Development of a Collagen Gel Sandwich Hepatocyte Bioreactor for Detecting Hepatotoxicity of Drugs and Chemicals

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Abstract

Understanding the hepatotoxicity of drugs and chemicals is essential for progress in academic research, medical science and in the development of new pharmaceuticals. Studying hepatotoxicity *in vitro* is a challenging task because hepatocytes, the metabolically active cells of the liver, are very difficult to maintain in culture. After just 24 hours, the cells detach from the plate and die, and even if they survive they usually do not express the metabolic functions which they have *in vivo*. It has been observed by others that culturing hepatocytes between two layers of collagen type I maintains *in vivo*-like morphology and also many drug metabolizing enzymes for weeks. In spite of the research examining drug metabolism in collagen sandwiches, there are very few studies evaluating this system for investigating hepatotoxicity. We cultured primary rat hepatocytes in the collagen sandwich configuration and our goal was to optimize this system for long-term studies and to examine toxicity of a variety of hepatotoxins. By measuring secretions of urea and albumin, and P4501A activity, we determined the optimal cell density to be 50,000 cells/cm^2. We also evaluated the need for epidermal growth factor (EGF) in our cultures, by comparing urea and albumin secretions in cultures grown with and without EGF. The cultures without EGF had significantly less secretion of both urea and albumin just two days after plating. Therefore, we decided to include EGF in the medium. The toxins we examined were aflatoxin B1, acetaminophen, carbon tetrachloride, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methyl methane sulfonate (MMS), cadmium, vinyl acetate and dimethylformamide (DMF). The cells were sensitive to aflatoxin B1, MMS, MNNG and cadmium. However, they were immune to acetaminophen, carbon tetrachloride, vinyl acetate and DMF. Our Western Blots showed that CYP1A, 2B and 3A were maintained in the culture for a week, but CYP2E1 was lost gradually over time. CYP2E1 is also the primary metabolic enzyme for acetaminophen, carbon tetrachloride and DMF. Thus, it is possible that the lack of toxicity is due to the loss of the enzyme responsible for the metabolism of these compounds. Immunity to vinyl acetate suggests that carboxylesterase is also lost in culture, since this enzyme is the one which converts vinyl acetate to acetaldehyde. The metabolism of acetaminophen was also examined with liquid chromatography and mass spectrometry. Liquid chromatography showed that acetaminophen is metabolized primarily to the sulfate and glucuronide metabolites. In order to investigate whether the glutathione adduct was formed, we synthesized the adduct and determined its retention time with liquid chromatography and its fragmentation pattern with mass spectrometry. We isolated the fraction with the same retention time from the medium of acetaminophen-treated cells, and showed that it contains a peak with the same mass to charge ratio and fragmentation pattern as the glutathione adduct. We also examined the

conditioned medium from the hepatocytes to investigate the secreted protein profile, which could potentially be used to find toxicity biomarkers. We were able to remove most of the albumin from the medium using an immuno-affinity column containing anti-albumin antibodies bound to protein A-agarose beads. Our proteomic analysis combined gel electrophoresis with Qstar LC-MS/MS, and we were able to identify 493 proteins. Of those 493 proteins, 59 were plasma proteins, 234 were intracellular proteins and 21 were extracellular matrix proteins. The other 179 proteins were classified as miscellaneous, and they included 13 embryonic proteins, 40 central system proteins, 42 proteins from organs other than the liver and the central nervous system, and the remaining 84 proteins had unknown functions. Several of these proteins have only been identified in stellate and endothelial cells previously, suggesting the presence of nonparenchymal cells in the culture. Furthermore, we also identified many structural matrix proteins, such as laminin-12, fibronectin precursor and five types of collagen, showing that hepatocytes in the sandwich secrete the necessary matrix proteins for adhesion. In summary, we have shown that the collagen sandwich is a suitable system for hepatotoxicity studies as well as proteomic sanalysis.

Thesis supervisor: Steven R. Tannenbaum

Note: The term "collagen" is used in this thesis to refer to collagen type I, unless otherwise noted.

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CHAPTER 1: *In vitro* methods to study hepatotoxicity: A literature review

1.0 Abstract

Understanding the hepatotoxicity of drugs and chemicals is essential for progress in the pharmaceutical industry, medical science and academic research. The study of hepatotoxicity is complicated by the difficulty of culturing hepatocytes *in vitro* due to a lack of understanding of the humoral and matrix requirements of these cells. A variety of *in vitro* models of the liver have been developed, such as perfused livers, liver slices, static cell cultures and three-dimensional perfused bioreactors. The static cell culture is the most commonly used system for hepatotoxicity studies. In this review we present the role of each system in the study of drug metabolism and toxicity. We will also discuss the application of primary cells and human cell lines in toxicity studies. Since the static cell culture has been used in so many studies, we will just highlight some of the hepatotoxicity studies with model. Finally, we will discuss the impact of genomics, proteomics and metabonomics on hepatotoxicity studies, specifically how these approaches have helped to understand differential expression under toxic conditions.

1.1 Introduction

Developing new systems and methods for studying liver toxicity has important applications in medical science, academic research and the pharmaceutical industry. The liver is one of the major detoxifying organs in the body and it is especially susceptible to foreign agents, such as drugs and environmental pollutants, and their metabolites [1]. For this reason, the liver is the primary organ targeted during early toxicity screening studies and nonclinical safety assessment. It has been shown that 40% of the failures during preclinical trials are due to toxicity [2]. Furthermore, only one out of five drugs, which reach clinical trials, also reach the market place, and many of them fail due to hepatotoxicity. This has serious financial implications for the pharmaceutical industry, since the cost of bringing a drug to the market today is estimated to be $800 million. In fact, even though investment in drug development has tripled in the last 10 years to $30 billion, there are fewer drugs coming to the market. This phenomenon is thought to be due to increasing costs of developing drugs and to withdrawal of drugs during the post-marketing period because of adverse drug reactions. Thus, developing a system which would eliminate compounds earlier in the pipeline would be very beneficial [2].

Based on a survey between 1961 and 1992 from France, Germany, UK and USA, 23 out of 181 market withdrawals due to safety were because of hepatotoxicity [3]. Hepatoxicity during post-marketing also leads modification of labeling and stricter guidelines for prescriptions, limiting the market potential of the drug. In fact, development of hepatitis is a common side-effect of drugs. According to Zimmerman et al.[4] 10% of hepatitis is caused by drugs, and in patients over 50, exposure to drugs

accounts for 40% of hepatitis. Additionally, 15-25% of fulminant hepatic failure is due to adverse reaction to drugs.

In spite of the data generated by previous pharmaceutical and clinical research it is still not trivial to estimate the appropriate doses in humans based on animal studies. One reason is that there are significant species differences in the drug metabolism enzymes, as well as in the kinetics of the metabolic reactions. There are also significant polymorphisms in drug-metabolizing enzymes among humans. For example, West African populations have CYP2D6 variants, and some populations of Australian descent have variants of CYP2D6, CYP2C9 and CYP2E1 [5]. Furthermore, many instances of hepatotoxicity are due to an immune-mediated iodisyncratic response, which would be almost impossible to predict based on animal models.

In order to understand the metabolism of novel compounds, there has been a growing interest in the development of *in vitro* systems for studying hepatotoxicity. Hepatocytes, the metabolically active cells in the liver, are one of the most challenging cells to maintain *in vitro*. When plated on plastic or collagen-coated dishes, they have been shown to detach and lose their metabolic capacities within 24 hours. The needs of hepatocytes *in vitro* are not well-understood, but it is known that hepatocyte-specific gene regulation is influenced by both soluble and insoluble factors [6]. Soluble factors include hormones, and cytokines, and insoluble factors refer to cell-cell and cell-matrix contacts. There is currently no universally accepted protocol for maintaining hepatocytes, since as we will discuss, most factors can either stimulate regeneration or differentiation, but not both. In fact, the factors, which lead to hepatocyte differentiation usually suppress differentiation, and vice-versa. For example, long-term cultures of hepatocytes usually

requires a mitogenic compound, such as epidermal growth factor (EGF), which stimulates DNA synthesis. However, it has also been shown that addition of EGF to hepatocyte cultures leads to a down-regulation of the metabolic enzymes, specifically CYP1A, CYP2B [7;8], CYP2C11 [9] and CP3A [10]. Cell density also has a profound influence on cell proliferation and metabolism. Plating cells at low density increases cell proliferation, but reduces the expression and inducibilty of metabolic enzymes [11]. Therefore, when designing *in vitro* systems for detecting hepatotoxicity, these parameters need to optimized so that cells can be sustained without losing metabolic functions. Once the system has been designed, one still has to establish the experimental endpoints, which would be most relevant to the particular compound. Optimizing *in vitro* testing of compounds also involves determining the appropriate doses and the relevant time-points.

As we will discuss, the parameters of *in vitro* systems also depend on the type of toxin studied. While the variety of toxins is so complex, that no strict categorization can be applied, they are usually separated according to whether or not they need to endogenously activated for toxicity. The compounds, which are metabolically activated usually cause toxicity through formation of electrophiles, nucleophiles, free radicals or macromolecular binding [12]. Several commercial assays have been developed to measure toxicity, including measuring for leakage of intracellular enzymes, such as lactate dehydrogenase (LDH) and aspartate amino transferase (AST) as well as reduction of the tetrazolium salts, XTT and MTT. Furthermore, secretion of urea and albumin are also used as markers to asses the health of hepatocytes. Although these commercial assays can be used to measure cell viability, detection of toxicity should also include experimental endpoints known to be relevant to the mechanism of toxicity for a specific

compound. We will discuss some compound-specific endpoints, such as DNA-adducts and protein nitrotyrosine adducts.

We will also review the influence of the "-omics" revolution (genomics, proteomics and metabonomics) on the study of hepatoxicity. Application of genomics and proteomics technologies in toxicology have opened up the field of "toxicogenomics" and "toxicoproteomics." These technologies have facilitated very rapid collection of data to analyze differential gene, protein and metabolite expression, potentially enhancing the development of diagnostics and therapeutics. We will also discuss how the integration this data with systems biology has the potential to facilitate screening of drugs and prediction of metabolism and toxicity.

1.2 *In vitro* models of the liver

The *in vitro* systems, which are most commonly used to study drug metabolism and liver toxicity are (a) perfused livers, (b) liver slices, (c) static cell cultures, (d) flow-through bioreactors, and (e) cell-free systems such as microsomes, liver cytosol fractions and S9 fractions. Although perfused livers and liver slices have been used to study hepatotoxicity, static cell cultures are the most wide-spread system for toxicity studies. We will discuss many of the important parameters in cell cultures, including composition of the medium and the extracellular matrix, cell-density and the effects of co-culturing with nonparenchymal cells.

1.2.1 The isolated perfused liver

The isolated perfused liver is considered to be the closest system to *in vivo* experiments, since it maintains the three-dimensional architecture, the bile flow and the hemodynamics of the liver. Besides its role as an *in vitro* model, it has also been used in clinical settings to treat patients with hepatic coma [13;14]. Although it is expensive to maintain and it can only be used for a few hours, the isolated perfused liver has been used for various hepatotoxicity studies, especially for compounds, which could affect the bile flow. For example, the toxicity of the immunosuppressant drug, cyclosporine, has been studied in isolated perfused livers from rats. It has been shown that this drug can lead to significant liver injury, including cholestasis, and leakage of lactate dehydrogenase and glutamate dehydrogenase [15]. The isolated perfused liver has also been useful in studying the effects of metals, such as cadmium, mercury and copper [16]. These metals were found to decrease the bile flow, but they also decreased ATP content, glutathione content and increase the release of lactate dehydrogenase. Furthermore, copper also led to significant lipid peroxidation, as measured by malondialdehyde content. Release of oxidized glutathione (GSSG) into the bile has been used by several groups as a marker of oxidative stress by aromatic amines and nitroarenes, and it has been concluded that the carcinogenic doses are probably not sufficient to cause acute injury or oxidative stress in the liver [17;18]. The toxicity acetaminophen has also been studied in this system in order to determine how decreased metabolism due to hypothyrodism affects toxicity by this compound. Interestingly, it has been found that hypothyrodism protects the liver from injury by acetaminophen, even when the expression of CYP2E1 is the same as in the control groups [19]. In summary, the isolated perfused liver can be a useful system

for hepatotoxicity studies especially for studying changes in bile flow. As the above studies show, this system has also been used to study oxidative damage and release of intracellular enzymes. While the isolated perfused liver can be a good model for the bile flow and the three-dimensional architecture of the liver, it is very short-lived and it is probably best-suited for compounds which are expected to have toxic effects within a few hours.

1.2.2 Liver slices

Liver slices have been prepared since 1923 [20] and they have been used in a variety of toxicity studies because they maintain the cell-cell and cell-matrix interactions found in liver. The disadvantage of this system in comparison with the isolated perfused liver is that it cannot be used to analyze bile flow. It is also short-lived, primarily due to poor oxygen and nutrient diffusion from the medium. Liver slices are expected to be viable for a few hours, although some groups have found them to be metabolically active for two days [13;21].

Liver slices from rats, guinea pigs, male cynomolgus monkeys, and humans have been used to study unscheduled DNA synthesis (UDS) after exposure to the mutagens aflatoxin B1 (AFB1), 2-acetylaminofluorene (2-AAF) and 6-aminochrysene (6-AC) in order to determine species differences in response to these toxins [22]. UDS was induced by all three compounds in rats, guinea pigs, and humans. Although UDS was observed in monkeys by AFB1 and 6-AC, 2-AAF did not induce DNA synthesis possibly due to a lack of CYP1A2 in this species. A comparison of AFB1 metabolism between humans and rats was also carried out by Heinonen et al. [23], who showed that the rates of metabolism were similar among rats and humans, but there were significant inter-indivual

differences among the three human liver slices observed. This is an interesting result, since it suggests that species differences in metabolism of alfatoxin B1 in liver slices might be less significant than inter-individual differences within the same species.

Environmental pollutants such as cadmium and the herbicide, paraquat, have also been examined in liver slices. The toxicity of cadmium was investigated by Chan et al. [24], who demonstrated that pre-induction with zinc sulphate protected the slices from a decrease in glutathione concentration. The protective effect of zinc sulphate against cadmium toxicity has also been observed in hepatocytes by Moffat et al. [25]. Paraquat has been shown cause significant toxicity within six hours of exposure around the central vein [26]. The role of superoxide in the toxicity of this compound was examined by showing that addition of a superoxide dismutase inhibitor increased lactate dehydrogenase leakage. Inflammation caused by exposure to paraquat was also visualized by histological examination of the liver slices.

Studies of tissue slices are not limited to liver slices. Toxicity caused by a number of halogenated compounds, including the liver toxin carbon tetrachloride, has been examined in slices of rat liver, kidney spleen and testes in order to examine how well the LD_{50} correlated with doses shown to cause lipid peroxidation [27]. Interestingly, the doses only correlated for a few of the compounds, suggesting that lipid peroxidation alone is not sufficient for lethality. In summary, these studies show that liver slices have been applied in a number of different hepaotoxicity studies, and can be useful for studying DNA damage, oxidative damage and histology.

1.2.3 Static cell-cultures

Static cell cultures are currently the most popular *in vitro* systems for studying hepatotoxicity. The most commonly cultured cells are freshly isolated hepatocytes, since cryopreserved cells and cell lines do not express the necessary metabolic enzymes. However, cryopreserved cells and cell lines can be induced to express many of the cytochrome P450's, by growing the cells in the presence of phenobarbital, dexamethasone, rifampicin and 3-methylcholanthrene [28;29]. We will discuss the role of human cell lines in toxicity studies in a later section.

In order to facilitate adhesion of cells to the surface, most cultures are plated on dishes coated with extracellular matrix proteins, such as collagen, fibronectin and laminin. Culturing hepatocytes on these structural matrix proteins has been found to also maintain cell morphology and the metabolic functions much better and for longer than plating them on plastic. Culturing hepatocytes in a sandwich configuration is even more suitable for maintaining metabolically active cells, and hepatocytes in this configuration have also been shown to maintain biliary secretion. The most common matrices for sandwich cultures are collagen type I and Matrigel, an extract derived from the basesement membrane of the Engelbroth- Holm-Sworm mouse sarcoma [30]. Both collagen-collagen and collagen-Matrigel sandwiches have been shown to maintain morphology and albumin secretion for several weeks [31], and they are also inducible for both CYP1A2 and CYP3A4 in systems with human hepatocytes [32]. Collagen-Matrigel sandwiches have been shown to have some advantages over collagen-collagen sandwiches, such as expression of the gap junction protein connexin 32 and the expression of the epidermal growth factor receptor[31] . However, the disadvantages of

Matrigel are that it is significantly more costly than collagen, and there are many batch-to-batch variations. Thus, most studies in a sandwich culture have been carried out in collagen-collagen sandwiches. Cells in a collagen sandwich have been shown to maintain their polygonal morphology [33-35] and remain viable in culture for up to six weeks. In addition, they maintain CYP1A and CYP3A, sulfo-and glucuronsyltransferases, as well as glutathione S-transferase [36;37]. In fact, hepatocytes in the sandwich configuration have been shown to maintain biliary exretion [38-40] and this efflux has been show to be inhibited in a dose-dependent manner by cyclosporine A, a known blocker of bile secretion.

The composition of medium is also a very important factor in the maintenance of long-term cultures. Components in the medium can influence regeneration of hepatocytes as well as up-regulation or suppression of metabolic enzymes. In the absence of serum, long-term cultures of hepatocytes require insulin, as well as a mitogen such as EGF [41]. While EGF has been shown to maintain viable hepatocytes for weeks, it has also been shown to significantly decrease CYP2C11 [9] and CP3A [10] activities in hepatocytes and specifically CYP1A and CYP2B in collagen sandwiches [7;8]. This effect can lead to lack of toxicity from compounds metabolized by these enzymes. In contrast, Phenobarbital suppressed hepatocyte growth and secretion of structural matrix proteins, while maintaining expressions of CYP1A, 2B and 2E1 [42]. Thus, the choice of medium should be influenced by the type of application, such as the life-time of the culture system, and which toxins will be tested in the system.

There have also been attempts at establishing stable co-culture systems with nonparenchymal cells, especially endothelial, Kupffer and stellate cells. These non-

parenchymal cells play very important roles in the survival and differentiation of hepatocytes as well as in the mediation of toxicity. Endothelial cells line the sinusoidal wall and can play a role in the reorganization of hepatocytes in culture. They also secrete cytokines, nitric oxide and matrix components. Kupffer cells are the hepatic macrophages and they mediate inflammation and innate immunity through secretion of signaling molecules and antigen presentation. In the healthy liver, stellate cells are responsible for storing vitamin A and maintaining the extracellular matrix. When the liver is injured, stellate cells transform into myofibroblast cells to mediate the inflammatory response [43]. Co-culturing of nonparenchymal cells is challenging because the medium and matrix composition has to satisfy the needs of two different cell types. Co-cultures with endothelial cells has been achieved by Hirose et al. [44] and Harimoto et al.[45] and they observed that co-cultures maintenained cell junctions, secretion of extracellular matrix and hepatocyte morphology better than hepatocytes grown alone. Co-culture with stellate cells can also stimulate DNA synthesis [46] and maintain CYP450 activities [47]. Hepatocyte function can also be stimulated with conditioned medium from nonparenchymal cells. Kang et al. [48] exposed Matrigel and collagen gel sandwiches to conditioned medium from a culture of nonparenchymal cells and found an increase in DNA synthesis as well as an improvement in hepatocyte morphology. As these studies show, hepatocyte function in culture can be altered by a variety of matrix components, medium components and non-parenchymal cells, and the design of hepatotoxicity experiments with hepatocytes needs to take all these factors into consideration.

Hepatotoxicity studies in primary hepatocytes

It would be impossible to give a complete overview of all the hepatotoxicity studies with primary hepatocytes, because they have been used in a variety of toxicity studies. Most studies use hepatocytes from rat and mice, although we did find some references with primary human hepatocytes. In this section, we will highlight some examples of hepatotoxicity studies in primary hepatocytes with model toxins, particularly with acetaminophen and carbon tetrachloride, in order to demonstrate some of the advantages and disadvantages of this system.

Acetaminophen, an over-the-counter analgesic, has caused significant concern because of its easy accessibility and its history as a common suicidal agent. It has been used as a model toxin to study acute liver failure and the role and of biotransformation in hepatotoxicity [4]. Acetaminophen (APAP) can be metabolized by CYP1A2, CYP3A4 and CYP2E1 and converted to the reactive N-acetylbenzoquinone imine (NABQI), but in humans this conversion occurs primarily by CYP2E1 [49]. NABQI is detoxified by conjugation with glutathione, but after the glutathione is depleted NABQI can bind to cytosolic and mitochondrial proteins [50]. In cultured mouse hepatocytes 5 mM APAP led to oxidant stress and ultimately necrosis, but this toxicity was attenuated by pretreating the cells with N-acetylcysteine, a precursor of glutathione [51]. APAP has also been shown to cause DNA strand breaks, although the exact mechanism for this is not known. It has been shown recently that addition of the antioxidants, N-acetylcysteine, polyphenol silibin and α-tocopherol, protected primary rat hepatocytes from APAP-induced DNA strand breaks, but not APAP-induced toxicity [52]. The role of oxidant stress in DNA strand breaks was also demonstrated by an increase in malondialdehyde

production at the doses which caused DNA damage. Experiments with acetaminophen have also been useful in determining the effect of growth factors on toxicity. The growth factors EGF and HGF are known to suppress metabolic enzymes, but they have also been shown to offer protection against APAP toxicity in rat hepatocytes [53].

It is well-known that consumption of ethanol together with APAP leads to hepatotoxicity, due to the induction of CYP2E1 by alcohol [12;54]. Interestingly, caffeine has also been shown to increase APAP hepatotoxicity in mice and rats [55;56]. DiPetrillo et al. [57] investigated the effect of caffeine on APAP toxicity in cultured rat hepatocytes. They observed that ethanol treatment alone did not increase APAP toxicity, although it did induce CYP2E and CYP3A. However, when ethanol-pretreated hepatocytes were exposed to caffeine, there was a significant increase in APAP toxicity. In addition, caffeine increased the formation of the glutathione adduct (an indirect measure of NABQI formation) three-fold compared to cells exposed to just ethanol and APAP. These results could have important implications for human consumption of APAP with caffeine, which is found in many beverages and medications.

Carbon tetrachloride, which was used previously as a dry cleaning agent and refrigerant, has also been widely studied due to its severe hepatotoxic effects. It is converted to a free radical after undergoing reductive deholgenation by cytochrome P450's to form a carbon-centered free radical [58], that initiates lipid peroxidation and leads to morphological changes. Hepatocyte cultures lend themselves very well to microscopic investigations, allowing for detailed examination of structural changes after toxin treatment. While light microsopy has been used widely to study gross microscopic changes, the development of more advanced microscopy methods such as

epiflourescence microscopy, scanning electron microscopy, and laser-scanning confocal microscopy have significantly enhanced morphological studies. These techniques have made it possible to perform immunologic detection, to visualize ultrastructural changes and to recreate a cell or even a tissue section in three dimensions.

Toxicity following carbon tetrachloride treatment was investigated in hepatocyte cultures with light microscopy and it was observed that carbon tetrachloride leads to significant morphological alterations, which result from the peroxidation of polyunsaturated fatty acids in cellular membranes and affect the endoplasmic reticulum, the Golgi apparatus, the plasma membrane and the mitochondria [59;60]. Carbon tetrachloride is also known to be a fast-acting toxin and *in vivo* administration results in morphological changes appearing within 15 minutes after exposure. Thus, carbon tetrachloride is a good model compound for studying morphological changes in hepatocyte culture. In fact, carbon tetrachloride has been observed to cause significant ultrastructural changes in membranes 5 minutes after treatment. Scanning and transmission electron microscopy which magnify cells many thousand-fold can be used to visualize the effects of carbon tetrachloride, which include blebbing of the plasma membrane, smoothing out and degranulation of the rough endoplasmic reticulum 24 hours after treatment [59].

Microcystin-LR, a potentially lethal toxin produced by aquatic organisms, has been shown to exert its toxicity by inhibiting serine/threonine protein phosphatases, specifically types 1, 2A and 3. Similarly to carbon tetrachloride, microcystin has been shown to lead to lipid peroxidation, as well as production of reactive oxygen species and depletion of glutathione in cultured rat hepatocytes [61]. Using laser-scanning confocal

microscopy, it has been shown that microcystin exposure in hepatocytes leads to cell shrinkage, nuclear condensation, collapse of the actin cytoskeleton and cell blebbing, suggesting that microcystin causes cell death through apoptosis [62;63].

Confocal laser scanning microscopy (CLSM) combined with fluorescent detection of monochlorobimane (a lipophilic glutathione-specific probe, which is only fluorescent when bound to thiols) has also been applied to measure intracellular glutathione distribution [64]. This new technique has used to monitor depletion of glutathione by inhibitors such as buthionine sulfoximine, and known glutathione depletors, such as menadione, in rat hepatocytes. Since glutathione plays an important role in detoxification, this new method could be used to study the role of glutathione in the metabolism of novel compounds.

CLSM has also been used to evaluate the viability of cryopreserved hepatocytes. McKay et al. [65] used a vital stain carboxyfluorescein diacetate, which is deacetylated by intracellular esterases to form a fluorescent carboxyfluorescein (CF). CF cannot diffuse through membranes and is retained inside the cells. CLSM imaging showed higher CF retention for cells cultured in Williams' medium, versus Chee's medium, prior to freezing. Interestingly, the imaging suggested better retention of CF inside organelles, suggesting that the membranes of the organelles are less sensitive to freezing than the cell membranes, which is an important consideration when using cryopreserved cells for cell culture.

Since hepatocytes in culture do not express all the metabolic enzymes, *in vitro* experiments are sometimes combined with *in vivo* experiments by pretreating animals with inducers before hepatocyte isolation. It has been shown that carbon tetrachloride

toxicity is potentiated in rat hepatocytes isolated from animals treated with phenobarbital. In addition, the levels of total CYP450 were very close to those *in vivo* [66]. On the other hand, pretreating the animals with a protective agent such as alpha-tocopherol, significantly protected hepatocytes from necrosis due to carbon tetrachloride compared to cells from untreated rats. Lipid peroxidation, which is usually measured by monitoring malondialdehyde formation with thin layer chromatography, was also found to be significantly reduced in hepatocytes from treated rats [67].

Isolation of hepatocytes from treated rats has also been used for studying the anticancer drug, Yondelis. This drug, has been shown to be effective in treating breast and ovarian cancer, but it also causes hepatotoxicity in rats [68]. However, it has been observed that pretreatment of rats with dexamethasone, protects against hepatotoxicity, probably due to the upregulation of CYP3A, which metabolizes the drug. However, toxicity of Yondelis was not ameliorated in hepatocytes isolated from dexamethasone-pretreated rats. Thus, upregulation of the enzymes is not the only mechanism responsible for the protection against hepatotoxicity, suggesting that in order to study drugs with unknown mechanism, *in vivo* experiments are necessary for complete understanding of toxicity pathways.

Hepatoxicity in human cell lines

The HepG2 hepatoma cell line is one of the most popular human cell lines for hepatoxicity studies. This cell lines has been used to study the mechanisms of toxicity of a variety of toxins such as 7H-Dibenzo[c,g]carbazole (DBC) and aflatoxin B1 [69]. DBC, N-heterocyclic aromatic hydrocarbon, has been classified as carcinogenic and

hepatotoxic in several species. By exposing HepG2 cells to DBC it has been shown that at doses lower than 10 μM, DBC forms DNA adducts and leads to apoptotic cell death. At doses higher than 80 μM, there were fewer DNA adducts, and necrotic cell death was apparent from cell swelling and loss of membrane integrity. In contrast to the biphasic death curve of DBC, exposure to AFB1 resulted in a concentration-dependent increase in apoptosis.

The cytotoxicity of ethanol and arachidonic acid was studied in transduced HepG2 cells expressing CYP2E1 [70]. Toxicity by both compounds led to apoptosis, as shown by activation of caspases I and III. Furthermore, apoptosis was prevented by transfection with a plasmid containing Bcl-2. Thus, HepG2 can be used as a model cell line for studying toxicity and apoptosis by hepatotoxins. HepG2 cells have also been used to study the toxicity of pharmaceuticals such as troglitazone [71], an antihyperglycemic agent, and diclofenac [72], an anti-inflammatory drug, both of which have been shown to cause adverse hepatic reactions in patients. Troglitazone was found to cause significant mitochondrial damage as revealed by ultrastructural analysis, as well as a decrease in ATP levels and mitochondrial membrane potential. Diclofenac was actually more toxic to freshly isolated hepatocytes than to HepG2 cells, probably due to drug-metabolizing capacity of primary cells. Although diclofenac was shown to lead to mitochondrial damage in HepG2 cells, their decreased sensitivity in comparison to fresh hepatocytes suggests that HepG2 cells are better suited for testing drugs not requiring metabolism. However, as mentioned previously, HepG2 cells can be transduced to express certain CYP450's, making it possible to test toxicity of indirect toxins.

Another human hepatoma cell line (HLE), was engineered to express 2E1[73]. It was observed that a 3mM dose of CCl_4 caused almost 90% death in engineered cell lines but only a 20% death in the non-engineered controls. It was also shown that the gene expression of the heat shock protein HSP70, a marker of oxdative stress, increased more in the engineered cell lines than in the control cells after CCl_4 exposure. Mace et al.[74] engineered several human cell lines from THLE, a non-tumorigenic human cell line. These cells retained most of their phase II enzymes, but lacked the cytochrome P450's. The engineered cell lines were transfected with human CYP's 1A2, 2A6, 2B6 and 3A4. The sensitivity of these cell lines to aflatoxin B1 was, 125-, 2-, 2, and 15-fold higher than in the non-engineered cells. This result is consistent with *in vivo* metabolism studies since it is known that in humans CYP1A2 is the primary enzyme and CYP3A4 is the secondary enzyme responsible for the metabolism of aflatoxin. The DNA adducts were also quantified and it was found that nanomolar doses caused DNA adduct formation. The primary adducts were AFB1-N7 guanine, -pyrimidyl, and –diol. In addition, all three cell lines showed a dose-dependent accumulation in S-phase but they all had different abilities to recover from the S-phase [75]. These results have biological significance, since the most common lesions *in vivo* are the N7-guanine and the pyrimidyl, also known as AFB1-formamidopyrimidine (FAPY) [76].

Recently, a new human cell line, BC2, was derived from HepG2 and it has been shown that this cell line can maintain a number of drug-metabolizing enzymes, such as CYP1A1/2, 2E1 and 3A4, for up to five weeks in culture [77]. This cell line has also been shown to be sensitive to several toxins including acetaminophen and acetylsalicylic acid [78].

1.2.4 Three-dimensional and flow-through bioreactors

Although static hepatocyte cultures are inexpensive and practical, they lack the three-dimensional cell-cell contacts and the constant flow of oxygen and nutrients, which are present in the liver. In order to remedy this issue, several perfused bioreactors were developed. The earliest flow-through bioreactors were hollow fiber bioreactors, which are used today to sustain patients with acute liver failure [79], but they have also been tested as *in vitro* models of drug metabolism. Hollow fibers have been shown to maintain hepatocyte morphology such as bile canaliculi and intercellular junctions even seven days after culture [80]. Nyberg et al. [81] cultured gel-entrapped rat hepatocytes perfused in a hollow fiber reactor and showed that the hepatocytes in this system maintained several metabolic activities, including sulfation, glucuronidation, P4503A activity. Powers et al. [82;83] have developed a perfused three-dimensional bioreactor, consisting of primary rat hepatocytes seeded onto channels etched into a silicon scaffold. After seeding, the cells rearranged themselves into tissue-like structures, and it was observed that pre-aggregation of cells into spheroids prior to seeding improved morphogenesis. This system was also able to maintain rates of albumin and urea secretion for 15 days after isolation. There are also a few limited studies on the application of perfused systems for drug metabolism studies. Bader et al. [84] developed a flow-through collagen sandwich bioreactor with primary rat hepatocytes. They have shown that the metabolic profile of the antihypertensive drug, urapidil, includes all the metabolites found *in vivo*, suggesting this system can be used for drug metabolism studies.

In addition to all the considerations in the design static culture experiments, design of perfused reactors needs to take several addition parameters into account. The shear stress associated with the flow can lead to cell detachment, thus it can be advantageous to coat the surfaces with a matrix protein such as collagen to enhance cell adhesion. This strategy also ensures cell adhesion to the desired areas [83]. On the other hand, some shear stress can be beneficial, since it has been shown to stimulate metabolic functions and morphogenesis [85;86]. Furthermore, the flow of the medium needs to be controlled, in order to ensure an even distribution of oxygen and nutrients in the system. One of the considerations with metabolism studies is that the perfusion introduces significantly more liquid volume, which dilutes the concentration of drugs and their metabolites. This dilution factor complicates the detection of metabolites in the effluent medium, especially if an on-line detection system is desired. Therefore, design of perfused bioreactors for metabolism and hepatotoxicity studies should consider the parameters mentioned above to ensure more physiological conditions and maximum sensitivity.

1.2.5 Subcellular fractions

Subcellular fractions, such as microsomes, are widely used for studying metabolic activation of drugs. Microsomes, the packets of enzymes on the endoplasmic reticulum, contain the P450's and the necessary cofactors for metabolism of drugs[13]. Micrsomes are particularly useful for comparing species differences in the bioactivation of drugs by P450's. Human-derived liver microsomes have also been used to study the metabolisms of aflatoxins M1 and B1 in order to compare the metabolism of the two compounds [87].

It was observed that CYP1A2 contributed to over 95% of the AFB1 activation. This result is physiologically relevant DNA binding studies also showed that CYP1A2 was responsible for 95% of the AFB1 adduct formation [88].

Other subcellular factions such as liver cytosol and the S9 fraction have also been used for drug metabolism studies. The liver cytosolic fraction is isolated by differential centrifugation of a liver homogenate [89]. The advantage of this fraction is that it contains phase II enzymes such glutathione S-transferase and N-acetyltransferase. Although additional cofactors, such as acetyl coenzyme A, are necessary for this system, liver cytosolic fractions are available commercially and are easy to use. The S9 fraction of the liver contains the cytosols and the microsomes, thus it combines the metabolic capacities of the two systems. However, the S9 fraction also requires additional cofactors for phase II activities, as well as an NADPH-regenerating system to maintain the CYP450 enzyme activities.

1.3 The "-omics" revolution

1.3.1 Genomics

The genomics revolution, which began with the genome sequencing project, aims at identifying all the genes involved in biological processes. The identification of the complete genome sequences of multiple organisms made it possible to engineer DNA microarrays, which can measure gene expression of hundreds or even thousands of genes simultaneously. DNA microarrays consist of a solid support with oligonucleotides or

cDNA complementary to the sequences under investigation. The microarrays are incubated with fluorescently labeled samples, and hybridization is detected using fluorescence. The data shows the up or down-regulation of the genes and allows for comparison of gene expression levels between different biological samples. Boess et al. [90] evaluated gene expression in two cell lines, hepatocytes in a conventional monolayer, hepatocytes in a sandwich and liver slices. Overall, liver slices were the most similar to the *in vivo* liver, but even this system has decreased expression of several genes, including many of the drug metabolizing genes. It was also observed that gene expression changes most dramatically right after isolation and then stabilizes during the culture. This has implications for drug metabolism studies, since this data suggests that *in vitro* systems have significantly lower metabolic activities, even though some metabolic capacity is maintained throughout the life of culture.

Using a special toxicological microarray, which contained 900 genes, Baker et al. [91] studied the temporal changes (between 4 and 72 hours) in genes relevant to toxicological studies and confirmed the studies with RT/PCR. Clustering analysis showed that there was a time-dependent dedifferentiation response of the hepatocytes. Although many of the cytochrome P450's were down-regulated, their gene expression changed at different time-points. The expression of the phase II enzymes was more varied with UDP-glucuronosyl transferase and GST pi being upregulated, while the expressions of sulfotransferase and GST alpha were repressed.

DNA microarrays been also applied in toxicological studies to identify the pathways involved in toxicity mechanisms, opening up the fields of toxicogenomics The aim of toxicogenomics to identify a global gene expression profile in order to determine

the differential gene expression between two samples [2]. Toxicogenomics has been used to study differential gene expression caused by hepatotoxins including aflatoxin B1, 3-methylcholanthrene and dimethylnitrosamine.

Toxicogenomics in vivo

Gerhold et al.[92] exposed rats to several inducers of CYP450 genes, namely 3-methylcholanthrene (3MC), phenobarbital, dexamethasone or clofibrate and measured the expression of several drug metabolizing genes including CYP's, glutathione transferases, sulfotransferases, and drug transporters genes. It was observed that that the responses of the genes agreed with the published observations. For example, 3MC induced CYP1A1/2 and CYP2B1 and several phase II enzymes including UGT1A6, GSTA1, GSTA2 and GSTM1, all of which have been observed in previous research to be induced by 3MC in rat hepatocytes.

Ellinger-Ziegelbauer et al. [93] compared the expression profiles induced in rats by four genotoxic hepatocarcinogens: dimethylnitrosamine, 2-nitrofluorine, aflatoxin B1 and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. As expected, the expression profiles among the four different toxins showed several similarities including up-regulation of DNA damage, oxidative stress and fibrosis response genes. Furthermore, there was a significant increase in the tumor suppressor protein p53. It is encouraging, that compounds which are known to act through the same pathways lead to similar changes in the gene expression profiles in the rat liver. Similar results were found by Waring et al. [94] who tested 15 different hepatotoxins including carbon tetrachloride, dimethylformamide and arsenic, and analyzed the histopathologic and gene expression caused by these agents. Treatment with these agents resulted in significant liver injury

including cirrhosis, hypertrophy and also hepatocellular damage such as necrosis and DNA damage. The gene expression results correlated well with the histopathological results, suggesting that genomics analysis can be used to evaluate hepatotoxicity changes *in vivo*.

Toxicogenomics in vitro

In vitro systems have decreased gene expression compared to the liver, but experimental data suggests that they are still suitable for toxicogenomics studies. Waring et al. [95] treated rat hepatocytes with several hepatotoxins such as carbon tetrachloride and dimethylformamide in order to study differential gene expression. The changes in gene expression in the hepatocytes correlated well with the histopathological changes observed in the *in vivo* experiment. Furthermore, compounds with similar toxicity mechanisms led to overlapping changes in gene expression. These changes were not identical, however, suggesting that each compound might have a unique genomic signature.

Most microarrays contain many thousands of targets, therefore special microarrays have been developed for particular applications, which contain a smaller set of genes. De Longueville et al [96] used a special microarray containing 59 genes, which were thought to be relevant to toxicology. The chip included the CYP450's, stress response genes and genes involved in apoptosis. Eleven toxins, divided into 4 categories (CYP 450 inducers, necrosis inducers, cholestasis inducers and steatosis inducers) were tested on primary rat hepatocytes. Changes in gene expression correlated well with the known mechanisms of toxicity for these compounds, and clustering analysis showed that compounds with similar mechanisms cause similar types of changes in gene expression.

The toxicogenomics of carbon tetrachloride and ethanol were investigated in HepG2 cells in order to determine similarities and differences in the differential gene expression [97]. It was shown that carbon tetrachloride up-regulated genes involved in extracellular transport and cell signaling, such as apolipoprotein A-II and IL-6, and ethanol down-regulated genes involved in stress response and metabolism, such as DNA mismatch repair protein PMS2 and mutY. Changes in genes involved in the cell cycle was common to both compounds, such as the down-regulation of MAP kinase 3 and c-myc binding protein MM-1.

In order to compare toxicogenomics *in vitro* to *in vivo,* Jessen et al. [98] tested five different hepatotoxins in rats and rat liver slices. Qualitatively, there was 80% concordance between the two systems but the *in vitro* systems had a much lower response, suggesting that *in vitro* experiment might not give the complete global genomics profile.

1.3.2 Proteomics

Proteomics, defined as the high-throughput separation, display and identification of proteins, is a developing field which has the potential to enhance understanding of biological processes by identifying the full range of proteins in biological samples [99-101]. One of the major advantages of using proteins as biomarkers is that they are secreted in body fluids and many of them change during a disease state. With the advances in technology, especially in mass spectrometry and liquid chromatography, it is now possible to quickly characterize proteins in biological samples and to identify some possible targets for screening and diagnostics.

The methods for proteomics analysis are still under development, but most protocols rely upon separation of proteins by gel electrophoresis, digesting the proteins with a proteolytic enzyme such as trypsin, and identifying the peptide masses with mass spectrometry. The most common ionization techniques for identifying peptides are matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). MALDI instruments, which are usually coupled to time-of-flight (TOF) spectrometers, are especially well-suited for peptide mass fingerprinting. However, this approach usually requires the identification of at least four peptides per protein [102]. For protein mixtures, which usually require LC-MS or LC-MS/MS, ESI is the standard method of ionization. The development of the nanospray around 1999 [103] allowed injections in the range of 20 nl/min, which made it possible to perform several stages of tandem mass spectrometry from sample quantities as small as 1 μl [104]. In general, the detection limit for proteins and peptides is between femtomoles and picomoles, however the sensitivity depends on the purity of the sample and the instrument.

Identification of proteins in biological samples usually requires the removal of high-abundance proteins, such as serum albumin and immunoglobulins, before gel electrophoresis [105;106]. The removal of these proteins is usually achieved by immunoaffinity columns, and has allowed for more sensitive and higher resolution of proteins in serum [106;107]. The sensitivity of the proteomics analysis is also influenced by the sample collection method. Witzmann et al.[108] showed that one can obtain a higher yield of cellular proteins when the proteins are directly solubilized in the cell culture, rather than extracting them after cell scraping. In fact, they found the latter method resulted in a 35% loss of the total protein content.

The identification of proteins involved in disease or toxicological processes has opened up the new field of "toxicoproteomics," with the potential of discovering proteins, which can be used as markers of toxicity in biological samples. Biological fluids such as serum, urine, spinal fluid and even exhaled breath can be used as proteomics samples to diagnose and prevent disease [104]. One of the diseases that proteomics aims to study is cancer. Comparison of normal tissues with tumor tissues has already revealed a number of differences in protein expression. Heat shock proteins, in particular, have been found to be differentially expressed in liver tumors [109], oral tongue squamous cell carcinoma [110] and ductal carcinoma of the breast [111].

It is well-known that growth factors in the medium can significantly change the expression of several proteins, especially those involved in differentiation and regeneration. Chevalier et al. [112] analyzed the protein profiles in primary hepatocytes treated with either epidermal growth factor (EGF) or the peroxisome proliferator, nafenopin, in the presence of tumor necrosis factor alpha (TNF-α). Both EGF and nafenopin stimulate DNA replication, but through different pathways. The proteomic profiles in response to these agents were quite different, and some of the proteins induced by nafenopin did not involve the peroxisomes at all. These types of analyses can help to understand the mechanisms of growth control in hepatocyte cultures.

Proteomics analysis has also been used to gain a better understanding of the effects of toxin exposure. Ruepp et al. [113] characterized the protein profile in the mouse liver after acetaminophen exposure and found a significant decrease in protein abundance in several mitochondrial proteins, such as the heat shock proteins, just 15 minutes after exposure. Large doses of acetaminophen have been found to bind

covalently to proteins, particularly the cysteine residues, which leads to significant centrilobular necrosis [114]. Oxidant stress mediated by nitric oxide has also been shown to be involved in acetaminophen toxicity, which can be detected by measuring nitrotyrosine adducts in proteins [114;115]. Qiu et al. [116] investigated the target proteins for acetaminophen binding by treating mice with radio-labeled acetaminophen, isolating liver proteins and separating the proteins with two-dimensional gel electrophoresis. Using MALDI mass spectrometry, they identified 20 proteins, including glutathione peroxidase, aldehyde dehydrogenase and glutathione S-transferase, which were radiolabeled, suggesting that these proteins formed acetaminophen adducts. The effects on protein expression of several other hepatotoxins was also investigated by Man et al.[117], who found that the steroid cyproterone acetate and dexamethasone both upregulate haptoglobin, α1-antitrypsin and carboxylesterase in rat livers. Such analyses could therefore be used to determine whether toxins act through shared pathways.

Fountoulakis et al. [118] identified several proteins, which changed in the liver after rats were dosed with carbon tetrachloride. They found that two stress proteins, catalase and uricase, were up-regulated, while α2-macroglobulin and senescence marker protein decreased. Interestingly, the changes in the gene expression were different, with an increase in DNA damage and stress reponse genes and a decrease in some of the P450's. While the changes in gene and protein expression are consistent with the mechanism of toxicity, they do not correlate, probably due to different regulation of protein and gene expressions. It is also possible that the proteomic and the genomic analyses did not give a global profile, and many of the affected genes and proteins were not identified.

One challenge in proteome analysis is relative quantitation of protein in two different samples. A recently-developed method to accomplish this goal, is the isotope-coded affinity tag (ICAT) labeling method. The ICAT uses a special biotinylated isotope-coded affinity tag to label proteins on their cysteine residues [119]. This approach is based on labeling proteins from different samples with two isotopically different labeling reagents. The two samples are combined, digested and isolated using an avidin affinity column. The proteins are separated by reverse-phase microcapillary liquid chromatography and analyzed with ESI mass spectrometry [120;121] . The relative quantities of identical peptides from different samples are then calculated from the ratio of the signal intensities. This method was further developed by combining it with multidimensional chromatography in order to be able to analyze low-abundance proteins [122]. This method, which combined cation exchange, biotin affinity and reverse phase chromatography, has been used to decrease the complexity of samples and to reveal lower-abundance proteins.

1.3.3 Metabonomics

The field of metabonomics aims at analyzing the global metabolic profile under cetain conditions. Metabonomics has the power to identify toxicity when genomics and proteomics do not, since toxicity sometimes does not change the protein or gene expressions. For example, toxicity might be due to induction or inhibition of enzymes or macromolecular binding. The most common technology to study metabolic profles is ^{1}H-NMR spectroscopy. In order to determine differences in samples, the data from NMR spectroscopy is analyzed with pattern recognition methods, multivariate statistical analysis and principal component analysis (PCA) [123]. Biological fluids such as urine

and serum are very rich in metabolites and are therefore well-suited for metabonomics analysis. Exposure to toxins usually creates characteristic metabolic fingerprints, which can be used as reference for studying novel compounds. For example, Nicholson et al. [124] show the characteristic NMR spectra caused by exposure to the renal toxins puromycin, uranyl nitrate, 2-bromoethanamine and the liver toxin hydrazine. Using NMR and pattern recognition studies, the cholestatic hepatotoxin, alpha-naphthylisothiocyanate (ANIT), has been shown to increase bile acids, choline and decrease in glucose and glycogen [125]. A previously unknown metabolite in the urine of acetaminophen-treated rats was also identified with ^{1}H-NMR spectroscopy. This metabolite, 5-oxoproline (5OXP, pyroglutamic acid), was also shown to be result of the depletion of sulphur-containing molecules such as cysteine and glutathione [5]. Acetaminophen was also shown to increase peroxisomal activity, lipid triglycerides and monounsaturated fatty acids, and it also decreased in polyunsaturated acids, suggesting that its toxicity is mediated through mitochondrial injury [126]. NMR spectra can thus be used to identify toxic compounds and the target organ for toxicity. Metabonomics has also been applied at determining biomarkers for other diseases such as renal damage [124] and coronary heart disease [127] for which no reliable diagnostic methods have been found.

1.3.4 Systems Biology

The integration of genomic, proteomic and metabonomic data, requires sophisticated modeling tools to determine causes and possibly cures of human disease. This approach has applications in drug metabolism and discovery, which require combining information from several different levels of drug screening. Such *in silico*

integration tools are used widely in the pharmaceutical industry to evaluate possible metabolism and toxicity pathways for new drugs. Currently, it is not possible to fully understand the characteristics of a specific compound, but a lot of the technology is already in place and incorporation of new data could lead to more reliable tools in the near future [128].

There are three technologies, which form the backbone of this analysis. The first one aims at predicting metabolism and toxicity based on structure. Extensive databases in the literature have been compiled to build quantitative structure activity relationship (QSAR) models. Using data from over 2000 compounds several models have been developed to determine possible substrates for the CYP450's [129]. These tools, however, are not sufficient for the prediction of drug metabolism, since many other pathways are also involved in biotransformation and endogenous factors also influence the kinetics and the metabolic fluxes.

The second technology involves integration of the data generated by genomics, proteomics, metabonomics as well as transcriptomics. As we have discussed earlier, the proteomic, genomic and metabonomic profile of many compounds has already been studied in their respective target organs. Since these technologies are so recent, currently there is not sufficient data to be able to determine differential expression under toxic conditions. However, the methods for genomics, proteomics and metabonomics are well-established and databases of expression profiles for many compoundst are being developed.

In order to complete the systems biology analysis, the third tool used to integrate information involves databases combining all the cellular pathways. These databases

include information regarding signal transduction, metabolism, and protein interactions. Although many databases have been developed, they are far from being complete, since only 15% of the human genome has known function. The completion of these databases will require significantly more research, since data from animals will also be necessary to extrapolate data from animal experiments to clinical trials. In summary, systems biology is a promising approach for predicting potential drug toxicity and discovery of new therapeutics. Since many of the high-throughput assays have only been recently developed, there is not sufficient data to build the necessary databases and models. However, with the current progress of technology, systems biology is expected to be fruitful in the near future.

1.4 Conclusions

We have introduced several different *in vitro* systems used for studying hepatotoxicity. The isolated hepatocyte culture is the most popular system, but precision-cut liver slices and isolated perfused livers have also been used in toxicity studies. In static cell cultures primary rat hepatocytes are the most commonly used cells, but occasionally it is possible to obtain primary human hepatocytes. Human cell lines have not been used very frequently for hepatotoxicity studies since they lack many of the drug metabolizing enzymes necessary for bioactivation of toxins. However, it has been shown that cell lines transduced with certain cytochrome P450 genes are susceptible to toxins such as alfatoxin B1. Furthermore, there are promising cell lines, such as BC2, which have been found to be susceptible to acetaminophen and acetyl salicylic acid.

System parameters, such as extracellular matrix and medium composition, have a profound influence on the viability and functionality of hepatocytes. The extracellular matrix promotes adhesion to the surface, and in a sandwich culture it provides three-dimensional matrix contacts. Growth factors in the medium can promote cell regeneration, but growth factors have also been shown to suppress cytochrome P450 activity. Furthermore, low cell density promotes regeneration but down-regulates metabolism, whereas high cell density maintain metabolism but suppresses cell division.

Recently, several three-dimensional perfused bioreactors have also been developed to provide the hepatocytes with a three-dimensional architecture and continuously flowing medium. These systems have been shown to maintain viable hepatocytes, which are also metabolically active. On the other hand, perfused reactors can be more difficult to use for hepatotoxicity studies. Continuously flowing medium dilutes the drug as it enters the reactor, and it also dilutes the metabolites in the effluent. Nevertheless, perfused bioreactors show promise as systems to study hepatotoxicity, since they are more physiologic model than systems used currently.

High-throughput assays such as genomics, proteomics and metabonomics have been used to analyze differential expression of genes, proteins and metabolites under toxic conditions. These experiments have been useful in establishing "fingerprints" for toxins, which could be used in the future to study toxicity of novel compounds. Another essential component of hepatotoxicity studies is integration of these data sets. The aim of systems biology is to combine global expression profiles with structure-activity relationship and metabolic pathway networks. While this approach still requires a lot of

data in order to be functional, it has the potential to be able to predict drug metabolism and toxicity of novel compounds.

Chapter 2: Development of a Collagen Sandwich Bioreactor for Hepatotoxicity Studies

2.0 Abstract

We have designed a two-dimensional hepatocyte bioreactor for the detection of hepatotoxicity caused by drugs and chemicals. Hepatocytes were cultured in a collagen (I) sandwich configuration at a density of 50,000 cells/cm^2 and were exposed to eight different compounds. To detect toxicity we measured urea, albumin and the release of lactate dehydrogenase. The hepatocytes were sensitive to aflatoxin B1, cadmium and the alkylating agents N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) methyl methane sulfonate (MMS), but were resistant to acetaminophen, carbon tetrachloride, vinyl acetate and N, N-dimethylformamide (DMF). Our western blots showed that hepatocytes in the culture maintain CYP1A, 2B, 3A2 but gradually lose CYP2E1, which is the main metabolic enzyme for acetaminophen, carbon tetrachloride, and DMF. The metabolites of acetaminophen were identified using liquid chromatography and electrospray mass spectrometry. We found that the hepatocytes convert most of the acetaminophen to the glucuronide and sulfate metabolites and only formed a small amount of the glutathione adduct. This research shows that the collagen sandwich can be used for detecting hepatotoxicity as well as for identifying the major metabolites produced by hepatocytes.

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2.1 Introduction

Cultured hepatocytes rapidly lose liver-specific functions, which limits their use for studying drug metabolism and toxicity. This phenomenon is thought to be due to lack of proper extracellular matrix, cell-cell contacts and humoral factors in the *in vitro* systems, which are known to regulate gene expression in the liver [6;130;131]. Providing extracellular matrix proteins such as collagen [34;35;37;132] or complex substrata such as Matrigel ®[30;31;133], has been shown to enhance hepatic function in *in vitro* systems, especially when cells are cultured in a sandwich configuration. Extracellular matrix in a sandwich form provides anchorage for the cells on top and bottom [34;134], and it also stores signaling molecules such as heparan-sulfate proteoglycans secreted by the cells [135].

Research on collagen sandwiches has shown that hepatocytes in this system maintain drug metabolism [32;36;37;84], morphology [31;132;134] and bile secretion [39;40;136]. Furthermore, hepatocytes in this system maintain transcriptional activity for albumin at least as high as freshly isolated hepatocytes and significantly higher than hepatocytes cultured on a single layer of collagen [33]. Collagen sandwiches and collagen-Matrigel sandwiches are also inducible for both CYP1A2 and CYP3A4 in systems with human hepatocytes [32]. Kern et al. [37] demonstrated that both human and rat hepatocytes in collagen sandwiches express CYP3A and CYP1A activity, as well as glucuronosyltransferase, sulfotransferase and glutathione *S*-transferase activities. Bader et al.[137] also used collagen sandwich cultures of rat and human hepatocytes to study the metabolism of the drug urapidil. They found that the metabolites produced

corresponded to the *in vivo* metabolites in rats and humans respectively, suggesting that collagen sandwiches could be used to predict the metabolites of xenobiotics.

There have been many investigations to determine whether a collagen sandwich is the optimal static (non-perfused) culture system. Dunn et al. [34] showed that albumin secretion in a collagen sandwich is higher than in cultures overlaid with agarose, and is at least as high as in Matrigel cultures. Beken et al. [138] compared albumin secretion and the expression of glutathione S-transferases in collagen sandwiches made from rat tail collagen, commercially available collagen type I, and commercially available collagen supplemented with fibronectin, laminin and heparan sulfate proteoglycans, and found that after seven days of culture, there was no significant difference among the cultures. A possible explanation for this phenomenon is that hepatocytes cultured in collagen sandwiches secrete the necessary extra-cellular proteins, which are retained in the culture by the second layer of collagen.

It is thought that hepatocytes in a sandwich system function better than on a single layer of collagen because they can anchor themselves better to the matrix. In the liver hepatocytes are anchored to the proteins in the ECM via a class or receptors called integrins. Integrins are usually dimers of various α and β subunits, but most of the integrins in the liver contain the $\beta 1$ subunit. Moghe et al. [134] determined the distribution of $\beta 1$ integrins in hepatocytes cultured for seven days in collagen gel sandwiches. They showed that after the hepatocytes were overlaid with a second layer of collagen, most of the $\beta 1$ integrins were bound to the top layer of collagen. In addition to expression of $\beta 1$ integrins, hepatocytes in collagen gel sandwiches also form an extensive bile canalicular network enhancing their cell-cell contacts [38] . The maintenance of $\beta 1$

integrin and the formation of bile canaliculi show that hepatocytes in a collagen sandwich exhibit *in vivo*-like tissue structures.

One of the most critical cell culture parameters is oxygen tension and it was initially debated whether hepatocytes overlaid with collagen receive sufficient oxygen. However, data suggests that hepatocytes in this configuration have enough oxygen to survive and carry out drug metabolism functions. Beken et al. [138] showed that CYP1A and glutathione S-transferase activities are the same in cultures on teflon membranes (which are oxygenated from the top and the bottom) and plastic dishes which receive oxygen only from the top. Bader et al. [139] also investigated the effect of oxygen partial pressure by measuring the metabolism of urapidil in collagen sandwiches cultured on polystyrene and gas permeable supports. They found that there was no difference in the metabolism between the two culture types, and the rate of metabolism was only slightly higher at an oxygen partial pressure of 20% than at 10%. Maintaining a physiological pH in the medium is essential for cell survival, and it has also been shown that there are no significant variations in the pH of the medium when cells are cultured in collagen sandwiches [140].

In spite of the large body of literature examining the viability and the drug-metabolizing capacity of hepatocytes in collagen sandwiches there are very limited studies on the reliability of this system in toxicological studies. De Smet et al.[141] studied the biotransformation of trichloroethylene (TCE) in collagen gel sandwiches and found that TCE and its metabolites caused significant toxicity, showing that the sandwich system maintained CYP1A and 2B and possibly 2E1, the enzymes responsible for metabolizing TCE.

In this chapter we show the development of a long-term collagen sandwich for toxicity studies. The effects of cell density and epidermal growth factor (EGF) on urea and albumin secretions were evaluated in order to determine optimal plating density and medium composition. We also observed significant variations in the data and we examined the variability of urea secretions in over 20 experiments to determine the distribution of hepatocyte function from different isolations.

We have chosen aflatoxin B1 as a model compound to develop our toxicity assays because this compound is toxic at nanomolar doses and its toxicity can be detected with a variety of methods. The hepatocytes were also dosed with alkylating agents, cadmium, acetaminophen, carbon tetrachloride, vinyl acetate and N,N-dimethylformamide. The toxicities from the various compounds were compared across several isolations to determine the reproducibility of toxicity in the collagen sandwich. The cells were most susceptible to the direct toxins and aflatoxin and resistant to acetaminophen, carbon tetrachloride, vinyl acetate and dimethylformamide. The metabolism of acetaminophen was further investigated using liquid chromatography and mass spectrometry. We found that the cells covert acetaminophen primarily to the sulfate and glucuronide metabolites and only produce a small amount of the glutathione adduct, which is a detoxification product of the toxic benzoquinone metabolite (NABQI). Based on our data, it is likely that the cells are immune to acetaminophen, dimethylformamide and carbon tetrachloride due to the loss of CYP2E1 in the collagen sandwich.

2.2 Materials and Methods

(See Appendix I for detailed protocols)

2.2.1 Cell Culture

Plastic plates were coated with 550 μm thick layer of collagen type I (Cohesion Technologies Inc., Palo Alto, CA). The collagen concentration was 2 mg/ml dissolved in 10x phosphate buffered saline, which included 20 g/L glucose and 37 g/L sodium bicarbonate. The pH of the collagen was measured every time a collagen solution was prepared and was found to be around 7.4.

Hepatocytes were isolated from male Fisher 344 inbred rats (Taconic, Germantown, NY) with a protocol described in Powers et al. [82] and were immediately plated on collagen-coated 24-well dishes. The cell viability, measured with trypan blue exclusion, was typically 87-91 %. The medium was Hepatocyte Growth Medium (HGM) [142] without albumin to decrease background during protein analysis. The oxygen tension in the incubator was 20% and the carbon dioxide pressure was 5%. Cells were plated at 50,000/cm^2 in a 24-well plate with 300 μl of medium. One day after plating, the medium was removed and a second layer of collagen 280 μm thick was pipetted on the cells. The collagen gelled after 1 hour in the incubator and new medium was pipetted into each well.

2.2.2 Assays for Measuring Cell Viability

Albumin secretion- Albumin secretion was measured by enzyme-linked immunosorbent assay (ELISA) using sheep IgG fraction against rat albumin (ICN Pharmaceuticals, Costa Mesa, CA) and horseradish peroxidase- conjugated goat anti-rat IgG (Accurate

Chemical, Westbury, NY). The absorbance was measured at 450 nm with a Spectramax 250 microplate spectrophotmeter (Molecular Devices Corp., Sunnyvale, CA). Medium samples had to be diluted 20-50-fold before measurement, and the exact concentrations were calculated after comparison with the absorbances of albumin standards (ICN Pharmaceuticals, Costa Mesa, CA).

Urea secretion – Urea was measured with a colorimetric assay (Sigma, Kit #640-A), which uses the determination method of Berthelot. Urea was hydrolyzed by urease to form ammonia, which formed indophenol, after reaction with phenol nitroprusside and alkaline hypochlorite. Medium samples between 5 and 15 µl were incubated for 15 minutes at 37° C with 40 µl of urease, followed by 20 minutes of room temperature incubation with 80 µl each of nitroprusside and hypochlorite. The absorbances of samples were promptly measured at 570 nm, as indophenol only keeps its color for 1-2 hours.

P4501A – Activity of P4501A was measured with the EROD (ethoxyresorufin O-deethylase) assay. A 30 µM concentration of ethoxyresorufin (Molecular Probes, Eugene, OR), was added to cells together with dicumarol (Sigma), an inhibitor of DT-diaphorase. The concentration of ethoxyresorufin was chosen based on kinetics, which showed that 30 µM was in the Vmax region. Higher concentrations of ethoxyresorufin were avoided, as they caused a background fluorescence which interfered with measurements After a one-hour incubation, medium was removed from the cells and the fluorescent product, resorufin, was measured at excitation and emission wavelengths of 555 nm and 600 nm

respectively. Exact concentrations were calculated after comparison with resorufin standards (Molecular Probes, Eugene, OR).

Western Blots for P450's 1A, 2B, 3A and 2E – (performed by J. Kevin Leach) Cells were lysed in RIPA buffer containing, 50mM Tris-HCl, 0.5% deoxycholic acid, 0.1% SDS, and 1% Nonidet P40. Protein concentrations were determined using the Pierce BCA protein binding kit, and absorbance was measured at 570 nm. Aliquots of 10 μg of protein were run on a 10 or 15% polyacrylamide gel and transferred to a polyvinyldifluoride membrane filter. The membranes were treated with 5% blotting grade milk for 1 hour to block non-specific binding, rinsed and incubated with a panel of antibodies against different antigens. Immune complexes were detected with an enhanced chemiluminescence substrate (Perkin Elmer), and exposed to Kodak MR film.

XTT and Alamar Blue – XTT (Sigma), also known as 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilidem and Alamar Blue (Biosource International, Inc. Camarillo, CA) are tetrazolium salts, which are reduced in the mitochondria to form colorimetric/fluorescent products respectively. These salts were added directly to cultures as described in the company protocols. After a 24-hour incubation the absorbance and the fluorescence of the medium were assayed.

Lactate Dehydrogenase – Lactate dehydrogenase was measured with Promega Kit # G7890. Briefly, this assay is based on the observation that dying cells do not maintain membrane integrity and release cytoplasmic contents, including enzymes such as lactate

dehydrogenase, into the medium. Samples of 50 µl were removed from each well and combined with the substrate solution containing lactate, rezaurin, diaphorase and NAD^+ and incubated at room temperature in the dark for 10 minutes. After the reaction was halted with a stop solution, the fluorescence was assessed immediately at excitation of 555 nm and emission at 600 nm.

2.2.3 Light microscopy

All microscopic visualization took place directly in 4-well plates (Nalgene Inc., Rochester, NY) and pictures were visualized with a 12 bit camera connected to a Nikon eclipse microscope, model Te300. Light and fluorescence microscopy was perfomed at magnifications of 10x and 20x and visualized with Openlab software. Texas Red-X and DAPI filters were used to visualize fluorescently labeled cells.

Cells were fixed in 4% paraformaldehyde (Sigma) for 1 hour at room temperature and rinsed multiple times with PBS. For fluorescence microscopy cells were fixed, rinsed multiple times with PBS and permeabilized with 0.1% Triton X-100 (Sigma) for 10 minutes. In order to reduce non-specific binding, cells were also incubated for 30 minutes with 1% bovine serum albumin (BSA) in PBS. Nuclei cells were visualized by incubating cells for 30 minutes in the dark with 300 µl of 30 nM DAPI (Molecular Probes, Eugene, OR) and rinsing them with PBS to remove unbound dye. The actin cytoskeleton was visualized with Texas Red phalloidin (Molecular Probes, Eugene, OR). After fixation and permeabilization cells were incubated with 7.5 µl of dye dissolved in 300 µl of PBS for 30 minutes in the dark and then washed thoroughly with PBS. The

cells were also"double-stained" simulateneously with both DAPI and Texas Red phalloidin. In this case, the 300 µl of PBS on each sample contained 30 nM DAPI and 7.5 µl of phalloidin.

2.2.4 Toxin treatment

Preparation of toxins and exposure to cells – Aflatoxin B1 was prepared fresh from powder (Sigma) before each experiment. The 1 mg powder in the sealed container was combined with 0.5 ml of DMSO using a syringe and needle. A sample of the solution was then removed with the syringe, diluted 200-fold with DMSO and its absorbance was measured with a spectrophotmeter at 362 nm to determine the concentration. The stock solution of aflatoxin was dissolved into DMSO at 1000 times the desired concentrations, and the final dilution was made into medium to ensure a DMSO concentration of 0.1 %. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and acetaminophen were weighed out and dissolved in DMSO first and then in medium, maintaining the DMSO concentration 0.1% in all cultures. Methyl methane sulfonate (MMS), cadmium, vinyl acetate and N,N-dimethylformamide (DMF) were diluted straight into the medium.

One day after the second layer was placed on the cells, the cells were washed with 2 ml/well of Hanks's Buffer (Life Technologies, Carlsbad, CA) four times. The following day toxins were dissolved in the medium as described above and 300 µl of toxin-containing medium was added to each well. The medium was collected every two days, stored at –80°C until analysis and replaced with fresh medium. Samples for lactate dehydrogenase were taken 24 hours after toxin treatment and the cultures from which the

samples were taken were discarded. There were usually parallel long-term cultures for assessing urea and albumin secretions.

2.2.5 Metabolism of Acetaminophen

Cells were treated with 1 mM acetaminophen for 24 hours and the conditioned media were stored at -80°C until analysis. Metabolites were first separated with an Agilent 1100 HPLC using a 2x 150 mm C18 column and 10 mM TFA and acetonitrile solvents at a 100 µl/min flowrate. The fractions were collected for mass spectrometry analysis to confirm the structure of the metabolites. Mass spectrometry was performed on an Agilent XCT LC-MS/MS electrospray ion trap. The LC was performed with a 300 µm x 12 cm Vydac column with solvent A as 0.1% acetic acid and 5% acetonitrile and solvent B as 0.08% acetic acid, 5% methanol, 5% water 90% acetonitrile at a 4 µl/min flowrate.

To determine whether the glutathione adduct was produced in the cells, a standard was synthesized according to the protocol of Yan et al [143] and its retention time was determined with liquid chromatography and its structure was confirmed with mass spectrometry. The glutathione adduct was also identified in the sample by isolating the fraction with the appropriate retention time and confirming its molecular weight and fragmentation pattern with mass spectrometry.

2.3 Results

2.3.1 Cell Culture

Composition and thickness of collagen- In order to maximize mass transport throughout the gel our goal was to find the lowest concentration of collagen, which would solidify in the incubator within one hour. This concentration was determined to be 2 mg/ml and we found that this concentration of collagen layer was stable and remained solid throughout the culture.

Cell density- We have examined the effect of cell density on the activity of P4501A and the secretion of urea and albumin. As Figure 1a shows, P4501A activity reaches a maximum around 40,000 cells/cm^2. The effect of cell density on urea and albumin secretion was also examined and it was observed that the secretion of urea increases with increasing density, while the secretion of albumin reaches a maximum around 45,000 and decreases at the higher density (Figures 1b and 1c). In light of these results we decided to plate our cultures at 50,000 cells/cm^2.

Medium composition- We have examined the effect of EGF on the long-term viability of hepatocytes. Figure 2 shows the secretions of urea and albumin between cultures with no EGF and EGF at a dose of 20 ng/ml. Only 2 days after the beginning of the culture hepatocytes grown without EGF secreted about 50% less urea and albumin. By days 10 and 20, the cells with EGF secreted only a quarter as much albumin and urea respectively as the control cells. Since our goal was to develop long-term cultures, we decided to include EGF in our medium.

Microscopy – The light and fluorescence microscopy studies revealed that the hepatocytes did maintain their polygonal morphology, and many of them were binucleate as they are *in vivo* (Figures 3a-b). Several days into the culture the cells rearranged themselves into structures forming networks (Figure 3b). DAPI staining was also performed to determine whether there were any mitotic figures showing condensed chromosomes, indicating cell division. After two days in culture we scanned over 10 views with about a hundred cells in each view. Although we found many binucleate cells, we could not locate any mitotic figures (Figure 3c). This experiment was repeated at least twice using hepatocytes from other perfusions, but mitotic figures could not be found.

Variation among cultures- Systems which are based on primary cells usually have a significant variation among cultures due to inter-individual differences among the animals from which the cells are isolated. We have decided to evaluate the variation by comparing the function of cells from different cell isolations. The urea secretion of the control cells on day 6 from 22 isolations is shown in Figure 4. While most cultures contained 10-40 μg/ml urea, some of them had as much as 80 μg/ml or as low as 10 μg/ml. All of the cultures had viabilities between 87% and 92% and there was no correlation between the viability and the urea secretion. Therefore, one should expect almost 10-fold variation in urea secretion from different isolations.

Drug Metabolism – The presence of various CYP450's proteins was measured as a function of time with Western Blotting. Figure 5 shows the expression levels of CYP1A1, 2B1/2, 2E1 and 3A2 between days 2 and 7. As the figure shows the expression of 2E1 decreases over time, while the expression of the other enzymes increase with culture time (data from Dr. J. Kevin Leach).

2.3.2 Toxicity Data

Aflatoxin- We have exposed our cultures to doses ranging from 8 to 64 nM and measured the secretions of urea and albumin, reduction of XTT and Alamar Blue as well as the release of lacate dehydrogenase (Figures 6a-c). Albumin decreases by about 30% at 8 nM dose, but urea does not decrease significantly until a 16 nM dose. XTT and Alamar Blue decrease about 20% at 16 nM. Based on the albumin and urea data we determined that the LD_{50} was about 32 nM, and this dose was our positive control in many of the toxicity studies. The lactate dehydrogenase assay was adapted to collagen sandwiches using aflatoxin as the model compound. It was observed that toxicity cannot be detected until at least 20 hours after treatment and that the minimum sample size was 50 μl.

Toxicity of direct-acting compounds and acetaminophen -
The direct-acting compounds we tested were cadmium, methyl methane sulfonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). All three of these compounds showed significant and reproducible toxicity at 1 mM doses. The cells were resistant to a 1 mM dose of acetaminophen and this compound became our negative control for toxicity experiments. The data in Figures 7a-b shows the averages of at least three toxicity experiments for aflatoxin, acetaminophen, MMS, MNNG and cadmium. Urea levels decreased at least 50% compared to controls and albumin levels decreased about 70% compared to controls. In contrast, the release of lactate dehydrogenase was more varied, and indicated less than 50% death except for cadmium. Table 1 shows the values for the paired T-test values for aflatoxin, MMS, MNNG and cadmium in the urea, albumin and lactate dehydrogenase assays. Most of the values were below 0.05 (within 95% confidence interval) showing that the toxicity is reproducible and that the toxin-

treated cells have significantly less urea, albumin and more release of lactate dehydrogenase.

Toxicity of carbon tetrachloride, vinyl acetate and dimethylformamide - Cultures were exposed to 1 mM doses of vinyl acetate, dimethylformamide and a 5 mM carbon tetrachloride. The secretions of urea, albumin and the release of lactate dehydrogenase were measured 6 days after treatment. However, toxicity could not be detected for these compounds by any of these assays (Figure 8).

2.3.3 Comparing high and low metal medium

The above experiments were performed with the medium formulation published by Block et al [142]. However, the publication contained the following errors:

Compound	Erroneous concentration	Correct concentration
Nicotinamide	0.61 g/L	0.305 g/L
Zn Cl_2	0.544 mg/L	0.0544 mg/L
Zn SO_4*$7H_2O$	0.75 mg/L	0.075 mg/L
$CuSO_4$* $5H_2O$	0,2 mg/L	0.02 mg/L
Glutamine	5 mM	1 mM

Before we switched to the "correct" medium, which had less zinc chloride, zinc sulfate, copper sulfate, niacinamide and glutamine, we compared cell function and toxicity and in the two media (Figures 9a – 9c.) The urea and the albumin secretion was higher in the low metal medium, suggesting that the cells functioned better. However, the toxicity data was very similar in the two media. MMS was the only compound, which was more toxic in the "high metal" medium. All of the other compounds caused about the same changes in urea, albumin and LDH in the two types of media.

2.3.4 Metabolism of Acetaminophen

The primary metabolites of acetaminophen were identified using liquid chromatography and LC-MS/MS. The HPLC chromatogram for conditioned medium after a 24 hour incubation with 1 mM acetaminophen is shown on figure 10. The glucuronide and sulfate metabolites eluted at 5.5 and 7 minutes respectively and their structure was confirmed with mass spectrometry. The glutathione adduct was identified with mass spectrometry in the fraction collected between 13 and 14 minutes and its structure was confirmed with collision induced dissociation (CID) shown in Figure 11a. The fragments corresponding to the CID spectra were confirmed using Chemwindows software showing that the loss of glycine results in the 382 ion, the loss of g-glutamic acid corresponds to the 311 ion, and breaking the C-S bond gives rise to the 182 ion (Figure 11b).

2.4 Discussion

The design of a system to detect hepatotoxicity is still a major challenge because of the difficulty of mimicking a hepatic microenvironment *in vitro*. We have designed a collagen sandwich, which maintains viable hepatocytes for at least two weeks, and we have shown that they maintain many of their metabolic enzymes for at least one week. In addition, we found noninvasive toxicity markers which do not require destruction of the culture. Urea, albumin and lactate dehydrogenase can all be measured from conditioned medium, making it possible to measure toxicity from the same culture repeatedly.

The parameters we used to optimize the culture included collagen concentration, cell density, addition of EGF to the medium, and selection of important time-points for toxicity measurements. Cell density was one of our critical parameters as it has been shown that cell density influences intercellular communication through gap junctions and could have a profound effect on the expression of CYP's in hepatocyte cultures [10;11].

Although our cultures are stable, the cells do not function as they would *in vivo*. The microscopy images show that the hepatocytes are not dividing. This phenomenon has also been observed by De Smet et al.[7] who showed that hepatocytes in collagen sandwiches remain arrested in the G1 phase even in the presence of EGF. We have also observed a gradual loss of CYP2E1 in the cultures (Figure 5), which is probably responsible for the lack of toxicity of acetaminophen, carbon tetrachloride and dimethlformamide, all substrates for CYP2E1 [49;144;145].

Although we see an increase in the protein expression of CYP's 1A, 2B and 3A it is likely that the expression of these enzymes is still significantly lower than would be found in vivo. EGF has been shown to significantly decrease CYP2C11 [9] and CP3A [10] activities in hepatocytes and specifically CYP1A and CYP2B activities in collagen sandwiches [7;8]. This is significant, because carbon tetrachloride is also activated by CYP2B1/2 and possibly CYP3A, to form the trichloromethyl radical, CCl3* [145]. Furthermore, acetaminophen is also metabolized by CYP's 1A1/2, 2B1/2, 2C11/12 in addition to 2E1 to form the reactive N-acetylbenzoquinone imine (NABQI) [49]. Thus, the lack of toxicity by acetaminophen and carbon tetrachloride could be due partially to the down-regulation of metabolic enzymes by EGF. The other possibility is that the detoxifying enzymes, phenolsulfotransferase and UDP-glucuronosyltransferase, which

form the sulfated and glucuronidated metabolites respectively are upregulated (see Appendix II for a detailed metabolic pathway of acetaminophen). Rats are actually relatively immune to acetaminophen since they convert most of the compound to the sulfate and the glucuronide [146]. We have found that hepatocytes in collagen sandwiches exposed to 1 mM acetaminophen convert most of it to the sulfate and glucuronide metabolites although a small amount of the glutathione adduct was also formed. Dimethylformamide (DMF), a widely-used industrial solvent, is also a substrate for CYP2E1. Therefore the lack of sensitivity of hepatocytes to DMF was consistent with the other results, which showed a loss of CYP2E1 and immunity to other substrates of CYP2E1, such as acetaminophen and carbon tetrachloride. It was surprising that there was no toxicity following vinyl acetate exposure. Vinyl acetate is metabolized by carboxylesterase to form acetate and acetaldehyde which binds covalently to DNA and proteins. It was surprising that we did observe toxicity at a 1mM dose because it has been shown by others that a 0.5 mM dose increases intracellular pH in freshly isolated hepatocytes by 0.5, which could significantly perturb cellular function [147]. It is possible that the hepatocytes in the collagen sandwiches lost their carboxylase activity, in contrast to the freshly isolated hepatocytes, which probably maintained most of their metabolic enzymes.

Cadmium, MMS and MNNG are direct toxins, thus it was expected that the hepatocytes would experience toxicity after being exposed to high doses of these compounds. It is interesting that their toxicities could be detected with urea, albumin and the release of lactate dehydrogenase because they all act through different mechanisms. MNNG reacts with a cysteine residue (usually found on glutathione) in order to form a

highly reactive electrophlic intermediate [148], which attacks DNA bases at random via an S_N1 reaction. Thus, exposure to MNNG usually results in formation of electrophiles which leads to depletion of glutathione as well as lipid peroxidation [149]. MMS is truly a direct-acting compound and reacts with the more nucleophilic sites on DNA via an S_N2 reaction [25;150] to form alkylated bases. Cadmium is a heavy metal, which has been shown to cause hepatotoxicity and nephorotoxicity after chronic exposure. Cadmium has a long biological half-life in humans, and its toxicity results primarily from buildup in soft tissues and its binding to sulfhydryl groups [151].

One of the interesting results was that only MMS –treated cells behaved differently in the high and low metal media. This is especially surprising since addition of micromolar concentrations of zinc and copper are thought to increase metallothionein (MT) expression, which has been shown by Moffat et al. [25] to protect cells against MNNG and cadmium. Our concentration of $ZnSO_4$ was 2.6 μM in the high metal medium, a concentration that should double the MT expression. Therefore, it was expected that the higher concentrations of metal might actually protect the cells against MNNG and cadmium. However, it is likely that the copper in the medium initiated the formation of free radicals through Fenton chemistry leading to cell death, however it is not clear why MMS is the only toxin, which acted synergistically with the metals to increase toxicity.

Aflatoxin B1 is the only indirect toxin which was toxic in our cultures. In order for AFB1 to be toxic the compound needs to be oxidized by CYP's 2C11 and 3A2 [152] to form the AFB1 *exo*-8,9-epoxide, the only known genotoxic product of AFB1 [153]. This data suggests that the cells do maintain 3A2 and/or 2C11, suggesting that cells do

maintain one or both of these enzymes in the sandwich. (See Appendix II for a detailed metabolic pathway of aflatoxin B1.)

While it has been found that hepatocytes in collagen sandwiches maintain many drug metabolizing enzymes for up to two weeks, the levels of these enzymes are much lower than in the *in vivo* liver. Richert et al. [154] compared antioxidant status, glutathione content and drug metabolism in collagen and Matrigel sandwiches and three-dimensional Matrigel coated dishes with rat livers. They found that the cultures do maintain viable hepatocytes and the CYP's are inducible, but the basal levels of the CYP's were significantly lower than *in vivo*. Therefore, when toxicity by xenobiotics is monitored *in vitro*, it is also essential to monitor the activities of the relevant metabolic enzymes.

We have shown that albumin was the most reproducible marker of toxicity. It has already been observed by others that albumin expression decreases in hepatocytes in response to inflammation and it has been categorized as a "negative acute phase protein." Kang et al.[155] exposed hepatocytes in collagen sandwiches to IL-1β and IL-6 and measured the expression levels of urea and albumin. Both cytokines inhibited albumin synthesis by as much as 90%, whereas urea synthesis was inhibited consistently only by IL-1β. IL-6 inhibited urea synthesis at high doses but stimulated it at low doses. Thus, it is possible that the inconsistency we saw in the urea synthesis was due to inter-individual differences in the rats. This is especially likely, since the hepatocytes, which increased their synthesis of urea in response to MMS, MNNG and cadmium, were isolated from same rat. It is interesting, though, that the secretion of albumin is affected by the toxins even 6 days after treatment. Albumin expression is thought to be regulated by the hepatic

nuclear factor HNF1, and it has been shown that the presence of IL-6 and dexamethasone in the medium induce a nuclear protein (IL6DEX-NP), which binds to a promoter element adjacent to HNF1, decreasing transcription. We show in Chapter 3 that the control cells secrete a significant amount of α2-macroglobulin, a protein whose production is thought to be stimulated by IL-6 [156] It has been shown by Saad et al. [157] that hepatocytes in culture exposed to LPS start secreting IL-6 within 4 hours of exposure. Thus it is likely that hepatocytes in the collagen sandwich secrete IL-6, which modulates the expression of albumin.

Expression of albumin was examined by Whalen et al. [158] after treatment with LPS and it was found that the expression remained low up to 48 hours after treatment probably due to the decreased affinity of HNF1 during the acute phase. In our cultures we have observed decreased albumin expression up to 12 days after aflatoxin treatment. It is possible that the toxins in our culture caused permanent alterations within the cells, or that the necrosing cells started a chain of inflammatory processes, that the culture could recover from. Initially, we hypothesized that urea and albumin decrease due to cell death, but the increase in urea expression accompanied by decrease in albumin in some experiments suggest that the cells are specifically down-regulating albumin expression without dying. Incidentally, HNF1 also regulates the expression of glutathione S-transferase A2 (GSTA2) and a decrease in albumin can also be accompanied by a decrease in GST expression [158]. We have shown (Chapter 3) that control cells which are dying release large amounts of GSTA into the medium. Thus it is possible that toxin-treated cells would have decreased expression of GSTA, and upon dying would not

release as much of it into the medium as control cells. Thus, it is conceivable that the presence of GSTA in the medium could be used as a marker of toxicity.

2.5 Conclusions

We have shown that the collagen sandwich can be used for long-term culture of hepatocytes and we have optimized the collagen composition, cell density and demonstrated the need for EGF in long-term cultures. Our data shows that the collagen sandwich can be used as a system to detect toxicity of direct acting compounds such as MMS, MNNG and cadmium, but the hepatocytes are immune to the indirect toxins acetaminophen, dimethylformamide, vinyl acetate and carbon tetrachloride. This immunity is probably due to the loss of CYP2E1, the primary metabolic enzyme for acetaminophen, dimethylformamide and carbon tetrachloride. We examined the metabolites of acetaminophen and found that the cells formed primarily the sulfate and glucuronide metabolites. A small amount of the glutathione adduct was also formed showing that the cells also converted a small portion of acetaminophen to the toxic benzoquinoneimmine, but the amount was not enough to cause long-term toxicity in the culture. Cells were particularly susceptible to aflatoxin B1 at doses as low as 8 nM. This susceptibility suggests that the cells maintain CYP3A2, which is also shown in the Western blots. This study demonstrates that the collagen sandwich is a good model for studying toxicity of direct toxins, but it is not necessarily a good system for examining the effects of indirect toxins especially those requiring CYP2E1 for metabolism.

Figures

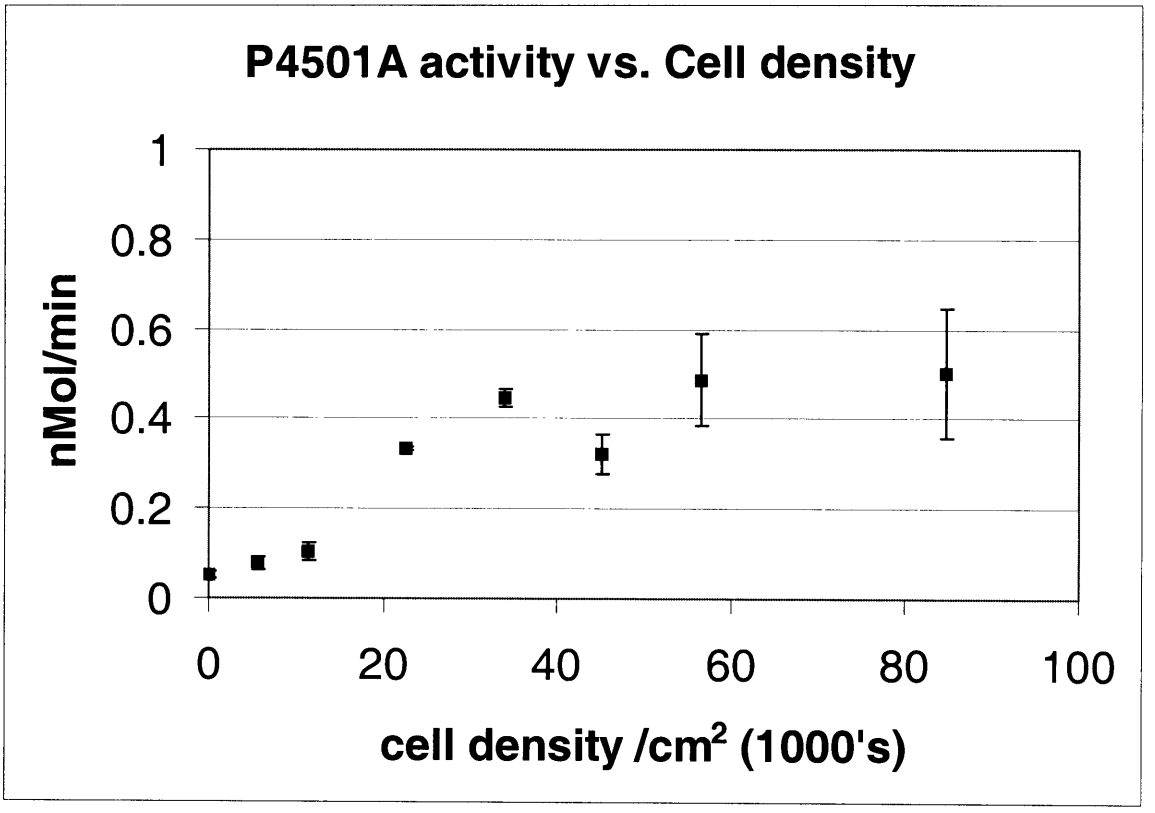


Figure 1a: EROD activity as a function of cell density in the collagen sandwich

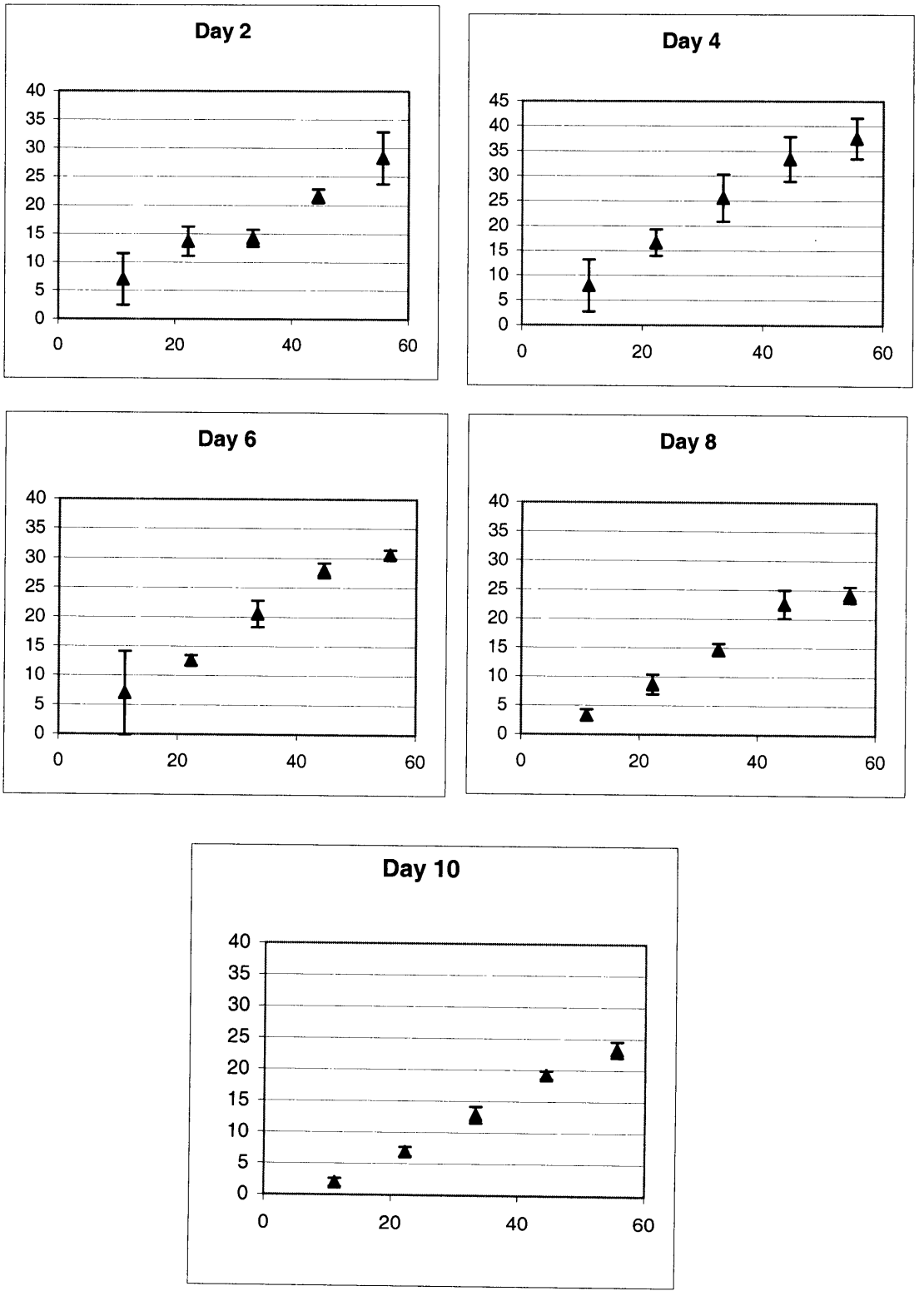


Figure 1b: Urea secretion as a function of cell density in the collagen sandwich.

X-axis : Cell density (1000's/cm^2); Y-axis: Urea (μg/ml)

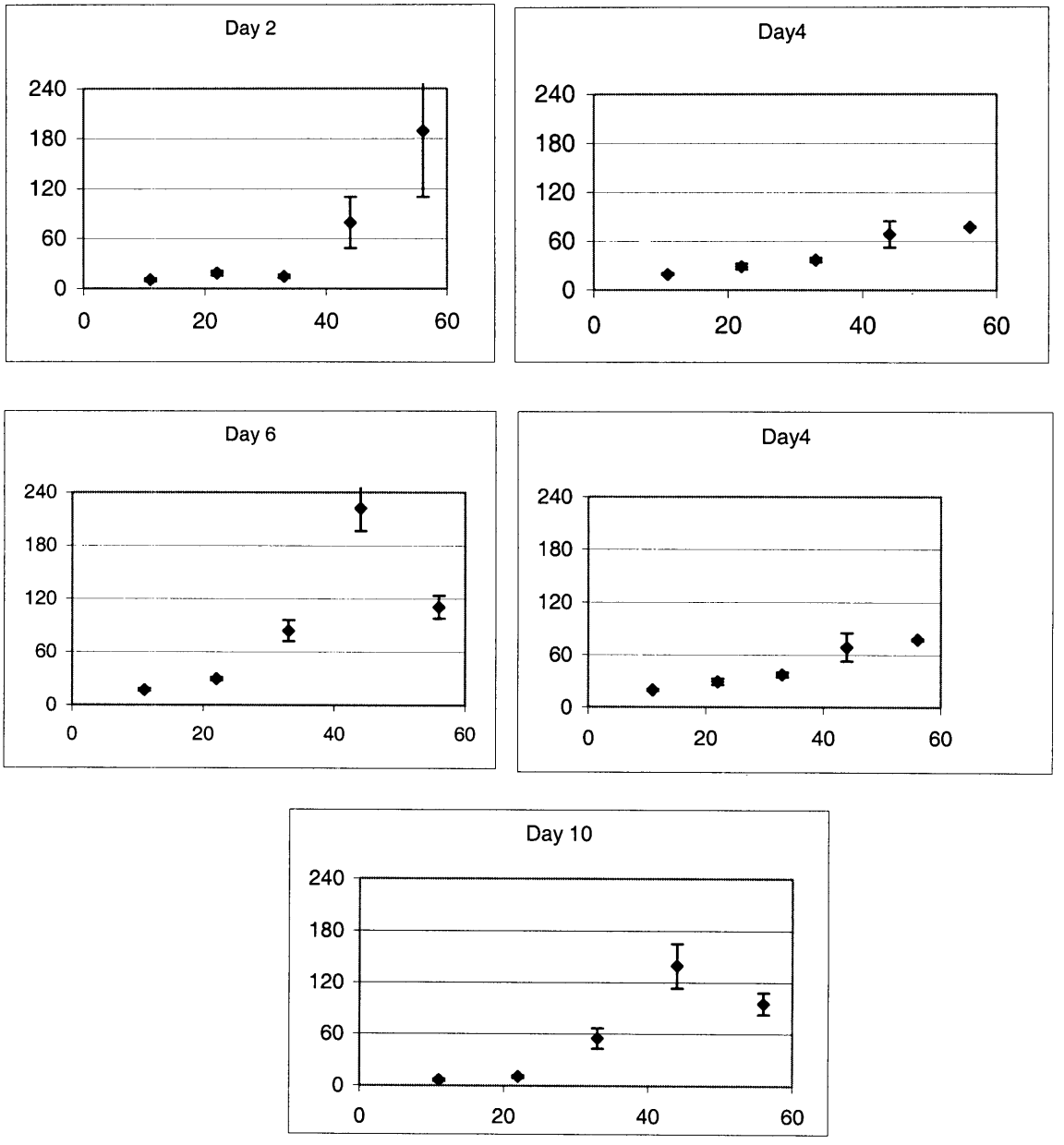
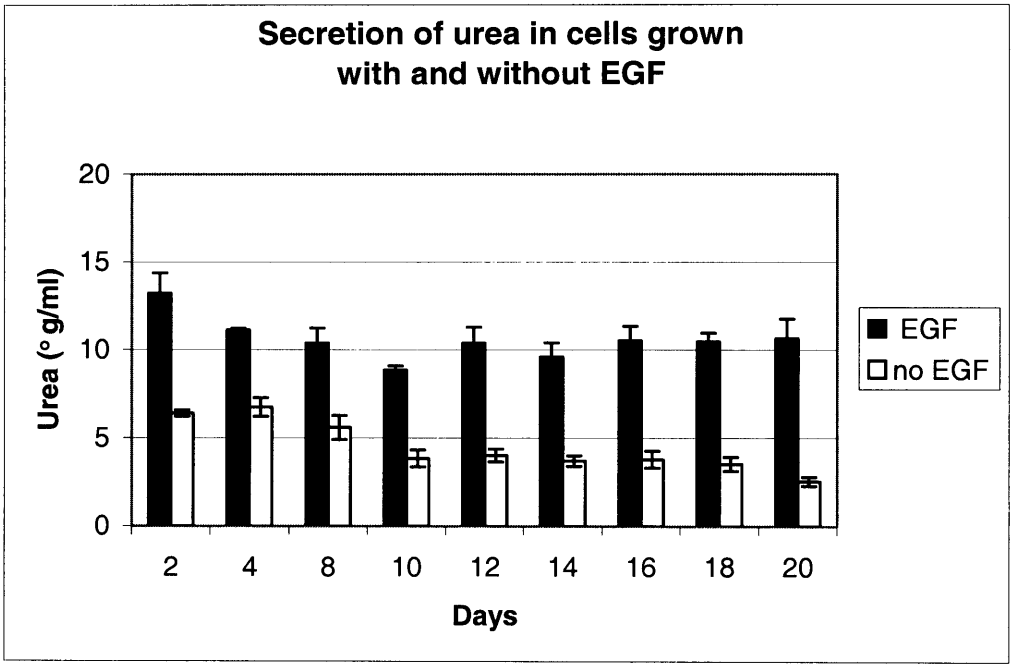


Figure 1c: Albumin secretion as a function of cell density in the collagen sandwich

X-axis : Cell density (1000's/cm^2); Y-axis: Albumin (μg/ml)



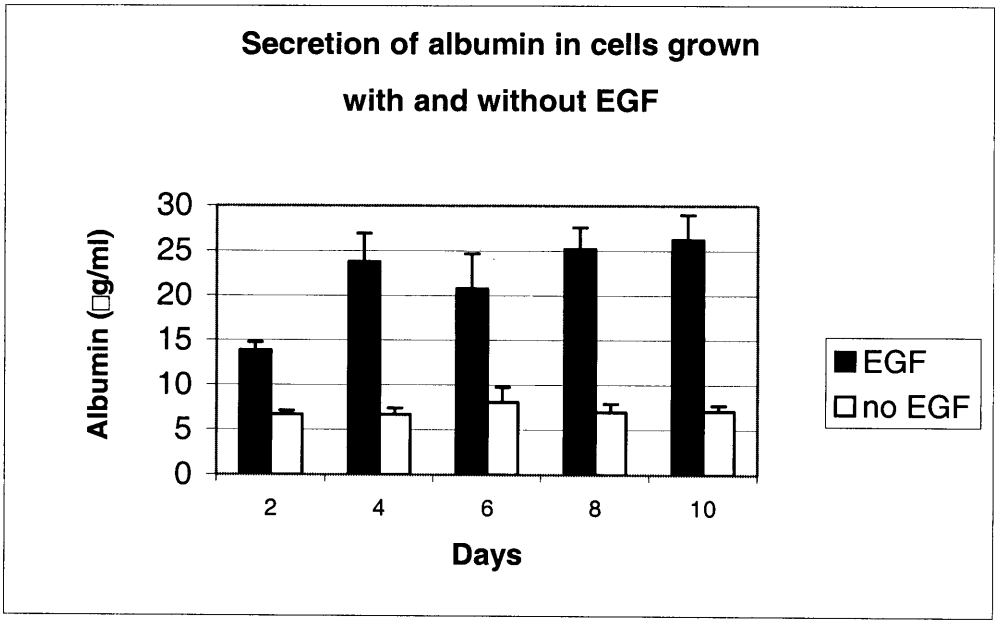


Figure 2: Effect of EGF on the secretion of urea and albumin

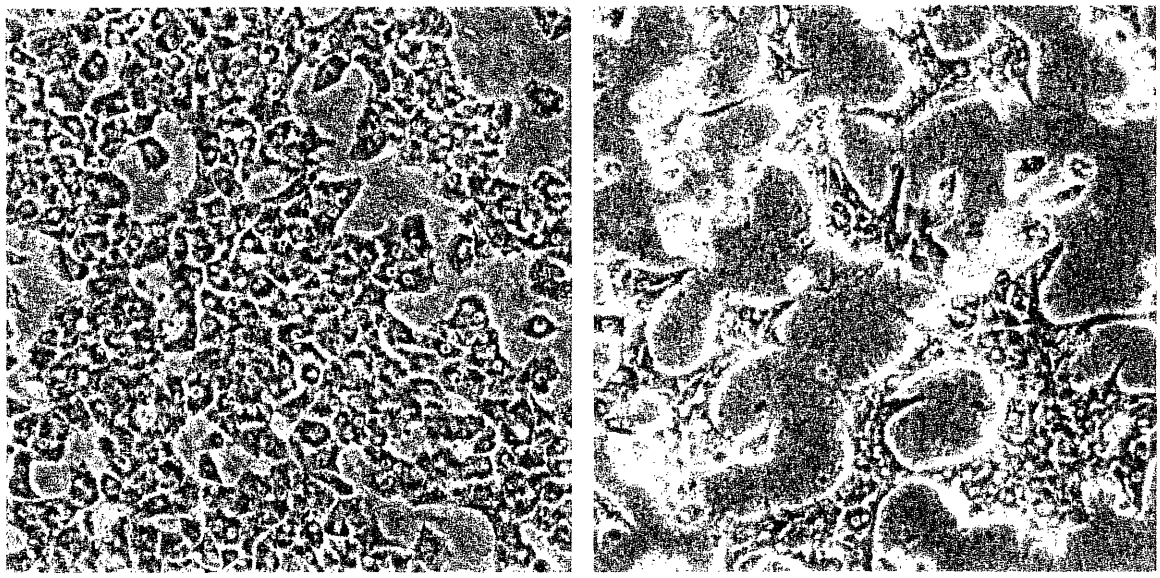


Table 3a: Light microscopy at 100x on day 2 (left) and day 5 (right)

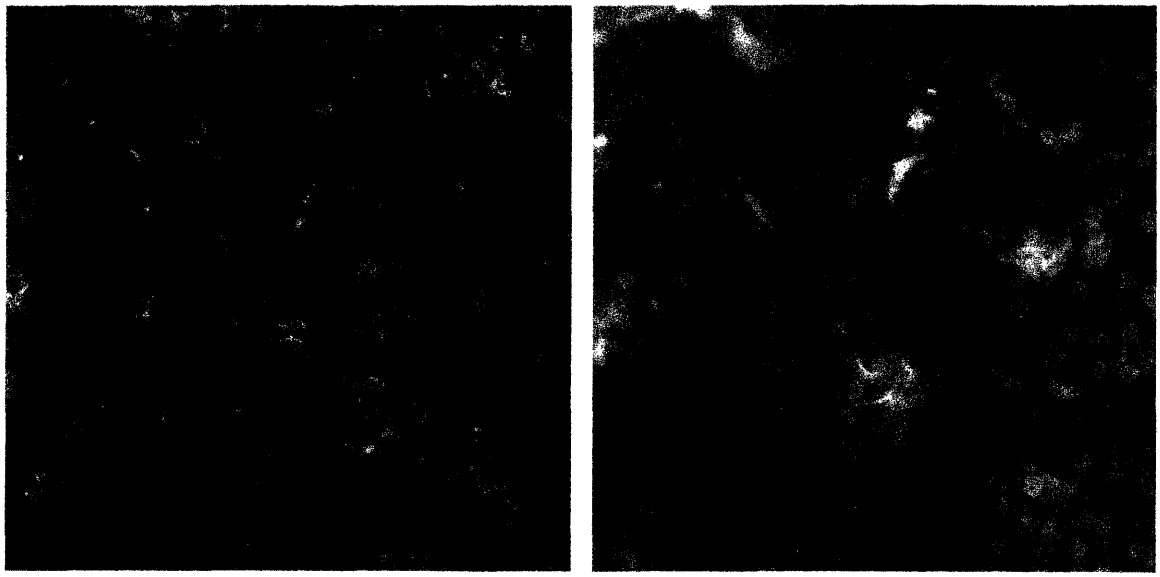
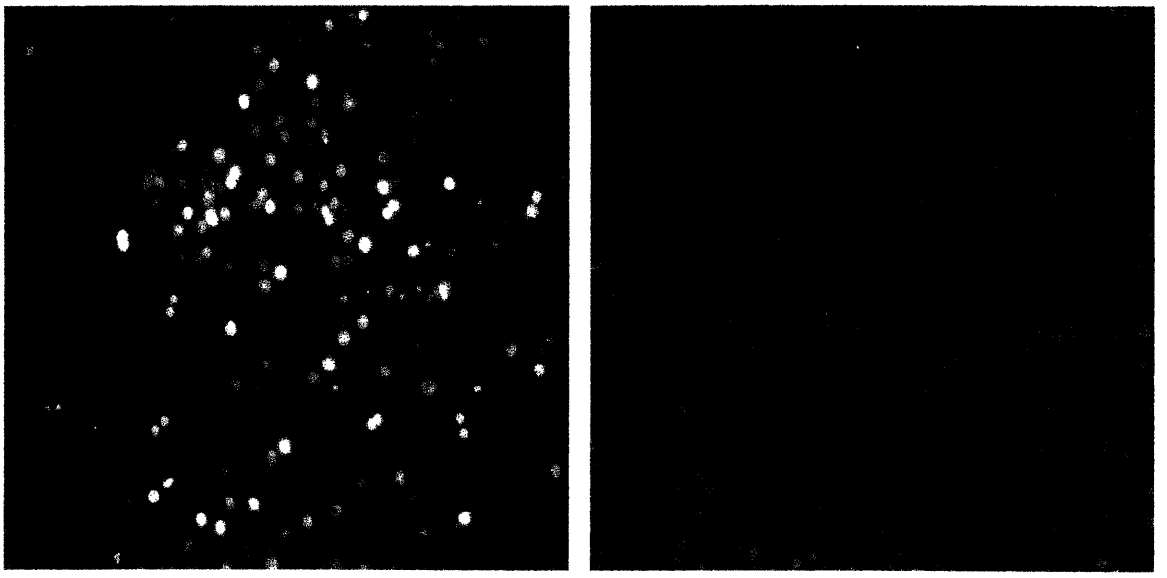


Figure 3b: DAPI and phalloidin staining on day 2 at 100x (left) and 200x (right) magnifications



3c: DAPI staining at 100x on day 2 to investigate presence of mitotic figures

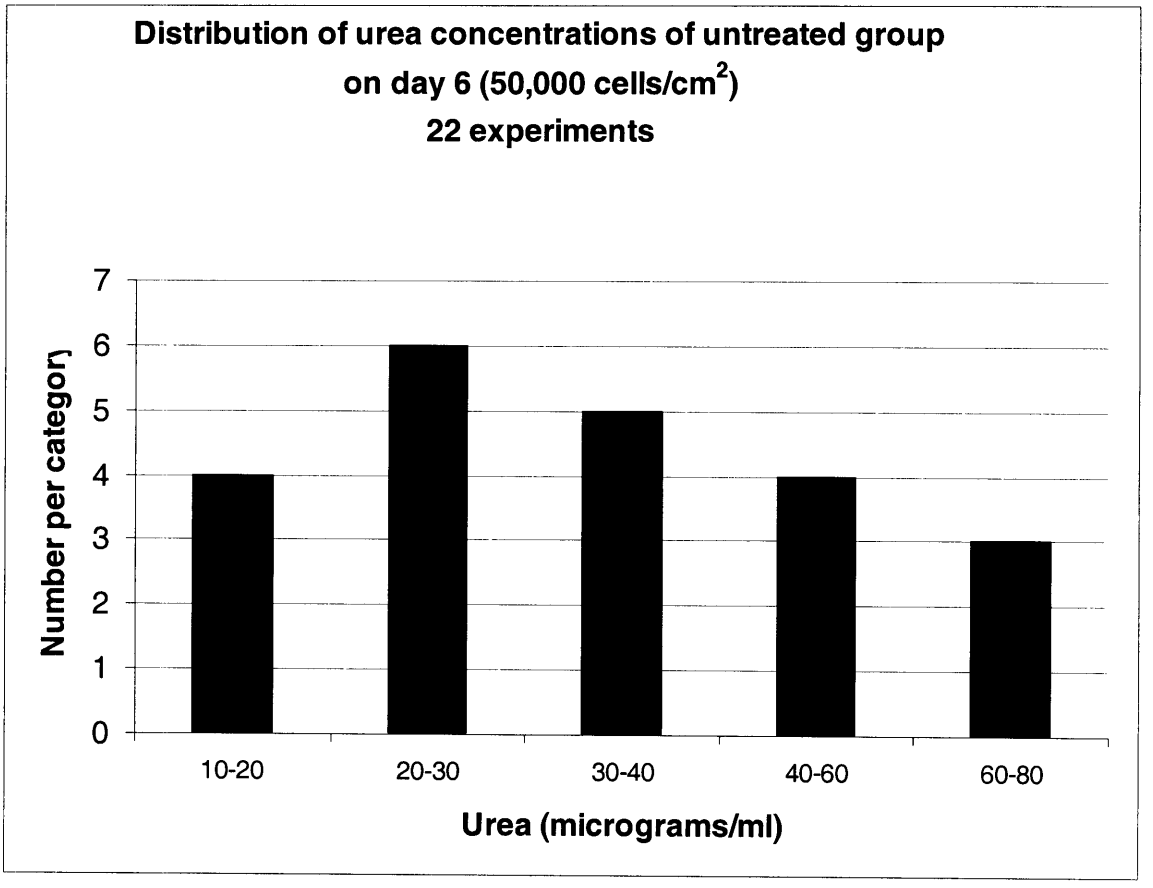


Figure 4: Variation in urea secretion in cultures isolated from different rats

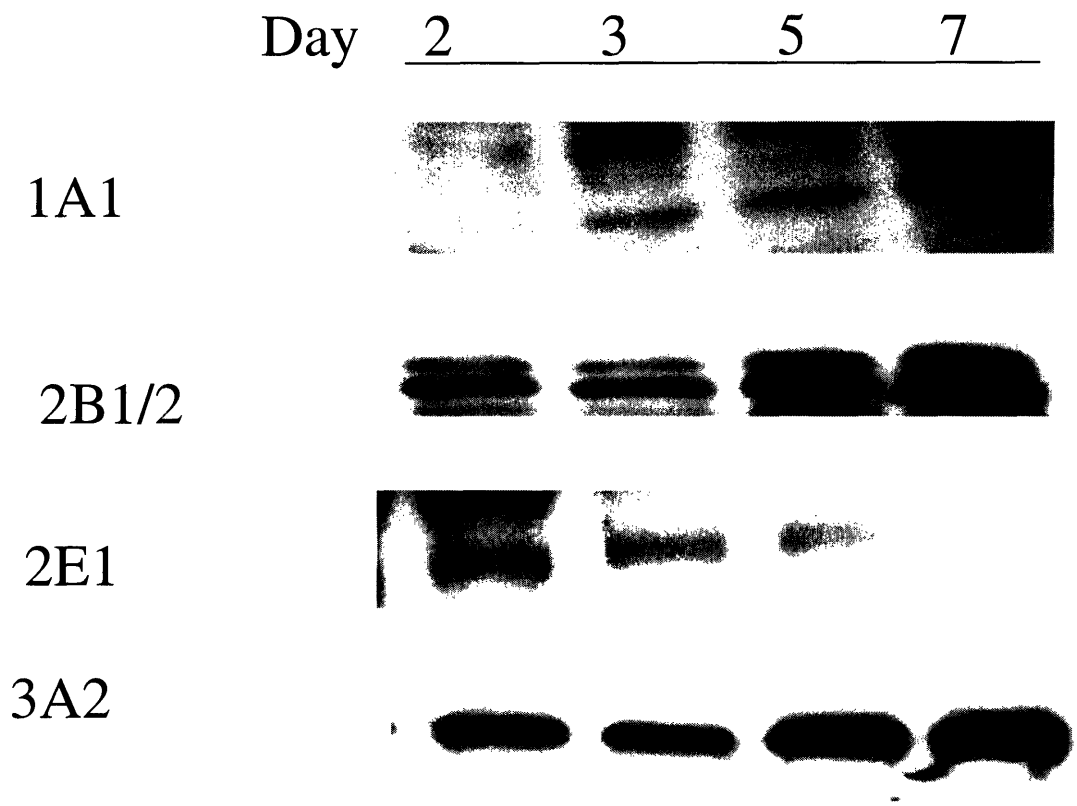


Figure 5: Expression of various CYP's as a function of time in the collagen sandwich

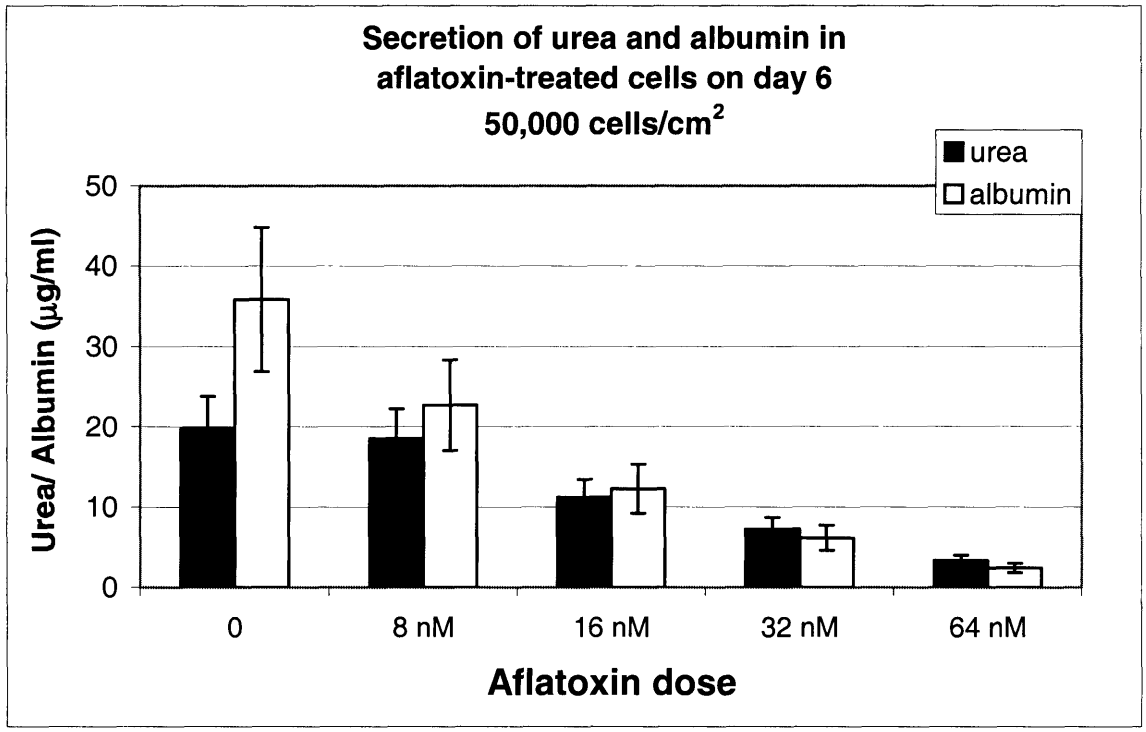


Figure 6a: Dose-response of aflatoxin B1 as measured by urea and albumin on day 6

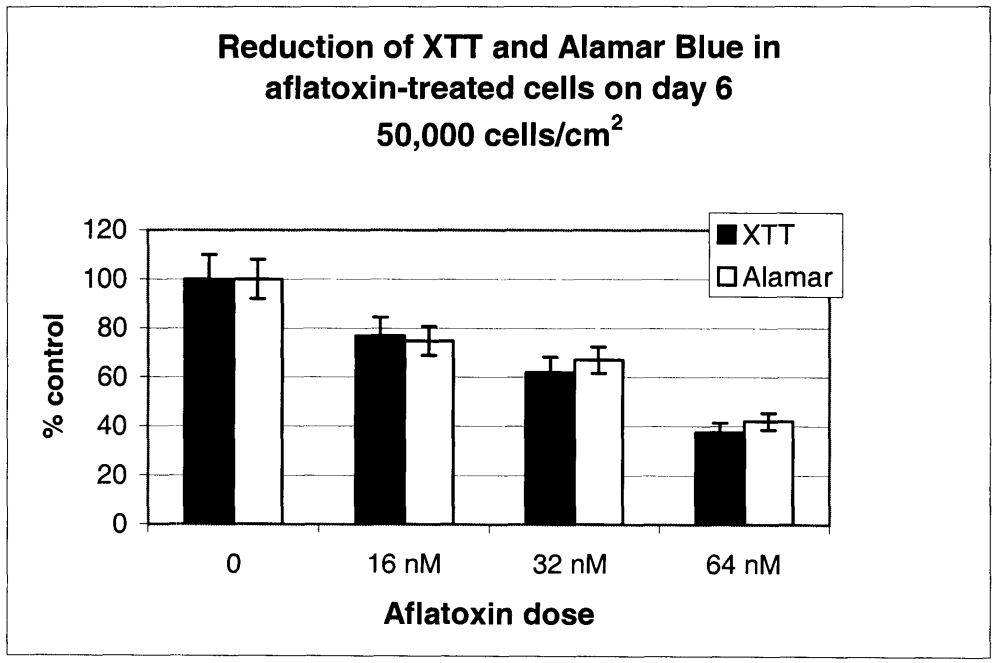


Figure 6b: Dose-response of aflatoxin B1 as measured by XTT and Alamar Blue day 6

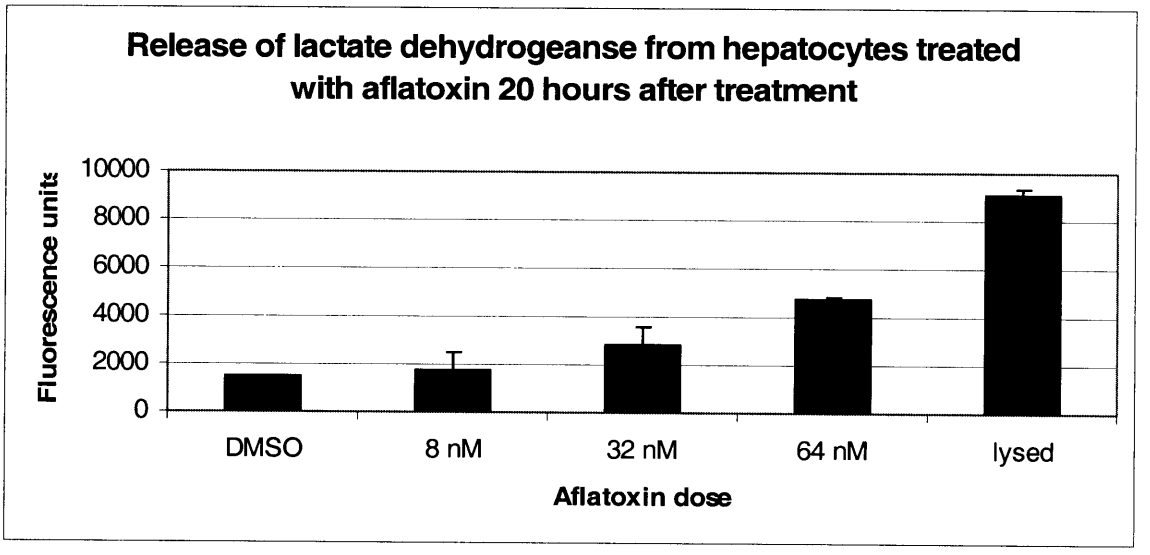


Figure 6c: Release of lactate dehydrogenase from cells as a function of aflatoxin concentration

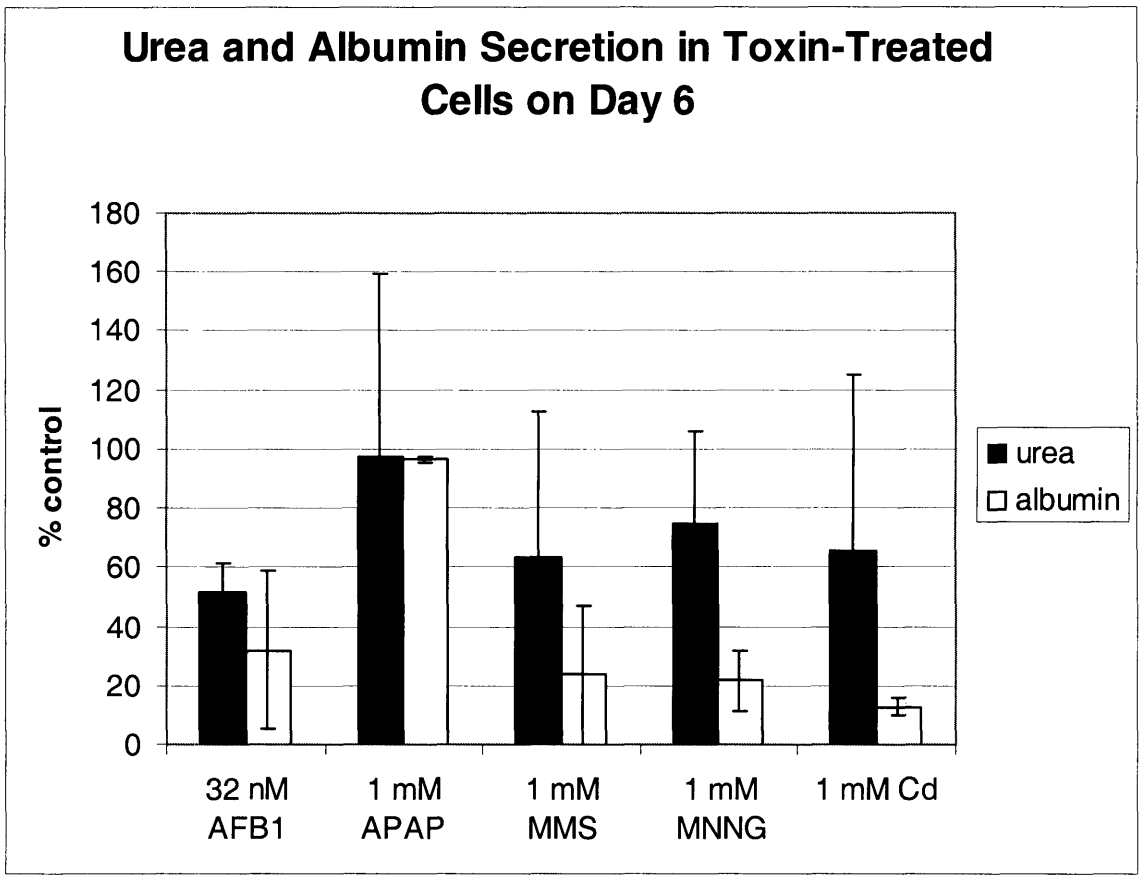


Figure 7a: Percent decrease of urea and albumin secretion after treatment with five different compounds (average of 3-5 experiments)

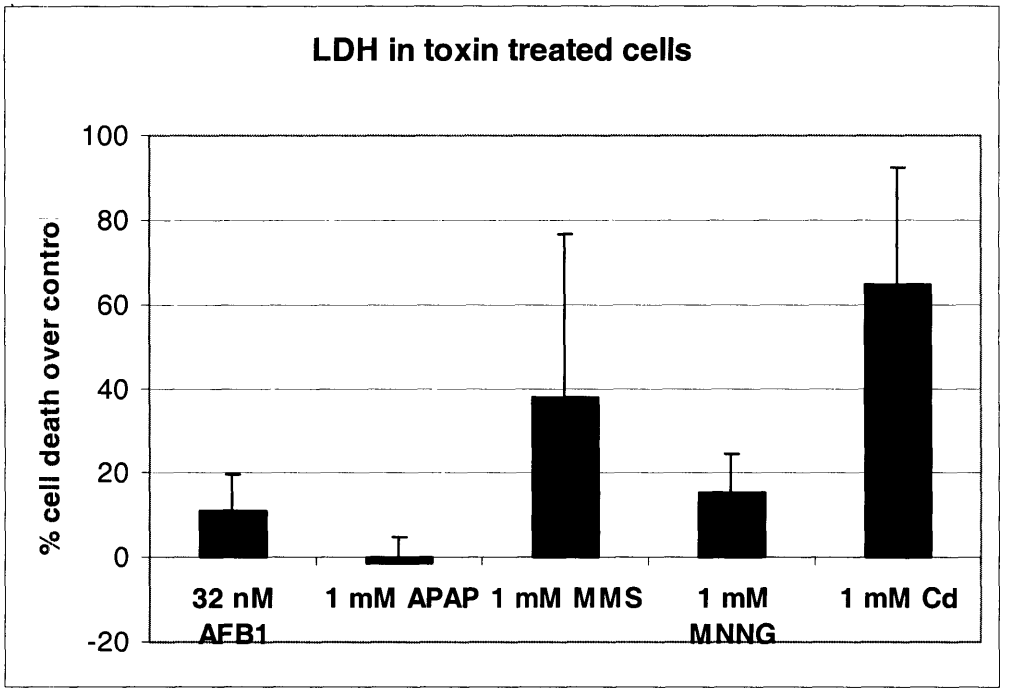


Figure 7b: Release of LDH after treatment with five different compounds (average of 2-5 experiments)

Paired T-test Values (n values in parentheses)			
	Urea	Albumin	LDH
Aflatoxin	0.0003 (20)	0.003 (8)	0.19 (6)
MMS	0.02 (15)	0.06 (7)	0.00003 (15)
MNNG	0.05 (17)	0.0004 (12)	0.011(11)
Cd	0.21 (11)	0.008 (7)	0.000003 (11)

Table 1: Paired T-test values for control and toxin-treated cells in the urea, albumin and LDH assays

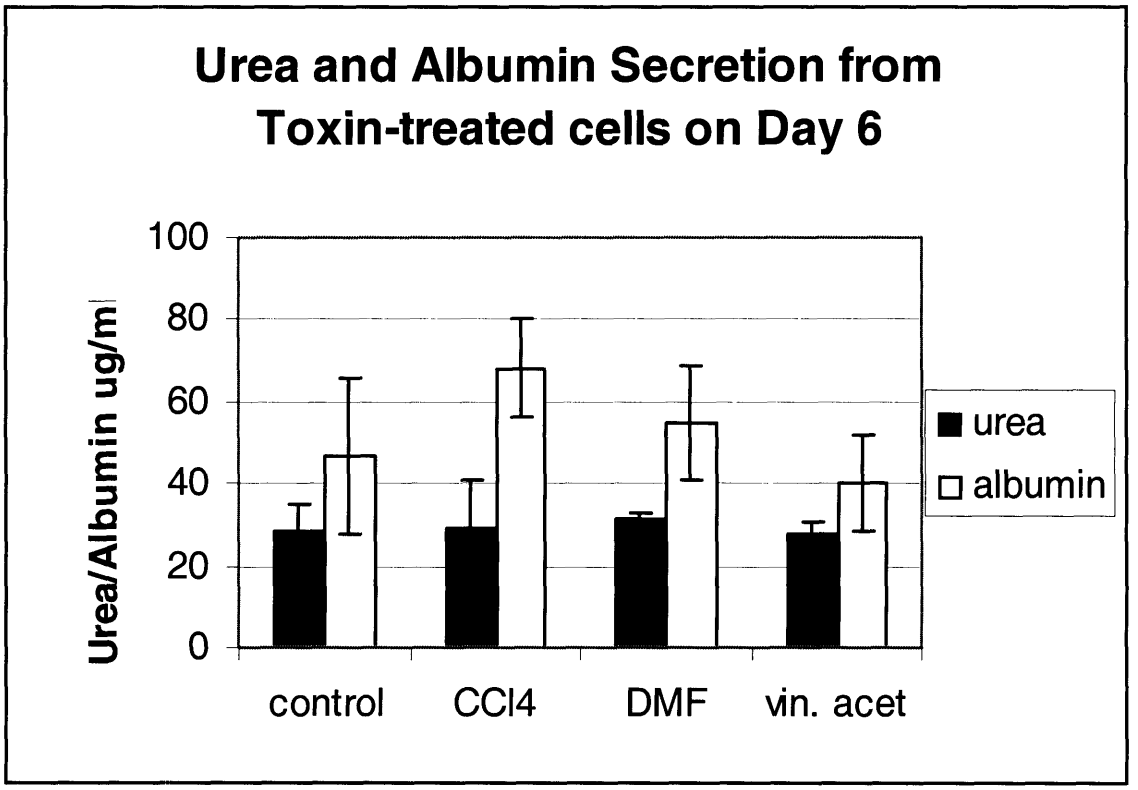
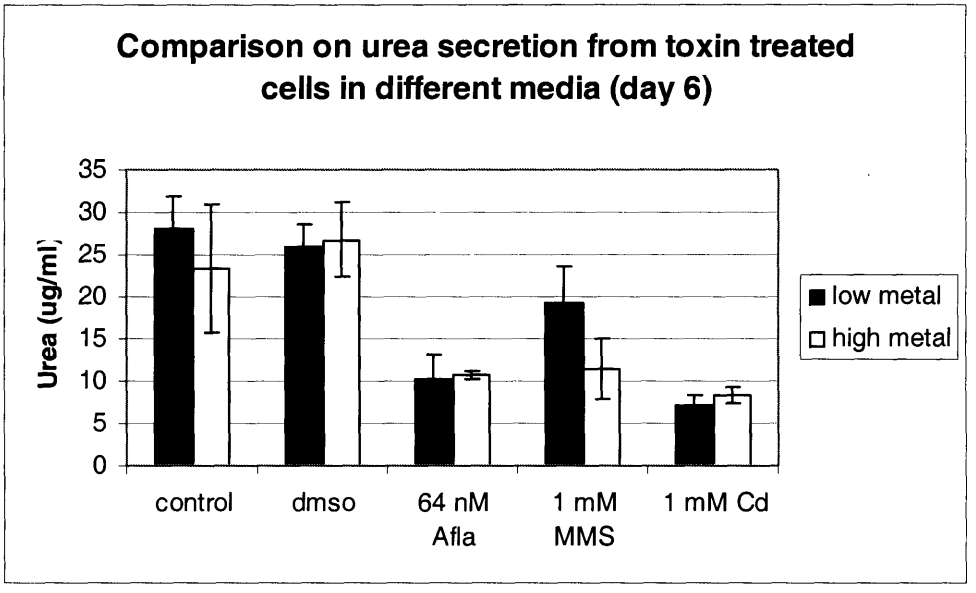


Figure 8: Urea and albumin secretions in control cells and cells treated with carbon tetrachloride, DMF and vinyl acetate



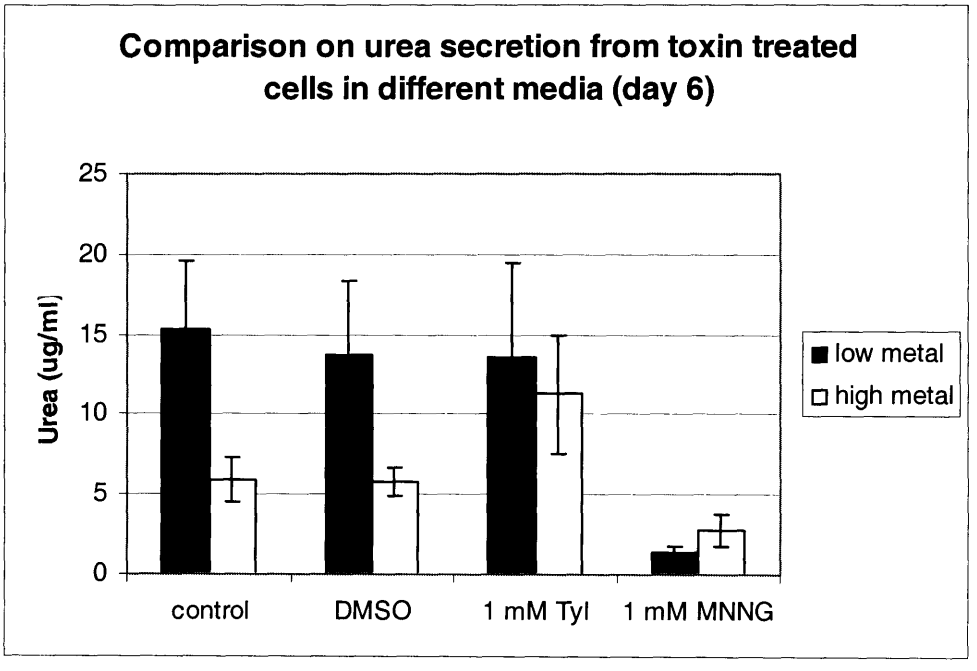
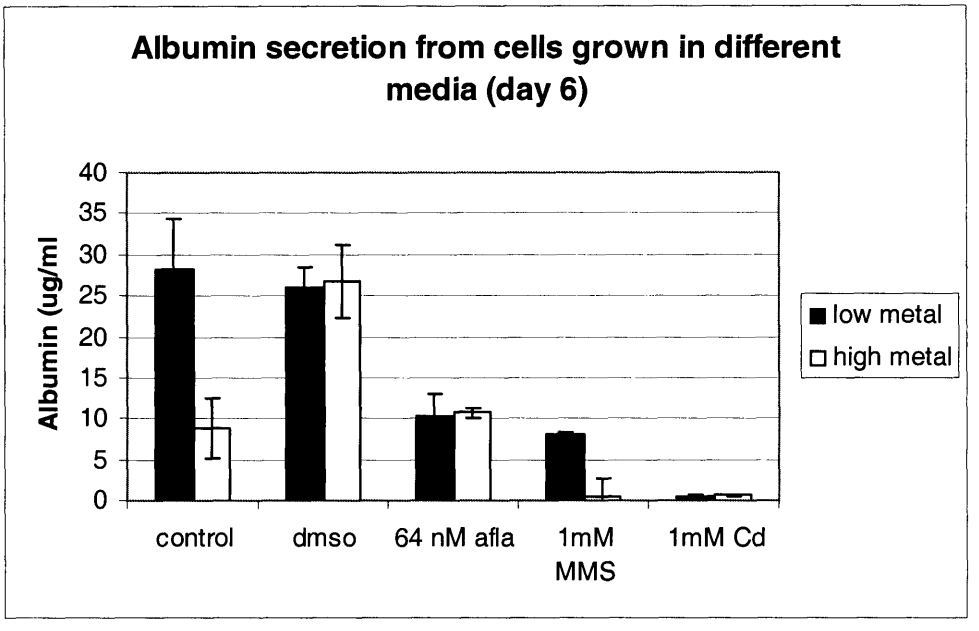


Figure 9a: Urea secretion of control and toxin-treated cells in low and high metal media



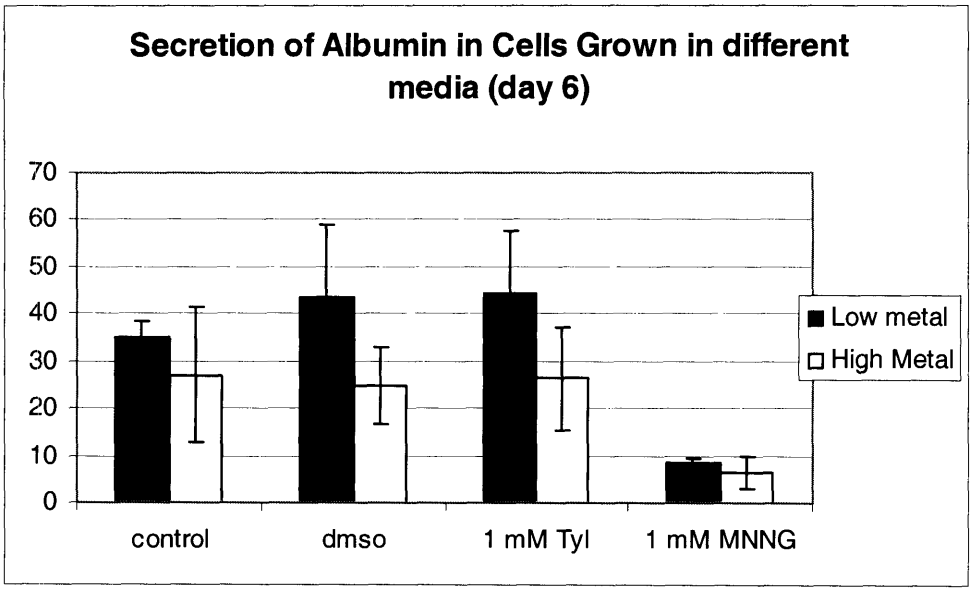
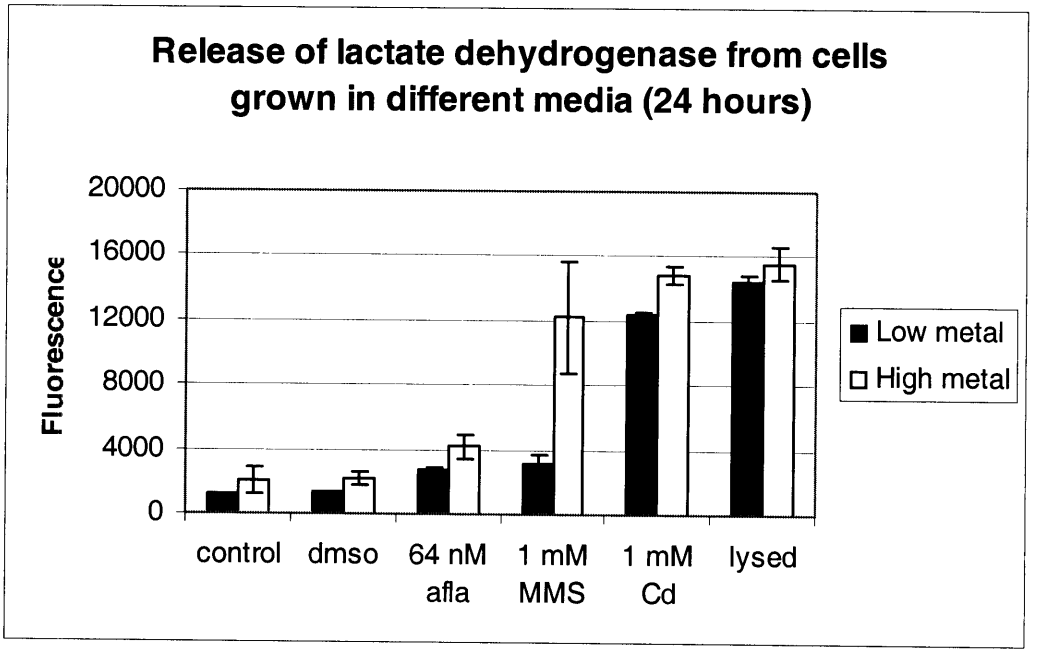


Figure 9b: Albumin secretion control and toxin-treated cells in low and high metal media



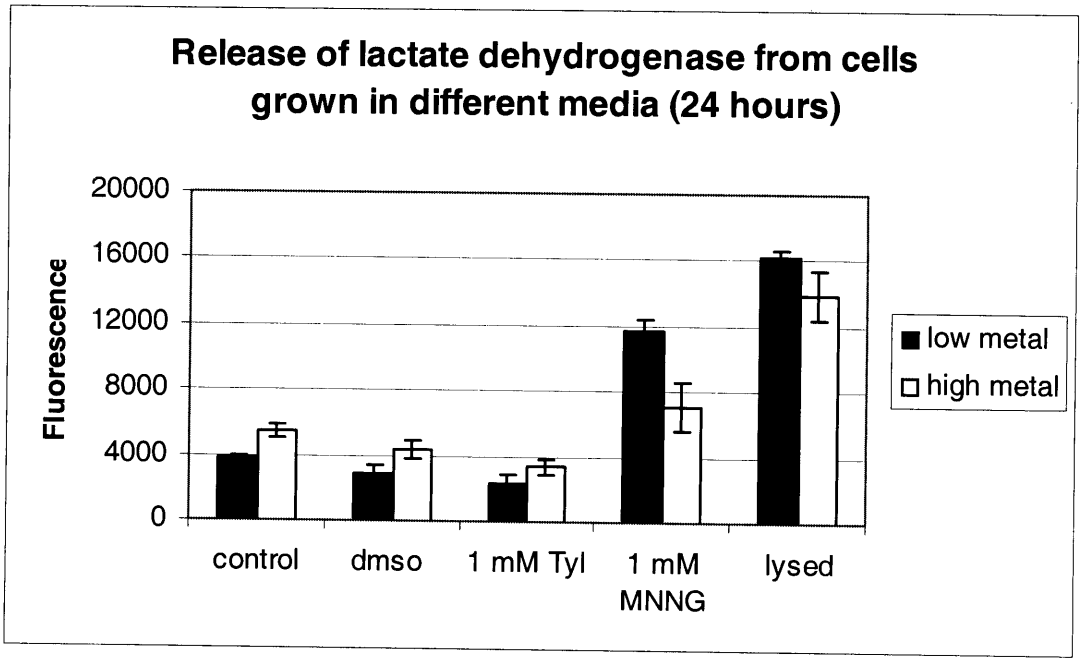


Figure 9c: Release of lactate dehydrogenase from control and toxin-treated cells

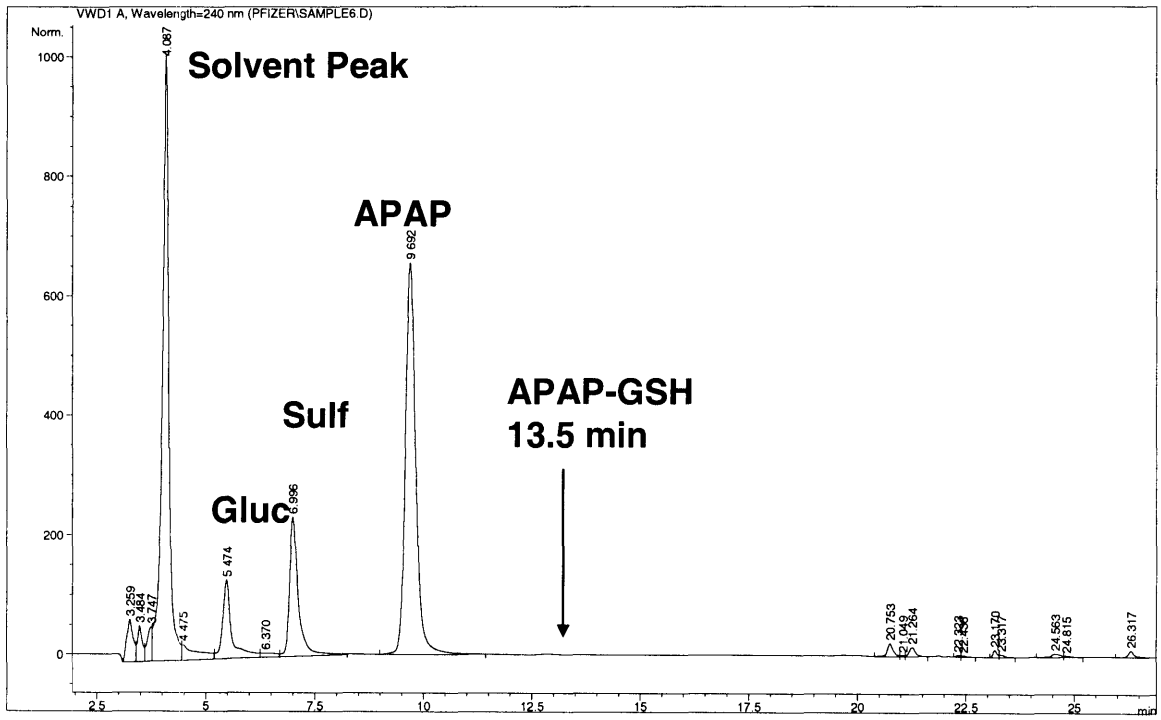


Figure 10: HPLC chromatogram of medium from cells incubated for 24 hours with acetaminophen

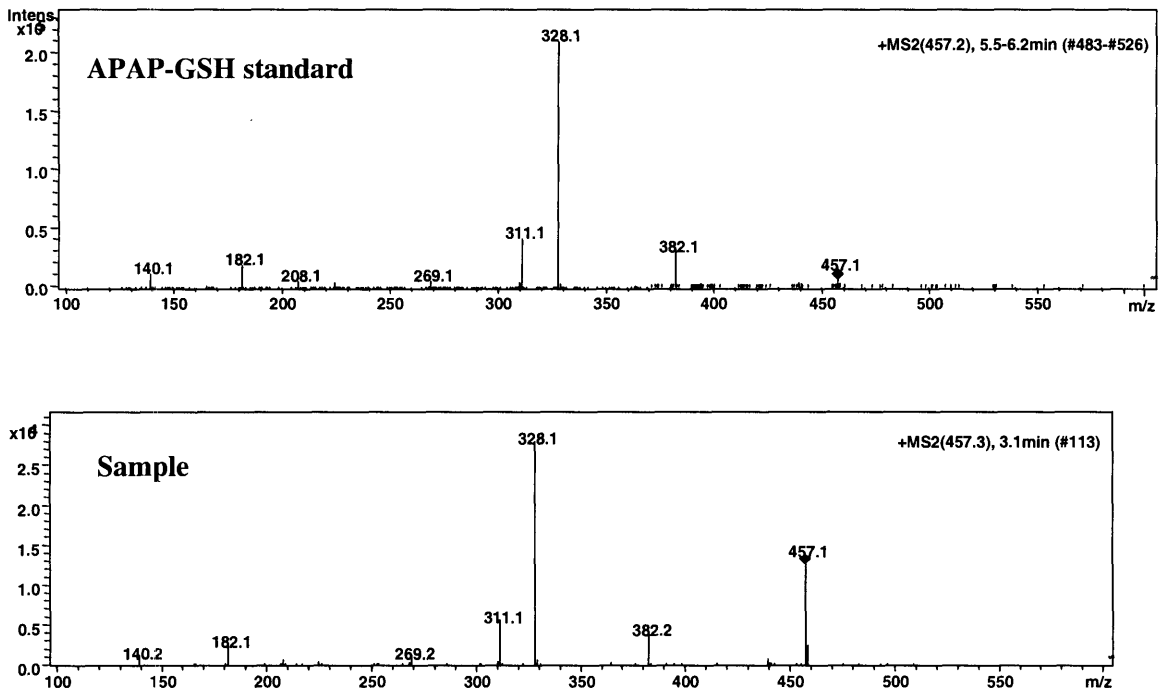


Figure 11a: Collision induced dissociation (CID) spectra of the APAP-GSH standard and the sample

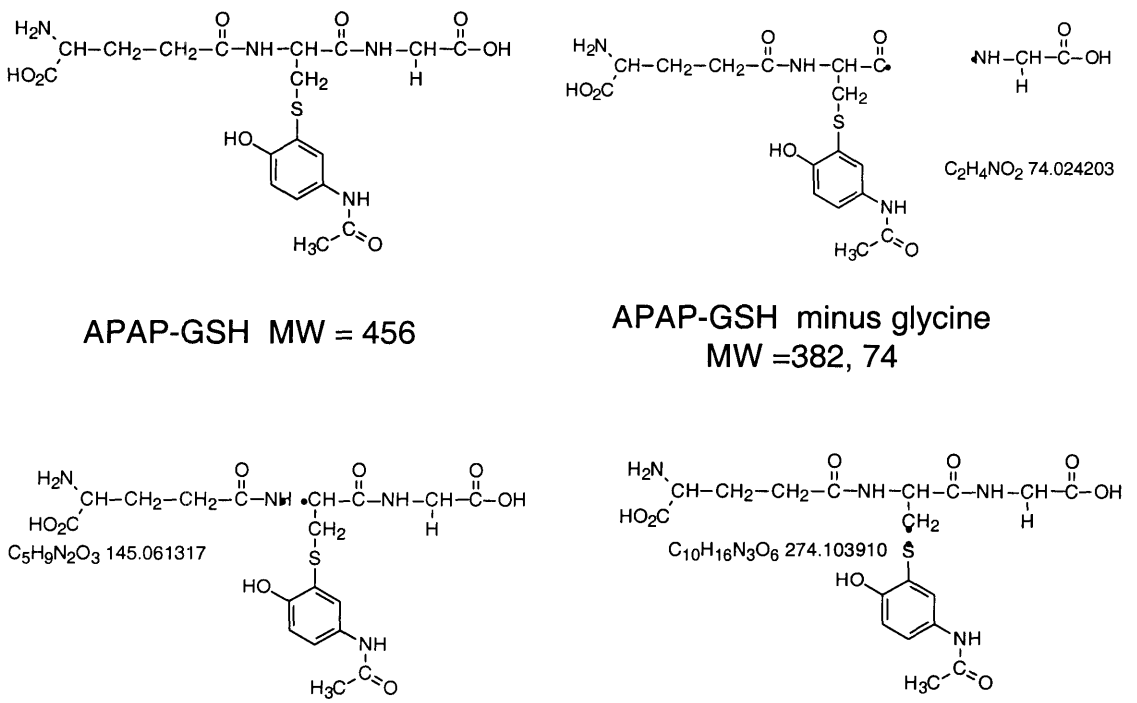


Figure 11b: Molecular weights of the fragmented ions of the acetaminophen-glutathione adduct

Chapter 3: Characterizing the proteome of conditioned medium from rat hepatocytes cultured in collagen sandwiches

3.0 Abstract

We have characterized the secreted proteome of primary rat hepatocytes cultured in a collagen gel sandwich configuration. After depletion of albumin from conditioned medium, we separated proteins on an 8-16% gradient gel and cut the lane into 30 slices. After in-gel digestion and peptide extraction on each slice we analyzed the samples with a Qstar LC-MS/MS mass spectrometer. We identified 890 peptides corresponding to 493 proteins. The protein profile included 59 plasma proteins, several structural extracellular matrix proteins, two heparan-sulfate proteoglycans and over 50 proteins identified previously as embryonic or neural proteins. We also analyzed conditioned medium from aflatoxin-treated cells and we have identified two proteins, α1-antitrypsin and α2-macroglobulin, whose secretions appear to be down-regulated in cells exposed to aflatoxin B1. Thus, these proteins could be potential biomarkers of aflatoxin toxicity. In summary, proteomics analysis of conditioned medium from collagen sandwiches can be used to evaluate hepatocyte function in culture and to establish a database to identify biomarkers of toxicity.

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3.1 Introduction

Proteomics, defined as the high-throughput separation, display and identification of proteins, is a developing field which has the potential to enhance understanding of biological processes by identifying the full range of proteins in biological samples [99-101]. One of the major advantages of using proteins as biomarkers is that they are secreted in body fluids and many of them change during a disease state. With the advances in technology, especially in mass spectrometry and liquid chromatography, it is now possible to quickly characterize proteins in biological samples and to identify some possible targets for screening and diagnostics.

Some of the most important technological advances which accelerate the development of proteomics are in the fields of (a) gel electrophoresis, (b) liquid chromatography, and (c) mass spectrometry. New technologies in these field focus primarily at increasing the speed and the sensitivity of protein identification. Furthermore, new techniques are being developed to understand the biological significance of the findings by estimating the relative protein abundances in samples [122].

The methods for proteomics analysis are still under development, but most protocols rely upon separation of proteins by gel electrophoresis, digesting the proteins with a proteolytic enzyme such as trypsin, and identifying the peptide masses with mass spectrometry. The most common ionization techniques for identifying peptides are matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). MALDI instruments, which are usually coupled to time-of-flight (TOF) spectrometers, are

especially well-suited for peptide mass fingerprinting. However, this approach usually requires the identification of at least four peptides per protein [102]. For protein mixtures, which usually require LC-MS or LC-MS/MS, ESI is the standard method of ionization. The development of the nanospray around 1999 [103] allowed injections in the range of 20 nl/min, which made it possible to perform several stages of tandem mass spectrometry from sample quantities as small as 1 μl [104]. In general, the detection limit for proteins and peptides is between femtomoles and picomoles, however the sensitivity depends on the purity of the sample and the instrument.

Identification of proteins in biological samples usually requires the removal of high-abundance proteins such as serum albumin and immunoglobulins before gel electrophoresis [105;106]. The removal of these proteins is usually achieved by immunoaffinity columns, and has allowed for more sensitive and higher resolution of proteins in serum [106;107]. The sensitivity of the proteomics analysis is also influenced by the sample collection method. Witzmann et al.[108] showed that one can obtain a higher yield of cellular proteins when the proteins are directly solubilized in the cell culture, rather than extracting them after cell scraping. In fact, they found the latter method resulted in a 35% loss of the total protein content.

The identification of proteins involved in disease or toxicological processes has opened up the new field of "toxicoproteomics," with the potential of discovering proteins which can be used as markers of toxicity in biological samples. Biological fluids such as serum, urine, spinal fluid and even exhaled breath can be used as proteomics samples to diagnose disease [104]. One of the diseases that proteomics aims to study is cancer. Comparison of normal tissues with tumor tissues has already revealed a number of

differences in protein expression. Heat shock proteins, in particular, have been found to be differentially expressed in liver tumors [109], tongue squamous cell carcinoma [110] and ductal carcinoma of the breast [111].

Proteomics analysis has also been used to gain a better understanding of the effects of toxin exposure. Fountoulakis et al. [118] identified several proteins, which changed in the liver after rats were dosed with carbon tetrachloride. They found that two stress proteins, catalase and uricase, were up-regulated, while α2-macroglobulin and senescence marker protein decreased. In a similar analysis, Ruepp et al. [113] characterized the protein profile in the mouse liver after acetaminophen exposure and found a significant decrease in the heat shock proteins, just 15 minutes after exposure. The effects on protein expression of several other hepatotoxins was also investigated by Man et al.[117], who found that the steroid cyproterone acetate and dexamethasone both upregulate haptoglobin, α1-antitrypsin and carboxylesterase in rat livers. Such analyses could therefore be used to determine whether toxins act through shared pathways. Proteomics analysis of plasma has also been used to predict toxicity in other organs. For example, the renal toxicity caused by 4-Aminophenol (4-AP), D-serine and cisplatin, has been studied by analyzing differences in the plasma proteome of treated and control rats, and it has been found that T-kininogen levels in the plasma correlated well with kidney toxicity [159].

Many plasma proteins are produced by hepatocytes, but to our knowledge no other group has categorized the secreted proteome of hepatocytes. The closest analysis was performed by Fountoulakis et al. [102], who studied the proteomics profile of the rat liver. Using two-dimensional electrophoresis followed by MALDI-MS they identified

273 intracellular proteins. In this chapter we present an in-depth analysis of the proteins in the conditioned medium of hepatocytes cultured in a collagen sandwich. This study is not only useful in understanding the functioning of hepatocytes *in vitro*, but it also facilitiates subsequent toxicity studies by establishing a database of proteins present in the medium of control cells. Using this information, we have gained understanding regarding the hepatocytes' ability to secrete plasma and extracellular matrix proteins. We have shown that the hepatocytes in culture produce a number of plasma proteins as well as structural matrix proteins such as collagens, laminin and fibronectin. Furthermore, we have also identified several proteins, which have not been observed previously in the liver.

3.2 Methods and Materials

3.2.1 Identification of acute-phase proteins

Sample preparation

Medium was collected every second day after toxin treatment and albumin was measured in each sample with ELISA (see Chapter 2). Samples from aflatoxin-treated and control cells were normalized so that the total amount of albumin was the same in both samples. This approach of normalizing the samples according to albumin secretion, allowed for the identification of proteins specifically down-regulated by aflatoxin. The media were first dialyzed against double distilled water at 4°C and were then concentrated in a vacuum to 20 μl.

Western blotting

Gel electrophoresis was performed on 10% Tris-HCl gel with Protean II mini-gel apparatus (Biorad, Hercules, CA). After gel electrophoresis proteins were transferred to a PVDF membrane at a constant voltage of 30 volts for two hours at room temperature. The membrane was blocked overnight in 5% milk. Primary antibodies for α-l-antitrypsin and α2-macroglobulin conjugated to horseradish peroxidase (Research Diagnostics Incorporated, Flanders, NJ) were diluted 1000 and 10,000-fold respectively. The membranes were incubated with antibodies for one hour and were washed with a solution containing 0.5% Tween and 0.1% Triton-X 100. The membranes were incubated with a chemiluminescent substrate, SuperSignal (Pierce Biotechnology, Rockford, IL) for five minutes. To visualize the proteins the membranes were exposed to x-ray film for 5-10 seconds.

3.2.2 Characterization of the secreted protein profile

Silver staining – (performed by Saraswathi Mandapati)

In order to visualize the complete protein profile, 50 µl of medium were concentrated and run on a 10% acrylamide 1-D SDS PAGE. Silver staining was performed as described by Shevchenko et al. [160]. Briefly, after electrophoresis the gel was fixed in 50% methanol-5% acetic acid for 30 min and washed with plenty of 50% methanol for 10min and then with plenty of water for 10 min. It was incubated for 1 min in 0.02% sodium carbonate and washed twice with water for 1 min each time. The gel was incubated in chilled 0.1% silver nitrate solution for 20 min at 4 °C. It was then rinsed thoroughly

twice with water for 1 min each time and developed in 0.04% formaldehyde in 2% sodium thiosulfate solution with rocking. The staining was stopped with 5% acetic acid and then gels were stored in 1% acetic acid at 4 °C for a day or two prior to enzymatic digestion.

Albumin removal (with Vadiraja Bhat)

Two and a half milliliters of conditioned medium were removed from hepatocytes after nine days in culture. During this time the medium was changed five times (on days 1,2,3,5 and 7). The culture was also washed four times with Hanks Buffer (Invitrogen, Carlsbad, CA) prior to the medium exchange on day 2 in order to remove contaminating proteins from the isolation procedure (see Chapter 2 for cell culture techniques). The medium was concentrated to 100 μl using a 3K cutoff centrifugation filter (Millipore, Billerica, MA). In order to remove the albumin, column was prepared by pipetting 200 μl of Protein-A agarose (Santa Cruz Biotechnology, Santa Cruz, CA) into a 0.45micron filter unit and adding 100 μl of rabbit anti-rat polyclonal antibody (Research Diagnostics Inc) for 2 hours and mixed continuously. Then, the column was washed 4 times with 500 μl of PBS. The next step was to add 200 μl of sample to column and to incubate for 2 hours while mixing continuously. The sample was recovered by centrifugation and the columns were washed 2 more times with 500 μl PBS. The sample and the washing solutions were concentrated to 30 μl using centricon before being loaded onto the gel. The gels were 8-16% gradient gels (Biorad, Hercules, CA). After the gel finished running it was rinsed for 10 minutes with water and then stained for an hour with Simply Blue

stain (Invitrogen, Carlsbad, CA). In order to visualize the bands the gel destained for an hour with water.

In-gel digestion

The gel was rinsed with water twice, 10 minutes each time. The gel was divided into 30 bands of equal width and each band was further cut into 1x1 mm cubes and placed into a 0.65 ml tube. During the following washing procedure all volumes were 200 µl. First, the gel pieces were rinsed with water for 15 minutes and vortexed continuously. Then, they were washed with water/acetronitrile 1:1, and vortexed for 15 minutes. Next, acetronitrile was added and vortexed until the gel pieces turned sticky white. The gel pieces were rehydrated in 100 mM ammonium bicarbonate and vortexed, but the liquid was not removed. After 5 minutes equal volume of acetonitrile was added and vortexed for 15 minutes. Then, all the liquid was removed and the gel pieces were dried down completely in a speed-vac.

The next step was to reduce and alkylate the proteins. First, the gel pieces were rehydrated in 1 mg/ml Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) in 100 mM ammonium bicarbonate and they were incubated at room temperature for 10 minutes while vortexing, in order to reduce the cysteine disulfide bonds. To alkylate cysteines, the solvent was removed and 55 mM (10 mg/ml) of iodoacetamide in 100 mM ammonium bicarbonate was added and incubated for 45 minutes in the dark. The gel pieces were subsequently washed with ammonium bicarbonate for 5 minutes and acetonitrile was added for 15 minutes. If the stain was not completely gone, the washing with ammonium

bicarbonate and acetonitrile was repeated. Finally, the gel pieces were dried down completely in a speed-vac.

In order to digest the proteins, the gel pieces were rehydrated in a trypsin solution (54 µl of 50 mM ammonium bicarbonate + 6 µl of trypsin) and kept on ice for one hour. Then, the tubes were placed at 37°C overnight. The next day the peptides were extracted using the following protocol: first, 20 µl of 10% TFA was added to the tubes to stop the reaction. Then, the tubes were sonicated for 30 minutes. The supernatant was removed and saved in a tube. In order to extract the peptides, 200 µl of 50 mM ammonium bicarbonate was added to the gel pieces. The gel pieces were sonicated again for 30 minutes and the supernatant was removed and saved in the tube. Afterwards, 200 µl of acetonitrile/water (1:1) with 0.1% TFA was added and the gel pieces were sonicated for 30 minutes and the supernatant was removed and combined with the supernatant from the ammonium bicarbonate extraction. The acetonitrile/water extraction was repeated once more. The supernatants were all combined in one tube and concentrated to 10 µl. The desalting of the peptides was carried out using zip tips (Millipore, Billerica, MA). All solutions were made in double-distilled water. The wetting and elution solutions were 60% acetonitrile with 0.1% TFA. The equilibration solution was 0.1% TFA. The washing solution was 5% methanol with 0.1% TFA. After the peptides were eluted, they were concentrated down to 1 µl.

Mass spectrometry

Mass spectrometry was carried out with a Qstar LC-MS/MS nanospray (Applied Biosystems, Foster City, CA) connected to an Agilent 1100 high performance liquid

chromatographer. The chromatography was carried out using a 75 μm x 10 cm C_{18} column and a 150 nl/min flowrate. Solvent A was 0.1% acetic acid with 5% acetonitrile and solvent B was 0.08% acetic acid with 5% methanol in 95% acetonitrile. The gradient was linear from 0-80% B from up to 90 minutes, and decreased linearly down to 0% B by the end of the run, which was 150 minutes. In order to analyze the samples, we injected 0.5 μl of sample.

Data Analysis

Peptides were autovalidated with Spectrum Mill software (Agilent, Palo Alto, CA) and proteins identified by only one peptide were manually validated using the NCBIR and the TREMBL protein and genebanks.

3.3 Results

3.3.1 Down-regulation of acute-phase proteins

A previous collaboration with Dr. Saraswathi Mandapti showed that there were two bands on silver-stained gels which had much lower intensity in the lanes of the samples from the aflatoxin treated cells. By comparing the bands with the molecular weight standards we estimated that one protein was 55 kDa and the other one was 180 kDa. The proteins were subsequently identified with mass spectrometry as α1-antitrypsin and α2-macroglobulin (unpublished results).

In this research we confirmed the down-regulation of these proteins with Western blotting. Figures 1a and 1b show the Western blots of conditioned medium for α1-

antitrypsin and α2-macroglobulin respectively on day 6. The down-regulation of α1-antitrypsin could already be detected on day 2, but the decrease in α2-macroglobulin could only be detected 6 or more days after aflatoxin exposure, because that was the earliest timepoint when the control cells produced a detectable amount of α2-macroglobulin. As the figures show, a 6 nMolar dose of aflatoxin does not change the secretion of these proteins significantly. A 15 nM dose decreases α1-antitrypsin at least 50%, and almost completely down-regulates α2-macroglobulin. A 30 nM dose decreases α1-antitrypsin to undetectable levels. Western blots were also performed twenty days after aflatoxin B1 exposure, and we observed that the hepatocytes exposed to the toxin had not regained the ability to secrete these proteins.

3.3.2 Characterization of the secreted proteome

Depletion of albumin from conditioned medium

Figure 2 shows the SDS-PAGE gel of the samples after albumin depletion. Lane 1 is 2.5 ml of hepatocyte medium concentrated into 20 μl. Lanes 2 and 3 are replicates of medium depleted of albumin as described in the methods section. As the gels show, most of the depletion is at 60 kDa, which corresponds to the molecular weight of albumin.
We cut lane 2 into 30 slices and performed in-gel extraction and peptide extraction on each slice.

Following in-gel digestion, the peptides were analyzed with Qstar LC-MS/MS. The total ion chromatogram of slice 14, the fraction with the most number of proteins (214 peptides, 108 proteins) is shown in Figure 3. From the 30 slices we identified 890 peptides belonging to 493 distinct proteins. The peptides were validated using Spectrum

Mill software (Agilent, Palo Alto, CA), and public database searches. The complete list of proteins is shown in Appendix IIIa. Using the molecular weight markers we made a calibration curve to relate the gel slices with the molecular weights of proteins that they would be expected to contain (Figure 4a). We have also mapped the distribution of peptides along the SDS-gel (Figure 4b, complete list in Appendix IIIb) and highlighted the regions where the peptides would be expected to be found based on the molecular weights of the proteins. The number of peptides and proteins are also shown on the top of Appendix IIIb.

Protein Profile

Of the 493 proteins, 47% were intracellular or membrane proteins, 13 % were plasma proteins, 6% were matrix proteins and 35% were miscellaneous (Figure 5a). The miscellaneous proteins were further subdivided. Of the 179 miscellaneous proteins, 49% had unknown functions or were not in the databases, 23% were only identified previously in the central nervous system origin, 22% were only found in organs other than the liver and the central nervous system (e.g. muscle and kidney) and 7% were considered embryonic proteins (Figure 5b). We also identified 59 plasma proteins and determined that twenty of these proteins were protease inhibitors, 7 were from the complement cascade, 4 were proteases with the rest including metal-binding proteins and apolipoproteins (Figure 5c). Almost a quarter of the intracellular proteins were membrane proteins, with the other proteins originating from various organelles including mitochondria, lysosomes, Golgi and the nucleus (Figure 5d). Several matrix proteins were also identified, including 5 types of collagen, fibronectin, laminin, agrin, and we also found several cell-matrix adhesion proteins (Table 1).

Silver Staining

Figure 6 shows a silver-stained gel of conditioned medium. Lane 1 is the marker, lane 2 is from control cells, lane 3 is from cells treated with aflatoxin and lane 4 is from a collagen sandwich without cells (just medium incubated on top of collagen.) As the figure shows, lane 4 has only one significant band, which we identified later as corresponding to transferring (a component in our medium).

3.4 Discussion

3.4.1 Down-regulation of acute-phase proteins

We have shown that exposing hepatocytes in collagen sandwiches to aflatoxin B1 leads to a decrease in the secretion of αl-antitrypsin and α2-macroglobulin. Both proteins are glycoproteins produced by the liver during inflammation in order to inhibit proteases which are secreted during the acute-phase response [161;162]. The presence of these proteins in the medium of control cells suggest that the untreated cells are stressed. It would be expected that the secretion of these acute-phase proteins would increase after exposure to a toxin, however, our results show that the opposite is true.

Our initial hypothesis was that exposure to aflatoxin decreased secretion of all proteins, however, our Western blots are normalized to secreted albumin. In other words, the samples were loaded on the gel so that the total amount of albumin was the same in all samples. Thus, a decrease in the acute-phase proteins shows that these proteins are specifically down-regulated after aflatoxin B1 exposure. Since both of these proteins are glycoproteins, which require glycosylation in order to be secreted, we hypothesized that

aflatoxin inhibited glycosylation and secretion of these proteins. It has been shown that antibodies used in Western blotting should still recognize the unglycosylated forms of these proteins [163;164]. Thus we performed Western blots on cell lysates, but we could not detect either of these proteins. Therefore, it is unlikely that the proteins are down-regulated at the glycosylation/secretion level. The most likely explanation for our observation is that aflatoxin impairs the transcription of these proteins.

3.4.2 Characterizing the secreted protein profile

We have characterized a comprehensive proteomics profile of conditioned rat hepatocyte medium, including plasma, extracellular and intracellular proteins. This analysis established a proteomics database for future toxicity studies in hepatocyte cultures. The advantage of a proteomics analysis of conditioned medium is that the culture does not need to be destroyed for analysis. Thus, this protocol can be adapted to other culture systems, such as flow-through bioreactors, which are expensive to set up. For example, with our proteomics analysis we were able to detect lactate dehydrogenase, which we used in the previous chapter to measure toxicity by a variety of compounds.

The proteomics analysis has also enhanced our understanding about the function of hepatocytes in collagen sandwiches by showing that they secrete a variety of plasma and matrix proteins. Our data also suggest that hepatocytes in the collagen sandwich are in stress. Many of the identified proteins are specific to tissue repair and are upregulated by the cytokines IL-6 and TGF-β. It has been shown by others that both

IL-6 [157] and TGF-β [165] can be secreted by hepatocytes in culture. Thus, it is possible that the stress experience by hepatocytes during the cell isolation procedure and the culture conditions leads to secretion of these cytokines and production of acute-phase associated proteins.

Plasma proteins

We have shown that hepatocytes secrete at least 59 plasma proteins in the collagen sandwich. Almost half of these proteins, such as the proteases, the protease inhibitors, and the complement proteins are usually associated with the acute phase. We also detected a significant amount of α2-macrogobulin, which is usually stimulated by IL-6 [156] and several other proteins associated with the acute phase, including alpha-1-acid glycoprotein, alpha-1-antitrypsin and proteins from the complement cascade.

The hepatocytes also secreted a number of normal plasma proteins such as selenoprotein P, Cc1-8 (iron-binding), ceruloplasmin precursor (copper-binding), and apolipoproteins, showing that the cells are secreting many of the proteins that they would *in vivo*. We also detected the presence of two proteins known to be produced specifically during tissue repair. The first protein was hepatocyte growth factor activator protein, which is usually produced as a zymogen and converted to its active form during tissue injury, in order to activate hepatocyte growth factor (HGF) [166]. HGF usually acts as a mitogen on hepatocytes during liver injury [167], and the presence of the activator of this protein suggests the cells are experiencing stress. The second protein was connective tissue growth factor (CTGF), a known mediator of inflammation, which participates in tissue repair [168]. CTGF can be produced by a number of cell types in

the liver including fibroblasts, endothelial cells and stellate cells [169]. It is usually upregulated by TGF-β, but its production can also be stimulated by EGF [170]. It is possible that EGF in our medium is responsible for the production of CTGF. In summary, the presence of EGF, a potent mitogen, and the stress experienced by the cells is most likely responsible for the presence of proteins associated with tissue repair and the acute-phase.

Extracellular matrix and membrane proteins

Our work clarified some of the mystery surrounding the survival of hepatocytes in collagen sandwiches. Beken et al.[138] compared albumin secretion and the expression of glutathione S-transferases in collagen sandwiches made from rat tail collagen, commercially available collagen type I, and commercially available collagen supplemented with fibronectin, laminin and heparan sulfate proteoglycans, and found that after seven days of culture, there was no significant difference among the cultures. They hypothesized hepatocytes cultured in collagen sandwiches secrete the necessary extra-cellular proteins, which are retained in the culture by the second layer of collagen.

We have shown that hepatocytes do secrete a number of structural matrix proteins including fibronectin precursor, laminin type 12 and several types of collagen. The cells also produced talin, which is responsible for linking integrin receptors to the actin cytoskeleton and also plays a role in the migration of various cell types [171].

Our conditioned medium also contained two heparan sulfate proteoglycans (HSPG). The first HSPG was a precursor to agrin, a proteoglycan found in the basement membrane at neuromuscular junctions, which binds to dystroglycan, part of the dystrophin-glycoprotein complex [172]. We were also able to identify dystroglycan, which was a surprising result, because both agrin and dystroglycan are found at very low levels in the liver [172]. In fact, dystroglycan has only been detected in the liver from lysates of stellate cells, and its expression is usually up-regulated during liver fibrosis [173]. Since our cell preparation is estimated to have about 5% non-parenchymal cells, and our other data suggests that the cells are experiencing stress, it is plausible that stellate cells in our culture are responsible for the secretion of agrin precursor and dystroglycan. The second HSPG was syndecan-4 precursor, which is primarily expressed in the liver on the membranes of endothelial cells. These two HSPG's in the medium suggests the presence of nonparenchymal, specifically stellate and endothelial, cells in the culture.

Interestingly, we also detected NG2, a chondroitin sulfate proteoglycan, which is usually associated with incompletely-differentiated or immature cells [174]. NG2, originally though to be associated with precursor cells in the brain, is now known to be expressed in cartilage, muscle and bone [175], but to our knowledge it has not been isolated from liver. It is also known to bind to collagen VI [176], which we have also identified in the medium. Although this data does not assert that the culture contains undifferentiated cells, it has been shown that hepatocyte isolation by collagenase-perfusion and addition of EGF to the medium can cause a down-regulation of transcription of hepatocyte-specific genes leading to a dedifferentiated phenotype [177].

We also identified some other proteins expressed specifically during inflammation. One of these proteins was thrombospondin-1, which is usually found near hepatocytes in congenital hepatic fibrosis tissue [178] and it is also considered to be an up-regulator of TGF-β [179]. Our conditioned medium also contained annexin II, a membrane protein, which is usually not detected in hepatocytes of normal adult livers. It has been found in the hepatocytes of embryonic livers, hepatocytes cultured in the presence of EGF [180] and in proliferating hepatocytes after liver damage [181]. Thus, the nature of the proteins found in the medium are consistent with our culture conditions since we included EGF in the medium and the hepatocytes were isolated by a collagenase perfusion.

One of the questions that arose was whether the matrix proteins we identified could be contaminants from the collagen preparation. Figure 6 shows a control experiment where we analyzed medium incubated with just collagen (lane 4). This experiment suggests that the only major protein in lane 4 is transferrin, which is a protein we add to the medium. While this experiment does not rule out the possibility that there are contaminants from the collagen, it shows that medium incubated with only collagen is free from major contaminants. In order to strengthen this result, a future experiment could include performing mass spectrometry analysis on that sample to characterize the complete protein profile.

Miscellaneous proteins

More than a third, or 179, of our 493 proteins were classified as miscellaneous. About half of these proteins have unknown functions, which is attributable to the incomplete annotation of the rat protein database. It was very surprising that almost a

quarter of the miscellaneous proteins are thought to be of central nervous system (CNS) origin. One of these proteins, reelin, has been regarded until recently as a protein involved in brain development, but it is now known to be also produced by the liver [182]. In fact, it has been shown that reelin is produced by stellate cells during hepatic tissue repair, for example following CCl_4 exposure [183]. Thus, the presence of reelin suggests not only the occurrence of tissue repair, but also active protein secretion by stellate cells. Stellate cells are actually known to involved in the remodeling of the extracellular matrix, especially during the inflammatory process [184]. It is possible that the presence of the other CNS-associated proteins are also produced by stellate cells since there is evidence to suggest that stellate cells share the same lineage as neural cells [185].

We also identified 13 embryonic proteins, including spindling-like protein, fetuin-B precursor and procollagen C-proteinase 3. It is unclear why the cells produced proteins associated with embryogenesis. It is possible that there are immature cells in the culture, as suggested also by the presence of NG2. The other possibility is that EGF caused dedifferentiation of the cells, or maybe these are normal proteins that have not been detected in liver previously. It is interesting to note that when we checked the presence of these CNS and embryonic genes on a commercial rat gene chip, we could only find 3 genes on the chip: translation initiation factor eIF-2B, postsynaptic density protein and the embryonic spindlin-like protein. Thus, with the current gene chips it is not possible to do a genomics analysis on these proteins. One possible experiment for future studies could be to compare the proteome of the liver with the sandwich to determine whether these CNS and embryonic proteins are also present in the liver. In

summary, this data shows that the proteomics approach can be used to detect a variety of proteins, even ones whose expression cannot yet be analyzed with gene chips.

3.5 Conclusions

We have shown that the collagen gel sandwich can be used as a system for proteomics analysis. We detected 493 proteins from conditioned, which included a number of plasma proteins, matrix proteins and intracellular proteins. Many of these proteins are produced during the inflammatory state, suggesting that the cells in the collagen sandwich are stressed. We also detected several proteins, which are thought to be produced in the liver by nonparenchymal cells during inflammation and tissue repair. Furthermore, we also identified a number of proteins not detected previously in the liver, including a number of embryonic and neural proteins.

In addition, we have shown that two acute-phase proteins, αl-antitrypsin and α2-macroglobulin, are down-regulated by aflatoxin B1. The presence of these acute-phase proteins in the medium of control cells is consistent with the rest of the proteomics data, which suggests that the cells are in an inflammatory state. We have shown that the secretion of these proteins is unchanged at a 6 nM, but decreases at 15 nM exposure. We have observed that the down-regulation of these proteins is very reproducible, thus, their absence from medium could be used as a marker of aflatoxin toxicity. Future studies could include an investigation of how these proteins are affected by expsosure to other toxins. In summary, we have shown that proteomics analysis on conditioned medium

from collagen sandwiches can be used evaluate hepatocyte function and to identify potential biomarkers of toxicity.

Figures

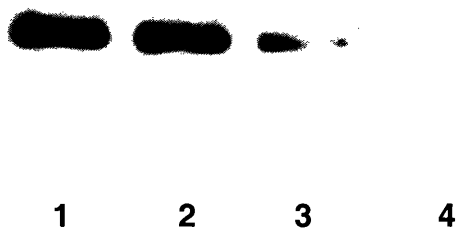

Figure 1a: α1-antitrypsin Western blot of conditioned medium on day 6.
Lane 1: control, lane 2 : cells treated with 6 nM aflatoxin, lane 3: 15 nM, lane 4: 30 nM.

Figure 1b: α2-macroglobulin Western blot of conditioned medium on day 6. The upper bands are α2-macroglobulin, the lower bands are albumin. Lane 1: control; lane 2: 6 nM aflatoxin; lane 3: 15 nM aflatoxin

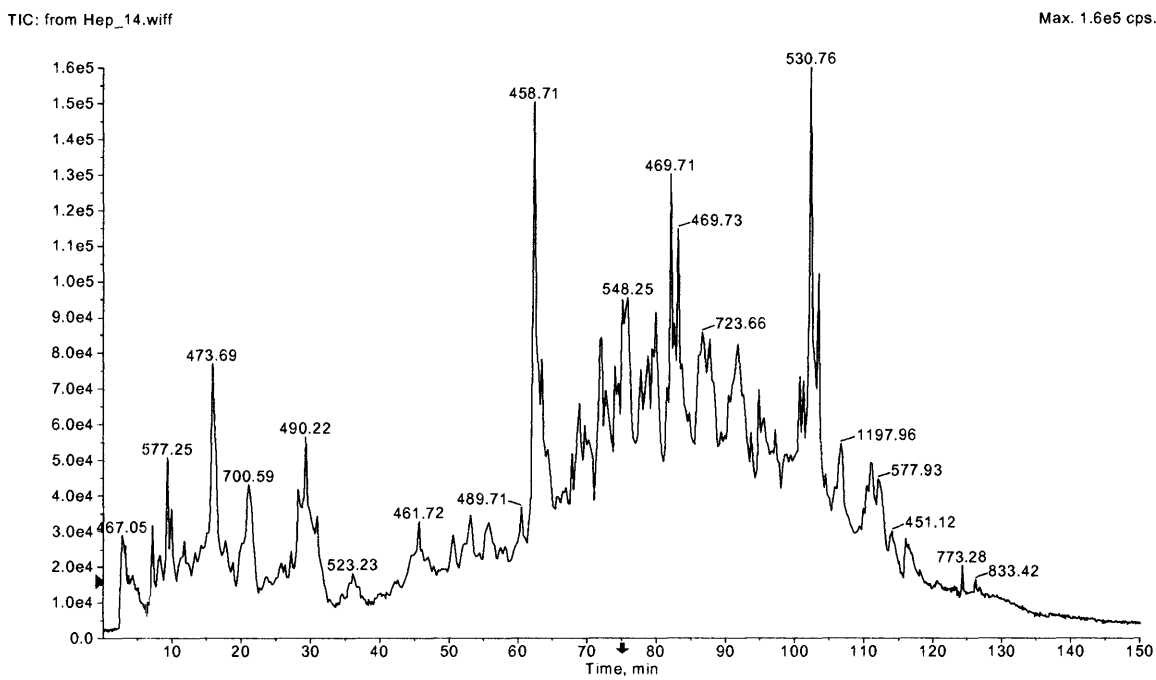

Figure 3: Total ion chromatogram of fraction 14, the slice with the most number of peptides (214 peptides, 108 proteins)

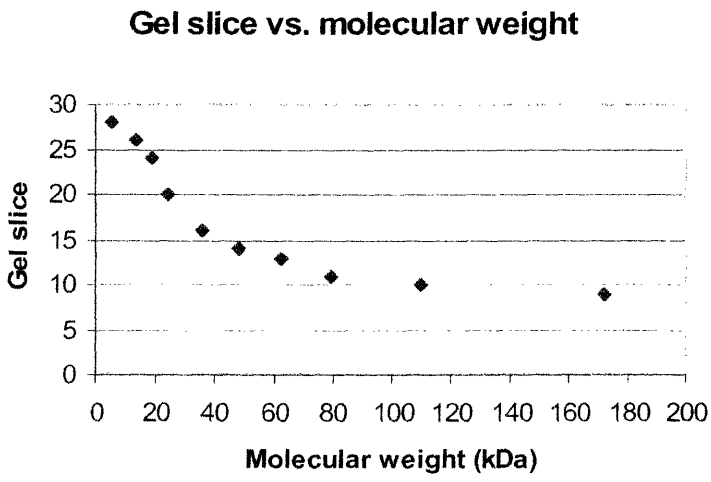

Figure 4a: Calibration curve for relating molecular weight of proteins with the gel slice number

																													Mol. Weight Markers (kDa)
							172		79			62.4		36			24.5			19				13.5		5.3			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	**Proteins (MW, kDa)**
29	0	9	1	4	11	12	22	9	2	0	0	1	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	Fibronectin precursor (273)
20	0	0	0	0	0	0	1	1	3	0	2	0	0	3	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	XP_346863 (186)
7	0	0	0	1	3	0	13	3	0	0	2	0	0	0	0	7	4	0	0	0	0	0	0	0	0	0	0	0	0	Alpha-1-macroglobulin (163)
5	0	3	0	1	3	1	5	5	1	7	6	10	11	4	1	5	1	1	0	0	1	0	0	0	2	0	0	0	0	CC1-8 (107)
0	0	0	0	0	0	0	2	1	1	0	2	21	18	5	2	5	4	1	0	1	3	4	3	1	2	1	0	1	1	Alpha-1-antitrypsin (46)
1	0	1	0	1	1	0	1	1	0	1	0	1	5	3	5	6	1	0	0	0	0	1	0	0	1	0	0	0	0	Cytokeratin-8 (54)
1	0	0	0	0	0	0	17	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	alpha-1 prot. inh. 3 (165)
0	0	0	0	0	0	0	0	0	0	0	0	9	13	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ser. Prot. inhibitor 3 (45)
0	0	0	0	0	0	0	0	0	0	1	5	6	3	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	T-kininogen II prec. (48)
0	0	0	0	0	1	0	0	1	0	3	9	6	4	3	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	Hemopexin precursor (51)
0	0	0	0	0	0	0	0	2	0	0	1	0	3	1	1	4	10	4	1	2	1	1	0	1	6	1	0	0	0	Apolipoprotein E (36)

Figure 4b: Distribution of peptides across slices 1-30. The blue squares indicate the slices where peptides were found. The red squares highlight the regions where peptides would be expected to be found based on the molecular weight of the proteins.

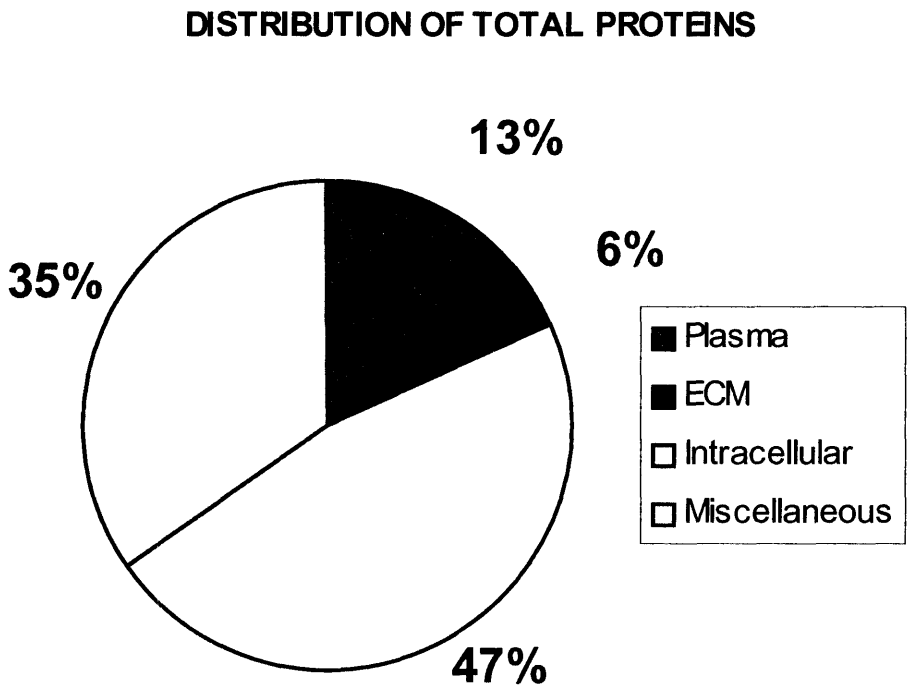

Figure 5a: Distribution of identified proteins in conditioned medium. 47% of the proteins were intracellular, 35% were miscellaneous, 13% were plasma proteins and 6% were extracellular matrix (ECM) proteins.

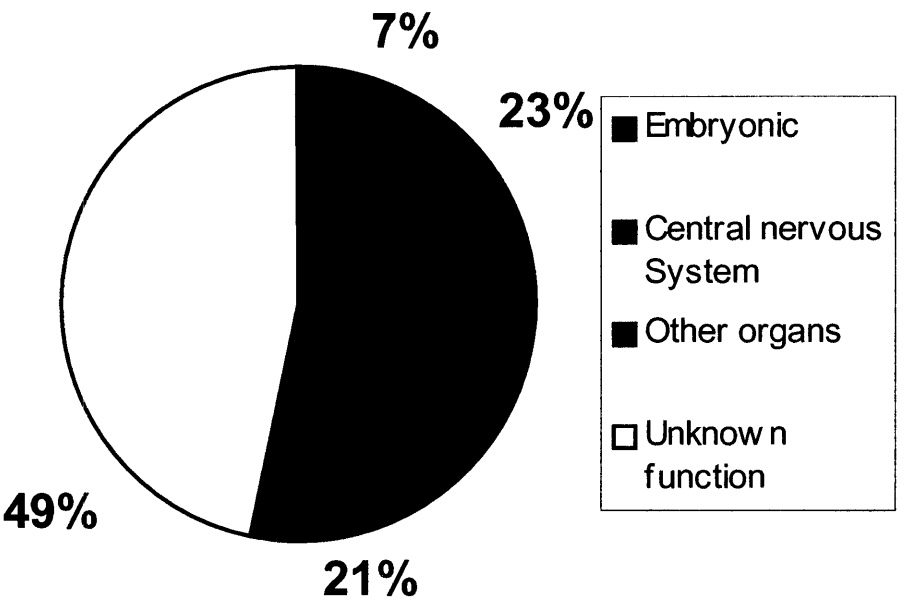

Figure 5b: Distribution of miscellaneous proteins. 49% of the proteins have unknown functions, 23% are considered to be central nervous system (CNS) proteins, 22% are from organs other than the liver and the CNS and 7% are embryonic proteins.

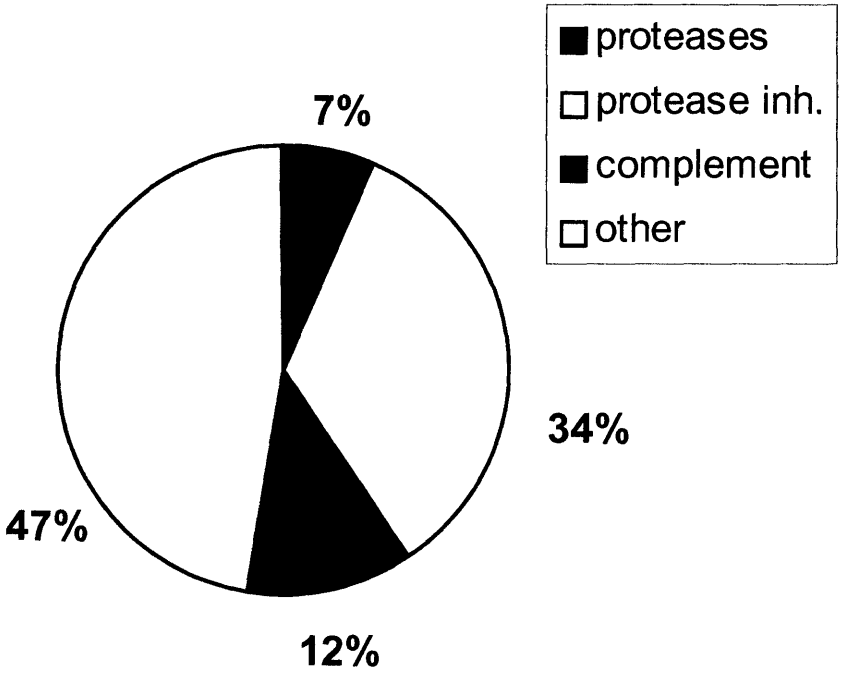

Figure 5c: Distribution of plasma proteins in conditioned medium. 20 proteins(34%) were protease inhibitors, 7 (12%) were from the complement cascade, 4 (7%) were proteases and the remaining 28 proteins (47%) included metal-binding proteins, apolipoproteins, fibrinogen and hemoglobin.

- **Cytoplasmic: 111**
- **Mitochondrial:11**
- **Nuclear/rib: 37**
- **Microsomal: 12**
- **Lysosomal: 9**
- **Golgi: 3**
- **Membrane: 54**

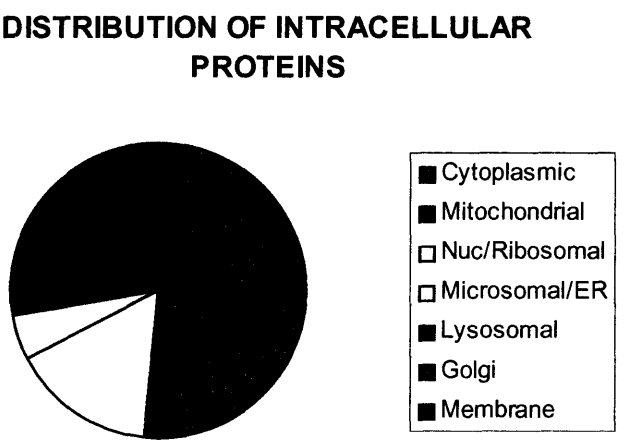

Figure 5d: Distribution of intracellular proteins across the various organelles

Structural matrix proteins	**Membrane Proteoglycans**	**Cell-matrix adhesion proteins**	**Other**
Fibronectin precursor	Agrin precursor (HSPG)	AC2-120	Alpha-amylase
Laminin type 12	Syndecan-4 Precursor (HSPG)	Dystroglycan	MMP-27
Collagen Types (I, IV,V,VI,XVIII)	Chondroitin sulfate proteoglycan, NG2	Talin	Thrombo-spondin -1

Table 1: Categorization of extracellular matrix-associated proteins

Figure 6: Silver-stained gel of conditioned medium (50 µl, day 8). Lane 1: marker; lane 2: control cells; lane 3: cells treated with aflatoxin; lane 4: medium incubated with collagen.

(Experiment performed by Saraswathi Mandapati)

Chapter 4: Summary and conclusions

In this thesis we developed a collagen sandwich bioreactor for studying hepatotoxicity by drugs and chemicals. A collagen sandwich is made by plating primary rat hepatocytes on collagen-coated dishes and overlaying the cells with another layer of collagen a day after plating. As we learned from previous work by others, the collagen sandwich maintains morphology and many phase I and phase II enzymes in hepatocytes. In spite of the extensive literature on the maintenance of drug metabolism in collagen sandwiches, there are very few studies regarding the use of this system to study toxicity.

Our first goal was to optimize the system parameters, particularly the cell density. Thus, we measured urea, albumin and P4501A activity at different cell densities. P4501A activity reached a maximum around 40,000 cells/cm^2, urea increased with increasing density, and the secretion of albumin reached a maximum around 45,000 cells/cm^2. In light of these results we decided to plate our cultures at 50,000 cells/cm^2. We also examined whether epidermal growth factor (EGF) should be added to our medium. It has been shown that EGF promotes regeneration but it also down-regulates the expression of some cytochorme P450's. We found that in the absence of EGF both urea and albumin secretion decreased very rapidly. Therefore, we decided to include EGF, even though it might compromise some metabolic functions.

The next task was to adapt toxicity assays to our system. Aflatoxin B1 was our model compound for protocol development, because we could detect its toxicity with a variety of assays including urea and albumin secretion, release of lactate dehydrogenase and the reduction of XTT and Alamar Blue. We determined the LD_{50} of aflatoxin B1 to be 32 nM, and this dose was our positive control for toxicity experiments.

The other toxins we tested we acetaminophen, carbon tetrachloride, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methyl methane sulfonate (MMS), cadmium, dimethyl formamide (DMF), and vinyl acetate. We found that the cells were sensitive to MMS, MNNG and cadmium. However, the cells were resistant to 1 mM doses of acetaminophen, DMF and vinyl acetate, as well as a 5mM dose of carbon tetrachloride. MMS, MNNG and cadmium are direct-acting compounds, which require no metabolism to be toxic, thus we expected the cells to experience toxicity. On the other hand, DMF, carbon tetrachloride and acetaminophen require CYP450 metabolism for activation. Vinyl acetate also needs to be metabolized by carboxylesterase to form acetaldehyde, which binds to proteins and DNA. Our Western Blots showed that CYP's 1A, 2B, 3A are maintained for seven days in culture, but CYP2E1 is lost gradually over time. Interestingly, CYP2E1 is also the primary metabolic enzyme for DMF, carbon tetrachloride and acetaminophen, suggesting that lack of toxicity is due to a decrease in this enzyme. Lack of toxicity by vinyl acetate suggests that carboxylesterase is also lost in culture.

The metabolism of acetaminophen was examined in more detail with liquid chromatography and mass spectrometry. Liquid chromatography showed that most of the parent compound was converted to the sulfate and glucuronide metabolites. We also wanted to examine whether the glutathione adduct was produced, since this metabolite is used as a marker for the formation of the toxic benzoquinoneimmine (NABQI) adduct. We synthesized the glutathione adduct and determined its retention time on liquid chromatography and the fragmentation pattern with mass spectrometry. After isolating the fraction with the same retention time from the medium of acetaminophen-treated

cells, we examined it with mass spectrometry. We were able to locate a peak with the same mass to charge ratio and the same fragmentation pattern as the glutathione-adduct standard. This data suggests that the cells do make the toxic NABQI adduct, but they do not experience toxicity, probably because it is detoxified before it causes significant damage. The other possibility is that there is toxicity, but it cannot be detected with our methods, which included measuring the secretions of urea and albumin and the release of lactate dehydrogenase.

In summary, we have shown that the collagen sandwich is a suitable system for hepatotoxicity studies. The hepatocytes maintain urea and albumin secretion as well as P4501A for at least two weeks. The cells were sensitive to four different toxins, but were immune to four other toxins, three of which were substrates for CYP2E1. We have also shown a gradual loss of CYP2E1 over time in culture, suggesting that the collagen sandwich might not be a good system for examining toxicity of compounds, which are substrates of 2E1.

Analysis of conditioned medium from aflatoxin-treated cells revealed that the secretion of two acute-phase proteins, α1-antitrypsin and α2-macroglobulin were down-regulated by aflatoxin B1. This experiment motivated a global investigation of the proteins in conditioned medium. One of the major challenges of this investigation was that albumin gave a very high background, complicating the identification of less abundant proteins. We were able to deplete most of the albumin with immuno-affinity columns consisting of anti-albumin antibodies bound to protein-A agarose beads. Following one-dimensional SDS-PAGE of albumin-depleted medium we cut the lane into 30 slices and performed in-gel digestion with trypsin on each slice. Mass spectrometry

analysis with Qstar LC-MS/MS revealed the presence of 493 proteins in the medium. Of those 493 proteins, 59 were plasma proteins, 234 were intracellular proteins and 21 were extracellular matrix proteins. The other 179 proteins were classified as miscellaneous, and they included 13 embryonic proteins, 40 central system proteins, 42 proteins from organs other than the liver and the central nervous system, and the remaining 84 proteins had unknown functions.

A more detailed analysis of the proteins suggested that the hepatocytes were in a stressed state. Our plasma proteins included acid-1-glycoprotein, α1-antitrypsin, α2-macroglobulin, hepatocyte growth factor activator and connective tissue growth factor, which are produced in the body during the acute-phase. We also identified laminin-12, fibronectin precursor and five types of collagen, showing that the cells in the sandwich produce the necessary structural matrix proteins. The extracellular matrix proteins also contained inflammation-specific proteins such as thrombospondin-1 and dystroglycan. Some proteins, such as the chondroitin sulfate proteoglycan NG2, have only been identified in immature cells, suggesting that we either identified a novel adult liver protein or that our culture contains stem cells. Furthemore, a number of the proteins have only been identified previously in stellate cells and endothelial cells. For example, reelin was previously considered a central nervous system protein, but it has been shown recently that stellate cells produce this protein during inflammation. We also identified syndecan-4, a heparin sulfate proteoglycan, which has only been identified on the surface of endothelial cells. Thus, the proteomics approach has revealed the presence of nonparenchymal cells in the culture. In addition, we have shown that hepatocytes in the

collagen sandwich secrete many matrix proteins, which has been hypothesized before, but it has been not explicity shown.

In summary, the collagen sandwich is a good system for proteomics studies. The medium is protein-rich and contains plasma, intracellular and extracellular matrix proteins from both parenchymal and nonparenchymal cells. The proteomics approach also has the potential to study differential protein expression of toxin-treated and control cells, which can be used to understand mechanisms of toxicity.

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Appendix I: Detailed Protocols

Protocol for Making Collagen Sandwiches for Hepatocytes

Ingridients of the collagen gel solution:

1. 80% 2 mg/ml collagen solution dissolved in 0.01 N HCl (3mg/ml in 0.01 N HCl is commercially available from Cohesion Technologies tel: (877) 264-3746; we make the further dilution of the collagen ourselves)
2. 10% water
3. 10 % Dora's special 10 x PBS (see ingredients below)
4. 20 µl of 1 N HCl per 5 ml of collagen solution

For one 24-well plate mix:
1.7 ml of 3mg/ml commercial collagen solution
1.2 ml of water
325 µl of Dora's 10x PBS
9µl of 1N HCl

The pH of the gel solution should be physiological and it should be measured with pH paper every time a gel is prepared. The gel solution can be stored for 4-6 hours in 4^{o} C.

One difficulty, which can arise with collagen sandwiches, is that the culture does not have medium during the time the second layer is gelling. To circumvent this problem, glucose is included in the special PBS solution, such that the concentration of glucose in the gel solution is exactly the same as the concentration of glucose in the medium (2g/L). A second problem is that while the collagen is gelling the pH of the gel solution might change. We avoid uncertainty in the pH by pre-equilibrate the PBS buffer with the atmosphere of the incubator. For added buffering, we add sodium bicarbonate to the gel solution such that the concentration in the gel will be the same as the concentration in the medium (3.7 g/L).

Dora's 10 x PBS:
10x PBS (commercial)
Dissolve glucose and bicarbonate in the 10x PBS such that the concentrations will be:
20 g/L glucose
37 g/L sodium bicarbonate

To equilibrate with the incubator put the PBS in a tissue culture plate and leave it in the incubator for a minimum of half an hour before making the gel solution. This only needs to be the done before the first use of the PBS. Afterwards, the PBS can be stored at room temperature without need for further equilibration.

Procedure for preparing the collagen sandwich in 24-well plates:

First layer:

1. Pipette 110 µl of collagen solution on the bottom of each well. For better spreading, pipette the solution into the middle of the well. Tap the bottom of the plate gently against the surface in the hood and rotate the plate so the solution can spread.
2. Put plates in the incubator and allow a minimum of 1 hr for gelation. (Can be prepared the night before)

Seeding cells:

1. Spread 90,000-100,000 cells evenly in 300µl of medium over the plate.
2. Leave cells in the incubator overnight to allow proper attachment of the cells to the collagen.

Second layer:

1. Aspirate off medium
2. Add 55 µl of gel solution and gently tilt the plate to assure even spreading.
3. Leave in the incubator for 1 hour, by which time the solution should have gelled completely.
4. Add 300µL medium to the cells and put back in the incubator for another night to give cells enough time to attach to the second layer. Measurements can already be performed the following day.

To make the gel solutions for plates of other sizes, scale the amount of collagen down proportional to the plate surface area.

For reference:

Plate size	Area	Bottom layer of collagen	Top layer of collagen
35 mm or 6-well	9.61 cm2	600 µl	300 µl
24-well	1.76 cm2	110 µl	55 µl
96-well	0.32 cm2	20 µl	10 µl

HGM (Hepatocyte Growth Medium) Preparation

Reference: Block et al., J Cell Biol. (1996) 132(6): 1133-1149

Updated with the correction concentrations for niacinamide, glutamine, $ZnCl_2$, $ZnSO_4 * 7H_2O$, $CuSO_4$

Base Medium:

DMEM, low glucose, pyridoxine HCL, sodium pyruvate, no glutamine, no phenol red; GIBCO catalog #11054-020 (500 mL) – *store in refridgerator*

Add to Base Medium:

1. **0.015 g L-Proline**; 0.03 g/L in medium; Sigma catalog #P-4655
2. **0.05 g L-Ornithine**; 0.1 g/L in medium; Sigma catalog #O-6503
3. **0.305 g Niacinimide**; 0.305 g/L in medium; Sigma catalog #N-0636
4. **0.5 g D-(+)-Glucose**; 2 g/L in medium (already had 1 g/L); Sigma catalog #G-7021
5. **1 g D-(+)-Galactose**; 2 g/L in medium; Sigma catalog #G-5388
6. **500 µL Trace Metals Solution – $ZnCl_2$, $ZnSO_4 * 7H_2O$, $CuSO_4 * 5H_2O$, $MNSO_4$**; 0.054 mg/L, 0.075 mg/L, 0.020 mg/L, 0.025 mg/L in medium; (*dissolve into 50 mL PBS – 0.0272 g $ZnCl_2$, 0.0375 g $ZnSO_4 * 7H_2O$, 0.01 g $CuSO_4 * 5H_2O$, 0.00125 g $MNSO_4$*)

!!!!!! Sterile Filter Medium After Step 6 !!!!!!!

Add to Sterile Filtered Medium:

7. **5 mL Penicillin/Streptomycin (sterile)**; Sigma catalog #P-0781; (*dispense stock into 5.1 mL aliquots, store at –20C)*
8. **2.5 mL L-Glutamine (sterile)**; 1.0 mM in medium; GIBCO catalog #25030-081 (100 mL); (*dispense 200 mM stock into 12.6 mL aliquots, store at –20C*)
9. **500 µL Insulin-Transferrin-Sodium Selenite (sterile)**; 5 mg/L – 5 mg/L – 5 µg/L in medium; Roche catalog #1074 547 (50 mg); 1213 849 (250 mg); (*dissolve 50 mg or 250 mg powder in 5 mL or 25 mL sterile MilliQ water, dispense into 505µL aliquots, store at –20C)*
10. **400 µL Dexamethasone (sterile)**; 0.1 µM in medium; Sigma catalog #D-8893; (*dissolve 1 mg in 1 mL EtOH using sterile syringe and needle; after powder is dissolved, add 19 mL PBS, mix thoroughly, and dispense into 405 µL aliquots; store at –20C, expires 3 months after date of reconstitution*)
11. **200 µL Epidermal Growth Factor (EGF) (sterile)**; 20 ng/mL in medium; Becton Dickinson #354010 (800-343-2035); (*dissolve 100 µg powder in 2 mL sterile*

MilliQ water, dispense into 205 μL aliquots, store ay –20C, expires 3 months from date of reconstitution)

Assay for Measuring Urea

Materials:

Urease (Sigma # 640-5), Phenol Nitroprusside Solution (Sigma # 640-1), Alkaline Hypochlorite Solution (Sigma #640-3) and Urea Nitrogen Standard Solution (Sigma # 535-30)
HGM medium (all the experiments below were performed with albumin-free HGM)
Spectramax 96 well plate reader, 96-well plates, multi-channel pipettor

Protocol:

1) Pipetting the samples

Pipette 15 μl of each sample into a well on a 96 well plate. Leave two columns empty for the standards.

2) Preparation of Standards

Dilute Urea Standard to 0.05 mg/ml and make six serial two-fold dilutions of it. In row A, pipette the blank, water, and and then pipette the standards in the rows below going from least to most concentrated. Pipette an equal volume of standards as samples.

3) Coverting Urea to Indophenol

Add 40 μl of urease to each well with a multipipettor and mix well by pipetting up and down three times. Change tips before every new row. Leave the plate in the 37 C box for 15 minutes. Then add (in this order) 80 μl of phenol nitroprusside and 80 μl of alkaline hypochlorite to each well and pipette up and down 3 times after adding each solution. Leave the plate at room temperature for 20 minutes, and then read the plate at 570nm. Indophenol only keeps its blue color for one-two hours.

Measuring Albumin in Collagen Gel Sandwiches

Materials:

Sheep IgG fraction to rat albumin ("unconjugated") [ICN cat #55729]
Horseradish peroxidase conjugated goat to rat albumin antibody [Accurate chemical Cat #YNGRAALBP]
Rat albumin, purified [ICN Cat# 55952]
Nunc Immunosorp 96-well plates (from VWR)

Solutions/Buffers:

Wash Buffer: PBS-Tween (2L)

200 ml 10xPBS
1800 ml milliq water
1 ml tween-20
adjust pH to be between 7.35 and 7.5

Bicarbonate buffer – pH 9.6

Name	MW	Conc. needed	Amount in 500 ml
Na_2CO_3 Sodium Carbonate, anhydrous	106	15.0 mM	0.795 grams
$NaHCO_3$ Sodium Bicarbonate	84	35.0 mM	1.470 grams
NaN_3 Sodium Azide	65	30.8 mM	1.000 gram

Substrate Buffer – pH 5.0

Name	MW	Conc. Needed	Amount in 500 ml
$C_6H_8O_7$-H_2O Citric Acid, monohydrate	210.1	61 mM	6.4 grams
$NaHPO_4$ Sodium Phosphate, dibasic (Disodium Phosphate)	142	90.8	5.54 grams

Stop Solution

Name	MW	Conc. Needed	Amount in 500 ml
NaF Sodium Fluoride	41.99	76.21	1.6 grams

Procedure:

1. **Coating plate with unconjugated antibody**:
    a. For each 96-well plate, dissolve 100 µl of unconjugated antibody in 11 ml of bicarbonate buffer.
    b. Pipette 100 µl into each well. Incubate at 37 C for 1 hour (or overnight at 4°C)
    c. Wash each well three times with PBS –tween.

2. **Blocking**:
    a. Pipette 200 µl of 1% gelatin (dissolved in PBS-tween) into each well.
    b. Incubate at 37C for 1 hr.
    c. Wash three times with PBS tween.
    d. Fill rows B-H with 100 µl of PBS-tween, and row A with 195 µl PBS-Tween.

3. **Preparing standards**: The "standard" aliquots in the freezer have to be diluted 7500-fold with PBS-tween before loading them into the plate in step 4. (I usually do the dilution in two steps: first 15-fold, then 500-fold)

4. **Loading samples**:
    a. Load 5 µl of each sample/standard into row A. The template in the plate reader is set up so that the standards should be loaded into columns 1 and 12 and the samples into columns 2-11.
    b. After loading standards and samples mix row A well, and pipette 100 µl from row A into row B.
    c. Change tips. Mix well and pipette 100 µl into row C.
    d. Continue procedure until 100 µl has been added to row G. Change tips, and take out 100 µl from row G and dispose of it.
    e. Leave Row H blank. Incubate for 1 hour at 37C.
    f. Wash three times with PBS.

    Note: This assay is very sensitive, and therefore most samples will have to be diluted (10-fold for the controls) before loading 5 µl into row A.

5. **Conjugated antibody**:
    a. Add 15 µl of conjugated antibody to 11 ml of PBS-tween (enough for 1 plate).
    b. Pipette 100 µl into each well. Incubate at 37C for 1 hour. Wash three times with PBS-tween.

6. **Substrate**:
    a. Dissolve 1 ABTS tablet into 40 ml of substrate buffer. Protect the solution from light with aluminum foil.

b. Right before using it, add 13.2 µl of hydrogen peroxide.
c. Add 100 µl of the solution to every well and incubate at room temp for 45 min.
d. While incubating keep the plates covered with aluminum foil to protect them from light.
e. Do not wash the plates after this step. Add 100 µl of stop solution to every well and read at 405 nm.

EROD (Ethoxyresorufin O-deethylase) assay

Materials:

Ethoxyresorufin (R-352) and Benzyloxyresorufin (R-441) and the Resorufin standard (R363) can be ordered from Molecular Probes: 1-800-438-2209. Dicumarol (M-1390) is from Sigma : 1-800-325-3010.

Protocol:

1) Preparing and storing stock solutions

Both ethoxyresorufin and benzyloxyresorufin are dissolved in DMSO to a final concentration of 1-5mM. The exact concentration can be checked by diluting the stock solution to about 10 µM in methanol and measuring the absorbance at 464nm. The extinction coefficient is 23,000. The stock solution should be sterile filtered and aliquoted out in 50-100µL portions and stored at 80°C. Before each assay the concentration of the stock should be measured. Ethoxyresorufin is not very soluble and some of the aliquots are more concentrated than the others.
Dicumarol is dissolved in 100mM NaOH to a concentration of 10 mM and stored in the fridge. It does not need to be aliquoted.

2) Preparation of the substrate solution

Right before the assay, dilute ethoxyresorufin/benzyloxyresorufin into medium to a final concentration of 30µM. Dilute dicumarol into the medium to a final concentration of 10µM. Make an extra 5-10ml of this solution for the standards.

3) Addition of substrate solution to cells

In a 24-well plate there are 100,000 cells in each well with 300 µL of medium. For the EROD/BROD assays, remove the medium and add 300 µL of the above solution and incubate for 1 hr in the incubator.

4) Preparation of standards

During this hour make up the standards. The resorufin stock solution is in DMF at 200µM (wrapped in aluminum foil). Check the concentration of the stock by diluting it into a solution with pH 9 (e.g. sodium borate) and measuring the absorbance at 464 nm. The extinction coefficient is 54,000. Dilute the stock to 200nM in the substrate solution. Do two-fold serial dilutions in the substrate solution until the concentration of resorufin

reaches 3nM. Prepare 500µL of each standard. The blank is the substrate solution made up for the EROD/BROD assay .

On a black 96-well plate:

Pipette 200µl of the blank into columns A and B in row 1. Pipette 200µL of the 3nM standards into columns A and B in row 2. Continue pipetting the standards in duplicates into rows 3-12 from least to most concentrated.

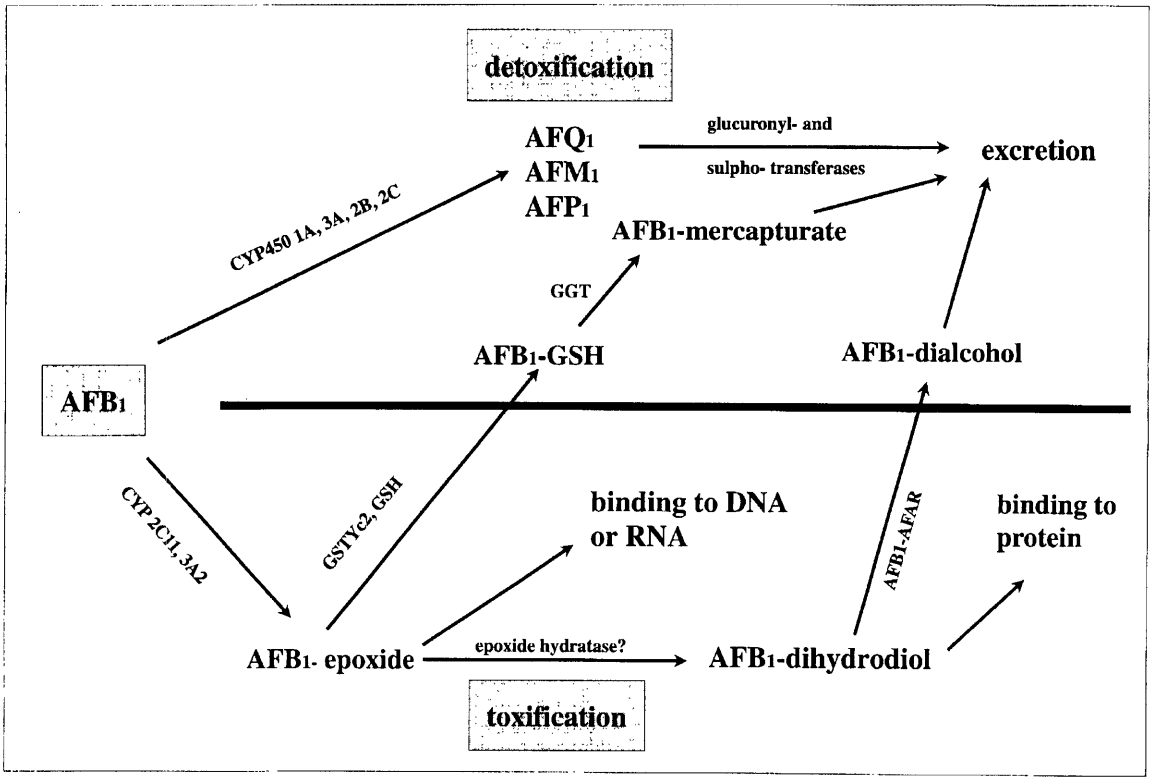
5) Collection of samples and reading the plate

When the 1 hr. incubation is over, pipette 200 µL from each well into the 96-well plate. The cells can be reused after the EROD/BROD assay by removing the remaining substrate solution and replacing it with medium.
The 96-well plate is then read on a fluorimeter with excitation wavelength of 555nm and emission of 600nm.

Phalloidin (and/or DAPI) staining of hepatocytes

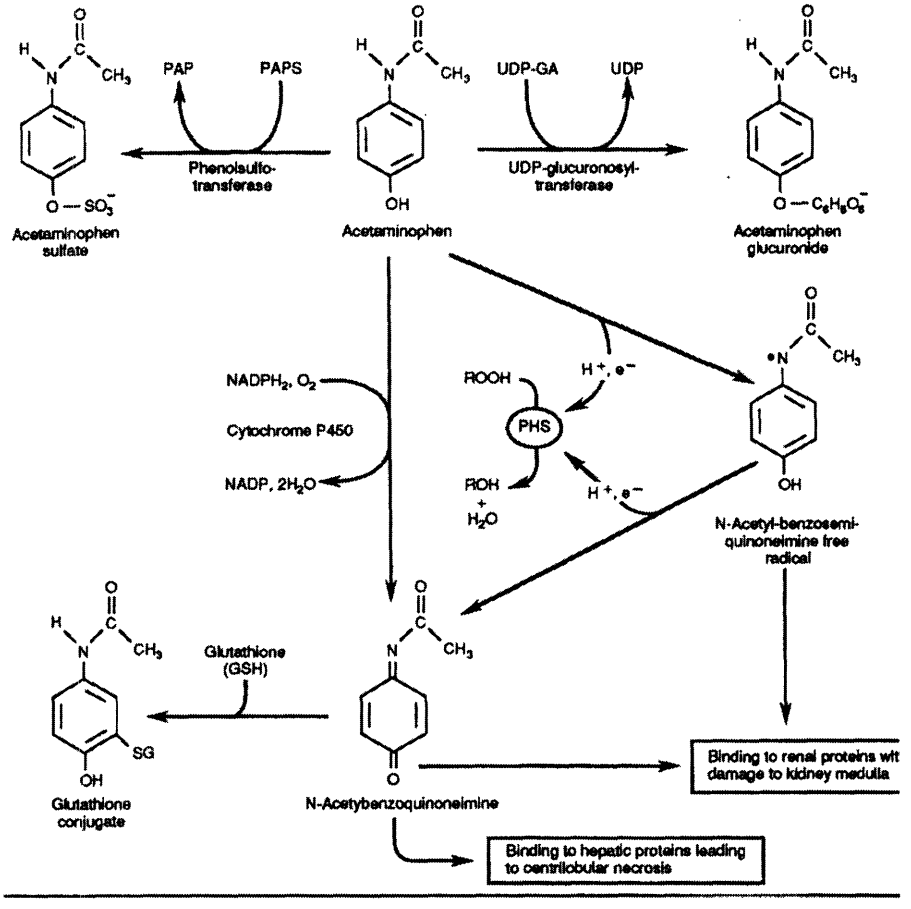
Washing: Wash cells with pre-warmed PBS
Fixing: Fix cells for 1 hour in 2% paraformaldehyde in 0.1 M phosphate buffer at room temperature. Cells can be held at 0.5% paraformaldehyde
Wash: Rinse cells twice with Hanks buffer (30 min/wash)
Permeabilizing: 0.1% Triton X-100 for 5 minutes
Blocking: Incubate for 20 minutes with 1% BSA in Hanks Buffer
Staining: Add 2.5 ul of stain to 100 ul of 1% BSA in Hanks buffer and incubate for 20 minutes (cover with aluminum).
Washing: Wash three times with Hanks buffer
DAPI: If we are also doing DAPI stain, add DAPI after the phalloidin has been washed off for 5 minutes
Washing: Wash three times with Hanks buffer

Appendix II: Detailed Metabolic pathways for aflatoxin B1 and acetaminophen



Detailed metabolism of aflatoxin B1

Reference: Manson *et al.* Carcinogenesis vol 18 (9) pp.1729-1738, 1997



Detailed metabolism of acetaminophen in the liver

Reference: Casarett & Doull's Toxicology, Curtis Klaassen, editor. page 133. McGraw-Hill, New York, 1996.

Appendix III: List of proteins identified by the proteomics approach

APPENDIX IIIa: Complete list of proteins identified by proteomics analysis

SECRETED

Comments	#pep	score	MW	Protein name
Plasma proteins				
transport protein	3	36.7	69335.8	Afamin precursor (Alpha-albumin) (Alpha-Alb)
protease inhibitor	12	176.6	165327.1	alpha-1 proteinase inhibitor III, variant 1 precursor
synthesized during acute phase	3	58.7	23575.3	ALPHA-1-ACID GLYCOPROTEIN PRECURSOR (OROSOMUCOID)
protease inhibitor	13	212.8	46136.1	ALPHA-1-ANTIPROTEINASE PRECURSOR (ALPHA-1-ANTITRYPSIN)
protease inhibitor	17	271.9	167160.1	alpha-1-macroglobulin precursor
serine protease inhibitor	7	102.6	46465.3	alpha-2 antiplasmin
protease inhibitor	1	15.78	163702	Alpha-2-macroglobulin precursor (Alpha-2-M)
alpha-1-microglobulin	4	66.43	38851.3	AMBP prot Inter-alpha Trypstatin]
protease inhibitor	4	60.4	30062.3	apolipoprotein A-I precursor
apolipoprotein precursor	6	82.85	44465.6	apolipoprotein A-IV precursor
Transport protein	1	9.48	21635	Apolipoprotein D precursor (ApoD)
apolipoprotein	8	140	35763.7	Apolipoprotein E
protease with trypsin activity	6	92.1	42475.1	Ba1-647
beta chain of MHC	1	17.9	13720	Beta-2-microglobulin precursor
Iron binding	17	269.4	107412.3	Cc1-8
copper binding	1	15.53	120841.3	Ceruloplasmin precursor (Ferroxidase)
glycoprotein, associated with apo	8	135.9	51464.7	Clusterin
in complement cascade	3	43.62	77701.5	Complement component 1, s subcomponent
in complement cascade	1	9.56	134448.5	complement component 5
trypsin activity	6	78.98	83810.8	complement component C2
in complement cascade	1	10.98	67298.1	Complement factor I precursor (C3B/C4B inactivator)
secreted by endothelial cells	3	42.84	37837.5	connective tissue growth factor
belongs to serpin family	1	16.23	52376.8	Dipeptidyl-peptidase I precursor (DPP-I) (DPPI) (Cathepsin C) (Cathepsin J)
present after inflammation	4	54.01	60490.5	fibrinogen alpha subunit
present after inflammation	5	63.41	54303.6	Fibrinogen beta chain precursor [Contains: Fibrinopeptide B]
present after inflammation	4	63.16	50632.9	Fibrinogen gamma chain precursor
can also be lysosomal	1	16.41	35830.2	Gamma-glutamyl hydrolase precursor (Gamma-Glu-X carboxypeptidase)
hemoglobin	1	11.61	15328.6	Hemoglobin alpha-1 and alpha-2 chains
hemoglobin chain	1	13.61	15992.5	hemoglobin beta chain, minor

serum glycoprotein	11	150.9	51291.1	Hemopexin precursor	
protease inhibitor	1	15.23	54552.1	Heparin cofactor II precursor (HC-II) (Protease inhibitor leuserpin 2)	
peptidase family	1	15.55	74199.7	hepatocyte growth factor activator	
transport protein	4	54.36	59049.3	histidine-rich glycoprotein 1	
binds insulin	1	15.75	29628	insulin-like growth factor-binding protein 1 precursor	
carrier of hyaluronan in serum	4	52.72	103607.8	inter-alpha-inhibitor H4P heavy chain	
protease inhibitor	4	53.09	99098.1	Inter-alpha-trypsin inhibitor heavy chain H3 precursor (ITI heavy chain H3)	
serpin family	3	51.56	48021.3	kallistatin	
probably from endothelial cells	1	11.88	130648.8	nitric-oxide synthase (EC 1.14.13.39)	
belongs to serpin family	1	15.34	90536.3	plasminogen protein	
protease inhibitor	2	32.38	84798.7	Polymeric-immunoglobulin receptor precursor (Poly-Ig receptor) (PIGR)	
protease inhibitor	4	57.58	50286.4	Protein Z-dependent protease inhibitor precursor (RASP-1)	
transport of selenium	1	11.18	41583	selenoprotein P	
serine protease inhibitor	7	104.2	55611.6	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1	
protease inhibitor	9	154.8	45554.6	serine proteinase inhibitor 3	
albumin precursor	9	125.6	68719.2	Serum albumin precursor [Contains: Neurotensin-related peptide (NRP)]	
similar to lipocalin	1	15.43	22399.9	similar to 1700013L23Rik protein	
serine-type endopeptidase inhibit	5	75.85	97227.9	similar to Ac2-248	
protease inhibitor	3	42.73	52058.9	similar to alpha-2-antiplasmin	
apolipoprotein	5	81.06	44143.4	similar to beta-2-glycoprotein I	
in complement cascade	2	25.05	87848.9	similar to complement component 1, r subcomponent	
in complement cascade	1	20.61	62093.2	similar to complement component 8 alpha subunit	
immunoglobulin heavy chain vari	1	10.57	25138.7	similar to immunoglobulin heavy chain variable region	
neutrophil chemotactic activity	1	13.09	20890.1	similar to LECT2	
binds insulin-like growth factor	2	27.33	30349.3	similar to mac25	
belongs to serpin family	1	17.25	42301.8	similar to SPI6	
protease inhibitor	11	154.4	47524.2	T-kininogen II precursor (Major acute phase protein) (Alpha-1-MAP)	
Thyroid hormone binding protein	2	35.93	15747.9	transthyreti Transthyretin (prealbumin, amyloidosis type I)	
serum transport protein	8	124	53544.9	Vitamin D-binding protein precursor (DBP) (Group-specific component)	
stimulates lipid dregradation	2	28.6	33712.3	ZN-alpha-2-glycoprotein	
Transport protein	6	74	Transcobalamin II precursor (TCII) (TC II)		plasma

CYTOPLASMIC

intracellular	1	18.55	14303.5	14.5 kDa translational inhibitor protein (Perchloric acid soluble protein)
intracellular	4	48.86	45112.5	4-hydroxyphenylpyruvate dioxygenase
intracellular	1	11.07	62600	5'-AMP-activated protein kinase,catalytic alpha-1 chain(AMPK alpha-1 chain)
Also mitochondrial nuclear	1	18.71	163792.9	Ab2-450 With a
intracellular	5	70.55	41751	actin beta
intracellular	1	10.71	86454.3	actin-filament binding protein Frabin
intracellular	10	138.6	104915.6	Actn4 protein
intracellular	1	17.92	47538.3	Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase)
intracellular	1	9.24	185854.9	adenylate cyclase (EC 4.6.1.1), cytosolic
intracellular	1	12.18	36747.3	Aflatoxin B1 aldehyde reductase member 1 (AFB1-AR) (rAFAR1)
intracellular	1	12.1	75938.8	A-kinase anchoring protein AKAP150
intracellular	2	29.9	36506.1	Alcohol dehydrogenase [NADP+] (Aldehyde reductase)
intracellular	1	13.91	54459	Aldehyde dehydrogenase 1A1 (Aldehyde dehydrogenase, cytosolic)
intracellular	1	9.9	8447.5	alkyl-dihydroxyacetonephosphate synthase
intracellular	3	36.28	34973.3	Arginase 1 (Liver-type arginase)
intracellular	1	15.29	51391.1	argininosuccinate lyase
Can also be nuclear	2	23.22	46428	aspartate transaminase (EC 2.6.1.1), cytosolic
can play role in cell proliferation	1	10.45	32969.3	BCNT
intracellular	1	19.93	44976.7	Betaine--homocysteine S-methyltransferase
intracellular	1	10.09	61259.9	Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1B
probably nuclear	1	9.65	41638.7	Calcium/calmodulin-dependent protein kinase type I (CAM kinase I)
intracellular	2	35.05	29431.6	Carbonic anhydrase III (Carbonate dehydratase III) (CA-III)
intracellular	1	15.1	22802.5	cyclophilin B
intracellular	1	10.16	2736.2	cysteine proteinase homolog (clone PCR33)
intracellular	11	183.7	54035.1	cytokeratin-8
cytoskeletal protein	1	14.48	36483.3	cytosolic malate dehydrogenase
chaperone	3	45.79	15578.3	dismutase
intracellular	1	14.82	95284.6	Elongation factor 2 (EF-2)
intracellular	2	26.39	54174.2	EZRIN
involved in electron transport	1	9.99	37196.7	glucokinase-associated dual specificity phosphatase
intracellular	1	11.16	59376	Glucose-6-phosphate 1-dehydrogenase (G6PD)
intracellular	2	30.24	25988.3	glutathione S-transferase alpha
intracellular	2	28.96	25914.1	Glutathione S-transferase Yb-1 (Chain 3) (GST Yb1) (GST M1-1)
intracellular	1	19.12	52344.8	Glutathione synthetase (Glutathione synthase) (GSH synthetase) (GSH-S)
intracellular	5	80.11	54589.8	hepatic multiple inositol polyphosphate phosphatase
intracellular	4	62.53	16960.1	keratin 18
intracellular	2	20.71	74324.1	Lamin A

intracellular	4	57.69	36450.7	L-lactate dehydrogenase A chain (LDH-A) (LDH muscle subunit) (LDH-M)
intracellular	1	12.95	18194.8	Low molecular weight phosphotyrosine protein phosphatase ACP1/ACP2
involved in calcium regulation	1	14.01	64286.9	M-phase inducer phosphatase 2 (Dual specificity phosphatase Cdc25B)
intracellular	1	12.55	184063.7	Munc13-2 protein
intracellular	1	11.5	17283.1	Nucleoside diphosphate kinase B (NDK B) (NDP kinase B) (P18)
intracellular	2	27.97	22109.5	Peroxiredoxin 1 (Thioredoxin peroxidase 2)
intracellular	1	9.69	86306.3	PHEX protein
intracellular	1	11.29	61403.5	Phosphoglucomutase (Glucose phosphomutase) (PGM)
anti-apoptotic protein	1	14.45	14987.3	profilin
intracellular	1	10.9	40314.2	Protein-tyrosine phosphatase, non-receptor type 7
intracellular	1	9.29	8193.9	protein-tyrosine-phosphatase
intracellular	1	10.42	72480.8	Rab proteins geranylgeranyltransferase component A 1 (Rab escort protein 1)
intracellular	2	39.57	33390	regucalcin
intracellular	2	27.47	49905.7	Ribonuclease inhibitor
intracellular	1	18.37	52532.4	selenium-binding protein
intracellular	1	15.24	38588.6	similar to 14-3-3 protein sigma
intracellular	1	11.94	65199.8	similar to 2410004L15Rik protein
intracellular	1	9.45	72904.9	similar to actin monomer-binding protein
intracellular	1	13.84	34500	similar to Aldo-keto reductase family 1 member E1
heat shock protein	1	9.58	27664.1	similar to Alpha enolase (2-phospho-D-glycerate hydro-lyase)
intracellular	1	12.26	187466.5	similar to ATP-binding cassette transporter sub-family A member 8a
intracellular	1	12.26	187466	similar to ATP-binding cassette transporter sub-family A member 8a
intracellular	1	11.28	487486.9	similar to axonemal dynein heavy chain 8 long form
intracellular	1	11.33	51106.5	similar to Carbohydrate kinase-like protein
intracellular	1	11.05	218239.8	similar to CCG1
intracellular	3	41.91	38710.6	similar to cytokeratin 13
intracellular	1	16.59	119180	similar to d PYK2 C terminus-associated protein
intracellular	1	9.55	15991.4	similar to DnaJ (Hsp40) homolog, subfamily D, member 1
involved in maintaining cytoskele	2	26.55	39915.5	similar to eukaryotic translation elongation factor 1 alpha 2
intracellular	1	10.07	31582.5	similar to FH1/FH2 domains-containing protein
intracellular	1	10.34	33251.1	similar to g glutaredoxin
also nuclear	1	11.67	25655.6	similar to group XIII secreted phospholipase A2
intracellular	3	43.66	71130.8	similar to Heat shock cognate 71 kDa protein
intracellular	1	9.11	124630.4	similar to HECT type E3 ubiquitin ligase
intracellular	1	9.81	542936	similar to Herc2
intracellular	4	60.18	64757	similar to Keratin, type II cytoskeletal 1
intracellular	1	15.28	60349.1	similar to Keratin, type II cytoskeletal 4 (Cytokeratin 4)

intracellular	1	13.3	149958.3	similar to kinesin-related protein KIF27A
intracellular	1	10.28	679812.8	similar to macrophin 1 isoform 4
plays role in B. and T cell develop	1	11	38668.5	similar to MAP-1 protein
intracellular	1	10.43	78521.7	similar to myotubularin
intracellular	1	12.91	31133.1	similar to NADP+-specific isocitrate dehydrogenase
intracellular	1	13.92	33738.5	similar to neurocalcin delta
intracellular	1	11.23	77595.3	similar to n pore membrane and/or filament interacting like protein 1
intracellular	1	9.64	20876.3	similar to Nspc1 protein
maybe nuclear	1	9.67	146112.1	similar to nuclear factor related to kappa B binding protein
most likely membrane protein	1	13.56	36093.5	similar to olfactory receptor MOR114-8
intracellular	1	11.12	65651.6	similar to Phenylalanyl-tRNA synthetase beta chain
intracellular	1	11.09	11000.5	similar to Phosphoglycerate mutase 1
intracellular	1	11.41	43292.5	similar to PROHIBITIN (B-CELL RECEPTOR ASSOCIATED PROTEIN 32)
probably cytoplasmic	1	9.43	176598.8	similar to protein kinase
intracellular	1	11.39	78818.7	similar to protein phosphatase 1, regulatory (inhibitor) subunit 15B
intracellular	1	11.24	44512.1	similar to putative E1-E2 ATPase
intracellular	1	9.81	120303.9	similar to putative protein kinase
intracellular	1	9.22	34895.7	similar to RAB5B, member RAS oncogene family
intracellular	2	20.82	344849.7	similar to Ran-binding protein 2
intracellular	1	17.8	67146.6	similar to F breast carcinoma-associated antigen BCAA
intracellular	1	9.56	39938.9	similar to RP2 protein, testosterone-regulated - ricefield mouse (Mus caroli)
intracellular	1	10.19	49092.8	similar to s semaphorin cytoplasmic domain-associated protein 3A
ATP-binding protein	1	14.06	59089.2	similar to Spindlin-like protein 2 (SPIN-2)
intracellular	1	14.06	70424.4	similar to TOB3
intracellular	1	9.21	141128.1	similar to Ubiquitin specific protease 25
most likely transcription regulator	1	14.23	89952.4	similar to uridindiphosphoglucosepyrophosphorylase 2
intracellular	1	10.86	63442.2	sulfhydryl oxidase
intracellular	1	11.33	97948.8	testosterone-regulated prominin-related protein
intracellular	1	16.09	37460.3	Transaldolase 1
intracellular	4	56.1	71187	transketolase
intracellular	1	11.94	104570.3	vp165
MITOCHONDRIAL				
	6	89.78	56353.8	ATP synthase beta chain, mitochondrial precursor
	3	43.15	61416.3	unnamed protein product /glutamate dehydrogena
	3	32.13	35655.7	Malate dehydrogenase, mitochondrial precursor
	1	19.53	39886.3	Ornithine carbamoyltransferase, mitochondrial precursor (OTCase)

	1	17.01	192249	hypothetical protein XP_346596
	1	16.47	28321.6	PRx III
	1	16.19	47314.5	Aspartate aminotransferase, mitochondrial precursor (Transaminase A)
	1	15.95	164580.8	Carbamoyl-phosphate synthase [ammonia], mitochondrial precursor
	1	15.81	47824.2	Acyl-CoA dehydrogenase
	1	11.7	54038.4	Dihydrolipoamide dehydrogenase
	1	9.36	51060.6	Import inner membrane translocase subunit TIM44, mitochondrial precursor

NUCLEAR/RIBOSOMAL

	3	43.37	50163.9	Alpha-tubulin
	1	13.48	30853.5	CArG-binding factor A
	1	10.2	43917.3	Class B basic helix-loop-helix protein 3
x	1	9.38	38175.4	deoxyribonuclease II
involved in nucleic acid binding	1	9.97	65631.6	FMRP isoform 18
	3	49.05	140231.8	hypothetical protein XP_346447
	1	10	77048.6	nucleolin
	1	9.76	53103	putative RNA methylase
	1	10.6	123247.6	replication factor C
	1	13.56	23758.5	similar to 60S ribosomal protein L10 (QM protein homolog)
	1	9.92	33754.5	similar to 60S ribosomal protein L10 (QM protein homolog)
	1	9.52	24986.4	similar to 60S ribosomal protein L19
	1	10.58	16681	similar to 60S RIBOSOMAL PROTEIN L29 (P23)
	1	10.17	100466.4	similar to Chromodomain-helicase-DNA-binding protein 1 (CHD-1)
	1	10	138375.9	similar to Chromosome-associated kinesin KIF4A (Chromokinesin)
	1	10.21	117381.8	similar to cysteine-rich protein NFX-1
	1	10.22	81819.6	similar to Dual-specificity tyrosine-phosphorylation regulated kinase 4
	1	9.91	16057	similar to C GLE1 (yeast homolog)-like, RNA export mediator
	1	11.89	40269.6	similar to h factor inhibiting HIF1
	1	9.71	145944.3	similar to Ifi204 protein
nuclear	1	10.27	17457.9	similar to K20D4
involved in transcription	1	9.54	220223.6	similar to KIAA1803 protein
	1	11.75	167123.6	similar to Msx2 interacting nuclear target protein
	1	13.51	233113.9	similar to Osa1 nuclear protein
	1	10.24	155283.7	similar to polymerase (RNA) III (DNA directed) (155kD)
	1	10.76	126673.9	similar to putative transcription factor ALF-4
	1	9.88	30313.4	similar to ribosomal protein L7, cytosolic

	1	10.34	21558.4	similar to ribosomal protein S2
helicase family	2	27.47	46440.7	similar to translation initiation factor eIF-4A II - mouse
	1	9.18	129840.7	similar to treacle
	1	9.56	40041.2	similar to TSC-22-like
involved in DNA excision repair	1	10.52	130412.1	similar to XPG
	1	12.32	108026.5	similar to zinc finger proliferation 1
	2	21.16	139501.6	similar to zinc finger protein Sall1
	1	9.32	90019.6	stat5b
	1	11.42	242307.3	transcription factor IIIC alpha chain
	1	10.43	85576.3	tripartite motif protein 28

MICROSOMAL/ER

found in ER	1	11.05	72347.3	78 KD GLUCOSE-REGULATED PROTEIN PRECURSOR (GRP 78)
	1	11.98	36996.7	Calumenin precursor (Crocalbin) (CBP-50)
microsomal	2	26.01	62130.5	carboxylesterase (EC 3.1.1.1) ES-10 precursor, microsomal
	1	14.82	28575	Endoplasmic reticulum protein ERp29 precursor (ERp31)
	1	12.42	9266.6	Immunoglobulin heavy chain
	2	21.51	51825.8	microsomal stress 70 protein ATPase core
phosphorylated in ER in respons	1	10.72	47048.8	phosphatidylinositol 5-phosphate 4-kinase gamma
ER, golgi and endosomes	1	10.48	119357.2	phospholipase D (EC 3.1.4.4) 1b
microsomal	3	40.34	56623.7	Protein disulfide isomerase A3 precursor (Disulfide isomerase ER-60)
ER	2	28.98	56864.5	protein disulfide-isomerase (EC 5.3.4.1) precursor [validated]
also mitochondrial	1	17.8	67146.6	similar to RBP1-like protein isoform 1
	1	13.06	62679.1	STERYL-SULFATASE PRECURSOR (STEROID SULFATASE)

LYSOSOMAL

	2	26.49	37470.2	Cathepsin B precursor (Cathepsin B1) (RSG-2)
	2	25.5	37686.7	prepro-cathepsin L
	1	17.77	53458.8	alpha-L-fucosidase (EC 3.2.1.51) precursor
	1	17.56	55114.6	Dipeptidyl-peptidase II precursor (DPP II) (Dipeptidyl aminopeptidase II)
	1	13.56	81851.1	similar to ATP-binding cassette transporter
	1	13.3	57110.3	similar to protective protein precursor - mouse
	1	10.68	44443.4	N-acylsphingosine amidohydrolase (acid ceramidase)

	1	9.63	44622.9	Cathepsin D
	1	9.37	44695.6	Sialidase 1 precursor (Lysosomal sialidase) (N-acetyl-alpha-neuraminidase 1)
GOLGI				
calcium-binding protein in Golgi	7	105.2	53507	Nucleobindin 1 precursor (CALNUC) (Bone 63 kDa calcium-binding protein)
x	1	11.44	58913.9	Formimidoyltransferase-cyclodeaminase
in Golgi	1	14.31	42075.3	45 kDa calcium-binding protein precursor (Cab45)
MEMBRANE PROTEINS				
membrane protein	1	9.77	21823.5	heparin-binding fibroblast growth factor receptor gamma precursor
membrane protein	1	14.7	41684	MHC class Ia protein
membrane protein	3	51.33	51981.8	Angiotensinogen precursor [Contains: Angiotensin I (Ang I)
membrane protein	3	44.34	47413.1	Lactadherin precursor (Milk fat globule-EGF factor 8) (MFG-E8)
membrane protein	2	33.74	55945.9	CD44 antigen precursor (Phagocytic glycoprotein I) (PGP-1) (HUTCH-I)
membrane protein	2	27.74	56158.9	receptor-like protein tyrosine phosphatase kappa extracellular region
membrane protein	2	27.39	65598.9	Interleukin-1 receptor accessory protein precursor
membrane protein	2	25.96	59661.6	similar to Carboxypeptidase N 83 kDa chain (Carboxypeptidase N regulatory sub
membrane protein	2	22.65	65022.3	Activated leukocyte cell adhesion molecule
membrane protein	2	21.17	46095.2	serine proteinase inhibitor 2.4
membrane protein	2	20.32	45658.7	apyrase
membrane protein	1	16.56	44568.6	Ckb protein
membrane protein	1	16.47	43118.9	similar to Vat1 protein
membrane protein	1	15.14	336588.4	Kalirin-12a
membrane protein	1	14.7	41684	MHC class Ia protein
ser/thr kinase family	1	12.62	164115.4	similar to alpha 1 type XVI collagen precursor
presents for antigens	1	12.47	98715.7	Epithelial-cadherin precursor (E-cadherin) (Uvomorulin) (Cadherin-1)
membrane protein	1	11.7	38760.6	annexin II
membrane protein	1	11.56	123684.3	similar to protein tyrosine phosphatase, receptor type, Q
membrane protein	1	11.45	220386.7	similar to scavenger receptor cysteine-rich protein type 12 precursor
membrane protein	1	11.34	35985.9	similar to cell surface protein
membrane protein	1	11.3	93764.3	MT-protocadherin
membrane protein	1	11.29	41011.7	similar to cell surface receptor FDF03

membrane protein	1	11.24	44512.1	similar to putative E1-E2 ATPase
not in database	1	11.14	57918.1	similar to Man9-mannosidase
membrane protein	1	11.1	40393	similar to Vesicular integral-membrane protein VIP36 precursor
also in golgi	1	10.95	26718.8	similar to MLN64 N-terminal domain homolog
also in golgi	1	10.92	49581.1	G protein-coupled receptor 135
	1	10.91	58289.3	similar to matrix metalloproteinase 19
	1	10.87	93261.9	similar to D-glucuronyl C5-epimerase
	1	10.66	64142.1	Interleukin-1 receptor-like 2 precursor (Interleukin-1 receptor-related protein 2)
also found in golgi	1	10.58	22243.7	similar to beta-amyloid binding protein
	1	10.56	119258.7	protein tyrosine phosphatase, receptor-type, M
	1	10.44	51437.9	T-cell surface glycoprotein CD4 precursor (T-cell surface antigen T4/Leu-3)
	1	10.42	51547.1	Adenylyl cyclase-associated protein 1 (CAP 1)
	1	10.37	304288.3	Inositol 1,4,5-trisphosphate receptor type 3
	1	10.33	33113.1	similar to beta-1,4-galactosyltransferase V
also in ER	1	10.24	126601	alpha D integrin
also in golgi	1	10.2	140018.1	similar to MLSN1- and TRP-related protein 1
only identified in testes	1	10.2	89673.2	similar to Tm9sf1 protein
	1	10.18	58461.9	similar to beta-1,3-N-acetylglucosaminyltransferase 5
	1	10.15	125358.3	similar to desmoglein 4 preproprotein
also in golgi	1	10.13	168142.8	similar to protocadherin beta 3
	1	9.96	307060.2	Inositol 1,4,5-trisphosphate receptor type 2
	1	9.89	37226	similar to olfactory receptor MOR31-8
also in ER	1	9.83	46353.4	Interleukin-1 receptor, type II precursor (IL-1R-2)
	1	9.77	42085.9	Zinc finger DHHC domain containing protein 2
	1	9.69	27405.5	protein-tyrosine kinase (EC 2.7.1.112), receptor type ron-like
	1	9.66	34205.5	similar to protocadherin 11 X-linked isoform c
	1	9.56	74258.9	similar to junctophilin type 2
	1	9.27	39426.6	similar to connexin39
	1	9.11	79842.1	similar to olfactory receptor MOR160-4

EXTRACELLULAR MATRIX AND ASSOCIATED PROTEINS

essential part of ECM	36	577.3	272512.6	Fibronectin precursor (FN)
heparan sulfate proteoglycan	7	107.5	208647.4	Agrin precursor
metalloprotease	4	57.15	129672.1	thrombospondin 1
type of collagen	3	51.51	174398.5	collagen, type XVIII, alpha 1

involved in cell adhesion	3	48.39	257726.9	similar to Ac2-120
essential part of ECM	2	30.01	167286.4	similar to laminin gamma-1 chain precursor - mouse
Hydrolyzes polysaccharides	1	19.63	58830.3	alpha-amylase
collagen	1	17.26	137954.1	similar to Collagen alpha1
links cytoskeleton to ECM	1	15.67	96707	dystroglycan 1
type of collagen	1	12.92	140963.3	collagen, type V, alpha 2
type of collagen	1	12.13	357705.9	similar to a collagen VI, alpha-3 polypeptide
essential part of ECM	1	11.3	415471.2	laminin, alpha 5
essential part of ECM	1	10.11	183652.8	similar to laminin 12 gamma 3 chain
metalloprotease	1	10.04	55265.5	similar to π matrix metalloprotease MMP-27
connects cell to matrix	1	9.96	269516.4	similar to talin
heparan sulfate proteoglycan	1	9.95	15799.1	agrin precursor
similar to laminin	1	9.95	125786.7	similar to laminin gamma 2 chain precursor
type of collagen	1	9.22	167863.8	similar to alpha-3 type IV collagen
found in immature cells	2	21.44	251474.1	chondroitin sulfate proteoglycan NG2
heparan sulfate proteoglycan	1	17.39	21961.9	Syndecan-4 precursor (Ryudocan core protein)

MISCELLANEOUS

Brain/Nervous System

usually found in brain or the nerv	1	17	22618.1	similar to RIKEN cDNA 1810013B01
usually found in brain tissue	1	16.79	34129	similar to hypothetical protein
brain and embryonic tissues	1	13.15	40696.3	similar to RIKEN cDNA C330005L02
brain tissue	1	13.09	119684.3	similar to mKIAA0357 protein
usually found in retina and brain,	1	11.84	64608.9	similar to nyctalopin
brain and muscle	1	11.82	44282.1	Dual specificity mitogen-activated protein kinase kinase 2
brain and testis	1	11.79	102784.5	similar to methyl-CpG binding domain protein 6
from brain	1	11.75	277807.7	similar to Cohen syndrome 1 protein
brain tissue	1	12.36	101310.6	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (E-NPP 2)
brain tissue	1	12.34	75306.3	arachidonate 12-lipoxygenase (EC 1.13.11.31)
brain tissue	1	12.74	178242.3	similar to brain-specific angiogenesis inhibitor 2
brain tissue	1	12.33	43900.8	similar to Vacuolar ATP synthase subunit C (V-ATPase C subunit)
brain tissue	1	12.23	225979.2	similar to KIAA0912 protein
found primarily in the brain	1	9.55	36283.9	brain mitochondrial carrier protein-1
found in neural cells	2	26.89	48760.4	similar to Protein disulfide isomerase A6 precursor
from neuronal precursor cells	1	12.61	68228.4	similar to ash2 (absent, small, or homeotic)-like
only identified in brain	1	10.88	60034.1	similar to CGI-09 protein
only identified in bone marrow	1	9.49	304196.6	similar to Hypothetical protein KIAA0233

only identified in brain	1	11.01	104910.6	similar to KIAA1582 protein
only identified in brain	1	10.82	178597.8	similar to KIAA0339 protein
only identified in brain	1	10.68	57292.1	similar to RIKEN cDNA 2410008G02
only identified in brain	1	10.13	177811.7	similar to KIAA1009 protein
only identified in brain	1	9.38	127249.1	similar to calsyntenin-1 protein
only identified in brain	1	9.38	33678.5	Translation initiation factor eIF-2B alpha subunit
only identified in brain and testis	1	10.77	60243.5	similar to hypothetical protein FLJ25333
only identified in eye	1	11	114385.1	similar to Glycyl-tRNA synthetase
only identified in cerebellum	1	9.17	85754.5	similar to RIKEN cDNA A730063F07 gene
only identified in nervous system	1	9.94	43962.6	similar to Veph-A
only identified in neurons	1	9.75	185927	postsynaptic density protein
only identified in neuropeptide sig	1	11.15	47971.5	similar to carcinoma associated protein-like protein
predominantly found in brain	1	9.69	270937.4	beta spectrin, beta_SpIII_sigma1
primarily found in nervous system	1	9.37	456493.8	similar to sacsin
only identified in eye	1	9.45	76039.8	similar to hypothetical protein FLJ23322
specific to brain	1	9.89	291476.3	similar to Brain-specific angiogenesis inhibitor 1 precursor
specific to nervous system	1	10.17	135350.9	vesicle associated protein 1
neural specific protein	1	10.05	88497.8	BMP/Retinoic acid-inducible neural-specific protein-3
neuronal protein	1	10.62	85145.6	similar to neuronal transmembrane protein Slitrk5
probably in pituitary	1	10.11	56450.8	pituitary adenylate cyclase-activating polypeptide type I receptor precursor
ECM in brain	3	3	42.59	Reelin precursor
ECM A368protease in brain	3	42.59	387533.5	Reelin precursor
Embryonic				
only identified in embryo	1	11.44	95334.8	similar to RIKEN cDNA 2600017A12
only identified in embryo	1	11.21	181895.3	similar to Nck-interacting kinase-like embryo specific kinase
only identified in embryo	1	10.5	59638	similar to RIKEN cDNA 1200014P05
only identified in embryo	1	10.12	46353.7	similar to expressed sequence AL022641
only identified in embryo	1	9.2	222341.7	similar to Kielin
involved in embryonic cell cycle	1	14.06	59089.2	similar to Spindlin-like protein 2 (SPIN-2)
involved in stem cell differentiatio	1	10.61	97660.3	GERp95
embryonic brain	1	11.85	198567.3	microtubule-associated protein MAP2
embryonic junction protein	1	9.39	104498.3	similar to desmocollin type 2
usually found in embryonic tissue	1	16.74	27199.5	procollagen C-proteinase 3
embryonic protein	1	9.13	23060	beta ig-h3
fetal brain	1	12.09	350168.9	ryanodine receptor 3
embryonic cell differentiation	2	32.38	41532.8	Fetuin-B precursor (IRL685)

From other organs

found in sperm	1	10.19	61103.2	similar to spermatid-specific thioredoxin
from colon tumors	1	12.47	237365.5	similar to colon cancer antigen NY-CO-45
from small intestine	1	12.71	42510.2	neuropeptide Y/peptide YY-Y2 receptor
from testis	1	11.66	26247.9	similar to polycythemia rubra vera 1
involved in meiosis in testis	1	10.56	28046.4	kinesin-like protein KIF2
involved in presenting antigens tc	1	9.27	58076.6	similar to histocompatibility 28
mostly in brain and kidney	1	13.56	129368.5	similar to HSPA12A
muscle protein	2	26.74	13155.9	similar to Myosin light chain alkali, smooth-muscle isoform (MLC3SM)
only identified in breast tumor	1	9.11	45477.9	similar to cytokine receptor like molecule 3
only identified in developing male	1	9.51	68566.7	similar to alpha-3 type IX collagen
only identified in lung	1	11.62	89399.5	similar to 1200003M09Rik protein
only identified in mammary gland	1	9.21	116923.7	similar to putative serine/threonine protein kinase MAK-V
only identified in muscle	1	11.05	69277	similar to titin isoform N2-A
only identified in muscle tissue	1	10.73	52656.6	Myosin-binding protein H (MyBP-H) (H-protein)
only identified in ovaries	1	11.31	58254.1	similar to Breast cancer associated protein BRAP1
only identified in prostate	1	10.13	108586.9	similar to RN49018
only identified in skin	1	11.55	85753.4	similar to cDNA sequence AF155546
only identified in testes	1	9.9	83705.9	similar to RIKEN cDNA 4832415H08 gene
only identified in testes	1	9.43	19445.5	similar to RIKEN cDNA 1700011A15
only identified in testis	1	10.98	104767.7	similar to bromodomain-containing female sterile homeotic-like protein
only identified in testis	1	10.81	116278.8	similar to hypothetical protein FLJ36090
only identified in uterus	1	10.9	24466	similar to RIKEN cDNA 2610528H13
only identified in uterus	1	9.83	60531.2	similar to hypothetical protein FLJ10057
primarily identified in gastric cells	1	9.16	52669	similar to gasdermin
secreted by neutrophils	1	9.3	3339	defensin NP-4
role in egg/sperm fusion	2	26.4	34296.2	Secreted acidic cysteine rich glycoprotein
found in testis	2	22.56	70390	similar to plastin 1 (I isoform)
cartilage component	2	28.27	68966.9	similar to matrilin-4 precursor
primarily in cartilage	1	11.92	46483.3	proteoglycan link protein 2 precursor
primarily in placenta, uterus	1	10.32	84743.4	similar to lysyl oxidase-related protein C

Multiple locations

lipid transport into, out of, within c	7	92.86	536026.7	Ac1-060
Membrane-associated, cytoplasn	6	74.07	47420.2	Transcobalamin II precursor (TCII) (TC II)
Membrane-associated, cytoplasn	3	46.61	50046.5	calcium binding protein NEFA
anti oncogene	1	12.34	57398.7	similar to putative protein kinase
calcium binding, unknown locatio	2	24.95	719257.1	similar to 4921510J17Rik protein
can have multiple functions	1	9.12	197290.7	similar to putative pheromone receptor
colon and brain tissue	1	12.65	44738.3	similar to ubiquitous alpha-2,3-sialyltransferase VI
up regulated during adipocyte dif	1	11.75	55204.4	similar to adipocyte-specific protein 4
usually found in prostate	1	15	117769.9	similar to STAT4
secreted or cytoplasmic protein	2	29.17	51105.5	similar to Peptidoglycan recognition protein-like
secreted or membrane protein	2	29.62	80741.4	proprotein convertase subtilisin/kexin type 9
Unknown Function				
unknown function	25	360.6	186325.3	hypothetical protein XP_346863
unknown location	2	34.04	48826.8	pre-procarboxypeptidase R
unknown location	3	30.11	461426.4	similar to highwire
unknown function	2	29.76	55960.4	Aa1018
unknown function	2	35.33	53498.3	hypothetical protein XP_346679
unknown function	1	17.3	116462.2	Ba2-693
unknown function	1	16	51969.7	hematopoietic lineage switch 2 related protein
unknown function	1	15.54	65615.1	similar to cDNA sequence BC004044
unknown function	1	15.41	95977.9	hypothetical protein XP_346609
unknown function	1	15.39	20819.7	Unknown (protein for MGC:72954)
unknown function	1	15.03	49801.3	Unknown (protein for MGC:73008)
unknown function	1	14.35	132077.4	similar to hypothetical protein MGC15523
unknown function	1	13.93	34889.5	luteinizing hormone/chorionic gonadotropin receptor
unknown function	1	13.86	74208.6	similar to hypothetical protein MGC28705
unknown function	1	13.79	45011.9	hypothetical protein XP_345594
unknown function	1	13.75	6697.4	unnamed protein product
unknown function	1	13.46	28264.9	similar to hypothetical protein MGC2650
unknown protein	1	12.78	26746.9	similar to hypothetical protein MGC15677
unknown protein	1	12.68	34148.2	similar to hypothetical protein MGC3279 similar to collectins
unknown function	1	12.25	32363.8	similar to PEBP2a1 protein
unknown function	1	11.88	32144.4	LRRGT00077
unknown function	1	11.11	41353	similar to RIKEN cDNA 9630046K23

unknown function	1	11.06	18972.7	similar to CG18661-PA
unknown function	1	11.84	50848.4	similar to ALEX3 protein
unknown function	1	12.52	121743.4	s-tomosyn isoform
unknown function	1	11.52	43455.1	similar to RIKEN cDNA 1700001E04
unknown function	1	11.45	32227.2	unnamed protein product
unknown function	1	11.05	54439.8	similar to RIKEN cDNA 4932432K03
unknown function	1	10.91	12743.8	Unknown (protein for MGC:72998)
unknown function	1	10.91	128322.1	similar to CG14803-PA
unknown function	1	10.71	117791.1	similar to mKIAA1122 protein
unknown function	1	10.69	133381	similar to hypothetical protein C130031J23
unknown function	1	10.84	124717.4	similar to RIKEN cDNA C230075L19 gene
unknown function	1	10.52	43266.7	similar to CG13379-PA
unknown function	1	10.52	31783.6	similar to RIKEN cDNA 1810003N24
unknown function	1	10.34	57906.2	hypothetical protein XP_346941
unknown function	1	10.29	304103.7	similar to putative protein family member (XC177)
unknown function	1	10.29	78849.3	similar to mDia interacting protein-1
unknown function	1	10.29	20168.6	similar to C184M protein
unknown function	1	10.23	29201	similar to FLJ00089 protein
unknown function	1	10.21	46300.9	LRRGT00048
unknown function	1	10.04	91215.5	similar to RIKEN cDNA 0710001G09
unknown location	1	10.02	58350.2	similar to GARG-39
unknown function	1	9.94	40476	similar to hypothetical protein MGC45594
unknown function	1	9.93	57832.4	similar to hypothetical protein FLJ20729
unknown function	1	9.93	65821.1	similar to BC282485_1
unknown function	1	9.82	74689.1	similar to GLP_171_8870_6279
unknown function	1	9.8	26834	similar to RIKEN cDNA 4931426K16 gene
unknown function	1	9.78	1086949.9	similar to bullous pemphigoid antigen 1-b
unknown function	1	9.74	18990.5	similar to hypothetical protein MGC23918
unknown function	1	9.59	58556.6	similar to RIKEN cDNA E030002O03
unknown function	1	9.09	80883.7	similar to Mpo protein
unknown function	1	9.03	40086	similar to hypothetical protein FLJ22693
unknown location	1	13.22	189401.2	similar to metalloprotease disintegrin 12 protein
cellular location unknown	1	9.19	49459.5	similar to FLJ00364 protein
unknown cellular location	1	11.32	72724.6	similar to microtubule-interacting protein that associates with TRAF3
unknown cellular location	1	11.29	58099.8	chaperonin delta subunit
unknown cellular location	1	11.19	18604.5	similar to diphosphate dimethylallyl diphosphate isomerase 2
unknown cellular location	1	11.15	15186.3	c-met/hepatocyte growth factor receptor

unknown cellular location	1	11.04	138899.1	similar to polycystin-1L1
unknown cellular location	1	10.97	32847.1	similar to T-cell activation protein phosphatase 2C
unknown cellular location	1	10.67	283021.5	similar to type VII collagen
unknown cellular location	1	10.32	104185.8	similar to adaptor-related protein complex 2, beta 1 subunit
unknown cellular location	1	10.18	153460.3	similar to cofactor required for Sp1 transcriptional activation, subunit 2
unknown cellular location	1	10.07	59188.2	similar to INNER EAR-SPECIFIC COLLAGEN PRECURSOR
unknown cellular location	1	9.7	26558.8	similar to alpha-catenin-like protein
unknown cellular location	1	9.69	371947.2	similar to huntingtin interacting protein B isoform 1
unknown cellular location	1	9.67	22261.2	alpha-crystallin chain A, minor component
unknown cellular location	1	9.61	180894.8	similar to amylo-1,6-glucosidase, 4-alpha-glucanotransferase isoform 1
unknown cellular location	1	9.61	68572.6	similar to amyotrophic lateral sclerosis 2 (juvenile) chromosome region
unknown cellular location	1	9.58	150815.9	similar to putative adapter and scaffold protein
unknown cellular location	1	9.45	218572.5	similar to dedicator of cyto-kinesis 1
unknown function	1	11.27	102377.5	similar to KIAA0738 gene product
unknown location	1	13.08	144974.6	similar to JNK interacting protein-3a

Not in Database

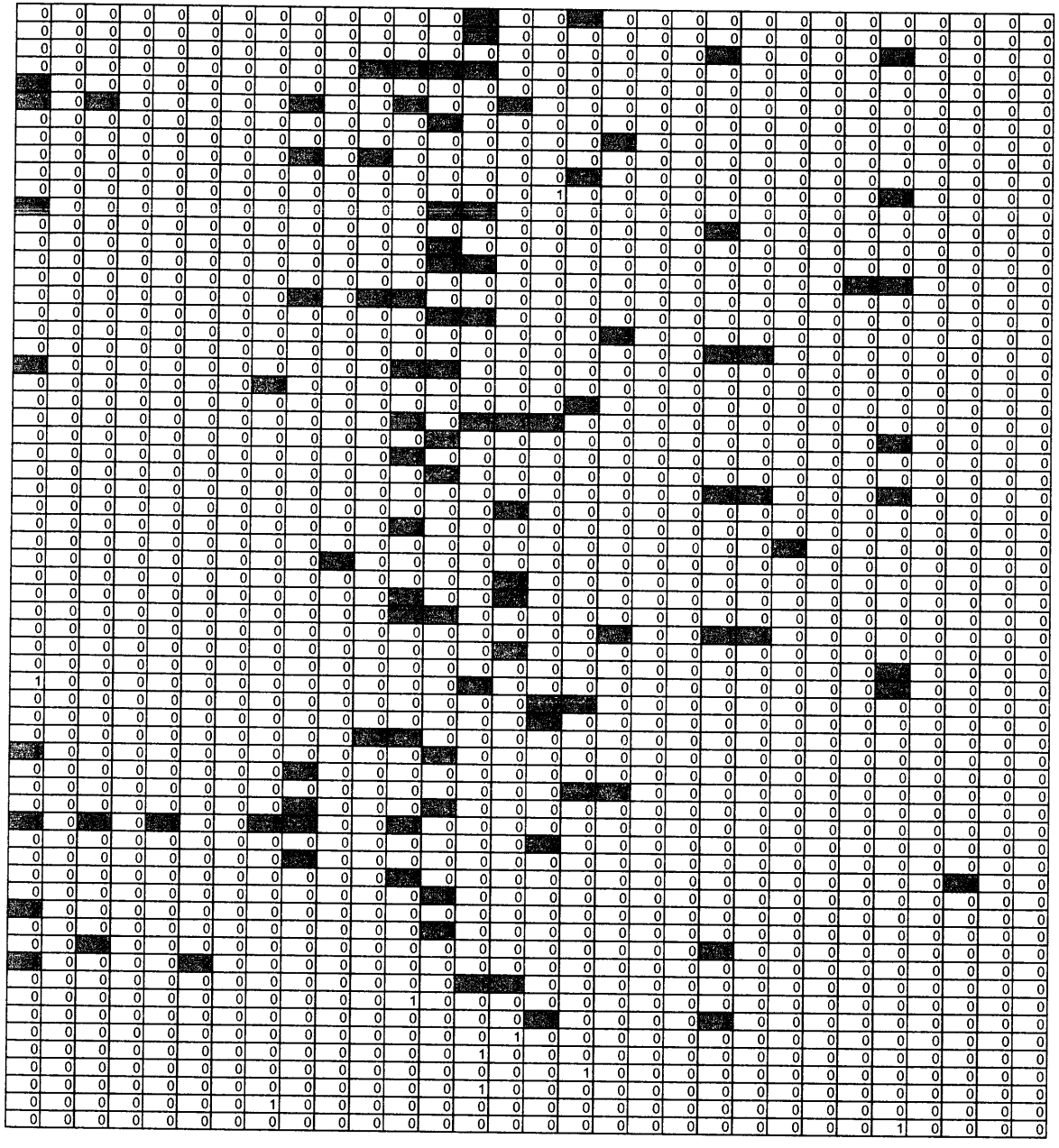
not a database	1	9.2	63858	sphingosine-1-phosphate phosphohydrolase
not a database	1	9.14	29625.5	hypothetical protein XP_228902
not in database	1	9.43	58535.1	similar to cyclic nucleotide phosphodiesterase PDE7A2
not in database	1	9.27	45670.8	extracellular signal-regulated kinase 1b
not in database	1	9.25	12097	similar to DNA segment, Chr 10, ERATO Doi 214, expressed
not in database	1	9.09	57946.7	similar to putative protein at the X chromosomal L1cam locus
not in database	1	9.04	66697.6	similar to p66 alpha homolog
not in database	1	9.04	127597.9	similar to CG5882-PA
not in database	1	9.02	102734	LSC
not in database	1	9.01	52367.1	similar to GLP 618 12970 14472 like (4J465)

SUMMARY

TOTAL PROTEINS		Miscellaneous	
Plasma	59	Embryonic	13
ECM	21	Central nervous	40
Intracellular	234	Other organs	42
Miscellaneous	179	Unknown func.	84
		Total	179

Appendix IIIb: Distribution of peptides across the SDS-gel

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	#	entry_name
121	0	37	2	34	45	28	105	104	44	49	108	162	214	80	67	84	79	15	5	25	24	14	6	13	53	6	1	1	1		**total peptides in each fraction**
56	0	21	2	29	26	17	38	53	32	29	74	79	108	46	38	42	34	9	2	18	19	5	4	11	34	6	1	1	1		**total proteins in each fraction**
	0									0	0						0	0	0	0	0	0	0	0	0	0	0	0	0	1	Fibronectin precursor (FN)
	0	0	0	0	0	0				0		0					0	0	0	0	0	0	0	0	0	0	0	0	0	2	hypothetical protein XP_346863
	0	0	0			0			0	0		0	0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	3	alpha-1-macroglobulin precursor
	0		0																0	0	1	0	0	0		0	0	0	0	4	Cc1-8
0	0	0	0	0	0	0				0									0											5	ALPHA-1-ANTIPROTEINASE PRECURSOR (ALPHA-1-ANTITRYPSIN)
	0		0			0			0		0							0	0	0	0		0	0		0	0	0	0	6	cytokeratin-8
	0	0	0	0	0	0			0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	alpha-1 proteinase inhibitor III, variant 1 precursor
0	0	0	0	0	0	0	0	0	0	0	0				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	serine proteinase inhibitor 3
0	0	0	0	0	0	0	0	0	0								0	0	0	0	0	0	0	0	0	0	0	0	0	9	T-kininogen II precursor (Major acute phase protein) (Alpha-1-MAP)
0	0	0	0	0		0	0		0						0			0	0	0	0	0	0	0	0	0	0	0	0	10	Hemopexin precursor
0	0	0	0	0	0	0	0		0	0		0											0				0	0	0	11	Apolipoprotein E
0	0	0	0	0	0	0	0		0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	Actn4 protein
0	0	0	0	0	0	0				0												0					0	0	0	13	Clusterin
0	0	0	0	0	0	0	0		0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	Serum albumin precursor [Contains: Neurotensin-related peptide (NRP)]
0	0	0	0			0	0	0	0	0	0						0	0	0	0	0	0	0	0	0	0	0	0	0	15	Vitamin D-binding protein precursor (DBP) (Group-specific component) (GC-globulin)
0	0		0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	Agrin precursor
0	0	0	0	0	0	0	0	0	0	0						0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	Nucleobindin 1 precursor (CALNUC) (Bone 63 kDa calcium-binding protein)
0	0	0	0	0	0	0	0		0		0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1
0	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	19	alpha-2 antiplasmin
	0	0	0		0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	Ac1-060
0	0	0	0	0	0	0	0	0	0	0	0	0			0			0	0	0	0	0	0	0	0	0	0	0	0	21	Ba1-647
0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	22	ATP synthase beta chain, mitochondrial precursor
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	23	apolipoprotein A-IV precursor
0	0	0	0	0	0	0	0	0	0	0	0			0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	24	similar to beta-2-glycoprotein I
0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25	hepatic multiple inositol polyphosphate phosphatase
0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	26	complement component C2
0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27	similar to Ac2-248
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28	Transcobalamin II precursor (TCII) (TC II)
0	0	0	0	0	0	0	0	0	0	0	0	0	0				0	0	0	0	0	0	0	0	0	0	0	0	0	29	actin beta
0	0	0	0	0	0		0	0	0	0	0	0	0	0	0			0	0			0	0	0	0	0	0	0	0	30	AMBf Inter· Trypstatin]
0	0	0	0	0	0	0	0	0	0	0	0	0		0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	31	Fibrinogen beta chain precursor [Contains: Fibrinopeptide B]
0	0	0	0	0	0	0	0	0	0	0	0	0				1	0	0	0	0	0	0	0	0	0	0	0	0	0	32	Fibrinogen gamma chain precursor
0	0	0	0	0	0	0	0	0	0	0	0	0					0	0	0	0	0	0		0		0	0	0	0	33	keratin 18
0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0				0	0		0	0	0	0	34	apolipoprotein A-I precursor
	0		0			0			0	0		0	0	0		0	0	0	0	0	0	0	0		0	0	0	0	0	35	similar to Keratin, type II cytoskeletal 1 (Cytokeratin 1) (67 kDa cytokeratin)
0	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	36	ALPHA-1-ACID GLYCOPROTEIN PRECURSOR (OROSOMUCOID) (OMD)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	37	L-lactate dehydrogenase A chain (LDH-A) (LDH muscle subunit) (LDH-M)
0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	38	Protein Z-dependent protease inhibitor precursor
0	0	0	0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	39	thrombospondin 1
0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40	transketolase
0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	41	histidine-rich glycoprotein 1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0		0	0	0	0	0	0	0	0	42	fibrinogen alpha subunit
0	0	0	0	0	0	0				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	43	Inter-alpha-trypsin inhibitor heavy chain H3 precursor (ITI heavy chain H3)
0	0	0	0	0	0	0	0			0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	44	inter-alpha-inhibitor H4P heavy chain
0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	45	kallistatin
0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	46	collagen, type XVIII, alpha 1
0	0	0	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	47	Angic Angi· Angiotensin III (Ang III) (Des-Asp[1]-angiotensin II)]
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	0	48	similar to Zinc finger protein 37 (Zfp-37)
0	0	0	0	0	0	0		0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	49	hypothetical protein XP_346447
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	4-hydroxyphenylpyruvate dioxygenase
0	0	0	0	0	0	0		0			0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	51	similar to Ac2-120
0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	52	calcium binding protein NEFA
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	53	dismutase
0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	54	Lactadherin precursor (Milk fat globule-EGF factor 8) (MFG-E8)
0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	55	similar to Heat shock cognate 71 kDa protein
0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	56	Complement component 1, s subcomponent



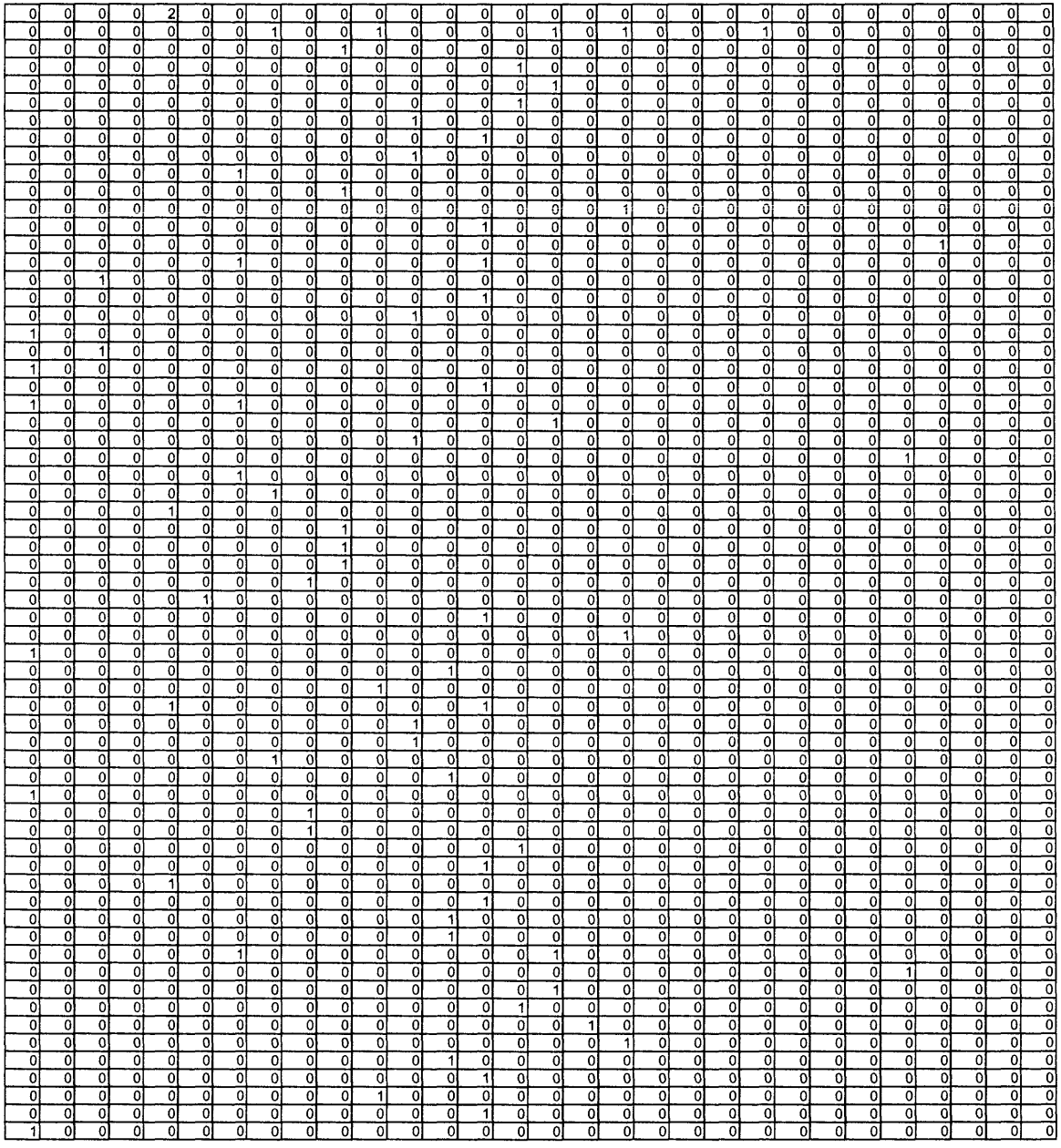
57 Alpha-tubulin
58 unnamed protein product
59 connective tissue growth factor
60 similar to alpha-2-antiplasmin
61 Reelin precursor
62 similar to cytokeratin 13
63 Protein disulfide isomerase A3 precursor (HIP-70) (Q-2)
64 regucalcin
65 Afamin precursor (Alpha-albumin) (Alpha-Alb)
66 Arginase 1 (Liver-type arginase)
67 transt Transthyretin (prealbumin, amyloidosis type I)
68 hypothetical protein XP_346679
69 Carbonic anhydrase III (Carbonate dehydratase III) (CA-III)
70 pre-procarboxypeptidase R
71 CD44 antigen precursor (Phagocytic glycoprotein I) (PGP-1) (HUTCH-I)
72 cystatin C
73 Polymeric-immunoglobulin receptor precursor (Poly-Ig receptor) (PIGR)
74 Fetuin-B precursor (IRL685)
75 Malate dehydrogenase, mitochondrial precursor
76 glutathione S-transferase alpha
77 simila PAM rpm 1
78 similar to laminin gamma-1 chain precursor - mouse
79 Alcohol dehydrogenase [NADP+] (Aldehyde reductase)
80 Aa1018
81 propr neural apoptosis-regulated convertase 1
82 similar to Peptidoglycan recognition protein-like
83 protein disulfide-isomerase (EC 5.3.4.1) precursor [validated]
84 Glutathione S-transferase Yb-1 (Chain 3) (GST Yb1) (GST M1-1) (GST class-mu 1)
85 ZN-alpha-2-glycoprotein
86 similar to matrilin-4 precursor
87 Peroxiredoxin 1 (Thioredoxin peroxidase 2)
88 receptor-like protein tyrosine phosphatase kappa extracellular region
89 Ribonuclease inhibitor
90 similar to translation initiation factor eIF-4A II - mouse
91 Interleukin-1 receptor accessory protein precursor (IL-1 receptor accessory protein)
92 similar to mac25
93 similar to Protein disulfide isomerase A6 precursor (Protein disulfide isomerase P5)
94 similar to Myosin light chain alkali, smooth-muscle isoform (MLC3SM)
95 similar to eukaryotic translation elongation factor 1 alpha 2
96 Cathepsin B precursor (Cathepsin B1) (RSG-2)
97 Secreted acidic cysteine rich glycoprotein
98 EZRIN
99 carboxylesterase (EC 3.1.1.1) ES-10 precursor, microsomal
100 similar to Carboxypeptidase N 83 kDa chain (Carboxypeptidase N regulatory subunit)
101 prepro-cathepsin L
102 similar to complement component 1, r subcomponent
103 similar to 4921510J17Rik protein
104 aspartate transaminase (EC 2.6.1.1), cytosolic
105 Activated leukocyte cell adhesion molecule
106 similar to plastin 1 (I isoform)
107 microsomal stress 70 protein ATPase core
108 chondroitin sulfate proteoglycan NG2
109 serine proteinase inhibitor 2.4
110 similar to zinc finger protein Sall1
111 similar to Ran-binding protein 2
112 Lamin A
113 similar to complement component 8 alpha subunit
114 apyrase
115 Betaine--homocysteine S-methyltransferase
116 alpha-amylase
117 Ornithine carbamoyltransferase, mitochondrial precursor (OTCase)
118 Glutathione synthetase (Glutathione synthase) (GSH synthetase) (GSH-S)
119 Ab2-450
120 14.5 kDa translational inhibitor protein (Perchloric acid soluble protein)

0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	121 selenium-binding protein
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	122 similar to complement component 8 beta subunit
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	123 Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase) (AdoHcyase)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	124 Beta-2-microglobulin precursor
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	125 similar to GLUTATHIONE S-TRANSFERASE 8 (GST 8-8) (CHAIN 8) (GST CLASS-ALPHA)
1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	126 simil: brea: brea: Rb-binding retinoblastoma-binding protein 1-like 1
0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	127 alpha-L-fucosidase (EC 3.2.1.51) precursor
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	128 Dipeptidyl-peptidase II precursor (DPP II) (Dipeptidyl aminopeptidase II)
0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	129 Syndecan-4 precursor (Ryudocan core protein)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	130 Ba2-693
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	131 similar to Collagen alpha1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	132 similar to SPI6
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	133 Peroxiredoxin 5, mitochondrial precursor (Prx-V) (Peroxisomal antioxidant enzyme)
0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	134 hypothetical protein XP_346596
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	135 similar to RIKEN cDNA 1810013B01
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	136 similar to hypothetical protein CAB56184
0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	137 procollagen C-proteinase 3
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	138 simil: PYK2 C terminus-associated protein
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	139 Ckb protein
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	140 PRx III
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	141 similar to Vat1 protein
0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	142 Gamma-glutamyl hydrolase precursor (Gamma-Glu-X carboxypeptidase) (Conjugase) (GH)
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	143 Ab1-046
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	144 Dipeptidyl-peptidase I precursor (DPP-I) (DPPI) (Cathepsin C) (Cathepsin J)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	145 Aspartate aminotransferase, mitochondrial precursor (Transaminase A)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	146 Transaldolase 1
0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	147 hematopoietic lineage switch 2 related protein
0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	148 Carbamoyl-phosphate synthase [ammonia], mitochondrial precursor
0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	149 Acyl-CoA dehydrogenase, short/branched chain specific, mitochondrial precursor
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	150 Alpha-2-macroglobulin precursor (Alpha-2-M)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	151 insulin-like growth factor-binding protein 1 precursor
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	152 dystroglycan 1
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	153 hepatocyte growth factor activator
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	154 simil: clone MGC:7673
0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	155 Ceruloplasmin precursor (Ferroxidase)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	156 similar to 1700013L23Rik protein
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	157 hypothetical protein XP_346609
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	158 Unknown (protein for MGC:72954)
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	159 plasminogen protein
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	160 Peroxiredoxin 6 (Antioxidant protein 2) (1-Cys peroxiredoxin) (1-Cys PRX)
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	161 argininosuccinate lyase
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	162 similar to Keratin, type II cytoskeletal 4 (Cytokeratin 4) (Cytoskeletal 57 kDa keratin)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	163 similar to 14-3-3 protein sigma
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	164 Heparin cofactor II precursor (HC-II) (Protease inhibitor leuserpin 2)
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	165 Kalirin-12a
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	166 cyclophilin B
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	167 Unknown (protein for MGC:73008)
1	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	168 similar to STAT4
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	169 Elongation factor 2 (EF-2)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	170 Endoplasmic reticulum protein ERp29 precursor (ERp31)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	171 MHC class Ia protein
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	172 cytosolic malate dehydrogenase
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	173 profilin
0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	174 similar to hypothetical protein MGC15523
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	175 similar to inter-alpha trypsin inhibitor, heavy chain 1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	176 45 kDa calcium-binding protein precursor (Cab45) (Stromal cell-derived factor 4) (SDF-4)
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	177 similar to uridindiphosphoglucosepyrophosphorylase 2
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	178 similar to Spindlin-like protein 2 (SPIN-2)
1	0	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	179 similar to TOB3
0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	180 M-phase inducer phosphatase 2 (Dual specificity phosphatase Cdc25B)
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	181 lutein LH/CG receptor
1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	182 similar to neurocalcin delta
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	183 Aldehyde dehydrogenase 1A1 (Aldehyde dehydrogenase, cytosolic) (ALDH class 1)
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	184 similar to hypothetical protein MGC28705

0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	185 similar to Aldo-keto reductase family 1 member E1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	186 hypothetical protein XP_345594
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	187 unnamed protein product
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	188 hemoglobin beta chain, minor
0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	189 similar to olfactory receptor MOR114-8
0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	190 similar to ATP-binding cassette transporter
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	191 similar to HSPA12A
0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	192 similar to 60S ribosomal protein L10 (QM protein homolog)
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	193 similar to Osa1 nuclear protein
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	194 CArG-binding factor A
1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	195 similar to hypothetical protein MGC2650
0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	196 similar to kinesin-related protein KIF27A
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	197 similar to protective protein precursor - mouse
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	198 similar to metalloprotease disintegrin 12 protein
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	199 similar to RIKEN cDNA C330005L02
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	200 similar to mKIAA0357 protein
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	201 similar to LECT2
1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	202 similar to 4930431B09Rik protein
0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	203 similar to JNK interacting protein-3a
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	204 STERYL-SULFATASE PRECURSOR (STEROID SULFATASE)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	205 Low molecular weight phosphotyrosine protein phosphatase ACP1/ACP2
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	206 collagen, type V, alpha 2
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	207 similar to NADP+-specific isocitrate dehydrogenase
0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	208 similar to hypothetical protein MGC15677
0	0	1	0	0	0	0	1	1	0	0	2	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	209 similar to brain-specific angiogenesis inhibitor 2
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	210 neuropeptide Y/peptide YY-Y2 receptor
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	211 similar to hypothetical protein MGC3279 similar to collectins
1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	212 similar to ubiquitous alpha-2,3-sialyltransferase VI
0	0	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	213 simila collagen XVI, alpha-1 polypeptide
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	214 simila ash2 (absent, small, or homeotic, Drosophila, homolog)-like
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	215 Munc13-2 protein
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	216 s-tomosyn isoform
1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	217 similar to colon cancer antigen NY-CO-45
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	218 Epithelial-cadherin precursor (E-cadherin) (Uvomorulin) (Cadherin-1)
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	219 Immunoglobulin heavy chain
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	220 similar to Siglec-G
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	221 Ector Nucleotide pyrophosphatase (NPPase)]
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	222 arachidonate 12-lipoxygenase (EC 1.13.11.31)
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	223 similar to putative protein kinase
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	224 similar to Vacuolar ATP synthase subunit C (V-ATPase C subunit)
1	0	0	0	0	0	0	0	1	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	225 similar to zinc finger proliferation 1
0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	226 similar to ATP-binding cassette transporter sub-family A member 8a
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	227 similar to PEBP2a1 protein
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	228 similar to KIAA0912 protein
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	229 Aflatoxin B1 aldehyde reductase member 1 (AFB1-AR) (rAFAR1)
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	230 similar to sporulation-induced transcript 4-associated protein
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	231 simila collagen VI, alpha-3 polypeptide
0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	232 A-kinase anchoring protein AKAP150
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	233 ryanodine receptor 3
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	234 similar to RalGDS-like factor
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	235 Calumenin precursor (Crocalbin) (CBP-50)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	236 similar to 2410004L15Rik protein
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	237 vp165
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	238 proteoglycan link protein 2 precursor
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	239 simila factor inhibiting HIF1
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	240 nitric-oxide synthase (EC 1.14.13.39)
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	241 LRRGT00077
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	242 microtubule-associated protein MAP2
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	243 similar to ALEX3 protein
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	244 similar to nyctalopin
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	245 Dual specificity mitogen-activated protein kinase kinase 2 (MAP kinase kinase 2)
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	246 similar to methyl-CpG binding domain protein 6
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	247 similar to Cohen syndrome 1 protein
0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	248 similar to Msx2 interacting nuclear target protein

0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	249 similar to adipocyte-specific protein 4
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	250 Dihydrolipoamide dehydrogenase
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	251 annexin II
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	252 similar to group XIII secreted phospholipase A2
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	253 similar cell surface receptor
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	254 similar to 1200003M09Rik protein
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	255 Hemoglobin alpha-1 and alpha-2 chains
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	256 similar to protein tyrosine phosphatase, receptor type, Q
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	257 similar to cDNA sequence AF155546
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	258 similar to RIKEN cDNA 1700001E04
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	259 Nucleoside diphosphate kinase B (NDK B) (NDP kinase B) (P18)
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	260 unnamed protein product
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	261 similar to scavenger receptor cysteine-rich protein type 12 precursor
0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	262 similar to RIKEN cDNA 2600017A12
0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	263 Formimidoyltetrahydrofolate cyclodeaminase (Formiminotetrahydrofolate cyclodeaminase)]
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	264 transcription factor IIIC alpha chain
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	265 similar to PROHIBITIN (B-CELL RECEPTOR ASSOCIATED PROTEIN 32) (BAP 32)
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	266 similar to protein phosphatase 1, regulatory (inhibitor) subunit 15B
1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	267 similar to cell surface protein
1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	268 testosterone-regulated prominin-related protein
0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	269 similar to Carbohydrate kinase-like protein
0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	270 similar interleukin 13 receptor alpha 1-binding protein-1
0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	271 similar to Breast cancer associated protein BRAP1
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	272 laminin, alpha 5
0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	273 MT-protocadherin
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	274 Phosphoglucomutase (Glucose phosphomutase) (PGM)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	275 similar to cell surface receptor FDF03
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	276 chaperonin delta subunit
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	277 similar to axonemal dynein heavy chain 8 long form
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	278 similar to KIAA0738 gene product
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	279 similar to putative E1-E2 ATPase
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	280 similar pore steerin 3
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	281 similar to Nck-interacting kinase-like embryo specific kinase
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	282 similar to diphosphate dimethylallyl diphosphate isomerase 2
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	283 selenoprotein P
0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	284 Glucose-6-phosphate 1-dehydrogenase (G6PD)
0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	285 c-met/hepatocyte growth factor receptor
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	286 similar to carcinoma associated protein-like protein
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	287 similar to Man9-mannosidase
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	288 similar to Phenylalanyl-tRNA synthetase beta chain (Phenylalanine--tRNA ligase beta chain)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	289 similar to RIKEN cDNA 9630046K23
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	290 similar to Vesicular integral-membrane protein VIP36 precursor
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	291 similar to Phosphoglycerate mutase 1 (Phosphoglycerate mutase isozyme B) (PGAM-B)
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	292 5'-AMP-activated protein kinase, catalytic alpha-1 chain (AMPK alpha-1 chain)
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	293 similar to CG18661-PA
0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	294 similar conn CMH cardiomyopathy, dilated 1G (autosomal dominant)
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	295 similar to RIKEN cDNA 4932432K03
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	296 78 KD GLUCOSE-REGULATED PROTEIN PRECURSOR (GRP 78)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	297 similar to CCG1
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	298 similar polycystic kidney disease 1 like 1
0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	299 similar to KIAA1582 protein
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	300 similar to Glycyl-tRNA synthetase
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	301 similar to MAP-1 protein
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	302 similar to bromodomain-containing female sterile homeotic-like protein
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	303 Complement factor I precursor (C3B/C4B inactivator)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	304 similar to T-cell activation protein phosphatase 2C
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	305 similar to MLN64 N-terminal domain homolog (STARD3 N-terminal like protein)
0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	306 G protein-coupled receptor 135
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	307 similar to matrix metalloproteinase 19
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	308 Unknown (protein for MGC:72998)
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	309 similar to CG14803-PA
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	310 similar to RIKEN cDNA 2610528H13
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	311 Protein-tyrosine phosphatase, non-receptor type 7 (Protein-tyrosine phosphatase LC-PTP)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	312 similar to CGI-09 protein

0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	313 similar to D-glucuronyl C5-epimerase
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	314 sulfhydryl oxidase
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	315 similar to RIKEN cDNA C230075L19 gene
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	316 similar to KIAA0339 protein
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	317 similar to hypothetical protein FLJ36090
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	318 similar to hypothetical protein FLJ25333
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	319 similar to putative transcription factor ALF-4
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	320 Myosin-binding protein H (MyBP-H) (H-protein)
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	321 phosphatidylinositol 5-phosphate 4-kinase gamma
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	322 similar to mKIAA1122 protein
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	323 actin-filament binding protein Frabin
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	324 similar to hypothetical protein C130031J23
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	325 N-acylsphingosine amidohydrolase (acid ceramidase)
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	326 similar to RIKEN cDNA 2410008G02
0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	327 similar to type VII collagen
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	328 Interleukin-1 receptor-like 2 precursor (Interleukin-1 receptor-related protein 2) (IL1R-rp2)
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	329 similar to neuronal transmembrane protein Slitrk5
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	330 GERp95
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	331 replication factor C
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	332 similar to 60S RIBOSOMAL PROTEIN L29 (P23)
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	333 similar to beta-amyloid binding protein
0	0	0	0	0	0	0	1	0	0	0	1	2	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	334 similar to immunoglobulin heavy chain variable region
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	335 protein tyrosine phosphatase, receptor-type, M
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	336 kinesin-like protein KIF2
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	337 similar to CG13379-PA
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	338 similar to RIKEN cDNA 1810003N24
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	339 similar to XPG
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	340 similar to RIKEN cDNA 1200014P05
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	341 phospholipase D (EC 3.1.4.4) 1b
0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	342 BCNT
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	343 T-cell surface glycoprotein CD4 precursor (T-cell surface antigen T4/Leu-3)
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	344 similar to myotubularin
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	345 tripartite motif protein 28
0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	346 Rab proteins geranylgeranyltransferase component A 1 (Rab escort protein 1) (REP-1)
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	347 Adenylyl cyclase-associated protein 1 (CAP 1)
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	348 Inositol 1,4,5-trisphosphate receptor type 3 (Type 3 inositol 1,4,5-trisphosphate receptor)
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	349 similar to ribosomal protein S2
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	350 hypothetical protein XP_346941
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	351 simil glutaredoxin
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	352 similar to beta-1,4-galactosyltransferase V
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	353 simil adap clathrin-associated/assembly/adaptor protein, large, beta 1
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	354 similar to lysyl oxidase-related protein C
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	355 similar to putative protein family member (XC177)
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	356 similar to mDia interacting protein-1
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	357 similar to C184M protein
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	358 similar to macrophin 1 isoform 4
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	359 similar to K20D4
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	360 similar to polymerase (RNA) III (DNA directed) (155kD)
0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	361 alpha D integrin
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	362 similar to FLJ00089 protein
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	363 similar to Dual-specificity tyrosine-phosphorylation regulated kinase 4
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	364 similar to cysteine-rich protein NFX-1
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	365 LRRGT00048
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	366 similar to MLSN1- and TRP-related protein 1
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	367 Class B basic helix-loop-helix protein 3 (bHLHB3)
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	368 similar to Tm9sf1 protein
0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	369 simil semaphorin cytoplasmic domain-associated protein 3A
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	370 similar to spermatid-specific thioredoxin
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	371 simil hum chro cofactor re vitamin D receptor-interacti...
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	372 similar to beta-1,3-N-acetylglucosaminyltransferase 5
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	373 vesicle associated protein 1
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	374 similar to Chromodomain-helicase-DNA-binding protein 1 (CHD-1)
0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	375 cysteine proteinase homolog (clone PCR33)
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	376 similar to desmoglein 4 preproprotein



377 similar to RN49018
378 similar to KIAA1009 protein
379 similar to protocadherin beta 3
380 similar to expressed sequence AL022641
381 similar to laminin 12 gamma 3 chain
382 pituitary adenylate cyclase-activating polypeptide type I receptor precursor
383 Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1B
384 similar to FH1/FH2 domains-containing protein (Formin homolog overexpressed in spleen)
385 similar to INNER EAR-SPECIFIC COLLAGEN PRECURSOR (SACCULAR COLLAGEN)
386 BMP/Retinoic acid-inducible neural-specific protein-3
387 simil matr matrix metalloprotease 27
388 similar to RIKEN cDNA 0710001G09
389 similar to GARG-39
390 nucleolin
391 similar to Chromosome-associated kinesin KIF4A (Chromokinesin)
392 glucokinase-associated dual specificity phosphatase
393 FMRP isoform 18
394 similar to talin
395 Inositol 1,4,5-trisphosphate receptor type 2 (Type 2 inositol 1,4,5-trisphosphate receptor)
396 agrin precursor
397 similar to laminin gamma 2 chain precursor
398 similar to Veph-A
399 similar to hypothetical protein MGC45594
400 similar to hypothetical protein FLJ20729
401 similar to BC282485_1
402 similar to 60S ribosomal protein L10 (QM protein homolog)
403 simil GLE hGLE1
404 alkyl-dihydroxyacetonephosphate synthase
405 similar to RIKEN cDNA 4832415H08 gene
406 similar to olfactory receptor MOR31-8
407 similar to Brain-specific angiogenesis inhibitor 1 precursor
408 similar to ribosomal protein L7, cytosolic
409 Interleukin-1 receptor, type II precursor (IL-1R-2)
410 similar to hypothetical protein FLJ10057
411 similar to GLP_171_8870_6279
412 similar to putative protein kinase
413 similar to Herc2
414 similar to RIKEN cDNA 4931426K16 gene
415 similar to bullous pemphigoid antigen 1-b
416 Unknown (protein for MGC:72610)
417 Zinc finger DHHC domain containing protein 2
418 heparin-binding fibroblast growth factor receptor gamma precursor
419 putative RNA methylase
420 posts citron
421 similar to hypothetical protein MGC23918
422 similar to Ifi204 protein
423 similar to alpha-catenin-like protein
424 beta spectrin, beta_SpIII_sigma1
425 PHEX protein
426 protein-tyrosine kinase (EC 2.7.1.112), receptor type ron-like
427 simil hunti hunti HSPC069
428 similar to nuclear factor related to kappa B binding protein
429 alpha-crystallin chain A, minor component
430 simil protc protc protocadherin-S
431 Calcium/calmodulin-dependent protein kinase type I (CAM kinase I)
432 similar to Nspc1 protein
433 Cathepsin D
434 simil glyc amyl Amylo-1,6-glucosidase, 4-alpha-...
435 simil partit partitioning-defective 3-beta
436 similar to RIKEN cDNA E030002O03
437 similar to Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase)
438 similar to putative adapter and scaffold protein
439 similar to RP2 protein, testosterone-regulated - ricefield mouse (Mus caroli)
440 complement component 5

0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	441 similar to junctophilin type 2
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	442 similar to TSC-22-like
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	443 brain mitochondrial carrier protein-1
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	444 similar to DnaJ (Hsp40) homolog, subfamily D, member 1
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	445 similar to KIAA1803 protein
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	446 similar to 60S ribosomal protein L19
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	447 similar to alpha-3 type IX collagen
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	448 similar to Hypothetical protein KIAA0233
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	449 Apolipoprotein D precursor (ApoD)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	450 similar to actin monomer-binding protein
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	451 similar to dedicator of cyto-kinesis 1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	452 similar to hypothetical protein FLJ23322
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	453 similar to cyclic nucleotide phosphodiesterase PDE7A2
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	454 similar to protein kinase
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	455 similar to RIKEN cDNA 1700011A15
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	456 similar to desmocollin type 2
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	457 deoxyribonuclease II
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	458 similar to calsyntenin-1 protein
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	459 Translation initiation factor eIF-2B alpha subunit (eIF-2B GDP-GTP exchange factor)
0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	460 Sialidase 1 precursor (Lysosomal sialidase) (N-acetyl-alpha-neuraminidase 1)
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	461 similar to sacsin
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	462 Import inner membrane translocase subunit TIM44, mitochondrial precursor
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	463 stat5b
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	464 defensin NP-4
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	465 protein-tyrosine-phosphatase
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	466 similar to connexin39
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	467 similar to histocompatibility 28
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	468 extracellular signal-regulated kinase 1b
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	469 similar to DNA segment, Chr 10, ERATO Doi 214, expressed
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	470 adenylate cyclase (EC 4.6.1.1), cytosolic
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	471 similar to RAB5B, member RAS oncogene family
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	472 similar to alpha-3 type IV collagen
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	473 similar to putative serine/threonine protein kinase MAK-V
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	474 similar to Ubiquitin specific protease 25
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	475 similar to Kielin
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	476 sphingosine-1-phosphate phosphohydrolase
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	477 similar to FLJ00364 protein
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	478 similar to treacle
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	479 similar to RIKEN cDNA A730063F07 gene
0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	480 similar to gasdermin
0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	481 hypothetical protein XP_228902
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	482 beta ig-h3
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	483 similar to putative pheromone receptor
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	484 similar to olfactory receptor MOR160-4
0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	485 similar to HECT type E3 ubiquitin ligase
0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	486 similar to cytokine receptor like molecule 3
0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	487 similar to Mpo protein
0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	488 similar to putative protein at the X chromosomal L1cam locus
0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	489 similar to p66 alpha homolog
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	490 similar to CG5882-PA
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	491 similar to hypothetical protein FLJ22693
0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	492 LSC
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	493 similar to GLP 618 12970 14472 like (4J465)
121	0	37	2	34	45	28	105	104	44	49	108	162	214	80	67	84	79	15	5	25	24	14	6	13	53	6	1	1	1	