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Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation but is strongly changed after hepatocyte plating

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Abbreviations:

CYP : cytochrome P450, BSA : Bovine Serum Albumin, PBS: Phosphate Buffered Saline, FCS: Fetal Calf Serum, DMEM: Dulbeco's Modified Eagle Medium, EDTA : , UW: University of Wisconsin solution, UGT : UDP-glucuronosyltransferase,

Abstract

Isolated primary human hepatocytes are a well-accepted system for evaluating pharmacological and toxicological effects in humans. However, questions remain regarding how culturing impacts the liver-specific functions of the hepatocytes. In addition, cryopreservation could also potentially affect the differentiation state of the hepatocytes. The first aim of the present study was to compare gene expression in freshly isolated primary hepatocytes to that of the liver of origin, and to evaluate the expression changes occurring following cryopreservation/thawing, both when maintained in suspension and after plating. The second aim of the present study was to evaluate gene expression in hepatocytes following cold storage of suspensions up to 24h, compared to freshly isolated hepatocytes in suspension. Our results show that the gene expression in freshly isolated human hepatocytes in suspension after isolation is similar to that of the liver of origin. Furthermore, gene expression in primary human hepatocytes in suspension is not affected by hepatocyte cold storage and cryopreservation. However, the gene expression is profoundly affected in monolayer cultures after plating. Specifically, gene expression changes were observed in cultured relative to suspensions of human hepatocytes that are involved in cellular processes such as phase I/II metabolism, basolateral and canicular transport systems, fatty acid and lipid metabolism, apoptosis, and proteasomal protein recycling. An oxidative stress response may be partially involved in these changes in gene expression. Taken together, these results may aide in the interpretation of data collected from human hepatocyte experiments and suggest additional utility for cold storage and cryopreservation of hepatocytes.

Introduction

The liver serves as the primary site of detoxification of exogenous and endogenous compounds in the systemic circulation. Other biological and physiological functions include the production and secretion of critical blood and bile components, such as albumin, bile salts, and cholesterol. The liver is also involved in protein, steroid, and fat metabolism, as well as vitamin, iron, and sugar storage.

One of the most complex functions specific to liver is its ability to metabolize an enormous range of xenobiotics. Many drugs present in the blood are absorbed by hepatocytes, where they can be metabolized via phase I and II biotransformation reactions. Information concerning hepatic drug uptake and metabolism, phase I and phase II induction, drug interactions affecting hepatic metabolism, as well as hepatotoxicity are essential for pharmacology and toxicology of a given drug. Due to major species differences both in the catalytic activities and regulation of enzymes involved in drug metabolism, many of these evaluations can only be accurately investigated with human tissue. Since intact cells more closely reflect the environment to which drugs are exposed in the liver, isolated primary human hepatocytes are a well-accepted system for evaluating pharmacological and toxicological effects in humans (Gomez-Lechon et al., 2004; O'Brien et al., 2004; Liguori et al., 2005) and have been described as one of the best *in vitro* models for the prediction of *in vivo* metabolism in humans (Lave et al., 1999; Li, 2001). This has prompted research groups, including ours, to optimize the isolation and culture conditions of human hepatocytes from both surgical liver resections and non-transplantable livers (Richert et al., 2004; LeCluyse et al., 2005; Donato et al., 2005).

Sparse availability of high quality human liver tissue for research purposes, the demand for standardized cell populations, and the need for proper storage of cells for future

research investigations have resulted in the development of cryopreservation techniques (Alexandre et al., 2002; Garcia et al., 2003; Roymans et al., 2005). However several problems exist with the use of thawed human hepatocytes. For instance, cryopreserved hepatocytes have displayed a substantial variability between donors both in terms of recovery of viable hepatocytes and in their capacity to attach after thawing (Alexandre et al., 2002; Blanchard et al., 2005). This has profound consequences in terms of hepatocyte differentiation rates in culture and responses to xenobiotics since it has been found that isolated hepatocytes in culture display markedly different gene expression patterns depending on attachment status (Waring et al., 2003). In addition, it has previously been shown that constitutive expression and inducibility of the cytochrome P450s (CYPs) is dependent on cell-density (Hamilton et al., 2001). Also, studies have shown that cultured thawed hepatocytes in monolayers appeared smaller than their freshly isolated hepatocyte counterparts in monolayers, suggesting a variability in the resistance to cryopreservation of the various liver hepatocyte populations (Alexandre et al., 2002).

The first aim of the present study was thus to compare gene expression in freshly isolated hepatocytes to the liver of origin, and to evaluate the changes occurring following cryopreservation/thawing, both when maintained in suspension and after plating. The second aim of the present study was to evaluate gene expression in hepatocytes following cold storage as suspensions up to 24h, compared to freshly isolated hepatocytes in suspension. A pictorial depiction of the intended study is represented in Figure 1.

Materials and methods

2.1. Materials

Bovine Serum Albumin (BSA), Phosphate Buffered Saline (PBS), Fetal Calf Serum (FCS), Dulbeco's Modified Eagle Medium (DMEM) and TriZol™ were purchased from Invitrogen-Life technologies–France). Collagenase type IV, Percoll, and EDTA were purchased from Sigma (St Louis, MO, USA).

2.2. Human hepatocyte isolation

Adult normal liver samples were obtained from 3 patients undergoing partial hepatectomy for primary or secondary tumours. All experimental procedures were done in compliance with French laws and regulations and were approved by the National Ethics Committee. **Table 1** depicts the patient's demographics. Liver resections were handled, a small portion of liver was suspended in RNALater™ and frozen at -80°C. Hepatocytes were isolated by a two-step collagenase perfusion as recently described (Richert *et al.*, 2004). After Percoll purification, cell viability was estimated by Trypan blue exclusion. Part of freshly isolated human hepatocytes were kept directly in TriZol™ (0.5mL TriZol™ for 1 million cells) and frozen at -80°C.

2.3. Human hepatocyte cold storage or cropreservation and thawing

Part of the freshly isolated human hepatocytes was kept in UW solution at 4°C for 3 or 24 hours. Another portion of the freshly isolated human hepatocytes was immediately subjected to cryopreservation as previously described (Alexandre *et al*, 2002).

Human hepatocytes after cold storage (3 and 24 hours timepoints) were resuspended in DMEM and washed one time before having been subjected to percoll purification. The cell

pellet obtained was suspended in TriZol™ (0.5mL TriZol™ for 1 million cells) and frozen at -80°C. (**see Figure 1**)

2.4. Human hepatocyte culture

Human hepatocytes, either directly after isolation or after cryopreservation/thawing were subjected to percoll purification and suspended in DMEM supplemented with FCS (5%), insulin (4 mg/L), hydrocortisone (10^{-6} mol/L) and gentamycin (50 mg/L). Some of the thawed hepatocyte cells were directly suspended in TriZol™ and frozen at -80°C. Freshly isolated hepatocytes and the remaining thawed hepatocytes were plated onto 60 mm dishes at a density of 3.5×10^{6} cells/dish. At each time point of culture (24h, 48h and 72h for the fresh hepatocytes and only 24h for the thawed hepatocytes), hepatocyte cells were scraped in TriZol™ (0.5mL TriZol™ for 1 million cells) and frozen at -80°C. (**see Figure 1**)

RNA extraction and microarray analysis

Total RNA was isolated from the TRIzol™ extracts using the procedure from Invitrogen. Optical density at 260 nm determined RNA concentration. RNA quality was accessed using an Agilent Technologies bioanalyzer before proceeding to microarray sample preparation. Microarray analysis was performed using the standard protocol provided by Affymetrix Inc. (Santa Clara, CA) and as previously described, starting with 5 μg of total RNA (Liguori, et al. 2005). Fragmented, labeled cRNA was hybridized to an Affymetrix human genome U133A array, which contains sequences corresponding to roughly 22,200 transcripts at 45°C overnight.

Microarray data analysis

The microarray scanned image and intensity files were imported in Rosetta Resolver™ gene expression analysis software version 5.0 (Rosetta Inpharmatics, Kirkland, WA). Resolver's

Affymetrix error model was applied and ratios were built for each step in the study using freshly isolated human hepatocytes as the baseline. Using Rosetta Resolver, a p-value is calculated for every fold change, using the Rosetta Resolver error model. For all of the gene changes shown, the p-value is displayed in the figure legend. If the p-value is greater than the listed value, the fold change is shown as 1.0 (or black on a heatmap). Gene ontologies were deciphered using NETAFFX™ or GenMAPP (Liu et al 2003; Dahlquist et al 2002).

Results

Study Design

Gene expression analysis was conducted for three distinct human donors according to the study design as revealed in **Figure 1**. Briefly, microarray analysis was performed on human liver and freshly isolated primary human hepatocytes as well as hepatocyte monolayers from 24-72 hrs. Additionally, a portion of the primary hepatocytes was cryopreserved, and these cells underwent genomic analysis immediately after thaw and again after culture for 24 hrs. Some of the hepatocytes were stored in a cell suspension of UW solution, and subsequent gene expression analysis was conducted at the 3 hour and 24 hour timepoints. For data analysis purposes, the freshly isolated primary human hepatocytes were considered a control and were used as a baseline for ratio builds.

Primary Human Hepatocyte Morphology, Viability, and Attachment

Figure 2 displays light micrographs of different stages of the study for hepatocytes from donor 2. **Table 2** exhibits the percent recovery of viable primary human hepatocytes subsequent to cold storage and cryopreservation. For all donors, viability declined upon continued cold preservation in UW solution up to 24 hrs, the loss of cells being more evident for donor 1 than for donor 2 and donor 3. Also, viability was decreased upon cryopreservation (liquid nitrogen), with recovery being equivalent for donor 2 and donor 3, about 50 %, higher than donor 1 (10%). **Table 3** illustrates that primary human hepatocyte attachment efficiency declined subsequent to cryopreservation. Again, attachment rate of plated viable thawed hepatocytes was lower for donor 1 than for donor 2 and 3. As also seen in **Table 3**, when considering total cell protein level of attached thawed hepatocytes, the values were very similar for the 3 donors, being respectively 0.85, 0.99 and 0.83 mg protein/dish. After thawing, this total cell protein level was lower, respectively 53% (0.45

mg/dish; donor 1), 60% (0.59 mg/dish; donor 2) and 82% (0.68 mg/dish; donor 3) from that of the respective freshly isolated attached total protein level.

Gene Expression Analysis

Using freshly isolated primary human hepatocytes as the baseline, microarray analysis on human liver and primary human hepatocytes at various stages of preparation revealed relatively few gene expression changes for intact human liver, hepatocytes from 3 and 24 hr cold storage, and thawed primary hepatocytes post-cryopreservation (**Figure 3**). However, the gene expression profiles from primary human hepatocytes after plating for 24-72 hrs both from fresh and cryopreserved preparations result in a markedly increased number of differentially regulated transcripts relative to freshly isolated primary hepatocytes. The gene expression changes in plated hepatocytes relative to freshly isolated hepatocytes were, in general, consistent over time for all donors (**Figure 3**). A supplemental MS Excel table is included with this communication detailing all gene expression changes (relative to freshly isolated human hepatocytes) with at least +/- 1.5-fold change and p-value of 0.05 or less. The fold changes for probe sets with p-values greater than 0.05 are represented as "1."

Hepatic phase I and II biotransformation reactions are critical to xenobiotic metabolism. Examination of CYP gene expression reveals a down-regulation of most transcripts upon plating of the primary hepatocytes relative to freshly isolated primary hepatocytes, whereas the CYP expression level is predominately unchanged in samples from intact liver, cold storage, and immediately post-cryopreservation (**Figure 4A**). Most of the major CYP isoforms (CYP1, 2, 3, 4) have a reduced expression level upon plating of the primary hepatocytes, with the exception of CYP2C18, which was slightly up-regulated upon plating for all donors. Additionally, there are some cytochrome P-450 genes (CYP1B1, CYP20A1, CYP51A1, CYP2C18, CYP21A2, and CYP26A1) that had elevated levels of

expression upon primary hepatocyte plating, especially for donor 2 (**Figure 4B**). However, many of these described CYPs vary from donor to donor and are not induced in every donor nor at every plating timepoint. Many gene products for the phase II conjugation system, including transcripts encoding several UDP-glucuronosyltransferases (UGTs) initially exhibited reduced levels of expression upon plating, while remaining unchanged in samples from liver, cold storage, and post-cryopreservation (**Figure 4C, 4D**). Most of the Phase II genes that were regulated for the plated human hepatocytes were decreased quite consistently across all three donors evaluated.

Hepatic transport is a critical function for the uptake and excretion of xenobiotic compounds. Microarray analysis revealed that the greatest effect on transporter mRNA levels was seen in plated hepatocytes. **Figure 4E** shows that many of the solute carrier family of transporters have reduced levels of transcripts with respect to freshly isolated hepatocytes. For instance, SLC22A7 (solute carrier family 22), an organic anion transporter, was down-regulated after plating for all donors. Alternatively, there are transcripts such as SLC38A1, which is responsible for transport of glutamine (Gu et al. 2001), that have elevated levels upon primary hepatocyte plating. Most transporter mRNA levels remained unchanged for human hepatocytes in cold storage and immediately after cryopreservation, suggesting that the transporter gene expression changes result from plating of the primary hepatocytes. Furthermore, many transporter genes, such as ABCG5, SLCO2B1, and ABCB11, were consistently regulated across all three donors, while the expression of others, including ABCC3, SLC38A2, and SLC4A4, varied quite extensively from donor to donor.

Another important function of the liver pertains to its role in lipid and fatty acid metabolism. In particular, several genes involved with fatty acid degradation exhibited a trend of down-regulation. For the 24 hr. primary hepatocyte monolayer for donor 1, genes encoding enzymes such as carnitine palmitoyltransferase II, carnitine/acylcarnitine

translocase, acyl-CoA dehydrogenases, and long chain fatty acid CoA ligase 5 were all down-regulated relative to freshly isolated primary hepatocytes (**Figure 5A**). Additionally, a gene for short chain 3-hydroxy-acyl-CoA dehydrogenase (HADHSC, probe ID 201035_at), an essential enzyme for mitochrondrial β-oxidation catalyzing the conversion of 3-hydroxyacyl-CoAs to 3-ketoacylCoAs, was strongly reduced. Furthermore, for many transcripts, a trend of down-regulation is apparent with genes involved in lipid metabolism (as defined by gene ontology) for primary hepatocyte monolayers (**Figure 5B**). Intact human liver, primary hepatocytes in cold storage, and post-cryopreservation primary hepatocytes show the fewest gene expression changes for lipid metabolism.

The proteasome is the cellular machinery responsible for protein regulation, degradation, and recycling, and it serves a vital function in maintaining homeostasis. It is a multi-subunit ubiquitin dependent holoenzyme with a core complex responsible for hydrolytic activity (20S core) and a regulatory complex with ATPase activity (19S regulator) that mediates identification and proper trafficking of peptide substrates into the active site (Meiners, et al. 2003). There was a significant up-regulation in the expression level of genes that encode for proteasome subunits upon plating of primary hepatocytes for all donors (**Figure 5C**). More specifically, genes such as PSMD12 (a non-ATPase regulatory subunit), PSMD8 (a non-ATPase regulatory subunit), and PSMC6 (a subunit with ATPase activity), were all significantly overexpressed upon plating of the human hepatocytes (data not shown). This effect was largely not observed in samples from intact liver, primary hepatocyte cold storage, and post-cryopreservation, which suggests the possibility that some aspect of the plating process may play a role in this enhancement of expression.

Apoptosis is a type of cell death that has distinct morphological and biochemical characteristics and that is under strict regulation. As displayed in **Figure 5D**, the expression level of several key transcripts associated with induction of apoptosis were increased in

primary hepatocyte monolayers relative to freshly isolated hepatocytes. For instance, caspases, which are enzymes involved in the execution phase of programmed cell death, such as CASP7 (all donors) and CASP3 (donor 1 in 24 hr monolayer and donor 3) were significantly overexpressed in the plated human hepatocytes. Furthermore, other prominent players in apoptotic cell death exhibited enhanced expression in plated human hepatocytes, such as PARP, an activator of cellular proliferation and of various nuclear proteins involved with DNA repair mechanisms. Also, BID, involved in apoptosis activation via mitochondrial cytochrome c release, and *c-myc*, a transcription factor that assists with cell cycle regulation, were induced in samples of plated human hepatocytes for most donors and timepoints. The expression of these pro-apoptotic genes was changed upon cultivation of the primary hepatocyte monolayer, but was largely unchanged in samples from intact liver, cold storage, and post-cryopreservation.

Discussion

Gene expression in human hepatocytes in suspension after isolation is similar to that of the liver of origin

The relatively few genes that were regulated in the intact livers versus freshly isolated hepatocytes tended to be down-regulated, suggesting that some of these expression changes may result from the fact that the liver is composed of a heterogeneous cell population while primary hepatocyte cultures are a more homogenous population. These small changes in gene expression in hepatocytes following their isolation are consistent with our recent observation that CYP-dependent and UGT-dependent activities are equivalent or only slightly decreased in microsomes prepared from human hepatocytes after their isolation compared to liver microsomes from the liver of origin (data not shown).

Gene expression in human hepatocytes in suspension is not affected by hepatocyte cold storage and cryopreservation

The equivalent gene expression profile observed in suspension of freshly isolated human hepatocytes, after cryopreservation/thawing and after cold-storage, are also in line with our recent observations that metabolic capacity of human hepatocytes in suspension after cold storage or after cryopreservation was intact and clearance predictions were equivalent to suspensions of freshly isolated hepatocytes from the same donor (Blanchard et al., 2005). Shitara et al. (2003) also observed that the transporter systems in human hepatocytes were functional after cryopreservation. Interestingly, gene expression for primary human hepatocytes in cold storage and cryopreservation is similar to that of the liver of origin, which could have implications for hepatocyte model systems being translated to intact liver.

The recovery of viable hepatocytes after a 24h cold storage is at least equivalent to that after cryopreservation (see **Table 2**), suggesting that cold storage could be an alternative for human hepatocyte short-term shipping and storage. The data also suggest that CYP expression levels remain relatively stable at baseline levels upon cold storage of human hepatocytes. In addition, few gene expression changes were evident for critical hepatocellular processes including transport, lipid metabolism, phase II metabolism, and protein processing, degradation, and recycling (proteasome subunits). Furthermore, human hepatocytes after cryopreservation and in cold storage may be less prone to initiating and undergoing apoptosis as evidenced by few expression changes for genes involved in apoptosis induction for these samples.

Gene expression in human hepatocytes is strongly changed after plating

Significant gene expression changes occur upon plating of hepatocytes. It has been previously shown that major cytochrome P450 dependent enzyme activities decline in culture, and this is associated with decreases in both gene and protein expression, symptomatic of a dedifferentiated state (Madan et al., 2003). The data of the present communication thus confirm these observations and highlight that some other CYP isoforms are, in contrast, up-regulated following plating. We (Richert et al., 2002; Binda et al., 2003) have previously suggested that the decline in CYP expression could be related to oxidative stress occurring during time in culture. Several genes associated with an cellular oxidative stress response, such as superoxide dismutase 2, glutathione reductase, p53, and peroxiredoxin, are over-expressed upon plating of human hepatocytes (data not shown). The present study therefore presents further evidence that oxidative stress occurs during plating, and also further highlights that pathways leading to apoptosis are activated as well, most probably, at least in part oxidative stress related. In support of this, it has been reported that reactive oxygen species mediate apoptosis in hepatocytes (Rauen et al., 1999; Ishihara et al, 2005).

UDP-glucuronosyltransferases (UGTs) are a family of Phase II enzymes critical for xenobiotic biotransformation reactions. The findings from this study indicate that the mRNA levels of several members of this class are reduced upon primary hepatocyte plating at the 24 hour timepoint. At 72 hours, there appears to be a slight trend toward increasing levels of mRNA relative to the 24 or 48 hour timepoint for some UGT genes, especially for donor 2. This reflects a similar tendency observed in other reports with primary rat hepatocytes (Li et al., 1999; Zurlo et al., 1996). Furthermore, in activity measurements, UGTs retain basal metabolizing capabilities upon primary rat hepatocyte plating compared to whole liver (Richert et al., 2002). Additionally, cryopreservation did not affect the gene expression of UGTs in this study, in concordance with other studies (Li et al., 1999).

Hepatocellular transporter systems are essential regulators of homeostatis, are involved in drug disposition and bile formation, and may be critical for toxicological and pharmacological studies (Chandra and Brouwer 2004). The data indicate that the expression of many transporter sytems, especially those in the solute carrier family series, is decreased upon plating of the primary human hepatocytes. Messenger RNA from major basolateral transporters, such as OATP2 (SLC22A7) and NTCP (SLC10A1), have been previously shown to be markedly reduced upon plating of primary rat hepatocyte cultures (Rippin et al. 2001; Luttringer, et al, 2002). Our results also support this trend, and furthermore offer evidence of down-regulation of additional basolateral transporters, such as OCT1 (SLC22A1) and MRP6 (ABCC6) upon plating of primary human hepatocytes, in agreement with studies from other laboratories (Jigorel et al., 2005).

Hepatic canicular transport proteins incorporate a variety of ATP-dependent membrane associated pumps that encompass the ATP binding cassette superfamily, including the well studied MDR1 or p-glycoprotein (Rippin, et al. 2001; Chandra and Brouwer 2004). In primary rat hepatocytes, MDR1b (ABCB1) and MRP3 (ABCC3) transcription products have been observed to increase upon plating and cultivation (Luttringer et al., 2002). Similarly, in plated primary human hepatocytes, we observe over-expression of ABCB1 mRNA relative to freshly isolated hepatocytes, which could result in more informed interpretation for studies involving xenobiotic metabolism of ABCB1 substrates. Other canicular pumps, such as ABCB11 and ABCB4, tend to be under-expressed in plated primary human hepatocytes for these evaluated donors.

To our knowledge, the present study is the first report on drastic changes in the expression of genes involved with fatty acid and lipid metabolism in primary human hepatocytes after plating as determined by microarray analysis. A large number of lipid metabolism transcripts are under-expressed in monolayers of primary human hepatocytes

relative to their freshly isolated counterparts. This, in part, could be related to an oxidative stress response since both arachidonic acid mobilization and phospholipase A2 activity were found affected by oxidative stress (Babenko et al., 1998). Even more relevant to the present results, a direct link between oxidative stress and ApoB100 degradation and VLDL secretion has been recently reported (Pan et al., 2004).

It has been well documented that free fatty acids can have dramatic direct or indirect effects on expression of genes involved in lipid metabolism via regulation of nuclear transcription factors such as the PPARs, SREBPs, LXRs, or HNF (Jump, 2002; Swagell, et al., 2005). Although no significant gene expression regulation of PPARs (alpha, beta, or gamma) or HNF1 was detected, LXRa (203920_at) was under-expressed for all donors, and SREBP1 (202308_at) was under-expressed for donor 1 and 3 only in the monolayer cultures relative to freshly isolated human hepatocytes (data not shown). Perhaps changes in the abundance and variety of fatty acids from intact liver to cell culture may be at least partially responsible for the observed changes in expression.

Interestingly, many proteasomal subunits, the proteolytic machinery responsible for degradation and recycling of ubiquitin-protein complexes, are over-expressed in primary human hepatocyte monolayers compared to freshly isolated hepatocytes. Increased expression of these subunits may be associated with an increased proteasome presence, and ultimately potentially elevated proteolytic activity relative to the native state (Hobler, et al., 1999; Fang et al., 2000). A recent communication highlights that mild oxidative stress may augment proteasome activity and expression in response to a heightened level of oxidatively damaged proteins (Elkon, et al., 2004), whereas severe oxidative stress may result in proteasome failure and in abolishment of proteasome activity. Therefore, it is possible that some degree of oxidative stress in plated human hepatocytes may drive, in part, the over-expression of these proteasomal subunits. Awareness of this potential increased proteolytic

activity and turnover may assist in some types of data interpretation for primary human hepatocyte studies.

Gene expression after plating is not affected by cold storage or cryopreservation

In the present study, we found that the gene expression pattern for the most of the major genes evaluated in plated cryopreserved/thawed human hepatocytes was similar to that of their respective plated fresh monolayers (24 hr timepoint) for all donors. One should be aware however that in the present study, the plating density of thawed hepatocytes was 50-80% of the plating density of their respective freshly isolated hepatocytes, and this could contribute to the similar pattern of gene expression for plated thawed human hepatocytes. Indeed, in a previous study, we found that human hepatocytes in culture displayed markedly different gene expression patterns depending on attachment status (Waring et al., 2003), in accordance with the observations of Hamilton et al. (2001), who described a decrease in constitutive levels of CYP3A4 in human hepatocytes that correlated with the plating density. Nonetheless, it is noteworthy that in the latter work, the fold induction of CYP3A4 observed following exposure to rifampicin was equivalent in cultures as long as the cell density was not reduced to more than 50% of standard density. Taken together, these results suggest that plated cryopreserved hepatocytes are a valid tool for drug metabolism and toxicological evaluations as long as the plating density is at least 50% of that of a confluent monolayer.

It is possible that the expression profiles for some genes in other human donors may behave differently than observed in this study due to the large degree of primary human hepatocyte donor to donor heterogeneity (Liguori et al., 2005). However, it is clear from these

results that plating of primary human hepatocytes leads to a vigorous change in gene expression from the native state, which may be a critical factor for some studies involving human hepatocytes. Taken together, these results may aide in the interpretation of data collected from human hepatocyte experiments and suggest additional utility for cold storage and cryopresevation of hepatocytes.

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Footnotes:

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LEGENDS TO FIGURES

Figure 1. Study design is illustrated. Three distinct human liver donors were evaluated using gene expression analysis along multiple steps of the human hepatocyte preparations. Gene expression changes were monitored using freshly isolated primary human hepatocytes (t=0hr.) as the baseline.

Figure 2.

Hepatocytes in suspension after isolation from donor 2 (A), in primary culture, 24h after seeding (B), 48h after seeding (C), 72h after seeding (D), and in primary culture 24h after thawing (E). All panels are at 40X magnification.

Figure 3. Global gene expression profiles for all of the primary human hepatocyte donors at various stages of preparation. Each horizontal lane in the heat map represents a different stage of human hepatocyte preparation for its corresponding donor. Each vertical lane represents a single Affymetrix probe set. For the heat maps in all the figures, sequence agglomerative cluster analysis, using average link heuristic criteria and Euclidean distance for similarity measure, was performed to group together genes with similarities in gene expression. Genes represented were regulated at least +/-2.0-fold with a p-value of 0.01 or less. Increases in mRNA level are represented as shades of red with decreases in shades of green. If the p-value for a particular gene expression change is greater than 0.01 (or other value as indicated for subsequent figures), the log(ratio) is represented as zero or black on a heat map.

Figure 4. A. Gene expression changes for the cytochrome P-450 transcripts present on the array. **B.** Observed gene expression changes for the major CYP isoforms (right hand panel)

and for those CYPs that were induced (left hand panel). The majority of CYPs were down-regulated upon plating the primary human hepatocytes, while cells in suspension or after cyropreservation showed few changes. **C.** Under-expression of phase II transcripts upon plating the hepatocytes. **D.** Gene expression changes for several UDP-glucuronosyltransferases (UGTs) during various stages of human hepatocyte preparation **E.** Regulation of transcripts associated with canicular and basolateral transporter systems in primary human hepatocytes. The greatest number of changes in transporter expression is observed in plated primary human hepatocytes. For all panels, genes were filtered for p-value of 0.05 or less.

Figure 5. A. Regulation of genes involved in the cellular fatty acid degradation pathway for 24 hour monolayer human hepatocytes from donor 1 using GenMAPP visualization software (Dahlquist et al., 2002). An over-expressed gene is colored in red and an under-expressed gene is colored in green. The quantitative fold change for each gene is shown as a number to the right of the gene box. The statistical cutoffs for a gene being designated as regulated are at least +/-2.0-fold change with a p-value of 0.01 or less. **B.** A global view of the gene expression changed for fatty acid and lipid metabolism transcripts (as defined by gene ontology designation) for the various stages of human hepatocyte preparation. For many of these probe sets, transcription is down-regulated. **C.** Expression profiles for genes that code for proteasomal subunits for the various stages of human hepatocyte preparation. These genes are overwhelmingly induced upon plating of human hepatocytes. For the heatmaps, genes were filtered for a p-value of 0.05 or less. **D.** A heat map for mRNAs which are associated with positive regulation of apoptosis (as defined by gene ontology). Many transcripts that code for proteins associated with apoptosis induction were induced upon hepatocyte plating.

DMD Fast Forward. Published on February 10, 2006 as DOI: 10.1124/dmd.105.007708
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Table 1. Characteristics of donors and yield of viable isolated hepatocytes.

	Age	**Gender**	**Disease**	**Size of resection (g)**	**Yield (Viable hepatocytes /g liver)**
Donor No. 1 Date: 3/16/04	54	F	Metastasis (colorectal)	83	3.86×10^6
Donor No. 2 Date: 2/24/04	32	F	Metastasis (breast)	34	7.41×10^6
Donor No. 3 Date: 2/18/04	52	F	Metastasis (ovary cancer)	70	19.8 x 106

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Table 2. Recovery of viable hepatocytes after cold preservation and cryopreservation/thawing	**Donor No. 1**	**Donor No. 2**	**Donor No. 3**
Recovery of viable cells after 3h in UW solution (%)	55%	97%	66%
Recovery of viable cells after 24h in UW solution (%)	35%	56%	61%
Recovery of viable cells after cryopreservation (%)	10%	54%	49%

Hepatocyte viability was assessed by trypan blue exclusion before and after cold- or cryo-preservation. From this, recovery of viable cells was calculated and expressed as percentage of viable cells before preservation.

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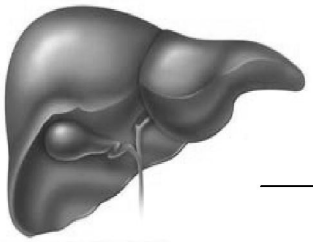
Table 3. Extent of attachment, 24 hours after seeding, of hepatocytes freshly isolated and after cryopreservation / thawing	**Donor No. 1**	**Donor No. 2**	**Donor No. 3**
Extent of attachment of viable freshly isolated hepatocytes (%)	92%	92%	87%
Cell proteins of attached freshly isolated hepatocytes (mg/dish)	0.85 mg/dish	0.99 mg/dish	0.83 mg/dish
Extent of attachment of viable thawed hepatocytes (%)	35%	55%	65%
Cell proteins of attached thawed isolated hepatocytes (mg/dish)	0.45 mg/dish	0.59 mg/dish	0.68 mg/dish

Hepatocytes were plated at a density of 3.5×10^6 viable cells/dish (see Materials and Methods section). After a 24 hour attachment period, monolayers were washed, cells were detached with trypsin, and counted. From this, the extent of attachment of viable seeded hepatocytes was calculated. In addition, the protein content of the cell monolayers was determined.

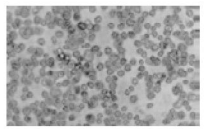
Figure 1 Experimental Design

Donation Dates
March 16, 2004
February 24, 2004
February 18, 2004



Human Liver Intact

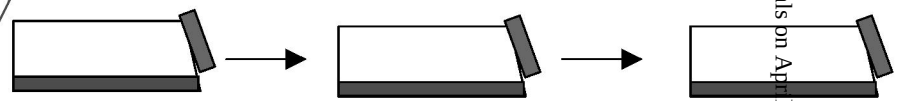
Used as baseline



Human Hepatocytes
Freshly isolated

Human Hepatocyte Cell Suspension (2-8ºC) UW 3 Hr.

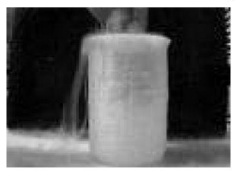
Human Hepatocyte Cell Suspension (2-8ºC) UW 24 Hr.



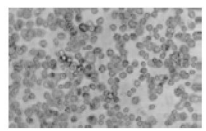
Human Hepatocyte Monolayer 24 Hr.

Human Hepatocyte Monolayer 48 Hr.

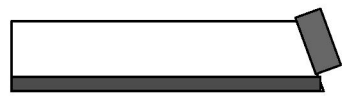
Human Hepatocyte Monolayer 72 Hr.



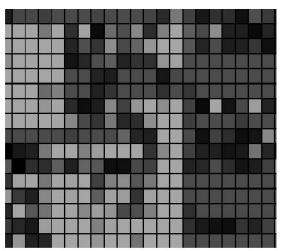
Human Hepatocytes
Frozen



Human Hepatocytes
Freshly Thawed



Human Hepatocytes
24 Hour Monolayer
postfreeze



Microarray Analysis
HG-U133A Chip (~22,200 Transcripts)

Figure 2

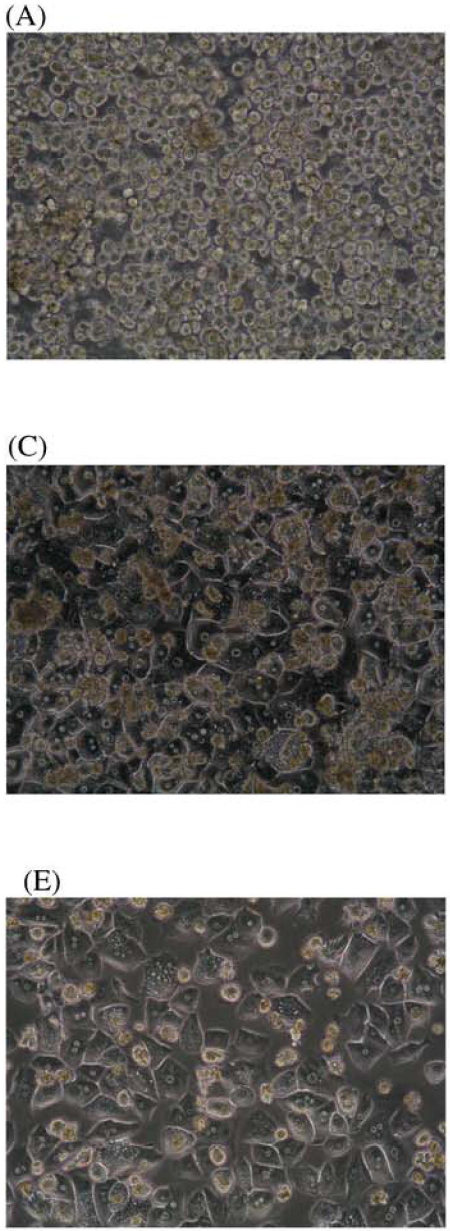


Figure 3

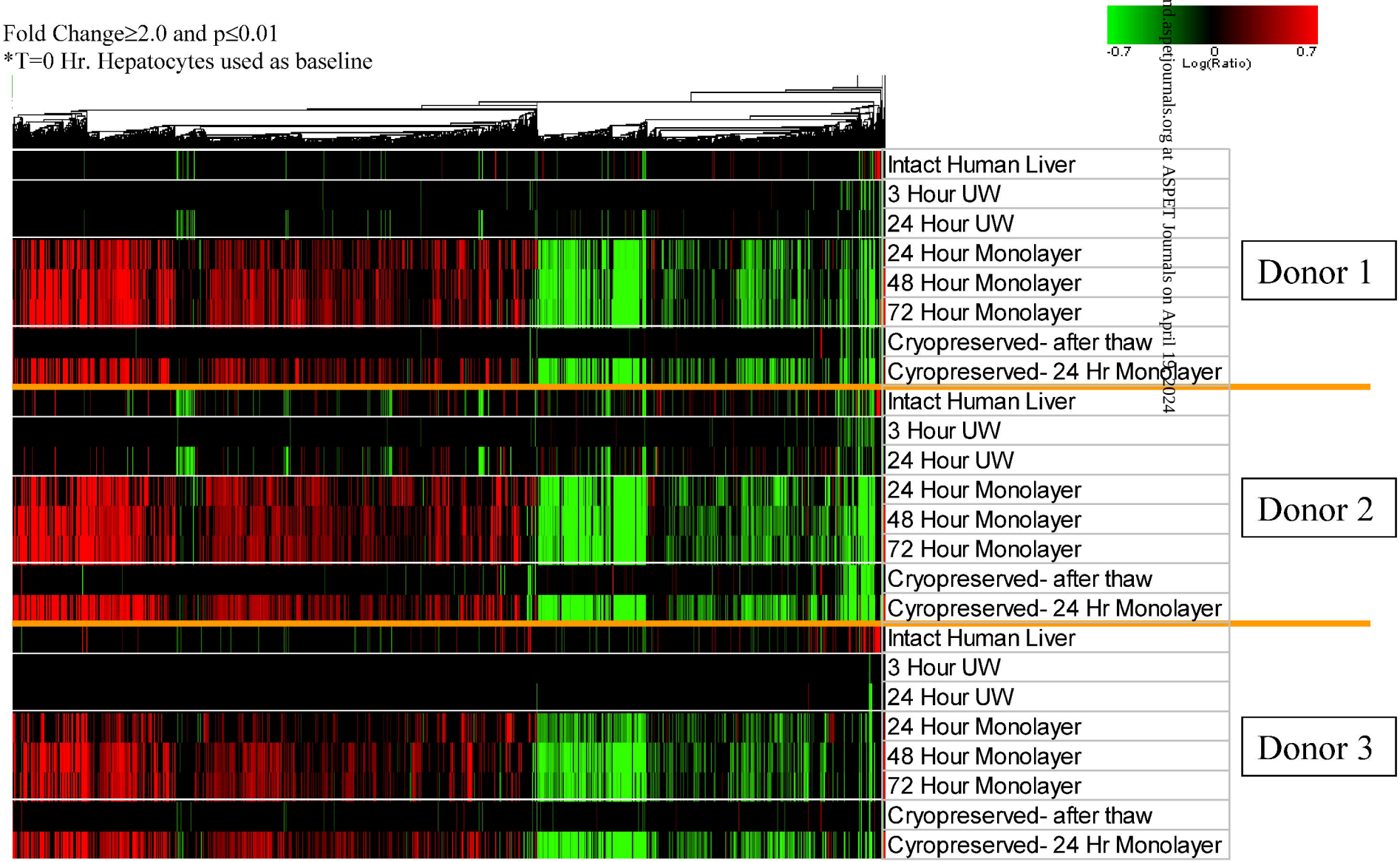
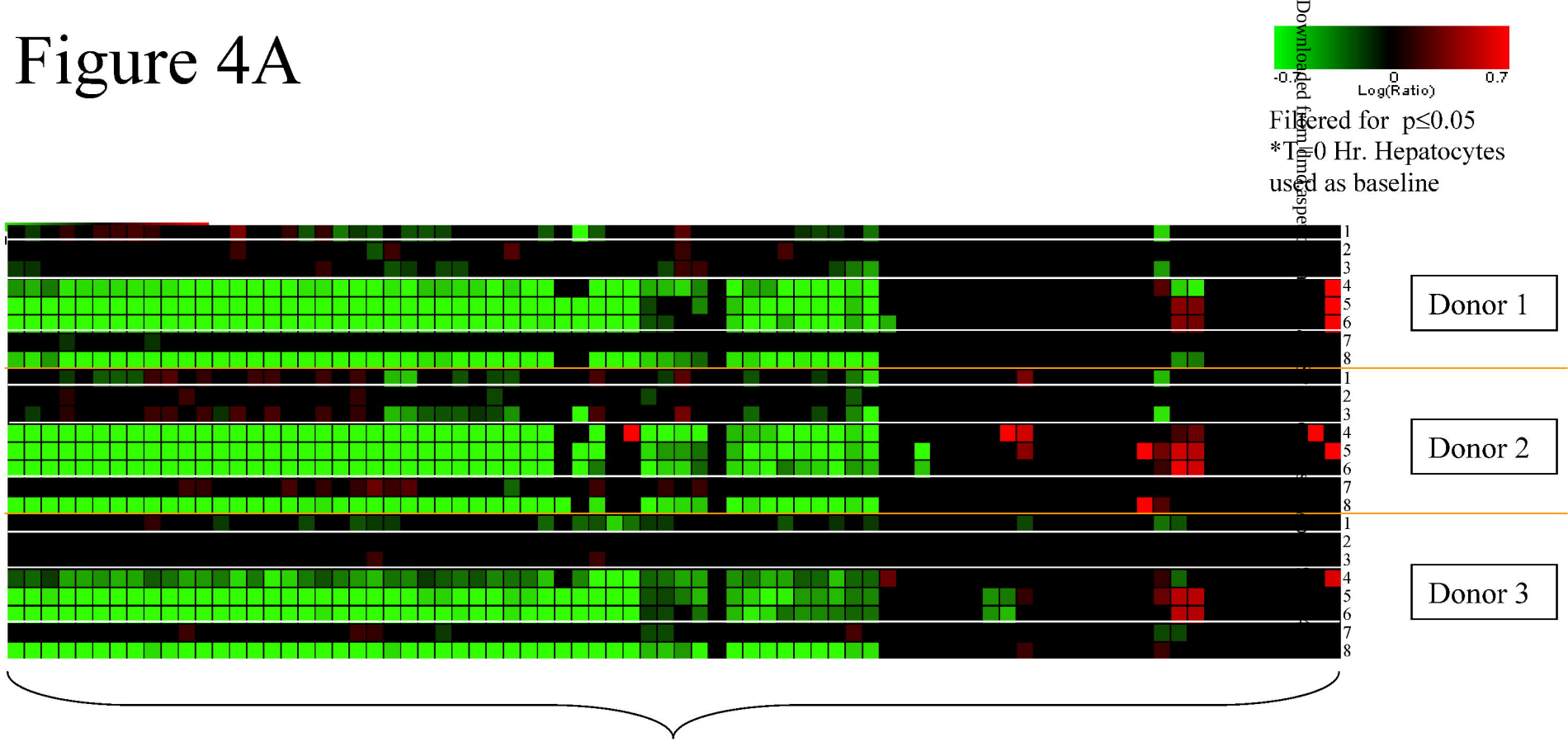
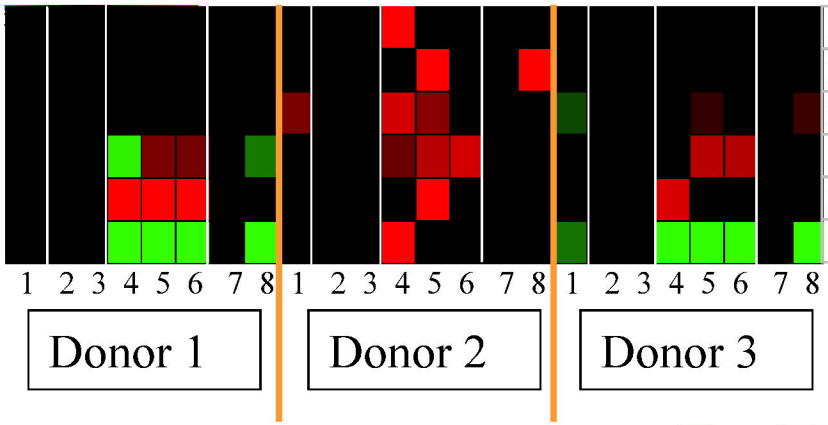


Figure 4A



All Cytochrome P450s on Array

1) Intact Human Liver
2) 3 Hour UW
3) 24 Hour UW
4) 24 Hour Monolayer
5) 48 Hour Monolayer
6) 72 Hour Monolayer
7) Cryopreserved- immediately after thaw
8) Cyropreserved- 24 Hr Monolayer



202437_s_at	CYP1B1
219565_at	CYP20A1
202314_at	CYP51A1
208126_s_at	CYP2C18
214622_at	CYP21A2
206424_at	CYP26A1

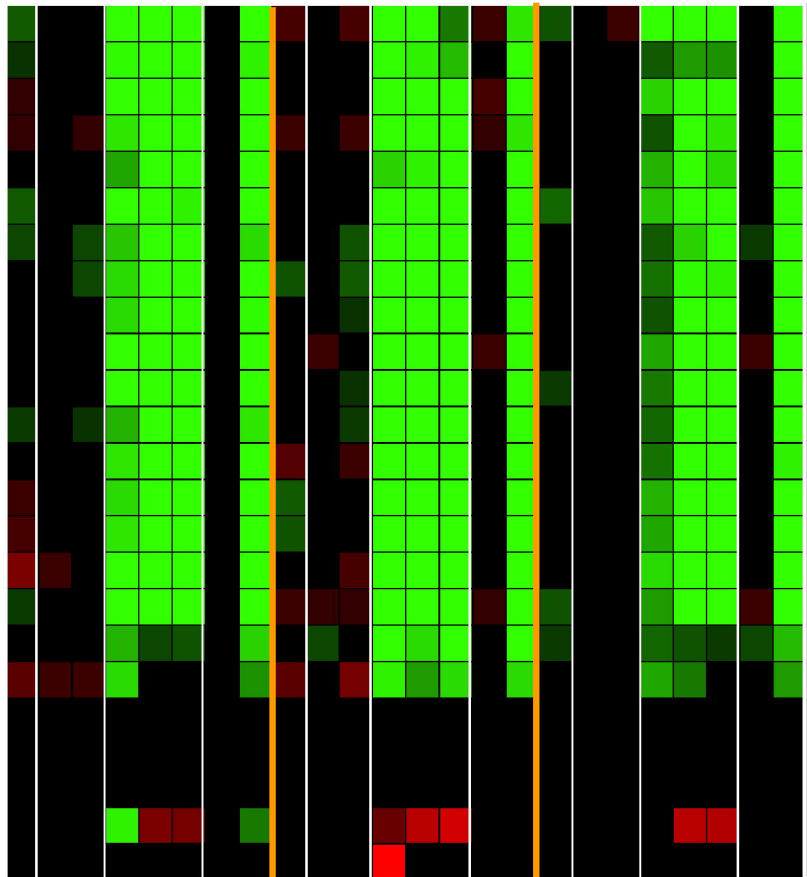
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

Donor 1 Donor 2 Donor 3

Induced CYPs

Filtered for $p \leq 0.05$
*T=0 Hr. Hepatocytes used as baseline

Major CYPs



205743_at	CYP1A1
220017_x_at	CYP2C9
207493_s_at	CYP2D6
206515_at	CYP4F3
206539_s_at	CYP4F12
216058_s_at	CYP2C19
211843_x_at	CYP3A7
211442_x_at	CYP3A43
208367_x_at	CYP3A4
217319_x_at	CYP4A11
208147_s_at	CYP2C8
209976_s_at	CYP2E1
206755_at	CYP2B6
211295_x_at	CYP2A6
207718_x_at	CYP2A7
210272_at	CYP2B7P1
207608_x_at	CYP1A2
205765_at	CYP3A5
214235_at	CYP3A5P2
208327_at	CYP2A13
216334_s_at	CYP2A7P1
210576_at	CYP4F8
208126_s_at	CYP2C18
202437_s_at	CYP1B1

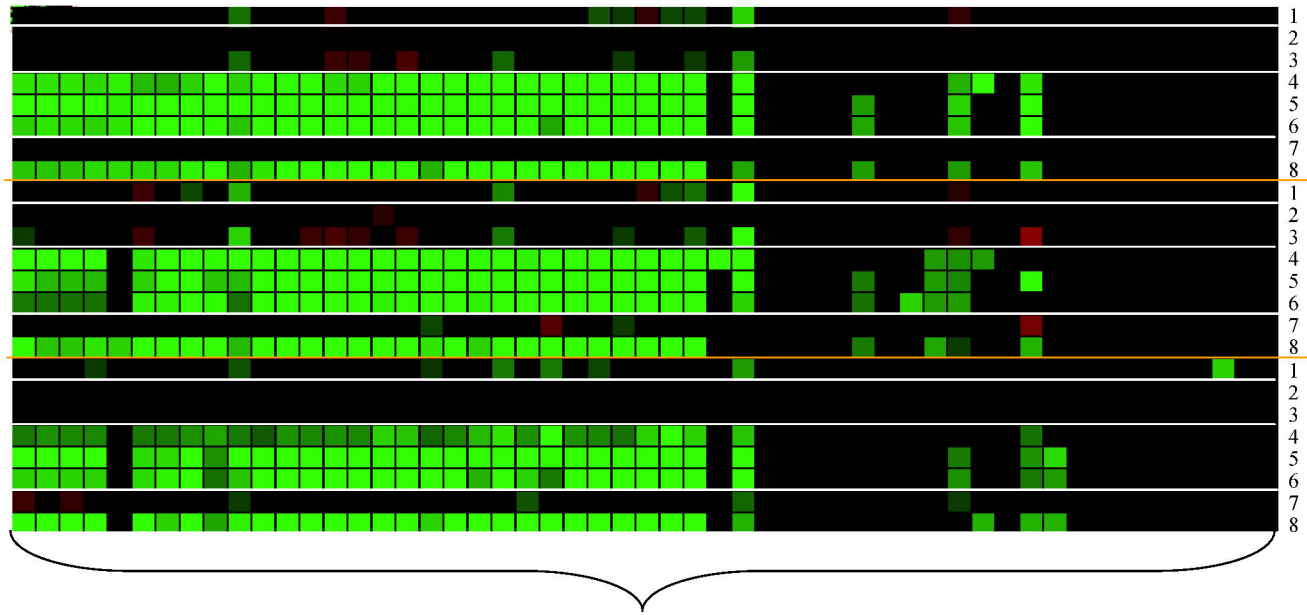
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

Donor 1 Donor 2 Donor 3

Figure 4B

1) Intact Human Liver
2) 3 Hour UW
3) 24 Hour UW
4) 24 Hour Monolayer
5) 48 Hour Monolayer
6) 72 Hour Monolayer
7) Cryopreserved- immediately after thaw
8) Cyropreserved- 24 Hr Monolayer

Figure 4C



Donor 1

Donor 2

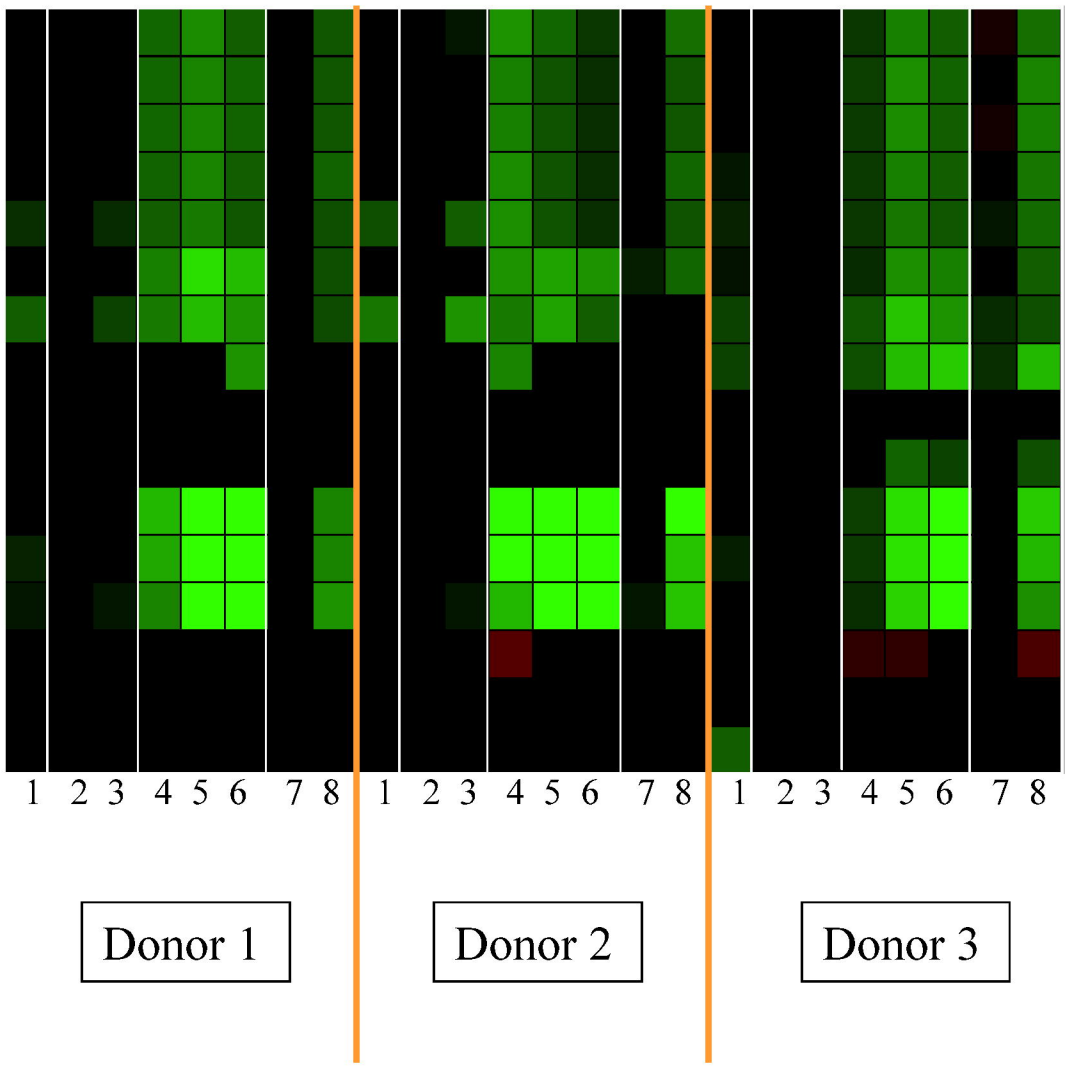
Donor 3

Phase II Metabolizing Enzymes

1) Intact Human Liver
2) 3 Hour UW
3) 24 Hour UW
4) 24 Hour Monolayer
5) 48 Hour Monolayer
6) 72 Hour Monolayer
7) Cryopreserved- immediately after thaw
8) Cyropreserved- 24 Hr Monolayer

Filtered for $p \leq 0.05$
*T=0 Hr. Hepatocytes used as baseline

Figure 4D



206094_x_at	UGT1A6
204532_x_at	UGT1A9
207126_x_at	UGT1A1
215125_s_at	UGT1A10
208596_s_at	UGT1A3
206505_at	UGT2B4
221305_s_at	UGT1A8
217175_at	UGT2B15
221304_at	UGT1A8
207958_at	UGT2A1
216687_x_at	UGT2B15
207392_x_at	UGT2B15
211682_x_at	UGT2B28
221765_at	UGCG
208358_s_at	UGT8
207245_at	UGT2B17

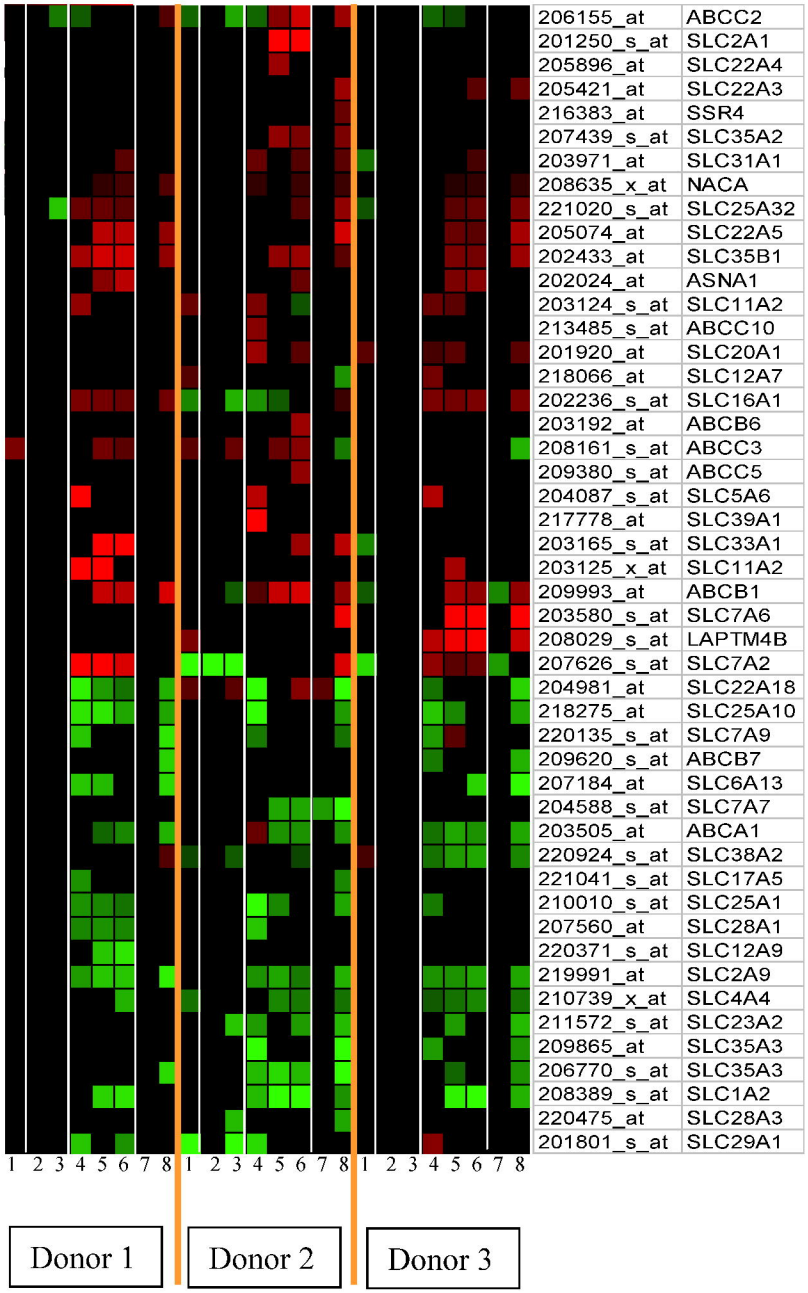
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

Donor 1 Donor 2 Donor 3

-1.60000 0 1.60000
Log(Ratio)

Filtered for p≤0.05
*T=0 Hr. Hepatocytes used as baseline

1) Intact Human Liver
2) 3 Hour UW
3) 24 Hour UW
4) 24 Hour Monolayer
5) 48 Hour Monolayer
6) 72 Hour Monolayer
7) Cryopreserved- immediately after thaw
8) Cyropreserved- 24 Hr Monolayer



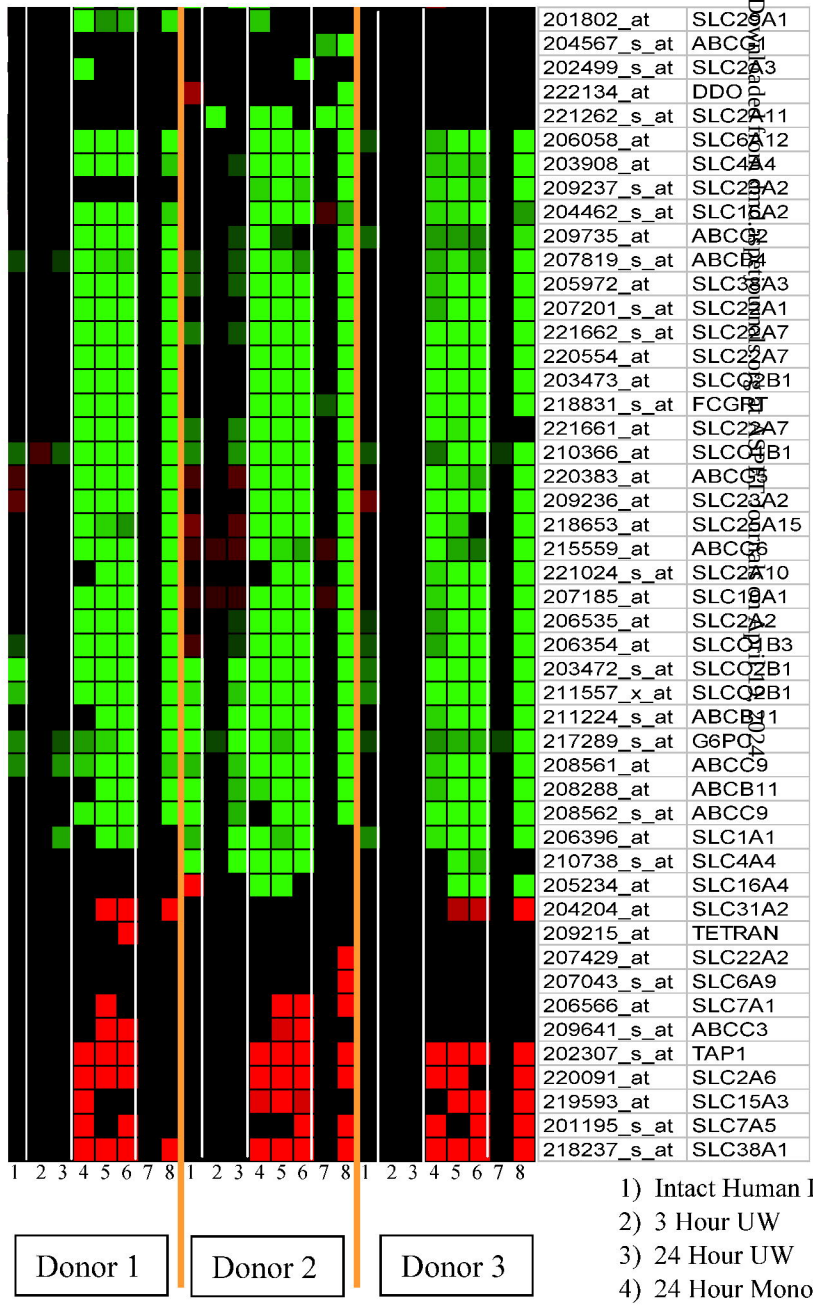


Figure 4E

1) Intact Human Liver
2) 3 Hour UW
3) 24 Hour UW
4) 24 Hour Monolayer
5) 48 Hour Monolayer
6) 72 Hour Monolayer
7) Cryopreserved- immediately after thaw
8) Cryopreserved- 24 Hr Monolayer

Figure 5A

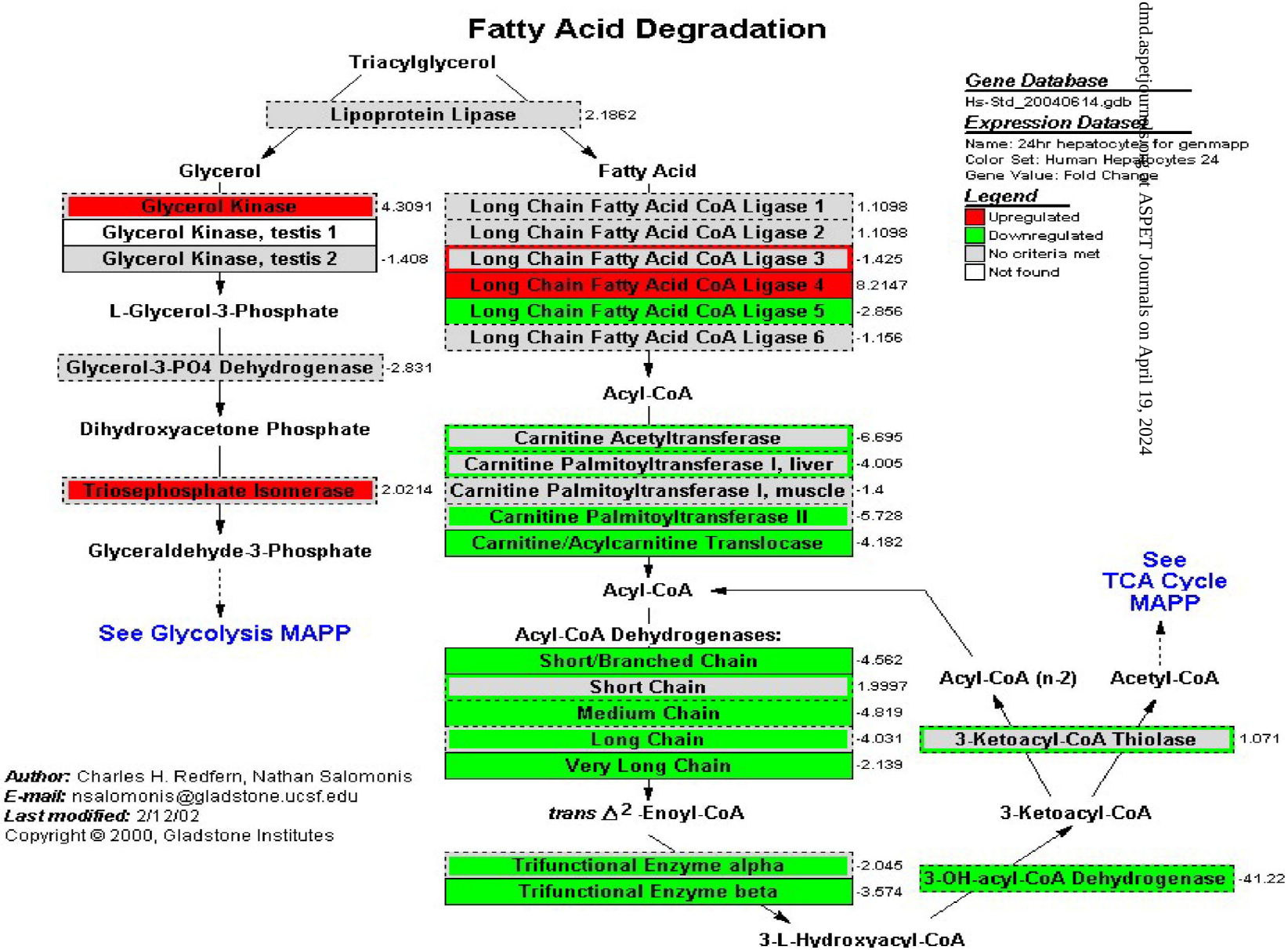
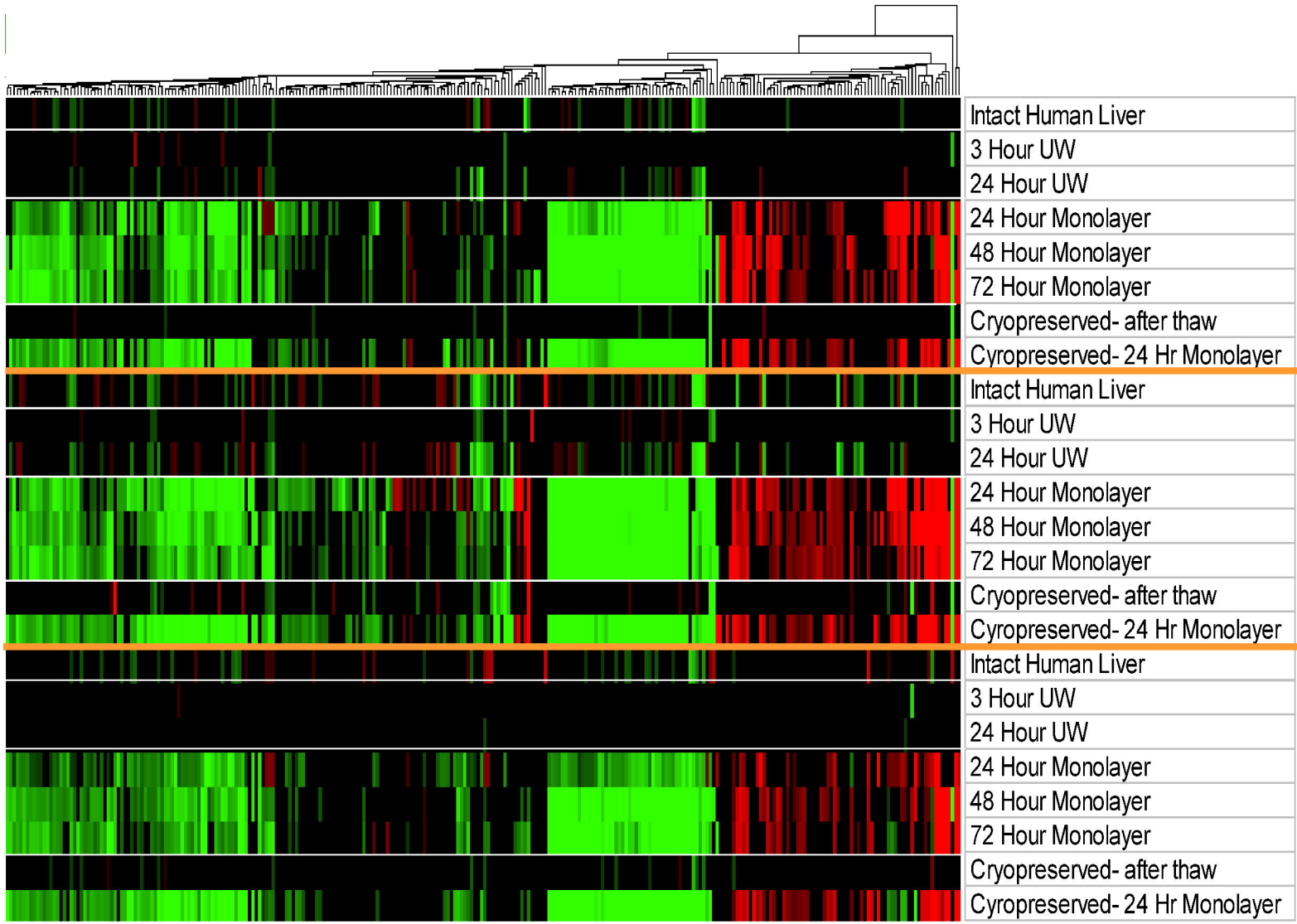


Figure 5B



Donor 1

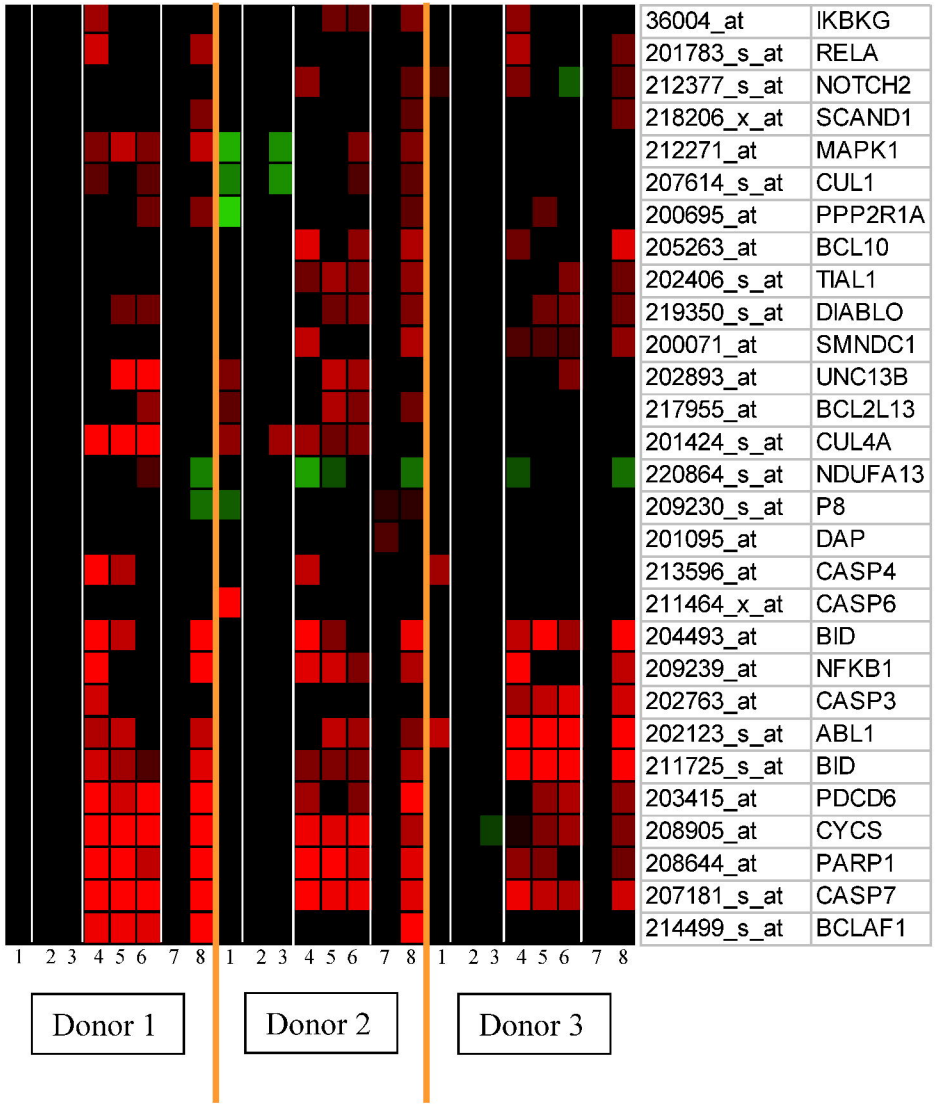
Donor 2

Donor 3

Filtered for $p \leq 0.05$

*T=0 Hr. Hepatocytes used as baseline

Figure 5C



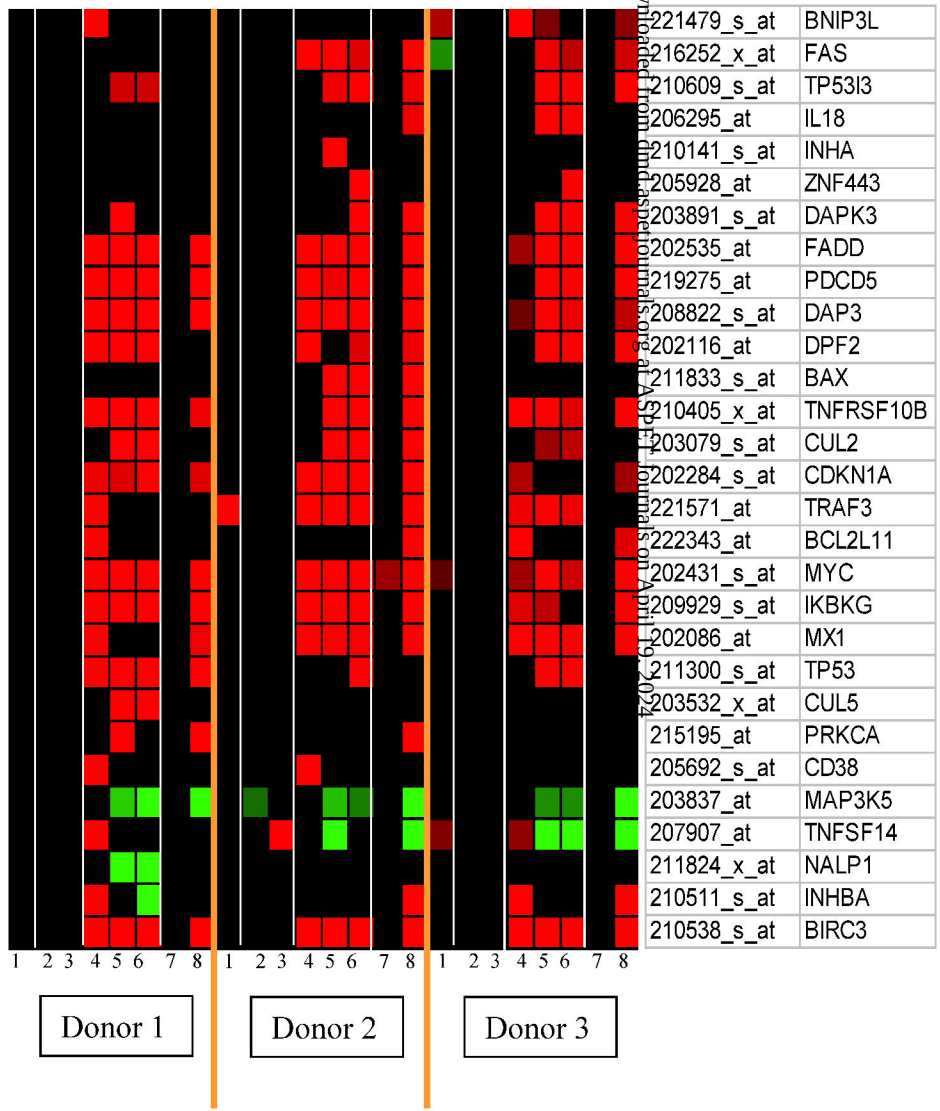


Figure 5D

Filtered for $p \leq 0.05$
*T=0 Hr. Hepatocytes used as baseline

1) Intact Human Liver
2) 3 Hour UW
3) 24 Hour UW
4) 24 Hour Monolayer
5) 48 Hour Monolayer
6) 72 Hour Monolayer
7) Cryopreserved- immediately after thaw
8) Cyropreserved- 24 Hr Monolayer