4.

RegeneMed 3D Liver (San Diego, CA) is a liver tissue coculture system used for screening hepatic absorption, distribution, metabolism, and elimination, using the transwell culture approach. NPCs are seeded in a nylon screen sandwich mesh insert with a 140-μm pore size and stabilized for a week, followed by incubation with hepatocytes to form a 3D liver tissue. Liver-specific functions, including production of albumin, fibrinogen, transferrin, and urea, can be maintained up to 3 months, and the induction of CYP1A1, 2C9, and 3A4 activity for up to 2 months. Co-culture with Kupffer cells allows for study of the inflammatory response as the release of proinflammatory cytokines can be observed with lipopolysaccharide (LPS) exposures. Basolateral transport-mediated drug uptake activity using [3H]-labeled estrone-3-sulphate as the tracer has been demonstrated in this system. Although images of bile canaliculi-like structures were presented, efflux transporter activity or bile excretion were not reported.

The 3D microtissue spheroid culture, 3D InSight, provided by InSpherio (Schieren, Switzerland), is a hanging drop co-culture system that uses gravity-forced cellular self-assembly of hepatocytes and NPCs into spheroids using a 96-well format. This format is well suited for high throughput applications and stable viability and liver-specific function, such as persistent albumin secretion, are preserved over 5 weeks. MDR1 and BSEP are expressed in this microtissue, evidence that these cultures exhibit cell polarization and bile canaliculi formation. Inflammation-mediated toxicity and chronic toxicity assays with acetaminophen and diclofenac have also been evaluated in this system. However, to date, there are no published data that demonstrate that this system can be applied to transporter assays.

Organovo (San Diego, CA) uses a 3D bioprinting technique to generate small-scale hepatic tissues using human primary cells in a platform called exVive3D Liver. This product can maintain stable viability and albumin secretion for up to 4 weeks and has rifampicin-inducible CYP3A4 activity, as midazolam biotransformation to 1-hydroxy-midazolam was increased by rifampicin pretreatment. As Kupffer cells are also present, this system can respond to immune stimulation (LPS) with the release of proinflammatory cytokines.

The 3D liver bioreactors designed by the Charité Universitätspolitische medizin Berlin are derived from bioartificial livers used in the clinic. This hollow-fiber and perfusion-based bioreactor provides a continuous mass exchange of culture media and controlled oxygenation with a scaffold for cells to maintain a physiologically relevant environment. This 3D bioreactor is co-cultured with hepatocytes and NPCs and can maintain albumin secretion and CYP activity (CYP1A, CYP2C9, and CYP3A4) for up to 2–3 weeks, as well as expression of canalicular transporters (MRP2, MDR1, and BCRP). Limitations of this system for use in pharmacokinetic and drug toxicity testing include a lack of zonation seen in liver tissues and low throughput, as only one condition can be evaluated per system.

The HiREL microdevice, an MPS platform provided by Hurel (Beverly Hills, CA), is an integrated and microfluidic system that assembles multiple units of microfluidic microscale cell culture analogs (μCCA) cultured from hepatic tissue or other organ tissues in parallel. The Hurel plastic biochips are connected to a fluid reservoir and pump system interconnected with a complex set of tubing that serves to recirculate the media. The hepatic co-culture system with NPCs forms a 2D monolayer and maintains high viability for up to 9 days, with higher expression of CYPs, SULT, and UGT, and in vivo-like hepatic clearance of diclofenac, indomethacin, and coumarin, compared with traditional static culture conditions. The formation of the bile canaliculi network was visualized by using carboxy-2’, 7’-dichlorofluorescein diacetate, a fluorescent substrate of BSEP. However, there was no evidence to demonstrate that this system can maintain other hepatic functions, such as albumin secretion, urea excretion, or transporter activity.

A multiple-well plate platform, LiverChip, by CN Bio Innovations (Hertfordshire, UK) uses a flow system driven by a pneumatic pump at the bottom of the plate. This system was designed to recapitulate the hepatic microenvironment in terms of fluid flow, oxygen gradient, and shear stress (145 μM to 50 μM at a flow of 0.25 mL/min). Compared with 2D static conditions, hepatocytes cultured in this system can maintain CYP activity (1A2, 2B6, 2C9, 2D6, and 3A4), albumin secretion, expression of phase II-UGT enzymes and transporters (MDR2, MRP2, and BCRP) for up to 7 days. Hepatocytes co-cultured with liver...
sinusoidal endothelial cells enriched NPC fractions can maintain high viability, CYP activity, albumin secretion, and urea excretion for up to 13 days.40 Kupffer cells incorporated in the model can release proinflammatory cytokines by LPS stimulation. The intrinsic clearance values of hydrocortisone and its metabolites generated in this system correlated well with human data, demonstrating that this system has great potential for high-throughput use in hepatic metabolic research.41

CellAsic (Hayward, CA) has a microfluidic liver sinusoid model with a microporous endothelial-like barrier that mimics liver sinusoids. This platform utilizes a 96-well plate format containing 32 small units that utilized a perfusion system with a flow of 10–20 nL/min and ~250 cells in each unit. Hepatocytes cultured in this system can maintain high viability for up to 7 days and respond to drugs.42 Other liver-specific functions and the characterization of transporters need to be further analyzed.

Vernetti et al.43 developed a human, 3D, microfluidic, four-cell, sequentially layered, self-assembly liver model based on an MPS device platform by Nortis (Woodinville, WA). The current sequentially layered, self-assembly liver model uses a co-culture of primary human hepatocytes along with human endothelial (EA.hy926), immune (U937), and stellate (LX-2) cells in physiological relevant ratios that are viable and functional for at least 28 days under continuous flow. This model can maintain canaliculi structure, phase I/II activity, albumin production, and urea excretion. In addition, by the integration of protein-based fluorescence biosensors, the system can be used in reporting drug-induced mechanistic toxicity, such as apoptosis and reactive oxygen species for high throughput screening (reviewed by ref. 33).

The above-described 3D or liver-on-a-chip platforms generally provide suitable microenvironments for recapitulating most in vivo hepatic functions compared with traditional 2D hepatocyte cultures. However, these state-of-the-art in vitro/ex vivo technologies still have limitations in accurately predicting human hepatotoxicity and first pass drug clearance. Further development of advanced 3D hepatic culture systems needs to consider the complexity of the liver, including the co-culture ratio between hepatocytes and NPCs, the source of hepatocytes, and zonation effects. Because the liver has a wide range of diverse functions, hepatic cells show large heterogeneity and plasticity of functions. Oxygen gradients, hormones, and ECM all can regulate zonal variations, which are reflected in drug biotransformation capabilities. It would be interesting if these zonation characteristics could be established and sustained on the 3D liver-on-chip systems.44

Although these 3D models have great potential in pharmacokinetic prediction of first pass drug clearance, additional “proof of concept” studies are needed to validate in vitro-to-in vivo correlations using selected clinical drugs. In addition, most current models are individual liver organ systems, lacking the gastrointestinal and/or renal transport/metabolism modules, which are needed to understand the whole profile of drug absorption, distribution, metabolism, and elimination in vivo.

Renal nephron microenvironment–proximal tubule
The status of renal in vitro microphysiological systems to study transporter function is not as well developed as the liver. As with the liver section, we review renal transporters in the proximal tubule and in vitro renal systems that have been used to study their function. Knowledge of the function of renal transporters in the proximal tubule is important for understanding the crucial role this organ plays in drug disposition. Demonstration that these transporters are functional in an in vitro renal system is critical for method validation and proper data interpretation.

The nephron is the functional unit of the kidney responsible for maintaining both the homeostasis of electrolytes as well as fluid volume.45 However, from a drug-development perspective the nephron is the primary site of renal handling involving any combination of filtration and phase III mediated-secretion/ reabsorption. Structurally, the nephron is a series of segmented tubules that individually play a critical role in its overall function. The proximal tubule, found in juxtaposition to the glomerulus, has been shown to be the primary site of transport-mediated reabsorption/secretion xenobiotics. Active vectorial transport of xenobiotics are achieved given the polarized configuration of the proximal tubule epithelial cells involving both transporters found on the brush-border containing apical side (facing urine) and the basolateral side (facing systemic circulation; Figure 1).

Basolateral transporters
The SLC transporters are the major family of multispecific transporters that mediate the proximal tubule uptake of both xenobiotic and endogenous substrates that are circulating within the blood. SLC transporters include the organic anion transporters (OATs; OAT1, OAT2, and OAT3), OCTs (OCT2 and OCT3), and OATP4C1.

Apical efflux transporters
The SLC transporters are also found on the apical side of the proximal tubule and are responsible for the removal of exogenous and endogenous substrates that have accumulated within the cell via diffusion, reabsorption, and/or uptake from the circulatory side. SLC transporters found on the apical side of the proximal tubule include the OAT4, multidrug and toxin extrusion (MATE) protein (1 and 2-K), urate anion exchanger 1, and OCTN1/2. Additionally, another superfamily of multispecific transporters found on the apical side includes the ABC transporters, which use ATP hydrolysis to drive molecules across cell membranes. The ABC transporters on the apical side include MDR1 or P-glycoprotein, BCRP, and MRP2/4.

Proximal tubule transport model–historical perspective and current status
In recent studies, there have been vast improvements of the modeling capabilities of xenobiotic transport using models and cell types that accurately recapitulate human physiology. Traditionally, researchers have used established immortalized cell lines from animals, including canine (MDCK), opossum, and porcine cells (LLC-PK1), as a cell source to optimize models.46–48 Transwell studies, using cells grown in monolayers on a permeable scaffold, are utilized to investigate the facilitated transport from one compartment to the other resembling the apical-basolateral relationship seen in vivo.49
Although established immortalized cell lines have remained as the gold standard for *in vitro* transport studies, there are drawbacks associated with their use, particularly the poor expression and/or absence of human-specific transporters.

To combat this problem, investigators have used molecular techniques to transf ect the established animal cell lines with specific human transporters. Additionally, investigators have immortalized primary renal cortex cells from human kidneys (HK-2) and have shown that not only do they retain proximal tubule cell phenotypes, but they also possess the functional aspect of transport and sensitivity to toxin. However, as demands increase to resemble the *in vivo* phenotype to its highest extent, there are shortcomings with the immortalized human cell lines that research groups have addressed. As models continue to become more advanced, there has been a widespread shift from immortalized cells to primary renal cells to more accurately represent the native *in vivo* microenvironment. Brown *et al.* has shown, using primary human proximal tubule cells grown on permeable filter supports, differentiated cells expressing a wide array of transporters as well as functional transport activity of OAT1/3, MRP2/4, OCT2, MDRI, and MRP2.

**Engineered transport modeling of the proximal tubule**

The utility of bioengineered models to replicate function of the kidney proximal tubule is summarized in Table 2. These have ranged from a macroscale designed for clinical use in patients with kidney failure to microphysiological scales designed to study biochemical and toxicological processes.

**Bioartificial renal tubule assist device**

Documented as one of the earliest systems to effectively model the microenvironment of the kidney, researchers developed the bioartificial tubule renal assist device (RAD) with the perspective of improving renal replacement therapies and outcomes of patients suffering from endstage renal disease. The RAD is described as a perfusion bioreactor system in which cells are grown in a monolayer on a permeable synthetic hollow-fiber membrane (270 μm inner diameter, 35 μm wall thickness, and 2.5 cm length). Earlier developments used MDCK cells as the cell source for system validations in which functional confluence and fluid transport was demonstrated using radiolabeled inulin. MacKay *et al.* observed >98.9% recovery of perfused radiolabeled inulin. The multilayer microfluidic device provided a fluidic proximal tubule-on-a-chip, combining a hemofiltration cartridge which resembles an efficient metabolic replacement of kidney function in both animals as well as a patient population with acute renal failure. The RAD system has not only served as a translational therapy targeted against renal failure but a foundational model when approaching recapitulation of the functional aspect of the proximal tubule microenvironment.

**Human kidney proximal tubule-on-a-chip**

Successful mimicry of the renal microenvironment is a complex undertaking given the 3D architecture of the proximal tubule and the fluidic environment to which it is exposed. A number of research groups have developed multilayer systems using polydimethyl siloxane scaffolds in combination with a porous membrane to which cells can culture in 3D. Jang and Suh developed their multilayer microfluidic device using two compartments (one static chamber and one flow chamber) separated by a porous membrane to which rat renal tubule cells were cultured and polarized on. The multilayer microfluidic device provided a fluidic microenvironment (fluid shear-stress of 1 dyne/cm²) similar to what has been observed in the nephron, which has been proposed to being a key component in tubule cell cytoskeletal reorganization and remodeling of functional complexes.

Leading the organ-on-chips focus at the Wyss Institute, the Ingber Laboratory has improved upon the multilayer microfluidic device by microfabricating a polydimethyl siloxane microfluidic device containing both an interstitial fluidic compartment and luminal flow channel (fluid shear-stress 0.2 dyne/cm²) separated by a porous membrane coated with extracellular matrix protein, collagen type IV. Using primary human kidney proximal tubule epithelial cells cultured within the device, the fluidic proximal tubule microenvironment was demonstrated by the presence of tight junction protein, ZO-1, as well as basolateral distribution of Na/K-ATPase and cytoplasmic expression of aquaporin 1.
Furthermore, acetylated tubulin staining was used to visualize primary cilia, a fluid shear stress mechanosensor important for regulation of tubular morphology. Functionally, the microfluidic device was used to evaluate albumin uptake, cellular alkaline phosphatase activity, and transepithelial glucose transport. Jang and Suh observed, in response to static cultures, a significant uptake of fluorescein isothiocyanate (FITC)-albumin, a significant level of alkaline phosphatase activity, and significant levels of glucose transported by fluidic cultures of proximal tubule epithelial cells. Additionally, the microfluidic device was used as a model of nephrotoxicity using the prototypical nephrotoxin, cisplatin (100 μM), in the absence and presence of cisplatin, which has been previously shown to suppress cisplatin-induced injury. Using lactate dehydrogenase (LDH) release and apoptosis, via annexin V staining and a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling assay, significant cisplatin-induced cellular injury was observed in static cultures compared with fluidic cultures as well as a significant decrease in injury when cisplatin was co-administered with cisplatin. Finally, to assess the transport capabilities of the microfluidic device, cellular accumulation of calcine-AM was evaluated in the presence and absence of the P-glycoprotein ATP-binding cassette membrane transporter inhibitor, verapamil (100 μM). Static cultures showed higher accumulation of calcine-AM (presence/absence of verapamil) in comparison with fluidic cultures lending evidence to the concept of the fluidic microenvironment enhancing transporter expression and improving the phenotype of the proximal tubule cells.

Using unique conditionally immortalizing proximal tubule epithelial cells (ciPTECs) previously characterized by their laboratory group, Jansen et al. functionally tested ciPTECs grown on hollow fiber membranes (HFMs) using microfluidics. Upon maturation of ciPTECs within the HFM, collagen IV ECM was visualized using immunocytochemistry lending evidence to renal lineage. Additionally, using FITC-inulin, transepithelial barrier function was significantly greater (and caused less FITC-inulin leakage) for cells containing HFM in comparison to cells with empty HFM. Morphologically, the mature cultured ciPTECs displayed well-developed organelles as well as cell-surface microvilli, a typical marker of the proximal tubule epithelial cell. To assess the polarity of the ciPTECs cultured in the HFM, confocal microscopy was implored using immunocytochemistry to observe the high expression of tight junction protein, ZO-1, and correct localization of basolateral transport protein, OCT2. Furthermore, OCT2 transport activity was measured using real-time fluorescent 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+) uptake in the absence and presence of OCT2 inhibitors, cimetidine (100 μM) and uremic toxin mix, showing significant inhibition of OCT2-mediated uptake.

Additional models of the human renal microenvironment

There are a number of groups currently developing outstanding models of the renal proximal tubule model that faithfully recapitulate the microenvironment, but have yet to assess the transport capabilities. Hoppensack et al. used highly proliferative human kidney-derived cells cultured in monolayers on small intestinal submucosa to show the correct phenotype of the proximal tubule using immunohistochemical staining as well as detecting basement membrane proteins and microvilli. Finesilver et al. prepared kidney micro-scaffolds and cultured HK-2 cells within the microstructures in comparison with traditionally cultured HK-2 cells. Cells grown within the kidney micro-scaffolds for 25 days showed a significant (>twofold) increase in expression of genes AQP-1, ATP1B1, LRP-2, CCL-2, SLC23A1, and SLC5A2 (except for 21–24 days). Additionally, the research group showed less cytokine release in response to stimulation for HK-2 cells grown in the kidney micro-scaffolds compared with cultures grown traditionally. Adler et al. and Kelly et al. are developing a microfluidic system that uses primary renal epithelial cells cultured in 3D on a collagen scaffold coated with ECM proteins. In response to cadmium chloride exposure, the microphysiological system showed sensitivity and expressed biomarkers (Heme oxygenase-1/kidney injury molecule-1) of injury showing the potential of the model to serve as a suitable predictor of toxicity ex vivo. In recent light, additional groups are interested in the toxicity modeling aspect, as evidenced by Kim et al. exposing fabricated microfluidic devices with MDCK cells cultured on porous membranes to gentamicin over time. In response to gentamicin exposure, the researchers observed a significant difference of injury between culture types (static/shear) of HK-2 cells as well as dosing regimens (D1 = bolus mimicking regimen; D2 = continuous infusion regimen).

FUTURE DIRECTIONS

Although great strides have been made in developing isolated hepatic and renal microphysiological systems, the integration of the two organ systems to model complex metabolic processes is the current focus of several groups. Our group has developed an integrated liver-kidney MPS system for identifying potentially nephrotoxic liver-metabolized chemicals by connecting a liver-on-a-chip with primary rat or human hepatocytes, to a kidney-on-a-chip with human proximal tubule epithelial cells in MPS devices developed by Nortis (Woodinville, WA). To test the hypothesis that first pass hepatic clearance of a nephrotoxic chemical might have significant importance in determining ultimate kidney toxicity, we utilized aristolochic acid I, a well-known nephrotoxin and carcinogen that undergoes extensive hepatic metabolism to form toxic metabolites. Our results provide mechanistic insights into the important role of hepatic biotransformation for the kidney-specific toxicity of AA-I toxicity. This integrated in vitro/ex vivo MPS model provides a novel approach for investigating the mechanisms that underlay pharmacokinetically and toxicologically important organ-organ interactions.

Other integrated MPS models include the H4tREL microdevice platform that assembles multiple culture units of hepatic tissue with other organ tissues in parallel. Used in combination with a mathematical modeling approach (pharmacokinetic/pharmacodynamic modeling), this novel platform provides improved predictability for drug biotransformation, clearance, and toxicity. The integrated discrete multiple organ co-culture platform that assembles multiple culture units of hepatic tissue with other organ tissues in parallel. Used in combination with a mathematical modeling approach (pharmacokinetic/pharmacodynamic modeling), this novel platform provides improved predictability for drug biotransformation, clearance, and toxicity. The integrated discrete multiple organ co-culture platform that assembles multiple culture units of hepatic tissue with other organ tissues in parallel. Used in combination with a mathematical modeling approach (pharmacokinetic/pharmacodynamic modeling), this novel platform provides improved predictability for drug biotransformation, clearance, and toxicity.
“wells-in-a-well” concept, interacting multiple cell types via the overlying medium. This model can mimic multiple organs in a human body interacting via the systemic circulation.\textsuperscript{72} The ultimate goal of accurate assessment of human drug toxicity will rely on the development of in vitro platforms with multiple organs, including the immune system, with each organ represented by multiple cell types and communication among organs achieved using human plasma or equivalent. It would be interesting to see more results of selected clinical drugs treated in these integrated models for validating in vitro-to-in vivo correlation.

There are still a number of technical challenges in multiple-organisms-on-chips development that have been important research areas for this field (reviewed by ref. 73). (1) Microfluidic volume problems are the physiological relevant scaling of fluid volumes and flow rates that are associated with individual organ microphysiological systems are difficult to acquire, as delicate and reliable engineering systems are required for the connection of reservoirs, pumps, and tubing to deliver accurate flow rates to the cultured cells. In addition, the determination of the physiologically relevant flow rate for hepatic cells cultured in microphysiological systems is difficult to determine given that the liver is a complex organ architecturally with varying fluid channel areas and perfusion rates that can expose hepatic cells to a wide range of shear forces. One approach to this problem would be to incorporate vascular systems within hepatic or renal chips to allow for cell-controlled flow rates between the vascular system and organspecific cells. (2) Universal cell culture media are individual cell types, especially primary cells, which require customized media for optimal cell culture performance. A universal culture media that can sustain multiple cell types from different organs will need to be developed to successfully co-culture cells from multiple organs with an optimal balance of nutrients, osmolality, pH, and supplements.

To address these challenges, the Defense Advanced Research Projects Administration, the National Center for Advancing Translational Sciences of the National Institutes of Health, the Food and Drug Administration, and the Environmental Protection Agency have funded several groups for MPS development and organotypic culture models for predictive toxicity and pharmacokinetic study.\textsuperscript{74,75} The European Commission is funding a Body-on-a-Chip project to many collaborated groups and supplements.

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