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Effects of moderate drinking during pregnancy on placental gene expression

Martina J. Rosenberg, **Christina R. Wolff**, **Ahmed El-Emawy**, **Miranda C. Staples**, **Nora I. Perrone-Bizzozero**, and **Daniel D. Savage***
Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA

Abstract

Many children adversely affected by maternal drinking during pregnancy cannot be identified early in life using current diagnostic criteria for fetal alcohol spectrum disorder (FASD). We conducted a preliminary investigation to determine whether ethanol-induced alterations in placental gene expression may have some utility as a diagnostic indicator of maternal drinking during pregnancy and as a prognostic indicator of risk for adverse neurobehavioral outcomes in affected offspring. Pregnant Long-Evans rats voluntarily consumed either a 0 or 5% ethanol solution 4 h each day throughout gestation. Ethanol consumption produced a mean maternal daily intermittent peak serum ethanol concentration of 84 mg/dL. Placentas were harvested on gestational day 20 for gene expression studies. Microarray analysis of more than 28,000 genes revealed that the expression of 304 known genes was altered twofold or greater in placenta from ethanol-consuming dams compared with controls. About 76% of these genes were repressed in ethanol-exposed placentas. Gene expression changes involved proteins associated with central nervous system development; organ morphogenesis; immunological responses; endocrine function; ion homeostasis; and skeletal, cardiovascular, and cartilage development. To date, quantitative real-time polymerase chain reaction analysis has confirmed significant alterations in gene expression for 22 genes, including genes encoding for three calcium binding proteins, two matrix metalloproteinases, the cannabinoid 1, galanin 2 and toll-like receptor 4, iodothyronine deiodinase 2, 11-β hydroxysteroid dehydrogenase 2, placental growth factor, transforming growth factor alpha, gremlin 1, and epithelial growth factor (EGF)-containing extracellular matrix protein. These results suggest that the expression of a sufficiently large number of placental mRNAs is altered after moderate drinking during pregnancy to warrant more detailed investigation of the placenta as a biomarker system for maternal drinking during pregnancy and as an early indicator of FASD. Furthermore, these results provide new insights into novel mechanisms on how ethanol may directly or indirectly mediate its teratogenic effects through alterations in placental function during pregnancy.

Keywords

Fetal alcohol spectrum disorder; Ethanol; Placenta; Microarray; qRT-PCR; Biomarker

Introduction

Heavy or binge patterns of drinking during pregnancy can cause profound morphological and neurological aberrations in offspring called fetal alcohol syndrome (FAS; Clarren and

* Corresponding author. Tel.: +1-505-272-8808; fax: +1-505-272-8082. dsavage@salud.unm.edu (D.D. Savage)..

Smith, 1978; Jones et al., 1973; Jones and Smith, 1973; Lemoine et al., 1968). Increasing evidence indicates that moderate drinking during pregnancy can cause subtle, long-term behavioral and cognitive impairments in the absence of the birth defects associated with FAS (Abel, 1995; Hanson et al., 1978; Shaywitz et al., 1980). These behavioral deficits may not become apparent until the educational years (Conry 1990; Hamilton et al., 2003; Jacobson et al., 1998; Streissguth et al., 1990) and may increase in severity as the child matures (Jacobson et al., 2004; Streissguth et al., 1991, 1994). Collectively, these observations led to an expansion of the diagnostic classification of prenatal alcohol-related effects to include alcohol-related neurodevelopmental disorder (Stratton et al., 1996) and, subsequently, fetal alcohol spectrum disorder (FASD) to encompass the entire range of fetal ethanol-affected children.

Current estimates suggest that at least 1% of the pediatric population has FASD (May et al., 2007, 2008; Sampson et al., 1997), and that a large majority of this group may have no physical evidence of prenatal alcohol effects at birth. In such cases, adverse neurobehavioral consequences may not be diagnosed for years, diminishing the beneficial prospect of earlier interventional opportunities. Thus, one of the critical challenges for the fetal alcohol research community is to develop more sensitive and reliable means to detect moderate drinking during pregnancy. Ideally, a reliable indicator of prenatal ethanol exposure may also serve as a predictor of functional damage to newborns, allowing earlier identification of children at risk for longer-term adverse neurobehavioral outcomes.

One approach to this clinical challenge has focused on the identification of biomarkers of alcohol consumption as a means to confirm maternal drinking during pregnancy. A relatively small number of studies on biomarkers of drinking during pregnancy have been reported (see review by Bearer, 2001a). Most of these efforts have been clinical studies of serum biomarkers and have focused either on measurements of ethanol, ethanol metabolites, compounds that chemically interact with ethanol, or a variety of proteins either directly involved in ethanol metabolism or impacted indirectly as a consequence of ethanol metabolism. A recurring theme in most of these clinical studies is that the sensitivity of these biomarkers is generally limited to heavy drinking, these ethanol-induced changes are often short-lived with abstinence, and specificity is impacted by confounding variables present in subject populations (Bearer, 2001).

Another biomarker that appears to have greater sensitivity and specificity for detecting drinking during pregnancy are the fatty acid ethyl esters (FAEEs) ethyl linolate, ethyl oleate, and ethyl arachidonate, which accumulate in maternal liver, placenta, fetal tissues, meconium, and hair after ethanol consumption (Bearer et al., 1999, 2003a; Bearer, 2001a; Kulaga et al., 2006). FAEEs have a half-life of approximately 7 days in mouse placenta (Bearer et al., 1992) and have been detected in umbilical cord blood and meconium from newborns of alcoholic mothers (Bearer et al., 1999, 2003b, 2005). However, FAEEs have also been found in some abstinent groups (Bearer, 2001a) and their sensitivity for moderate drinking during pregnancy is not firmly established at present, possibly indicating a need for more sensitive analytical approaches for detecting these compounds in clinical samples (Pichini et al., 2008).

One alternative strategy to the challenge of diagnosing drinking during pregnancy is to use a bottom-up approach, where biomarkers are first identified and validated in animal models of drinking during pregnancy and then, based on this information, pursue parallel human studies to assess clinical utility. This approach has four distinct advantages. First, it increases the prospects of identifying novel markers without the confounding variables associated with patient populations. Second, biomarker validation can proceed in a more systematic and controlled fashion over a shorter period of time and in a more cost-effective

manner. Third, an animal model system provides an opportunity to assess more directly how a biomarker signature may change as a function of ethanol dosing, patterns of ethanol exposure, and the influence of other interacting risk factors during pregnancy, such as concomitant exposure to nicotine, other drugs of abuse, stress, malnutrition, or heavy metals. How a biosignature pattern is altered by concurrent exposure to other risk factors would be critically important to the interpretation of data from clinical studies. Finally, an animal model system allows for direct correlation of biomarker patterns with markers of functional damage to the fetus, longer-term adverse neurobehavioral outcomes and, in the best-case scenario, provide insights about the mechanistic basis for the teratogenic damage—assessments that would be more difficult, if not impossible, to examine in a clinical study.

In the present study, we used a recently developed rat model of voluntary drinking during pregnancy that produces offspring with deficits in hippocampal synaptic plasticity and learning to study the effects of moderate drinking during pregnancy on placental gene expression. From a clinical standpoint, placenta has a number of advantages in consideration as a biomarker tissue given that relatively large quantities are readily obtainable by minimally invasive and inexpensive procedures that are generally ethically acceptable to both the mother and in clinical practice. To date, we have confirmed that moderate drinking during pregnancy significantly altered the expression of nearly two dozen genes, of which the protein products play important roles in placental function and fetal development.

Materials and methods

Materials

All reagents were acquired from Sigma Chemical Company unless indicated otherwise in parenthetical text.

Voluntary drinking paradigm

All procedures involving the use of live rats were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee. Four-month-old Long-Evans rat breeders (Harlan Industries, Indianapolis, IN) were single housed in plastic cages at 22°C and kept on a “reverse” 12-h dark/12-h light schedule (lights on from 2100–0900 h) with Harlan Teklad rodent chow and tap water ad libitum. After 1 week of acclimation to the animal facility, all female rats were provided 0.066% saccharin in tap water for 4 h each day from 1000 to 1400 h. The saccharin water contained 0% ethanol on the first and second day, 2.5% ethanol (vol/vol) on the third and fourth day, and 5% ethanol on the fifth day and thereafter. Daily 4-h consumption of ethanol was monitored for at least 2 weeks, and then, the mean daily ethanol consumption was determined for each female breeder. At the end of 2 weeks of daily ethanol consumption, females that drank less than 1 standard deviation below the mean of the entire group were removed from the study. The remainder of the females were assigned to either a saccharin control or 5% ethanol-drinking group and matched such that the mean prepregnancy ethanol consumption by each group was similar.

Subsequently, females were placed with proven male breeders until pregnant, as indicated by the presence of a vaginal plug. Female rats did not consume ethanol during the breeding procedure. Beginning on gestational day 1, rat dams were provided saccharin water containing either 0 or 5% ethanol for 4 h a day. The volume of 0% ethanol saccharin water provided to the controls was matched with the mean volume of saccharin water consumed by the 5% ethanol-drinking group. Daily 4-h ethanol consumption was recorded for each dam.

Maternal serum ethanol levels

A separate set of 12 rat dams was used to determine serum ethanol concentrations. These dams were run through the same voluntary drinking paradigm as described earlier, except that at the end of the 4-h ethanol consumption episode on each of three alternate days during the third week of gestation, each rat dam was briefly anesthetized with isoflurane. One hundred microlitres of whole blood was collected from the tail vein and immediately mixed with 0.2 mL of 6.6% perchloric acid, was frozen, and was stored at –20°C until assayed. Serum ethanol standards were created by mixing rat whole blood from untreated rats with known amounts of ethanol ranging from 0 to 240 mg ethanol/dL and then mixing 100-μL aliquots of each standard with perchloric acid and storing the standards frozen with the samples. Serum ethanol samples were assayed using a modification of the method of Lundquist (1959).

Tissue harvesting and RNA preparative procedures

On gestational day 20, rat dams were sacrificed, Caesarian sections were performed, and placental tissue was harvested rapidly. The position of each placenta within the uterine horn and the gender of the associated fetus were noted. The placenta was perfused with ice-cold saline to remove blood, frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated from the frozen tissue using the RNeasy kit following the manufacturer's instructions (Qiagen, Valencia, CA), and the yield was determined by spectrophotometry (Nanodrop, Wilmington, DE). Total RNA was assessed with an Agilent 2001 Bioanalyzer using RNA 6000 nanochips (Agilent Technologies, Santa Clara, CA). All samples had a RNA integrity number of 9.8 or higher, indicating high quality RNA (Schroeder et al., 2006).

Microarray analysis

RNA samples from individual placenta were labeled and analyzed separately on GeneChip Rat Genome 230 2.0 Arrays (Affymetrix Inc., Santa Clara, CA). Equal amounts of total RNA (5 μg) were converted into double-stranded cDNA using Superscript II (Invitrogen, Carlsbad, CA). The resulting cDNA was used for the in vitro synthesis of biotin-labeled cRNA using the ENZO Bioarray High Yield RNA Transcript Labeling Kit T7 (Enzo Diagnostics Inc., Farmingdale, NY). After a cleanup step, 15 μg of the antisense cRNA was fragmented for 35 min at 94°C and then used as a probe on the microarray. Immediately following incubation for 16 h at 45°C, the chips were washed and stained with streptavidin–phycoerythrin using a GeneChip Fluidics Station 400 (Affymetrix Inc.). Washing, staining, and scanning were carried out according to the standard Affymetrix protocol.

The raw data were analyzed with the Affymetrix Microarray Analysis Suite (MAS 5.0) and GeneSpring GX 7.3 software (Agilent Technologies, Santa Clara, CA), starting with a per-chip normalization. The microarray data are available from the National Center for Biotechnology Information's Gene Expression Omnibus at http://www.ncbi.nlm.gov/geo/ (Barrett et al., 2005; Edgar et al., 2002) under series accession number GSE18162. All samples had a scaling factor of less than 20 to achieve the same overall intensity (500 RFU). Raw data were adjusted using a pergene normalization step to the median to compare the relative expression profiles of genes that might be expressed at very different absolute levels. Next, samples from the ethanol-exposed group were normalized to the saccharin controls. Normalized data was prefiltered by expression level (> 100 RFU). In addition, only genes that were called “present” (i.e., by intensity of signal and specificity of hybridization to all of the corresponding oligonucleotide probes per set of each gene on the chip) in at least three of the seven samples were analyzed, thereby reducing false-positive calls and removing genes that were not reliably detected (McClintick and Edenberg, 2006). A principal component analysis of the samples demonstrated that all of them passed this

quality control step (data not shown). Significant changes in gene expression were defined using two filters: first by fold change of more than 2 and then by a Student's *t*-test multiple testing correction with a threshold of $P < .05$ for false discovery rate (Benjamini and Hochberg, 1995).

A global characterization of significant genes in gene ontology (GO) categories of biological processes, molecular function, and cellular compartment (Ashburner, Ball et al., 2000; Harris, Clark et al., 2004) was performed using the Gene Ontology Tree Machine tool of Vanderbilt University in Nashville, TN (http://bioinfo.vanderbilt.edu/gotm). Briefly, a list of differentially expressed genes was compared with a list of all genes represented on the Rat Genome 230 2.0 Array. Relatively enriched genes were identified using the GO hypergeometric distribution analysis. Categories were considered significant at $P < .01$.

Real-time quantitative polymerase chain reaction analysis

Total RNA was isolated and quantified as described earlier and stored in aliquots at –80°C until use. First-strand cDNA synthesis from 1 μg of total RNA was performed using Superscript II reverse transcriptase and oligo(dT) primer (Invitrogen, Carlsbad, CA). Gene expression levels in all samples were examined by quantitative real-time polymerase chain reaction (qRT-PCR) reactions using SYBR® green Supermix (BioRad, Hercules, CA) on an ABI 7300 system (Applied Biosystems, Foster City, CA). Using Primer 3 software (Rozen and Skaletsky, 2000), the primer pairs were designed to be exon-spanning if possible to ensure that no product was amplified from genomic DNA and were created to be specific for each gene (as verified by a BLAST search) to a region different from the one used by the oligonucleotides on the Affymetrix chip. Table A1 provides detailed information of the primer sets used in the qRT-PCR studies. In preliminary studies, the optimal concentration for each primer set was determined using 5 ng of template per reaction, and a dissociation curve analysis was performed to ensure that specific amplification was achieved. The amplification conditions consisted of an initial step at 50°C for 2 min, denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Controls included analysis of template-free reactions (both in the reverse transcription and in the PCR reaction), RNA not being reverse transcribed (to detect contamination with DNA in the RNA preparation) and samples treated with RNase A before reverse transcription reaction.

RNA samples were run in triplicate for the genes of interest and for the reference gene within the same experiment. Each experiment was performed three times. Triplicate cycle thresholds (Ct's) of all the experiments were averaged for each sample. The size of the amplicons and specificity of the primer set was verified on a 2% agarose Tris-acetate-EDTA (TAE) gel.

All data were normalized against β-actin as a reference gene. The expression of β-actin was similar in the saccharin and ethanol-exposed groups both in the microarray data and the qRT-PCR experiments. The mean Ct values for all samples were similar, making β-actin an appropriate control. Relative quantification of gene expression, that is, the relative amount of target RNA, was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Results

Voluntary drinking paradigm

Rat dams stably consumed an average of 2.82 ± 0.13 g of ethanol/kg body weight over the 4-h interval each day (approximately 16 mL of 5% ethanol in 0.066% saccharin water). This pattern and level of ethanol consumption produced a mean maternal serum ethanol concentration of 84.0 ± 5.5 mg/dL during the third week of gestation. Ethanol consumption

did not affect maternal weight gain during pregnancy (Table 1). Litter sizes and placental wet weights at harvesting were not different between the two experimental groups.

Microarray analysis of placental gene expression

The Rat Genome 230 2.0 Expression Array contains more than 31,000 probe sets (30,000 transcripts and variants) from more than 28,000 rat genes. Of those, about 53% were detected as being expressed in placental tissue, that is, "present" in at least three of seven samples. After applying our criteria of a minimum twofold change in expression and statistical significance ($P < .05$) based on a Student's *t*-test, 649 genes were identified as significantly altered in the placenta of ethanol-consuming dams compared with the saccharin controls. The whisker box plots shown in Fig. 1A illustrate that the distribution of signal intensities of altered genes among the placental samples within each of the two experimental groups was similar, with the alcohol-exposed samples showing an overall reduction in expression levels. Figure 1B shows an unsupervised hierarchical cluster analysis of expression profile similarity between the two experimental conditions for the 649 identified genes, indicating that the two groups can be clearly distinguished. After excluding expressed sequence tags and unidentified genes, 304 of the identified genes remained. A compilation of the 304 identified genes is available in Table A2.

In general, ethanol consumption repressed placental gene expression. About 76% of the 304 identified genes were downregulated in the placentas harvested from ethanol-consuming dams compared with the controls. Of the 304 selected genes altered by ethanol consumption, 147 were differentially regulated between two- to threefold; 115 genes displayed a three- to fivefold difference in expression; and 40 genes showed differences in expression greater than fivefold (Table 2), including genes encoding proteins involved in a wide array of biological processes associated with placental and fetal development.

Gene ontology analyses

Of 15,389 GO categories surveyed by GO analyses, 77 were significantly enriched in altered placental genes, that is, the odds ratio of experimentally observed differentially transcribed genes to expected genes in a given GO category was greater than 1 with *P* values less than .01. Those enriched categories were divided into three main groups (GO level 1): biological processes, molecular function, and cellular component (localization). The vast majority of enriched gene categories were found within the biological processes component. After applying an exclusion criterion of at least four ethanol-altered genes per category, several categories were significantly overpopulated with alcohol-altered gene products, including nervous system development; organ morphogenesis; immunological responses; ion homeostasis; and skeletal, cardiovascular, and cartilage development (Fig. 2). A prominent category of particular interest to us, within the context of biomarkers for fetal alcohol-related synaptic plasticity and learning deficits, was the nervous system development category, where 31 genes were significantly altered greater than twofold (Table 3) with an enrichment factor of 1.8 ($P < .002$).

Within the molecular function GO categories, five were overpopulated by alcohol-sensitive placenta genes after application of our exclusion criterion (Fig. 3). Most significant among these were the hormone activity category (enrichment factor of 3.9) and the calcium ion–binding category (enrichment factor of 2.1). It is worth mentioning some categories that failed to pass the exclusion criteria of more than four genes/term. Several functional categories consisting of only two to three members have the common theme of binding to protein families dealing with attachment, migration, and organization of cells (i.e., laminin, collagen, actinin). GO cellular component analyses revealed that altered genes coded for proteins at various cellular locations. Most of the proteins in significantly enriched GO

categories are in the extracellular region (95 observed genes, 53.12 expected genes, $P > .0005$).

Real-time polymerase chain reaction confirmation of differential gene expression

Quantitative reverse-transcription (RT-PCR) measurements were conducted to verify placental gene alterations observed in the microarray studies. Thus far, 38 placental genes have been evaluated using qRT-PCR. These genes were selected based primarily on either having a relatively high microarray fold change and/or specific interest related to known placental function or putative teratologic mechanisms of ethanol action, based on the literature. Table 4 summarizes the qRT-PCR results for these genes organized by the cellular location of action of their protein product. Within cellular location, genes are listed from most to least statistically significant. Overall, the mRNA expression values obtained for each gene using qRT-PCR qualitatively mirrored the directional change in the microarray data, but the quantitative differences varied considerably and, in general, were of smaller magnitude compared with the microarray fold-change data. These differences likely reflect technical differences between the two analytical platforms.

In general, qRT-PCR expression values less than 0.2 or greater than 5.0 correlated with statistically significant alterations in gene expression. Six exceptions to this observation were the genes for thyrotropin-release hormone (TRH), orosomucoid 1, matrix metalloproteinase 3 (MMP-3), secreted frizzled-related protein 5, small X-linked muscle protein, and the progesterone receptor. In each of these six cases, the qRT-PCR value was in the same direction as the microarray fold-change data, but greater variability among individual samples in one or both experimental groups and the small group sample sizes resulted in P values greater than .05 but less than .10.

Thus far, 22 of the genes examined were confirmed as significantly altered ($P < .05$) based on qRT-PCR analysis (Table 4). This group includes genes encoding for three isoforms of S100 calcium binding proteins (A4, B, and G), two isoforms of hemoglobin (ε1 and γA), galanin and the galanin 2 receptor, the cannabinoid 1 (CB1) and toll-like 4 receptors, iodothyronine deiodinase 2, 11-β hydroxysteroid dehydrogenase 2 (HSD2), placental growth factor, transforming growth factor (TGF)-α, gremlin 1, EGF-containing extracellular matrix protein, and MMP-2.

Discussion

The salient observation from this study is that intermittent consumption of moderate quantities of ethanol during pregnancy alters the expression of at least 22 placental genes. These alterations occur in the absence of any gross observable effects of ethanol on the mother's weight gain during pregnancy, the placenta at term, fetal litter size, or pup weight (Table 1). Nevertheless, adult offspring of this moderate prenatal ethanol exposure paradigm exhibits hippocampal synaptic plasticity deficits and performance deficits in learning paradigms (unpublished observations), indicating long-lasting functional brain damage in the absence of physical defects at birth. Taken together, these results suggest that placental gene expression may be a more sensitive indicator of moderate ethanol consumption than most current ethanol biomarker systems.

Although we are encouraged by the number of placental gene alterations confirmed to date, we have also identified at least five factors that may have contributed to variability in the results of this initial study that precluded the likely identification of a larger number of ethanol-induced placental gene changes. One factor is the impact of individual genetic variation in an outbred rat stock. However, the ability to identify altered genes in an outbred stock should be considered a strength of this paradigm, as it more accurately models the

human condition and promises that gene alterations that stand out will be a reliable tool for the detection of maternal drinking. A second putative factor contributing to variability may be the voluntary drinking paradigm itself. We endeavored to minimize this by screening female drinking behavior during the prepregnancy period and removing females from the study whose ethanol consumption was greater than 1 standard deviation below the mean of the entire group. Furthermore, although there were some small day-to-day variations in ethanol consumption by individual rat dams, the ethanol-exposed placentas used in this study were harvested from four ethanol-consuming dams whose mean daily ethanol consumption was within the standard error of the mean range shown in Table 1. A third factor relates to intrauterine variability in ethanol's effects (Mitchell et al., 2002). We strove to minimize this factor in our initial study by only selecting placentas attached to female fetuses, from litters with greater than nine pups (average litter size was 13), where at least one of the adjacent fetuses was male and the location of the selected fetal–placental unit was a least one position away from either the proximal or distal end of a uterine horn. Even with the incorporation of these selection criteria, it is likely that ethanol has variable effects within a litter and that this putative effect on gene expression will require more detailed investigation.

A fourth issue relates to the fact that we opted to examine whole placenta in this initial study. The placenta contains multiple cell types and, in some cases, it is clear that some genes are primarily expressed in more discreet regions within the placenta (Sood et al., 2006). Thus, sampling from whole placenta diminished our “signal to noise ratio” for detecting effects of ethanol on gene expression. For example, placental gene and protein expression of the CB1 receptor is primarily located in the syncytiotrophoblast layer near the surface facing the maternal boundary (Park et al., 2003). A similar distribution has been observed for 11β-HSD2 in preliminary in situ hybridization studies (unpublished observations). Although we were able to confirm ethanol-induced gene repression of both the CB_1 receptor and HSD2 in whole placenta (Table 4), the question remains as to how many genes whose expression is heterogeneously distributed across placenta were missed in an analysis of the effects of ethanol on whole placenta. Subsequent histological approaches using in situ hybridization to examine gene expression and immuno- and radiohistochemical approaches for quantitating protein will be required to better address this question. Finally, another factor contributing to variability is that we were limited in our ability to analyze a larger number of samples in a preliminary microarray analyses. It is likely that larger sample sizes would have resulted in more significantly altered genes based on the qRT-PCR analysis. Subsequent studies will use larger sample sizes to better address this point.

Considerable work remains to confirm the utility of placental gene alterations as a biomarker system, both for detecting ethanol consumption and a prognostic indicator of adverse neurobehavioral outcomes in the absence of morphological alterations. Systematic examination of altered gene expression as a function of different levels and patterns of ethanol consumption as well as the persistence of gene alterations after the last drinking episode are critical translational research questions to address. Further, how the presence of other common pregnancy risk factors impacts ethanol-induced alterations in placental gene expression patterns needs to be determined. For example, how will concomitant exposure to such factors as nicotine, other drugs of abuse, stress, malnutrition, or heavy metals modify a biomarker signature pattern? Data from such studies would be critical for interpreting altered patterns of placental gene expression in clinical studies.

The results of this initial study also provide intriguing insights into the implications of maternal drinking on placental function and putative mechanisms of ethanol teratogenesis. At a more global level, the GO analyses of biological processes (Fig. 2) indicated that ethanol has significant effects on genes associated with organ morphogenesis as well as

nervous, endocrine, and immune system development and function. This observation is consistent with a wealth of literature indicating that prenatal ethanol exposure affects organ development (Weinberg, 1994, Byrnes et al., 2003; Qiang et al., 2002; Taylor et al., 1999), particularly the development of these three highly susceptible and critically interactive organ systems. Altered expression of genes associated with vascular, skeletal, and cartilage development, although less investigated in the fetal alcohol research field to date, clearly merit additional study.

Of particular interest was the observation that a number of placental genes altered by moderate ethanol exposure are known to play critical roles in pattern formation during nervous system development. For example, interactions of the members of the TGFβ family, such as bone morphogenic protein (BMP)4 and the BMP4 antagonist chordin, help regulate polarity (i.e., back to front patterning) of the developing embryo (Chesnutt et al., 2004; Millet et al., 2001). Likewise, the products of the WnT and Notch signaling–related genes ASCL2, HeYL, SFRP4, SFRP5, and WnT1 are known to regulate cell fate during the induction of both the central and peripheral nervous system (Ciani and Salinas, 2005; Nakagawa et al., 2001). Further, both the products of SEMA3B, semaphorin 3B (Falk et al., 2005) and of THBS4, thrombospondin (Arber and Caroni, 1996) are important for axonal growth and guidance. It is also important to note that similar levels of these genes occur in both placenta and fetal brain (Genomics Institute of the Novartis Research Foundation website http://biogps.gnf.org). Taken together, these observations suggest that some changes in placental gene expression may be predictive of similar changes in gene expression in fetal brain, and that the placenta could serve as a window on brain development. Follow-up studies examining both placental and fetal brain gene expression in the same placental–fetal brain unit will directly address this supposition and, in the process, could strengthen the prospects of establishing meaningful cause–effect relationships that will further our understanding of ethanol's impact on early developmental processes.

GO analyses of molecular processes also suggested important effects of maternal ethanol consumption on endocrine mechanisms (Fig. 3). Of particular note are the systems that regulate corticosterone and thyroid hormone. The expression of 11β-HSD2 mRNA was significantly reduced by maternal ethanol consumption (Table 4). Placental HSD2 inactivates corticosterone, and the enzyme plays a critical role in regulating the levels of maternal corticosterone that cross the placenta and enter fetal circulation (Michael et al., 2003; Waddel et al., 1998). If reduced gene expression results in diminished HSD2 protein or enzymatic activity, the fetus may be exposed to abnormally high levels of corticosterone, which has been shown to have deleterious effects on brain development (Holmes et al., 2006; Welberg et al., 2000; Weinstock, 2007) and longer-term consequences (see review by Seckl and Holmes, 2007). In contrast to the neuroprotective effects of placental HSD2 during development, placental iodothyronine deiodinase 2 (DIO2) is responsible for the conversion of maternal thyroxine (T_4) to the triiodothyronine (T_3), the physiologically active form of thyroid hormone. Placental conversion of maternal T_4 to T_3 provides the only source of active thyroid hormone to the fetus through most of gestation in rodents (Morreale de Escobar et al., 1987). T_3 regulates the expression of a large number of molecules important in fetal development, including neurotropic factors (Alvarez-Dolado et al., 1994), cytoskeletal elements (Silva and Rudas, 1990), and extracellular matrix molecules, such as L1 (Alvarez-Dolado et al., 2000), which is also affected by relatively low levels of ethanol exposure (see review by Bearer, 2001b). Thus, if diminished DIO2 protein or activity follows from an ethanol-induced reduction in placental DIO2 gene expression (Table 4), the fetus may be subject to a broad array of immediate and prolonged neurodevelopmental consequences as a function of a hypothyroid environment during most of the prenatal period.

GO analysis of molecular processes also suggested important effects of ethanol exposure on calcium ion binding and various types of protein- and carbohydrate-binding interactions in placenta (Fig. 3). Of particular note was ethanol's impact on three of the S100 calcium binding proteins (Table 4). The members of the S100 family are multifunctional signaling proteins that influence with many cellular events. S100B, S100A4, and S100G appear to be involved in neurotrophic and/or neuroprotective processes (Donato 2007; Druse et al., 2007; Santamaria-Kisiel et al., 2006). The literature on the function of these proteins in placental tissue is sparse. However, S100B is highly abundant in the nervous system, predominantly in astroglia, exhibiting temporal and spatial concentration patterns during brain maturation. Although the mechanisms of action of these proteins are not completely understood, they have protective neurotrophic effects during brain development, and alterations may serve as early, quantitative indicators of fetal brain damage in some biological fluids, for example, cord blood (Michetti and Gazzolo, 2002). Of particular note is the observation that S100B acts as a trophic factor for the development of the brainstem serotonergic system, which is adversely affected by prenatal ethanol exposure (Druse et al., 1991; Zhou et al., 2001, 2005).

Other proteins whose gene expression was altered (Table 4) suggest that a number of additional placental functions important for fetal development may be compromised by maternal drinking during pregnancy. However, confirmation of this speculation will require quantitation of protein levels and function in placental tissues. Such studies will be challenging for a number of reasons, including the relative paucity of tools for quantitating these proteins by standard means. Many of these proteins are membrane associated and likely to be scarce enough to be difficult to detect by proteomic approaches. Further, a number of these proteins have not been studied in placental preparations and, in some cases, the function of these proteins is not well understood in any tissue type.

Nevertheless, these challenges do not diminish the diagnostic potential of altered placental gene expression as a biomarker of fetal alcohol exposure and fetal alcohol effects. Even in this preliminary report, a sufficiently large enough number of placental genes were altered by ethanol exposure to warrant more detailed investigation of placenta as a biomarker system. Given that these genes are also expressed in human placenta, it is reasonable to expect that these findings could translate into human studies of drinking during pregnancy. Further, the clinical relevance of our findings is underscored by the fact that these gene changes occur after moderate intermittent ethanol consumption during pregnancy, a level that causes functional brain damage and learning deficits in the absence of any observable dysmorphologic effects in rat offspring. With the growing realization that most of the children with FASD exhibit neurobehavioral deficits in the absence of dysmorphologic features, the discovery of more sensitive biomarkers of fetal alcohol effects becomes an increasingly important objective for earlier diagnosis and treatment of FASD.

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Appendix

Table A1

Primer sets used in quantitative real-time polymerase chain reaction validation of candidate placental genes listed in Table 4

Symbol	Gene name	Affymetrix ID	Forward primer	Reverse primer	Amplicon bp size	Position[a]
AFP	α-Fetoprotein	1367758_at	ACAGGGCGATGTCCATAAAC	TGCCATTGATGCTCTCTTTG	170	5538
ACTB	β-Actin	1398835_at	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC	97	3474
CNRNA7	Nicotinic cholinergic receptor α7	1387419_at	TATCACCACCATGACCCTGA	CAGAAACCATGCACACCAGT	81	121903
CNR1	Cannabinoid receptor 1	1369677_at	AGGAGCAAGGACCTGAGACA	TAACGGTGCTCTTGATGCAG	166	1197
CYP1A1	Cytochrome 450 1A1	1370269_at	TGAGGCTCAACTGTCTTCCAA	TCTTACTGCCCAGAAAGTCTGTC	189	5452
CYP2E1	Cytochrome 450 2E1	1367871_at	TGAGACCACCAGCACAACTC	CTTCATGGGGTAGGTTGGAA	216	6552
DIO2	Iodothyronine deiodinase 2	1385568_at	CTTCCTGGCGCTCTATGACT	ACACTGGAATTGGGAGCATC	189	69
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	1390112_at	CCGGGTTCCTTTTACTGTCA	CCACTTGGTAACCCTGAGGA	286	74674
FMO3	Flavin-containing monooxygenase 3	1368304_at	GAGAAACCAACCATGGCAGT	CTGGGGTCCTTGAGAAACAG	284	15144
GAL	Galanin	1387088_at	AGAGCAATATCGTCCGCACT	GTGTTGGCTTGAGGAGTTGG	216	2972
GALR2	Galanin receptor 2	1384667_x_at	GCTCTGCAAGGCTGTTCATT	GGGTGGCATACTGTCAGGTT	238	325
GREM1	Gremlin 1	1369113_at	GACAAGGCTCAGCACAATGA	CAGGTATTTGCGCTCTGTCA	159	108
Hand2	Heart and neural crest derivatives expressed 2	1369818_at	CAAGGCGGAGATCAAGAAGA	TGGTTTTCTTGTCGTTGCTG	81	94561
HBD	Hemoglobin, delta	1371102_x_at	ATGGCCTGAAACACTTGGAC	GCCCAACACAATCACAATCA	128	335
HBE1	Hemoglobin, gamma 1	1388270_at	GCCTCTGCCATAATGGGTAA	CCTGTACCTCAGCCGTGAAT	232	272
HBG1	Hemoglobin, epsilon 1	1388269_at	TGGGAAAAAGTGGACTTGGA	CCGAACCTAGAGACGTCAGC	178	45
HSD11B2	11β-hydroxysteroid dehydrogenase type 2	1368102_at	GCTATTGCACTGCTCATGGA	GCAATGCCATTCTGAGTGAA	235	4060
HSPA1B	Heat shock 70 kDa protein 1B	1368247_at	CAAGATCACCATCACCAACG	GCTGATCTTGCCCTTGAGAC	193	3326
IGFBP6	Insulin growth factor binding protein 6	1387625_at	CAGAGACCGGCAAAAGAATC	CTGCTTGCGGTAGAAACCTC	193	2779
INS	Insulin	1370077_at	CAGCACCTTTGTGGTTCTCA	CAGTGCCAAGGTCTGAAGGT	165	83
ITGA7	Integrin α7	1388240_a_at	TCGGGAACCCTATGAAGAGA	ATGAAGACATGAGCCCGAAC	160	19564
LBP	Lippopolysaccharide binding protein	1387868_at	AAGGCGCAAGTGAGACTGAT	AGTCGAGGTCGTGGAGCTTA	172	216
MMP10	Matrix metalloproteinase protein 10 (Stromolysin2)	1368713_at	GGATAAAGGCTTCCCGAGAC	TGTGATGATCCACGGAAGAA	111	6274
MMP2	Matrix metalloproteinase protein 2 (gelatinase A)	1370301_at	GGATACAGGTGTGCCAAGGT	TCGGTGAGAAAAATGCAGTG	141	37
MMP3	Matrix metalloproteinase protein 3	1368657_at	GATCGATGCAGCCATTTCTT	CACTTTCCCTGCATTTGGAT	235	9908
ORM2	Orosomucoid 2	1368731_at	TTCAGACCACAGACGACCAG	CATGCCCACATCTTTGACAG	254	650
PGF	Placental growth factor	1368919_at	TGCTGGGAACAACTCAACAG	CAGCGACTCAGAAGGACACA	159	1186
PGR	Progesterone receptor	1387563_at	GAGAGGCAGCTGCTTTCAGT	AAACACCATCAGGCTCATCC	117	42364
S1004A	S100 calcium binding protein A4	1367846_at	CAACGAGGGTGACAAGTTCA	TGCAGGACAGGAAGACACAG	182	1281
S100B	S100 calcium binding protein G	1386903_at	GGTGACAAGCACAAGCTGAA	TGGAGACGAAGGCCATAAAC	172	28294
S100G	S100 calcium binding protein B	1368339_at	CTCTGGCAGCACTCACTGAC	GCTGGGGAACTCTGACTGAA	164	24
SFRP4	Secreted frizzled-related protein 4	1368394_at	TATGACCGTGGAGTGTGCAT	CGATCAGGGCTCAGATGTTT	140	485
SFRP5	Secreted frizzled-related protein 5	1393069_at	TCCTCTGGACAACGACCTCT	CTTAATGCGCATCTTGACCA	163	484
SMPX	Small muscle protein, X-linked	1370165_at	AGCCTCCCAGAAGGAAAGAG	CCATTGAGAAAGCACGTCAA	212	15686
SPINK5	Serine peptidase inhibitor, Kazal type 5	1398688_at	TTAGAGCACCAGCTGAGCAA	GCCTTGTGGACATGACAGTG	202	30
TGFA	Transforming growth factor alpha	1387450_at	GGTTTTTGGTGCAGGAAGAG	GGCACCACTCACAGTGCTT	219	69269
TLR4	Toll-like receptor type 4	1387982_at	TCACAACTTCAGTGGCTGGA	GTCTCCACAGCCACCAGATT	176	5708
TRH	Thyrotropin releasing hormone	1368912_at	CAGAACGTCGATTCTTGTGG	TTCTCCCAAGTCTCCCCTCT	152	1515
TRPC4	Transient receptor potential cation channel C4	1369164_a_at	GATGGCGGACTTCAGGATTA	CAGGTGAGAATTGGCAGTGA	240	100275

[a] Position number refers to the first base of the target sequence from transcription start.

Table A2

Differentially regulated genes (304) in microarray experiment filtered by statistical significance and fold change greater than two, excluding expressed sequence tags (ESTs) and not annotated genes

Affymetrix ID	Symbol	RefSeq	Downregulated gene name	Fold Δ
1369113_at	GREM1	NM_019282	Gremlin 1, cysteine knot SuperFamily	–25.3
1385568_at	DIO2	NM_031720	Deiodinase, iodothyronine type II	–25.2
1387088_at	GAL	NM_033237	Galanin	–21.4
1368731_at	ORM1	NM_053288	Orosomucoid 1	–17.0
1388269_at	HBG1	NM_172093	Hemoglobin, gamma A	–16.2
1390112_at	EFEMP1	NM_001012039	EGF-containing fibulin-like extracellular matrix protein 1	–15.8
1369677_at	CNR1	NM_012784	Cannabinoid receptor 1	–15.5
1368304_at	FMO3	NM_053433	Flavin-containing monooxygenase 3	–14.2
1368394_at	SFRP4	NM_053544	Secreted frizzled-related protein 4	–13.9
1368912_at	TRH	NM_013046	Thyrotropin-releasing hormone	–13.3
1398688_at	SPINK5	XM_341607	Serine peptidase inhibitor, Kazal type 5	–10.7
1374558_at	ICOSLG	XM_574731	Inducible T-cell co-stimulator ligand	–9.31
1387563_at	PGR	NM_022847	Progesterone receptor	–9.21
1369625_at	AQP1	NM_012778	Aquaporin 1	–9.15
1367846_at	S100A4	NM_012618	S100 calcium binding protein A4	–8.78
1367627_at	GATM	NM_031031	Glycine amidinotransferase	–8.26
1370843_at	GNG8	NM_139185	Guanine nucleotide binding protein, gamma 8	–8.25
1369695_at	WT1	NM_031534	Wilms tumor 1	–8.14
1393069_at	SFRP5	XM_219887	Secreted frizzled-related protein 5	–7.86
1369164_a_at	TRPC4	NM_080396	Transient receptor potential cation channel C4	–7.63
1387450_at	TGFA	NM_012671	Transforming growth factor alpha	–7.62
1371102_x_at	HBD	NM_033234	Hemoglobin, delta	–7.35
1369817_at	HAND2	NM_022696	Heart and neural crest derivatives expressed 2	–6.97
1388270_at	HBE1	NM_001008890	Hemoglobin, epsilon 1	–6.91
1368919_at	PGF	NM_053595	Placental growth factor, VEGF-related protein	–6.46
1396407_at	GAS8	NM_001039030	Growth arrest-specific protein 8	–6.43
1380206_at	KIF5C	XM_221307	Kinesin family member 5C	–6.36
1367600_at	DES	NM_022531	Desmin	–6.23
1370157_at	PLN	NM_022707	Phospholamban	–6.03
1388138_at	THBS4	XM_342172	Thrombospondin 4	–5.70
1367794_at	A2M	NM_012488	Alpha2 macroglobulin	–5.62
1387656_at	SLC4A1	NM_012651	Solute carrier family 4, anion exchanger 1	–5.60
1368713_at	MMP10	NM_133514	Matrix metalloproteinase 10 (stromelysin 2)	–5.49
1370956_at	DCN	NM_024129	Decorin	–5.37
1380285_at	CHRD	NM_001024273	Chordin	–5.36
1368342_at	AMPD3	NM_031544	Adenosine monophosphate deaminase E	–5.18
1386903_at	S100B	NM_013191	S100 calcium binding protein B	–4.93

Affymetrix ID	Symbol	RefSeq	Downregulated gene name	Fold Δ
1370301_at	MMP2	NM_031054	Matrix metalloproteinase 2	–4.87
1387295_at	SLC6A12	NM_017335	Solute carrier family 6, betaine/GABA, member 12	–4.69
1387868_at	LBP	NM_017208	Lipopolysaccharide binding protein	–4.66
1382757_at	FOXL2	XM_345975	Forkhead box l2	–4.62
1368362_a_at	ASGR2	NM_017189	Asialoglycoprotein receptor 2	–4.49
1380432_at	CMAH	XM_341876	Cytidine monophosphate-n-acetylneuraminic acid hydroxylase	–4.46
1385182_at	PKP1	XM_222666	Plakophilin 1	–4.41
1387659_at	GDA	NM_031776	Guanine deaminase	–4.33
1390596_at	MLANA	XM_215234	Melan-A	–4.26
1387625_at	IGFBP6	NM_013104	Insulin-like growth factor binding protein 6	–4.26
1383792_at	SYTL1	NM_001025651	Synaptotagmin-like 1	–4.19
1389670_at	HOXA11	XM_575479	Homeobox A11	–4.19
1386921_at	CPE	NM_013128	Carboxypeptidase E	–4.17
1377311_at	EMX2	XM_574698	Empty spiracles homolog 2	–4.11
1388292_at	KCNJ3	NM_031610	Potassium inwardly-rectifying channel J3	–4.08
1387025_at	DYNC1I1	NM_019234	Dynein, cytoplasmic 1, intermediate chain 1	–4.04
1373032_at	MUSTN1	NM_181368	Musculoskeletal embryonic nuclear protein 1	–4.02
1372254_at	SERPING1	NM_199093	Serpin peptidase inhibitor, Clade G (C1 inhibitor)	–3.95
1368413_at	ABP1	NM_022935	Amiloride binding protein 1	–3.93
1368914_at	RUNX1	NM_017325	Runt-related transcription factor 1	–3.93
1372065_at	ART3	NM_001012034	ADP–ribosyltransferase 3	–3.92
1387004_at	NBL1	NM_031609	Neuroblastoma, suppression of tumorigenicity 1	–3.90
1388608_x_at	HBA2	NM_001013853	Hemoglobin, alpha 2	–3.90
1387419_at	CHRNA7	NM_012832	Nicotinic cholinergic receptor alpha 7	–3.89
1367992_at	SCG5	NM_013175	Secretogranin V (7B2 protein)	–3.88
1369773_at	BMP3	NM_017105	Bone morphogenetic protein 3	–3.88
1376198_at	ASAM	NM_173154	Adipocyte-specific adhesion molecule	–3.87
1389160_at	ERAF	XM_215059	Erythroid-associated factor	–3.86
1387200_at	OLIG1	NM_021770	Oligodendrocyte transcription factor 1	–3.84
1369430_at	BCMO1	NM_022862	Beta-carotene 15, 15’-monooxygenase 1	–3.74
1378745_at	PER3	NM_023978	Period homolog 3	–3.73
1368081_at	ABCA2	NM_024396	ATP-binding cassette, sub-family A (ABC1), member 2	–3.71
1369735_at	GAS6	NM_057100	Growth arrest-specific 6	–3.69
1391534_at	ELOVL2	XM_574001	Elongation of very long chain fatty acids-like 2	–3.64
1377867_at	QPCT	XM_233812	Glutaminyl-peptide cyclotransferase	–3.61
1367985_at	ALAS2	NM_013197	Delta-aminolevulinate, synthase 2	–3.60
1372649_at	HSPB7	XM_342966	Heat shock 27 kDa protein family member 7	–3.60
1369572_at	MCPT1	NM_017145	Mast cell protease 1	–3.58
1369464_at	ZP1	NM_133569	Zona pellucida glycoprotein 1	–3.57
1388569_at	SERPINF1	NM_177927	Serpin peptidase I, Clade F (alpha-2 antiplasmin)	–3.52
1393588_at	CLDN14	NM_001013429	Claudin 14	–3.51

Affymetrix ID	Symbol	RefSeq	Downregulated gene name	Fold Δ
1377643_at	HOXD10	XM_221510	Homeobox D10	–3.47
1367566_at	SCGB1A1	NM_013051	Secretoglobin 1A1	–3.46
1378898_at	DDX19A	NM_001005381	Dead box polypeptide 19A	–3.44
1368583_a_at	HRG	NM_133428	Histidine-rich glycoprotein	–3.44
1394316_a_at	TSPAN5	NM_001004090	Tetraspanin 5	–3.42
1391018_at	MYO5C	XM_236411	Myosin VC	–3.41
1372335_at	PCGF1	NM_001007000	Polycomb group ring finger 1	–3.38
1369926_at	GPX3	NM_022525	Glutathione peroxidase 3	–3.35
1369520_a_at	BCAT1	NM_017253	Branched-chain aminotransferase 1	–3.33
1377336_at	SEMA3B	XM_343479	Semaphorin 3B	–3.31
1388170_at	KCTD1	XM_214617	Potassium channel tetramerisation domain-containing 1	–3.32
1367847_at	NUPR1	NM_053611	Nuclear protein 1	–3.27
1368102_at	HSD11B2	NM_017081	Hydroxysteroid (11-beta) dehydrogenase 2	–3.22
1387982_at	TLR4	NM_019178	Toll-like receptor 4	–3.21
1379039_at	CMKLR1	NM_022218	Chemokine-like receptor 1	–3.21
1388204_at	MMP13	XM_343345	Matrix metalloproteinase 13 (collagenase 3)	–3.21
1378673_at	MITF	XM_001065525	Microphthalmia-associated transcription Factor	–3.19
1368167_at	CTSE	NM_012938	Cathepsin E	–3.16
1386637_at	FGL2	NM_053455	Fibrinogen-like 2	–3.14
1368338_at	CD52	NM_053983	CD52 molecule	–3.12
1397516_at	ALG2	XM_232987	Asparagine-linked glycosylation 2 homolog	–3.08
1382612_at	HOXA9	XM_001057018	Homeobox A9	–3.06
1393219_at	C2	NM_172222	Complement component 2	–3.06
1398398_at	HOXA10	XM_347220	Homeobox A10	–3.03
1377729_at	ELOVL4	XM_236476	Elongation of very long chain fatty acids-like 4	–3.03
1389408_at	RRM2	NM_001025740	Ribonucleotide reductase M2 polypeptide	–3.02
1388240_a_at	ITGA7	NM_030842	Integrin, alpha 7	–3.01
1387011_at	LCN2	NM_130741	Lipocalin 2 (oncogene 24p3)	–3.01
1368464_at	CLEC10A	NM_022393	C-type lectin domain 10A	–2.99
1398253_at	KAP	NM_052802	Kidney androgen-regulated protein	–2.99
1379345_at	COL15A1	XM_216399	Collagen, type XV alpha 1	–2.99
1367919_at	NUP210	NM_053322	Nucleoporin 210 kDa	–2.98
1370895_at	COL5A2	XM_343564	Collagen, type V, alpha 2	–2.98
1367998_at	SLPI	XM_215940	Secretory leukocyte peptidase inhibitor	–2.97
1367960_at	ARL4A	NM_019186	ADP-ribosylation factor-like 4A	–2.96
1370665_at	HYOU1	NM_001034028	Hypoxia up-regulated 1	–2.94
1367774_at	GSTA1	NM_031509	Glutathione s-transferase A1	–2.94
1391201_at	WDHD1	XM_223933	WD repeat and HMG-box DNA binding protein 1	–2.92
1390547_at	ST6GALNAC1	XM_221248	ST6	–2.91
1386889_at	SCD2	NM_031841	Stearoyl-coenzyme A desaturase 2	–2.90
1368893_at	CAP2	NM_053874	CAP, adenylate cyclase-associated protein 2	–2.87

Affymetrix ID	Symbol	RefSeq	Downregulated gene name	Fold Δ
1377772_at	TMEFF1	NM_023020	Transmembrane protein w/EGF-& follistatin-like domains 1	–2.84
1368860_at	PHLDA1	NM_017180	Pleckstrin homology-like domain A1	–2.83
1398304_at	FZD2	NM_172035	Frizzled homolog 2	–2.82
1368490_at	CD14	NM_021744	CD14 molecule	–2.82
1383862_at	CLEC2D	XM_342769	C-type lectin domain 2D	–2.79
1385665_at	ADAM19	XM_220328	ADAM metallopeptidase domain 19 (Meltrin beta)	–2.76
1386884_at	HTRA1	NM_031721	HTRA serine peptidase 1	–2.71
1375123_at	SOX4	XM_344594	SRY (sex determining region y)-box 4	–2.70
1370361_at	CGREF1	NM_139087	Cell growth regulator with EF-hand domain 1	–2.69
1368657_at	MMP3	NM_133523	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	–2.67
1368721_at	ASCL2	NM_031503	Achaete-scute complex homolog 2	–2.67
1393808_at	FA2H	XM_001073350	Fatty acid 2-hydroxylase	–2.66
1371081_at	RAPGEF4	XM_215985	RAP guanine nucleotide exchange F 4	–2.64
1371087_a_at	MAP6	NM_017204	Microtubule-associated protein 6	–2.64
1382809_at	CIRBP	NM_031147	Cold inducible RNA binding protein	–2.60
1367564_at	NPPA	NM_012612	Natriuretic peptide precursor A	–2.60
1370236_at	PPT1	NM_022502	Palmitoyl-protein thioesterase 1	–2.58
1370034_at	CDC25B	NM_133572	Cell division cycle 25 homolog B	–2.58
1388902_at	LOXL1	NM_001012125	Lysyl oxidase-like 1	–2.58
1387219_at	ADM	NM_012715	Adrenomedullin	–2.55
1370260_at	ADD3	NM_031552	Adducin 3, gamma	–2.52
1368681_at	PTHLH	NM_012636	Parathyroid hormone-like hormone	–2.51
1368021_at	ADH1C	NM_019286	Alcohol dehydrogenase 1C gamma polypeptide	–2.51
1377950_at	IIGP1	NM_001024884	Interferon-inducible GTPase 1	–2.50
1371989_at	HMGN3	NM_001007020	High mobility group nucleosomal binding domain 3	–2.47
1368970_at	CDH23	NM_053644	Cadherin-like 23	–2.44
1376973_at	SDCBP2	NM_001025692	Syndecan binding protein 2	–2.44
1392965_a_at	SMOC2	XM_214777	SPARC-related modular calcium binding 2	–2.43
1391279_at	SCIN	NM_198748	Scinderin	–2.42
1370384_a_at	PRLR	NM_001034111	Prolactin receptor	–2.41
1369977_at	UCHL1	NM_017237	Ubiquitin carboxyl-terminal esterase 11 (Ubiquitin thiolesterase)	–2.40
1384073_at	ADHFE1	NM_001025423	Alcohol dehydrogenase, iron-containing, 1	–2.39
1387972_at	MUCDHL	NM_138525	Mucin and cadherin-like protein	–2.39
1369012_at	INHBA	NM_017128	Inhibin, beta A (activin A, activin AB alpha polypeptide)	–2.38
1367816_at	HOP	NM_133621	Homeodomain-only protein	–2.37
1389746_at	NAGLU	XM_340905	N-acetylglucosaminidase, alpha	–2.35
1387873_at	WFDC1	NM_133581	WAP four-disulfide core domain 1	–2.34
1368503_at	GCH1	NM_024356	GTP cyclohydrolase 1	–2.33
1370633_at	CXCL1	NM_138522	Chemokine ligand 1	–2.33
1372042_at	CMTM3	XM_226200	CKLF-like marvel transmembrane domain containing 3	–2.33

Affymetrix ID	Symbol	RefSeq	Downregulated gene name	Fold Δ
1367705_at	GLRX	NM_022278	Glutaredoxin (thioltransferase)	–2.31
1374151_at	TM6SF1	NM_145785	Transmembrane 6 SuperFamily, member 1	–2.30
1397758_at	GNPTAB	NM_001007750	N-acetylglucosamine-1-phosphate Transferase, α & β subunits	–2.29
1379075_at	MBOAT2	XM_234011	Membrane bound O-acyltransferase domain containing 2	–2.29
1367568_a_at	MGP	NM_012862	Matrix GLA protein	–2.28
1370714_a_at	ST6GAL1	NM_147205	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	–2.27
1372715_at	SFXN1	NM_001012213	Sideroflexin 1	–2.27
1376697_at	CHST12	NM_001037775	Carbohydrate (chondroitin 4) sulfotransferase 12	–2.27
1368367_at	CUZD1	NM_054005	Cub and zona pellucida-like domains 1	–2.27
1385559_at	DNHD3	NM_001126292	Dynein, axonemal, heavy chain 2	–2.26
1383363_at	DIRAS2	XM_225214	DIRAS family, GTP-binding RAS-like 2	–2.25
1377573_at	CA5B	NM_001005551	Carbonic anhydrase VB, mitochondrial	–2.24
1388427_at	MXRA8	NM_001007002	Limitrin	–2.24
1370291_at	PDLIM3	NM_053650	PDZ and LIM domain 3	–2.24
1367722_at	DPP7	NM_031973	Dipeptidyl-peptidase 7	–2.23
1370026_at	CRYAB	NM_012935	Crystallin, Alpha B	–2.23
1369724_at	F13A1	NM_021698	Coagulation factor XIII, A1 polypeptide	–2.22
1372301_at	AEBP1	XM_223583	AE binding protein 1	–2.22
1368005_at	ITPR3	NM_013138	Inositol 1,4,5-triphosphate receptor 3	–2.22
1385013_at	WNT1	XM_235639	Wingless-type MMTV integration site 1	–2.22
1387588_at	EHD3	NM_138890	EH-domain containing 3	–2.21
1398311_a_at	KIDINS220	NM_053795	Kinase D-interacting substance of 220 kDa	–2.21
1370182_at	PTPRN2	NM_031600	Protein tyrosine phosphatase receptor N2	–2.21
1389423_at	DDR2	NP_113952	Discoidin domain receptor family 2	–2.21
1397164_at	POLA2	NM_053480	Polymerase (DNA-directed), alpha 2 (70 kDa Subunit)	–2.20
1390233_at	GLI2	NM_001107169	Gli-kruppel family member GLI2	–2.20
1390846_at	COL16A1	XM_345584	Collagen, type XVI, alpha 1	–2.20
1383630_at	DOK3	XM_225170	Docking protein 3	–2.20
1390882_at	HEYL	NM_001107977	Hairy/enhancer-of-split related with VRPW motif-like	–2.20
1367859_at	TGFB3	NM_013174	Transforming growth factor beta 3	–2.20
1387505_at	GNAI1	NM_013145	G-Protein, alpha inhibiting activity polypeptide 1	–2.19
1389651_at	APLN	NM_031612	Apelin, AGTRL1 ligand	–2.18
1374863_at	RBP7	XM_575960	Retinol binding protein 7	–2.16
1367823_at	TIMP2	NM_021989	TIMP metalloproteinase inhibitor 2	–2.16
1368292_at	DNM1	NM_080689	Dynamin 1	–2.16
1375033_at	CPT1C	XM_218625	Carnitine palmitoyltransferase 1C	–2.15
1374849_at	ADAMTS7	XM_236471	ADAM metallopeptidase with thrombospondin type 1 motif, 7	–2.15
1370342_at	KCNK2	NM_172041	Potassium channel K2	–2.15
1393245_at	PHYH	NM_053674	Phytanoyl-CoA 2-hydroxylase	–2.13
1371237_a_at	MT1E	NM_138826	Metallothionein 1E	–2.13

Affymetrix ID	Symbol	RefSeq	Downregulated gene name	Fold Δ
1369443_at	ANGPTL2	NM_022926	Angiopoietin-like 2	–2.13
1389739_at	NEURL2	XM_230848	Neuralized homolog 2	–2.11
1369640_at	GJA1	NM_012567	Gap junction protein, alpha 1, 43 kDa (Connexin 43)	–2.11
1377163_at	INHBB	XM_344130	Inhibin, beta b (Activin AB beta polypeptide)	–2.10
1388070_a_at	AKAP1	NM_053665	A Kinase (PRKA) anchor protein 1	–2.10
1381226_at	NAV1	XM_222662	Neuron navigator 1	–2.10
1370713_at	CDC2L1	NM_145766	Cell division cycle 2-like 1 (PITSLRE proteins)	–2.10
1375871_at	SLC35B1	NM_199081	Solute carrier family 35, member B1	–2.10
1378671_at	CREBBP	NM_133381	CREB binding protein (Rubinstein-Taybi syndrome)	–2.10
1398295_at	SLC29A1	NM_031684	Solute carrier family 29 (nucleoside transporters), member 1	–2.10
1369141_at	CSH1	NM_017363	Chorionic somatomammotropin hormone 1	–2.08
1396831_at	MAML3	XM_227165	Mastermind-like 3	–2.08
1375243_at	BTBD2	XM_576181	BTB (POZ) domain-containing 2	–2.08
1382511_at	E2F1	XM_230765	E2F transcription factor 1	–2.07
1368006_at	LAPTM5	NM_053538	Lysosomal-associated multispanning membrane protein 5	–2.07
1373970_at	IL33	NM_001014166	Interleukin 33	–2.07
1385587_at	MCOLN2	NM_001039005	Mucolipin 2	–2.07
1369194_a_at	CDKN2A	NM_053434	Cyclin-dependent kinase inhibitor 2A	–2.07
1379495_at	PLXDC2	NM_001108422	Plexin domain-containing 2	–2.07
1377699_at	BACH1	XM_221712	BTB and CNC homology 1	–2.06
1384667_x_at	GALR2	NM_019172	Galanin receptor 2	–2.05
1370202_at	HRASLS3	NM_017060	HRAS-like suppressor 3	–2.05
1387952_a_at	CD44	NM_012924	CD44 molecule	–2.04
1386953_at	HSD11B1	NM_017080	Hydroxysteroid (11-beta) dehydrogenase 1	–2.04
1368254_a_at	SPHK1	NM_133386	Sphingosine kinase 1	–2.04
1389115_at	EVPL	XM_221129	Envoplakin	–2.04
1371441_at	PEA15	NM_001013231	Phosphoprotein-enriched in astrocytes 15	–2.04
1369431_at	GALNT7	NM_053648	Polypeptide n-acetylgalactosaminyltransferase 7	–2.03
1386160_at	TCHH	XM_227373	Trichohyalin	–2.01
1389533_at	FBLN2	XM_232197	Fibulin 2	–2.01
1367882_at	MAP1A	NM_030995	Microtubule-associated protein 1A	–2.01
1387296_at	CYP2J2	NM_023025	Cytochrome P450 2J2	–2.01
1394451_at	ANXA1	NM_012904	Annexin A1	–2.01

Affymetrix ID	Symbol	RefSeq	Upregulated gene name	Fold Δ
1386980_at	APOM	NM_019373	Apolipoprotein M	2.00
1370259_a_at	PTHR1	NM_020073	Parathyroid hormone receptor 1	2.00
1369331_a_at	UNC13B	NM_031550	unc-13 homolog B	2.01
1391345_at	BMPER	NM_001135799	BMP binding endothelial regulator	2.02
1372690_at	RTN1	NM_181377	Reticulon 1	2.02
1398383_at	CYB561	XM_221030	Cytochrome B-561	2.02

Affymetrix ID	Symbol	RefSeq	Upregulated gene name	Fold Δ
1375726_at	LMO7	NM_001001515	LIM domain 7	2.02
1376799_a_at	CRLF1	XM_214312	Cytokine receptor-like factor 1	2.02
1370420_at	SRD5A1	NM_017070	Steroid-5 alpha-reductase	2.02
1368785_a_at	PITX2	NM_019334	Paired-like homeodomain transcription factor 2	2.03
1369756_a_at	SLC4A4	NM_053424	Solute carrier family 4, sodium bicarbonate cotransporter 4	2.03
1386943_at	PLLP	NM_022533	Transmembrane 4 SuperFamily, member 11	2.03
1385961_at	KLF5	NM_053394	Kruppel-like factor 5	2.04
1374273_at	CXADR	NM_053570	Coxsackie virus and adenovirus receptor	2.04
1377304_at	CDC26	NM_001013240	Cell division cycle 26 homolog	2.05
1375849_at	RGMA	NM_001107524	Rgm domain family, member a	2.05
1368247_at	HSPA1B	NM_212504	Heat shock 70 kDa protein 1B	2.06
1389735_at	RPS6KA6	XM_228473	Ribosomal protein S6 kinase, 90 kDa, polypeptide 6	2.08
1387298_at	PGA5	NM_021753	Pepsinogen 5, group I (Pepsinogen a)	2.12
1387232_at	BMP4	NM_012827	Bone morphogenetic protein 4	2.13
1392556_at	SHROOM3	XM_223229	Shroom family member 3	2.16
1383981_at	TRP53BP2	XM_223012	Tumor protein p53 binding protein 2	2.17
1394663_at	POLS	XM_225072	Polymerase (DNA-directed) Sigma	2.18
1392773_at	PCSK5	XM_342032	Proprotein convertase subtilisin/kexin type 5	2.20
1389276_at	L3MBTL3	XM_001062689	L(3)MBT-like 3	2.20
1383747_at	ECT2	XM_342220	Epithelial cell transforming sequence 2 oncogene	2.20
1387574_at	CHRNA2	NM_133420	Nicotinic cholinergic receptor alpha 2	2.22
1389066_at	DSCR1L1	NM_175578	Down syndrome critical region gene 1-like 1	2.22
1375729_at	EPHA4	XM_244186	EPH receptor A4	2.25
1369727_at	APOA2	NM_013112	Apolipoprotein A-II	2.29
1377379_at	IRF6	XM_344194	Interferon regulatory factor 6	2.33
1368339_at	S100 G	NM_012521	S100 calcium binding protein G	2.35
1386396_at	DUSP8	XM_341963	Dual specificity phosphatase 8	2.36
1367598_at	TTR	NM_012681	Transthyretin (prealbumin, amyloidosis Type I)	2.37
1371030_at	SPP2	NM_053577	Secreted phosphoprotein 2	2.39
1386873_at	TNNI1	NM_017184	Troponin I type 1	2.40
1393139_at	APOC2	XM_214872	Apolipoprotein C-II	2.40
1389234_at	VWF	XM_342759	Von Willebrand factor	2.41
1371059_at	PRKAR2A	NM_019264	CAMP-dependent protein kinase 2A	2.44
1370463_x_at	HLA-F	NM_001008829	Major histocompatibility complex, class I, F	2.44
1389648_at	RIPK4	XM_221619	Receptor-interacting serine-threonine kinase 4	2.44
1368280_at	CTSC	NM_017097	Cathepsin C	2.46
1384747_at	GPR137B	XM_237907	G Protein-coupled receptor 137B	2.51
1369837_at	GULO	NM_022220	Gulonolactone (L-) oxidase	2.56
1387396_at	HAMP	NM_053469	Hepcidin antimicrobial peptide	2.59
1368278_at	LGALS2	NM_133599	Lectin, galactoside-binding, soluble, 2 (galectin 2)	2.59
1367758_at	AFP	NM_012493	Alpha-fetoprotein	2.60
1386913_at	PDPN	NM_019358	Podoplanin	2.67
1382965_at	AMIGO3	NM_178144	Adhesion molecule with Ig-like domain 3	2.69

Affymetrix ID	Symbol	RefSeq	Upregulated gene name	Fold Δ
1367749_at	LUM	NM_031050	Lumican	2.77
1375183_at	ID4	NM_175582	DNA binding inhibitor 4, dominant negative helix-loop-helix prot.	2.80
1368442_at	F2	NM_022924	Coagulation factor II (thrombin)	2.85
1380621_at	FES	NM_001108488	Feline sarcoma oncogene	2.88
1379741_at	ATP6V0A4	XM_231615	ATPase, H+ transporting, lysosomal V0 subunit A4	2.92
1370086_at	FGG	NM_012559	Fibrinogen, gamma chain	2.92
1387414_at	DUOX2	NM_024141	Dual oxidase 2	2.94
1368316_at	AQP8	NM_019158	Aquaporin 8	2.97
1388190_at	APOB	NM_019287	Apolipoprotein B	2.98
1378866_at	ABLIM1	XM_217645	Actin binding LIM protein 1	3.11
1368527_at	PTGS2	NM_017232	Prostaglandin-endoperoxide synthase 2	3.16
1392703_at	TBX4	NM_001107034	T-box 4	3.18
1387565_at	TRPV6	NM_053686	Transient receptor potential cation channel V6	3.26
1370269_at	CYP1A1	NM_012540	Cytochrome P450 1A1	3.33
1369663_at	EPHX2	NM_022936	Epoxide hydrolase 2	4.10
1392948_at	CLIC6	NM_176078	Chloride intracellular channel 6	4.33
1393891_at	COL8A1	XM_221536	Collagen, type VIII, alpha 1	4.40
1370310_at	HMGCS2	NM_173094	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2	4.65
1368335_at	APOA1	NM_012738	Apolipoprotein A-1	4.88
1380134_at	VTCN1	NM_001024244	V-set domain-containing T-cell activation inhibitor 1	5.08
1370594_at	IGSF1	NM_175763	Immunoglobulin SuperFamily, member 1	5.99
1370077_at	INS	NM_019130	Insulin	6.67
1370165_at	SMPX	NM_053395	Small muscle protein, X-linked	13.7

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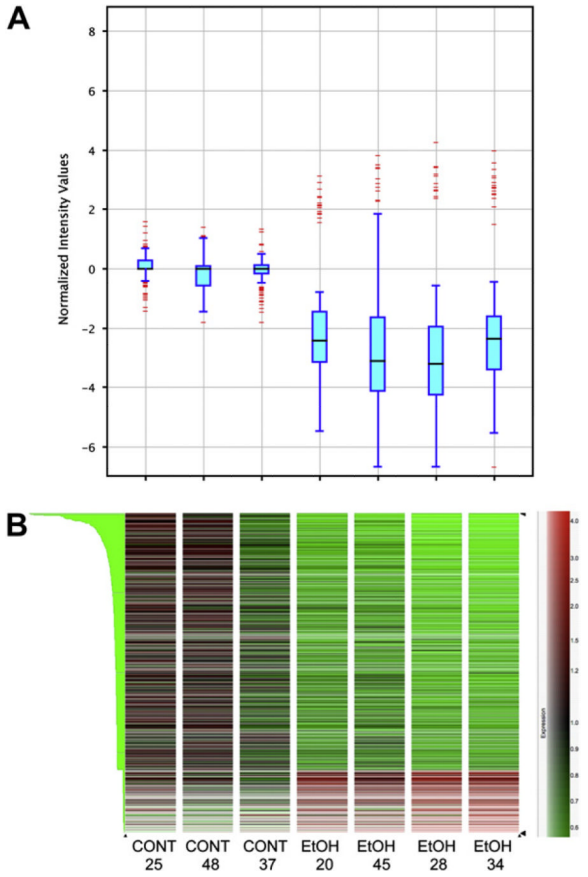

Fig. 1.

A. Box whisker plots of placental gene expression in control and ethanol-exposed placentas representing the minimum (end of the *bottom* whisker), the first quartile (*bottom* border of the box), the median (line through the box), the third quartile (*top* border of the box), and the maximum (end of the *top* whisker) of the distribution. The separately drawn points are outliers. Points are regarded as outliers if the minimum of their distance to the first and the third quartile is greater than 1.5 times the interquartile range (IQR = third quartile–second quartile). B. Heat map plots illustrating unsupervised hierarchical clustering on similarity in expression profiles across conditions of 643 genes list, filtered by twofold expression changes and statistical significance (Student's *t*-test). CONT = saccharin control group and EtOH = 5% ethanol treatment group. The numbers below the group labels indicate the litter number from which the placenta tissue was harvested. Each heat map corresponds to the whisker plot directly above it.

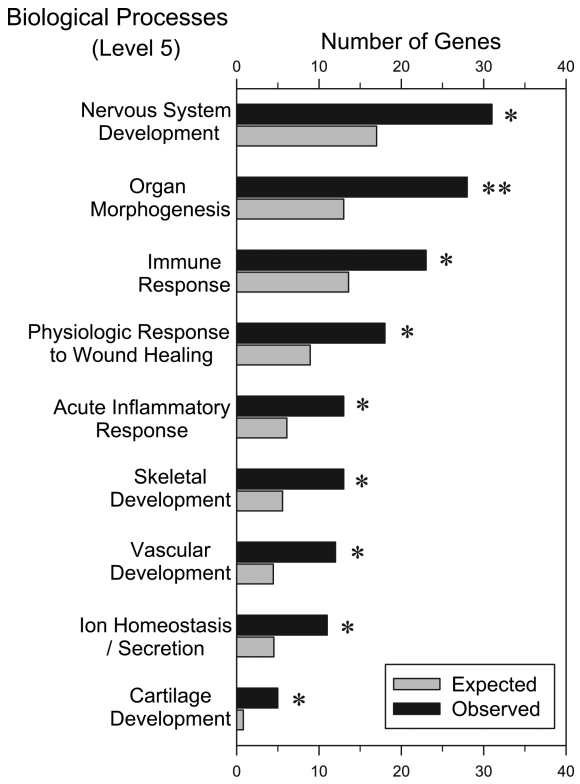

Fig. 2.

Selected enriched “biological processes” gene ontology (GO) level 5 categories. Data were analyzed using Gene Ontology Tree Machine GOTM software (Zhang et al., 2000). The distribution of differentially regulated genes (gray bars) in each GO category was compared with all genes on the Affymetrix Rat 230 2.0 array (black bars). Statistical significance was analyzed using the hypergeometric statistical test; $*P < .05$, $**P < .005$.

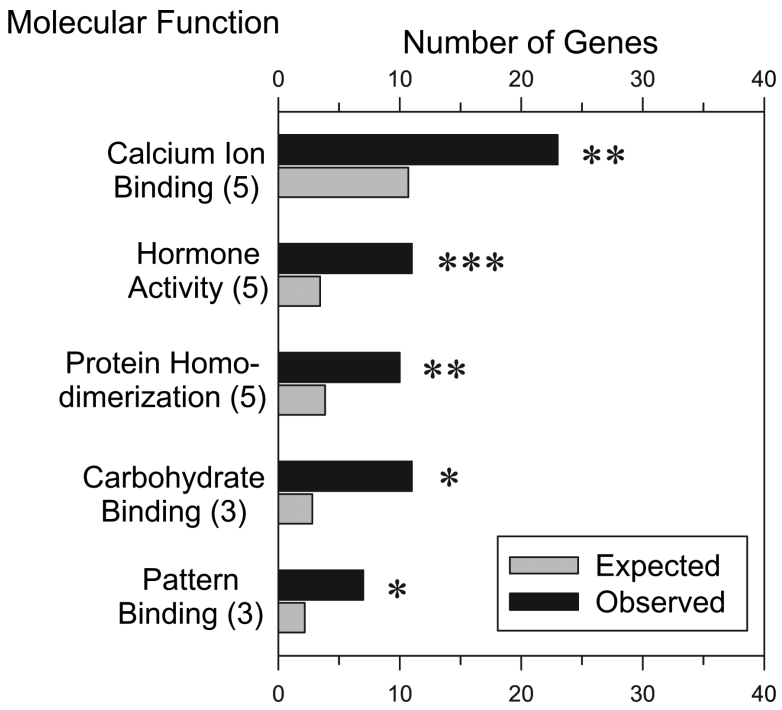

Fig. 3.

Selected enriched “molecular function” gene ontology (GO) categories. Numbers in parenthesis after the molecular function denote the GO category level. Data were analyzed using Gene Ontology Tree Machine GOTM software (Zhang et al., 2000). The distribution of differentially regulated genes (gray bars) in each GO category was compared with all genes on the Affymetrix Rat 230 2.0 array (black bars). Statistical significance was analyzed using the hypergeometric statistical test; *$P < .05$, **$P < .005$, ***$P < .0005$.

Table 1

Effects of the voluntary ethanol consumption paradigm

Outcome measure	Saccharin control	5% Ethanol
Maternal weight gain during pregnancy	120 ± 3[a] (44)	115 ± 4 (51)
Daily 4-h ethanol consumption	NA	2.82 ± 0.13[b] (51)
Maternal serum ethanol concentration	NA	84.0 ± 5.5[c] (24)
Fetal litter size	12.7 ± 0.8[d] (9)	12.9 ± 0.9 (13)
Placental wet weight	0.533 ± 0.049[e] (6)	0.513 ± 0.045 (6)

NA = not applicable; Numbers in parentheses indicate sample size; S.E.M. = standard error of mean.

[a]Mean ± S.E.M. grams increase in body weight from gestational day 1 through 21.

[b]Mean ± S.E.M. grams ethanol consumed/kg body weight/day.

[c]Mean ± S.E.M. mg ethanol/dL serum, 30 min after a 4-h drinking period.

[d]Mean ± S.E.M. number of fetuses/litter at gestational day 20.

[e]Mean ± S.E.M. grams/placenta (averaged from four placentas in each litter).

Table 2

Differentially regulated placental genes, filtered by statistical significance and fold change of more than five

Affymetrix ID	Symbol	RefSeq	Downregulated gene name	Fold Δ
1369113_at	GREM1	NM_019282	Gremlin 1, cysteine knot superfamily homolog	–25.3
1385568_at	DIO2	NM_031720	Deiodinase, iodothyronine type 2	–25.2
1387088_at	GAL	NM_033237	Galanin	–21.4
1368731_at	ORM1	NM_053288	Orosomucoid 1	–17.0
1388269_at	HBG1	NM_172093	Hemoglobin, gamma A	–16.2
1390112_at	EFEMP1	NM_001012039	EGF-containing fibulin-like extracellular matrix protein 1	–15.8
1369677_at	CNR1	NM_012784	Cannabinoid receptor 1	–15.5
1368304_at	FMO3	NM_053433	Flavin-containing monooxygenase 3	–14.2
1368394_at	SFRP4	NM_053544	Secreted frizzled-related protein 4	–13.9
1368912_at	TRH	NM_013046	Thyrotropin-releasing hormone	–13.3
1398688_at	SPINK5	XM_341607	Serine peptidase inhibitor, Kazal type 5	–10.7
1374558_at	ICOSLG	XM_574731	Inducible T-cell co-stimulator ligand	–9.31
1387563_at	PGR	NM_022847	Progesterone receptor	–9.21
1369625_at	AQP1	NM_012778	Aquaporin 1	–9.15
1367846_at	S100A4	NM_012618	S100 calcium binding protein A4	–8.78
1367627_at	GATM	NM_031031	Glycine amidinotransferase	–8.26
1370843_at	GNG8	NM_139185	Guanine nucleotide binding protein (G-protein), gamma 8	–8.25
1369695_at	WT1	NM_031534	Wilms tumor 1	–8.14
1393069_at	SFRP5	XM_219887	Secreted frizzled-related protein 5	–7.86
1369164_a_at	TRPC4	NM_080396	Transient receptor potential cation channel 4C	–7.63
1387450_at	TGFA	NM_012671	Transforming growth factor, alpha	–7.62
1371102_x_at	HBD	NM_033234	Hemoglobin, delta	–7.35
1369817_at	HAND2	NM_022696	Heart and neural crest derivatives expressed 2	–6.97
1388270_at	HBE1	NM_001008890	Hemoglobin, epsilon 1	–6.91
1368919_at	PGF	NM_053595	Placental growth factor	–6.46
1396407_at	GAS8	NM_001039030	Growth arrest-specific 8	–6.43
1380206_at	KIF5C	XM_221307	Kinesin family member 5C	–6.32
1367600_at	DES	NM_022531	Desmin	–6.23
1370157_at	PLN	NM_022707	Phospholamban	–6.03
1388138_at	THBS4	XM_342172	Thrombospondin 4	–5.69
1367794_at	A2M	NM_012488	Alpha-2-macroglobulin	–5.62
1387656_at	SLC4A1	NM_012651	Solute carrier family 4, anion exchanger, member 1	–5.60
1368713_at	MMP10	NM_133514	Matrix metalloproteinase 10 (Stromelysin 2)	–5.49
1370956_at	DCN	NM_024129	Decorin	–5.37
1380285_at	CHRD	NM_001024273	Chordin	–5.36
1368342_at	AMPD3	NM_031544	Adenosine monophosphate deaminase (isoform E)	–5.18

Affymetrix ID	Symbol	RefSeq	Upregulated gene name	Fold Δ
1380134_at	VTCN1	NM_001024244	V-set domain containing T-cell activation inhibitor 1	5.08
1370594_at	IGSF1	NM_175763	Immunoglobulin superfamily, member 1	6.00

Affymetrix ID	Symbol	RefSeq	Upregulated gene name	Fold Δ
1370077_at	INS	NM_019130	Insulin	6.67
1370165_at	SMPX	NM_053395	Small muscle protein, X-linked	13.7

Table 3

Thirty-one differentially regulated genes in the “Nervous System Development Ontology Category”

Affymetrix ID	Symbol	Fold Δ	Gene
1387088_at	GAL	–21.4	Galanin
1370843_at	GNG8	–8.25	G-protein gamma 8
1380172_at	KIF5C	–6.36	Kinesin 5C
1388138_at	THBS4	–5.70	Thrombospondin 4
1380285_at	CHRD	–5.36	Chordin
1386903_at	S100B	–4.93	S100 protein B
1387659_at	GDA	–4.33	Guanine deaminase
1368914_at	RUNX1	–3.93	Runt-related transcription factor 1
1377336_at	SEMA3B	–3.31	Sema domain, immunoglobulin domain (IG)
1367668_a_at	SCD2	–2.90	Stearoyl-coenzyme A desaturase 2
1368721_at	ASCL2	–2.67	Achaete-scute complex homolog 2
1370236_at	PPT1	–2.58	Palmitoyl-protein thioesterase
1376973_at	SDCBP2	–2.44	Syndecan binding protein 2
1369977_at	UCHL1	–2.40	Ubiquitin carboxy-terminal hydrolase 11
1369012_at	INHBA	–2.38	Inhibin beta A
1388427_at	MXRA8	–2.24	Limitrin
1385013_at	WNT1	–2.22	Wingless-related MMTV integration site 1
1390233_at	Gli2	–2.20	GLI-Kruppel Family member GLI2
1390882_at	Heyl	–2.20	Hairy/enhancer-of-split related with YRPW motif-like
1387271_at	PHYH	–2.13	Phytanoyl-CoA hydroxylase
1369640_at	GJA1	–2.11	Gap junction protein, alpha 1
1382511_at	E2F1	–2.07	E2F transcription factor 1
1384667_x_at	GALR2	–2.05	Galanin receptor 2
1368254_a_at	SPHK1	–2.04	Sphingosine kinase 1
1372690_at	RTN4RL1	–2.02	Reticulon 4 receptor-like 1
1386943_at	PLLP	2.03	Transmembrane 4 superfamily, member 11
1375849_at	RGMA	2.05	Rgm domain family, member A
1387232_at	BMP4	2.13	Bone morphogenic protein 4
1383981_at	TRP53BP	2.16	Transformation-related binding protein 53
1389066_at	DSCR1L1	2.22	Regulator of calcineurin 2
1382965_at	AMIGO3	2.68	Amphoterin induced gene ORF 3

The enrichment factor for this category was 1.8, as determined by the ratio between the expected number of genes to the observed number of altered genes ($P = .0016$).

Table 4

Relative quantification of mRNA using the comparative cycle threshold (C_t) method

Cellular location of protein product gene name	Relative mRNA levels[a]	P[b]	Gene ontology category[c]
Extracellular space			
Gremlin 1	0.05	.002	Organ morphogenesis
Matrix metalloproteinase 2	0.13	.011	Peptidase activity/blood vessel maturation
EGF-containing fibulin-like extracellular matrix protein 1	0.06	.013	Calcium ion binding
Galanin	0.08	.013	Nervous system development
Transforming growth factor alpha	0.12	.013	Regulation of cell cycle progression
Serine peptidase inhibitor, Kazal type 5	0.04	.046	Regulation of cell adhesion
Placental growth factor	0.09	.048	Progression through cell cycle
Thyrotropin-releasing hormone	0.06	.061	Neuropeptide hormone activity
Orosomucoid 1	0.03	.069	Acute-phase response
Insulin-like growth factor binding protein 6	0.32	.106	Regulation of cell growth
Matrix metalloproteinase 3	0.17	.213	Proteolysis
Matrix metalloproteinase 10 (stromelysin 2)	0.34	.402	Proteolysis
Insulin	3.58	.477	Insulin receptor signaling pathway
Alpha-fetoprotein	2.27	.707	Progesterone metabolism
Plasma membrane			
Cannabinoid receptor 1	0.13	.001	G-protein coupled receptor
Galanin receptor 2	0.33	.001	G-protein coupled receptor
Toll-like receptor 4	0.22	.018	Inflammatory response
Integrin α7	0.34	.024	Receptor activity/regulation of cell shape
Secreted frizzled-related protein 4	0.01	.037	Development
Lipopolysaccharide binding protein	0.16	.042	Lipid transport
Transient receptor potential cation channel 4	0.04	.043	Calcium ion transport
Secreted frizzled-related protein 5	0.04	.079	Development
Nicotinic cholinergic receptor, α2 subunit	1.39	.220	Neurotransmitter receptor
Nicotinic cholinergic receptor, α7 subunit	0.64	.438	Extracellular ligand-gated ion channel
Cytoplasm			
Hemoglobin, epsilon 1	0.15	.003	Oxygen transport
Hydroxysteroid (11β) dehydrogenase 2	0.09	.009	Oxidoreductase activity
Hemoglobin, gamma A	0.10	.010	Oxygen transport
Deiodinase, iodothyronine type II	0.02	.012	Hormone biosynthesis
S100 calcium binding protein A4	0.09	.021	Calcium ion binding
S100 calcium binding protein G	3.10	.029	Calcium ion binding
S100 calcium binding protein B	0.04	.038	Regulation of neuronal synaptic plasticity
Small muscle protein, X-linked	17.8	.058	Striated muscle contraction
Heat shock 70 kDa protein 1B	2.05	.092	Anti-apoptosis
Flavin-containing monooxygenase 3	0.77	.233	Oxidoreductase activity
Cytochrome P450 1A1	2.45	.549	Oxidoreductase activity
Cytochrome P450 2E1	0.95	.910	Oxidoreductase activity

Cellular location of protein product gene name	Relative mRNA levels[a]	P[b]	Gene ontology category[c]
Nucleus			
Heart and neural crest derivatives expressed 2	0.18	.012	Neural crest cell development
Progesterone receptor	0.06	.065	Steroid hormone receptor

[a] All mRNA levels were calculated relative to beta-actin using the formula $2^{-\Delta\Delta CT}$. Values < 1 are indicative for downregulation of expression, and values > 1 signify higher expression in the experimental group compared with the control group.

[b] The *P* value is calculated by comparing all control with all ethanol exposed samples and applying Student's *t*-test.

[c] Shown are selected levels of gene ontology; most genes fit in more than one category. Statistically significant genes with $P \leq .05$.