Metabolic Biomarkers of Prenatal Alcohol Exposure in Human Embryonic Stem Cell–Derived Neural Lineages

Jessica A. Palmer, Ashley M. Poenitzsch, Susan M. Smith, Kevin R. Conard, Paul R. West, and Gabriela G. Cezar

Background: Fetal alcohol spectrum disorders (FASD) are a leading cause of neurodevelopmental disability. The mechanisms underlying FASD are incompletely understood, and biomarkers to identify those at risk are lacking. Here, we perform metabolomic analysis of embryoid bodies and neural lineages derived from human embryonic stem (hES) cells to identify the neural secretome produced in response to ethanol (EtOH) exposure.

Methods: WA01 and WA09 hES cells were differentiated into embryoid bodies, neural progenitors, or neurons. Cells along this progression were cultured for 4 days with 0, 0.1, or 0.3% EtOH. Supernatants were subjected to C18 chromatography followed by ESI-QTOF-MS. Features were annotated using public databases, and the identities of 4 putative biomarkers were confirmed with purified standards and comparative MS/MS.

Results: EtOH treatment induced statistically significant changes to metabolite abundance in human embryoid bodies (180 features), neural progenitors (76 features), and neurons (42 features). There were no shared significant features between different cell types. Fifteen features showed a dose–response to EtOH. Four chemical identities were confirmed: L-thyroxine, 5′-methylthioadenosine, and the tryptophan metabolites, L-kynurenine and indoleacetaldehyde. One feature with a putative annotation of succinyladenosine was significantly increased in both EtOH treatments. Additional features were selective to EtOH treatment but were not annotated in public databases.

Conclusions: EtOH exposure induces statistically significant changes to the metabolome profile of human embryoid bodies, neural progenitors, and neurons. Several of these metabolites are normally present in human serum, suggesting their usefulness as potential serum FASD biomarkers. These findings suggest the biochemical pathways that are affected by EtOH in the developing nervous system and delineate mechanisms of alcohol injury during human development.

Key Words: Human Embryonic Stem Cells, Neural Progenitors, Fetal Alcohol Syndrome, 5′-Methylthioadenosine, L-Kynurenine, Indoleacetaldehyde, LC-MS, Metabolomics.

FETAL ALCOHOL SPECTRUM disorders (FASD) are a leading cause of neurodevelopmental disability and are associated with deficits in learning, motor skills, and executive functioning. The basis of alcohol injury to neurons includes altered proliferation, differentiation, and synaptogenesis and is incompletely understood (reviewed in Miller, 2006). Deeper understanding of the mechanisms underlying alcohol's toxicity could facilitate prevention, earlier detection, or repair of the resulting damage. However, implementation of such strategies is challenged by the limited availability of diagnostic biomarkers that could identify at-risk pregnancies and those with FASD.

The human embryo and the regulatory mechanisms directing its development are challenging to study. Human embryonic stem (hES) cells permit this study using an in vitro model. hES cells are pluripotent, self-renewing cells isolated from human embryos (Thomson et al., 1998). They give rise to all differentiated cell types in the human body and recapitulate embryonic development in vitro, including the faithful replication of neurodevelopment and differentiation of hES cells into neural precursors, neurons, and glia (Reubinoff et al., 2000; Zhang et al., 2001). Because they function similarly to in vivo counterparts (Ben-Hur et al., 2004; Zhang et al., 2001), hES cell-derived models are an ideal research tool to elucidate biochemical mechanisms underlying disease, including alcohol's effects on human neural development.

The human metabolome is the comprehensive set of endogenous small molecules. It is a product of health or disease states and can be measured on every level of complexity, for example, tissues, biofluids, and cells (Fiehn, 2002).

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Abnormal metabolite production in response to environmental, dietary, or disease insult provides mechanistic insights into these atypical processes. Metabolite composition of a biologic sample is very sensitive to environmental effects (Lindon et al., 2003). We have used hES cells to both discover biomarkers and establish predictive models of chemically induced developmental toxicity (Cezar et al., 2007; West et al., 2010).

Specific changes to metabolites identified in response to alcohol exposure could serve as biomarkers for FASD. Such biomarkers also outline the mechanisms underlying alcohol's pathogenicity, leading to novel treatment opportunities. The analysis of biomarkers in serum and amniotic fluid is an established approach to predict birth defects and premature births. The presence of fatty acid ethyl esters (FAEEs) in meconium is employed to identify alcohol exposure during pregnancy. However, FAEEs can be present in the meconium of unexposed infants (Chan et al., 2003) and do not provide information on the timing, duration, and dosage of exposure (Litten et al., 2010). Additionally, FAEEs do not provide information on the damage that may have occurred specifically to the developing brain and the mechanism of injury. Thus, there is a significant need for reliable biomarkers that can be used for FASD prevention, diagnosis, and treatment.

This study tested the hypothesis that alcohol exposure induces statistically significant changes to the abundance of small molecule metabolites in human neural progenitors and neurons. To test this, we treated hESC-derived embryoid bodies (EBs), neural progenitors, and neurons with 0, 0.1, or 0.3% ethanol (EtOH). We employed liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS) to identify and measure changes to secreted metabolite abundance in response to alcohol exposure. Here, we describe the metabolome of hES cell derivatives exposed to EtOH during 3 stages of neurogenesis.

MATERIALS AND METHODS

Ethanol Treatment

WA01 and WA09 hES cells, obtained from WiCell Research Institute (Madison, WI), were exposed to 0, 0.1, or 0.3% EtOH for 4 days at 1 of 3 stages of neurogenesis: days 0 to 4 (EBs), days 17 to 21 (neural progenitors), and days 28 to 32 (neurons) (Zhang et al., 2001) (outlined in Fig. 1). EtOH doses were selected to mimic fetal exposure to binge-like low and high EtOH levels, respectively. Each cell line, time point, and treatment included 5 biologic replications of 3 samples per treatment per replication. Additionally, at each time point, media controls were included containing appropriate media exposed to the plate conditions without cells.

Neural Differentiation

Stage 1: Embryoid Body Formation. hES cells grown in mTeSR1 media (Stem Cell Technologies Inc., Vancouver, BC) were dispersed following treatment with dispase (2 mg/ml; Invitrogen, Carlsbad, CA) and grown in suspension. EBs were cultured in EB formation media, containing Iscoves's Modified Dulbecco's Medium (1×; Invitrogen), supplemented with 15% heat-inactivated certified fetal bovine serum (Invitrogen) for 4 days in 90 mm low-binding dishes (Nalge Nunc International, Rochester, NY). EB formation media was changed every other day for neural progenitor and neuron differentiation experiments. For treatment, ~50 EBs were placed in each well of 24-well low-binding microplates (Nalge Nunc International) 24 hours after formation. During treatment, media and compound were replaced daily.

Stage 2: Neurospheres. On day 5 of suspension culture, media was switched to neural precursor media, which consisted of DMEM/F12 (1:1) supplemented with 1% N2 Supplement, 0.1 mM MEM nonessential amino acids (NEAA), 1% Insulin-Transferrin-Selenium-X Supplement, 20 ng/ml FGF2, 20 ng/ml EGF (all from Invitrogen), 2 μg/ml heparin, 0.5 μM retinoic acid, and 60 μM putrescine (all from Sigma-Aldrich, St. Louis, MO). Neurospheres were maintained in neural precursor media for 11 days, with media changed every other day.

Stage 3: Neural Progenitors. On day 17 of neural differentiation, neurospheres were dissociated into a single-cell suspension using 0.25% trypsin-EDTA (Invitrogen) containing 2% chick serum (Sigma-Aldrich). The cells were plated on precoated poly-L-ornithine and laminin 24-well plates (BD Biosciences, San Jose, CA) at 2×10^6 cells/well. Neural progenitors were maintained in neural progenitor media, which consisted of DMEM/F12 (1:1) supplemented with 1% N2 supplement, 0.2 mM MEM-NEAA, 500 ng/ml sonic hedgehog (R&D Systems, Minneapolis, MN), 100 ng/ml FGF8 (Invitrogen), and 10 ng/ml FGF4 (Invitrogen). Media was changed every other day during routine maintenance. Treatment began on day 18, media was replaced, and fresh compound added daily during the exposure period. Treated neural progenitors were fixed on day 22 for nestin and caspase-3 analysis.

Stage 4: Neural Differentiation. Beginning on day 22, neural progenitors were differentiated into neurons. Cells were cultured in

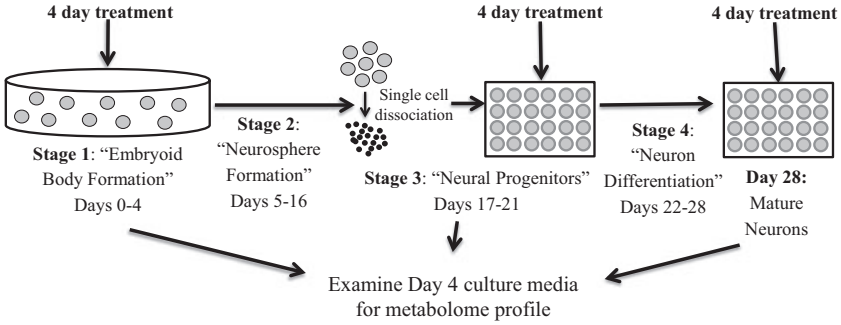

Fig. 1. Outline of human embryonic stem cell differentiation and treatment protocol used in this study.

neural differentiation media consisting of neurobasal medium supplemented with 1% N2 supplement, 0.1 mM MEM-NEAA, 0.5 mM L-glutamine, 1 μg/ml laminin, 20 ng/ml BDNF, 10 ng/ml GDNF (all from Invitrogen), 1 μM cAMP, 30 μM serotonin (5HT) (both from Sigma-Aldrich), and 50 ng/ml sonic hedgehog. Starting on day 28, neurons were treated for 4 days and media was replaced daily. Neurons were fixed on day 32 for caspase-3, synaptogenesis, and differentiation immunocytochemistry analysis.

Immunocytochemistry

Paraformaldehyde-fixed cells were immunostained using standard techniques. Primary antibodies included anti-nestin (MAB5326; Millipore, Billerica, MA), anti-β-III-tubulin (T8660; Sigma-Aldrich), anti-synaptophysin (ab52636; Abcam, Cambridge, MA), anti-SV2A (sc-28955; Santa Cruz Biotechnology, Santa Cruz, CA), anti-tyrosine hydroxylase (AB152; Millipore), and anti-cleaved caspase-3 (9661; Cell Signaling Technology, Danvers, MA). Secondary antibodies were conjugated with Alexa Fluor 488 (A11001; Invitrogen) or Alexa Fluor 594 (A21207; Invitrogen). For synaptophysin and SV2A, Triton X-100 was omitted to maintain the integrity of membrane structures. Positive staining of postmitotic neurons was defined as cells that co-expressed β-III-tubulin and an additional neuronal marker: synaptophysin, SV2A, or tyrosine hydroxylase. At least 300 cells/sample and 6 samples/treatment were counted. Statistical analysis was performed using a 1-way analysis of variance (ANOVA) with statistical significance defined as $p \leq 0.05$.

Sample Preparation

On the fourth day of treatment, spent media was collected from each sample, quenched with LC-MS-grade acetonitrile (final acetonitrile concentration = 40%), and stored at −80°C until preparation. For MS analysis, equal amounts of quenched sample and LC-MS-grade water were added to a Microcon centrifugal filter with a molecular weight cutoff of 3 kDa (Millipore). Each sample was centrifuged at 13,000×*g* for 3 hours at 4°C. The flow through was retained, dried using a vacuum concentrator, and stored at −80°C. Samples were reconstituted in 50 μl of 0.1% formic acid in water prior to MS analysis.

LC-ESI-QTOF-MS

LC-ESI-QTOF-MS was performed at Stemina Biomarker Discovery (Madison, WI) using an Agilent QTOF LC-MS system consisting of a 1200SL HPLC system and a G6520AA QTOF high-resolution mass spectrometer (Agilent Technologies, Wilmington, DE) capable of exact mass MS and tandem mass spectrometry (MS/MS) ion fragmentation. The flow through of each sample was analyzed using a 2.1 × 50 mm Zorbax 1.8 um C18-SB column (C18; Agilent Technologies). Ultraperformance liquid chromatography analysis was performed using a 20-minute gradient starting with ratio of 95% 0.1% formic acid in water (Solvent A) to 5% 0.1% formic acid in acetonitrile (Solvent B) and ending with 100% Solvent B at a flow rate of 500 μl/min. ESI was employed using a dual ESI source, with an Agilent isocratic pump continuously delivering an internal mass reference solution into the source at approximately 0.01 ml/min for mass correction. For positive-ion ESI, the reference solution consisted of 7H-purine (m/z 121.0509) and HP-0921 (m/z 922.0098). The reference solution for negative-ion ESI contained trifluoroacetic acid (TFA; m/z 112.9856) and the TFA adduct of HP-0921 (m/z 1033.9881). The mass range of the instrument was set at 70 to 1,600 Da. The sample order was randomized, and 5 μl of each sample was injected. A solvent blank (0.1% formic acid) was run after every 10 samples. All 3 time points were analyzed with positive-ion ESI. Additionally, negative-ion ESI was performed on the EB experiment samples. Data acquisition was performed with Agilent MassHunter Acquisition software using high-resolution exact mass conditions.

Data Analysis of LC-ESI-QTOF Metabolomics

Prior to data analysis, the total ion chromatogram (TIC) of each sample was carefully inspected for quality and reproducibility of the MS signal. If a sample's TIC abundance deviated by more than 25% from the median across the LC-MS gradient, the LC-MS analysis was repeated for that sample. The data were deisotoped and then converted into the open-source mzData format. Data analysis was performed using the open-source statistical programming and analysis software, R. The XCMS package (Smith et al., 2006) was used to analyze the LC-ESI-QTOF-MS resulting files using the Centwave algorithm for peak peaking (Tautenhahn et al., 2008). Retention time deviations across EB LC-MS samples were corrected using retcor loess regression and the obiwarp method for neural precursor and neuron samples. After retention time correction, the features were grouped using the density-based functions in XCMS. After the grouping function was performed, features missing in LC-MS samples were iteratively integrated using m/z and retention time windows based on the range of the feature group. Contaminant ions were removed by comparing spent media extracts with blank extraction samples. Statistical significance of individual mass features was performed under the null hypothesis that no difference exists in feature abundance between control and treated samples. Differential small molecule metabolites, or features, were determined using a complete block design ANOVA with the model "log2(abundance) ~treatment + replicate + cell line." Features were considered statistically significant if they had a p-value ≤ 0.05 in the treatment factor of the ANOVA model. Additionally, to be considered authentic, each feature was required to show a statistically significant alteration in both cell lines evaluated. The extracted ion chromatogram (EIC) of each statistically significant feature was then visually evaluated to confirm an observable difference between treated and control samples and to reduce the inclusion of spurious results.

For feature annotation, the neutral exact mass of each feature was queried against the public searchable databases METLIN (http://metlin.scripps.edu), The Human Metabolome Database (http://www.hmdb.ca), and the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/) for candidate identities. Measured mass features were considered a putative match to a small molecule present in the databases if their exact masses were within 20 ppm of the annotated database molecule (0.00002× mass). Statistically significant annotated features were confirmed using MS/MS.

Confirmation of Candidate Biomarkers by LC-ESI-QTOF-MS/MS

Analytical-grade chemical standards for thyroxine, 5′-methylthioadenosine (MTA), L-kynurenine, and indoleacetaldehyde (IAA) were purchased from Sigma-Aldrich for comparative MS. Chemical references were evaluated using identical sample preparation and chromatographic methods employed in the analysis of the original samples. Additionally, the 3 original samples with the highest abundance for each feature of interest were re-prepped and analyzed for comparison with the standard. Chemical references were dissolved in the appropriate basal media at 3 concentrations: 1, 0.1, and 0.01 mM. Additionally, a 0.1 mM solution was prepared for each standard in 0.1% formic acid, with the exception of thyroxine, which was solubilized in a 0.1 mM solution of 50/50 methanol and dichloromethane. Data acquisition for targeted MS/MS was performed by inputting the m/z value of the precursor ion of interest into the acquisition software with a value for the collision-induced dissociation collision energy and expected retention time range. A putative annotation was considered confirmed if the retention time, measured exact mass, and fragmentation pattern of the metabolite

reasonably match these data from chemical references. If published MS/MS data were available, the fragmentation pattern of the sample must additionally match the published MS/MS spectra.

RESULTS

Metabolomics

Embryoid Bodies. In culture medium from human EBs, we identified 3,246 total features using C18 chromatography followed by positive ionization ESI-QTOF. Of these features, 510 were considered secreted molecules, as defined by having at least a 10% increase in abundance in the cell samples compared with the media controls. This standard was used in defining secreted molecules for all data sets. In 0.1 and 0.3% EtOH-treated EBs, 70 and 62 secreted features were statistically significant, respectively, from control-treated cells ($p < 0.05$, Table S1). There were a total of 9 significant features in common between the 2 EtOH treatments (Table 1). One of the most challenging aspects of metabolomics is that >50% of the features identified do not have annotation in public databases yet. Of these 9 features, 2 had molecular weights consistent with a putative annotation of diethylphosphate and heptacarboxylporphyrin III. Three were tentatively assigned identities as tripeptides, and 4 additional small molecules were unannotated. Several of these identities remain putative given the unavailability of a commercially available chemical standard. We confirmed the identity of MTA (exact mass 297.0895) using MS/MS (Fig. 2, Table 2). MTA participates in polyamine synthesis and methionine metabolism. MTA levels were significantly decreased in 0.3% EtOH-treated EBs ($p = 0.0275$). MTA was also decreased in 0.1% EtOH-treated EBs, although the reduction was not statistically significant ($p = 0.1251$).

Using C18 chromatography and negative ionization ESI-QTOF, 1,009 total features were measured of which 295 were secreted. Of the secreted features, 21 and 27 were significantly altered in 0.1% and 0.3% EtOH-treated EBs, respectively, when compared to controls ($p < 0.05$, Table S2). Two secreted features were significantly altered in both EtOH treatment groups, and based on their exact mass and retention time, they were putatively identified as the methylated purine 7-methylinosine (exact mass 283.1060) and succinyladenosine (exact mass 383.1075,

Table 1. Metabolome Features Significantly Altered by both 0.1 and 0.3% Ethanol (EtOH) Treatment

Putative annotation	Neutral mass (Da)	Retention time (seconds)	*p*-Value	Percent fold change
Embryoid bodies				
Positive ionization				
Diethylphosphate	154.0404	371.07	0.1%: 0.0215	0.1%: 139%
			0.3%: 0.0021	0.3%: 81%
Unannotated EB 1	182.0717	372.69	0.1%: 0.0009	0.1%: 81.5%
			0.3%: 0.0010	0.3%: 58%
Unannotated EB 2	341.9227	440.64	0.1%: 0.0099	0.1%: −27%
			0.3%: 0.0102	0.3%: −46%
Unannotated EB 3	444.1747	35.43	0.1%: 0.0248	0.1%: 12%
			0.3%: 0.0392	0.3%: −40%
Unannotated EB 4	161.9918	31.39	0.1%: 0.0173	0.1%: 13%
			0.3%: 0.0464	0.3%: −16%
EB Tripeptide 1 Ala/Arg/Trp	431.2362	518.06	0.1%: 0.0099	0.1%: −27%
			0.3%: 0.0102	0.3%: −46%
EB Tripeptide 2 His/Pro/Trp	438.2000	373.32	0.1%: 0.0246	0.1%: −25%
			0.3%: 0.0023	0.3%: 26%
EB Tripeptide 3 Ile/Trp/Trp; Leu/Trp/Trp	503.2478	63.20	0.1%: 0.0236	0.1%: 0.7%
			0.3%: 0.0278	0.3%: −16%
Heptacarboylporphyrin III	754.2607	59.09	0.1%: 0.0066	0.1%: 33%
			0.3%: 0.0415	0.3%: 8%
Negative ionization				
7-Methylinosine	283.1060	21.07	0.1%: 0.0155	0.1%: −26%
			0.3%: 0.0081	0.3%: 20%
Succinyladenosine	383.1075	35.92	0.1%: 0.0380	0.1%: 67%
			0.3%: 0.0387	0.3%: 94.5%
Neural progenitors				
Unannotated hNP 1	746.0645	369.11	0.1%: 0.0367	0.1%: −6%
			0.3%: 0.0280	0.3%: −13%
Neurons				
hNN Tripeptide 1 Cys/His/Lys	386.1734	523.15	0.1%: 0.0001	0.1%: 212%
			0.3%: 1.8e-19	0.3%: 240%
Na+ adduct of hNN Tripeptide 1	408.1558	523.19	0.1%: 5.0e-11	0.1%: 161%
			0.3%: 3.6e-33	0.3%: 184%
K+ adduct of hNN Tripeptide 1	424.1308	523.13	0.1%: 4.8e-8	0.1%: 227%
			0.3%: 6.5e-31	0.3%: 256%

EB, embryoid body.

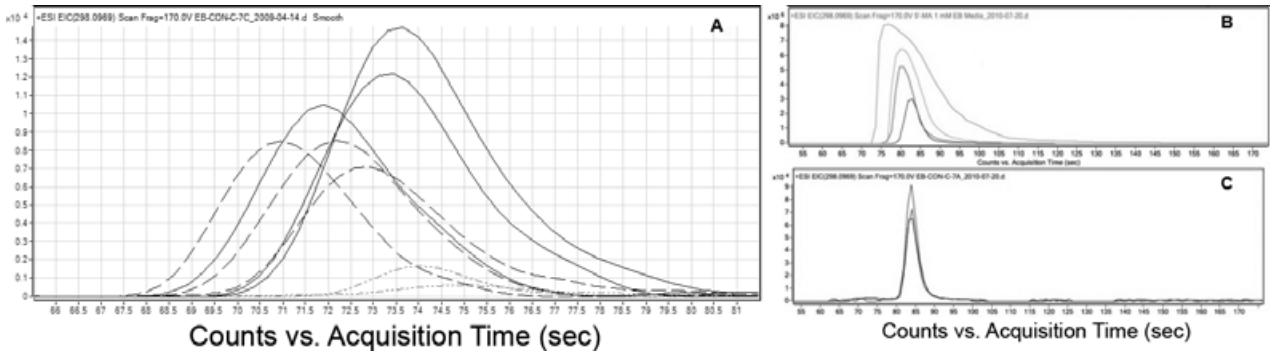

Fig. 2. Identification and abundance of 5′-methylthioadenosine (MTA) in ethanol (EtOH)-treated embryoid bodies. (**A**) Extracted ion chromatogram (EIC) in positive ionization mode compares the abundance of MTA in individual sample media plotted against its characteristic retention time following separation using quadrupole time-of-flight mass spectrometry. The solid lines represent control samples, dotted lines are 0.3% EtOH samples, and gray dashed are media control samples. (**B**) EIC of chemical standards for comparison. (**C**) EIC of 3 samples with the highest abundance of MTA. (**B**, **C**) show that the retention time of the samples and chemical standards is the same, confirming the compound's identity.

Table 2. Summary of MS/MS Confirmed Molecules at Various Differentiation Time Points

Compound	Exact mass (Da)	Retention time (seconds)	*p*-Value	Percent change
Embryoid bodies				
5′-Methylthioadenosine	297.0895	73.72	0.1251	0.1%: −27%
			0.0275	0.3%: −7.2%
Thyroxine	776.6867	449.72	0.0011	0.1%: 24%
			0.1427	0.3%: −6%
Neural progenitors				
L-Kynurenine	208.0848	33.77	0.0200	0.1%: 11%
			0.8951	0.3%: −18%
Neurons				
Indoleacetaldehyde	159.06842	30.67	0.0493	0.1%: 5.7%
			0.4735	0.3%: 3.5%

MS/MS, tandem mass spectrometry.

Table 1). Succinyladenosine levels were significantly increased in both 0.1 and 0.3% EtOH-treated EBs compared with untreated controls ($p = 0.0380$ and 0.0387, respectively). Succinyladenosine is a biomarker for adenylosuccinate lyase deficiency, but it has also been measured in the cerebrospinal fluid of children without this condition, using HPLC negative-ion ESI MS on a C18 column (Krijt et al., 1999). Unfortunately, no chemical standard was commercially available for chemical identity confirmation. Using negative ionization ESI-QTOF MS/MS, we also confirmed the identity of thyroxine (exact mass 776.6867; Fig. 3, Table 2). Thyroxine was significantly increased in 0.1% EtOH-treated EBs compared with controls (24% increase, $p = 0.0011$).

Neural Progenitors. At the neural progenitor differentiation stage, we measured 927 total features with C18 chromatography and positive ionization ESI-QTOF. Nearly half of the total features were considered secreted ($n = 434$). In comparison with untreated neural progenitors, 56 and 20 features were significantly different in 0.1 and 0.3% EtOH treatment groups, respectively ($p < 0.05$, Table S3). Between the 2 EtOH treatment groups, there was 1 significant feature in common, which had a decreased abundance in EtOH treatment (Table 1). It could not be assigned an identity and had neutral mass of 746.0645 Da. In addition, L-kynurenine (exact mass 208.0848), a tryptophan metabolite, was

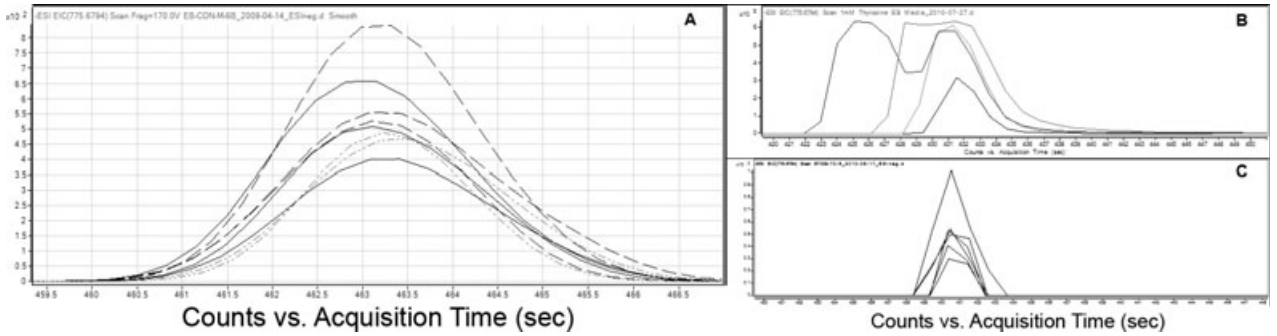

Fig. 3. Identification and abundance of thyroxine in ethanol (EtOH)-treated embryoid bodies (EBs). (**A**) Extracted ion chromatogram (EIC) in negative ionization mode compares the abundance of thyroxine in individual sample media plotted against its characteristic retention time following separation using quadrupole time-of-flight mass spectrometry. The solid lines are control samples, dotted lines are 0.1% EtOH samples, and gray dashed lines are media controls. (**B**) EIC of chemical standards for comparison. (**C**) EIC of the 3 samples with the highest abundance of thyroxine. (**B**, **C**) show that the retention time of the samples and chemical standards is the same, confirming the compound's identity.

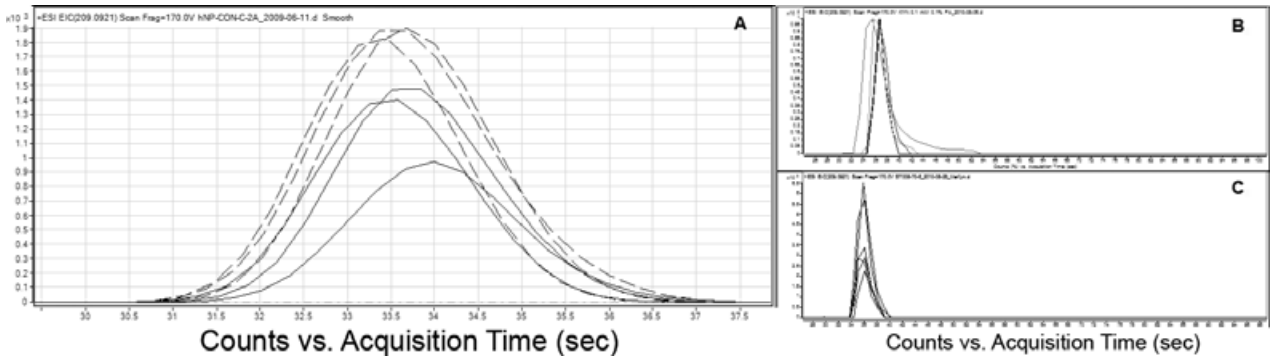

Fig. 4. Identification and abundance of L-kynurenine in ethanol (EtOH)-treated neural progenitors. (**A**) Extracted ion chromatogram (EIC) in positive ionization mode compares the abundance of kynurenine in individual sample media plotted against its characteristic retention time following separation using quadrupole time-of-flight mass spectrometry. The solid lines are control samples, dotted lines are 0.1% EtOH samples, and gray dashed lines are media controls. (**B**) EIC of chemical standards for comparison. (**C**) EIC of the 3 samples with the highest abundance of kynurenine. (**B**, **C**) show that the retention time of the samples and chemical standards is the same, confirming the compound's identity.

significantly increased in 0.1% EtOH-treated neural progenitors ($p = 0.0200$). The identity of kynurenine was confirmed using MS/MS (Fig. 4).

Neurons. In terminally differentiated neurons, 1,268 total features were measured by C18 chromatography and positive ionization ESI-QTOF. The total number of secreted features was 582. In 0.1 and 0.3% EtOH-treated neurons, 23 and 19 features, respectively, were statistically significant when compared with control samples ($p < 0.05$, Table S4). Three significant features were common to both EtOH treatment groups ($p < 0.0001$ for all vs. control neurons, Table 1). The EIC of the primary feature is shown in Fig. 5. These 3 features were tentatively identified as a tripeptide containing cys-his-lys (exact mass 386.1734), and its respective sodium (exact mass 408.1558) and potassium adducts (exact mass 424.1308). The amino acid sequence order could not be determined. We confirmed the identity of IAA (exact mass 159.06842), another metabolite in the tryptophan pathway, with MS/MS (Fig. 6). IAA was significantly increased in the media of 0.1% EtOH-treated neurons compared with controls ($p = 0.0493$). IAA levels were also increased in 0.3% EtOH-treated EBs compared with controls, although the increase was not statistically significant ($p = 0.47$).

Immunocytochemistry

To determine whether EtOH exposure affected the differentiation of these neural populations, we examined the expression of neural markers at the end of the 4-day alcohol exposure period. For the neural progenitors, we found no alcohol-related differences in their expression of nestin, an intermediate filament protein expressed in mitotic neural progenitors (Table 3). Control, 0.1%, and 0.3% EtOH treatments all exhibited >95% nestin expression in the progenitor cultures, confirming their neural progenitor identity. For the neurons, we observed no significant differences in the percentage of cells that co-expressed β-III-tubulin, demarcating neuronal populations, and tyrosine hydroxylase, a marker of dopaminergic neurons (Table 3, Fig. S1). Similarly, there were no significant differences in the percentage of cells that co-expressed β-III-tubulin and synaptophysin or SV2A; the latter are both integral membrane glycoproteins present on presynaptic vesicles.

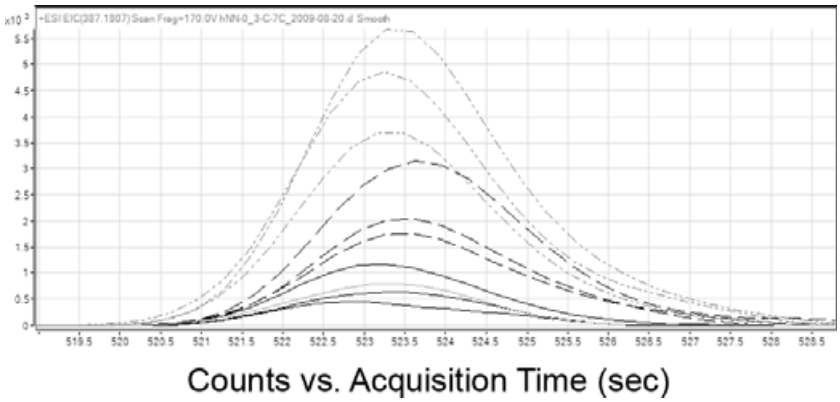

Fig. 5. Identification and abundance of an unknown feature with an exact mass of 386.1734 in ethanol (EtOH)-treated neurons. Extracted ion chromatogram in positive ionization mode compares the feature's abundance in individual sample media plotted against its retention time following separation using quadrupole time-of-flight mass spectrometry. The black solid lines are control samples, black dotted lines are 0.1% EtOH samples, gray dashed lines are 0.3% EtOH, and gray solid lines are media controls.

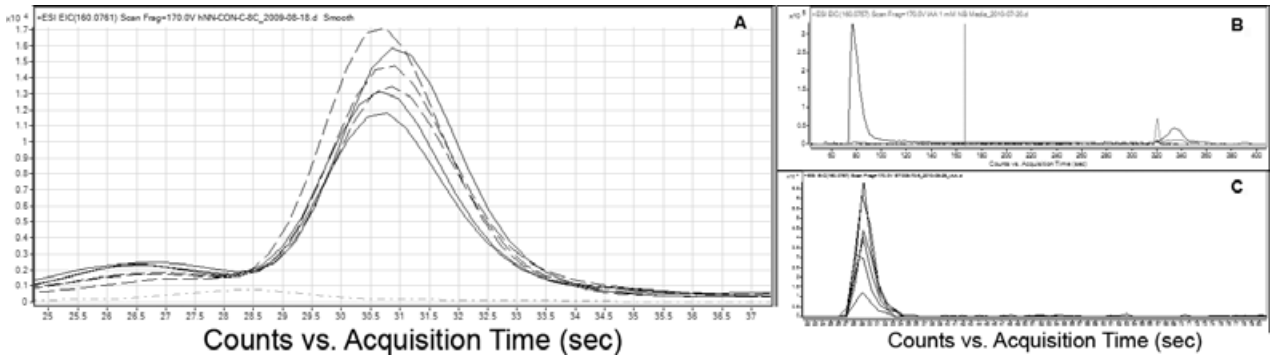

Fig. 6. Identification and abundance of indoleacetaldehyde (IAA). (**A**) Metabolomics Extracted ion chromatogram (EIC) of IAA in ethanol (EtOH)-treated neurons comparing abundance to retention time of the compound in various sample types. The solid lines are control samples, dotted lines are 0.1% EtOH samples, and gray dashed lines are media controls. (**B**) EIC of chemical standards for comparison. (**C**) EIC of the 3 samples with the highest abundance of IAA. (**B**, **C**) show that the retention time of the samples and chemical standards is the same, confirming the compound's identity.

Table 3. Distribution of Neural and Apoptosis Markers*

	0% EtOH	0.1% EtOH	0.3% EtOH	*p*-Value
Neural precursors				
Nestin	>95	>95	>95	n.s.
Caspase-3	10.0 ± 2.5	4.7 ± 0.6	6.5 ± 0.2	0.06
Neurons[a]				
Tyrosine hydroxylase	48.1 ± 8.4	46.3 ± 9.1	48.7 ± 10.4	0.98
Synaptophysin	82.7 ± 5.7	93.7 ± 3.4	90.5 ± 6.0	0.33
SV2A	68.4 ± 11.3	93.6 ± 2.3	85.4 ± 5.6	0.079
Caspase-3	5.1 ± 0.7	3.2 ± 0.5	3.4 ± 0.8	0.087

EtOH, ethanol.
*Mean ± SEM; comparisons using 1-way analysis of variance.
[a]% of β-III-tubulin-positive cells co-expressing the indicated marker.

We found modest EtOH effects on apoptosis of human neural cultures (Table 3, Fig. S2). For the neural progenitors, there was no statistically significant difference in the percentage of cleaved caspase-3-positive cells in controls and those treated with 0.3% EtOH ($p = 0.19$); there was a nonsignificant trend to reduced number of cleaved caspase-3-positive cells following treatment with 0.1% EtOH ($p = 0.06$). In the neurons (D32), we detected a statistically significant decrease in the percentage of caspase-3-positive cells in 0.1% EtOH-treated neurons compared with controls ($p = 0.03$), but not in those treated with 0.3% EtOH ($p = 0.10$).

DISCUSSION

In this study, hES cells were differentiated into neuronal lineages and exposed to either 0, 0.1, or 0.3% EtOH at 3 developmental stages, EBs, neural progenitors, and neurons, to model the effects of EtOH exposure on early human brain development. This in vitro model was coupled with metabolomics to test the hypothesis that EtOH exposure induces statistically significant changes to endogenous small molecules. We also sought to define specific small molecules and biochemical pathways that are altered in alcohol-induced developmental neurotoxicity, leading to FASD. Neural differentiation of hES cells is an important model to study the earliest effects of alcohol on human development given that the timing of neuronal and glial differentiation from hES cells is similar to lineage allocation in vivo (Zhang, 2006). Alcohol exposure during embryogenesis and early neural differentiation, a period when pregnancy may be unknown, leads to a higher incidence of craniofacial defects and mental disabilities (Ernhart et al., 1987; Guerri, 2002). There is a significant need to identify the biochemical pathways that play a causal role in alcohol-induced developmental neurotoxicity. Our study confirms that the exposure of hES cell derivatives to EtOH results in significant changes to the abundance of human endogenous metabolites.

No significant features were shared between the 3 cell types following EtOH treatment. This likely reflects the disparate maturation states of these differentiating cells. Approximately half of the features showed similar responses at both EtOH concentrations, although the magnitude of change did not always achieve statistical significance. Other features, such as thyroxine and kynurenine, were increased at 0.1% EtOH but had decreased abundance at 0.3%. This differential response likely reflects the cells' adaptive responses to the higher EtOH level over the 4-day culture period (e.g., Buck and Harris, 1991; Yakovleva et al., 2011). This interpretation is endorsed by the finding that 0.1% EtOH treatment produced appreciably more significant features (154) than did 0.3% EtOH (112), of which 15 were significant in both exposures. Many individuals with FASD experience chronic alcohol exposure and biomarkers that reflect those adaptive mechanisms are clinically relevant.

EBs are comprised of the 3 primary germ layers and thus model the earliest stages of differentiation. We identified statistically significant changes in multiple developmentally important metabolites between 0, 0.1, and 0.3% EtOH-treated EBs. The abundance of MTA was significantly decreased in 0.3% EtOH-treated EBs compared with untreated controls. MTA is produced in all mammalian cells during the synthesis of polyamines putrescine, spermidine, and spermine from decarboxylated S-adenosylmethionine (SAM), and its levels directly reflect the rates of polyamine synthesis (Avila et al., 2004; Sufrin et al., 1989). Inhibition of poly-

amine synthesis impedes embryo growth (Fozard et al., 1980; Mendez, 1989), whereas exogenous polyamines protect against embryotoxicants (Chirino-Galindo et al., 2009). Both acute and chronic EtOH exposure suppress polyamine synthesis, and its dysregulation was identified as a potential mechanism contributing to FASD (Desiderio et al., 1987; Sessa and Perin, 1997; Shibley et al., 1995; Thadani et al., 1977). The decline in MTA is consistent with those reports, and thus, secreted MTA may be a useful biomarker for alcohol's toxicity. MTA also supplies the sulfur atom for methionine synthesis within the methionine salvage pathway. Reductions in MTA might also contribute to the depleted methionine and SAM levels observed in chronic EtOH consumption (Finkelstein et al., 1974; Walcher and Miller, 2008). Importantly, because MTA is produced from SAM, its alteration may reflect a larger disruption of methyl group homeostasis within EtOH-exposed cells, an interpretation affirmed by the increased folic acid and polyglutamate content found in EtOH-treated EBs and neural progenitors (Tables S1 and S2). Methyl group supplements in the form of choline, betaine, and SAM can prevent or reverse EtOH's developmental toxicity both in vitro and in vivo (Seyoum and Persaud, 1994; Thomas et al., 2009, 2010). To our knowledge, there are no previous reports specifically linking MTA to EtOH exposure or FASD. Its metabolic links with polyamine and methyl metabolism make MTA an excellent potential biomarker in addition to offering mechanistic insights into FASD.

We additionally confirmed thyroxine as a putative biomarker of prenatal EtOH exposure during the embryogenesis stage. Thyroxine abundance was significantly increased in 0.1% EtOH-treated EBs compared with untreated controls. Thyroxine is 1 of the 2 hormones known as thyroid hormone secreted by the thyroid gland. In these cultures, thyroxine likely originated from the serum in the culture medium. Thyroxine signaling through its nuclear receptors is critical for normal central nervous system development and contributes to multiple processes including cell migration, dendrite/axon outgrowth, and synaptogenesis. A precise balance of thyroid hormone is necessary for proper brain development, and both deficiency and hyperthyroidism can cause significant and irreversible brain dysfunction (Bernal and Nunez, 1995; Lauder, 1977; Pharoah et al., 1971). Our studies show a 24% increase in exogenous thyroxine levels, suggesting an EtOH-induced perturbation to thyroxine metabolism and/or its uptake by the early embryo. This is consistent with prior demonstrations of altered thyroid hormone metabolism in animal models and infants with FASD (Cudd et al., 2002; Hannigan and Bellisario, 1990; Kornguth et al., 1979). In rat models, prenatal alcohol exposure (PAE) also alters the levels of iodothyronine deiodinase III and thyroid hormone receptor alpha-1 (Shukla et al., 2011). Thus, the significant increase in thyroxine detected herein suggests that EtOH exposure is likely to impair proper thyroid hormone metabolism in the early embryo, corroborating a proposed mechanistic pathway that EtOH may produce neurodevelopmental disorders by negatively affecting maternal–fetal hormonal homeostasis. Our study is the first to indicate a differential uptake or processing of maternal thyroxine by human embryos as a result of PAE. It is plausible to speculate that this detrimental effect may be mediated by functional impairment of the thyroid hormone-specific transporters Oatp1c1 (organic anion transporting polypeptide 1c1), monocarboxylate transporter (MCT) 8 and MCT10. Mutations to MCT8 actually increase the extracellular abundance of thyroxine in vivo, producing severe psychomotor retardation (Grijota-Martínez et al., 2011; Visser et al., 2008). There are currently no published studies examining the direct effects of EtOH exposure on thyroxine-specific transporters, which will be a focus of future studies.

EtOH exposure at 2 different stages of neurogenesis produced statistically significant changes to tryptophan metabolism. These changes are summarized in Fig. 7. These differences were not attributed to trivial effects of EtOH upon differentiation states, as the cellular type distribution between treatments did not differ. Tryptophan is metabolized primarily along the kynurenine pathway, and kynurenine levels were increased in neural progenitors treated with 0.1% EtOH, as were the levels of the tryptamine catabolite IAA in neurons exposed to 0.1% EtOH. Dysregulation of tryptophan metabolism is reported in several models of alcohol exposure. Kynurenine levels are increased in human plasma following acute EtOH consumption (Badawy et al., 2009), and tryptophan levels are significantly reduced in the plasma, brain, and liver of rat pups following gestational EtOH exposure (Lin et al. 1990). Serum levels of kynurenine are also perturbed in several cognitive disorders such as Down syndrome, bipolar disorder, and postpartum depression but had not been previously described in FASD. Kynurenine has additionally been identified in hES cells treated with valproate, a known inducer of neurodevelopmental defects (Cezar et al., 2007). Kynurenine was recently reported to activate the aryl hydrocarbon xenobiotic receptor (AhR) with an apparent Kd ~4 μM (Opitz et al., 2011). AhR activation adversely affects brain development and behavior (Latchney et al., 2011; Seo et al., 1999). Our finding of elevated kynurenine in EtOH-treated neural progenitors is a novel insight into the mechanisms underlying FASD and confers this metabolite a translational role as a candidate biomarker of FASD.

Our study is the first to indicate a direct effect of EtOH exposure on the human endogenous metabolite IAA. Tryptophan is critical for normal neurogenesis as the precursor to the neurotransmitter 5HT, and its availability has a direct effect on 5HT synthesis (Fernstrom and Wurtman, 1971). Development of the serotonergic system occurs earlier than other neurotransmitter systems, and its sensitivity to disruption by PAE is well documented (Druse et al., 1991; Maier et al., 1996; Sari et al., 2010). Elevated IAA decreased the activity of tryptophan hydroxylase, the rate-limiting enzyme in 5HT synthesis, by as much as 33% (Nilsson and Tottmar, 1987). Thus, elevations of kynurenine and IAA detected here

could be biomarkers for a metabolic diversion of tryptophan away from serotonin synthesis and toward these alternate metabolic pathways and may explain the reduced tryptophan and 5HT content of the EtOH-exposed fetal brain (Maier et al., 1996; Sari et al., 2010). In fact, an increase in the IAA-related 5-hydroxylindoleacetic acid was measured in the urine of rats chronically exposed to EtOH (Bonner et al., 1993), and IAA itself is amenable to detection in urine, which renders it a translational application in biofluids. Alternatively, we note that kynurenine also lies along the synthetic pathway of nicotinamide from its tryptophan precursor. EtOH oxidation places a high requirement for niacin and NADPH reducing equivalents, and one possibility is that EtOH's effects on cellular redox potential might be driving these changes in tryptophan metabolites. Whether one or both of these hypotheses underlie increased kynurenine levels in EtOH-exposed neural progenitors, serving as the cause of serotonergic dysfunction, will be elucidated in future mechanistic studies. Nonetheless, it is noteworthy that significant perturbations to the tryptophan pathway and its metabolites were consistent across different timelines of neural differentiation, neural precursors, and neurons.

In addition to the 4 confirmed metabolites with marked changes resulting from EtOH exposure, we identified multiple unknown compounds with statistically significant changes in abundance. Further experiments are required to determine the chemical formula of such compounds by ion fragmentation and other analytical platforms such as nuclear magnetic resonance. The fact that statistically significant small molecules are not annotated in public databases does not preclude their potential application as candidate diagnostic biomarkers for FASD. Given the highly sensitive, high-resolution nature of the MS detection system employed in this study, a measurable end point (exact mass) is determined, which can be used for continued chemical structure determinations. An important limitation to the lack of a chemical annotation is that these unannotated small molecules cannot be mapped to a biochemical pathway for mechanistic elucidation.

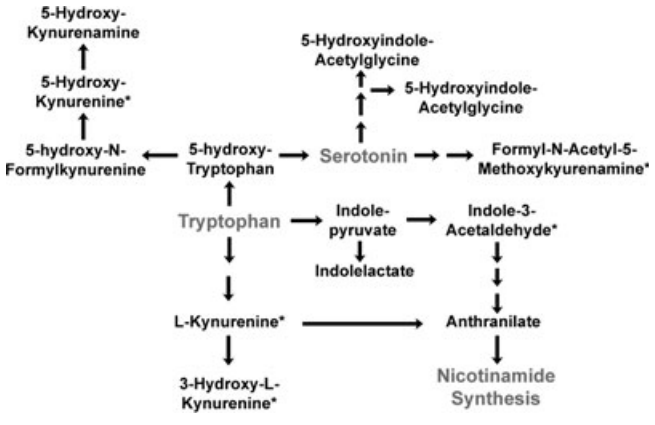

Fig. 7. Summary of ethanol (EtOH)-induced changes in tryptophan metabolism in human embryonic stem cells. Black font indicates metabolites that are detected in media of EtOH-treated human embryoid bodies, neural precursors, and neurons. Asterisk indicates those metabolites whose levels are significantly altered by EtOH exposure. Gray font indicates metabolites included as reference indicators, and these are not significantly altered by EtOH in the media. Note that 3-hydroxy-kynurenine and 5-hydroxy-kynurenine have identical molecular weights; therefore, it is unclear which one was altered by EtOH.

No changes in the overall cell differentiation ability of hES cells were measured in response to 0.1 or 0.3% EtOH (Table 3), at least for the culture conditions employed in this study and for the relatively short EtOH exposure period. There was a nonsignificant trend for an increased percentage of neurons expressing synaptophysin and SV2A following the EtOH treatment of mature neurons. Together with the multiple changes in the levels of tryptophan metabolites, these data may indicate an overall trend of increased synaptogenesis in response to EtOH exposure. In a finding that seemed counterintuitive, we found modest nonsignificant decreases in apoptosis frequency following EtOH treatment of neural precursors and neurons. Prock and Miranda (2007) reported a similar resistance to apoptosis in EtOH-treated mouse neural progenitors, a population substantially similar to those studied herein. Our findings endorse their interpretation that apoptosis in response to EtOH may be a differentiation state-specific response. Further study is required to understand why this population is resistant to apoptosis upon exposure to EtOH.

In conclusion, exposing hES cell-derived EBs, neural progenitors, and neurons to EtOH identified marked changes in biochemical pathways and metabolites critical for proper neurodevelopment. Our findings not only provide novel candidate diagnostic biomarkers for FASD but also corroborate independent studies that have proposed alterations to methionine, methyl group, thyroid hormone, and tryptophan metabolism as potential mechanisms underlying FASD. Although some of these metabolites, that is, kynurenine, have been described as putative biomarkers for other neurodevelopmental disorders, it is the integration of several of these endogenous small molecules from independent biochemical pathways, which may be used as a specific fingerprint test set or specific fingerprint for the diagnosis of FASD in biofluids. Metabolomics is able to measure simultaneous changes to multiple unrelated biochemical pathways, providing not only a biochemical signature specific to FASD but also mechanistic insight into the effects of PAE on early human embryogenesis. The identification of differential biomarkers in our in vitro model prior to analysis of biofluids allows for a more targeted approach in an in vivo model, which has multiple confounding factors, that is, maternal–fetal interactions. Biofluids are complex mixtures of systemic byproducts influenced by both endogenous and exogenous factors, for example, genetics, diet, and environment. Using our strategy, we can design specific analytical protocols to examine biofluids based on our confirmed metabolites. Our future efforts will examine whether molecules detected here are subject to similar changes in in vivo models and children diagnosed with FASD. The

biochemical pathways revealed by metabolomics of hES cell derivatives may also present new targets for the identification and development of novel therapies for FASD.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. SV2A/synaptophysin/TH expression at 40×.

Fig. S2. Cleaved caspase-3 expression in postmitotic neurons.

Table S1. Statistically significant secreted molecules in 0.1 or 0.3% EtOH-treated embryoid bodies. ESI positive mode.

Table S2. Statistically significant secreted molecules in 0.1 or 0.3% EtOH-treated embryoid bodies. ESI negative mode.

Table S3. Statistically significant secreted molecules in 0.1 or 0.3% EtOH-treated neural progenitors.

Table S4. Statistically significant secreted molecules in 0.1 or 0.3% EtOH-treated neurons.

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