Published in final edited form as:

Mutat Res. 2012 August 15; 746(2): 104–112. doi:10.1016/j.mrgentox.2011.12.017.

Sources of Variance in Baseline Gene Expression in the Rodent Liver

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Abstract

The use of gene expression profiling in both clinical and laboratory settings would be enhanced by better characterization of variation due to individual, environmental, and technical factors.

Analysis of microarray data from untreated or vehicle-treated animals within the control arm of toxicogenomics studies has yielded useful information on baseline fluctuations in liver gene expression in the rodent. Here, studies which highlight contributions of different factors to gene expression variability in the rodent liver are discussed including a large meta-analysis of rat liver, which identified genes that vary in control animals in the absence of chemical treatment. Genes and their pathways that are the most and least variable were identified in a number of these studies. Life stage, fasting, sex, diet, circadian rhythm and liver lobe source can profoundly influence gene expression in the liver. Recognition of biological and technical factors that contribute to variability of background gene expression can help the investigator in the design of an experiment that maximizes sensitivity and reduces the influence of confounders that may lead to misinterpretation of genomic changes. The factors that contribute to variability in liver gene expression in rodents are likely analogous to those contributing to human interindividual variability in drug response and chemical toxicity. Identification of batteries of genes that are altered in a variety of background conditions could be used to predict responses to drugs and chemicals in appropriate models of the human liver.

Keywords

toxicogenomics; baseline expression; microarray; fasting; sex; circadian rhythm; microbiota; life stage; diet

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Animal models are routinely used to assess the risk of exposure to drugs and chemicals for the human population. Whole genome sequencing and microarray technology are powerful tools that can be integrated into traditional toxicity testing strategies for enhanced predictive and mechanistic insights. Variations in study design from laboratory to laboratory or even within laboratories are typical for toxicogenomics studies, but the impact of these variations on gene expression in control animals has not been well characterized. A number of studies, described below, demonstrate the influence of individual study factors on global gene expression. One unique study is discussed in detail that simultaneously assessed the contribution of multiple study factors on global gene expression.

The ILSI Health and Environmental Sciences Institute Baseline Study [1]

The ILSI Health and Environmental Sciences Institute (HESI) Technical Committee on the Application of Genomics in Mechanism Based Risk Assessment generated a publicly accessible dataset of control animal microarray data to serve as a resource for analysis of baseline fluctuations in gene expression due to biological or technical factors. Datasets from control animals within toxicogenomics studies were obtained from HESI participants in the US and Europe. In order to harmonize the appropriate data format and content for the dataset, as well as to increase the feasibility of comparing signal data across multiple sites and conditions, rat liver and kidney gene expression measured on only Affymetrix arrays were collected. Importantly, information was collected on common variables in toxicity studies (e.g., dosing regimen) and other known confounding factors that could potentially affect sensitivity to chemicals in toxicity studies (e.g., strain, supplier, sex, diet, and age) [2].

The resulting large database formed a unique resource for understanding the relationships between study factors and gene expression and is available at both the Chemical Effects in Biological Systems Database (CEBS) [3] and ArrayExpress at European Bioinformatics Institute [4]. Signal data from a total of 536 microarrays was received from 48 studies carried out in 16 institutions. Each study contained a unique combination of treatments and handling conditions. Three Affymetrix rat expression array types were used (RGU34A (n = 192), RAE230A (n = 213), and RAE230 2.0 (n = 131)) for two tissues (liver (n = 396) and kidney (n = 140)), allowing for the creation of 6 tissue-array datasets. The dataset included 3 rat strains (Sprague-Dawley (n = 302), Wistar (n = 210), and F344/N (n = 24)) of both sexes (males (n = 436) and females (n = 100)).

The collected control animal microarray data was analyzed for the contribution of individual study factors to baseline variability in gene expression. Several multivariate techniques were applied separately on each of the six tissue-array data subsets for the study factors. Contributions to variability were assessed using Hotelling-Lawley (HL) and Variance Components [5] scores computed for each of the 17 study factors in each of the six tissue-array sets. The HL score is the Hotelling-Lawley trace statistic for a specific factor computed using the first 10 principal components as a multivariate response and standardized to be between 0 and 1 by dividing by the HL score for the maximal model consisting of all distinct factor combinations [1]. The VC score represents the proportion of variability captured by each study factor and is computed as a weighted average of variance proportion estimates computed by considering the first 10 principal components as separate univariate responses and weighting by the corresponding eigenvalues [1] [6]. Multivariate variability analysis of the baseline expression dataset revealed sex, fasting, and organ section as some of the most prominent factors associated with overall transcriptional changes, although most of the seventeen factors included in the multivariate analyses appeared to be a significant source of variability in at least one of the six tissue-array datasets. Fasting tended to be a stronger source of variability in liver than kidney. Given the heterogeneous structure of the kidney, it was not surprising that organ section was a more

prominent source of variation in kidney than in the more homogeneous liver, in agreement with a study that examined gene expression profiles in kidney slices [7]. While laboratory (or institution) was a clear source of variation, the specific cause could not be discerned by the analysis.

In an extension of the Boedigheimer et al. [1] study, methods for visualizing bias in the data and the effect bias adjustment had on results were examined [8]. A graphical approach was used to detect data bias by plotting the difference between the group mean and overall mean divided by the overall mean of gene expression (RI-plot). A locally weighted scatter-plot smoothing (loess) curve through the data was found to be a highly sensitive technique for visualizing bias in multiple arrays at one time. Interestingly, the loess curves tended to be sinusoidal and were further characterized by the difference in amplitude between the peaks and troughs. A striking negative association was found between the amount of bias in the low intensity regions and the number (or fraction) of probesets detected on an array. A high bias in this region implied not just an overall lower detection rate but lower detection throughout most of the signal intensity range. This finding suggested that for affected probesets, signal intensity is unrelated, or at least not highly related, to transcript abundance. Future work may reveal how to best deal with probesets that exhibit this behavior.

The average intensity of significantly regulated genes was overlaid on the RI plots; significant changes tended to be clustered near the peaks and troughs of the bias. The changes tended to be in the same direction as the bias. It was our assumption that many of these were artifacts because of our expectation that changes due to biological factors would be roughly random with respect to intensity. Despite these notable changes, normalization did not alter the majority of findings regarding major sources contributing to variance in the baseline expression data set. However, the change in the number of significant findings depended on the degree of bias correction. Based on these findings, examining data for smooth bias would be prudent when analyzing data generated at different times or in different laboratories. Plotting significant findings in the context of the bias can reveal potential artifacts that may be removed by loess correction. If the bias is small, then loess correction is likely to yield modest reductions in batch effects and perhaps allow a greater degree of variability to be attributed to experimental sources. If the bias is large, it implies there may be a qualitative difference in the assays and additional scrutiny should be placed on probesets that are near the detection limits. In the cases that were studied, the known sources of biological variability still manifested despite visible systematic bias. These findings bring validity to cross-study analysis of microarray data.

Individual gene variance analysis

Gene expression variance between individuals in control groups may be attributed to differences in environmental factors, genetic background, and measurement protocols. Expression variance that is consistently observed among control animals but cannot be readily attributed to any identified study factor can adversely affect the reliability and reproducibility of toxicogenomics data. A number of studies have identified genes with high or low inherent variability in the mouse or rat liver.

In an early study to assess natural differences in murine gene expression, Pritchard et al. [9] used a 5406-clone spotted cDNA microarray to quantitate transcript levels in the kidney, liver, and testis from 6 male C57BL/6 mice. Compared to the other two tissues, liver exhibited the least number of genes with statistically significant variance (~2%). Pathways involved in immune function, stress response, and hormone regulation had the most variable transcripts. In a recent study, Vedell et al. [10] carried out a comprehensive analysis of transcript abundance variation in four tissues (adipose tissue, heart, kidney, and liver) of

young adult male C57BL/6J mice. By analyzing multiple samples from the same tissue from individual mice, gene variance could be assessed within and between mice. The amount of variation was measured in more than 22,000 protein coding genes and analysis identified groups of genes with shared patterns of variation. Adipose tissue had the largest total and within-mouse variance while liver had the smallest total within-mouse variance, but had the greatest between-mouse variance. Within-mouse transcript variation likely reflected tissue heterogeneity as different samples taken from the same liver were likely to be more homogeneous than those taken from the same fat tissue. Genes with high variability between mice were associated with functions of circadian rhythm, growth hormone signaling, immune response, androgen regulation, lipid metabolism, and the extracellular matrix.

The HESI baseline project [1] identified a subset of probes for which there was evidence of high variance (ranked by variance in the top 5% of expressed targets either across tissue-array sets or for multiple probe sets per array) or relevance to toxicogenomics (defined as inclusion on the Affymetrix GeneChip Rat ToxFX 1.0 array). Using these criteria, 373 targets had reproducible high baseline variance in liver (n = 103), kidney (n = 121), or both tissues (n = 149). Gene pathways and functions most closely associated with high baseline variance were identified using annotation classification programs in the Database for Annotation, Visualization and Integrated Discovery (DAVID) [11, 12][11, 12][11, 12][11, 12]. Forty-three genes highly variant in control liver and kidney samples were enriched and uniquely present in the KEGG pathways for “antigen processing and presentation”, “androgen and estrogen metabolism”, “maturity onset diabetes of the young”, “metabolism of xenobiotics by cytochrome P450”, “glycine, serine, and threonine metabolism”, and “biosynthesis of steroids”. Gene ontology (GO) analysis identified molecular functions 1) monooxygenase or other heme binding activity, 2) cytokine, growth factor, or other receptor binding activity, 3) carbohydrate binding, and 4) transporter activity and biological processes including 1) lipid metabolism, 2) defense response, and 3) transport for the remaining highly variant genes.

From the rat and mouse studies discussed above, toxicology-relevant genes involved in the absorption, distribution, metabolism, and excretion of drugs and chemicals (e.g., members of the ABC and SLC transporter superfamilies, phase I and II enzymes) exhibited high baseline variability. Additional highly variable genes are involved with defense response. Similar classes of genes were observed in a recent study of variability in the human liver transcriptome [13]. Among 75 diverse individuals, the most variable hepatic genes were involved in drug and intermediary metabolism, inflammation, and cell cycle control. One contributing factor to high baseline variability may be increased sensitivity in transcriptional response to environmental cues. Therefore, it follows that some of most variable genes in control animals could also be significantly changed by exposure to drugs or chemicals. In fact ~20% of the genes altered by cisplatin-induced kidney injury [14] or regulated by the PPAR α activator clofibrate [15] exhibited high baseline variability as determined in the Boedigheimer et al. [1] study. Thus, genes with high baseline variability may be identified as statistically different between treatment groups of small size but could be false positives that are independent of treatment.

Genes with low inherent variability are of interest in toxicogenomics studies not only for data normalization but as potential biomarkers for chemical exposure. Low gene variance in liver, kidney, or both tissues were identified across tissue-array sets. A combined non-redundant list of 163 genes with the lowest variance in control animals was compiled for functional annotation analysis. GO analysis of this gene list identified associations with cellular component terms of mitochondrion or other organelle membranes and biological processes associated with transport or defense response. Many of these genes identified with low overall variance have “housekeeping” functions and are often used as controls in gene

expression studies (e.g., *Aldoa*, *Pgam1*, and *Rps2*) [16]. *Aldoa*, *Pgam1*, and *Rps2* described in Lee et al., 2002]. Genes that have low inherent variability in one tissue however, do not necessarily exhibit low variability in other tissues. For example, 8 genes expressed in both liver and kidney were highly variable in control kidney samples, but among the least variable in control liver samples.

Sources of variability

Sex-selective genes

Sex differences are known to exist in response to chemical exposure. For example, females exhibit a higher incidence of drug-induced liver injury [17]. These differences may be due to pharmacokinetic differences in expression of cytochrome P450 genes, phase II metabolism genes, and transporters such as ATP-binding cassette (ABC) transporters [18]. A number of studies have identified genes which exhibit sex selectivity in the mouse liver and have characterized the contribution of possible regulators genetically linked to sex differences [19] or shown experimentally to play important roles as mediators of growth hormone signaling such as *STAT5a*, *STAT5b*, and *HNF4α* [20–22]. Although sex should not be a source of variability in toxicity studies (because the sex of the animals is presumably known!), knowledge of sex differences in gene expression could be used to predict differential responses to chemicals and drugs (discussed below). Additionally, sex genes could be used as a bioset to examine masculinization or feminization after changes in environment including chemical exposure. As an example, the sex genes identified in the HESI baseline study were used to show that older male rats exhibited genomic features of feminization [23].

Prior to the HESI baseline study, very few studies had been carried out to identify sex selective genes in the rat liver. To determine the HESI baseline dataset's utility for identifying genes associated with different experimental factors, genes differentially expressed in males and females were analyzed using EPIG, a profile-based analysis method for Extracting Patterns and Identifying co-expressed Genes which uses signal to noise ratio, correlation and magnitude of change to categorize genes to all significant patterns found in the dataset [24]. Genes were derived from 14 studies carried out in 5 institutions and included datasets from 76 male and 58 female rat livers and 30 male and 28 female rat kidneys. There was no information about the female estrus cycle provided by the data submitters. A total of 854 or 863 genes exhibited significant differences between the sexes in one or more institutions in liver or kidney, respectively. About 30% of these genes exhibited significant differences across more than one institution. Overall, 265 sex-selective genes in liver and 305 genes in kidney showed excellent concordance between institutions in terms of sex-selective expression as well as magnitude of the difference between sexes. While 76 genes consistently exhibited similar sex-selective behavior in both liver and kidney, 22 genes showed reverse patterns of sex-selective expression between liver and kidney. Although the EPIG analysis was performed using microarray data from Wistar and Sprague-Dawley rats, gene expression was validated by RT-PCR using tissues from an independent study using male and female control F344/N rats confirming the robustness of the methods used to identify sex-selective genes and indicating that these genes may be common among different rat strains.

Sex-selective genes fell into a number of functional categories including “xenobiotic metabolism” and “response to xenobiotic stimulus” similar to previously described mouse studies. Many of these genes have been previously characterized as sex-selective, including members of the *CYP2A*, *CYP2C*, and *CYP3A* families [25]. Liver and kidney sex-selective genes also included those involved in “cellular lipid metabolism”, “lipid metabolism” and “fatty acid metabolism”, a number of which were identified in two microarray studies of

gene expression in the livers of male and female rats using much smaller cohorts of animals [26-28]. The sex-selective genes identified in liver were evaluated for common regulatory elements in their promoters. Many of the genes contained STAT1, STAT3, STAT5, and STAT6 sites. STAT1, STAT3, and STAT5 are activated by growth hormone (GH) and STAT5b plays a major role in determining sex-selective gene expression through GH-janus kinase pathways [25]. The sources of biological variability are summarized in Table 1.

Fasting

Overnight fasting is often used in toxicogenomics experiments to reduce variation in tissue gene expression due to differences in *ad libitum* feeding behavior prior to sacrifice. Due to the fact that fasting has known effects on gene expression (see below), some groups allow animals to be fed *ad libitum* up until the time of sacrifice. The HESI baseline study dataset was used to identify genes regulated by fasting in the rat liver. The transcript profiles from a total of 11 studies carried out at 5 institutions were used. Datasets were identified because the providers indicated that food was withheld prior to sacrifice. Although not specified, fasting was likely to have been ~12 to 18 hr in duration, i.e., overnight before sacrifice the next morning. The dataset included 115 liver samples from 85 males and 30 females derived from three strains of rats. Sixty-one animals were fed *ad libitum* (AL) and 54 were fasted. There were a total of 190 or 311 genes that were significantly different using EPIG or t-test, respectively, with ~95% concordance between the two methods. Most of the animals clustered into two distinct groups for AL fed and fasted. However, there were a number of AL animals that clustered with the fasted animals. An examination of the expression profiles from these animals indicated that many of the fasting responsive genes were altered, although not to the extent observed in the fasted animals in most cases. Thus, it is possible that these animals were subjected to fasting conditions for shorter duration either intentionally or because of housing conditions.

Functional categorization showed that fasting altered the expression of genes predominantly involved in lipid utilization, similar to the mouse liver [29]. It should be noted that comprehensive global analysis of fasting-responsive genes in rat liver remains largely uncharacterized. However, the impact of fasting on the interpretation of toxicogenomics studies has been discussed [30] [31].

Genes differentially regulated by sex or fasting state tended to have high baseline variability. This is not surprising, as these genes are presumably sensitive to nutritional or hormonal signals. Approximately 34% of the sex and fasting genes were included in the 500 RAE230A probe sets that have the highest baseline variability (3% of the total array content) and all sex or fasting genes were included among the top ~31% most variant probe sets.

Circadian Rhythm

Circadian rhythms (consisting of 24 hr cycles) have been observed in virtually all aspects of mammalian function from complex physiological processes to coordinated expression of genes [32]. The master clock which controls responses is present in the suprachiasmatic nucleus (SCN) located in the anterior part of the hypothalamus. Peripheral clocks found in other parts of the body are under control of this master clock. Circadian rhythms in genome-wide mRNA expression are regulated by components of this core clock mechanism, which in turn regulate various biological processes. The central clock anticipates the change in photoperiods, allowing the animal to be prepared for the upcoming period of activity and feeding. Disruption of circadian rhythms can be either the cause or the effect of various disorders including metabolic syndrome, inflammatory diseases, and cancer [32]. Circadian disruption accelerates diethylnitrosamine-induced liver carcinogenesis in mice [33].

Importantly, rhythmic variations in biological processes can affect response to drugs through changes in absorption/distribution, excretion, and protein binding. For example, Bruckner et al. [34] showed that indicators of liver damage in rats measured 24 hrs post dosing of carbon tetrachloride exhibited distinct maxima in both fed and fasted animals near the beginning of their dark/active cycle. Understanding the regulation of circadian rhythms in gene expression has been useful in both optimizing the dosing time for existing drugs and in the development of new therapeutics targeting the molecular clock [35, 36].

Circadian effects on the mouse hepatic transcriptome have been well documented; characterization of circadian gene networks has been facilitated using mice nullizygous for central controllers of circadian rhythm. Akhtar et al. [37] used a custom-made cDNA microarray to show that approximately 9% of the 2122 genes studied showed circadian cycling in the liver. Circadian regulation of genes was tissue specific, as rhythmic liver genes were not necessarily rhythmic in the brain, even when found to be expressed in the SCN. Surgical ablation of the SCN severely dampened or completely destroyed the cyclical expression of both well known circadian genes and novel genes identified by microarray analysis. Circadian programming of the transcriptome in a peripheral organ is imposed across a wide range of core cellular functions and is dependent on interactions between intrinsic, tissue-specific factors, and extrinsic regulation by the SCN central pacemaker. Oishi et al. [38] examined the dependence of circadian transcription in the liver on a major controller of circadian rhythm called *Clock*. More than 100 circadian genes were found to be dependent on *Clock*. Additionally, mice double nullizygous for Cryptochrome 1 and 2 genes (*Cry*; a class of blue light photoreceptor proteins) had elevated expression of most *CLOCK*-regulated genes during the upper range of normal oscillation. A deeper analysis of the genes regulated by *Clock* and *Cry* genes showed that *Clock* controlled the circadian transcription of one set of liver genes (typified by the transcription factors DBP, TEF, and *Usp2*) and partially with another set (including *mPer1*, *mPer2*, *mDec1*, *Nocturnin*, *P450* oxidoreductase, and *FKBP51*). These results demonstrated that *CLOCK* and *CRY* proteins are involved in the transcriptional regulation of many circadian output genes in the mouse liver, many of which regulate or are directly involved in drug metabolism.

Although we know less about the major controllers of the rat hepatic circadian rhythm, genome-wide studies have identified genes influenced by circadian rhythm that are drug targets and/or linked to drug and chemical metabolism. Kita et al. [30] examined relative levels of gene expression in the rat liver using a microarray with 8448 transcripts in the rat UniGene database (1928 known genes, 6520 unknown ESTs) as a function of time of day and also feeding regime, common variables in preclinical pharmacogenomic studies. Hundreds of genes (597 genes) were identified, including several in key metabolic pathways, whose relative expression levels were significantly affected by time of day. The expression of certain genes was further modified by feeding state. Boorman et al. [39] examined temporal hepatic gene expression in 48 untreated F344/N rats. The analysis identified numerous periodically expressed genes in these samples including period genes, clock genes, clock-controlled genes, and genes involved in metabolic pathways. Furthermore, rhythms in gene expression were identified for several circadian genes not previously reported in the rat liver. Overall the results of the mouse and rat studies demonstrate that circadian rhythm effects on liver gene expression should be considered a critical factor in planning toxicogenomics experiments.

In an effort to identify circadian genes that can influence drug action, including pharmacodynamic responses due to availability or functioning of drug targets, Almon et al. [40] examined the circadian variations in rat liver gene expression. Animals were maintained on a light/dark regimen consisting of 12-hr light/12-hr dark and 18 time points were analyzed within the 24-hr cycle. Of the more than 15,000 probe sets on these arrays,

265 exhibited oscillations with a 24 hr frequency. Five distinct circadian clusters were identified, with approximately two thirds of the transcripts reaching maximal expression during the dark/active period. Of the 265 probe sets, 107 were identified as having potential therapeutic importance.

The hypothalamic/pituitary/adrenal axis (HPA) is of particular importance to the active feeding period. The HPA effector hormones, glucocorticoids, are at high circulating levels during the light period in diurnal animals and during the dark period in nocturnal animals [41]. Changes in glucocorticoids are thought to directly regulate specific mRNAs [42]. Animals were exposed to corticosteroids to determine whether or not genes linked to circadian rhythm were also targets of glucocorticoid regulation. While some overlap was found, most genes were not targets of both conditions. Because synthetic glucocorticoids are a widely used class of drugs, future research may focus on the timing of glucocorticoid administration in humans.

In addition to the glucocorticoid-regulated genes, Almon et al. [40] identified several groups of genes that are linked to drug treatments. These include chemotherapies which regulate cell cycle and apoptosis. As noted by the authors, the rhythmic nature of the genes in cancer versus normal tissues is uncertain and targeting these genes with drugs in cancerous cells based on knowledge of circadian rhythm could potentially impact the balance between toxic and therapeutic effects. Knowledge of the circadian expression of drug targets in normal cells, however, has the potential to reduce drug toxicity while increasing efficacy toward killing dividing cancer cells.

Another group of circadian genes that were identified in the Almon et al. [40] study are closely associated with hypocholesterolemic drug strategies. Identified genes included *HMG-CoA reductase* (the target for statin drugs), *squalene epoxidase* (involved in cholesterol synthesis), and *Cyp7a1* (a fibrate drug target involved in the conversion of cholesterol to bile acids). This cluster of genes reached maximum expression 4 hr after the start of the dark/active period. Toxic side effects, which may be partially dictated by circadian gene expression, include destabilization of muscle membranes and the development of rhabdomyolysis. This information may impact when a patient is directed to take a statin (for example, avoid taking before bed).

Diet

A number of studies have explored the effects of dietary components on the hepatic transcriptome. These studies demonstrate that dietary components can regulate some of the same genes that are altered by chemical exposure. Two studies have examined the effects of protein quantity and quality. Endo et al. [43] showed that under two different states of protein intake (protein-free diet versus a casein diet for 1 wk), hundreds of genes were altered in the rat liver. Additionally, many genes were altered in the livers of rats fed a wheat gluten diet compared with those fed the casein diet. A subset of genes was associated with cholesterol biosynthesis and disposal pathways. In a second study, Iqbal et al. [44] compared liver gene expression in obese Zucker rats (fa/fa) on a low- or a high-isoflavone soy protein diet with animals on a casein diet. Many of the 62 genes identified were also targets of chemical treatment.

Two studies examined the effects of lipid components in the diet. In the first study [45], gene expression levels were examined in the livers of C57BL/6J mice fed mouse chow, an atherogenic diet containing cholesterol, cholate, and fat, or modified versions of the atherogenic diet in which cholesterol, cholate, or fat were omitted. Cholesterol was found to be required for induction of genes involved in acute inflammation, while cholate induced the expression of genes involved in extracellular matrix deposition in hepatic fibrosis. Elevated

circulating serum amyloid A levels and accumulation of collagen in the liver was found to require cholesterol and cholate, respectively. These biochemical measurements correlated well with the gene expression findings. In the second study, Takahashi et al. [46] examined the effects of fish oil diet on the gene expression profile in mouse liver. Gene expression was analyzed after 6 months of feeding a high-fat diet (60% of total energy intake) as either safflower oil or fish oil derived from tuna. The fish oil diet altered the expression of immune reaction-related genes, antioxidant genes (several *glutathione transferases*, *uncoupling protein 2*, and *Mn-superoxide dismutase*), and lipid catabolism-related genes. Gene expression changes due to fish oil mimicked changes seen with PPAR α activation, leading authors of the study to conclude fish oil must be regulating this nuclear receptor.

These studies demonstrate that diet can have an impact on liver gene expression. However, the type of diet is not always reported in published toxicogenomics studies or is listed as “standard laboratory chow”. A common laboratory chow is the Purina Laboratory Rodent diet 5001 (LRD-5001), which contains fish meal. This diet, not surprisingly, has been reported to contain varying and often high concentrations of methylmercury [47]. In contrast, the purified AIN-76A diet contains casein as a major protein source, as well as refined sources of minerals and vitamins in standard proportions with minimal variability from lot-to-lot.

In an important study, diet was shown to mask arsenic-induced gene expression in the mouse liver [48]. Microarray analysis demonstrated that LRD-5001 fed animals displayed a significantly higher hepatic expression of genes involved in xenobiotic metabolism including Phase I and II metabolism genes compared to AIN-76A fed animals. Remarkably, when the mice were exposed to 100 ppb arsenic in the drinking water, the LRD-5001 diet masked arsenic-induced gene expression changes that were observable in animals fed the AIN-76A diet. The masking of effects in the LRD-5001 diet was likely due in part to the high levels of a mixture of inorganic and organic arsenic measured at a total concentration of 390 ppb, while the AIN-76A diet contained ~ 20 ppb. These findings indicate that dietary effects may have a profound influence on the ability to study and interpret effects of xenobiotics and other treatments on drug metabolism. These results also promote the use of purified diets with known contaminant levels.

Life stage

While most toxicogenomic studies use young adult animals in acute or subchronic exposure studies, animals representative of different human *life stages* have been used to determine the effects of drugs or xenobiotics on gene expression and physiology. Pharmacokinetic differences between the fetus, neonates, juveniles, and the aged may alter responses to chemicals compared to adults. These differences may result in altered therapeutic drug efficacy and sensitivity to drugs and environmentally-relevant chemicals. Numerous phenotypic and genomic changes take place in the liver during early development including hematopoiesis. During the late fetal and neonatal stages, the liver initiates gene expression associated with liver maturation and architecture. In addition, hepatocytes begin expressing xenobiotic metabolizing enzymes and transporters (XMETs), including *CYP* genes [49-51]. Many environmental chemicals and drugs are known to cause unwanted effects in the embryo or fetus, including *in utero* death and birth defects, determined in part by XMET expression. Likewise, pharmacokinetic changes in the elderly can be attributed in part to decreases in liver volume and diminished hepatobiliary functions including decreased phase I drug metabolism capability [52]. Dramatic changes in the expression of some XMETs have been observed in the livers of aged rats [23, 53]. The elderly often are susceptible to disease and prescribed several drugs concurrently to treat these burdens. As a result, predicting response to chemicals and drugs in this population can be complicated.

In a recent study, genome-wide mRNA expression was measured in fetal (gestation day (GD) 19), neonatal (postnatal day (PND) 7), prepubescent (PND32), adult (reference population, 2 or 6 months), middle age (12 months), and old age (18 and 24 months)

C57BL/6J (C57) mouse livers (Lee et al., submitted). Fetal and neonatal life stages exhibited dramatic differences in XME mRNA expression compared to the relatively minor effects of old age. At all life stages compared to the adult, with the exception of PND32, under-expressed genes outnumbered over-expressed genes. Many altered XMETs were associated with the major metabolic and transport phases, including introduction of reactive or polar groups (Phase I), conjugation (Phase II) and excretion (Phase III). In the fetus and neonate, parallel increases in expression were noted in the dioxin receptor and Nrf2 components and their regulated genes while nuclear receptors and their regulated genes were generally down-regulated. Male-specific XMET suppression was observed at early (GD19, PND7) and to a lesser extent, later life stages (18 and 24 months). A number of female-specific XMETs exhibited a spike in expression during PND7. This study revealed dramatic differences in the expression of the XMETs, especially in the fetus and neonate, partially dependent on sex-dependent factors. These results are consistent with a number of studies examining the expression of individual XMETs at various *life stages* [54-57].

In summary, there are profound differences in xenobiotic metabolism gene expression during liver development. With some exceptions, the vast majority of genes exhibit decreased levels of expression compared to adults. Reduced xenobiotic expression and subsequent metabolic capacity could result in prolonged chemical effects including toxicity in the fetus. XMET expression may therefore be used to predict life stage- specific responses to environmental chemicals and drugs (discussed below).

Intestinal microbiota

Data accumulated over the last decade strongly support the hypothesis that the commensal intestinal flora has profound effects on the physiology of the host. There is an intimate relationship between the intestine and the liver. Nutrients and metabolites are transported from the intestine to the liver via the afferent pathways, the portal vein, and the lymphatic system. Conversely, the efferent bile ducts transport molecules from the liver to the intestine via the enterohepatic circulation system. A partially characterized array of metabolites circulates between the intestine and liver. For example, intestinal microbes can alter the profile of lipids that reach the liver to be metabolized [58].

While many of the effects are found locally in the intestine, recent work indicates that the flora contributes to multisystemic diseases including obesity and diabetes [59]. As an example, Fox et al. [60] demonstrated the impact of gut flora on liver cancer. Hepatocellular carcinoma (HCC) frequently results from synergism between chemicals and infectious liver carcinogens. The highest incidence of HCC is in regions endemic for the foodborne contaminant aflatoxin B1 (AFB1) and hepatitis B virus (HBV) infection. In the study, liver tumors were quantitated at 40 weeks in *Helicobacter hepaticus*-free C3H/HeN mice inoculated with AFB1 and/or HBV. Remarkably, intestinal colonization by *H. hepaticus* was sufficient to promote aflatoxin- and HBV transgene-induced HCC in the absence of bacterial translocation to the liver or induction of hepatitis. *H. hepaticus* in the intestinal mucosal layer activated nuclear factor-kappaB-regulated gene networks, associated with adaptive immunity in the liver. The results indicate that enteric microbiota define HCC risk in mice exposed to carcinogenic chemicals or hepatitis virus transgenes.

Two recent studies highlight the impact of gut flora on background gene expression in the mouse liver [61, 62]. In both microarray studies, gene expression was examined in the livers of mice that were germ free (GF) versus those that carried gut flora but were specific

pathogen free (SPF). In both studies the microbiota had extensive effects on hepatic gene expression, especially those involved in xenobiotic metabolism (although not necessarily in the same direction for the same genes). Physiological effects of the gene expression changes were confirmed in the Bjorkholm et al. study [62]. In GF mice, the liver to body weight ratios were lower and anesthesia effects were shorter compared to SPF mice. These studies indicate that differences in the colonizing microbiota can not only contribute to background differences in gene expression in the liver but, importantly, the microbiota may affect the ability of the rodent to respond to chemicals.

Lobe-specific responses

Compared to other tissues, the liver has a relatively uniform gross appearance. However, there is evidence of functional heterogeneity among individual liver lobes in the absence or presence of environmental factors. The physiological basis for these differences is likely due to left-right asymmetric portal blood flow during development [63], where fetal left liver lobe receives more nutrients than other regions of the liver [64]. Thus, differences between liver lobes may be programmed during development. As an example, the adult offspring of Wistar rat dams fed a low protein diet during pregnancy exhibited reduced fibrinogen mRNA and protein levels only in the left lobe of the liver [64].

The adult liver exhibits heterogeneity at a number of anatomical levels. The left lobe has one primary portal branch while the median lobe has two portal branches, one shared with the left lobe [65, 66]. On a microscopic level, a liver lobule consists of a number of different cell types including hepatocytes that are arranged along the sinusoids carrying blood that flows from the portal to the central veins. There is a gradient of nutrient levels and oxygen tension across the hepatic lobule. There are numerous examples of differential gene and protein expression across the lobule [67, 68]. These differences in structure across the liver contribute to different responses to disease states and chemical exposure. In human infants, iron accumulates more in the left hepatic lobe [69], an effect also seen in iron storage disease in adults [70]. Similarly, copper tends to accumulate more in the left hepatic lobe in newborn human infants [71], while in Wilson's disease more copper accumulates in the right hepatic lobe [72]. In rats treated with the initiating agent diethyl-nitrosamine (DEN), there is a higher incidence of DNA damage and carcinomas in the left and median lobes as compared to the right lobe [73]. Cirrhosis progressed more rapidly in the right lobe compared to the left [74]. In A/J mice infected with *Helicobacter hepaticus*, a lobe-specific distribution of the lipid peroxidation DNA adduct (cyclic pyrimidopurine N-1,N(2) malondialdehyde-deoxyguanosine (M1dG) adduct) was noted in both infected and control mice. The lowest level of the adduct was in the left lobe compared with the right and median lobes, while levels of another DNA adduct (8-hydroxy-deoxyguanosine) were significantly greater in the median compared with the left lobe at 12 weeks after treatment [75].

Only two studies have measured global transcript profiles in different lobes of the liver. In a study of acetaminophen toxicity in F344 rats, there were differences in the total number of differentially expressed genes in the left versus median lobes, as well as the total number and identity of genes that were differentially expressed uniquely in each of the lobes [76]. In Sprague-Dawley rats exposed to furan for up to 14 days, similar gene expression changes were observed in all lobes, but the magnitude of changes was more pronounced in the right lobe [77]. In summary, these examples underscore the importance of consistency in sampling the same lobe in toxicogenomics experiments.

Future directions

Variability in hepatic gene expression is inherent in human populations that have different genetic backgrounds, ages, diets, and exposures to chemicals, drugs, and disease. A

challenge for toxicologists is to predict chemical or drug toxicity in sensitive subpopulations. One way to approach this problem is to use gene or protein expression differences to predict differential response to chemicals or drugs in an experimental system that would be representative of different human subpopulations. Here, a number of factors that affect variability in hepatic gene expression in experimental animals were discussed which also have relevance to the human condition. To identify potentially sensitive subpopulations, investigators would need to have the following information: 1) quantitative differences in gene/protein expression and protein function between two populations (for example, a fetal versus an adult population), 2) knowledge of the functions of those proteins which exhibit expression differences, and 3) knowledge of the impact of those functions on chemical metabolism and toxicity.

Starting with defined gene or protein differences between two populations derived from microarray or proteomic studies, commercial databases (e.g., Ingenuity, MetaCore) could be queried to extract some of the known interactions between proteins and chemicals. Alternatively, a public repository of relevant information is found in the Comparative Toxicogenomics Database (CTD), which catalogs interactions between genes, chemicals, and diseases [78]. Preliminary work in our laboratory (Corton et al., unpublished) examined the usefulness of this database to identify chemicals predicted to have differential effects in two populations of mice. Using gene expression changes between the fetal and adult liver, we identified chemicals that interacted with proteins encoded by the altered genes. These chemicals can be extracted from the CTD by querying the database for genes/proteins that have effects on chemicals, drugs, or nutrients (for example, “gene X affects the metabolism of chemical Y”). Interactions can be further defined by genomic studies using the direction and degree of change in gene expression. Chemicals which exhibit similarities in the pattern of interacting genes/proteins could lead to numerous hypotheses of whether or not gene/protein expression differences between the populations can be used to predict chemical sensitivity. The challenge, however, is testing these hypotheses. Animal studies, although more relevant, would be tedious and costly. An attractive alternative would be to recapitulate the essential features of the populations of interest using cell models. This would allow more high-throughput assessment of hundreds or thousands of chemicals. Because of these advantages, there is intense interest in “next-generation” liver models that retain hepatic expression for extended periods of time but also retain other cell types, in addition to the hepatocytes that are important for liver toxicity. However, the technical hurdle to create *in vitro* models that retain the genomic and phenotypic differences of the population still needs to be overcome.

Future studies will capitalize on advances made by the HESI baseline study. The next effort will be the HESI's Genomics Committee's Baseline Animal and Reference Toxicogenomics Data Exchange (BARTDEX) Project. The BARTDEX project aims to facilitate cross-institution data sharing and to improve the usability of public databases. This project will involve a multiphase project starting with a collection of baseline array data for other species and organs. Importantly, reference toxicant data will be collected to assess the effect of different experimental factors on adaptation, toxicology, and/or recovery. It is hoped that there will be contributions of baseline array data and associated metadata (i.e., information about study factors) from rat heart, liver, kidney, blood, and muscle, as well as mouse liver. No array platform will be excluded from this study.

Concluding comments

Through the efforts of the HESI Committee on Genomics, a unique resource has been assembled that connects gene expression levels in the liver and kidney of rats from the control arms of numerous, diverse toxicogenomics studies to specific study meta-data [1]. One outcome from this project was to identify key descriptors that are essential to interpret

results of independent toxicogenomics studies. Variability in baseline gene expression was identified as an important factor. Sex, diet, tissue section, fasting, and life stage can all play a role in defining baseline gene expression. Even in cases where known experimental factors are controlled, there are genes that show inherent variability. Future experiments could be designed to uncover the factors that can account for this variability.

The observational nature of the HESI study hindered causal inference of the influence of all study factors on variability. While it was hypothesized that certain factors within a study may contribute disproportionately to the observed variance (e.g., the use of oil as a vehicle or a diet lower in protein or phytoestrogens), many of these factors could not be confirmed with the current dataset because of confounding factors. Hypotheses generated from these data could be tested by experiments specifically focused on the influence of specific factors of interest. This dataset is available for further data mining in the CEBS and EBI ArrayExpress public databases. Investigators are encouraged to use the control animal dataset for exploratory analysis of gene changes associated with the different study factors.

Acknowledgments

We would like to thank Drs. Brian Chorley and Mitch Rosen for their critical review, the HESI Genomics Technical Committee for supporting the work, and to members of the committee who contributed microarray data for this and on-going analyses. Some of the work described was carried out by a subgroup of the HESI Application of Genomics to Mechanism-Based Risk Assessment Technical Committee, funded through ILSI HESI. This research was supported by the Division of the National Toxicology Program, the Intramural Research Program of the National Institutes of Health (NIH) and National Institute of Environmental Health Sciences (NIEHS) [Z01 ES102345-04]. The information in this document has been supported by the U.S. Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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- We highlight contributions of different factors to gene expression variability.
- Life stage, fasting, sex, diet, and circadian rhythm are sources of variability.
- These factors also contribute to human interindividual variability in drug response.
- Genes linked to factors could be used to predict responses to drugs and chemicals.

Table 1

Factors which influence baseline gene expression in the rodent liver.

Factor	Species	References
Sex	Mice	[19-22]
	Rat	[1, 26-28]
Fasting	Mice	[29]
	Rat	[1]
Orcadian rhythm	Mice	[37,37]
	Rat	[30,38,39]
Diet	Mice	[44,45,47]
	Rat	[42,43]
<i>Life stage</i>	Mice	Lee et al., submitted
Intestinal microbiota	Mice	[60,61]
Liver lobe	Rat	[75,76]