

**Evaluation of a Multiwell Perfused Bioreactor System for the Long-term Culture of
Mouse Hepatocytes**

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ABSTRACT

VALERIE YURIEVNA SOLDATOW: Evaluation of a Multiwell Perfused Bioreactor System
for the Long-term Culture of Mouse Hepatocytes
(Under the direction of Ivan Rusyn, MD. Ph.D.)

Liver is the primary organ for xenobiotic metabolism and *in vitro* models that faithfully recapitulate its function are needed for medium/high-throughput toxicity studies. We evaluated a three-dimensional multiwell perfused bioreactor system as a model of murine liver. System parameters (flow rate, seeding procedure and cell density, scaffold matrix, and media composition) were varied to identify optimal conditions for mouse hepatocytes. Biochemical assays (pyruvate, lactate, urea, albumin, and intracellular ATP) indicated that the three-dimensional system improves functionality of hepatocytes cultured for 3-5 days. We also tested hepatocyte's basal and inducible metabolism using model toxicants acetaminophen and WY-14,643. Finally, we cultured non-parenchymal cells with mouse hepatocytes to determine whether it may improve the functionality of cells. This work demonstrates that the three-dimensional multiwell perfused bioreactor seeded with mouse hepatocytes is a useful model for short-term studies; however, improvements in the system are needed for it to become a promising sub-chronic model.

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LIST OF ABBREVIATIONS

APAP- Acetaminophen

ATP- Adenosine triphosphate

DMEM- Dulbecco's Modified Eagle Medium

DMSO- Dimethyl sulfoxide

DPBS- Dulbecco's Phosphate-Buffered Saline

EC₅₀- Concentration at 50% activity

ECM- Extracellular matrix

EGF- Epidermal growth factor

EGTA- Ethylene glycol tetraacetic acid

EPA – Environmental Protection Agency

FBS- Fetal bovine serum

GBS- Gey's buffered saline

GFAP- Glial Fibrillary Acidic Protein

GPE- Glycerophosphorylethanolamine

HBSS- Hanks' Balanced Salt Solution

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ICCVAM- Interagency Coordinating Committee on the Validation of Alternative Methods

ITS- Insulin-Transferrin-Selenium

LDH- Lactate dehydrogenase

NAD- β -Nicotinamide adenine dinucleotide hydrate

NAPQI- N-acetyl-p-benzo-quinone imine

NICEATM- National Interagency Center for the Evaluation of Alternative Toxicological Methods

NMEM- MEM non-essential amino acids

NPC- Non-parenchymal cell

NRC- National Research Council

NTP- National Toxicology Program

PECAM- Platelet-endothelial cell adhesion molecule

PEG- Polyethylene glycol

PFA- Paraformaldehyde

PPAR- Peroxisome proliferator-activated receptors

PVDF- Polyvinylidene fluoride

SEC- Sinusoidal endothelial cell

VEGF- Vascular endothelial growth factor

WEM- Williams' Medium E

WY-14,643- 4-Chloro-6-(2,3-xylidino)-2-pyrimidinythiol acetic acid

CHAPTER 1

LITERATURE REVIEW

Current Chemical Toxicity Testing

The gold standard toxicological approach for evaluating chemical toxicity involves complex *in vivo* studies which are both time consuming and costly. Due to the pressures from animal activist groups, time constraints, cost reduction plans, the ever increasing number of chemicals and combinations of chemicals that need testing, and the need for high-throughput testing, establishing workable *in vitro* culture systems has become a priority for the toxicology community. In addition, the predictivity of rodent *in vivo* testing of human toxic effects has become a matter of dispute in recent years, in part due to poor relevance of animal study results to heterogeneous human populations (Holmes et al., 2010; National Research Council, 2007). The use of *in vitro* systems for toxicological testing has many advantages including the reduction in animal numbers, the reduced cost of animal maintenance and care, the reduced experimental variability, the reduced quantity of chemical needed for testing, and the decreased time needed and increase in ease of evaluating metabolites (LeCluyse, 1996; DelRaso, 1993). *In vitro* systems also allow us to establish metabolic mechanisms of toxicity, measure enzyme kinetics, and establish dose-response relationships (DelRaso, 1993).

The U.S. National Toxicology Program (NTP) has established the Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) which works in conjunction with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to establish and validate alternative methods to *in vivo* toxicological

testing. The three main tenants for animal toxicity studies of these agencies are known as the three R's: refinement, reduction, and replacement. The ultimate goal is "the validation and regulatory acceptance of test methods that are more predictive of adverse human and ecological effects than currently available methods, supporting improved protection of human health and the environment" ("About the National Toxicology Program", 2011).

The landmark report released by the National Research Council (NRC), "Toxicity Testing in the 21st Century: A Vision and a Strategy" (National Research Council, 2007), identified the challenges of modern toxicology and provided strategies for developing alternatives to *in vivo* research. The Council supports the movement towards the use of *in vitro* systems instead of *in vivo* toxicological studies and, as requested by the U.S. Environmental Protection Agency (EPA), has developed a long-term objective of decreasing the use of *in vivo* studies for toxicity testing and proposed an initial strategy towards achieving that goal. In addition to the limitations mentioned above, the NRC acknowledges that *in vivo* study results cannot evaluate the much lower concentrations and mixtures of chemicals that humans are exposed to, lack information regarding modes and mechanisms of actions, and cannot account for human variability in responses and susceptibility (National Research Council, 2007). The Council asserts that *in vitro* work can elucidate cellular-response networks and toxicity pathways, modes and mechanisms of action, allow for high-throughput studies, enhance dose-response relationships, evaluate many more concentrations than *in vivo* work, use concentrations relative to human exposure, provide information for generation of pharmaco-kinetic and -dynamic models, and lead to genome based investigations into perturbations of toxicity pathways.

There are three important factors which hinder the ability to use animal models to predict human adverse effects and the National Research Council claims the use of *in vitro* models will allow us to address these factors. First, *in vivo* studies typically use high doses of compounds which are orders of magnitude greater than those humans are exposed to.

Dose-response relationships are complex so extrapolation from these high doses to lower, human, exposure levels is difficult and results in many inaccuracies. Second, *in vivo* studies examine the response of a standard laboratory animal to a toxicant. This response may or may not occur in humans. Even more important is that the human population is very heterogeneous and a single strain of animal cannot accurately predict the variability in responses seen in the human population. Finally, *in vivo* exposures in toxicity testing are usually composed of a single compound. While this allows for close examination of the results of that single compound, humans are constantly exposed to mixtures of compounds daily and the effects of these co-exposures need to be examined. Conducting *in vivo* studies using mixtures of chemicals would be a monumental task due to the number of different chemicals and combinations of chemicals as well as the time it takes to complete each *in vivo* study.

To overcome these limitations to *in vivo* studies, the National Research Council developed four criteria important to designing a new toxicity-testing paradigm: broaden the studies to include a greater number of chemicals and chemical mixtures as well as more end points and life-stages; decrease the time it takes to obtain results for risk assessment by reducing costs and length of tests and increasing efficiency and flexibility of said tests; reduce the number and suffering of animals involved in testing; and more closely examine mechanistic and dosimetry information to provide risk assessment organizations with a broader range of information. The major components of the proposed strategy to establish this paradigm are chemical characterization, toxicity testing, and dose-response and extrapolation modeling. The National Research Council has recognized the fact that this new toxicity-testing paradigm will require years of research and assay validation and may never fully eliminate the need for *in vivo* work; however the benefits of reducing the number of animal studies are well worth the effort.

One of the current major benefits of *in vitro* systems is their use in screening chemicals for prioritization purposes. The EPA ToxCast™ project was established mainly for the purpose of prioritization of chemicals for further toxicological study by using high-throughput *in vitro* assays to establish “toxicity signatures” of chemicals, identify toxicity pathways, and predict potential toxicity (Judson et al., 2009). To do this, ToxCast™ uses a large number of cell-free or cell-based *in vitro* high-throughput assays. The different assays measure perturbations in pathways that could lead to cellular toxicity and the results of ToxCast™ assays will be used to link particular genes to toxicity endpoints. Researchers hope to use data produced by the high-throughput assays of ToxCast™ to supplement data from genomics, proteomics, and metabolomic studies to further elucidate toxicity pathways (Judson et al., 2010). Unfortunately, the high-throughput screening results do not correlate well with *in vivo* toxicity and cannot account for biological processes related to toxicity such as exposure, biotransformation, toxicokinetics, and individual genetic diversity (Knudsen et al., 2011). Thus, other *in vitro* methods must be examined to supplement these high-throughput assays.

Hepatocyte Cultures as *In Vitro* Systems

The liver is the major source of metabolism and drug biotransformation, thus liver cells are a logical choice for toxicological and pharmacological testing. Many different *in vitro* liver models have been employed over the years with the hopes that the effects after chemical treatment will be predictive of *in vivo* responses. Tissue slices preserve the zonal characteristics of a hepatic lobule and contain functional bile canaliculi, however they decline rapidly in viability, are not homogenous in functionality, and functional activities cannot usually extend past 10 hours (Guillouzo, 1997; LeCluyse, 2001; Lerche-Legrand and Toutain, 2000). Microsomes can be obtained from frozen liver tissue and allow for high throughput studies, but they lack any cytosolic enzymes and are limited in their use

(Houston and Carlile, 1997; Rodrigues and Wong, 1997). Isolated perfused livers preserve liver structure and functionality of even bile canaliculi, however they are difficult to work with, lose functionality more quickly than tissue slices, and are limited in their throughput (Wolcoff et al., 1987; Brouwer and Thurman, 1996). Immortalized hepatic cell lines lack phenotypic stability and normal gene expression (Yu et al., 2001; Wooster et al., 1993; MacDonald et al., 1994). Suspensions of primary hepatocytes retain high levels of functionality but are also short-lived (Guillouzo et al., 1997; LeCluyse, 2001).

Due to the limitations of the subcellular-, tissue- and organ-based *in vitro* models, as well as the need for higher throughput, cell lines, stem cells, and primary isolated cells are currently the most popular cellular models. Immortalized cell lines, as mentioned, tend to be unable to maintain phenotypic characteristics and are genetically unstable (Yu et al., 2001; Wooster et al., 1993; MacDonald et al., 1994). Few lines are able to express any liver-specific functions and, of those, most are derived from tumors. Common immortalized lines in use are Fa2N-4, HepG2, Hep3B, PLC/PRFs Huh7, HBG, and HepaRG (Guguen-Guillouzo and Guillouzo, 2010). The HepG2 line was generated in the 1970s and expresses many liver-specific genes (Braiterman and Hubbard, 2009); however, the line varies between passages and as a result data can be difficult to interpret (Guguen-Guillouzo and Guillouzo, 2010). The more recently developed human hepatoma line, HepaRG, has retained the expression of many liver-specific functions as well as many cytochrome P450s, nuclear receptors, membrane transporters, and phase II enzymes (Aninat et al., 2006). HepaRG cells have a stable karyotype, can differentiate into either hepatocyte or biliary lineages, have a high proliferative capacity, and have shown to produce data that is both reproducible and consistent (Marion et al., 2010; Guguen-Guillouzo and Guillouzo, 2010; Lubberstedt et al., 2011). Unfortunately, the expression of liver-specific functions in HepaRG cells is still much lower than that of primary hepatocytes (Marion et al., 2010) and they came from a single donor, thereby reducing their predictive value for the human population.

Stem cells constitute a very small fraction of liver cells and evidence has shown that, when cultured, they can proliferate without losing phenotypic characteristics (Schmelzer et al., 2007). It has also been demonstrated that *in vitro* hepatic stem cells, or even undifferentiated embryonic stem cells, can directly differentiate into hepatocyte-like cells (Navarro-Alvarez et al., 2010). Unfortunately, these hepatocyte-like cells only express some of the many liver-specific features and express them in much lower levels than primary hepatocyte cultures and usually only under very specific culture conditions (Navarro-Alvarez et al., 2010; Snykers et al., 2006). In addition, not much is understood of the differentiation process of these cells, the cell populations are quite variable, and their morphology and liver-like function is not stable for more than a few days (Navarro-Alvarez et al., 2010; Guguen-Guillouzo and Guillouzo, 2010). For these reasons, stem cells are not yet a widely accepted option for toxicological and pharmacological studies.

Cultures of primary hepatocytes have been the gold standard for *in vitro* testing as they can maintain functional activities for 24-72 hours, can be used for enzyme induction and inhibition studies, allow for medium-throughput, and can easily examine interspecies and inter-individual differences in metabolism (LeCluyse, 2001; Sivaraman et al., 2005). Primary hepatocytes are used with the expectation that chemicals will affect an isolated cell in the same manner that they would affect the whole organ and will in this way be a predictive model (DelRaso, 1992). Traditional primary hepatocyte cell culture involves the method of plating cells on a rigid substratum in which they proceed to form a monolayer across the bottom of the culture plate well. In this method, primary hepatocytes undergo changes in cell morphology, structure, polarity, gene expression, and liver-specific functions (LeCluyse, 1996; Nelson et al., 1982; Dunn et al., 1989), a process known as de-differentiation. Hepatocyte de-differentiation is an important limitation to this *in vitro* system. As the isolated cells have lost their normal microenvironmental structure, cell to cell interactions, and cell membrane structures, response to chemical exposures can be

different than those occurring *in vivo*. Lack of proper absorption, distribution, metabolism, and excretion (ADME) properties as a result of cellular disconnect from the circulatory and other organ systems also make assessment of chemical exposure results difficult with primary hepatocytes (DeIRaso, 1993; LeCluyse, 2001). Finally, one of the most problematic disadvantages of the use of primary hepatocytes for toxicological research is the drastic decrease in cell functionality over time. Liver specific functions such as albumin production and cytochrome P450 expression decline quickly over the first 24-48 hours of culture as the cells begin to lose their differentiated status (LeCluyse, 1996; LeCluyse, 2001; Bader et al., 1992; Nelson et al., 1982). Extending culture longevity, both in terms of liver specific functions as well as basic cellular functionality, has been a great obstacle science has not yet been able to overcome.

Several reasons for poor hepatocyte function in long-term culture have been suggested (see above). Hence, many approaches to surmount this challenge have been proposed, including adjusting components of the culture medium, altering the extracellular matrix, changing the cell culture format (such as monolayer, spheroid, or 3D cultures), adding flow to the culture system, and culturing hepatocytes with other cell types, all of which are summarized in multiple reviews (Mitaka, 1998; LeCluyse, 2001; LeCluyse et al., 1996; Meng, 2010; Coecke et al., 1999; Griffith and Swartz, 2006). Although a number of investigators have found certain approaches to benefit hepatocyte culture in their own laboratories, difficulties in comparison of experimental results occur and continue to hinder the discovery of optimal culture conditions. In addition, differences in species, strain, and sex of animal vary greatly across experiments. Conditions of cell isolation are also variable as are the methods for cell purification, conditions of cell culture, and assay methods. Yields and viabilities of final cell preparations can differ depending on types of collagenase and perfusion methods used. Species differences in optimal culture conditions abound as

well. These factors must be taken into consideration when examining different culture systems and developing the proper procedures in one's own laboratory.

Three-dimensional Culture Systems

Three-dimensional culture of hepatocytes is a burgeoning field of study as scientists attempt to recreate the complex cellular microenvironment of the liver in hopes of extending primary hepatocyte culture longevity and functionality. 3D cultures range in complexity from monolayer sandwich culture and spheroids to more advanced systems involving porous materials, packed-bed reactors, hollow fibers, and perfusion flow.

The microenvironment of the hepatocyte is very important and can be complex to mimic. The importance of cell polarity has been stressed by many investigators and it has been suggested that without it hepatocytes will not function properly in culture (Dunn et al., 1991). Hepatocytes possess not only a single apical and basal surface, but have multiple apical surfaces (bile canalicular surfaces) and two basolateral surfaces (Dunn et al., 1991). For this reason, re-establishing cell polarity is not as easy as with other types of epithelial cells. Different extracellular matrices (ECMs) have been extensively evaluated in 2D and 3D hepatocyte culture in an attempt to re-establish and subsequently maintain hepatocyte cell polarity. Many configurations of matrices have been evaluated to determine optimal conditions for restoration of hepatocyte polarity. The other main limitations of traditional two-dimensional culture, namely reduction of gene activity and expression of genes involved in phases I, II, and III of drug metabolism, mainly occur within the first 24-48 hours and these cultures have been shown to have a low sensitivity of drug hepatotoxicity detection (Meng, 2010). The use of three-dimensional liver cultures can overcome deficits of the 2D culture system by providing models with more complex local environments.

Three-dimensional culture systems have evolved greatly within the last 10—20 years; initially composed of a simple sandwich configuration where hepatocytes would be

placed between two layers of matrix (traditionally collagen or Matrigel®). Collagen sandwiches increase and maintain albumin secretion, maintain polygonal morphology similar to that of *in vivo* cells, prevent loss of cell viability, increase basal enzyme activities, increase and maintain enzyme induction, and mimic *in vivo* biliary excretion rates (Bader et al., 1992; Dunn et al., 1989; LeCluyse et al., 2000; Mingoia et al., 2007; Liu et al., 1999). Some of these functions are retained for relatively long periods of 24 days to 7 weeks in rat hepatocytes. Investigators attribute these positive effects to cell polarity which was induced by the ECM (Dunn et al., 1989). Research has shown that sandwich culture prevents the formation of stress fibers in addition to increasing transferrin, fibrinogen, and bile salt secretion, and stabilizing urea secretion in rat hepatocytes over 42 days (Dunn et al., 1991). Mouse hepatocytes also respond well to the sandwich culture method. Genes responsible for liver-specific functions lose expression over time (90 hours) while those involved in gross cellular organizations increase in activity. In general, phase I enzyme gene expression decreases over time but phase II enzymes stay stable and data indicate that P450 gene expression in sandwich cultured mouse hepatocytes is more stable than in rat hepatocytes (Mathijs et al., 2009). The loss of phenotype that occurs despite culturing hepatocytes in a sandwich system has been suggested as a cause of the decline of metabolic enzyme activity in these cultures and that application of the sandwich culture model is best used for hepatobiliary mechanistic studies (Meng, 2010). Thus, other strategies to extend culture period and retain hepatocyte function have been employed.

Spheroid culture was the next 3D model evaluated for hepatocyte culture, with the assumption that cellular aggregates better mimic liver tissue characteristics. Working with self-forming hepatocyte spheroids was initially a complex undertaking involving an initial culture period for spheroid formation prior to the hepatocytes being available for any toxicological or pharmacological experimentation. In 1996, Wu and colleagues optimized a protocol for quick production of rat hepatocyte spheroids through use of spinner flasks (Wu

et al., 1996). They observed rat hepatocyte spheroid physiology and found that oxygen supply was critical in the proper formation of spheroids and that these cells maintained a more differentiated state than those of monolayer culture. Cyp1a2 and Cyp1a1 expression levels dropped rapidly in normal 2D monolayer culture in mouse hepatocytes while in 3D spheroids the levels stayed high over 5 days (Nemoto and Sakurai, 1992; Nemoto and Sakurai, 1993) which indicates that spheroid culture is preferred over monolayers for studies evaluating levels of gene expression. Spheroid culture of mouse hepatocytes can also maintain Cyp2b9 and Cyp2b10 for a number of days at levels equal to *in vivo* (Nemoto et al., 1995). Du et al. (2006) were able to develop a 3D monolayer culture configuration by conjugating cell adhesion peptides Arg-Gly-Asp (RGD) with galactose ligand and polyethylene terephthalate. They determined that the rat hepatocyte spheroid configuration was ideal; by using this new culture system cells would anchor well to the substrata and have a limited ability to spread while still keeping the functional characteristics of spheroids. Liver specific functions of cells on the new substratum, such as albumin secretion and urea synthesis, were similar to that of spheroids. These cells also exhibited greater sensitivity to acetaminophen-induced hepatotoxicity than those cultured on traditional 2D collagen. However, due to oxygen and nutrient diffusion difficulties, spheroid culture is limited in its ability to be used for bioartificial liver models or long-term culture, thus more 3D models were investigated.

Gelled materials have been devised to combine the complexity of a Matrigel®-type ECM with spheroid formation. The idea of culturing hepatocytes encapsulated within gel was originally developed for use in a bioartificial liver where semi-permeable hollow fiber cartridges were used as a scaffold for hepatocyte attachment (Meng, 2010). Miccheli and associates entrapped rat hepatocytes in alginate beads and placed them in a bioreactor system with a continuous flow of medium (Miccheli et al., 2000). In this system energy metabolism, viability, and redox state was stable within 3 hours and the proper shape,

microvilli, tight junctions, and bile canaliculi were reformed within 8 hours. The authors confirmed previous demonstrations that adequate supply of oxygen and substrates is critical for proper cellular reorganization in these systems. They observed increased glycerophosphorylethanolamine levels in the static cultures indicating problems with membrane phospholipid metabolism regulation contrasting low levels in the bioreactor system. Gels of agarose have been employed to sustain viability of mouse hepatocyte cultures for 21 days. Within these gels, hepatocytes typically form aggregates and excrete significantly higher amounts of albumin than their 2D counterparts (Ise, 1999). Shen et al. (2006) demonstrated that gel entrapped hepatocytes expressed higher levels of liver-specific functions and phase I metabolism, maintained higher intracellular ATP levels and mitochondrial membrane potential, and accumulated less lipids within the cytoplasm than those cultured in traditional 2D monolayer. In addition, measurements of phase I metabolic enzymes were similar to levels found *in vivo*. Furthermore, gel entrapped cultures have been able to accurately reflect hepatotoxicity in over twenty reference compounds (Lu et al., 2008). Some of these gel culture systems limit mass transfer of oxygen and nutrients while others require large amounts of reagents, thus models using a type of scaffolding to provide structural support and semi-permeable hollow fibers were developed. Some of these fiber systems incorporated gel entrapment while others did not. Investigators were able to obtain rat hepatocyte cultures with increased functionality through use of scaffolding composed of polyvinyl formal resin and nanofibers (Miyoshi et al., 1998; Chu et al., 2009). The scaffolding increased cell adhesion, even in high density, lower LDH release, and maintained biochemical functions such as albumin secretion, urea synthesis, and glycogen synthesis. Stabilization of albumin and urea levels after an initial decline was noted in a functional 20-day rat culture (Miyoshi et al., 1998). Shen and colleagues examined a simple micro-hollow fiber reactor system for rat hepatocytes and determined that functionality (in terms of urea synthesis and albumin secretion) was increased in this system when

compared to simple monolayer culture (Shen et al., 2007). The system increased metabolic capabilities as well, as indicated by greater sensitivity to chemical insult than seen in cells in monolayer culture.

Incorporating fluid flow to three-dimensional culture systems was an important addition to tissue engineering. Packed-bed, hollow fiber, or other type of “reactors” integrate micro-circulation of medium to recreate the fluid flow within the liver. Addition of flow to 3D cultures is important in combating the issues of poor oxygen and nutrient diffusion through spheroids and aggregates of cells. Systems incorporating shear flow have shown to promote round cell aggregates that are more similar to *in vivo* morphology and increase and maintain liver-specific functions (Kan et al., 2004). Flow has shown to increase and maintain a higher level of urea synthesis (Taguchi et al., 1996) and to increase albumin, lactate, and glucose secretion (Kim et al., 1998). Physiologic oxygen gradients can be established in these microfluidic systems and this can assist in elucidating the physiological mechanisms involved in toxicity (Allen et al., 2005). Furthermore, the organotypic physiological geometries and flow properties in these systems allow for the study of drug-drug and cell-cell interactions (Chao et al., 2009).

Although these methods of three-dimensional hepatocyte culture show improvement over traditional two-dimensional cultures they are still insufficient for long-term culture or use in clinical therapies (such as bioartificial livers) due to the continued loss of metabolic gene expression after cell isolation. Thus, it is no surprise that more complex 3D systems are being engineered. The 3-D culture systems under development use existing knowledge regarding the important cellular environment dynamics between extracellular matrix, micro-circulation, and cell type and density, to form a fully functional liver culture model that can be used for toxicological and pharmacological studies or even modified into a bio-artificial liver for clinical use. A few systems will be briefly discussed here.

HμREL® Biochip. Chao et al. (2009) published an evaluation of a novel microfluidic device, a HμREL® biochip (an earlier model of this system was previously described by Sin et al., 2004). Four biochips are enclosed in a polycarbonate housing connected, by tubing lines, to a fluid reservoir and peristaltic pump. Each biochip has one or more separate compartments in which different cells can be housed. The compartments are microfluidically connected, in a linear path, to allow for interaction between the cell types. A new design allows for separate microfluidic experiments to be run in parallel on a larger set of housing plates. Evaluation of the system using primary human hepatocytes indicated the system preserved cell viability and metabolic competency was at least as high as, and sometimes higher than, the traditional static culture conditions (Chao et al., 2009; Novik et al., 2010). The microscale system allows for microscopic imaging, oxygen sensing, physiologically realistic ratios of chamber sizes and liquid residence times in each compartment, physiological hydrodynamic shear stress, physiological liquid to cell ratios, and requires less media and cells. The model is severely limited, however, in that sample removal is difficult without disturbing the system dynamics, recirculation of the media involves a complex set of tubing lines and reservoirs, and cells on chips form monolayers and not physiological tissue constructs.

Hollow-Fiber Reactor. A hollow-fiber based bioreactor was used by Schmelzer et al. (2009) for the regeneration and culture of human hepatocytes. Cells are seeded into the extracapillary space and are surrounded by three independent capillary membrane systems. The capillary systems are composed of porous polyethersulphone and hydrophobic multilaminate hollow fiber membranes which allow for gas exchange. The capillary layers are interwoven around the extracapillary space and two of the capillary systems are perfused in a counter-current flow with either culture medium or plasma while the third allows for decentralized oxygenation and supply of nutrients. The bioreactor functions through use of a perfusion device that uses pressure-regulated pumps to control the

medium flow. A gas mixing unit is also used to provide the system with, and control the rates of, air, O₂, and CO₂. Human hepatocyte functionality was stable for up to four weeks in this bioreactor system. Although this bioreactor system allowed for a microenvironment more similar to *in vivo* conditions, the system was large and required high numbers of cells and large amounts of reagents. A microscale prototype version of this bioreactor was then developed (Schmelzer et al., 2010). In this smaller model, the bioreactor is comprised of four cell chambers each of which contains four compartments. The first compartment houses the cells, two contain culture medium, and the last provides the oxygen supply. All compartments are connected to provide the cells with a physiologically-based environment. This micro-bioreactor has thus far been used for human fetal liver cell culture and has sustained cell viability and differentiation. The prototype allows for small numbers of cells and limited reagent use, microscopic evaluation of the cells, and monitoring of oxygen concentrations. In addition, the counter-directional perfusion method is unique in providing a more physiologically similar flow. Limitations to this system include a lack of physiologic gradients normally seen in liver tissue, the complication of numerous tubing lines, and limited throughput as only four different culture conditions can be evaluated on a single system.

Single- and Multi-Well Perfused Bioreactor. Sivaraman et al. (2005) used a 3D bioreactor system to evaluate the functionality of rat hepatocyte spheroids. They had hypothesized that a system that included heterotypic cell interactions, fluid flow stresses, and microarchitecture similar to that *in vivo* would provide the incentive for cells to function as they do *in vivo*. This system housed spheroids of rat hepatocytes attached within the many channels of a silicon scaffold. Perfusion of the medium was directed both over the top of the channels as well as through the tissue in the channels. A high-resistance filter controlled the perfusion through the channels so flow was uniform through all channels of the scaffold. Flow was initially in a downward direction to trap the cells in the channels and

later, after the cells had adhered to the channel walls, the flow was reversed. Spheroids seeded in the bioreactor system had an increased and sustained functionality when compared to spheroids in both static culture and single cells seeded into the bioreactor system. Both urea synthesis and albumin excretion were increased and more stable with the spheroid bioreactors. This bioreactor system was scaled up easily to incorporate more channels (and thus, more cells) as well as up to 6 different reactors controlled by the same unit. The scaffolds promote tissue morphogenesis and allow for microscopic examination via light or two-photon microscopes. This cell culture system still lacks high-throughput capabilities, does not accommodate large numbers of cells, and needs to be examined with hepatocytes from other species.

Although much progress has been made over the traditional two-dimensional static culture system, we are still lacking a model in which all levels of liver-specific function can be maintained for long periods of time. Furthermore, many of the models have yet to establish whether the systems can function with hepatocytes from more than a single species.

Co-culture Systems

In the body, cells function by way of complex interactions and signals from other cells. For this reason, we can assume that cultures of solely hepatocytes may not properly represent *in vivo* functionality especially when examining drug related hepatotoxicity. Hepatocyte co-cultures are usually comprised of hepatocytes with one other cell type, often other liver cells (Ries et al., 2000; Higashiyama et al., 2004; Krause et al., 2009; Tukov et al., 2006), non-liver epi- or endothelial cells (Gebhardt et al., 1998; Talamini et al., 1998), fibroblasts or cell lines (Evenou et al., 2011; Bhatia et al., 1998; Kang et al., 2004). Co-cultures have also been prepared using hepatocytes and more than one additional cell type, such as hepatocyte-non-parenchymal cell (NPC) cultures. In these cultures the hepatocytes are cultured with the other liver cells: Kupffer cells (resident macrophages), sinusoidal

endothelial cells, and stellate cells (stromal cells). Co-culturing hepatocytes with other cell types have been shown to be one of the most successful techniques for maintaining hepatocyte function in *in vitro* models (Guguen-Guillouzo et al., 1983; Begue et al., 1984; Kaihara et al., 2000; Gueguen-Guillouzo and Guillouzo, 2010).

Griffith and colleagues demonstrated that hepatocytes seeded with bovine aortal endothelial cells could form three dimensional structures in a polymer scaffolding system with flow (Griffith et al., 1997). Within these structures hepatocytes bound to the substrata and the endothelial cells flattened and “covered” the hepatocytes. Other investigators have found increases in albumin secretion and urea synthesis in rat hepatocytes when cultured with mouse embryonic fibroblasts, 3T3-J2, or NIG-3T3 cells. Other benefits included retention of hepatic polygonal morphology, visible bile canaliculi, and distinct nuclei (Khetani et al., 2004). Mouse hepatocytes demonstrated better maintenance of albumin secretion and urea synthesis over a 7 day culture period when allowed to form 3D aggregates with mouse liver fibroblasts. The importance of the physical interactions between the two cell types was evident. Increased liver-specific functionality developed when the cells were in contact in the same culture rather than on different substrata (Jiman and Meifu, 2000). Results from a study culturing micropatterned hepatocytes (produced by seeding on a stencil and then removing the stencil) on a 3T3-J2 fibroblast feeder layer indicate that this type of co-culture can lead to increased and maintained albumin and urea production as well as retained hepatocyte morphology (Khetani and Bhatia, 2008). The importance of the heterotypic interface between the hepatocytes and fibroblasts further exemplifies the importance of cell-cell contacts in hepatocyte culture (Cho et al., 2010).

Not only can cell-cell contacts with fibroblasts, cell lines, and epi- and endothelial cells affect hepatocytes in culture, but the soluble factors excreted by other cell types have been shown to be extremely important as well. Cultures of rat hepatocytes in medium conditioned by cardiac endothelial cells functioned similarly to hepatocytes cultured under a

layer of endothelial cells and gel (Jindal et al., 2009). Hepatocytes were shown to modulate endothelial activation states as well (Jindal et al., 2011). Morin et al. (1988) observed similar increases in albumin secretion in culture systems in which the endothelial cells and hepatocytes were not in contact as cultures in which the cells were in contact. These non-contact co-cultures have had varied success and limited reproducibility between laboratories, however, thus cell-cell contacts continue to be one of the most important features of co-cultures (Coecke et al., 1999; Bhatia et al., 1999).

The addition of liver non-parenchymal cells to cultures of hepatocytes is required to reconstruct an organotypic environment that can approximate *in vivo* conditions. Many publications report on the benefit of non-parenchymal cells on hepatocyte function. Ries et al. (2000) described a hepatocyte-NPC co-culture model in which hepatocytes were seeded on top of the NPC layer (containing Kupffer, stellate, and sinusoidal endothelial cells). In their cultures, sinusoidal endothelial cells survived and maintained their fenestrae for 6 days. Other cell types remained viable for up to 14 days. They were able to demonstrate that not only did the presence of the NPCs maintain hepatocyte differentiation, but the hepatocytes contributed to survival of the NP cells. Experiments of human hepatocytes with non-parenchymal cells, in a system with flow, have shown to have greater drug metabolic capabilities and better maintenance of that activity than cultures composed solely of hepatocytes (Novik et al., 2010). Other cultures of hepatocytes with non-parenchymal cells found that stimulation of hepatocyte DNA replication after treatment with known hepatotoxicant WY-14,643 was enhanced compared to cultures of hepatocytes alone, indicating that the co-cultures result in more *in vivo*-like responses to treatment (Karam and Ghanayem, 1997). Tukov et al. (2006) examined the effects of Kupffer cells cultured in contact with rat hepatocytes. They found that by adding Kupffer cells to the cultures they could mimic *in vivo* drug-inflammation interactions thus providing a model which could be useful in predicting such interactions before *in vivo* studies were necessary. Co-cultures of

rat primary hepatocytes and stellate cells on a poly (DL-lactic acid) surface quickly formed spheroidal aggregates and maintained liver-specific functions for over 2 months.

Cytochrome P450 enzyme induction was maintained for almost the whole culture period (Riccalton-Banks et al., 2003). Stellate cells have also been shown to promote cell proliferation when in contact with hepatocytes and maintain hepatocyte differentiation when cultured without direct contact (Higashiyama et al., 2004).

It has been suggested that any bio-artificial device composed of primary cells will require non-parenchymal cells in order to maintain tissue-specific functions (Soto-Gutierrez et al., 2010). Bioartificial liver devices are being developed to not only assist in the treatment of liver failure but also for use in drug metabolism and toxicological studies (Sharma et al., 2011). Whether or not a bioartificial liver or other type of three-dimensional model would best predict *in vivo* responses to chemical treatment remains to be seen; however the identification of micro-environment requirements, such as the presence of non-parenchymal cells, is critical to the development and application of these systems.

CHAPTER 2

OBJECTIVES

Inter-individual Variation in *In Vitro* Systems

The National Research Council Committee on Toxicity Testing and Assessment of Environmental Agents recognizes that *in vitro* tests need to take a more prominent position in the toxicity-testing paradigm. Although they developed a strategy containing different tiers of evaluation which include extensive *in vitro* work, their proposal is based on the use of human cell lines. The committee does admit, however, there are many limitations to the use of such cell lines (NRC, 2007). As mentioned previously, cell lines lack phenotypic characteristics of cells *in vivo* and expression of liver-specific functions is limited. One of the most important limiting factors of cell line use is the lack of inter-individual variability found in these lines. Primary cells, then, become a more preferred method of *in vitro* evaluation.

Human hepatocyte cultures are useful in toxicological and pharmacological studies as well as risk assessment as they eliminate species differences in response to chemicals, however initial levels of metabolic capacity as well as induction ability can vary greatly between human donors (Novik et al., 2010; LeCluyse, 2001). To reduce this inter-individual variability and accurately predict the human population scale of responses, reproduction of the experiment using multiple donors or combining cells from donors would be required (Novik et al., 2010). An animal model which could account for the range of human variability would be a great resource in eliminating this predicament.

Previous Studies Using Multi-Strain Mouse Models of Hepatocytes

Our laboratory has previously utilized an *in vivo* inbred mouse diversity panel to model the variability in drug responses seen in the human population (Harrill et al., 2009). In that model, 36 inbred mouse strains were used to demonstrate the variability seen in response to administration of acetaminophen and identify specific genes involved in these different responses. When the results from experimentation with mice were validated by a human cohort, investigators determined the mouse strain panel could be used in prescreening studies to identify individuals susceptible to drug or environmental pollutant toxicities (Harrill et al., 2009b; Bradford et al., 2011).

Subsequently, our laboratory investigated the possibility that these strain differences could be modeled *in vitro*. The functionality of hepatocytes from 15 inbred mouse strains was initially examined and significant differences in gene expression levels were found. Experiments were then conducted on three of the 15 strains to determine if the model would accurately reflect strain differences in response to treatment with four reference compounds. Differences were noted, although the findings did not correlate well to the *in vivo* study due to changes in the ranking of sensitivity and resistance to acetaminophen treatment (Martinez et al., 2010). Nevertheless, the results indicate that a panel of inbred mice can be a valuable tool in examining inter-individual variation in toxicity responses.

Hypothesis and Goals

Powers et al. (2002) and Sivaraman et al. (2005) demonstrated that rat hepatocyte cultures, as well as co-cultures with endothelial cells, could present phenotype, albumin and urea levels, and gene expression more similar to *in vivo* tissue than either 2D or spheroid cultures when placed in a 3D bioreactor that provided the cells with continuous perfusion of medium. Their work led to development of a 3D multiwell perfused bioreactor system

designed for increased ease of use as well as increased throughput (Domansky et al., 2010). This system conferred the same types of benefit to rat cultures and co-cultures as the previous flow systems. Therefore, our hypothesis was that it could easily be applied for use with mouse hepatocytes. The successful long-term culture of mouse hepatocytes in this system would afford the opportunity to apply our genetically diverse mouse panel model to a more complex, *in vivo*-like environment for more accurate *in vitro* responses to chemical treatment.

Our goals were to 1) optimize the bioreactor system for mouse hepatocyte culture, 2) use the system to evaluate responses to chemical treatment in a mouse diversity panel, and 3) establish co-cultures of hepatocytes and non-parenchymal liver cells to further elucidate the contribution of the other cell types in toxicity responses.

CHAPTER 3

CHARACTERIZATION OF MOUSE HEPATOCYTES IN THE BIOREACTOR SYSTEM

PART I: Materials and Methods

Reagents and Solutions

Type IV and Type IA collagenase, Percoll, dexamethasone, fetal bovine serum (FBS), Williams' Medium E (WEM), pyruvic acid, lactate dehydrogenase, triethanolamine, β -Nicotinamide adenine dinucleotide hydrate and reduced dipotassium salt (NAD and NADH), hydrazine monohydrate, trypan, Optiprep™, adenosine triphosphate (ATP), D-glucose, fructose, and ethylene glycol tetraacetic acid (EGTA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibiotic-Antimycotic solution, Insulin-Transferrin-Selenium (ITS) solution, sodium pyruvate, GlutaMAX™, TRIzol®, Hanks' Balanced Salt Solution (HBSS) (without calcium and magnesium), HBSS (containing calcium and magnesium), Dulbecco's Phosphate-Buffered Saline (DPBS), Dulbecco's Modified Eagle Medium (DMEM), MEM non-essential amino acid solution, and rat anti-Glial Fibrillary Acidic Protein (GFAP) and anti-mouse platelet-endothelial cell adhesion Molecule 1 (PECAM-1 or CD-31) fluorochrome conjugated antibodies were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), PureCol Collagen, Biocoat tissue culture plates, and glycine, were purchased from Fisher Scientific (Waltham, MA, USA). Matrigel™ was purchased from BD (Franklin Lakes, NJ, USA). Anti-mouse F4/80 antigen PE fluorochrome conjugated antibody was purchased from eBioscience (San Diego, CA, USA).

Animals and Preparation for Surgery

Hepatocytes were isolated from male C57Bl/6J and CD-1 mice aged 4-17 weeks and non-parenchymal cells were isolated from male C57Bl/6J and CD-1 mice aged 6-20 weeks (purchased from Jackson Laboratories, Bar Harbor, ME and Charles River, Wilmington). Mice were housed by strain, no more than five per cage, in polycarbonate microisolator or Tecniplast ventilated cages. Animals were kept on an alternating 12-hour light and dark cycle and food (Purina Prolab® RMH3000, Purina Mills, Inc., Richmond, IN, USA) and water were provided *ad libetum*. All procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill, NC. Prior to surgery, mice were anesthetized by an injection of Nembutal® (100mg/kg, sodium pentobarbital, Lundbeck Inc., Deerfield, IL).

Isolation and Culture of Hepatocytes

Upon reaching a surgical plane of anesthesia, mice were placed in a supine position and the liver was perfused in situ in a two-step collagenase perfusion method modified from Seglen (1976) and Klaunig et al. (1981). A cannula was inserted through the right atrium of the heart into the superior vena cava, the portal vein was cut, and the liver was perfused with HBSS, containing 0.5mM EGTA but without calcium and magnesium, for 4 minutes followed by HBSS, containing calcium, magnesium, and 0.1-0.4mg/mL collagenase, for 7-12 minutes. Upon visual confirmation of digestion, the livers were flushed with a supplemented WEM buffer (see Appendix A for formulation; HEPES, GlutaMAX™, dexamethasone, ITS, Antibiotic-Antimycotic) containing 10% FBS. Livers were then excised and placed into same medium and gently dissociated by removing the capsule and gently shaking the liver. The cell suspension was filtered through a 100µm nylon cell strainer (BD Falcon, Fisher Scientific) and yield and viability were analyzed on a hemocytometer by trypan blue exclusion. Cell suspensions of greater than 80% viability and good cell quality were taken

through the remainder of the isolation procedure. Following filtering, cells were purified by three centrifugation steps; 50 x g for 3 minutes, 68 x g for 4 minutes, and 50 x g for 3 minutes. At each step the supernatant was removed and the pellet resuspended in the supplemented WEM. For the second spin a 50% (v/v) isotonic Percoll gradient was used to isolate only the viable hepatocytes from the suspension. Upon completion of the purification, the trypan blue exclusion test was once again used to determine the final yield, viability, and cell quality of the preparation. Only cell preparations of more than 90% and good cell quality (as determined through visual examination of cell membrane integrity, extent of membrane blebbing and cytoplasm vacuolization, amount of cell clumps, and amount of debris) were used for experiments. Cell suspensions were diluted to desired seeding densities (unless otherwise noted, 150,000 cells per well for 24 well plates and 600,000 cells per bioreactor scaffold), seeded onto appropriate substrata and subsequently placed into an incubator set at 37°C and 5% CO₂. Cells were allowed to attach for 4 hours at which point the seeding medium was removed and replaced with feeding medium (same as seeding medium minus the FBS). Culture medium was removed and replaced with fresh medium daily.

Isolation of Non-parenchymal Cells

Upon reaching a surgical plane of anesthesia, mice were placed in a supine position, blood was flushed from the liver, and cells were isolated using a slight modification of the methods reported by DeLeve (1994). Briefly, a 27g butterfly needle was inserted into the portal vein, the inferior vena cava was cut, and the liver was perfused in situ for 45 seconds with Gey's buffered saline (GBS; see Appendix B for formulation and vendor information). Upon completion, the liver was removed and rinsed three times with GBS buffer before being placed into a petri dish and dissociated using a scissors and forceps. The liver pieces were then placed into a 100mL beaker with collagenase solution (Sigma Type IA

collagenase). The beaker was placed into a 37°C waterbath and swirled every 4-5 minutes for 30 minutes. The liver slurry was filtered through a 297µm filter (Spectra/Mesh, Spectrum Labs, Rancho Dominguez, CA) and placed into a 50mL conical tube with GBS. Following filtering, the cells were purified by three 4°C centrifugations steps; 400 x g for 15 minutes, 1400 x g for 15 minutes, and 400 x g for 15 minutes. For the first and last spin the supernatant was removed and the pellet resuspended in GBS buffer. For the second spin the cell suspension was mixed with Optiprep™ to form a 17% final Optiprep™ solution with 1mL of GBS layered on top. Non-parenchymal cells were collected from the interface of the GBS and Optiprep™ solution. Upon completion of the purification cells were evaluated by trypan blue exclusion and a cell count was taken. All NPC preparations had a final viability of no less than 98%. Cell suspensions were diluted to desired seeding densities in WEM with 10% FBS and seeded onto appropriate substrata containing recently seeded hepatocytes, and subsequently placed into an incubator set at 37°C and 5% CO₂. Medium was changed at 4 hours post hepatocyte seeding (usually approximately 2 hours post NPC seeding).

Evaluation of Non-parenchymal Cell Populations

In most cases, an aliquot of cells was removed prior to suspension dilution and this aliquot was centrifuged for 14,000 rpm for 4 minutes, fixed with 2% PFA, and stored in 0.1% Tween at 4°C. Within 7 days, these cells were removed from storage, centrifuged for 4,000 rpm for 4 minutes, incubated for 45-60 minutes with fluorochrome labeled antibodies, rinsed by centrifugation two to three more times (4,000 rpm for 4 minutes), filtered, and evaluated by flow cytometry. Cells were analyzed on a Beckman Coulter (Dako) CyAn ADP (Beckman Coulter, Brea, CA). Data were analyzed using Cyflogic software (CyFLo Ltd, Turku Finland). Briefly, forward and side scatter characteristics were used to exclude cell debris and hepatocytes from the analysis. An unstained population was used to set a gate and all

events outside that gate (along a single axis) were considered positive for the defined population.

Evaluation of Cellular Morphology

Cells in 2D culture were examined and photographed by phase microscopy daily (100X and 200X). Bioreactor cells were examined briefly on the first day of culture by inverted microscope to determine attachment efficiency and continuation of the experiment (40X). Cells in each bioreactor scaffold were also examined and photographed by phase microscopy (40X, 100X, 200X) on the day of harvest for the scaffold.

Evaluation of Functionality

Cell culture medium was collected daily from each bioreactor reactor-reservoir pair and from 3 to 4 wells per culture condition in 2D cultures. Media samples were centrifuged at 14,000 rpm for 4 minutes and supernatants were collected and stored at -20°C until completion of the experiment. At this time, all samples were thawed at 4°C and assays were performed using a Beckman Coulter DTX880 Multimode Detector plate reader (Beckman Coulter, Brea, CA). Media samples were used to determine production of pyruvate and lactate, urea synthesis and albumin production. Pyruvate and lactate were assessed by standard enzymatic procedures (Bermeyer, 1988). Urea synthesis was evaluated using the BioAssay Systems (Hayward, CA) QuantiChrom Urea Assay Kit. Albumin production was measured using the Active Motif (Carlsbad, CA) Albumin Blue Fluorescent Assay Kit. Intracellular ATP was measured in some experiments at pre-determined timepoints. ATP was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) according to manufacturer's protocol and using standards made prior to the experiment start and stored at -20°C. For RNA analysis, culture

medium was removed and replaced with 0.1mL/well TRIzol® (for 2D cultures) or 0.3mL/scaffold (for 3D cultures) and stored at -80°C until experiment completion.

Preparation of Treatment Chemicals

Stock concentrations of both acetaminophen (APAP) and 4-Chloro-6-(2,3-xylidino)-2-pyrimidinythiol acetic acid (WY-14,643) (purchased from Sigma Aldrich and Chemsyn Science Laboratories, Lenexa, Kansas, USA, respectively) were prepared by dissolving the compounds in DMSO so as to obtain concentrations 200-fold higher than treatment concentrations. Stock concentrations were stored in aliquots at -80°C until the day before treatment. On the day before treatment, an aliquot of stock solution was removed and thawed to room temperature. Working solutions for each concentration and day of treatment were prepared from this stock aliquot to allow for treatment solutions to have a final DMSO content of 0.5%. These working solutions were stored at 4°C until day of treatment at which time the working solutions were diluted in cell culture medium.

mRNA Isolation and Gene Expression Analysis by RT-PCR

Cells stored in TRIzol® were removed from culture plate wells or bioreactor scaffolds by use of cell scrapers (TPP, Fisher Scientific). In order to obtain enough RNA from 2D cultures, cells from 3-4 wells were combined for evaluation. Suspensions were placed into 1.5mL tubes and homogenized using an insulin syringe with 30 gauge needle (BD, Fisher Scientific). The RNA was extracted from the cell lysates by use of the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) with slight modifications to the original protocol. Briefly, chloroform was added to cell lysates and mixed well. Lysates were centrifuged at 4°C for 15 minutes at 12,000 rpm. The upper aqueous phase was transferred to a new 1.5mL tube, mixed with equal parts EtOH, and transferred to an RNeasy Mini spin column. The remainder of the protocol was according to manufacturer recommendations. RNA

concentrations and quality were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). From these RNA concentrates, 1µg was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. The resulting cDNA was stored at -20°C until the time of RT-PCR analysis at which point it was thawed at 4°C prior to use. Real time PCR was performed using the following probes (Applied Biosystems): albumin (Mm00802090_m1), Cyp2e1 (Mm00491127_m1), Cyp4a10 (Mm01188913_g1), UGT1a1 (Mm02603337_m1), Cyp3a11 (Mm00731567_m1). The assay was performed in a 96-well RT-PCR plate (Roche, Indianapolis, IN) and processed using the Roche LightCycler® 480 System. Each plate contained GUSB (a housekeeping gene) as well as experimental genes. The cycle threshold (Ct) for each sample was determined as the time when the PCR reaction leaves the exponential phase and enters the final plateau phase. The ΔC_t was calculated relative to the control gene (GUSB) as fold change compared to the housekeeping gene.

Statistical Data Analysis

Time-course biochemical assays, gene expression data, and concentration-response curves were plotted by GraphPad software (version 4.0, Prism, La Jolla, CA). Data are represented as mean values +/- the standard deviation for at least two replicates per condition unless noted otherwise. Either t-tests or one- or two-way ANOVA was used for statistical comparison and a p value of less than 0.05 was determined to indicate statistical significance.

PART II: Bioreactor System Design

The 3D multiwell perfused bioreactor system evaluated here was developed by our collaborators at MIT, in the laboratory of Linda Griffith (see review of the system in Domansky et al., 2010). The system is composed of a multi-well plate in which isolated open-well bioreactors are housed in pairs (a reactor well and a reservoir well) and interactions are controlled by a micropump (Figure 1). Within each reactor well is a scaffold containing numerous channels all of which are coated prior to each experiment with an extracellular matrix. The medium is pumped from the reservoir well into the reactor well and is reoxygenated by flowing over a shallow surface channel before returning to the reservoir well (Domansky et al., 2010).

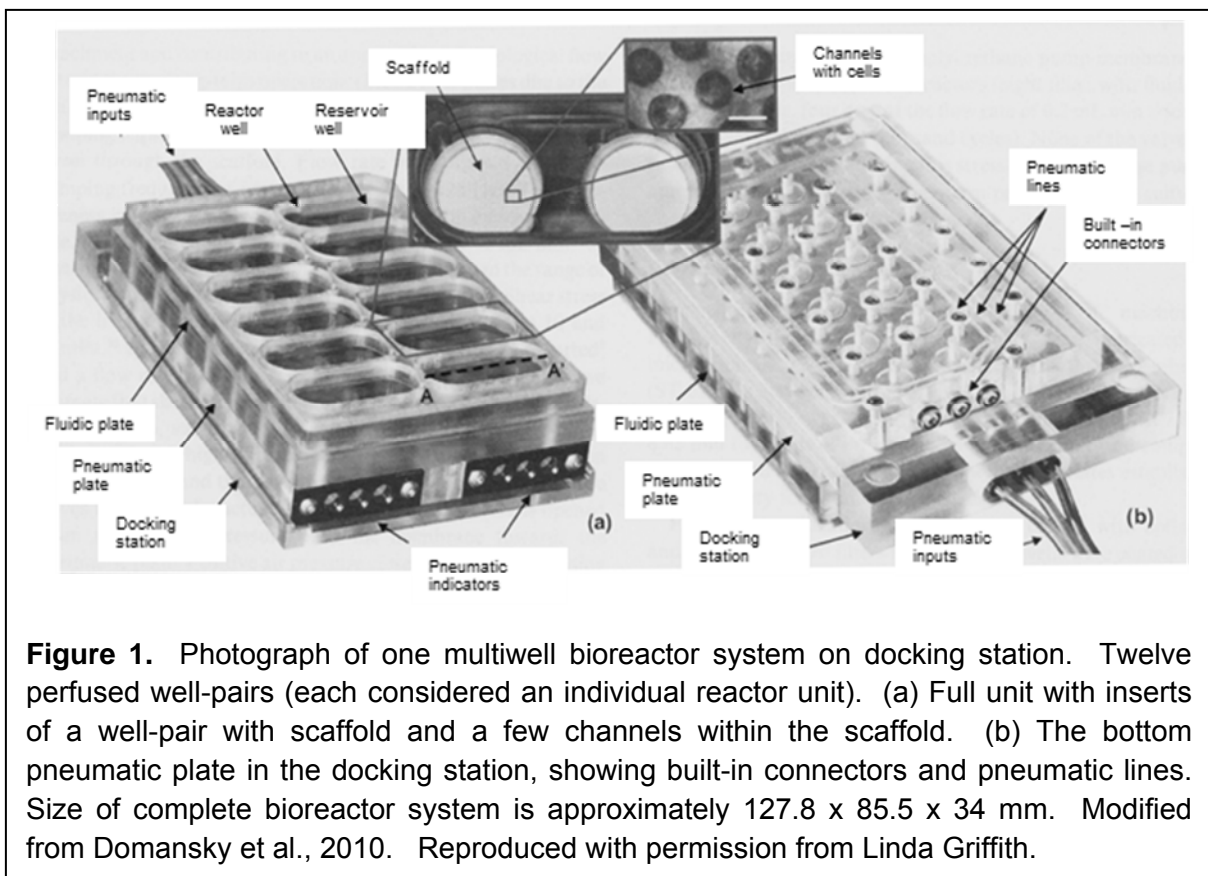
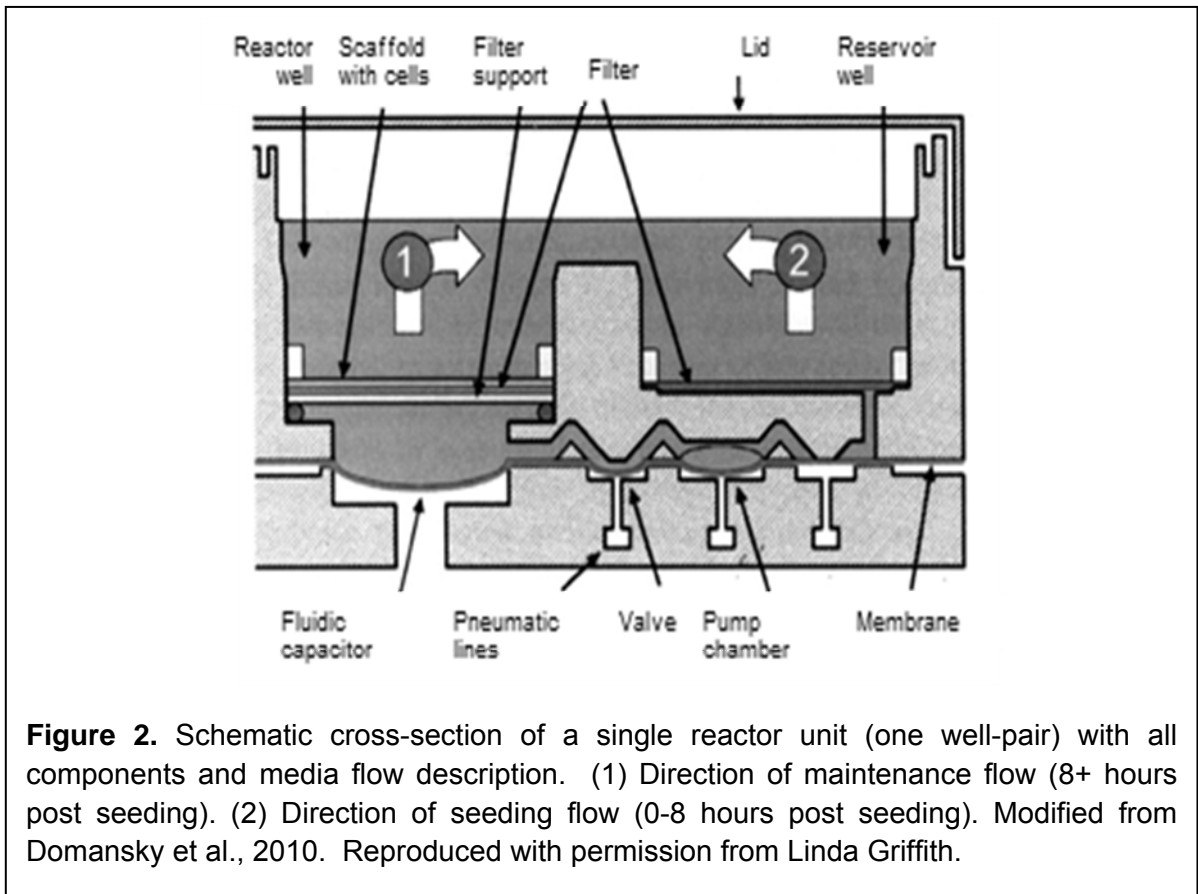


Figure 1. Photograph of one multiwell bioreactor system on docking station. Twelve perfused well-pairs (each considered an individual reactor unit). (a) Full unit with inserts of a well-pair with scaffold and a few channels within the scaffold. (b) The bottom pneumatic plate in the docking station, showing built-in connectors and pneumatic lines. Size of complete bioreactor system is approximately 127.8 x 85.5 x 34 mm. Modified from Domansky et al., 2010. Reproduced with permission from Linda Griffith.

The system is composed of two plates – a clear acrylic bottom plate and a yellow polysulfone fluidic top plate. The bottom plate functions as the pneumatic plate; the vacuum

and air are pumped through small channels in the plate. The diaphragm pumps built into the plate are controlled by an electro-pneumatic controller located outside the incubator. A very thin polyurethane membrane separates the fluidic plate from the pneumatic plate and prevents the medium from entering the pumps. The top fluidic plate has 12 pairs of wells which are fluidically isolated from one another. The top of the fluidic plate is molded to allow for a normal 2D multiwell plate lid to fit, thus preventing contamination of the wells.



The scaffold well of each well pair contains a scaffold placed on top of a hydrophilic PVDF filter, polysulfone filter support, and viton O-ring and all are held in place by a polypropylene retaining ring (Figure 2). There is also a PVDF filter in the reservoir well. The scaffold filter is used to trap the cells within the channels of the scaffold immediately upon downward flow at seeding. After the flow is reversed, any unattached cells will be trapped in

the reservoir well filter. During the remainder of the experiment dead cells that detach from the scaffold will be trapped on the reservoir well filter as well.

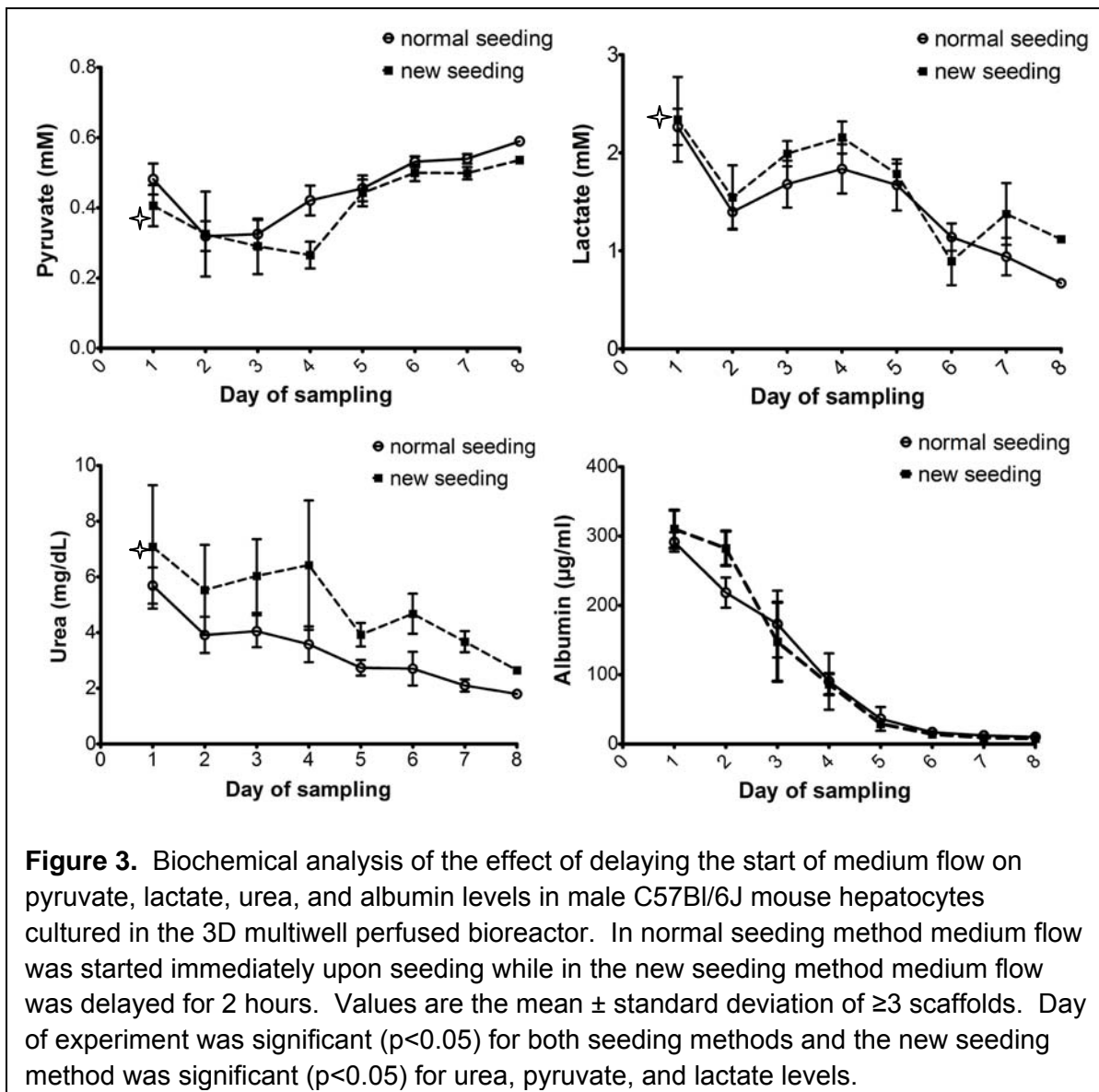
For each experiment, all components, except the acrylic pneumatic plate, are autoclaved (or, for polystyrene scaffolds, sterilized under UV light). The two plates are secured together, by 30 assembly screws and washers, to a pre-specified torque. Each bioreactor is then flow tested to make sure the plates are properly secured and that all well pairs are functioning properly and within range. Scaffolds were coated with an extracellular matrix and air bubbles were removed from the channels. All components are loaded into the bioreactors; the bioreactors are filled with medium and then placed into the incubator. The pumps are set and flow of medium continues for a minimum of 12 hours before medium is replaced with fresh medium and cell suspensions are seeded. Twenty-four hours after initial cell seeding, filters in the reservoir wells are removed and replaced.

PART III: Determining Operating Conditions for the 3D Multiwell Perfused Bioreactor

Evaluation of Changes in Seeding Method

Cellular attachment to the extracellular matrix is an important factor in functional activity in long-term cultures. Proper attachment leads to re-establishment of cell polarity which has been suspected to result in positive cell effects such as increased viability and maintenance of proper morphology (Dunn et al., 1989). For this reason, we examined the possibility that the downward flow immediately after seeding could be preventing the cells from establishing the necessary orientation and attachment required for proper functionality.

First we examined how the timing of the downward flow could impact the hepatocyte cultures. We evaluated the start of flow at two timepoints: seeding cell suspension into the multiwell bioreactor and immediately starting downward flow vs seeding cell suspension into the multiwell bioreactor and starting downward flow after two hours. For both multiwell bioreactors, as normal, media was changed four hours post seeding. Flow direction was changed to upwards flow 8 hours post seeding for the first bioreactor and 10 hours post seeding for the second bioreactor (see Appendix C). Albumin excretion and urea synthesis was evaluated daily. Two-way ANOVA was used to examine significance of the seeding



method and day of experiment. Figure 3 demonstrates that day of sampling had a significant effect on the albumin, urea, lactate, and pyruvate values ($p < 0.0001$). The new seeding method was significantly ($p < 0.0001$) better than the normal seeding in terms of urea synthesis, but it did not produce a significant effect on albumin production. The new seeding method was also significant in producing higher lactate ($p = 0.0034$) and lower pyruvate ($p = 0.0017$) levels.

Next, we evaluated a different method of seeding using a more concentrated cell suspension (and a lower amount of media – 0.5mL) and leaving the cultures static for 2 hours before adding more media and starting the downward flow or starting upward flow after 2 hours. These two methods were compared to the normal seeding of adding 1mL cell suspension and immediately beginning downward flow (see Appendix C). Analysis showed that day of experiment had a significant effect on lactate, pyruvate, and urea levels for each seeding method ($p < 0.0001$; Figure 4). The seeding method using concentrated cells and beginning with an upward flow 2 hours post seeding was significantly higher in terms of pyruvate levels ($p = 0.0387$), but not for lactate or urea production ($p = 0.1728$ and 0.3458 respectively).

Evaluation of Changes in Seeding Density

The impact cell density has on hepatocyte function has been studied in monolayer culture. Mouse hepatocytes were shown to prefer a lower seeding density of $1.0\text{--}1.25 \times 10^6$ cells per well than that of human or rat (1.75×10^6 cells per well; using 6 well plates) and were more sensitive to high seeding densities (Swift and Brouwer, 2010; Wolf et al., 2008; Yue et al., 2009). The hepatocytes showed an increase in cell death and decreased bile canaliculi network formation in high density seeding methods (Swift and Brouwer, 2010). For this reason, we examined the effect a lower seeding density may have on hepatocyte functionality in the multiwell bioreactor system.

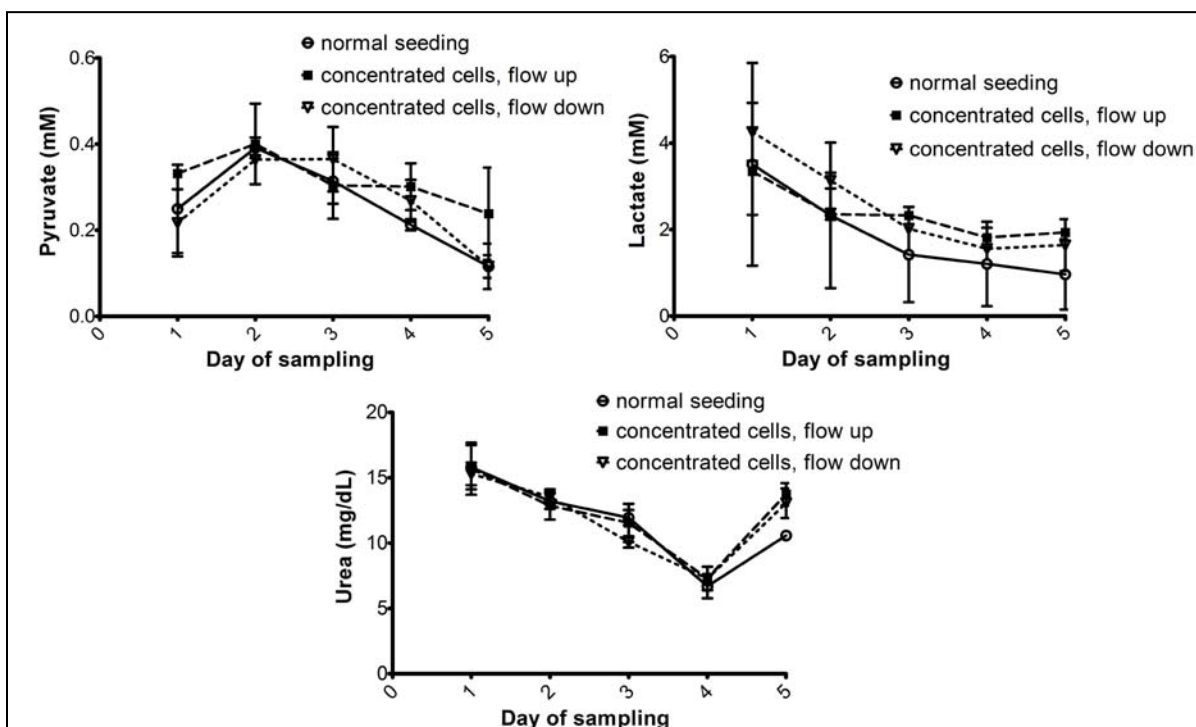
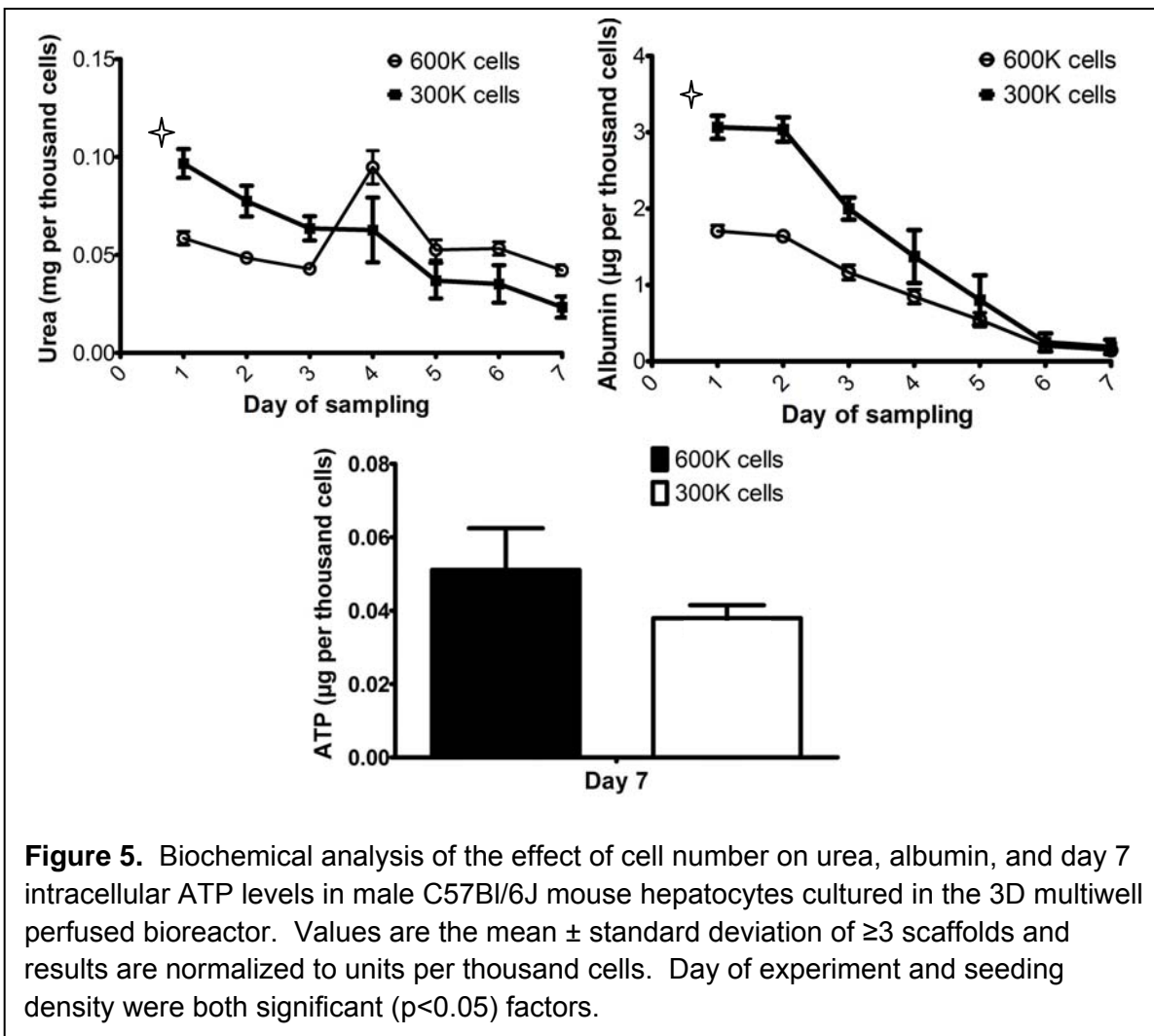


Figure 4. Biochemical analysis of the effect of seeding method on pyruvate, lactate, and urea levels in male C57Bl/6J mouse hepatocytes cultured in the 3D multiwell perfused bioreactor. Values are the mean \pm standard deviation of ≥ 3 scaffolds. Day of experiment was significant ($p < 0.05$) for all seeding methods and the concentrated cells with upward flow 2 hours post seeding is method was significant ($p < 0.05$) for pyruvate levels.

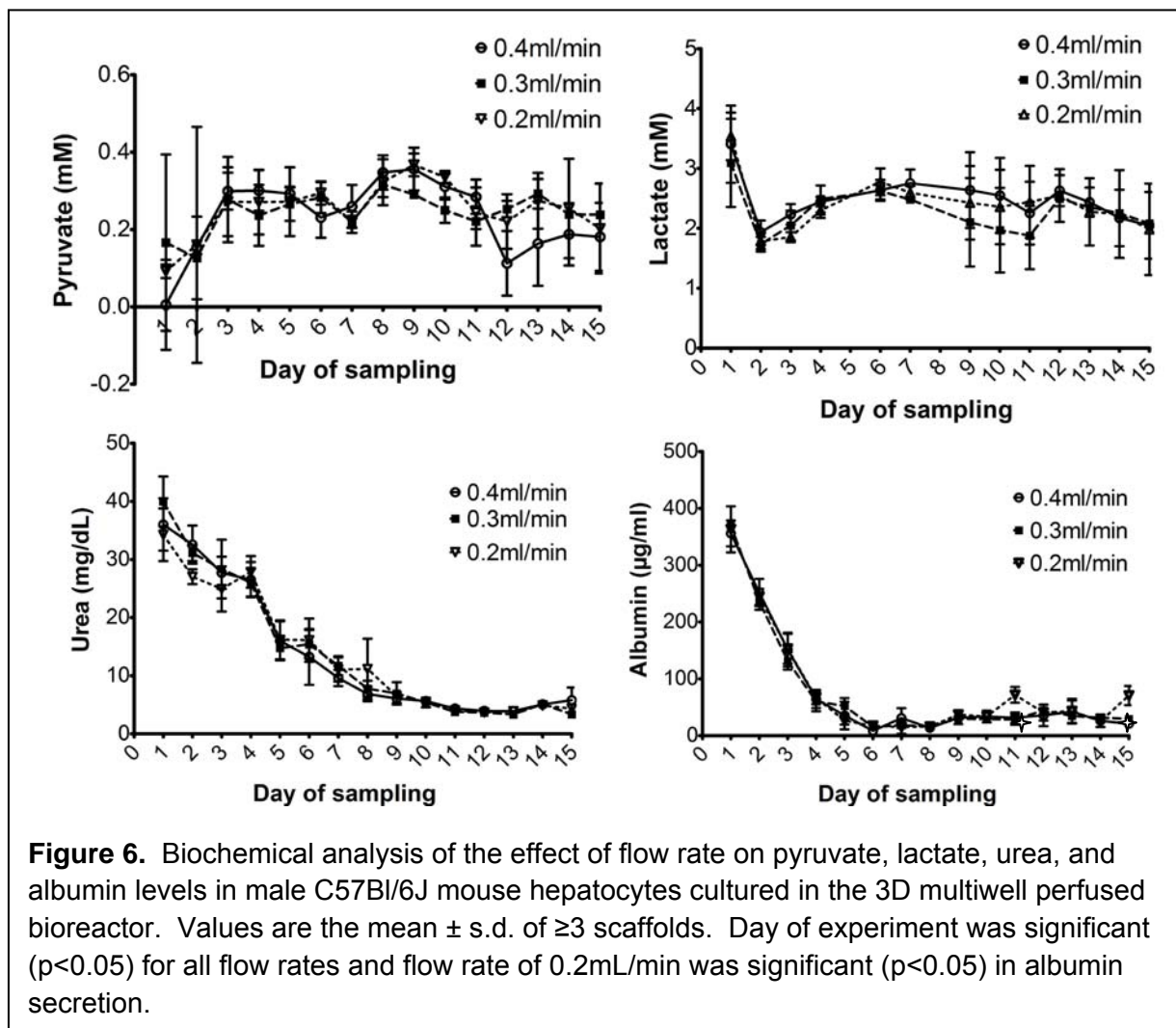
Initial experiments evaluating cell seeding density in the multiwell bioreactor systems had already been completed prior to my entry into the project. These previous experiments examined seeding densities of 0.25, 0.5, 0.6, 0.8, and 1.0 million cells per reactor well. Results (not shown) indicated that 0.6 million cells per reactor well allowed for high attachment efficiency and cell viability. This density was, from that point forward, to be considered “normal” seeding density. One experiment attempted to evaluate cell health with a lower seeding density with the hypothesis that the high seeding density may restrict available oxygen as well as increase cell death within the tissue formations. To determine the validity of this argument, an experiment comparing two seeding densities (0.3 and 0.6 million cells per reactor well) was conducted. By evaluating the urea production and albumin secretion per thousand cells, a 2-way ANOVA test showed that both day of

experiment as well as cell density were significant factors ($p < 0.0001$; Figure 5). The urea production was initially higher in the lower seeding density wells, but over time became less than that in the higher density wells. The albumin secretion was much greater at the first three days of the experiment for the lower cell density, however by the end of the experiment the levels were nearly identical. ATP on day 7 was examined for each cell density. The seeding density of 300,000 cells per bioreactor well had a lower amount of ATP per thousand cells but the difference was not significant (t-test $p = 0.1286$).



Evaluation of Flow Rate

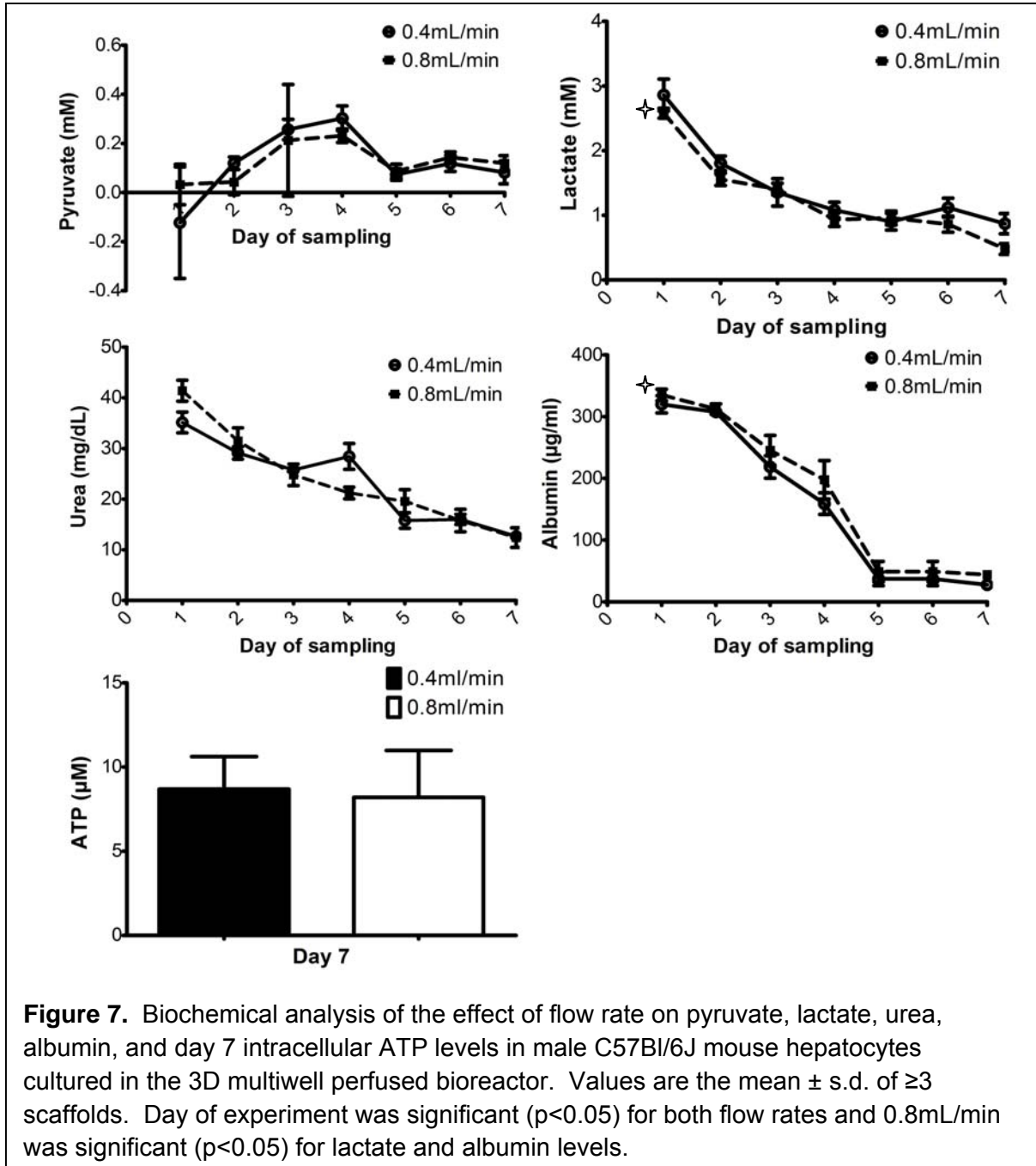
Flow rate of approximately 0.25 mL/min was previously established for rat hepatocytes in the multiwell bioreactor system (Domansky et al., 2010). This rate was determined to allow an inlet concentration within the physiologic range of *in vivo* hepatocytes as well as produce a gradient through the tissue by controlling outlet concentrations to be almost hypoxic (Domansky et al., 2010). For rat hepatocytes flow rate had been optimized to limit shear stress and provide adequate oxygenation to the cells. Flow rates had not been fully evaluated with mouse hepatocytes, thus we examined whether the more metabolically active mouse cells required a higher flow rate than that used for the



Secondly, we evaluated the difference between flow rates of 0.4 and 0.8 mL/min.

Figure 7 shows that day of experiment produced a significant effect in terms of lactate and pyruvate production, urea synthesis, and albumin secretion (2-way ANOVA $p < 0.0001$).

Flow rate had a significant effect on lactate production and albumin secretion (2-way ANOVA $p < 0.0001$), although effects were opposite. Measurements indicated that the higher



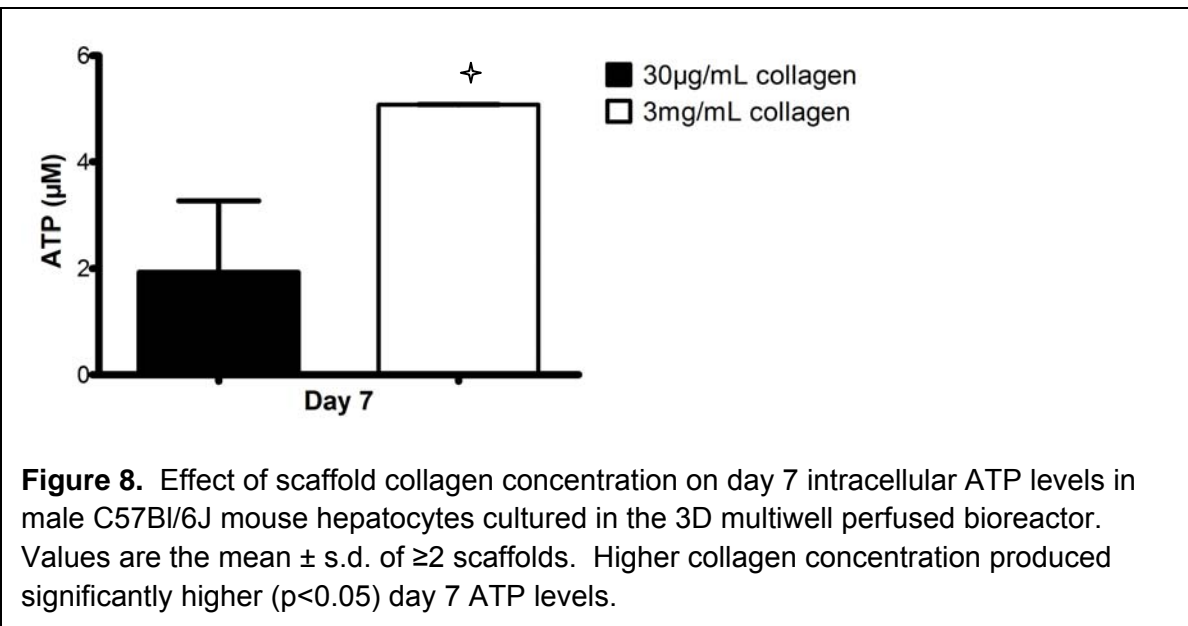
flow rate consistently resulted in lower lactate and higher albumin levels. Flow rate was not a significant factor for pyruvate production or urea synthesis ($p=0.7867$ and $p=0.1854$, respectively). Intracellular ATP production, measured on day 7 of the experiment, showed no significant difference between the two flow rates ($p=0.8175$).

Evaluation of Changes in Scaffold Matrix

The effects that different extracellular matrices have on hepatocyte cultures has been studied and reviewed in depth (Skett, 1994; LeCluyse et al., 1996; Griffith and Swartz, 2006). Two matrix configurations have been established which are considered the best monolayer culture conditions available at this point in time – single Matrigel® layer and sandwich culture (collagen or Matrigel®) (Bader et al., 1992; Dunn et al., 1989; LeCluyse et al., 2000; Liu et al., 1999; Dunn et al., 1991). The matrix used in previous experiments for rat hepatocytes in our multiwell bioreactor system, and has shown to support increased urea synthesis and albumin production, is a single collagen layer (30µg/mL) (Domansky et al., 2010). The scaffolds are soaked in this coating prior to being placed into the bioreactor wells. A single layer of collagen has been shown to be quite inadequate when culturing hepatocytes in 2D, however, resulting in poor morphology, and faster decline in P450 expression (LeCluyse et al., 1996; LeCluyse, 2001; Richert et al., 2002, Meng, 2010; Dunn et al., 1989; Bader et al., 1992). Thus, experiments were performed to determine if a more complex matrix would increase hepatocyte functionality as well as increase longevity of cultures. The majority of protocols for coating plates with collagen describe using a collagen concentration of 1 to 3mg/mL (Vinken, 2006; LeCluyse et al., 2000; Beken, 1998). We first compared 30µg/mL to 3mg/mL collagen concentrations. Subsequent experiments also compared a 2mg/mL Matrigel® coating.

In the first experiment, the 3mg/mL collagen appeared to significantly increase the amount of urea produced by the cells (even though overall urea levels began much lower

than normal and then increased to day 4, opposite normal trends) (data not shown). Based on this, additional wells were added to subsequent experiments to determine if this was a repeatable observation. Urea production was slightly higher again compared to 30 μ g/mL collagen, however albumin excretion did not differ between the two conditions (data not shown). ATP production on day 7 showed a significant difference between the two conditions, namely the higher collagen concentration resulted in higher ATP production (Figure 8). When photomicrographs of the wells were examined (Figure 9), however, wells containing the higher collagen matrix concentration appeared to have retained a larger number of cells which could account for the higher ATP levels (as levels per cell may not be different). Noticeably, all scaffolds had lower than normal number of cells by culture day 7.



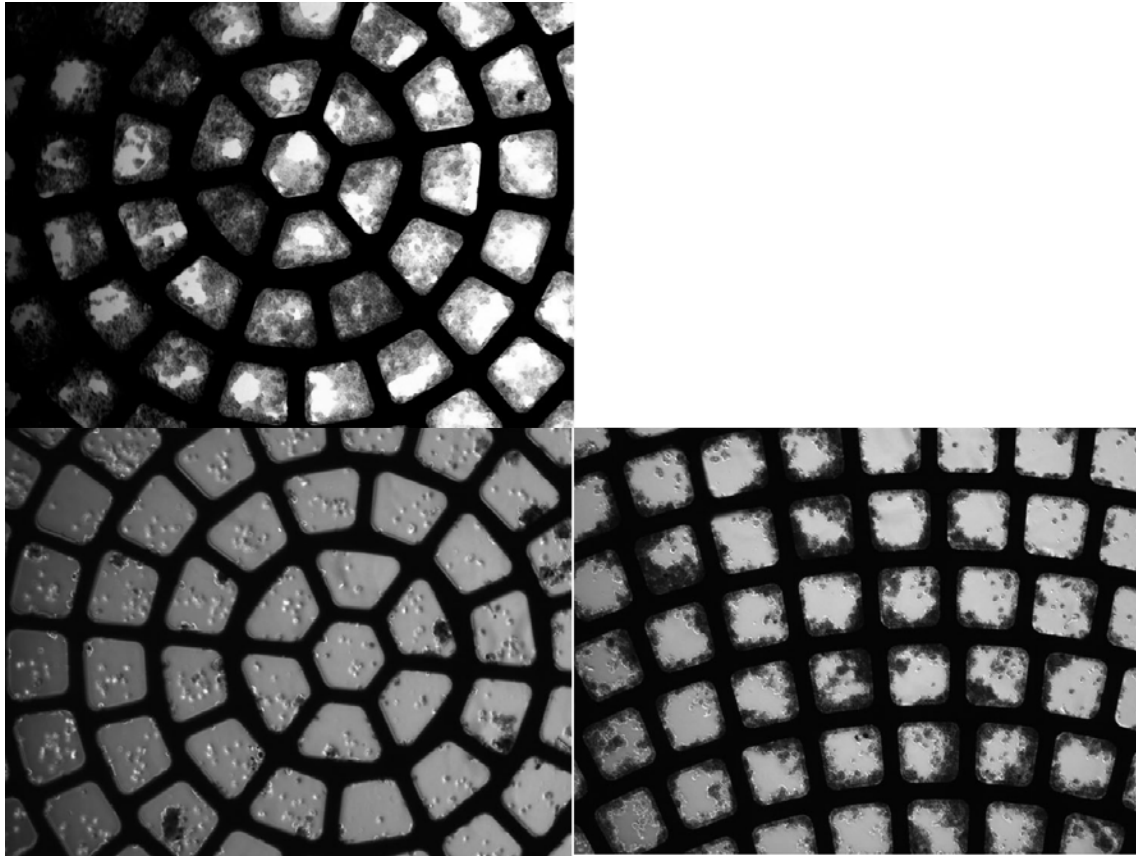


Figure 9. Comparison of bioreactor scaffolds on days 1 (above) and 7 (below) of culture. The number of cells was much greater on culture day 1 (representative picture for both ECM conditions) than on day 7. On day 7 of culture, scaffolds coated with 30 µg/mL collagen matrix (left) have less cells than those coated with 3 mg/mL collagen matrix (right) which may have led to an imprecise assessment of intracellular ATP levels between the two ECM conditions.

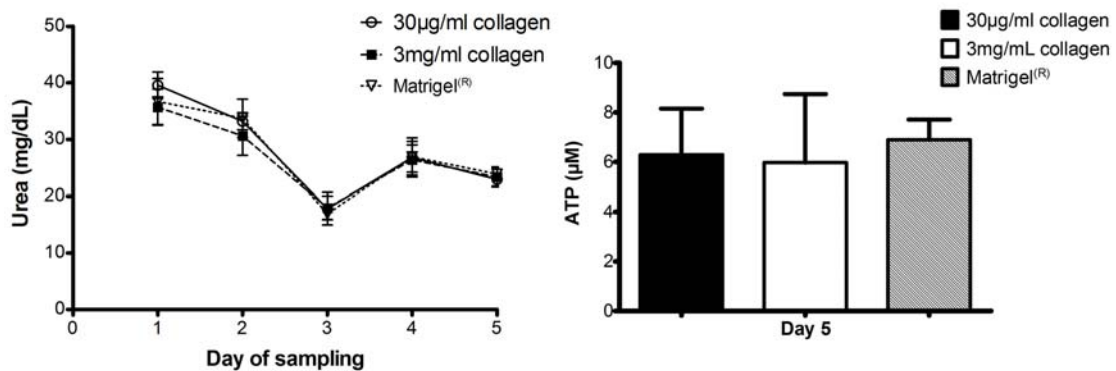


Figure 10. Comparison of urea synthesis and day 5 intracellular ATP levels among scaffold matrices (30 µg/mL collagen, 3 mg/mL collagen, and 2 mg/mL Matrigel®). Male C57Bl/6J mouse hepatocytes were cultured in the 3D multiwell perfused bioreactor. Values are the mean ± s.d. of ≥3 scaffolds. Day of experiment was significant ($p < 0.05$) for urea levels of all matrix conditions.

A repeat of the experiment demonstrated the same trend in urea production. Another experiment compared matrix configurations of 30 μ g/mL collagen to 3mg/mL collagen and 2mg/mL Matrigel®. Urea synthesis and day 5 ATP production did not differ among the matrix conditions (Figure 10).

These results were not expected due to the known benefit of matrigel gel on 2D cultures. Photomicrographs of scaffolds were taken to assess visual morphology as well. Again, many scaffolds had lost a majority of cells by day 5 (Figure 11). Tissue structure within scaffold channels coated with 2mg/mL Matrigel® mainly consisted of cells forming a ring along channel walls with a central opening for the passage of medium. This

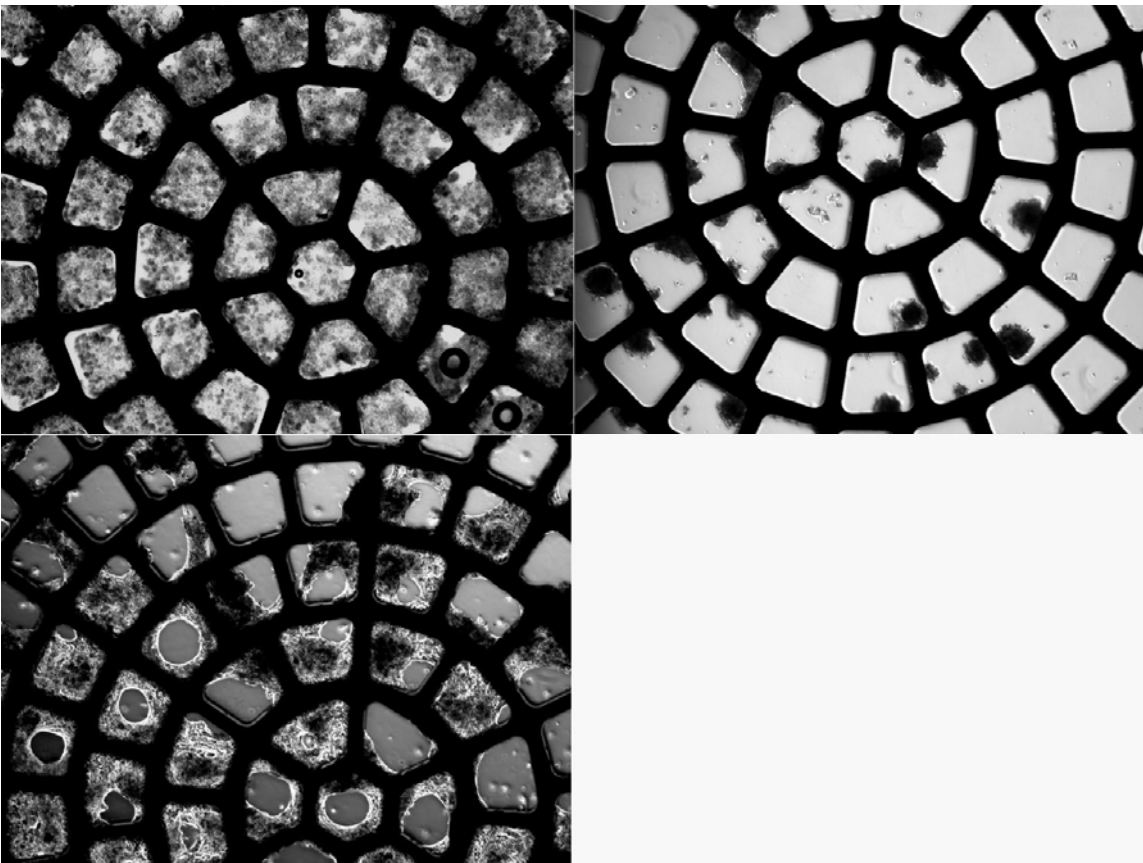
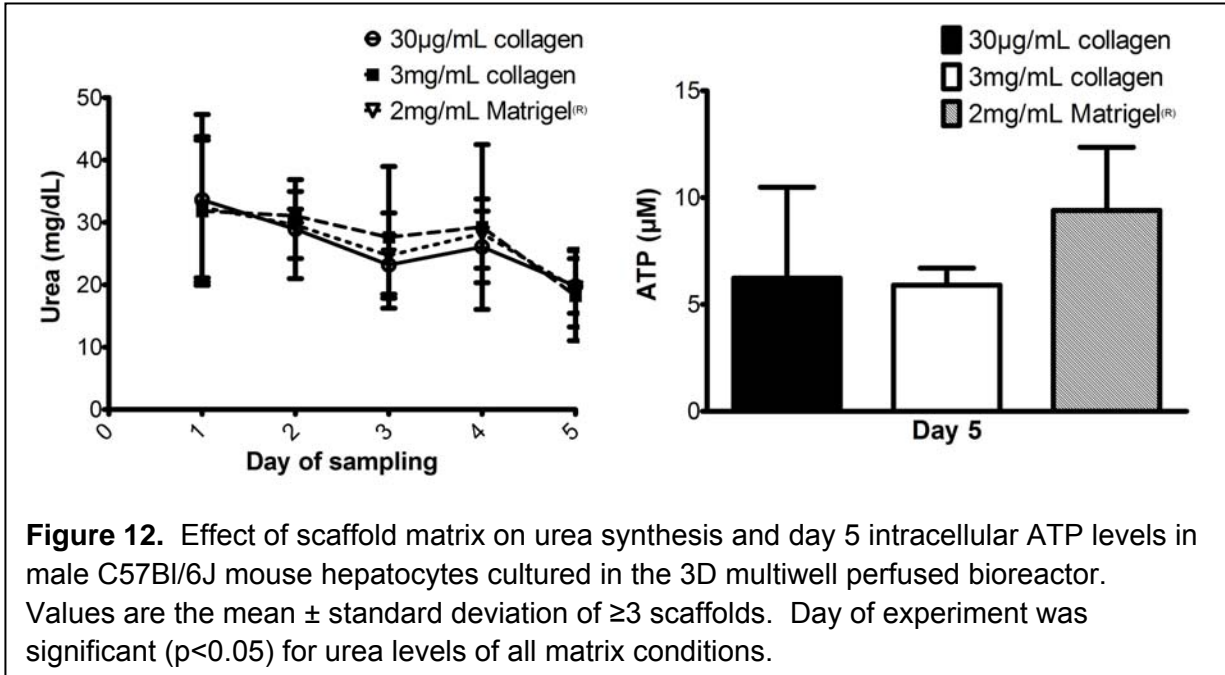


Figure 11. Two separate scaffolds coated with 2mg/mL Matrigel® on culture day 1 (top left) and day 5 (top right). Note that scaffolds have fewer cells on culture day 5. For the duration of the experiment, tissue morphology within the channels of many scaffolds coated with 2mg/mL Matrigel® resembled in vivo structure with cells gathered around a central opening that allows for movement of fluid (bottom).

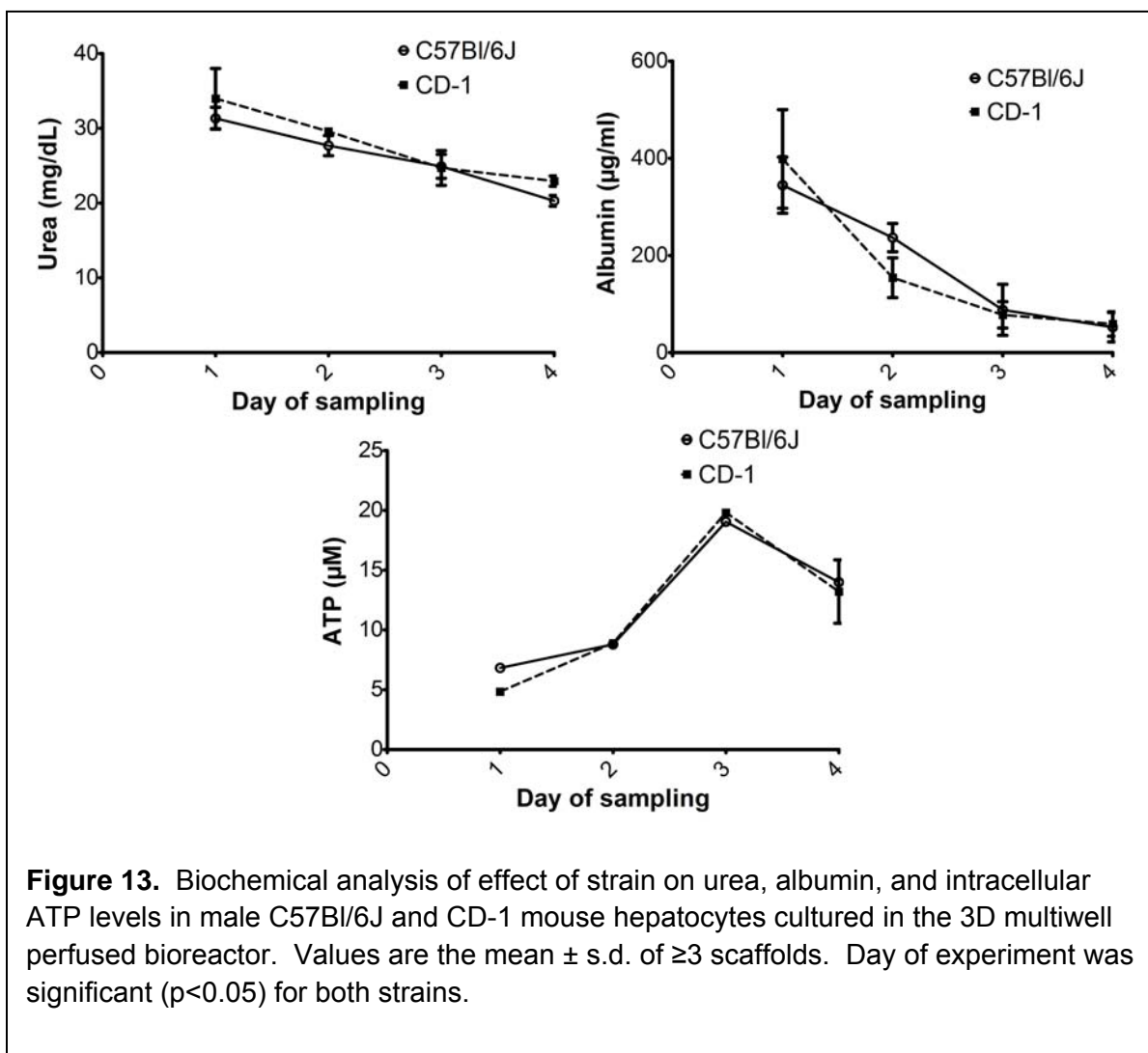
morphology occurred for the duration of the experiment.

Combination of experimental results indicated that the Matrigel® scaffold coating could have a slightly beneficial function as indicated by ATP values, although urea production seemed to remain unchanged (Figure 12).



Evaluation of Mouse Strain

Two strains were compared to ensure that the lack of increased functionality and longevity of the mouse cultures in the 3D bioreactor system was not a strain specific phenomenon. CD-1 mice were compared to the standard C57Bl/6J mice being used for all experiments. Two experiments were performed, and results of biochemical assays of both showed no change in albumin secretion, urea synthesis, or ATP production between the strains (Figure 13). Two-way ANOVA showed that day was a significant factor in determining urea synthesis, albumin secretion, and ATP production ($p = 0.0002$, $p < 0.0001$, and $p = 0.0421$ respectively). Strain was not a significant factor in any of the three biochemical assays.



Evaluation of Media Composition

Media composition has been the topic of numerous publications and general formulations for hepatocyte culture have been in use for years. Soluble factors such as serum, pyruvate, proline, glutamine, EGF, insulin, and glucocorticoids have all been shown to confer benefit to hepatocyte cultures (Tushnet and Mueller, 2006; Dunn, 1991; Kidambi et al., 2009; Klaunig et al., 1981; Tomita et al., 1995; Tamaki, 2005; Talamini, 1998; LeCluyse et al., 2000; Dunn, 1989; Laishes, 1976; Yamada 1980; Runge-Morris, 1998). Non-physiological chemical substances have also been found to sustain hepatocyte function in

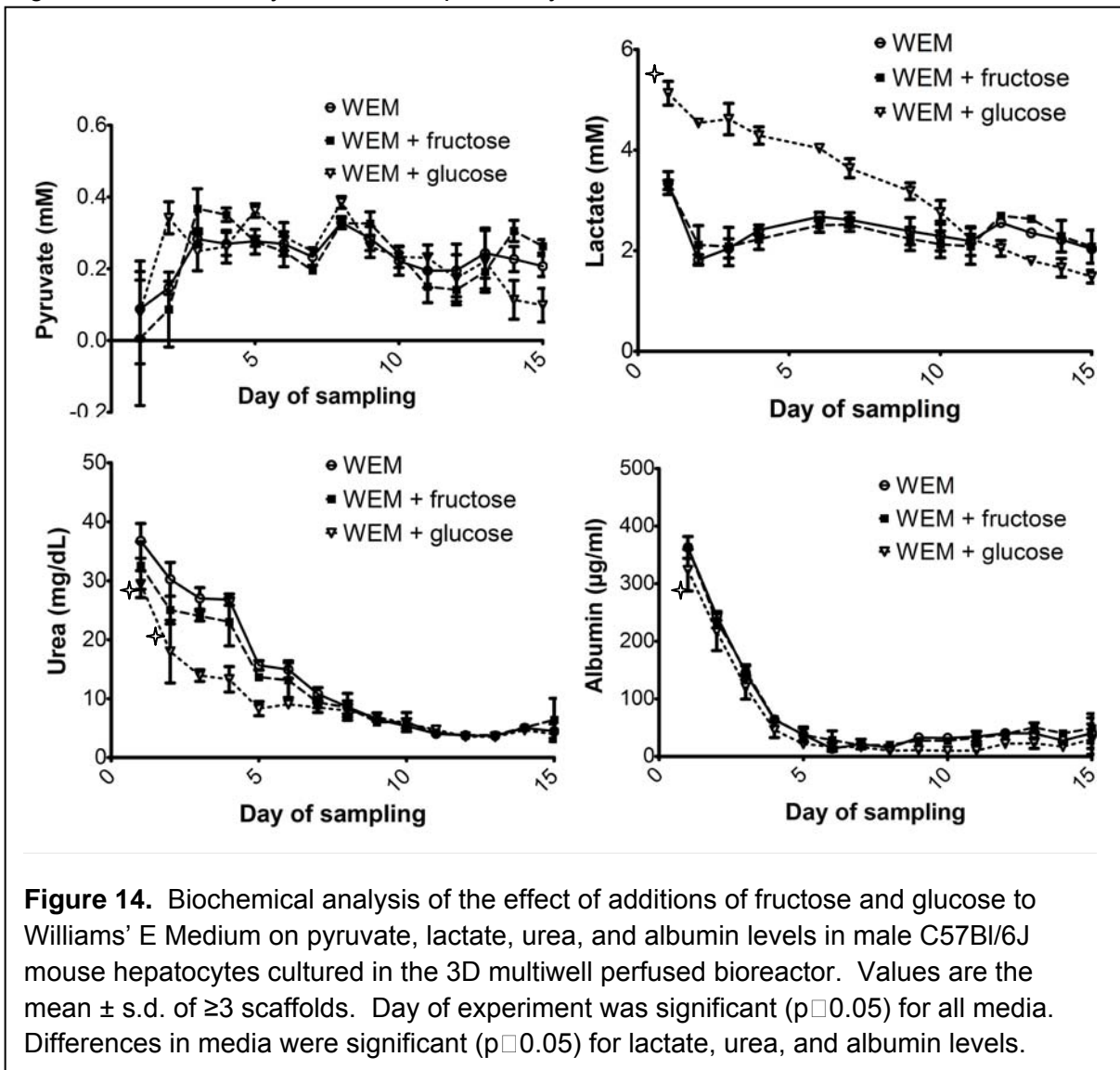
culture. Such substances include nicotinamide, metapyrone, selenium, dimethyl sulfoxide (DMSO), and Phenobarbital (LeCluyse, 1996; Nemoto et al., 1995; Rogiers et al., 1990; Kost and Michalopoulos, 1991). DMSO is perhaps one of the best known chemical modulators for hepatocyte culture and has been shown to stabilize liver-specific functions in hepatocytes, however its method of action is not well understood (LeCluyse et al., 1996; Parafita et al., 1983; Kost and Michalopoulos, 1991). For our purposes, media additions were always restricted to physiological substances (a single exception is our normal media composition containing selenium) as it is our opinion that chemical additions necessary to sustain a functional *in vitro* system is a poor model for toxicological predictivity.

Evaluation of Media in Two-Dimensional Culture

Numerous media experiments were primarily performed in two-dimensional hepatocyte cultures as the multiwell bioreactor system is limited in the number of culture conditions it is able to evaluate at one time (data not shown). The primary basal medium used was Williams E Medium (WEM), however a few other basal media were tried. In addition to the normal supplements, other additions included an amino acid mixture (L-tryptophan, 0.02mg/mL; L-phenylalanine, 0.1mg/mL; L-methionine, 0.05mg/mL), MEM non-essential amino acids solution (1% v/v), D-glucose (20mM, 100mM), fructose (1mM, 10mM), nicotinamide (10mM), FBS (5%), lactic acid (0.5, 1.0, 2.0, 4.0, 6.0, 15mM), sodium pyruvate (additional 1.0, 2.0, 15mM), EGF (10ng/mL), and ITS (double normal concentration). Dulbecco's Modified Eagle Medium (DMEM) and DMEM-F12 were also tried as basal media with the same supplements we normally add to the WEM. Of the 2D media combinations evaluated, only those showing possible positive effects were experimented with in the bioreactor system. These media additions included D-glucose (20mM), fructose (1mM), lactate (1mM, 2mM), and MEM non-essential amino acids (1% v/v). The effects produced by substituting basal medium DMEM for WEM were also examined.

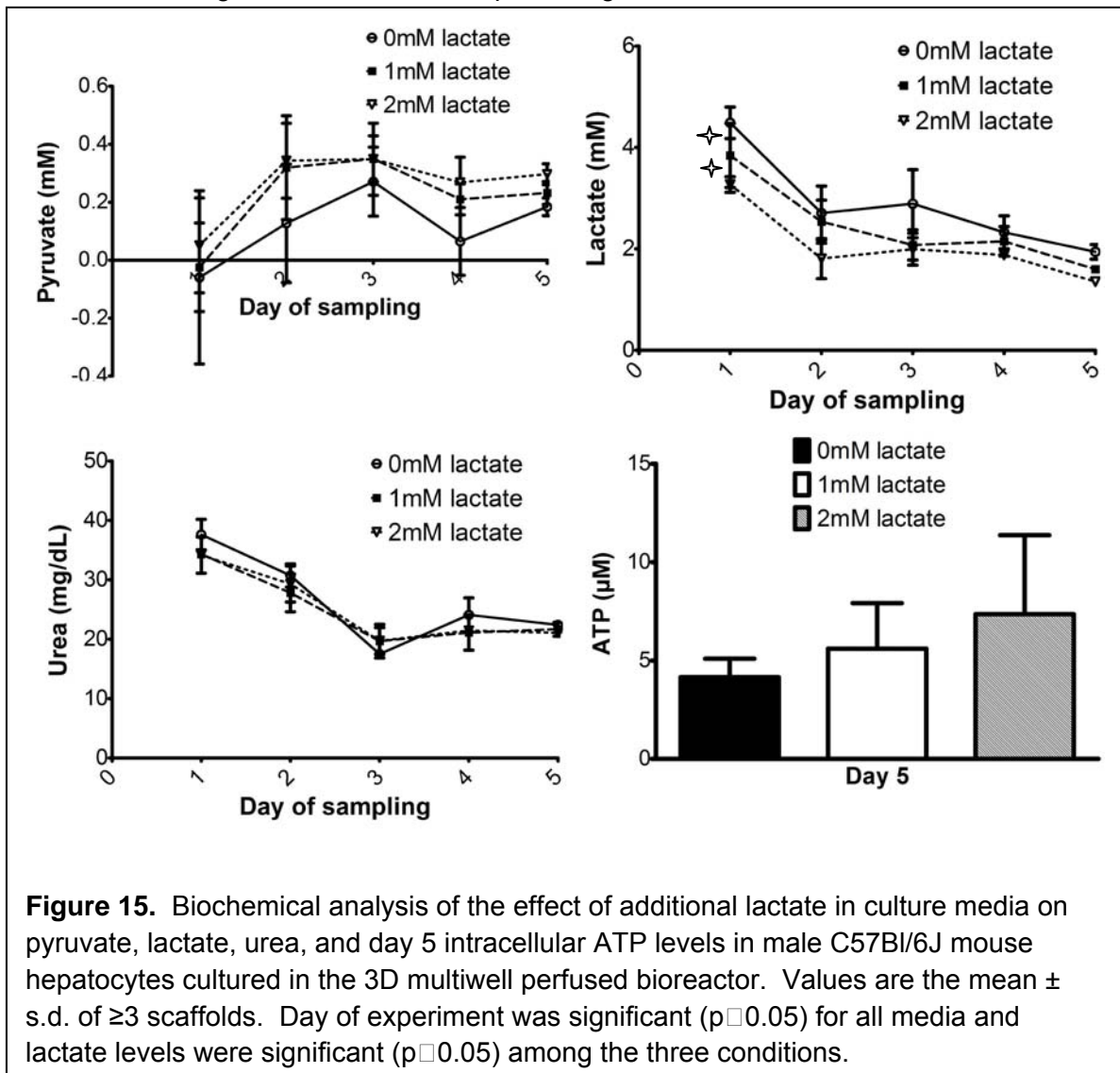
Evaluation of Media in Three-Dimensional Culture

We first examined the addition of D-glucose (20mM) and fructose (1mM) to the culture medium. We found significant differences in biochemical assay results. Day of culture was a significant factor in assay values, as we have demonstrated in the other culture conditions (Figure 14). Media was a significant factor in albumin secretion, urea synthesis, and lactate levels ($p < 0.0001$, $p = 0.0001$, and $p < 0.0001$ respectively). Cells that were fed with media supplemented with D-glucose had lower albumin secretion and urea synthesis levels throughout the whole culture periods. Lactate levels in these cells were higher as well until day 11, at which point they became lower than the other culture



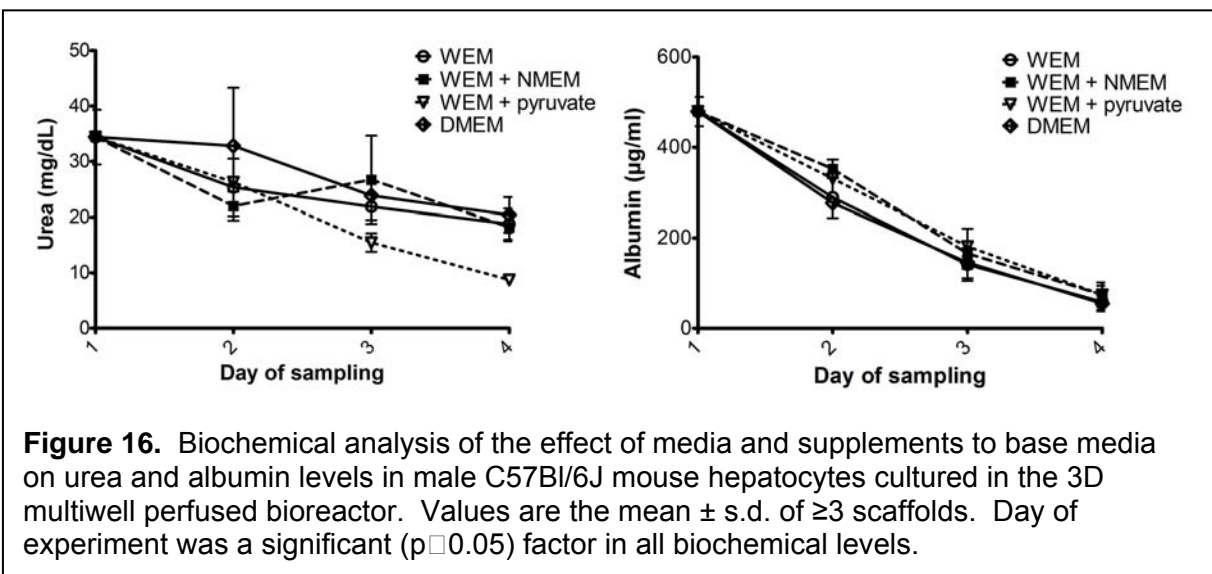
conditions. Pyruvate levels did not vary significantly among the media conditions ($p=0.9383$). The cells fed with media containing fructose did not vary significantly in biochemical analysis from cells fed with regular media except in urea synthesis where levels were significantly lower for the majority of the culture period ($P=0.0015$).

When we evaluated the addition of lactic acid to the media, no significant difference was seen in urea or pyruvate between the cells which received increases of 0mM, 1mM, or 2mM lactic acid to the media ($p=0.1733$ and $p=0.0937$ respectively; Figure 15). A significant difference was seen between the lactate levels in the three conditions ($p=0.0234$), with cultures receiving additional lactic acid producing lower amounts of lactate. Albumin was



not measured in this experiment. On day 5 of the experiment, ATP measurements varied greatly among all the scaffolds but no significant difference was found between the different lactate conditions (1-way ANOVA $p=0.3837$).

Next, we examined the addition of MEM non-essential amino acids (NMEM) or sodium pyruvate (final concentrations of 1% v/v and 20mM respectively) to our basal medium as well as the use of Dulbecco's Modified Eagle Medium (DMEM) as the basal medium. In this experiment, within the multiwell bioreactor system, day was a significant factor in both urea synthesis and albumin secretion, however media composition was not a significant factor in either biochemical assay ($p<0.0001$ for day and $p=0.0698$ for urea and $p=0.1190$ for albumin; Figure 16). Although the decrease in urea seen in the cells fed with the addition of sodium pyruvate to the medium is significant if days 3 and 4 are analyzed separately, the overall analysis lacks significance if the values are compared to the WEM group ($p=0.0520$).



Discussion and Summary of Optimization and Culture Conditions to Use in Treatment and Co-culture Experiments

Three-dimensional culture systems have been under development for years. Many show promise in terms of increasing liver-specific functions and extending culture longevity. The multiwell perfused bioreactor system produced in the Griffith laboratory at MIT has successfully extended the culture period as well as increasing functionality for rat and human hepatocytes (Powers et al., 2002; and Griffith Lab, MIT, unpublished data). Mouse hepatocytes are known to be difficult to culture, often de-differentiating in as little as three days of culture when non-physiological factors such as DMSO are not available (Mathijs et al., 2009). We evaluated the effects that changes in bioreactor parameters may have on the culture of mouse hepatocytes to establish a set of parameters that would cause the hepatocytes to not only increase in functionality but also extend the differentiated state for more than a few days. Pyruvate levels were used as a measure of cellular respiration and lactate levels were used as a measure of anaerobic status. Urea synthesis occurs as a result of the metabolism of nitrogen-containing compounds and is unique to the liver thus allowing it to be a marker of liver function. Albumin secretion is another liver-specific process which can be used as a marker of synthetic functionality. Intracellular ATP levels are commonly used as a measure of cell viability and general health. These measurements were all used to determine the optimal culture conditions for mouse hepatocytes in the bioreactor system.

Day of experiment was consistently a significant factor in the decrease in biochemical functionality. This was expected in two-dimensional culture due to previous experiments within our laboratory (Martinez et al., 2010), however it was not expected to be as significant in the three-dimensional multiwell bioreactor system. We had anticipated that one, or more, of the changes in operating conditions would decrease the change in liver-specific functions over time, resulting in a lack of significance of day of experiment.

Experiments conducted examining seeding method and density evaluated the time and direction of flow of media as well as a lower seeding density per scaffold. None of the changes resulted in significant increases in cell functionality or culture longevity. Although not statistically significant, intracellular ATP, per thousand cells, was slightly higher in the more heavily seeded scaffolds.

Channels within the scaffolds are individual functional units of the bioreactor system and represent a single capillary bed of the liver (Powers et al., 2002). The important factor of media flow in the system then determines the shear stress and oxygen availability for each “capillary bed”. A low flow rate (but not too low of a rate) would keep medium concentration within a physiologic range at the bottom of the scaffold and produce an oxygen gradient through the tissue. A flow rate that is too fast will limit the oxygen saturation of the media at the air-liquid interface of the surface channel thus decreasing the overall oxygen available in the media when it travels to the scaffold well. The reduction of available oxygen will lead to hypoxic conditions for all cells within the system. This type of flow rate will also cause shear stress in cells which has been shown to cause changes in cell function and morphology of hepatocytes (Tanaka, 2006). Flow rates of 0.2-0.4 mL/min are targeted to match those found *in vivo* and shear stress in the system is designed not to exceed 30mPa, well within the range found in liver sinusoids (Domansky, 2010; Lalor, 1999). Experiments conducted with rat liver cells showed adequate oxygenation of the media with flow rates above 0.25 mL/min (Domansky, 2010). Flow rates examined with mouse hepatocytes were 0.2, 0.3, 0.4, 0.8 mL/min. The low flow rate of 0.2mL/min showed a statistically significant increase in albumin secretion. Statistical significance resulted from two day-long increases late in the experiment period. The unexplained phenomenon of two isolated days of higher albumin production in the 0.2 mL/min flow rate bioreactor was determined to be an aberration as the increase in function was not sustained for more than 24 hours. No other biochemical assay showed a significant difference between flow rates

and this lack of significant differences was surprising considering the importance of proper flow rate in providing the cells with optimal oxygen and shear stress.

Scaffold matrix experiments indicated, primarily by intracellular ATP results, that the low collagen concentration of 30µg/mL may not be the optimal matrix condition for mouse hepatocytes in the bioreactor system. The use of Matrigel® as a scaffold matrix tended to produce a slight increase in intracellular ATP levels as well as a cellular conformation that was preferable as it more closely mimicked the physiological characteristics of hepatocytes and sinusoids. Namely, cells tended to attach to the walls of the channels and leave a circular opening in the middle of the channel for media flow.

Previous work in our lab has shown that strain differences exist in biochemical processes, gene expression, and response to chemical treatment (Martinez et al., 2010). For this reason, we tested another strain of mouse, CD-1, in the bioreactor system to confirm that the C57Bl/6J strain itself was not the cause of rapid de-differentiation. Mouse strain did not seem to affect hepatocyte functionality or longevity of cultures in the bioreactor system as indicated by biochemical assays.

Media composition does not appear to be a significant factor in how mouse hepatocytes perform in the bioreactors. Media supplementation conferred benefit in certain assays, but not others (i.e. adding glucose to the medium caused a decrease in urea and an increase in lactate production but did not affect albumin or pyruvate secretion). We saw a trend in increasing ATP with increasing lactic acid concentration in the medium, however due to variability between scaffolds the trend was not significant. Finally, changing the basal medium to DMEM did not result in any significant change in biochemical function.

Based on the results of all optimization experiments, the culture conditions for subsequent experiments were chosen either by using the parameter that gave higher functionality or, when no difference was seen, the parameter that correlated with prior experiments (ex. Flow rate; Table 1).

Seeding Method	(seed and immediately start downward flow, change media after 4 hours, change flow direction after 8 hours)
Seeding Density	600K per scaffold
Flow rate	0.4mL/min
Scaffold ECM	2mg/mL Matrigel®
Mouse Strain	Varied depending on experiment
Media Composition	WEM with additions of HEPES, Anti-Anti, GlutaMAX, dexamethasone, ITS (see Appendix A)

Table 1. Bioreactor parameters to be used for chemical treatment and co-culture experiments. Parameters were chosen based on optimization of bioreactor parameters for mouse hepatocytes.

PART IV: Evaluation of Metabolic Capacity of Mouse Hepatocytes in the 3D Bioreactor System through Use of Chemical Treatment

Experimental Design

Normal biochemical analysis revealed that the 3D multiwell perfused bioreactor system was not sufficient in itself to enhance the longevity of mouse hepatocyte cultures. We determined this through evaluation of urea production, albumin secretion, and ATP production. As in the 2D formats, mouse hepatocytes lost functionality within 3-5 days. To confirm that this loss of functionality extended to loss of metabolic capacity, hepatocyte response to treatment was evaluated using two well-known reference compounds: acetaminophen (APAP) and 4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthiol acetic acid (WY-14,643). APAP is metabolized by key cytochrome P450 enzymes and results in cytotoxicity.

The use of APAP as a reference compound allows for the evaluation of hepatocyte metabolic competence through measurement of expression levels of these cytochromes; low expression levels means loss of metabolic capacity and less cytotoxicity. Treatment with WY-14,643 provides evidence regarding the ability of hepatocytes to have metabolism induced. Maintenance of expression levels indicates cells have retained metabolic induction capabilities. The 12 wells of each multiwell bioreactor system were split into two groups – the wells of the first group were treated on day 1 and those of the second group were treated on day 3 (see Appendix D). Scaffolds were removed and examined 24 hours post treatment for cellular morphological features, intracellular ATP measurements, and RNA harvesting.

Evaluation of Hepatocyte Response to Acetaminophen in the 3D Culture System

First we wanted to confirm that the C57Bl/6J mouse strain would properly represent the ability of the bioreactor system to accommodate treatment experiments. We initially performed an experiment using both the C57Bl/6J and CD-1 strains of mice to confirm that there was no major difference in metabolic capacity between the two strains (as determined through treatment profiles) in the bioreactor system. We seeded one strain in each of two bioreactors and treated half of wells with 7mM APAP on day 1 and the second half of wells on day 3. We examined daily albumin secretion and urea synthesis as well as ATP production after treatment. For both strains of mice there was a decrease in both urea synthesis and albumin secretion on day 2 for cells treated with APAP (Figure 17). On day 4 there was no difference between non-treated and treated cells in urea and albumin levels. Both strains also showed a loss of ATP content after treatment on the first day of culture, however this loss was not seen after treatment on day two. This lack of cellular toxicity later in the culture period indicates that the hepatocytes have lost metabolic capacity by day

three. These results also demonstrate that loss of metabolic capability was similar in both strains, thus we used C57Bl/6J mice for the remainder of treatment experiments.

Subsequently, dose-dependent relationships and gene expression analysis was explored by treating C57Bl/6J mouse hepatocytes in the bioreactor system with APAP and WY-14,643. Two experiments examined the effects of APAP treatment, each performed with cells from separate isolations. Two bioreactors were seeded at the same time with cells from a single isolation. The cells in the first bioreactor were treated with APAP on day 1 and harvested for RNA and ATP on day 2. Two scaffolds were treated with plain media, media with 0.5% DMSO (the vehicle for the compounds), or one of four concentrations of APAP

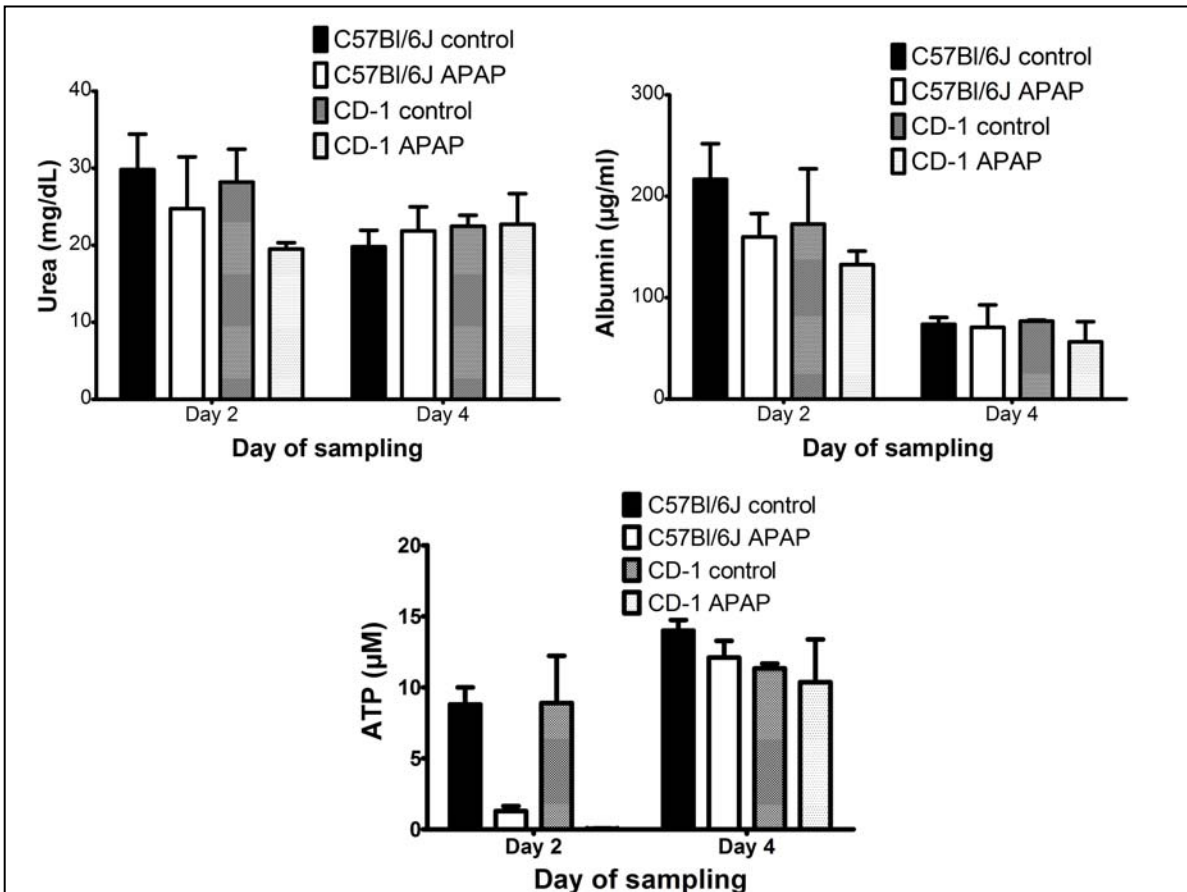
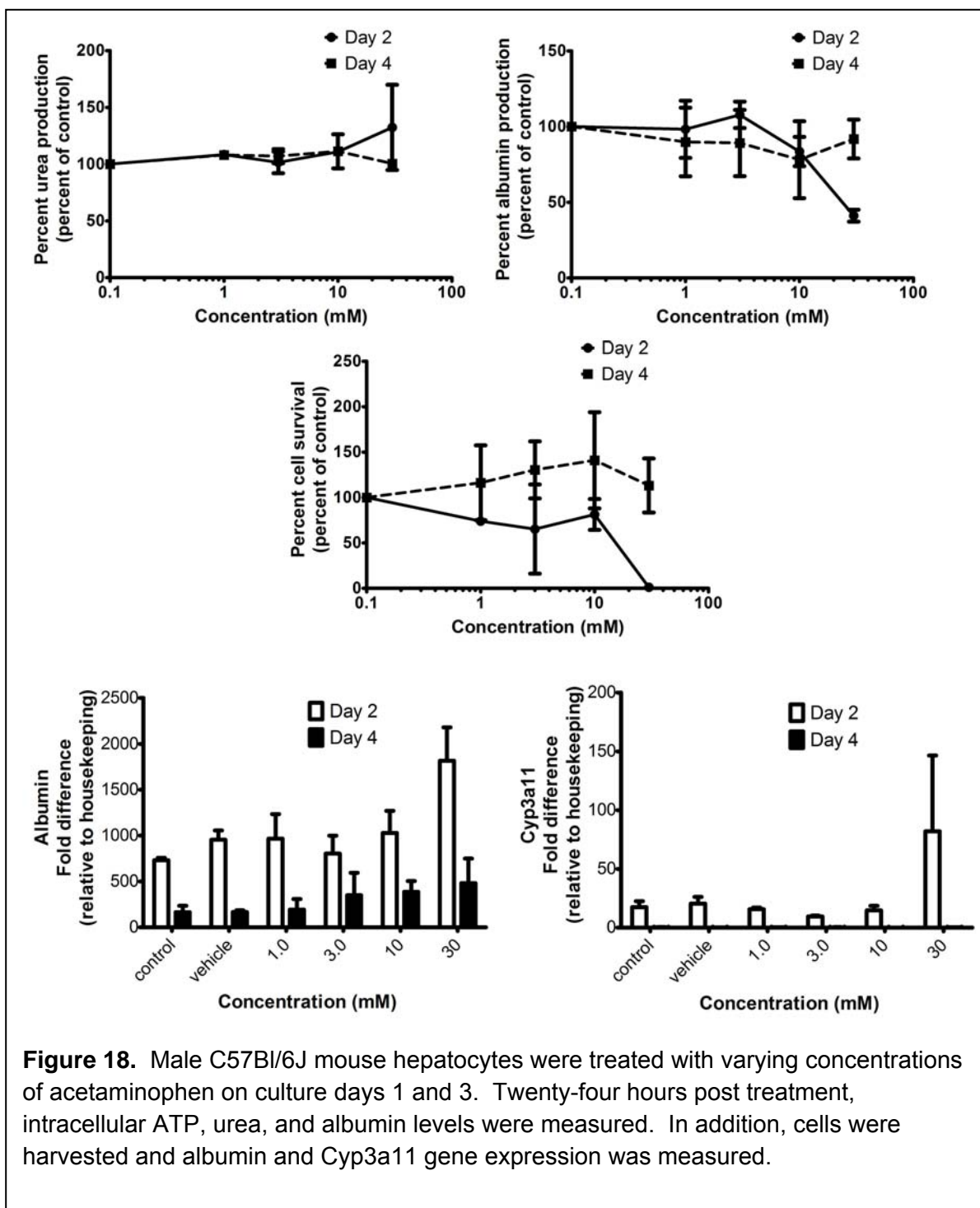


Figure 17. Urea, albumin, and intracellular ATP levels on days 2 and 4 of culture in hepatocytes of two mouse strains after treatment with 7mM APAP. Hepatocytes were isolated from male C57Bl/6J and CD-1 mice. Values are the mean \pm s.d. of ≥ 3 scaffolds. No significant change was noted between the two strains.

(1.0, 3.0, 10.0, 30.0mM). Upon harvest, one scaffold was used for RNA while the other was used to evaluate ATP levels. The second bioreactor was set up in a similar manner to the first, but treatment was not administered until day 3. Cells were also seeded onto 2D plates and treated under the same conditions as controls for cell health, morphology, and comparison to previous laboratory data (data not shown). No concentration dependent change was seen in urea production on either of the treatment days. There was a concentration dependent change in albumin secretion for treatment on day 1, but not on day 3. A concentration dependent decrease in ATP production was seen for treatment on day 1, but not for treatment on day 3 (Figure 18). The EC_{50} varied greatly between the two treatment days (from 9.603mM to well above 30mM). The decrease in ATP production indicates a loss in cell viability which positively correlates to metabolic functionality.

The scaffolds removed for RNA analysis showed a slight increase (although not significant) in gene expression at high concentrations on day 2 indicating induction of the liver specific albumin and Cyp3a11 genes. On day 4, the levels of Cyp3a11 were virtually undetectable, and expression of albumin had decreased drastically. On day 4 there was a concentration dependent increase of expression of albumin. Day was a significant factor in determining albumin expression ($p < 0.0001$) as was chemical concentration ($p = 0.049$).



Evaluation of Hepatocyte Response to WY-14,643 in the 3D Culture System

Next, the response of mouse hepatocytes cultured in the bioreactor system to treatment with WY-14,643 was evaluated. The set up of the bioreactor systems was the same as that in

the APAP experiments; however this experiment was replicated three times. Variability between scaffolds of the same condition was great, however standard deviation of data decreased with the addition of each subsequent experiment. Concentrations of WY-14,643 were 0.1, 0.3, 1.0, 3.0mM.

Figure 21 shows that day was not a significant factor in determining treatment related responses in relation to urea synthesis, however the concentration response was evident ($p=0.0258$). Neither day nor treatment concentration was a significant factor in determining albumin secretion levels. There was virtually no difference between concentrations on day 3 treatment.

Both day 1 and day 3 treatment resulted in a significant concentration dependent ATP decrease ($p=0.0359$), however there was not much change between the EC_{50} for day 1 and day 3 (1.981mM and 2.635mM respectively).

Both day and concentration were significant factors in Acox1 expression ($p=0.0234$ for day and $p=0.0584$ for concentration; Figure 19). Gene expression analysis showed a decrease in expression at the high treatment concentrations on day 2, due to cell toxicity. The expression on day 4 is not as variable among the concentrations. Gene expression for Cyp4a10 was significantly affected by day of treatment ($p=0.0157$), as is evident by the drastic decrease in expression at all concentrations from day 2 to day 4. On day 2, gene expression increased with increased treatment concentration except at the highest two concentrations and on day 4 all levels were low. Due to large variability between experiments the difference in expression was not significant ($p=0.0537$).

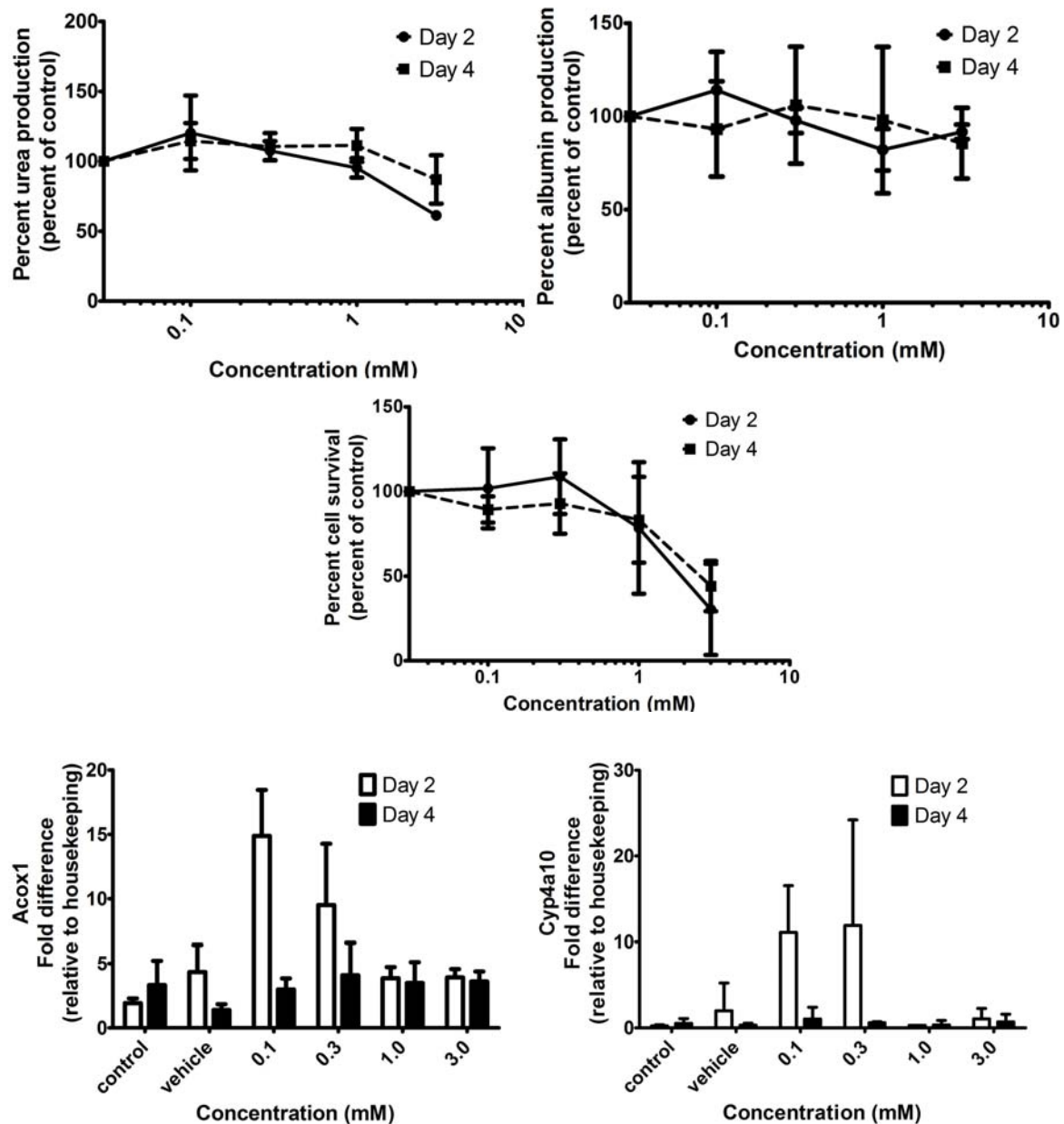


Figure 19. Mouse hepatocytes were treated with varying concentrations of WY-14,643 on culture days 1 and 3. Twenty-four hours post treatment, intracellular ATP, urea, and albumin levels were measured. In addition, cells were harvested and Acox1 and Cyp4a10 gene expression was measured.

Discussion of the Evaluation of the Bioreactor System through Use of Chemical Treatment

In our hepatotoxicity experiments, hepatocytes were isolated and allowed to adapt to the culture environment for either a 24 or 72 hour period. An adaptation period has been reported to be important in the evaluation of hepatotoxicity in culture systems as it allows the cells to adjust to and stabilize within their new environment (Mathijs et al., 2009). The two different periods of treatment afford us the ability to examine the effect of culture time on hepatocyte response to treatment. Four concentrations of each chemical were used as treatment as well as vehicle and non-treated groups in each experiment. Basic biochemical functions of urea synthesis and albumin secretion were examined in relation to treatment concentrations as was intracellular ATP production and gene expression.

Treatment with APAP resulted in an inverse relationship between concentration and intracellular ATP on day 2. As ATP content directly correlates to cell viability, and thus survival, we are able to establish a dose-dependent model of cytotoxicity with APAP treatment in the bioreactor system. On day 4, however, the response is not as well developed indicating that metabolic capacity of the cells has decreased (as noted as an increase in EC_{50} value). The EC_{50} for the hepatocytes in the bioreactor system was slightly lower than that of the corresponding 2D culture (results not shown) confirming our biochemical results indicating the bioreactor system can increase functionality for short-term culture. We saw a similar relationship to chemical concentration and albumin secretion on day 2 but not on day 4. This again indicates that metabolic functionality is lost over culture period and thus cytotoxicity is not as prevalent later in the culture period. No concentration-dependent change was seen in urea production on either day of assessment. Gene expression of both albumin and Cyp3a11 decreased greatly from day 2 to day 4 of culture, an occurrence noted previously in other publications (Mathijs et al., 2009). A slight, and

unexplained, increase in gene expression of albumin with increase in treatment concentration was seen.

Treatment with WY-14,643 produced a concentration dependent response in intracellular ATP content on both treatment days and the EC_{50} was similar for both days. This indicates that time in culture is not as significant in WY-14,643 toxicity as in APAP toxicity. EC_{50} was slightly lower in the bioreactor system than in the corresponding 2D cultures (data not shown), again indicating that a slight metabolic benefit was conferred by the 3D culture system. WY-14,643 concentration did not significantly affect albumin secretion for either culture day, however urea synthesis was concentration dependent. Expression of both Acox1 and Cyp4a10 was concentration dependent on day 2 indicating induction of these genes by treatment with WY-14,643. The low levels measured in the 1.0 and 3.0mM concentrations are due to cell toxicity. As we see a decrease in gene expression from day 2 to day 4 we know that the cells are losing their gene activity levels over the culture period even though toxicity to the chemical remains.

Thus, we have determined that the bioreactor culture system is not able to extend liver-specific gene expression in mouse hepatocytes to longer than 72 hours.

PART V: Evaluation of Biochemical Function and Metabolic Capacity of Co-cultures in the Bioreactor System

Experimental Design

Co-cultures of hepatocytes with other cell types have been the subject of investigation for decades. Numerous cell types from many different areas of the body have been cultured with hepatocytes including fibroblasts (Langenbach, 1979; Michalopoulos et al., 1979), endothelial cells (Morin and Normand, 1986; Kim and Rajagopalan, 2010), and cell lines (Mendoza-Figueroa. 1988; Donato, et al., 1990; Talbot, 1994). Researchers have even used cells from a different species than that of their hepatocytes (Jongen, 1987;

Donato et al. 1991; Goulet et al., 1988; Griffith, 1997). These co-cultures have shown to increase and maintain liver-specific functions in hepatocytes. The focus in our lab, however, is on maintaining a similar culture environment to that of the liver *in vivo*. To do so, we examined cultures of liver non-parenchymal cells (NPCs) and hepatocytes.

Our initial experiment used cell preparations containing Kupffer, stellate, and sinusoidal endothelial cells. Two separate isolations were performed to obtain the hepatocyte and NP cell suspensions. The hepatocytes were isolated and seeded into the bioreactor system prior to the isolation of the non-parenchymal cells. The NPCs were seeded approximately 2 hours after the hepatocytes were placed in the multiwell bioreactors (see Appendix E for the flow diagram). The number of hepatocytes seeded into each scaffold was decreased to 450,000 cells so as to not overload the scaffolds with cells and to allow for comparisons between conditions. Wells of hepatocytes alone contained 450,000 cells; wells containing hepatocytes and NPCs were seeded with 450,000 hepatocytes and either 49,500, 193,500, or 450,000 NPCs (equaling ratios of 10:0, 9:1, 7:3, and 1:1 hepatocytes to NPCs).

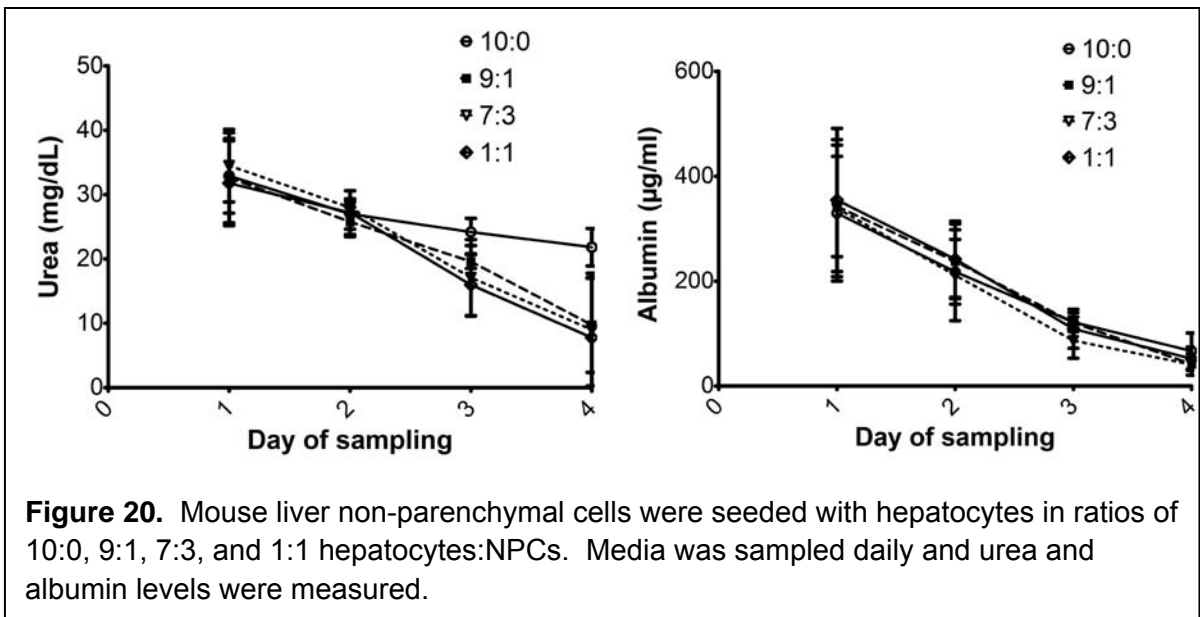
Biochemical Analysis and Chemical Treatment of Co-cultures in the 3D System

Flow cytometry revealed the non-parenchymal cell populations to consist of, on average, 32% endothelial cells, 37% Kupffer cells, and 69% stellate cells (although the actual stellate percentage was most likely much lower, the high percentage due to non-specific binding of our antibody; Table 2). The average yield of NPCs per isolation was 11.4 million. Data is expressed as the mean and standard deviation of the three experiments, each experiment using two or more scaffolds per condition. No difference in hepatocyte urea or albumin production was demonstrated with the addition of non-parenchymal cells ($p=0.0684$ and $p=0.9184$ respectively; Figure 20).

Experiment #	Total cells (visual count)	% cells of flow count that are usable -NPCs	CD31 (endothelial cells)	F4/80 (Kupffer cells)	GFAP (stellate cells)
1	18 million	22%	41%	32%	44%
2	13 million	12%	26%	41%	94%
3	3.3 million	No cells available for flow analysis			

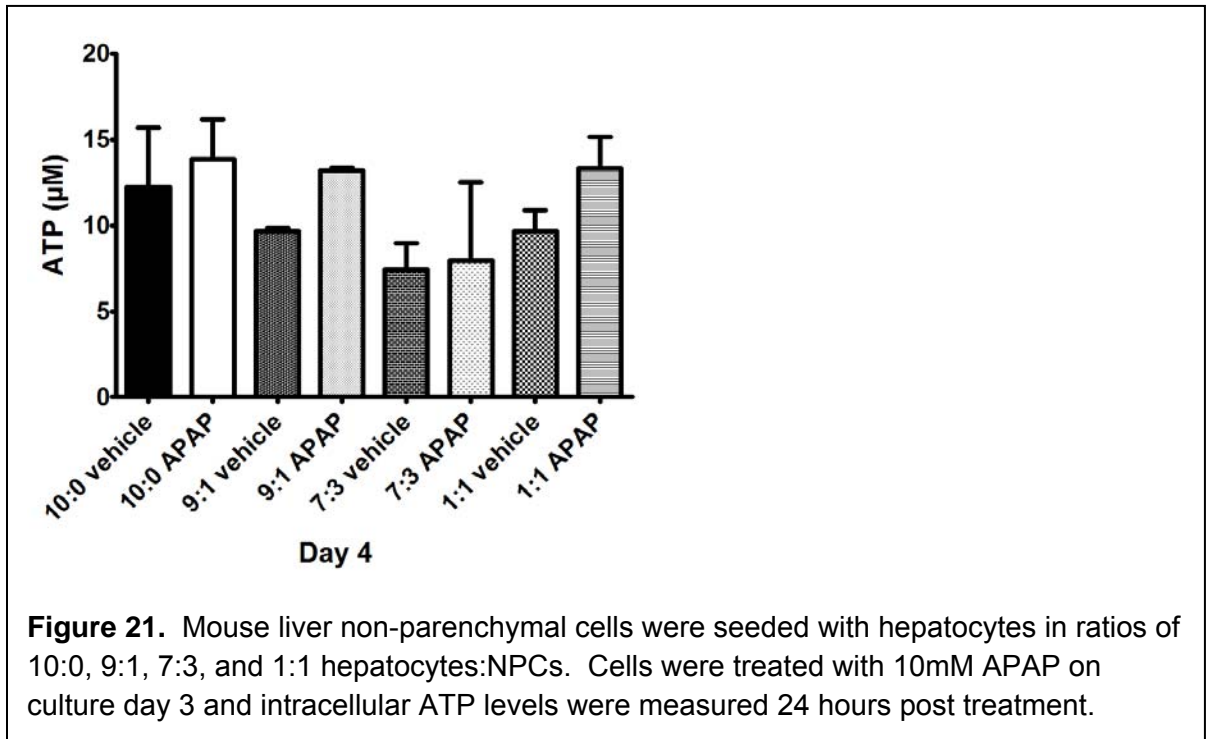
Table 2. Non-parenchymal cell isolation flow cytometry results.

As demonstrated, mouse hepatocytes within the multiwell bioreactor system lose metabolic functionality by culture day 3. This loss of functionality can be measured by the lack of cellular toxicity in response to treatment with acetaminophen. To determine if the presence of non-parenchymal cells could extend the metabolic functionality of our hepatocytes through culture day 3, we treated our co-cultures with 10mM APAP. Cells were treated with 10mM APAP on day 3 of culture by methods used in previous hepatocyte



culture experiments. Intracellular ATP was measured 24 hours post treatment, on culture day 4. The addition of the other liver cell types did not result in maintenance of enzyme

function; there was no decrease in cell viability after treatment (Figure 21). The increase in viability seen in all treatment groups (although not significant in most groups) is unexplained.



Discussion of Co-culture Experiments

Many different cell types have been cultured in conjunction with hepatocytes and were found to enhance functionality and lengthen culture period. Previous research has indicated that hepatocytes, when cultured with other liver cells, maintain higher levels of phase I and II metabolizing enzymes (Rogiers et al., 1990; Conner et al., 1990; Rogiers and Vererusse, 1993), are able to maintain levels or slow the rapid decline of albumin secretion (Guguen-Guillouzo, 1983; Mesnil, 1987), have an increased tolerance to oxidative stress (Mertens et al., 1993), and maintain other liver-specific functions.

Our experiments indicate that the addition of Kupffer, stellate, and sinusoidal endothelial cells to hepatocyte cultures do not increase or maintain liver-specific functions such as albumin secretion and urea synthesis in the multiwell bioreactor system. There is a drastic decrease in both liver-specific functions within the first three days of culture. Neither

does the addition of non-parenchymal cells to the culture system result in a maintenance or induction of metabolizing enzymes. After treatment on the third day of culture there is no decrease in ATP, thus no loss of cell viability, indicating a lack of metabolic capacity of the hepatocytes.

Results of numerous experiments demonstrate co-cultures increase hepatocyte functionality and viability, therefore we expected to see an increase in at least one of our measured parameters (urea synthesis, albumin secretion, or metabolic capacity). Although we cannot positively identify the cause of our negative results, we attribute our results to crude non-parenchymal cell suspensions, variable cell quantities, and sub-optimal culture conditions. Our non-parenchymal cell isolation procedure leaves us with a suspension of non-parenchymal cells, red blood cells, and cell debris. Although the debris and red blood cells should not hinder attachment of our NPCs to our scaffolds or hepatocytes, we cannot fully discern the impact such a crude preparation may have on our cultures. Our flow cytometry data indicate that our cell population in this final suspension consists of a large number of stellate cells. Since the liver is comprised of only 15% stellate cells (van Berkel, 1979) and recovery of these cells through isolation procedures commonly yields only 10% of the population (Alpini et al., 1994), we expected stellate cells to comprise less than 5% of our NPC population. We attribute the high percentage of stellate cells to non-specific binding by our antibody, GFAP; we saw a significant decrease in number of stellate cells when we titrated the antibody (data not shown). Therefore, we cannot give a precise account of the cell distribution within our NPC preparations and we must question our flow cytometry results. Elimination of the majority of events measured as stellate cells from our flow cytometry analysis leaves a substantial number of events which compose the NPC population but are not positive for any antibody, leaving us with an “unknown” population of non-parenchymal cells.

After we concluded our experiments we determined that our culture conditions were not optimal for the survival of the different non-parenchymal cells. For example, investigators have demonstrated that sinusoidal endothelial cells require serum to maintain viability; vascular endothelial cell growth factor (VEGF) and other paracrine factors influence survival as well (Braet et al., 1994; Krause et al., 2000; DeLeve et al., 2004). Although experiments have shown that in a three-dimensional system with flow co-cultures of hepatocytes with SECs can result in survival of the endothelial cells without the use of serum and VEGF (Hwa et al., 2007), this has not been established with mouse liver cells. Perhaps mouse SECs require the use of serum or VEGF even in the presence of hepatocytes and other non-parenchymal cells. Our results indicated that co-cultures of mouse liver cells are not beneficial to the hepatocytes, however due to the many variables in our non-parenchymal cell cultures we cannot conclude this with certainty and further experimentation is required.

CHAPTER 4

Discussion, Limitations, and Future Directions

Discussion

A significant effort has been made in the last few years by research and regulatory agencies, such as the NTP and NRC, to promote the use of *in vitro* assays and decrease the use of *in vivo* work. *In vitro* liver models are valuable in drug and chemical safety testing as well as for mechanistic research. A variety of *in vitro* liver models exist; liver tissue slices, microsomes, isolated perfused livers, immortalized hepatic cell lines, and suspensions and cultures of primary hepatocytes. Cultures of primary hepatocytes have been considered the best of these options, however long-term culture of fully functioning cells has been difficult to obtain. For this reason, much effort has been spent throughout the years defining optimal culture conditions and new culture systems for hepatocytes.

It has been difficult to develop functional hepatic tissue *in vitro* and much time has been spent over the last two decades attempting to engineer a proper system which would recreate the microenvironment of the liver, allow tissue formation similar to that of *in vivo*, retain liver-specific functionality, and maintain cellular viability. In an ideal system the cells would mimic physiological states and may include different cell types, allowing for experimentation in liver tissue replacement or toxicological and pharmacological studies.

Our multiwell bioreactor system incorporates specialized scaffolding, which allows for three-dimensional tissue constructs, and dynamic flow of medium to establish a physiologically relevant culture model that can be used for study of hepatic pathophysiology and chemical toxicity evaluation (Powers et al., 2001). The system improved functionality of

rat and human liver cells as well as extending the life of the cultures (Domansky et al., 2010; Griffith laboratory, MIT, unpublished data). Our goal was to optimize this system for mouse hepatocytes for use as multi-strain toxicogenetic models of hepatotoxicity. Our experimentation determined that this system could, in fact, increase functionality of mouse hepatocytes for an initial period of three to five days, however functionality could not be maintained over long-term culture.

In order to determine the optimal culture conditions for mouse hepatocytes within the bioreactor system, we examined basic parameters such as seeding method, seeding density, flow rate, scaffold extracellular matrix, and media composition and their effect on biochemical functions in the hepatocytes. Most of these parameters have been studied extensively in traditional two dimensional hepatocyte culture, although not often using mouse cells. Minor differences in initial functionality were seen for some of the parameters, however long-term differences were not significant. None of the changes in parameters could stop or even slow the process of murine hepatocyte de-differentiation that typically occurs after three to five days in culture. Minimal experimentation was performed combining changes in more than one parameter; however even data from those experiments did not show significant differences in cell functionality or culture longevity (data not shown).

As evident in previous experiments (Martinez et al., 2010), time in culture is a significant factor in biochemical functionality of hepatocytes. Over the culture period, cells cannot maintain stable lactate and pyruvate production and they lose their ability to secrete albumin and synthesize urea. This downward trend in functionality was consistent throughout all optimization experiments. Reproducibility between experiments, in terms of albumin and urea secretion and synthesis rates, was high indicating that the cell isolations and cultures continue to be reproducible processes in our laboratory. Loss of cell functionality after day 3 of experiment is consistent with other published results examining two-dimensional mouse hepatocyte culture indicating that our culture system was at least as

effective as the traditional culture system in terms of maintaining liver-specific functions over time (Nemoto et al., 1989; Nemoto et al., 1991; Klaunig et al., 1981).

Although biochemically the bioreactor system did not seem to provide a significantly better culture system in terms of longevity, gene expression and chemical treatment were evaluated to determine if the system would benefit the mouse hepatocytes in these ways. Using the optimal (or, if differences were minimal, the easiest culture method) conditions for mouse hepatocytes in the multiwell bioreactor system, cells were treated with both acetaminophen and WY-14,643 and cellular response to these treatments was examined.

Concentration-dependent cytotoxicity (measured by intracellular ATP content) occurred with both chemicals on culture day 2. On day 4, however, only treatment with WY-14,643 resulted in a similar cytotoxicity profile. Cytotoxicity to APAP occurs as a result of metabolism of the compound into a reactive metabolite, NAPQI, by cytochromes 1a2, 2e1, and isoforms of cytochrome 3a (Zhang et al., 2002; James et al., 2003). Therefore, the lack of cytotoxicity on day 4 indicates a loss of metabolic capacity by said cytochromes. Cytotoxicity to treatment with WY-14,643 occurs as a result of the binding of the compound to nuclear receptor PPAR α leading to its translocation into the nucleus whereupon it acts as a transcription factor for genes involved in fatty acid catabolism (Ioannides, 2008). This leads to the formation of peroxisomes within the cytoplasm of the hepatocytes and can lead to cellular toxicity. The maintenance of similar toxicity on day 4 of the experiment for WY-14,643 indicates that cells have not lost the ability to have certain metabolic conditions induced. Gene expression analysis for both treatment experiments produced results indicative of loss of gene expression and inducibility of said expression over time in culture.

Curiously, for both treatments, EC₅₀ values of the corresponding 2D cultures did not correlate well with that of previous experiments performed within this laboratory (Martinez et al., 2010). One can only speculate as to the reason for the increase in EC₅₀ in these experiments when compared to those past; however differences in the location and

personnel performing the liver perfusions and cell isolations could account for the lack of correlation. The increased seeding density used in the most recent experiments may have also had an effect on metabolism.

We expected that the presence of non-parenchymal cells in our culture system would increase hepatocyte functionality. Unfortunately, we did not find an increase in biochemical or metabolic functionality and metabolism capabilities were not extended through the third day of culture. In retrospect, our co-cultures of parenchymal and non-parenchymal liver cells were not characterized fully and culture conditions were suspect. Further optimization of isolation procedure, flow cytometry analysis, and culture conditions needs to be conducted before we can conclude that mouse hepatocytes are truly unresponsive to the presence of other liver cells.

As reported previously by other authors, expression of key liver-specific and metabolic genes and functions could be retained by mouse hepatocytes for up to three days in culture (Martinez et al., 2010; Mathijs et al., 2009; Tamaki et al., 2005; Nemoto et al., 1991; Nemoto et al., 1989). We were able to show those same results in our experiments with the multiwell perfused bioreactor, however we unexpectedly found that the 3D system could not maintain expression over a longer culture period than the traditional 2D cultures. Although not entirely successful, our research is important to understanding the differences between rat and mouse hepatocytes as well as the unique requirements of mouse liver cells for successful *in vitro* culture. In addition, we have demonstrated that the multiwell perfused bioreactor system has a positive effect on mouse hepatocyte culture and continued experimentation is required to improve the system and tailor its design to the specific needs of this distinctive cell type.

Limitations

Although the multiwell perfused bioreactor system is a favorable culture system for rat and human hepatocytes, it is not ideal for mouse hepatocytes. The rapid de-differentiation and loss of hepatocyte function results in a culture system that affords only a few days of use.

Although extending longevity of mouse hepatocyte culture is one of our ultimate goals, the system has limitations that need to be addressed if the system is to replace traditional two-dimensional culture. One limitation is the variability between scaffolds. The fact that each scaffold is an independent culture system with its own flow and medium can be advantageous in that it allows different conditions to be examined within a single bioreactor; however the independence of each well is also a drawback as the variability between scaffolds undergoing the same culture conditions is great. We have shown how attachment can vary greatly between scaffolds. A large number of scaffolds is needed to reduce standard deviation which hinders the ability of this system to evaluate multiple culture conditions. The multiwell bioreactor system is also limited by the complex nature of the system. When multiple culture conditions are evaluated a greater number of scaffolds is needed to accommodate each assay and each timepoint. The amount of space, reagents, and cells required for each experiment is much greater than in the traditional 2D culture system. Consequently, if the system is not able to maintain higher levels or stabilize the liver-specific functions of mouse hepatocytes for long-term culture, then the effort and materials required make the perfused multiwell system an inefficient culture method.

Although cultures containing non-parenchymal cells have demonstrated benefits to hepatocyte function (Ries, 2000; Novik, 2010), our results did not corroborate this. Further research into specific factors needed for the survival of co-cultures indicates that our culture medium lacked components that have been shown to be instrumental in NPC survival. Our medium composition may have selectively inhibited certain cell populations within our

cultures making our co-cultures inaccurately defined. Isolation of the NPCs resulted in very impure cell preparations that contained a large amount of cell debris and red blood cells. In the future, elutriation or different perfusion methods should be employed to obtain pure preparations. A more pure preparation would allow for more consistent non-parenchymal cell yields and cell populations. The inability to visualize our co-cultures in the bioreactor system hindered our ability to properly assess our cultures. In the future, use of a specialized microscope would allow for immunostaining and imaging of cells within the scaffolds. This would be a valuable tool in assessing the number and distribution of NPCs within the culture system.

Although as currently designed, the multiwell perfused bioreactor system appears to be a complicated system which does not afford much use for mouse hepatocyte culture. Slight modifications to the system or the use of more specific cultures of non-parenchymal cells and hepatocytes could lead to production of a valuable model for liver physiology.

Future Directions

The paradigm shift from *in vivo* to *in vitro* toxicological studies necessitates the availability of predictive models, of which hepatocyte culture appears to be a viable option. The rapid de-differentiation and subsequent loss of liver-specific functionality severely limits the utility of a mouse hepatocyte culture system. The theory that the long-term cultivation of mouse hepatocytes without use of exogenous substances is an unattainable goal is incongruous with the evidence that conditions exist that support the maintenance of rat and human hepatocytes for long culture periods. The small amount of research performed using mouse hepatocytes, most likely due in part to the small number of cells obtained from each animal, is a contributing factor to our lack of knowledge about optimal culture conditions for this species. A concerted effort must be made by a number of researchers to optimize culture conditions for mouse hepatocytes. In doing so, perfusion, isolation, and culture

methods can be standardized making experimental results from laboratories all over the world more comparable.

Continued research in the areas of 3D models and co-cultures is imperative to develop a model that is able to recreate the liver microenvironment and provide predictive results for toxicological and pharmacological studies. In the near future, our lab will investigate a new multiwell perfused bioreactor system and cultures using patterned hydrogels. The new bioreactor system modifies the channel length of the current system which will directly affect dynamics between oxygen mass transfer and consumption within the system. The PEG-fibrinogen hydrogels aim to reduce global cell density and improve the extracellular matrix environment that plays an important role in hepatocyte culture. Ideally, the hydrogels will then be used in the bioreactor system to provide the flow dynamics which have been shown to increase and maintain performance of rat and human hepatocytes. An improved ECM and increased oxygen availability in the multiwell perfused system may provide an environment more conducive to mouse hepatocyte long-term culture.

A multi-strain mouse model of hepatocytes that can retain functionality long-term would be a monumental achievement and has the potential to eliminate the need for much of the current *in vivo* toxicology testing. Additionally, the availability of a model which can mimic the range of responses to a compound in the human population is desperately needed. Research in our lab has provided evidence to suggest that this type of model is capable of supplying valuable and relevant toxicity information. Not only is interindividual variation within mouse strains a viable option for examining human drug metabolism, but the rise in humanized mouse lines allows for improved risk assessment. The humanized mouse can be used in the cases where there are species differences in response to xenobiotics such as differences in P450 expression and nuclear receptor ligand binding affinities seen, among others, with human CYP3a4 and rodent CYP3a (Cheung and Gonzalez, 2008).

In conclusion, future experimentation in our laboratory with improved co-culture conditions, a new multiwell perfused bioreactor system, and an improved extracellular matrix will be important in identifying the requirements of mouse hepatocytes in culture and may lead to a useful model for toxicological and pharmacological research.

APPENDIX A

Media Formulation

Base Medium was Williams' E Medium (Sigma W1878). Supplements below were added to each 500mL of base medium.

Chemical name	Amount to add	Final concentration	Stock concentration	Order information
HEPES	5mL	10mM	1M	Fisher Scientific
GlutaMAX™	5mL	2mM	200mM	Gibco 35050-061
Dexamethasone	0.4mL	0.1µM	125µM	Sigma D8893
Insulin- Transferrin- Sodium Selenite	5mL	1.72µM 69µM 0.1µM	0.2mM 6.9mM 8.5µM	Gibco 41400-045
Antibiotic- Antimycotic (penicillin, streptomycin, amphotericin B)	5mL	100 units/mL 100µg/mL 0.25µg/mL	10,000units/mL 10,000µg/mL 25µg/mL	Gibco 15240-062

Table S1. Supplements added to each 500mL of Williams' E Medium.

APPENDIX B
GBS Formulation

The additives below were added to 1L of purified water to make a 10X stock solution of GBS
(Formulations courtesy of Dr. Lori DeLeve, USC, Los Angeles).

Chemical name	Amount to add	Order information
Potassium chloride (KCl)	3.7g	Acros Organics 196770010
Potassium phosphate monobasic (KH ₂ PO ₄)	0.3g	Sigma P5655
Magnesium chloride (MgCl ₂ ·6H ₂ O)	2.1g	Fisher Biotech BP214
Magnesium sulfate (MgSO ₄)	0.3425g	Fisher Biotech BP213
Sodium phosphate dibasic (NaHPO ₄)	1.1975g	Fisher Biotech BP332
Glucose	10g	Sigma G-7021
Sodium chloride (NaCl)	70g	Mallinckrodt Chemicals 7581-06
Calcium chloride (CaCl ₂ ·H ₂ O) (only for calcium containing GBS)	2.25g	Sigma C3881

Table S2. Formulation for 1L of 10X GBS solution.

To make a 1X solution, sodium bicarbonate was added to 10X stock GBS (with or without calcium) and brought up to 1L with purified water.

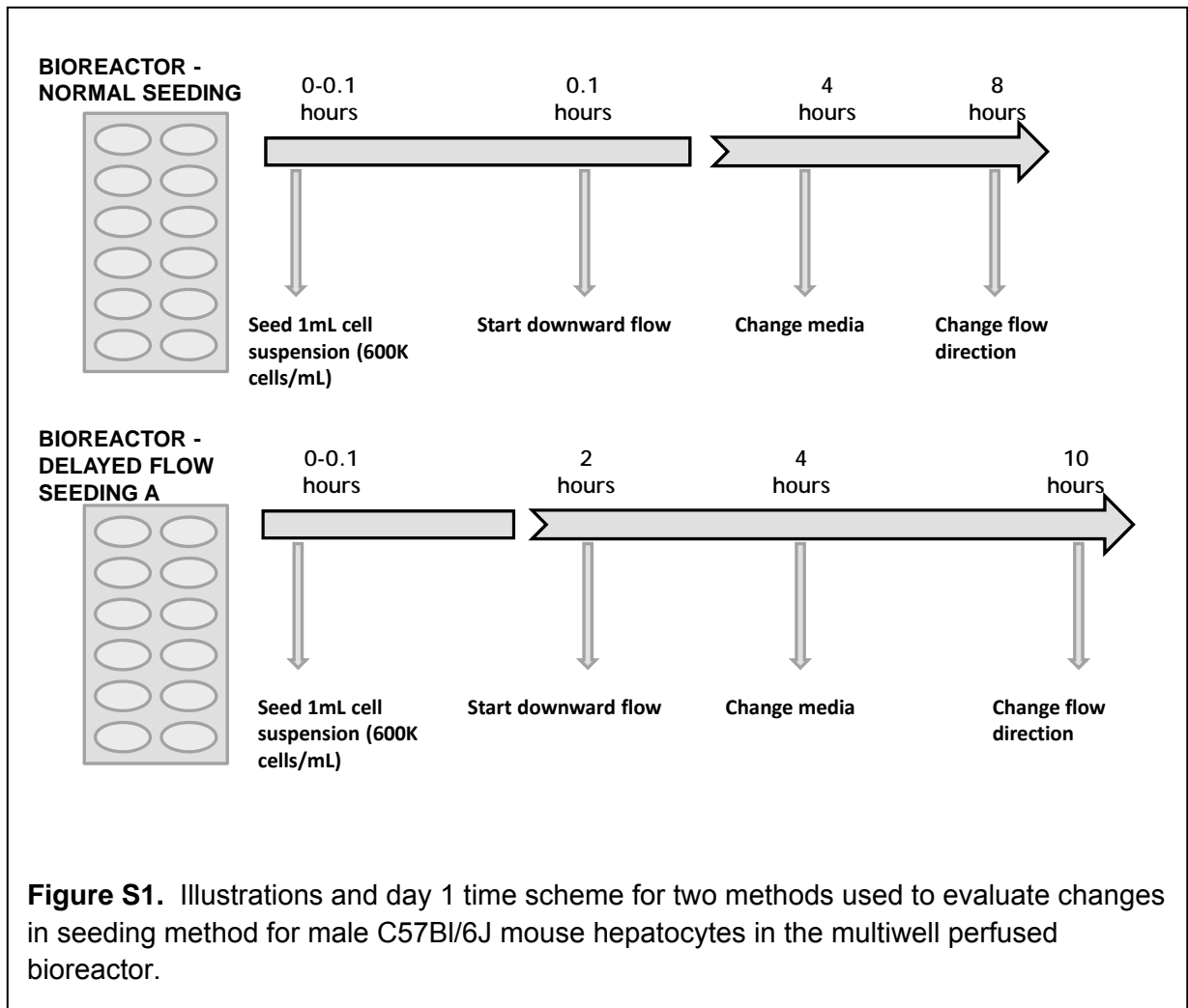
Sodium bicarbonate (NaHCO ₃)	2.27g	Mallinckrodt Chemicals 7412
10X GBS	100mL	

Table S3. Formulation for 1L of 1X GBS solution.

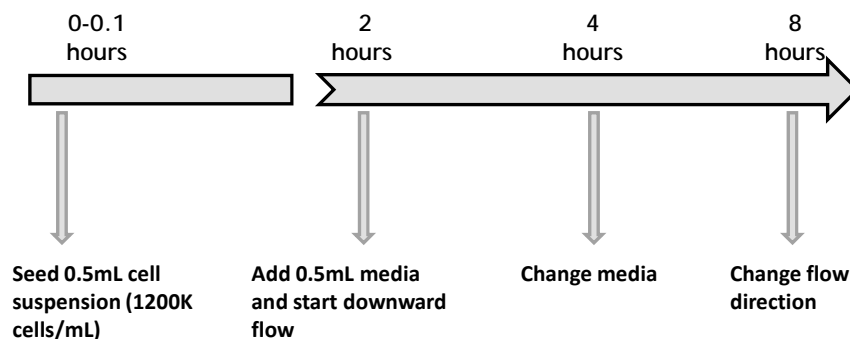
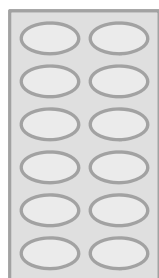
APPENDIX C

Seeding Methods Layouts

Illustrations and culture day 1 schemes for seeding method experiments.



**BIOREACTOR -
DELAYED FLOW
SEEDING B**



**BIOREACTOR -
DELAYED,
OPPOSITE FLOW
SEEDING**

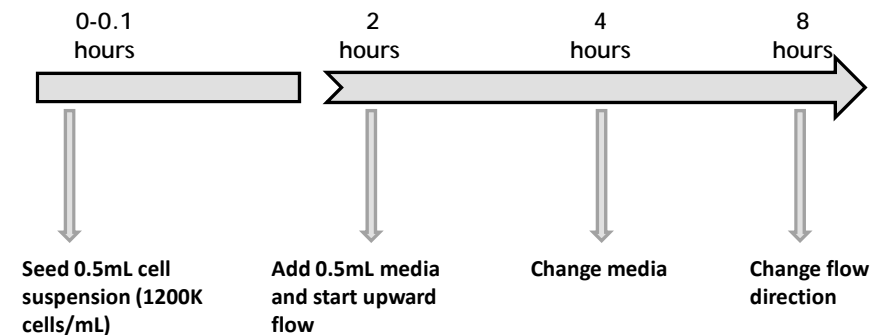
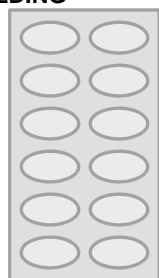


Figure S2. Illustrations and day 1 schemes for two additional methods used to evaluate changes in seeding method for male C57Bl/6J mouse hepatocytes in the multiwell perfused bioreactor.

APPENDIX D

Treatment Experiments - Layout

Illustrations of the experimental design and culture scheme for mouse hepatocytes in 2D culture and the 3D multiwell perfused bioreactor system.

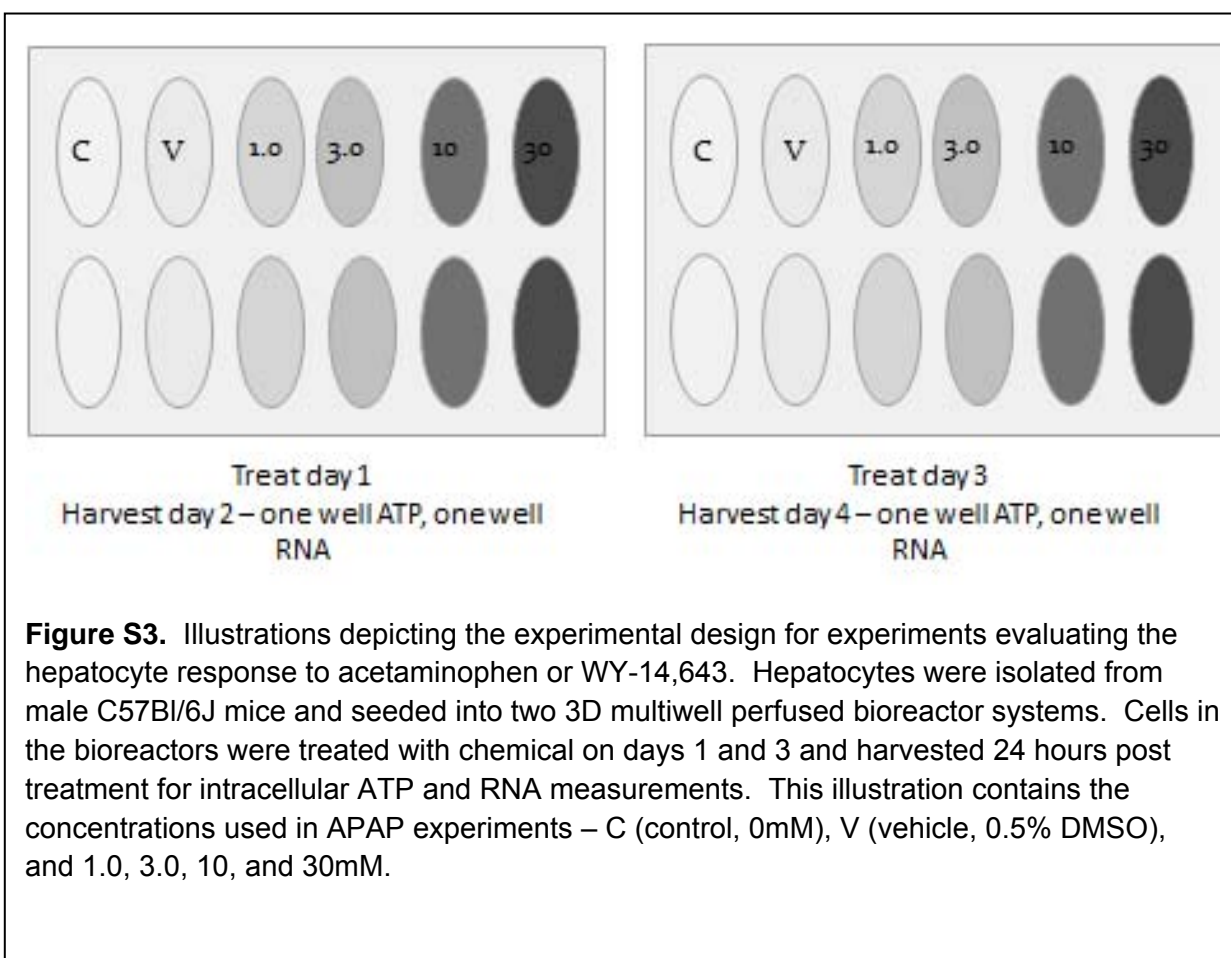
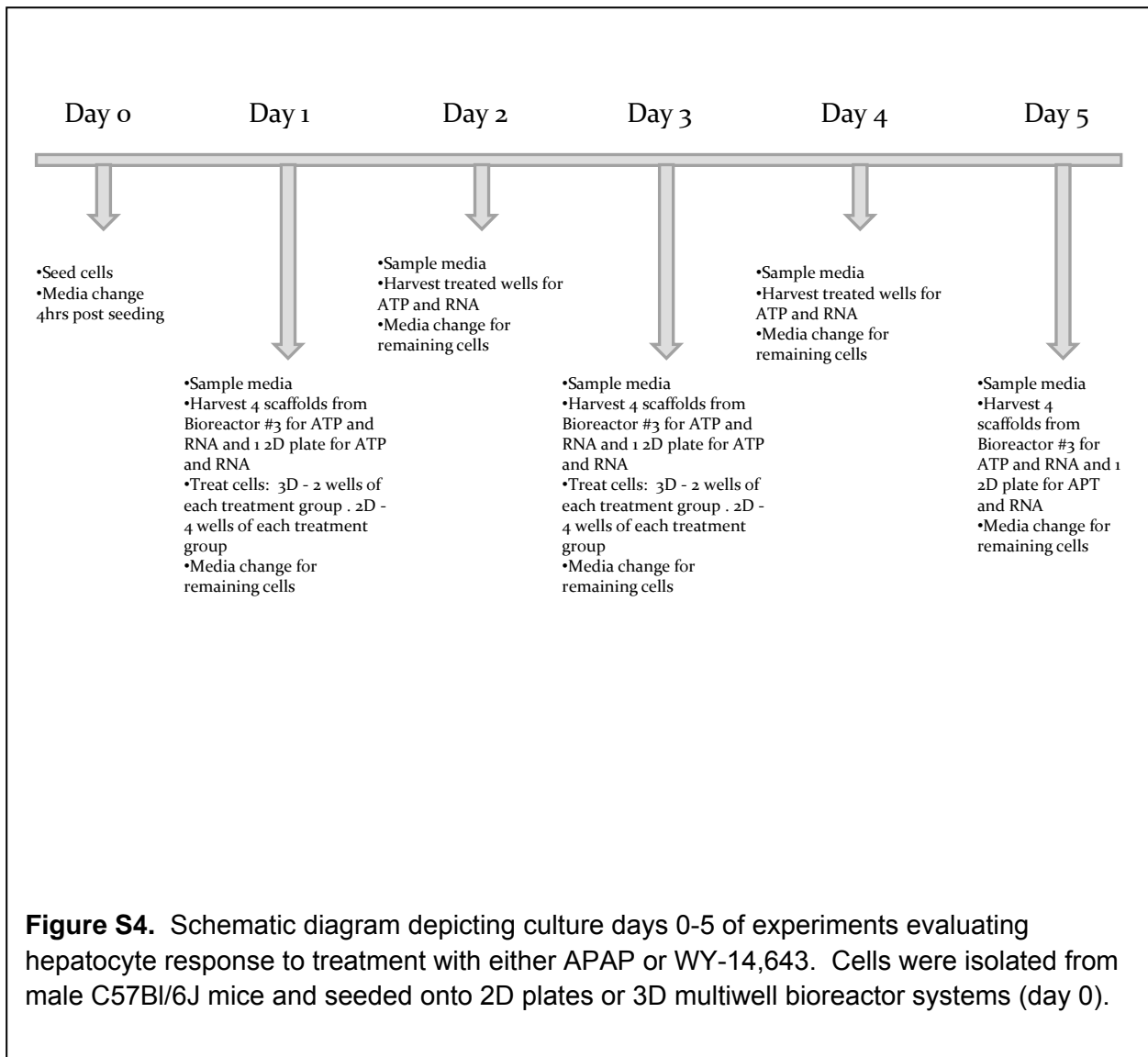


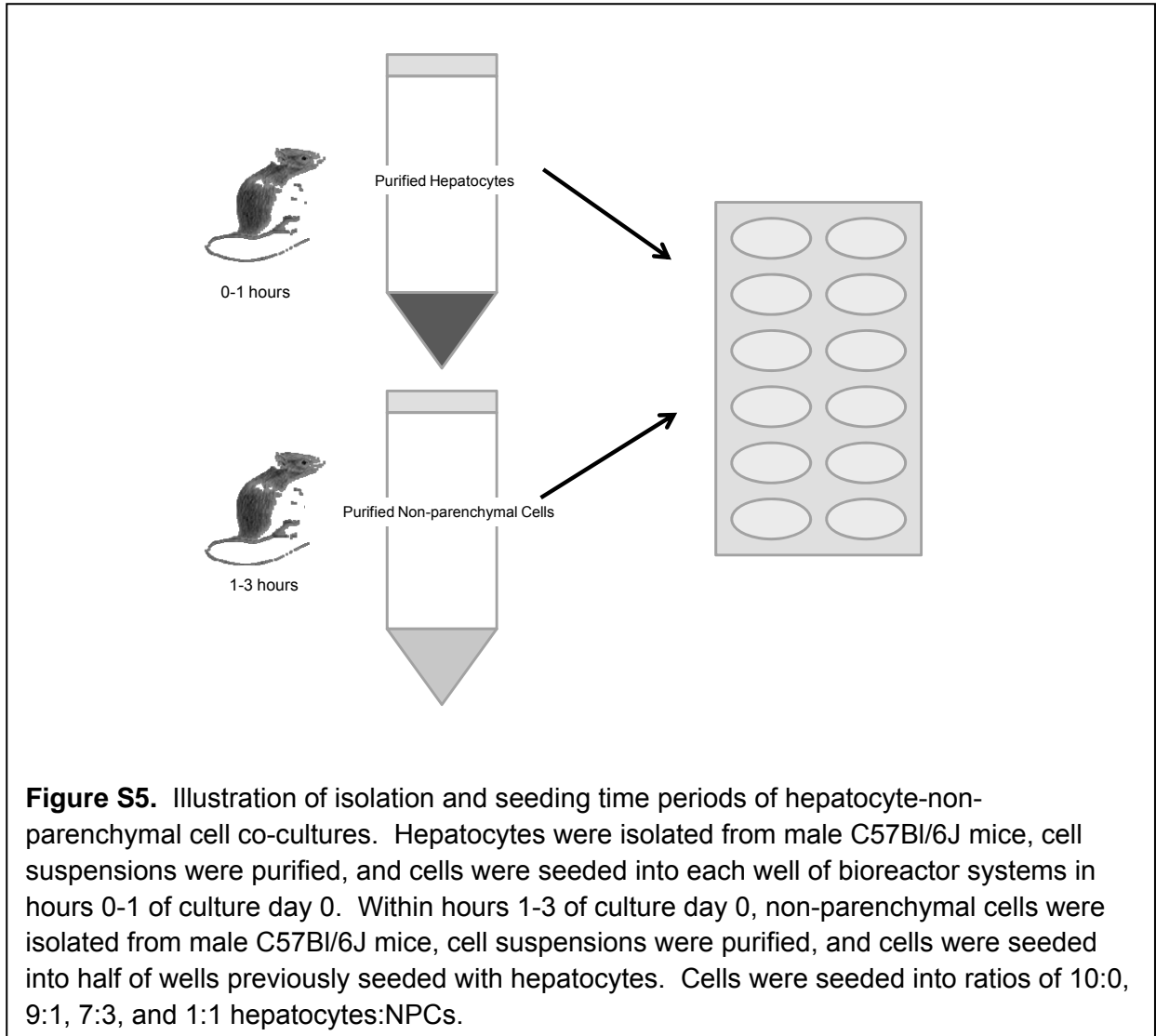
Figure S3. Illustrations depicting the experimental design for experiments evaluating the hepatocyte response to acetaminophen or WY-14,643. Hepatocytes were isolated from male C57Bl/6J mice and seeded into two 3D multiwell perfused bioreactor systems. Cells in the bioreactors were treated with chemical on days 1 and 3 and harvested 24 hours post treatment for intracellular ATP and RNA measurements. This illustration contains the concentrations used in APAP experiments – C (control, 0mM), V (vehicle, 0.5% DMSO), and 1.0, 3.0, 10, and 30mM.

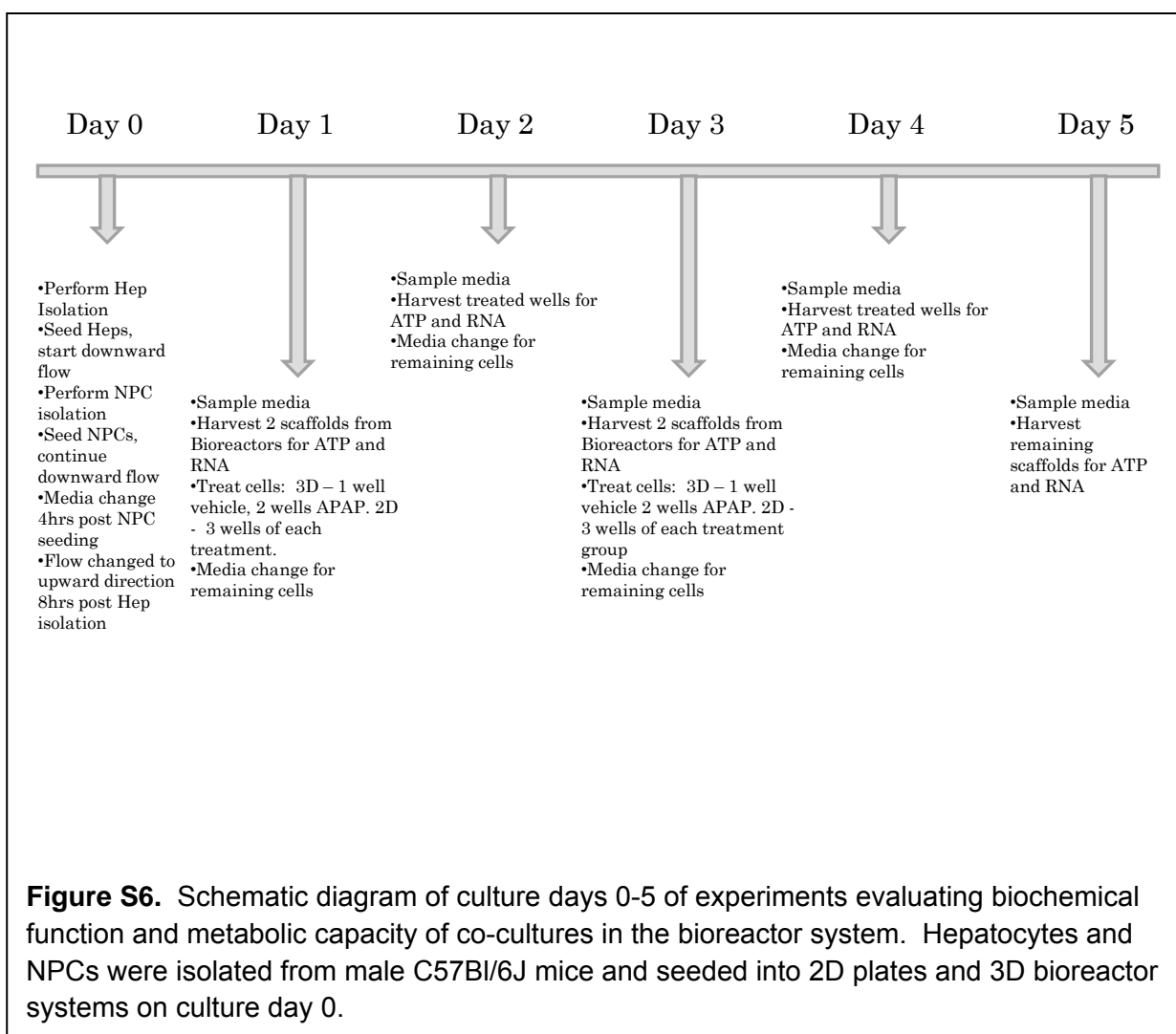


APPENDIX E

Co-culture Experiments - Layout

Illustrations of the isolation and culture schemes for hepatocyte-non-parenchymal co-cultures.





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