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Molecular Changes during Neurodevelopment following Second-Trimester Binge Ethanol Exposure in a Mouse Model of Fetal Alcohol Spectrum Disorder: From Immediate Effects to Long-Term Adaptation

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Key Words
Fetal alcohol spectrum disorder · Neurodevelopment · Mouse · Gene expression · Microarray · microRNA

Abstract
Fetal alcohol spectrum disorder (FASD) is an umbrella term that refers to a wide range of behavioral and cognitive deficits resulting from prenatal alcohol exposure. It involves changes in brain gene expression that underlie lifelong FASD symptoms. How these changes are achieved from immediate to long-term effects, and how they are maintained, is unknown. We have used the C57BL/6J mouse to assess the dynamics of genomic alterations following binge alcohol exposure. Ethanol-exposed fetal (short-term effect) and adult (long-term effect) brains were assessed for gene expression and microRNA (miRNA) changes using Affymetrix mouse arrays. We identified 48 and 68 differentially expressed genes in short- and long-term groups, respectively. No gene was common between the 2 groups. Short-term (immediate) genes were involved in cellular compromise and apoptosis, which represent ethanol's toxic effects. Long-term genes were involved in various cellular functions, including epigenetics. Using quantitative RT-PCR, we confirmed the downregulation of long-term genes: *Camk1g*, *Ccdc6*, *Egr3*, *Hspa5*, and *Xbp1*. miRNA arrays identified 20 differentially expressed miRNAs, one of which (miR-302c) was confirmed. miR-302c was involved in an inverse relationship with *Ccdc6*. A network-based model involving altered genes illustrates the importance of cellular redox, stress and inflammation in FASD. Our results also support a critical role of apoptosis in FASD, and the potential involvement of miRNAs in the adaptation of gene expression following prenatal ethanol exposure. The ultimate molecular footprint involves inflammatory disease, neurological disease and skeletal and muscular disorders as major alterations in FASD. At the cellular level, these processes represent abnormalities in redox, stress and inflammation, with potential underpinnings to anxiety.

Introduction

Over the years, clinical and experimental research has established that maternal consumption of alcohol during pregnancy leads to neurodevelopmental abnormalities in children characterized by the umbrella term: fetal alcohol spectrum disorder (FASD) [1, 2]. FASD characterizes

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maternal alcohol exposures ranging from a single exposure to heavy, consistent use during pregnancy [3]. Heavy prenatal alcohol exposure can lead to a diagnosis of fetal alcohol syndrome (FAS). FAS is the only medically diagnosable form of FASD, and is diagnosed when facial dysmorphology, growth restriction and central nervous system/neurodevelopmental abnormalities are present [3–5]. The overarching term FASD may represent individuals who have some, but not all, of the FAS characteristics. The prevalence of FASD is approximately 1 in 100 individuals in North America [6]. The condition is lifelong, and there is no cure.

Studies on the C57BL/6J (B6) mouse [1, 7, 8], have identified FASD-relevant behavioral and molecular changes following prenatal ethanol exposure. To this end, we have established a number of FASD-related growth trajectories and developmental abnormalities in B6 mice [9, 10]. Often, lifelong FASD-relevant abnormalities are attributed to changes in brain gene expression [11–13]. We have further shown that these abnormalities are persistent into adulthood, and may be maintained by changes in gene expression, microRNAs (miRNA) and methylation [13–15]. The question remains: how are the altered transcriptomes attained and maintained in the adult brain following prenatal alcohol exposure? The answer to this question is likely complex. To our knowledge, a comparison of the immediate and lifelong transcriptomic alterations in prenatally exposed individuals has not been completed. Such an analysis will help to distinguish between early fetal responses to ethanol and the molecular events that maintain changes in gene expression into adulthood, thereby leaving behind a molecular 'footprint'.

The mechanistic basis of FASD likely involves an immediate toxicological effect, as well as altered expression of specific genes. miRNAs are important epigenetic regulators of various cellular processes. They are responsible for fine regulation of gene expression, thereby 'tuning' the cellular phenotype during delicate cellular processes such as development and differentiation [16]. During neurodevelopment, miRNAs are expressed in neurons and show distinct expression patterns within the developing central nervous system [17, 18]. Although miRNAs have been established in other psychopathologies [19, 20] their role in FASD may also be relevant, given that miRNAs are ethanol responsive [21, 22].

To the best of our knowledge, no study has attempted to follow the trajectory of molecular adaptation in brain gene expression following prenatal ethanol exposure. In this study, we report the effect of second-trimester binge ethanol exposure on short-term (immediate, gestational day, GD 16) and long-term (adult, postnatal day, PD 70) changes in brain gene expression. We have assessed the altered functional networks, including 'hub' network molecules (nodes with high intramodule connectivity: ≥10 interactions) [13, 23]. The results identified changes in molecular processes that were altered immediately following neurodevelopmental ethanol exposure and transformed into a stable footprint over time. Although the immediate effects are related to ethanol toxicity, long-term changes affect specific pathways and involve epigenetic (miRNA:mRNA) interaction in developmental adaptation.

Materials and Methods

Animals and Treatment Groups

C57BL/6J (B6) mice were originally obtained from Jackson Laboratories (Bar Harbor, Me., USA) and subsequently bred at the Health Sciences Animal Care Facility at the University of Western Ontario. Males and females were housed in separate (same-sex) and standardized cages with free access to food and water. They were kept on a light/dark cycle of 14/10 h at a temperature of 21–24°C with 40–60% humidity. All animal protocols were approved by the Animal Use Subcommittee at the University of Western Ontario (London, Ont., Canada) and complied with the ethical standards established by the Canadian Council on Animal Care.

Eight-week-old B6 females were time-mated with 8- to 12-week-old B6 males. Throughout gestation, dams were housed in individual cages. To model acute alcohol (binge) exposure in mid-gestation, dams were injected subcutaneously with 2.5 g/kg of ethanol in 0.15 M saline at 0 and 2 h on GD 14 and 16 (long-term group) or GD 16 (short-term group) [13]. This dosage has been proven sufficient to induce FASD-relevant neurodevelopmental and behavioral abnormalities [4, 10, 24, 25]. In adult mice, the first injection of 2.5 g/kg is roughly equivalent to the resulting blood alcohol concentration (BAC) of 250 mg/dl; the second dose given at 2 h raises the BAC to approximately 500 mg/dl [4, 26]. Given that mice metabolize ethanol 5 times faster than humans do, the BAC would fall within an equivalency range of 200–300 mg/dl for humans. Matched control dams were injected with saline using the same paradigm. Dams for the short-term group were sacrificed 4 h after injections using CO_2 asphyxiation and cervical dislocation. Dams from the long-term group were allowed to give birth. The resulting pups were raised to PD 70, which represents maturity. At PD 70, the mice were sacrificed by CO_2 asphyxiation and cervical dislocation.

RNA Isolation and Microarray Hybridization

A total of 12 whole brains (n = 6 control, n = 6 ethanol) were dissected from fetuses at GD 16 (short-term). Another 12 whole brains (n = 6 control, n = 6 ethanol) were dissected from adult males at PD 70 (long-term). Tissue was snap-frozen in liquid nitrogen and stored at –80°C until further use. Total RNA was isolated from each brain using Trizol® (Invitrogen, Carlsbad, Calif.,

USA). Quality and quantity of RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, Calif., USA) and a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Del., USA). Equal concentrations of RNA from 3 male brains (from 3 separate mothers) were pooled for each treatment to reduce litter effects. Each treatment group had 2 biological replicates (n = 12 mice per group, total n = 24). All RNA labeling and hybridization steps were performed at the London Regional Genomics Centre (Robarts Research Institute, London, Ont., Canada). Briefly, single-stranded complementary DNA (sscDNA) was synthesized using 200 ng of total RNA using the Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript WT Expression Arrays (Applied Biosystems, Carlsbad, Calif., USA) and the Affymetrix GeneChip WT Terminal Labeling kit and hybridization manual (Affymetrix, Santa Clara, Calif., USA). First-cycle cDNA was transcribed in vitro to cRNA and used to synthesize 5.5 μg of single-stranded cRNA that was subsequently end labeled and hybridized for 16 h at 45°C to Affymetrix Mouse Gene 1.0 ST arrays. Liquid-handling steps were performed by a GeneChip Fluidics Station 450 and arrays were scanned using the GeneChip Scanner 3000 using Command Console v1.1 (Affymetrix).

Microarray Data Analysis

Probe level (CEL) data were generated using Affymetrix Command Console v1.1 and probes were summarized to gene level data using Partek Genomics Suite software v6.6 (Partek Inc., St. Louis, Mo., USA). Array data analyses for the 8 arrays (n = 4 short-term, n = 4 long-term) were performed separately. Data were background corrected, quantile normalized and summarized using the GeneChip-Robust Multiarray Average algorithm to take into account probe GeneChip content, and then $\log_2$ transformed [27, 28]. Partek was used to determine gene level ANOVA p values and fold changes. Given that this injection protocol produces subtle changes in gene expression [13], genes with a ±1.2-fold change and a $p < 0.05$ were considered for downstream analysis. All data files from the array experiments were deposited in the National Center for Biotechnology Information Gene Expression Omnibus and can be found under accessions GSE43324 (short-term) and GSE34469 (long-term). A partial analysis of the long-term arrays was included in an earlier publication that assessed the effect of alcohol at the first-, second- and third-trimester human equivalents of neurodevelopment [13]. Singular Partek analysis of second-trimester long-term arrays has not been previously performed.

miRNA Expression Arrays

The RNA from PD 70 males was also used for miRNA expression analysis. Briefly, equal amounts of RNA from 3 males were pooled, each for 2 biological replicates (n = 4 arrays). All sample labeling, hybridization and processing was performed at the London Regional Genomics Centre. One microgram of total RNA was labeled using the Flash Tag Biotin HSR kit (Genisphere, Hatfield, Pa., USA) and hybridized to Affymetrix miRNA 2.0 arrays for 16 h at 45°C. Probe level (CEL) data were generated using Affymetrix Command Console. Probes were summarized to gene level data in Partek using the Robust Multiarray Average algorithm [29]. Partek was also used to determine differences between control and ethanol samples using one-way ANOVA and corresponding p values and fold changes. miRNAs were filtered using a ±1.2-fold change and $p < 0.05$. miRNA array results are deposited in GEO under accession GSE34413, and have been partially reported on in our previous manuscript [15]. The list of differentially expressed miRNAs, along with target filtering results (see below), have not been previously reported.

Functional Network Analysis

To evaluate genetic networks that may be involved in ethanol response, we used Ingenuity Pathway Analysis v.9 (IPA; Ingenuity Systems, Redwood, Calif., USA, www.ingenuity.com) to identify functional genetic networks. The gene lists (±1.2-fold change, $p < 0.05$) from short- and long-term groups were analyzed separately. IPA builds an interacting pathway of molecules (genes/proteins) that have been reported to interact together. Based on the number of molecules that interact in a specific function, networks are sorted based on biological processes. Networks with a cutoff score >3 ($p < 0.001$) were reported. A higher network score indicates increased significance of the network.

miRNA Target Filter and Pathway Analysis

Data from the long-term group were analyzed using IPA's miRNA Target Filter function (Ingenuity Systems) to predict interactions between genes and miRNAs (±1.2-fold change, $p < 0.05$). Results were filtered based on a moderate or high confidence of interaction and an inverse miRNA to target mRNA expression relationship [15, 30, 31].

Quantitative RT-PCR Confirmation of Specific Genes

RNA extracts not used for array hybridization were used to independently confirm the expression of selected genes by quantitative RT-PCR (qRT-PCR). For confirmation of specific genes, cDNA was synthesized using 2 μg of total brain RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, Calif., USA) according to the manufacturer's protocol. Primers and FAM-labeled probes for *Camk1g*, *Ccdc6*, *Egr3*, *Homer1*, *Hsp90aa1*, *Hspa5*, *Tbxa2r* and *Xbp1* (long-term), and *Ccl3*, *Ccnt1*, *Gpr50*, *Lair1*, *Pip5k1b*, *Rybp*, *Slitrk2* and *Trdn* (short-term) were obtained from Applied Biosystems Inventoried Assays and used as per the manufacturer's instructions. Reactions were multiplexed with β-actin using gene-specific primers and a VIC-labeled probe. Reactions were carried out following a standard ramp speed protocol using 10-μl volumes. PCR cycling conditions consisted of a 10-min initiation at 95°C, followed by 40 cycles consisting of a 15-second denaturation at 95°C and an annealing and extension cycle at 60°C for 60 s. All long-term experiments included 5 ethanol biological replicates (1 sample was discarded due to RNA degradation), 6 control biological replicates, and 3 technical replicates per sample. All short-term experiments included 6 biological replicates per treatment and 3 technical replicates per sample. Relative expression was calculated according to the comparative C_T method [32] using StepOne™ v2.0 software (Applied Biosystems) and analyzed using Statistical Package for the Social Sciences v.16 (SPSS Inc., Chicago, Ill., USA). All PCR data were reported as mean ± SEM relative expression values. Significant differences were assessed using a Student t test.

Quantitative RT-PCR Confirmation of miR-302c

For confirmation of miR-302c, cDNA was reverse transcribed from 1 μg of RNA using the Applied Biosystems TaqMan™ miRNA Reverse Transcription kit (Foster City) and sequence-spe-

Table 1. Top IPA networks identified in short-term analysis of DEGs

Network	Focus molecules, n	Top function	Input genes	Score
1	13	cellular compromise, cell death, free radical scavenging	*Bcap31, Hpca, Tdg, mir-99, Zc3h18, Crem, mir-379, mir-337, Lair1, Jmy, Ccl3, Nmbr, Rybp*	28
2	5	cellular development, cellular growth and proliferation, hematological system development and function	*Ccnt1, Zmat5, Trdn, Sprr2a, Pip5k1b*	8

Number of focus molecules indicates the number of genes that were differentially expressed (Partek: ±1.2-fold change, $p < 0.05$) in fetal brain (GD 16) as a result of ethanol exposure in the second-trimester equivalent. Top function represents the name of the network in IPA. Score is equal to the negative exponent of the respective p value such that a score of 3 corresponds to a p value of 10E-3. Network 1 score of 28 is equivalent to p = 10E-28. Network 2 score of 8 is equivalent to p = 10E-8.

cific step-loop reverse transcription primers from TaqMan miRNA Assays (Foster City, Calif., USA) according to the manufacturer's protocol. Primers and miRNA-specific TaqMan probes for miR-302c were selected using the Applied Biosystems (Carlsbad) search engine to identify previously characterized TaqMan miRNA Assays. snoRNA 202 was chosen as an endogenous reference gene [15, 33]. The target (5 biological samples) and control (6 biological samples) reactions were run in separate tubes on the same plate for each sample. Three technical replicates were used for both the endogenous reference gene and gene of interest for each sample. Quantitative PCR reactions were performed on the Applied Biosystems StepOne RT-PCR System 2.0 according to the manufacturer's protocol. Fold change was calculated using the 2^ΔΔCt method [32, 34, 35], and statistically analyzed using Applied Biosystems DataAssist™ Software v3.0. Statistical significance was assessed using an unpaired Student t test.

Results

Prenatal Ethanol Exposure Causes Overall Gene Expression Changes in the Developing Brain

We identified changes in gene expression in the brains of resulting progeny following in utero ethanol exposure at GD 16 (immediate effect) and at GD 14 and 16 (PD 70, long-term effect) compared to their matched controls. Using Partek, this difference was assessed with a ±1.2-fold change and $p < 0.05$. The numbers of differentially expressed genes (DEGs) in the short- and long-term analyses were 48 and 68, respectively. There was no overlap among DEGs between the short- and long-term groups. DEGs from the short-term gene list ranged from a +1.43-fold increase (SLIT- and NTRK-like family, member 2 or *Slitrk2*) to a –1.91-fold decrease (triadin or *Trdn*). Of the 48 genes identified in the short-term group, 30 (63%) were upregulated. DEGs in the long-term group ranged from a +1.34-fold increase (predicted gene 9875 or *Gm9875*) to a –1.78-fold decrease (stromal cell-derived factor 2-like 1 or *Sdf2l1*). In the long-term group, only 27 of the 68 genes (40%) were upregulated.

Immediate Ethanol Exposure during the Second Trimester Alters Genetic Networks and Cellular Processes in the Fetal Brain

IPA was used to investigate networks that were disrupted following immediate ethanol exposure. The list of 48 DEGs was inputted into IPA using the Core Analysis function to identify enriched networks of interest. Two significantly disrupted networks, with a score >3, are reported in table 1. The top immediately altered network is shown in figure 1. This network contained DEGs involved in cellular compromise, cell death (apoptosis) and free radical signaling. An analysis of hub genes, which included the identification of any network molecules with ≥10 interactions, resulted in the identification of huntingtin (HTT), amyloid-β precursor protein (APP), nuclear factor of κ light polypeptide gene enhancer in B cells (NFkB), tumor protein 53 (TP53) and tumor necrosis factor (TNF; fig. 1).

Analysis of processes that were altered in the fetal brain following immediate ethanol exposure in the second trimester was completed using IPA. The top 5 functional annotations in each IPA category of Diseases and Disorders, Molecular and Cellular Functions, and Physiological System Development and Function, were reported (table 2). As with IPA network analysis, disrupted functions were largely involved in cellular maintenance and cell cycle function. Additionally, immune response and skeletal formation was altered in the fetal brain following immediate ethanol exposure (table 2).

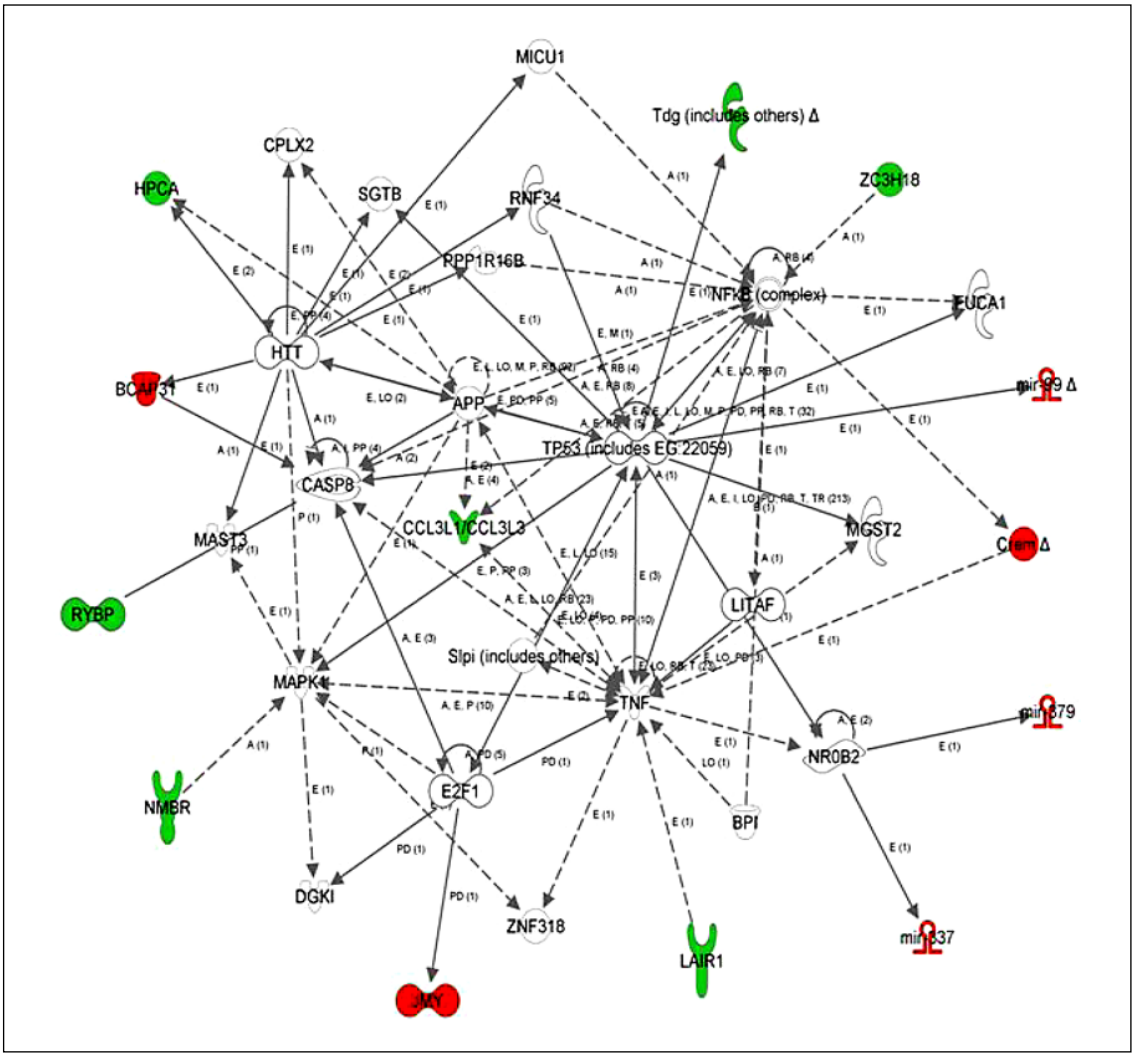

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Fig. 1. First network resulting from IPA of short-term DEGs. Network name: cellular compromise, cell death and free radical scavenging. Highlighting (red and green; color in online version only) represents input genes. Red indicates downregulation and green indicates upregulation in ethanol mice vs. control mice. DEGs were identified using Partek Genomics Suite with a ±1.2-fold change and $p < 0.05$. A single-headed arrow indicates the action of 1 gene on another. For instance, Tp53→Tdg means that p53 interacts directly with TDG by transcriptionally regulating its expression [84]. A double arrow-headed line indicates that the 2 genes act on each other. Dashed lines indicate an indirect interaction, and solid lines indicate direct interactions. E = Expression; L = proteolysis; LO = localization; PD = protein-DNA binding; A = activation; P = phosphorylation/dephosphorylation; PR = protein-RNA binding; PP = protein-protein interaction; I = inhibition; M = biochemical modification; RB = regulation of binding; T = transcription; TR = translocation. Numbers in brackets indicate the number of PubMed references that reported interactions between molecules.

Long-Term Ethanol Exposure during the Second Trimester Alters Genetic Networks and Cellular Processes in the Adult Brain

Three long-term genetic networks resulting from IPA analysis are reported in table 3. Only those networks with an IPA score >3 were identified. Most DEGs in these long-term networks were downregulated. This is contrary to results from the short-term (immediate) group. In the short-term analysis, the number of up- and downregulated genes was approximately evenly distributed within the top network. Long-term networks 1 and 3 are shown in figures 2 and 3, respectively. The top network is involved in inflammatory disease, neurological disease, and skeletal and muscular disorders. Analysis of hubs with ≥10 interactions identified mitogen-activated protein kinases (P38 MAPK, Jnk), extracellular signal-regu-

Table 2. Functional characterization of short-term DEGs using IPA

Name	p value	Genes, n	Genes	Function annotation
Diseases and disorders				
Developmental disorder	1.14E-03–3.29E-02	5	*mir-379, mir-99, Tdg, Trdn, Crem*	hypertrophy of skeletal muscle
Hereditary disorder	1.14E-03–2.29E-03	2	*mir-379, mir-99*	nemaline myopathy
Skeletal and muscular disorder	1.14E-03–4.12E-02	5	*mir-379, mir-99, Trdn, Crem, Ccl3*	muscular hypertrophy
Connective tissue disorder	1.34E-03–4.12E-02	3	*mir-379, mir-99, Ccl3*	adjuvant arthritis
Dermatological diseases	1.34E-03–1.34E-03	2	*mir-379, mir-99*	dermatomyositis
Molecular and cellular functions				
Cell cycle	2.10E-03–4.93E-02	1	*Crem*	arrest in G2/M phase
Cellular assembly/organization	2.10E-03–2.29E-02	1	*Pip5k1b*	formation of actin comet
Cellular function/maintenance	2.10E-03–3.11E-02	2	*Pip5k1b, Ccl3*	formation of microvilli
Antigen presentation	4.20E-03–8.38E-03	1	*Ccl3*	migration of dendritic precursors
Cellular movement	4.20E-03–4.93E-02	2	*Ccl3, Ccnt1*	Th1 cell transmigration
Physiological system development and function				
Nervous system development/function	2.10E-03–1.05E-02	1	*Ccl3*	astrocyte/microglia accumulation
Tissue development	2.10E-03–2.70E-02	3	*Ccl3, Bcap31, mir-99*	embryonic stem cell adhesion
Hematological system development/function	4.20E-03–4.93E-02	3	*Ccl3, Ccnt1, Lair1*	T lymphocyte trafficking
Hematopoiesis	4.20E-03–1.05E-02	1	*Ccl3*	migration of dendritic precursors
Immune cell trafficking	4.20E-03–4.93E-02	2	*Ccl3, Ccnt1*	natural killer cell migration

Table 3. Top IPA networks identified in second-trimester long-term analysis of DEGs

Network	Focus molecules, n	Top network function	Input genes	Score
1	13	inflammatory disease, neurological disease, skeletal and muscular disorders	*Cdkn1a, Dnajb11, Hist1h3a, Hspa5, Itga4, Pdia4, Pdia6, Ptpn22, Sdf2l1, Smarca5, Spred2, Xbp1, Fkbp2*	24
2	11	cardiovascular system development and function, cellular movement, cell-to-cell signaling and interaction	*Hsp90aa1, Dynlt1, Cbfa2t3, Prdx1, Dnahc3, P4ha1, Manf, Tbxa2r, Gchfr, Hist3h3, Slc9a3r2*	20
3	9	amino acid metabolism, lipid metabolism, molecular transport	*Zhx2, Fosl2, Tufm, Homer1, Klk1b11, Vps13b, Camk1g, Egr3, Tnnc1*	15

Number of focus molecules indicates the number of genes that were differentially expressed (Partek Genomics Suite: ±1.2-fold change, $p < 0.05$) in adult brain (GD 14 and 16) as a result of ethanol exposure in the second-trimester equivalent. Top function represents the name of the network in IPA. Score is equal to the negative exponent of the respective p value such that a score of 3 corresponds to a p value of 10E-3. Network 1 score of 24 is equivalent to p = 10E-24. Network 2 score of 20 is equivalent to p = 10E-20. Network 3 score of 15 is equivalent to p = 10E-15.

lated kinases (ERK1/2), and X-box binding protein 1 (XBP1, down-regulated; fig. 2). Network 3 is involved in amino acid metabolism, lipid metabolism and molecular transport. An analysis of hubs with ≥10 interactions identified FBJ murine osteosarcoma viral oncogene homolog (FOS), Serum response factor (c-fos serum response element-binding transcription factor, SRF), CREB binding protein (CREBBP) and HTT. The HTT hub molecule was common to immediate and long-term exposure groups.

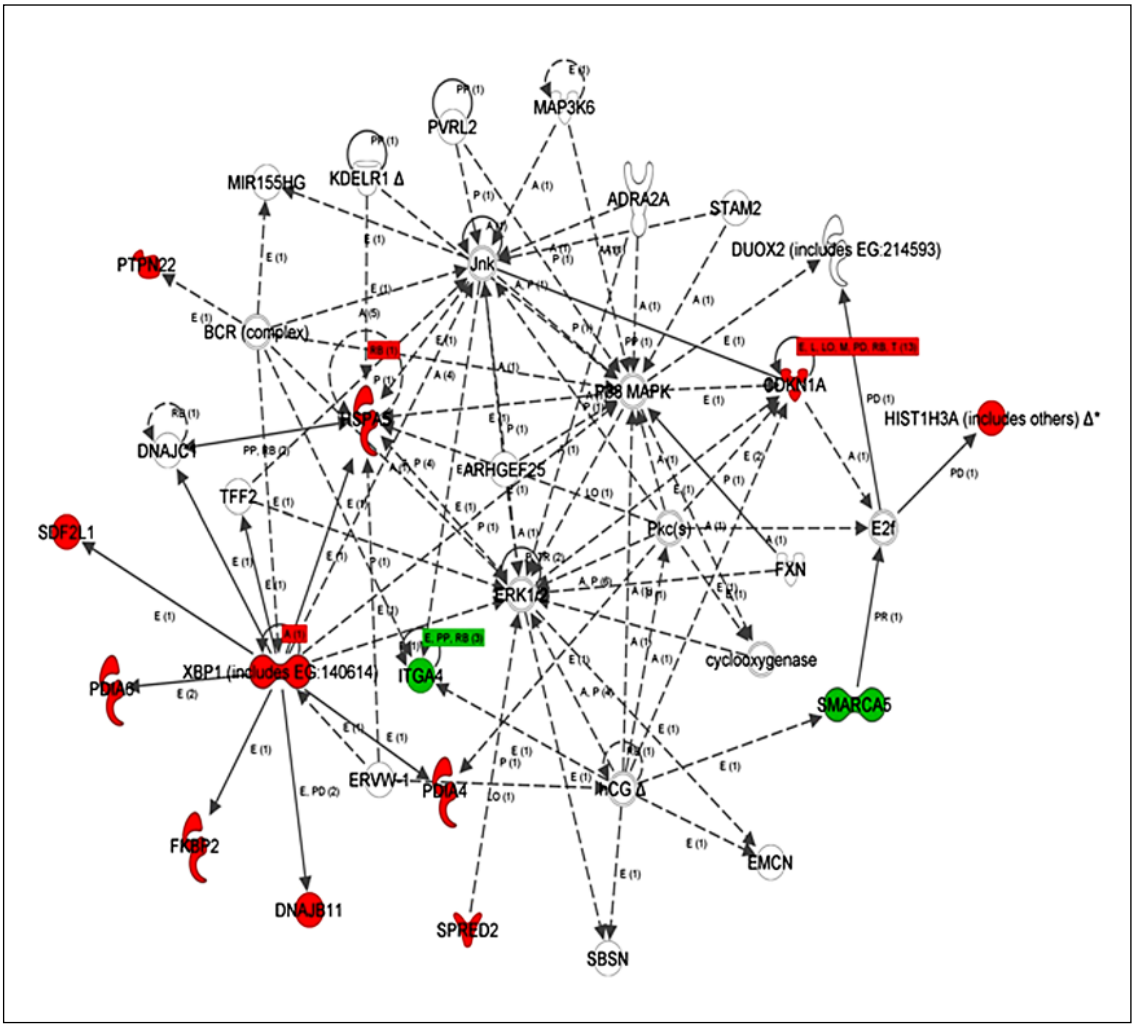

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Fig. 2. First network resulting from IPA of long-term DEGs. Network name: inflammatory disease, neurological disease and skeletal and muscular disorders. Highlighting (red and green) represents input genes. Red indicates downregulation and green indicates upregulation in ethanol mice vs. control mice. DEGs were identified using Partek Genomics Suite with a ±1.2-fold change and $p < 0.05$. A single-headed arrow indicates the action of 1 gene on another. A double arrow-headed line indicates that the 2 genes act on each other. E = Expression; L = proteolysis; LO = localization; PD = protein-DNA binding; A = activation; P = phosphorylation/dephosphorylation; PR = protein-RNA binding; PP = protein-protein interaction; I = inhibition; M = biochemical modification; RB = regulation of binding; T = transcription; TR = translocation. Numbers in brackets indicate the number of PubMed references that reported interactions between molecules.

Functional IPA analysis resulted in the top 5 processes categorized in Diseases and Disorders, Molecular and Cellular Functions, and Physiological System Development and Function (table 4). Similar to IPA network analysis, the top disrupted functions were involved in neurological disease and skeletal and muscular disorders. Among the top functions, cellular maintenance, cell death and cell-mediated immune response were altered. These processes (not the genes) were previously identified in short-term (immediate) analysis as well.

Prenatal Ethanol Exposure during the Second Trimester Causes Alterations in miRNA Expression in the Brain that are Maintained into Adulthood

Differential expression in brain miRNA expression was assessed using arrays only for the long-term group. This analysis involved comparison of ethanol and matched control mice using a ±1.2-fold change cutoff and $p < 0.05$. The results showed that 20 *Mus Musculus*-specific miRNAs were differentially expressed (table 5). The expression ranged from a +1.39-fold increase (miR-2145)

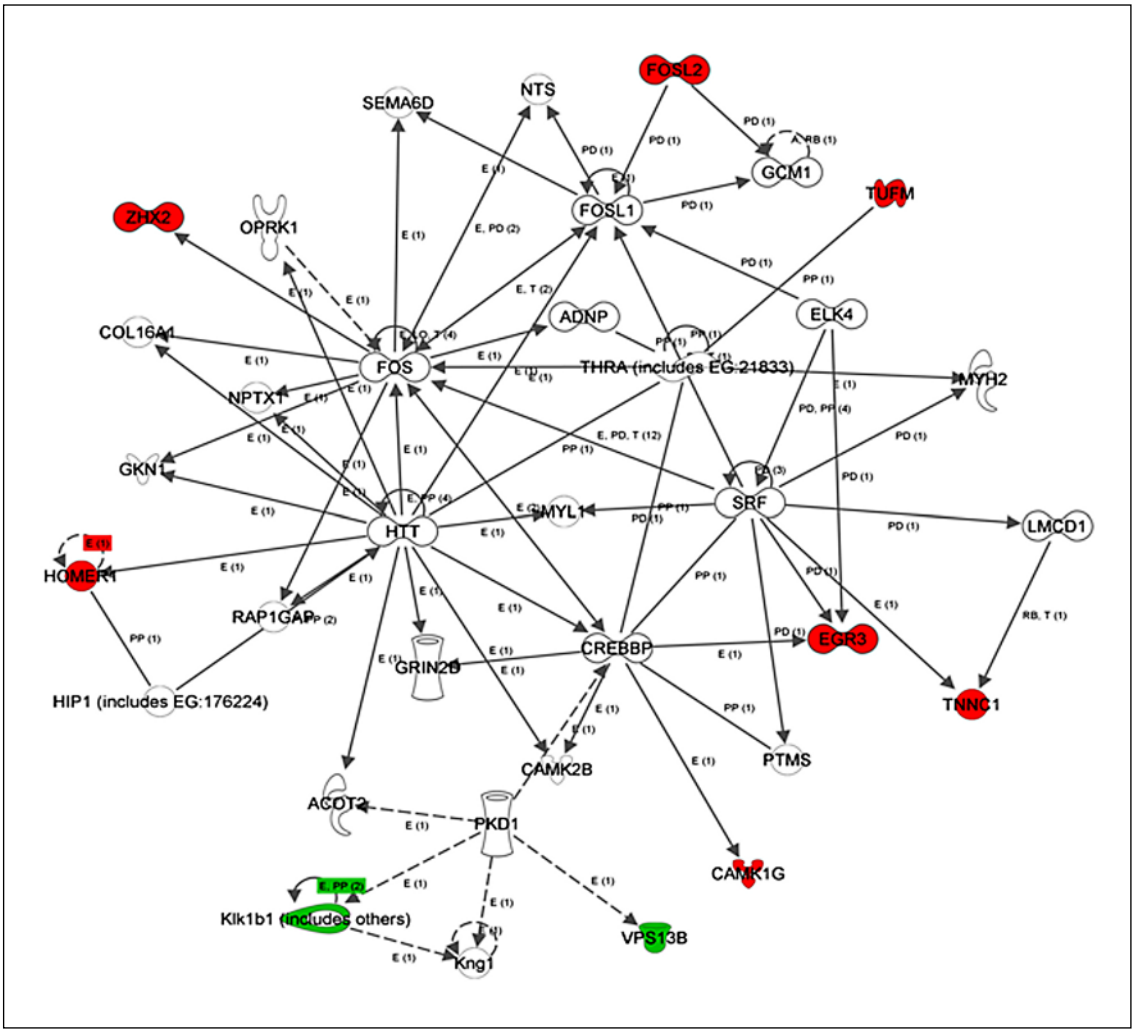

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Fig. 3. Third network resulting from IPA of long-term DEGs. Network name: amino acid metabolism, lipid metabolism and molecular transport. Highlighting (red and green) represents input genes. Red indicates downregulation and green indicates upregulation in ethanol mice vs. control mice. DEGs were identified using Partek Genomics Suite with a ±1.2-fold change and $p < 0.05$. A single-headed arrow indicates the action of 1 gene on another. A double arrow-headed line indicates that the 2 genes act on each other. Dashed lines indicate an indirect interaction, and solid lines indicate direct interactions. E = Expression; L = proteolysis; LO = localization; PD = protein-DNA binding; A = activation; P = phosphorylation/dephosphorylation; PR = protein-RNA binding; PP = protein-protein interaction; I = inhibition; M = biochemical modification; RB = regulation of binding; T = transcription; TR = translocation. Numbers in brackets indicate the number of PubMed references that reported interactions between molecules.

to a –1.90-fold decrease (miR-466c-3p) in ethanol-exposed versus matched control mice. Only 7 of the 20 miRNAs (35%) in this list were upregulated in ethanol-treated mice compared to matched controls.

The long-term gene expression and miRNA lists were subjected to supplementary target filtering analysis in IPA; 6 miRNAs were implicated in the results: miR-146b, miR-208b, miR-302c, miR-335, miR-449 and miR-455. All miRNAs in target filtering analysis, except for miR-302c, were downregulated. There were 10 genes associated in this analysis, all of which were downregulated (data not shown). The only opposing mRNA:miRNA expression pattern included miR-302c (upregulated) and coiled-coil domain containing 6 (*Ccdc6*, down-regulated). Because miR-302c was the only miRNA identified with an inverse miRNA:mRNA relationship, it was subjected to confirmation using qRT-PCR (–0.62-fold, $p = 0.04$; fig. 4a). Target Scan analysis of miR-302c binding to the 3′ untranslated region of *Ccdc6* was predicted using TargetScanMouse v.5.2 (TargetScanMouse, Cambridge, Mass., USA; fig. 4b).

Table 4. Functional characterization of long-term DEGs using IPA

Name	p value	Genes, n	Genes	Function annotation
Diseases and disorders				
Inflammatory disease	8.78E-05–3.11E-02	9	*Dnajb11, Hspa5, Itga4, Ptpn22, Xbp1, Prdx1, Fosl2, Egr3, mir-342*	SLE; multiple sclerosis
Neurological disease	8.78E-05–2.20E-02	13	*Egr3, Galnt7, Hist1h2bn, Homer1, Xbp1, Dnajb11, Hspa5, Itga4, Ptpn22, Dynlt1, Hsp90aa1, Tufm, P4ha1*	neuromuscular disease; schizophrenia
Skeletal and muscular disorders	8.78E-05–4.33E-02	14	*Egr3, mir-342, Ptpn22, Hsp90aa1, Prdx1, Cdkn1a, Fosl2, Tbxa2r, Dnajb11, Hspa5, Itga4, Xbp1, Dynlt1, Homer1, P4ha1*	SLE; osteosarcoma
Cancer	8.78E-05–4.93E-02	8	*Cdkn1a, Prdx1, Itga4, mir-342, Hsp90aa1, Hist1h3a, Hspa5, Ccdc6*	incidence of lymphoma
Hematological disease	2.78E-03–4.93E-02	6	*Cdkn1a, Itga4, Prdx1, Hspa5, Xbp1, Tbxa2r*	chronic B-cell leukemia
Molecular and cellular functions				
Cellular development	5.24E-04–4.93E-02	12	*Cdkn1a, Ccdc6, Xbp1, Tbxa2r, Egr3, marca5, Klk1b11, Hsp90aa1, Itga4, Ptpn22, Fosl2, Cbfa2t3*	plasmacytoid dendritic cell development
Cellular function and maintenance	5.24E-04–4.63E-02	10	*Egr3, Cdkn1a, Hsp90aa1, Itga4, tpn22, Hspa5, Tbxa2r, Slc9a3r2, Xbp1, Prdx1*	ER stress response
Cell cycle	6.28E-04–4.33E-02	3	*Cdkn1a, Ptpn22, Xbp1*	hepatocytes polyploidization
Cell death	8.15E-04–4.93E-02	14	*Smarca5, Cdkn1a, Hspa5, Xbp1, Egr3, Tbxa2r, Ptpn22, Ccdc6, Itga4, Prdx1, Slc9a3r2, Camk1g, Manf, Hsp90aa1*	cell death of immune cells
Cellular compromise	1.44E-03–4.33E-02	5	*Hspa5, Cdkn1a, Prdx1, Hsp90aa1, Xbp1*	stress response of cells
Physiological system development and function				
Lymphoid tissue structure/ development	4.30E-04–3.84E-02	10	*Egr3, Cdkn1a, Hsp90aa1, Itga4, Ptpn22, Xbp1, Tbxa2r, Spred2, Cbfa2t3, Prdx1*	development of plasmacytoid dendritic cells
Organ morphology	4.30E-04–4.93E-02	13	*Cbfa2t3, Cdkn1a, Prdx1, Ptpn22, Tbxa2r, Fosl2, Tnnc1, Hspa5, Spred2, Homer1, Xbp1, Klk1b11, Itga4*	Organization of cerebellum
Tissue morphology	4.30E-04–4.93E-02	12	*Itga4, Xbp1, Tbxa2r, Smarca5, Cdkn1a, Fosl2, Klk1b11, Ptpn22, Spred2, Cbfa2t3, Prdx1, Egr3*	abnormal morphology of cholinergic neurons
Cell-mediated immune response	5.24E-04–2.19E-02	5	*Cdkn1a, Egr3, Hsp90aa1, Itga4, Ptpn22*	metabolism of thymocytes
Embryonic development	5.24E-04–4.93E-02	10	*Cdkn1a, Spred2, Smarca5, Itga4, Egr3, Ptpn22, Fosl2, Xbp1, Hspa5, Otub2*	cell death of embryonic tissue

Quantitative RT-PCR Confirmation of Array Results

We chose to confirm the changes in expression of 16 genes (8 short-term, 8 long-term) using qRT-PCR. No genes from the short-term group were confirmed (p = 0.218–0.684). For the long-term group, we were able to confirm the downregulation of calcium/calmodulin-dependent protein kinase IG (*Camk1g*, 1.66-fold, p = 3.0e–4), *Ccdc6* (1.19-fold, p = 0.049), early growth response 3 (*Egr3*, 1.29-fold, p = 0.026), heat shock 70 kDa protein 5 (glucose-regulated protein 78 kDa, *Hspa5*, 1.61-fold, p = 0.029), and *Xbp1* (1.54-fold, p = 0.018). Albeit statistically significant, qRT-PCR results for homer homolog 1

Table 5. A list of differentially expressed miRNAs in second-trimester long-term array analysis

miRNA	Fold change	p value
miR-10b	–1.47	0.002
miR-1194	–1.30	0.006
miR-146b	–1.24	0.049
miR-184	–1.44	0.001
miR-1942	1.38	0.029
miR-1952	1.25	0.018
miR-1964	1.26	0.032
miR-208b	–1.37	0.040
miR-2145	1.39	0.029
miR-302c	1.27	0.011
miR-335-5p	–1.35	0.038
miR-342-5p	–1.27	0.025
miR-343	1.27	0.018
miR-369-5p	1.29	0.003
miR-449b	–1.27	0.043
miR-455	–1.48	0.037
miR-466b-3p	–1.35	0.038
miR-466c-3p	–1.90	0.030
miR-466e-3p	–1.49	2.5e-4
miR-684	–1.28	0.021

miRNAs are listed by numerical order. Data generated using a ±1.2-fold change and $p < 0.05$ in Partek Genomics Suite.

(Drosophila, *Homer1*, 0.74-fold, $p = 0.005$) and heat shock protein 90 kDa alpha (cytosolic), class A member 1 (*Hsp90aa1*, 0.89-fold, $p = 0.025$) were opposite to array results. Thromboxane A2 receptor *(Tbxa2r)* was not statistically significant ($p = 0.079$). We attributed this inconsistency to the sensitivity of the technology, as well as subtle changes in the arrays results.

Discussion

In this study, we compared gene expression changes between immediate and long-term ethanol-exposed B6 mice. Genes disrupted immediately following ethanol exposure were different from genes disrupted in adulthood (maintained). DEGs affected immediately following ethanol exposure represented the toxic effects of ethanol. Long-term DEGs were implicated in neurodevelopmental pathways relevant to FASD. Although behavioral pathways were not directly altered, changes to developmental processes may, indirectly, alter behaviors. These changes may be maintained by epigenetic mechanisms, and represent an adaptation to ethanol.

Immediate Ethanol Exposure Affects Genes in the Fetal Brain Involved in Toxicity and Apoptosis

The results demonstrated that prenatal ethanol exposure has an immediate effect on the fetal brain transcriptome. Given a short time of exposure (2 h), the major source of this subtle alteration may be attributable to alcohol toxicity and apoptosis of sensitive cell types, which can result in a novel composition of surviving cells. Early responding DEGs may provide an initial stress response to ethanol [36, 37], including a combination of random changes and an adjustment of physiological processes by alterations in defense genes. The result is that ethanol may disrupt specific genes and processes that are sensitive to external cues such as cell growth, proliferation or differentiation [38, 39].

Network analysis of short-term (immediate) ethanol exposure resulted in a network associated with free radical scavenging (fig. 1). Free radical scavenging is a mechanism by which ethanol may exert its effects on the fetal brain. The involvement of oxidative stress in FASD is well established in the literature [40–42]. Even a brief exposure to ethanol during gestation can produce an imbalance in the brain's intracellular redox state [43]. Such imbalances correlate with FASD-relevant spatial learning deficits [44]. We have previously identified a delay in spatial learning in second-trimester ethanol-exposed mice [10]. Our results could explain some of the FASD-relevant behavioral deficits resulting as a consequence of damage to cellular redox.

IPA network analysis involving genes affected in the short term identified hubs, one of which included HTT (fig. 1). HTT is involved in neuronal migration and survival during brain development [45]. This hub molecule was found to interact with two factors, hippocalcin (HPCA) and B-cell receptor-associated protein 31 (BCAP31). *Bcap31* is involved in a variety of cellular processes including apoptosis. Another hub, TP53, is a well-known apoptotic factor that may mediate the effects of prenatal ethanol exposure. Given that apoptotic events are altered following ethanol exposure during early neurulation [46], identifying TP53 as a central hub in our IPA network provides evidence for its potential role in FASD. This gives evidence for the immediate apoptotic and toxic effects of ethanol on neurodevelopment.

Potential Mechanisms Involved in Adaptation to Prenatal Ethanol Exposure in the Adult Brain

This research has identified functional networks affected over long-term ethanol exposure (fig. 2, 3). DEGs

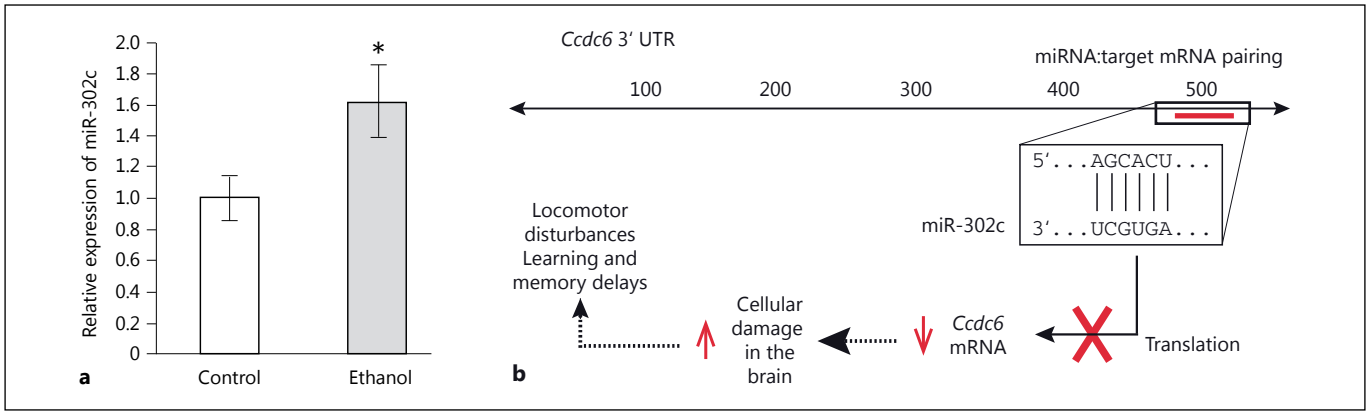

Fig. 4. Quantitative and bioinformatics analysis of miR-302c. **a** Bar graph of relative expression of miR-302c between ethanol and control samples. Error bars represent relative expression ± SEM. Statistical significance assessed using a two-tailed t test. * $p < 0.05$. Biological replicates: n = 5 control, n = 6 ethanol. Technical replicates: n = 3. **b** The molecular interaction between miR-302c and *Ccdc6*, and their potential implication for FASD-relevant cellular processes and behaviors. Alignment sequences of miRNA 302c (miR-302c) and coiled-coil domain containing 6 *(Ccdc6)* were identified using TargetScanMouse 5.2 (http://www.targetscan.org/mmu_50/). Binding of miR-302c to *Ccdc6* was predicted using TargetScanMouse. Red arrows indicate expression. A red 'X' indicates blocking. Dashed arrows indicate correlation. Dark red line represents miR-302c. UTR = Untranslated region.

involved in these networks represent genes that are maintained into adulthood. Such disrupted genes were relevant to neurological diseases, including systemic lupus erythematosus (SLE; table 4). *Xbp1* and *Egr3* have been previously associated with schizophrenia given their roles in unfolded protein response in neurons and in neuromuscular synaptic transmission, respectively [47, 48]. A number of genes were also involved in endoplasmic reticulum (ER) stress response. Alterations to ER processing have been implicated in a variety of neurodevelopmental disorders [49, 50]. Heat shock proteins (HSPA5 and HSP90AA1) are involved in ER-related processes such as folding and assembly of proteins. Disruptions in unfolded protein response are relevant to certain neurological diseases that share similarity to FASD, such as autism [51, 52].

Among the top long-term networks, XBP1 was identified as a hub molecule that appeared to downregulate other DEGs (fig. 2). Downregulation of *Xbp1* has been reported to regulate a subset of ER-resident chaperone genes (e.g. *Dnajb11*) in the unfolded protein response [53]. *Xbp1* is highly expressed in neurites during neurodevelopment through the induction of brain-derived neurotrophic factor [54], which has been implicated in our previous reports of FASD [13]. Figure 5 illustrates a network-based model of *Xbp1*'s effect on other DEGs, cellular processes and potential anxiety-related consequences. We have previously reported abnormalities in anxiety-related behaviors using this model [10]. This

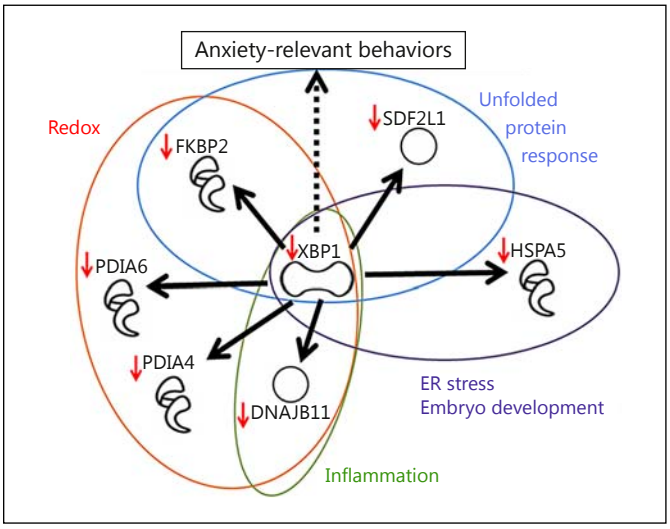

Fig. 5. A network-based model for the interaction of second-trimester disrupted molecules and their implication in FASD-relevant cellular processes and behaviors [47]. Black arrows indicate a molecule's interaction. A dashed arrow represents a correlation between a molecule and a specific behavior. Red arrows indicate downregulation. Redox = Reduction/oxidation. Molecular symbols are generated from IPA.

network-based model supports our findings that disrupted cellular processes (via dysregulation genes) may be directly affected following fetal alcohol exposure, while behavioral deficits may be a secondary, indirect result.

Adaptation of Neurodevelopmental Changes in Gene Expression following Prenatal Ethanol Includes Regulation by miRNAs

The effect of prenatal ethanol during neurodevelopment also includes miRNAs (table 5). These results follow recent literature [55, 56]. Our findings argue that these affected miRNAs may regulate fine-tuning of gene expression posttranscriptionally, which has been reported by others [57, 58]. Given that miRNAs can lead to long-term changes in gene expression [15, 16] and play important roles during development [59–61], we explored their role in the maintenance of FASD-related abnormalities. This analysis included target prediction and confirmation of miR-302c and its target gene, *Ccdc6*, by qRT-PCR (fig. 4). We recognize that our results do not show a direct miR-302c:*Ccdc6* interaction, and a luciferase assay could be performed to verify this relationship [62]. The miR-302 cluster has been identified as a key family of miRNAs involved in embryonic stem cell-specific cell-cycle regulation and reprogramming of human and mouse embryonic fibroblasts [63, 64]. miR-302c contributes to regulation of p21 (CKDN1A) expression in human embryonic stem cells, thus illuminating its role in DNA damage. Dolezalova et al. [65] reported that members of the miR-302 family were direct contributors to regulation of p21 expression in human embryonic stem cells using luciferase assays to confirm miRNA microarrays, qRT-PCRs and Western blots. Given that *Cdkn1a* and miR-302c were differentially expressed in our studies, their dysregulation could contribute to cell cycle arrest and DNA damage in the brain [65, 66]. Since fetal alcohol exposure may lead to DNA damage in the brain [46], the disruption of cell cycle checkpoint genes following exposure may lead to maintenance of FASD-relevant neurodevelopmental deficits in adults. Our target filtering approach demonstrates that 1 miRNA can affect multiple targets and may therefore play a role in altered transcriptomics in prenatal alcohol exposure (fig. 4b). Future research is needed to fully understand the role of epigenetic processes in the development and maintenance of neurodevelopmental disorders, including FASD.

Immune Response May Be Affected Immediately following Prenatal Ethanol Exposure and into Adulthood

Certain genes may play key roles in mediating the immediate effects of alcohol exposure (fig. 6; table 2). Specifically, *Ccl3* is implicated in innate immune response via its role in T lymphocyte trafficking. CCL3 is essential for the recruitment of dendritic cells to sites of microbial infection [67]. Its alteration may account for immune deficiencies, including impaired adaptive immune responses to viral infection, in neonates and adults with FASD [68, 69]. Hubs in the short-term network including TNF and NFkB act on the upregulated factor, CCL3 (fig. 1). TNF, NFkB and APP are involved in the regulation of hippocampal synaptic plasticity and synapse formation [70, 71]. Studies suggest that NFkB and APP also have important roles in learning and memory in mice by modulation of synaptic plasticity and growth of dendrites [70, 72, 73]. We have previously identified APP as a hub gene that was differentially methylated in adulthood [15]. These results suggest that synapse formation is largely affected immediately following ethanol exposure.

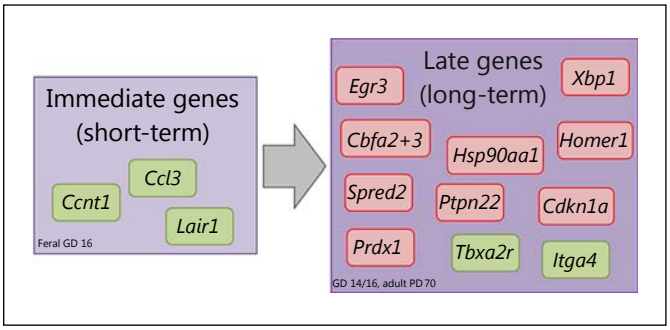

Fig. 6. Immune response-related genes altered in the short-term (immediate genes) and long-term (late genes) brains following binge ethanol exposure in the second trimester. DEGs identified using Partek Genomics Suite. Upregulated (green) and downregulated (red) genes are shown for each group.

Immune response genes are also altered in the adult brain that has been exposed to ethanol prenatally (fig. 6). IPA functional analysis implicated SLE as a top function under 'Inflammatory Disease' (table 4). T cells are defective in individuals with SLE [74]. Researchers have reported lowered T cell numbers in fetal mice exposed to ethanol; however, the ability of T cell levels to 'catch up' to normal levels was reinstated in adulthood [75]. This reflects a form of the speculated epigenetic adaption. Weinberg and colleagues [76] have reported on the influence of early-life fetal alcohol exposure and its impact on the reprogramming of the HPA-axis throughout life and subsequent vulnerability to illnesses later in life. We suspect that such genetic reprogramming, which may contribute to immune system deficits, is regulated by epigenetic modifications, including the action of miRNAs. In our study, the top IPA network is relevant to immunity and includes XBP1 (fig. 2). Mice deficient in XBP1 show increased bacterial burden once infected with a pathogen

[77]. Herein, *Xbp1* may be an important player in maintaining a compromised immune system into adulthood. Lowered expression of other genes resulting from down-regulation of *Xbp1* may represent an important cascade of events that together lead to compromised immunity in FASD (fig. 5, 6).

Limitations

Although this research is novel in the field of neurodevelopmental disorders and epigenetics, there are some caveats to our study. As mentioned, we did not directly observe an interaction between miR-302c and *Ccdc6*. This could be accomplished using a luciferase reporter assay, as performed by other researchers [78, 79]. Given this limitation, we can only conclude a speculative relationship between miR-302c and *Ccdc6*, and our findings would require further investigation. Another caveat to our study includes the inconsistency between fetal and adult brains. We used males for long-term expression studies because female estrous cycles may confound results, particularly in the expression of hormone receptors [80, 81]. Further, it has been reported that spatial reference memory, an FASD-relevant trait, varies with the estrous cycle in B6 mice [82]. In our short term studies, we did not differentiate between male and female fetal brains due to the unimportance of estrous cycle during this developmental time. However, we do recognize that the inconsistencies between brain tissues may confound our results. The next caveat was that long-term injections were given on 2 days (GD 14 and 16), whereas short-term injections were given on 1 day (GD 16). Only 1 day of injection was considered for our short-term exposure paradigm in order to immediately observe the apoptotic effects of alcohol following maternal exposure. We recognize that inconsistencies in treatment may confound results. However, our primary objective was to observe the effects of ethanol during the second-trimester equivalent in mice, both immediately following exposure and into adulthood. Although these caveats exist, our results provide evidence for epigenetic mechanisms in the long-term maintenance of abnormalities resulting from maternal alcohol exposure. Furthermore, the effects of alcohol immediately following exposure in the fetus are different from the effects of alcohol following long-term adaptation in the adult brain. Another consideration in our approach was the administration of ethanol noncontingently. We have previously used a self-administration model [9, 14], which has yielded a different set of DEGs. Given the nature of ethanol administration, these two paradigms would likely lead to differing maternal BACs, thereby generating different expression results. A final caveat to our study was the disuse of miRNAs for the short-term group. However, given that miRNAs may fine-tune gene regulation, our primary focus for this study was to evaluate miRNAs as a potential mechanism that could lead to maintenance of long-term gene expression changes.

Conclusions

Genes affected immediately following prenatal ethanol exposure are different than genes that show alterations following development and into adulthood. Most of the immediate effects are driven by ethanol's toxicity. The surviving cells may adapt by alterations in gene expression that may involve epigenetic changes [15, 83]. This report supports a role for miRNAs in establishing a novel molecular footprint in adults, and shows potential for interaction between miR-302c and *Ccdc6*. Disrupted functional networks are involved in inflammation, neuronal formation and DNA repair. Such networks are anchored by key hubs. These transcriptomic alterations may maintain subsequent FASD-relevant behavioral dysfunctions.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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