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Alcohol induced epigenetic alterations to developmentally crucial genes regulating neural stemness and differentiation

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Abstract

Background—From studies using a diverse range of model organisms, we now acknowledge that epigenetic changes to chromatin structure provide a plausible link between environmental teratogens and alterations in gene expression leading to disease. Observations from a number of independent laboratories indicate ethanol has the capacity to act as a powerful epigenetic disruptor and potentially derail the coordinated processes of cellular differentiation. In this study, we sought to examine whether primary neurospheres cultured under conditions maintaining stemness were susceptible to alcohol-induced alterations of the histone code. We focused our studies on trimethylated histone 3 lysine 4 and trimethylated histone 3 lysine 27, as these are two of the most prominent post-translational histone modifications regulating stem cell maintenance and neural differentiation.

Methods—Primary neurosphere cultures were maintained under conditions promoting the stem cell state and treated with ethanol for five days. Control and ethanol treated cellular extracts were examined using a combination of quantitative RT-PCR and chromatin immunoprecipitation techniques.

Results—We find that the regulatory regions of genes controlling both neural precursor cell identity and processes of differentiation exhibited significant declines in the enrichment of the chromatin marks examined. Despite these widespread changes in chromatin structure, only a small subset of genes including *Dlx2*, *Fabp7*, *Nestin*, *Olig2*, and *Pax6* displayed ethanol induced alterations in transcription. Unexpectedly, the majority of chromatin modifying enzymes examined including members of the *Polycomb Repressive Complex* displayed minimal changes in expression and localization. Only transcripts encoding *Dnmt1*, *Uhrf1*, *Ehmt1*, *Ash2l*, *Wdr5*, and *Kdm1b* exhibited significant differences.

Conclusions—Our results indicate primary neurospheres maintained as stem cells *in vitro* are susceptible to alcohol-induced perturbation of the histone code and errors in the epigenetic program. These observations indicate that alterations to chromatin structure may represent a crucial component of alcohol teratogenesis and progress towards a better understanding of the developmental origins of FASDs.

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Keywords

Epigenetic Programming; Bivalent Genes; Chromatin; Fetal Alcohol Syndrome; Neural Stem Cells

INTRODUCTION

Studies using a broad range of model systems representing multiple tissue types reveal exposure to alcohol significantly alters the developmental trajectory of progenitor cells and fundamentally compromises histogenesis (Camarillo and Miranda, 2008; Crabb et al., 2011; Crews et al., 2006; Gong and Wezeman, 2004; Hipp et al., 2010; Ieraci and Herrera, 2007; Mo et al., 2012; Roitbak et al., 2011; Vangipuram and Lyman, 2010; 2012; Vemuri and Chetty, 2005). These observations are highly suggestive that ethanol interferes with the ability of differentiating cells to properly engage the molecular systems necessary to execute cellular differentiation and patterning. Once established by lineage-specific transcription factors, the identity of each developing cell type is maintained through unique alterations in the way in which the DNA encoding each gene becomes packaged within the nucleus (Hemberger et al., 2009). From studies using a diverse range of model organisms, we now acknowledge that epigenetic changes to chromatin structure provide a plausible link between the environment and lasting alterations in gene expression leading to disease (Feil and Fraga, 2011).

The coordinated recruitment of activating and repressive chromatin modifying enzymes to developmentally crucial loci is absolutely essential for differentiating cells to exit the transcriptional networks promoting pluripotency and establish lineage-specific patterns of gene expression (Hemberger et al., 2009). Work from a number of independent laboratories have demonstrated exposure to ethanol is associated with genome-wide/gene specific changes in DNA methylation, (Downing et al., 2011; Garro et al., 1991; Haycock and Ramsay, 2009; Liu et al., 2009; Ouko et al., 2009; Zhou et al., 2011) alterations of post-translational histone modifications (Kim and Shukla, 2005; Pal-Bhadra et al., 2007; Park et al., 2005), and a profound shift in epigenetically sensitive phenotypes (Kaminen-Ahola et al., 2010). Collectively, each of these observations indicate ethanol has the capacity to act as a powerful epigenetic disruptor and derail the coordinated processes of cellular differentiation. Understanding how ethanol impacts the epigenetic processes by which stem or progenitor cells maintain potency and execute lineage-specific programs of differentiation is fundamental to determining both the molecular mechanisms of alcohol induced teratogenesis and the developmental origins of fetal alcohol spectrum disorders (FASDs).

While several models of prenatal alcohol exposure have examined epigenetic alterations within the context of stem cell differentiation, it remains unclear whether progenitor cells are themselves sensitive to alcohol-induced epigenetic alterations or if the processes of differentiation sensitize cells for brief developmental periods. In studies using animal models, correlation of acute ethanol exposures with major periods of organ growth strongly indicate that different tissues are largely susceptible to ethanol-induced teratogenesis during specific developmental windows (Becker et al., 1996). These results would suggest progenitor cells are able to faithfully maintain chromatin structure, while the reorganization that occurs as cells differentiate creates an epigenetically labile period, where ethanol can perturb the developmental program. To begin to address this question, we sought to examine static cultures of neural stem cells for changes in key post-translational histone modifications within the regulatory regions of developmentally crucial genes. We find that alcohol profoundly alters the epigenetic landscape of neural stem cells, imparting a lasting signature of exposure. These alcohol-induced alterations of the epigenetic programs

regulating fetal stem cell maintenance and differentiation are likely to have persistent, organizational effects upon subsequent neuronal maturation and potentially explain some of the pattering defects observed with the central nervous systems of FASD children.

MATERIALS AND METHODS

Neurosphere Stem Cell Culture

All animal procedures were approved and conducted in accordance with the Institutional Animal Care and Use Committee at TAMHSC. Culture and media preparations for neurospheres have been described previously (Camarillo and Miranda, 2008). Briefly, fetal cerebral cortical neuroepithelial stem cells isolated from gestational day 12.5 C57BL/6 mice were cultured as neurospheres in T25 flasks in media containing GlutaMAX™ DMEM F-12 (Invitrogen, Carlsbad, CA. Cat# 11330-032), 20ng/ml FGF basic, 20ng/ml EGF (Invitrogen, Carlsbad, CA Cat# 53003-018), 0.15ng/ml LIF, 1x ITS-X (Invitrogen, Carlsbad, CA. Cat# 51500-056), 0.85 units/ml heparin, and 20nM progesterone (Sigma, St. Louis, MO. Cat#P6149). Neurospheres were typically incubated for a total of 5 days at 37°C, 5% CO₂ in a humidified environment before passage. Medium was changed every 2–3 days depending on the level of confluence.

Ethanol treatment

Neurospheres were cultured in T25 flasks, with a parafilm (VWR) sealed lid to prevent ethanol evaporation. Dosing for ethanol followed previously published studies (Camarillo and Miranda, 2008), and utilized 60 mg/dL (13mM), 120 mg/dL (26mM), and 320 mg/dL (70mM) of ethanol (Sigma). Control samples for non-ethanol treated stem cells were concurrently cultured during the same experimental time course and in the same culture conditions. Neurospheres received fresh medium containing either control or ethanol treatments every 2–3 days. On day 5 of treatment, cells were isolated and cellular extracts collected for either Chromatin Immunoprecipitation or RNA analysis.

Chromatin Immunoprecipitation (ChIP) Analysis

Cultured neurospheres were grown to 80% confluence, washed twice in warm PBS, trypsinized then resuspended in warm growth medium containing 0.1 volume of crosslinking solution (Kondo et al., 2004). Subsequently, Chromatin Immunoprecipitation (ChIP) reactions were performed as described previously (Golding et al., 2010), which was followed by DNA purification using a Qiagen PCR Cleanup kit. Antibodies used in the immunoprecipitation of modified histones and Ezh2 were anti-Rabbit IGG (Santa Cruz SC-2027), anti-Trimethyl Histone H3 Lysine 4 (Millipore 04–745), anti-Trimethyl Histone H3 Lysine 27 (Millipore 17–622), and anti-Ezh2 (Millipore 17–662). Antibodies for modified histones were used at 1 µg/ChIP reaction while antibodies to Ezh2 were used at 2 µg/ChIP reaction. The concentration of IgG was adjusted from 1 µg to 2 µg as appropriate. For these experiments, a minimum of 3 independent experimental replicates were conducted. Cellular extracts from each of these replicates were subjected to 3 independent chromatin immunoprecipitations for each of the post-translational histone modifications examined. DNA precipitated from these ChIP reactions were analyzed by qPCR and results normalized to 1% of the total input. Analysis of candidate promoter regions was carried out using the DyNAmo Flash SYBR Green qPCR Mastermix (Fisher Scientific, Pittsburgh PA. Cat # F-415L) following the manufacturer's instructions. Primers used to examine the relative enrichment of candidate gene promoter regions are described in Supplementary Table 1. Reactions were performed on either a StepOnePlus Real Time PCR system (Applied Biosystems, Foster City CA.) or a CFX384 Real Time System (BioRad Hercules CA).

RNA Isolation and Reverse Transcription

Cultured cells were grown to 80% confluence, washed twice in warm PBS, and dissociated with 1X trypsin (Accutase Millipore Cat# SCR005). Cells were spun down, washed once in cold PBS, and RNA isolated using Trizol (Invitrogen, Carlsbad, CA. Cat # 15596026) according to the manufacturer's protocol. 1 µg of purified total RNA was treated with amplification grade DNaseI (Sigma, St. Louis, MO. Cat# AMPD1) according to the manufacturer's protocol, and 250ng RNA seeded into a reverse transcription reaction using the SuperScriptII kit (Invitrogen, Carlsbad, CA. Cat# 18064-071) by combining 1 µl random hexamer oligonucleotides (Invitrogen, Carlsbad, CA. Cat# 48190011), 1 µl 10 mM dNTP (Invitrogen, Carlsbad, CA. Cat# 18427-013), and 11 µl RNA plus water. This mixture was brought to 70°C for 5 minutes then cooled to room temperature. SuperScriptII reaction buffer, DTT, and SuperScriptII were then added according to manufacturer's protocol and the mixture was brought to 25°C for 5 minutes, 42°C for 50 minutes, 45°C for 20 minutes, 50°C for 15 minutes, then 70°C for five minutes.

Real-Time PCR Amplification of cDNA

Primers were designed using the NetPrimer software tool (www.premierbiosoft.com/netprimer) or identified from previously published research references (Supplemental Table 1.). Real-time PCR analysis of mRNA levels was carried out using the DyNAmo Flash SYBR Green qPCR Mastermix (Fisher Scientific, Pittsburgh PA. Cat # F-415L) following the manufacturer's instructions. Reactions were performed on a StepOnePlus Real Time PCR system (Applied Biosystems, Foster City CA.) or a CFX384 Real Time System (BioRad Hercules CA).

Statistical Analysis of Real Time PCR - Expression and ChIP Data

For analysis of gene expression, the measured CT (Cycle Threshold) experimental values for each transcript were compiled and normalized to the geometric mean of the reference genes Phosphoglycerate kinase 1 (*Pgk1* - NM_008828), Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh* - NM_008084), and Hypoxanthine-phosphoribosyl transferase (*Hprt* - NM_013556). From our previous studies of fourteen candidate reference genes in ethanol-exposed neurosphere cultures, *Pgk1*, *Gapdh* and *Hprt* were identified as being the most stable across all alcohol treatments (Vandesompele et al., 2002). Normalized expression levels were calculated using the $\Delta\Delta CT$ method described previously (Schmittgen and Livak, 2008). Values from these calculations were transferred into the statistical analysis program Graph Pad and an Analysis of Variance (ANOVA) run to assay differences between experimental treatments. For samples with p values greater than 0.05 we applied Tukey's HSD analysis for multiple comparisons and have marked statistically significant differences with the lower case letters. Samples not connected by the same letter are significantly different. For quantitative analysis of candidate gene regulatory region enrichment, ChIP samples were normalized to 1% input, and data analyzed using the formula previously described (Mukhopadhyay et al., 2008). The cumulative mean from each of the 3 independent experiments were calculated and the standard error of the mean derived. The statistical analysis package Graph Pad was used to measure statistical significance between the % input for the control and ethanol treated samples using a paired student's *t*-test.

RESULTS

Ethanol Alters the Promoter Regions of Key Neurogenic Regulators

Within the unique transcriptional environment of embryonic stem cells, several developmentally crucial genes are co-marked with both activating and repressive histone

modifications (Bernstein et al., 2006; Lim et al., 2009; Pan et al., 2007). Specifically, histone 3 lysine 4 trimethylation, which is typically associated with gene activation, co-exists with the repressive trimethyl state of histone 3 lysine 27. These uniquely marked loci are termed bivalent domains and generally encode transcription factors directing tissue-specific programs of differentiation (Fisher and Fisher, 2011). This same unique signature is found, albeit less frequently, in neuronal and other tissue specific progenitor stem cell types (Lim et al., 2009). While these bivalently marked genes are generally not expressed, they are thought to be “primed” for either rapid activation or silencing during differentiation. As differentiation progresses, the regulatory regions of bivalent genes commit to one of these two post-translational modifications in a cell-lineage dependent manner and resolve their chromatin structure towards either an active or silent conformation. The correct resolution of these chromatin marks, and their faithful maintenance during differentiation is a fundamental element of normal histogenesis (Hemberger et al., 2009).

Given the reported impact ethanol has upon cellular differentiation, both *in vitro* and *in vivo*, we hypothesized that exposure to alcohol would disrupt the distribution of these two vital post-translational histone marks. To address this question, primary neurosphere cultures were maintained under conditions promoting the stem cell state and treated with 320 mg/dL (70mM) ethanol for five days. This concentration of alcohol is representative of the blood alcohol levels obtainable in episodic binge drinking and chronic alcoholics (Centers for Disease Control and Prevention (CDC), 2004; White et al., 2006). Control and ethanol treated cellular extracts were immunoprecipitated with antibodies recognizing the specific chromatin modifications trimethylated histone 3 lysine 4 (H3K4 Me3) and trimethylated histone 3 lysine 27 (H3K27 Me3) using methods previously utilized by our lab (Golding et al., 2010). To assess alcohol-induced changes in chromatin structure, DNA fragments isolated from these chromatin immunoprecipitations were examined by quantitative PCR. qPCR reactions were carried out measuring the relative enrichment of candidate gene promoter regions relative to 1% of the total input. We began by examining the promoter regions of eight genes with established roles regulating neural stem cell biology (Kuegler et al., 2010; Zhou et al., 2011). As can be seen in Fig. 1a, all of our candidate genes exhibited significant changes in at least one of the post-translational marks examined, while two (*Igf1* and *Smarca2*) displayed changes in both. It is worth noting that a recent study examining alterations in DNA methylation within a similar neural stem cell model also identified these two candidates (Zhou et al., 2011). We next sought to determine the impact of ethanol upon the chromatin state of key genes regulating neuronal patterning. *Homeobox* genes operate at the hub of multiple growth factor signaling pathways controlling neurogenesis and therefore represent excellent candidates to examine in relation to the patterning defects seen in FASD studies. As can be seen in Fig. 1b, *Dlx2*, *Dlx5*, *HoxA1*, *HoxA7*, *Msx1*, *Msx2*, *Nanog*, *Nkx2.1*, *Nkx2.2* and *Pax6* all displayed alterations in at least one of the post-translational marks examined while *Dlx1* and *Dlx3* did not display any statistically significant changes ($p < 0.05$).

Ethanol Disrupts the Bivalent State

Interestingly, within our neurosphere stem cells *Dlx2* and *Nkx2.2* were equally marked by both histone 3 lysine 4 and histone 3 lysine 27 trimethylation and thus appeared to have retained an ES-cell like bivalent signature. Given the importance of this chromatin state to subsequent differentiation, and its alteration upon exposure to ethanol, we next set out to determine if other bivalent genes were present in neural stem cells and became modified upon exposure to alcohol. To this end, we examined ten other candidate bivalent genes identified in ES cells (Rugg-Gunn et al., 2010). Of these, the *Vdr* promoter was the only other region to retain a bivalent signature within our neurosphere cultures, and it too exhibited significant changes in chromatin structure, namely a loss in H3K4 Me3 ($p =$

0.0057) (Fig. 1c). The chromatin status of an intergenic region of chromosome six was utilized as a negative control.

The Effect of Ethanol on Gene Transcription

To determine if the observed alterations in chromatin structure were accompanied by changes in gene expression, we collected RNA from ethanol exposed neurosphere stem cells and examined the mRNA levels of our candidate genes relative to the geometric mean of *Gapdh*, *Hprt1*, and *Pgk1* transcript levels. For these experiments, ethanol dosing followed previously published studies (Camarillo and Miranda, 2008), and utilized 60 mg/dL (13mM), 120 mg/dL (26mM), and 320 mg/dL (70mM) of ethanol. We began by examining the relative mRNA levels of six established neural markers. *Fabp7*, *Nestin*, and *Tuj1* were abundantly expressed within our neurosphere cultures while transcripts encoding *Gfap*, *Gli3*, and *Olig2* were detected at residual levels. Of these six, mRNAs encoding *Fabp7*, *Gfap*, *Nestin*, and *Olig2* all demonstrated significant changes for at least one of the exposure levels tested (Fig. 2a). We next examined the relative mRNA levels of candidate genes identified in Fig. 1. Of the twenty-one candidate genes examined in figure 1, only six were expressed within our neurosphere stem cells. Interestingly, while *Nkx2.2*, *Smarca2*, *Sox2* and *Vdr* all exhibited significant changes in chromatin structure, none of these candidates demonstrated detectable alterations in mRNA transcript levels (Fig. 2b). In contrast, *Dlx2* and *Pax6* both displayed significant differences in mRNA levels relative to the control ($p = 0.015$ and $p = 0.02$). Of note, a similar degree of *Pax6* mRNA repression was recently reported in a human radial glia progenitor cell model of ethanol exposure (Mo et al., 2012). Collectively, these results indicate that while select candidates display altered levels of gene expression, the transcriptional control of many genes remains unperturbed despite changes in the distribution of H3K4 Me3 and H3K27 Me3 within their regulatory regions.

Ethanol Alters Global Histone 3 Lysine 27 Trimethylation

To help determine if the chromatin alterations observed in Fig. 1 were gene specific or reflective of larger, genome-wide changes to the epigenome, we next sought to examine the chromatin status and expression of transposable elements. Mammalian genomes contain large amounts of parasitic nucleic acids that originate from retrotransposons and retroviruses that invaded the ancestral germline (Mandal and Kazazian, 2008). While protein coding genes represent a little less than 2% of our genetic information, as much as 45% of mammalian genomes are composed of endogenous retro-elements, making them excellent candidates to examine as an assessment of global alterations to the histone code. While transposable elements and protein coding genes are clearly regulated through distinct biochemical pathways, their overall abundance makes them suitable for use as an estimator of the genomic condition. Using control and 320mg/dL treatments outlined above, we immunoprecipitated cellular extracts with antibodies recognizing H3K4 Me3 and H3K27 Me3 and seeded precipitated DNA fragments into qPCR assays measuring enrichment of the *Long Interspersed Nuclear Element 1 (LINE1)*, *Intra-cisternal A particle (IAP)*, *Mus D*, and *Etn* families of transposable elements. Each of these families have 660,000, 1300, 100 and 240 respective copies per haploid genome (Coffin et al., 1997; Mandal and Kazazian, 2008). These experiments revealed significant declines in the levels of H3K27 Me3 for all transposable element families examined, suggesting the global distribution of this mark had become altered as a consequence of *in vitro* ethanol exposure (Fig. 3a). Given the observed role of Polycomb group complexes and by proxy, H3K27 Me3 in maintaining the repressed state of transposable elements (Golding et al., 2010) we had anticipated a reduction in this mark would be correlated with a proportional increase in *LINE1*, *IAP*, *MusD* and *Etn* transcription. Surprisingly, only the *LINE1* family and 60mg/dL *IAP* group displayed statistically significant changes ($p = 0.001$ and $p = 0.002$ respectively) and transcript levels for both of these transposable elements were decreased (Fig 3b). For the *LINE1* family, we

verified our observations using three independent primer sets measuring multiple regions of the *LINE1* transcript.

Expression of Epigenetic Modifying Enzymes in Alcohol Treated Neurospheres

To pursue a potential mechanistic basis for the observed epigenetic alterations, we again collected RNA from ethanol exposed neurosphere stem cells and examined the mRNA levels of transcripts encoding several of the most prominently studied epigenetic modifying enzymes. Cells were exposed to control, 60 mg/dL, 120 mg/dL, and 320 mg/dL experimental treatments and mRNA levels normalized to the geometric mean of *Gapdh*, *Hprt1* and *Pgk1* as above. Of the thirty candidates evaluated (including *Smarca2* in Fig 2b.) only *Dnmt1*, *Uhrf1*, *Ash2l*, *Wdr5*, *Ehmt1* and *Kdm1b* exhibited statistically significant changes (Fig. 4). *Dnmt1*, *Uhrf1* and *Ehmt1* are important components of the molecular machinery regulating genomic DNA methylation (*Dnmt1* and *Uhrf1*) and trimethylation of histone 3 lysine 9 (*Ehmt1*) (Sharif et al., 2007). Interestingly, all three demonstrate increased mRNA levels within ethanol-exposed cultures, consistent with the hyper-suppressed state of the *LINE1* transposable elements (Fig. 3b) (Meissner et al., 2008; Mikkelsen et al., 2007; Yoder et al., 1997). *Ash2l*, *Wdr5* and *Kdm1b* all serve to regulate H3K4 Me3 and display mixed changes in relative expression. Unexpectedly, our results indicate that the Polycomb Repressive Complex 2 (PRC2) members *Eed* and *Ezh2* did not demonstrate statistically significant changes in expression. This was surprising given the unique role of this enzyme complex in establishing and maintaining the repressive H3K27 Me3 mark.

Polycomb Complex Localization and Altered noncoding RNA Levels in Alcohol Treated Neurospheres

In an effort to help explain the dramatic alterations in H3K27 Me3 levels, we next set out to examine PRC2 localization within ethanol exposed neurosphere stem cells. As mammalian genomes are devoid of canonical *Polycomb* DNA binding elements, we began our investigations by looking at noncoding RNAs, which are hypothesized to regulate PRC2 recruitment. Work over the past decade has revealed the transcriptomes of eukaryotic cells are far more complex than was originally postulated. The noncoding complement of the genome vastly outsize the protein-coding and includes transcripts of well-defined function, as well as those to whose function we can only speculate (Guttman et al., 2011). Studies of long intergenic noncoding RNAs have indicated this class of transcripts plays a fundamental role in shaping the fetal epigenome by directing PRC2 localization during stem cell maintenance and differentiation (Guttman et al., 2011). Studies by Guttman and colleagues recently identified several noncoding RNAs in ES cells, which based upon the genes they regulate, were postulated to have roles in regulating neural stem cell differentiation (Guttman et al., 2011). We therefore sought to examine the impact of ethanol exposure upon these noncoding transcripts. Of the eight candidate neural stem cell differentiation-associated noncoding RNAs tested, only linc1354 was detected within our neurosphere stem cell model (Fig. 5a). To examine the impact of ethanol exposure we again utilized control, 60 mg/dL, 120 mg/dL, and 320 mg/dL experimental treatments and normalized linc1354 RNA levels to the geometric mean of *Gapdh*, *Hprt1* and *Pgk1* measurements. As can be seen in Fig. 5b, ethanol induced a significant reduction in linc1354 levels across all ethanol concentrations tested ($p = 0.0022$). Given the previously characterized association between noncoding RNAs and PRC2 proteins, as well as the role dynamic changes in *PRC2* localization play during neural development, we next chose to examine *EZH2* occupancy within the regulatory regions of the candidate genes studied in Fig. 1 (Rinn et al., 2007). From these twenty-one candidate genes, only nine were bound by *EZH2* and with the exception of *Dlx1* ($p = 0.0256$), none demonstrated significant differences in enrichment relative to the untreated control (Fig. 5c). No enrichment of *EZH2* was observed using

primers amplifying the negative control, intergenic region of chromosome 6 (data not shown).

DISCUSSION

In this study we sought to examine whether primary neurospheres cultured under conditions maintaining stemness were sensitive to alcohol induced alterations of the histone code. We focused on trimethylated histone 3 lysine 4 and trimethylated histone 3 lysine 27, as these are two of the most prominent post-translational histone modifications regulating stem cell maintenance and neural differentiation (Bernstein et al., 2006; Lim et al., 2009; Pan et al., 2007). We find that the regulatory regions of a number of genes controlling both precursor cell identity and neural differentiation exhibited significant alterations in the enrichment of these chromatin marks. Our results clearly indicate primary neurospheres maintained as stem cells *in vitro* are not refractory to alcohol induced perturbation of the histone code, and that maintained stem cells as well as differentiating neurospheres (Zhou et al., 2011) are susceptible to errors in the epigenetic program.

Alterations of Bivalent Genes

Within many tissue precursor cell types, bivalent genes are maintained in a poised confirmation and hypothesized to hold a maturation “tipping point” between formation of a specific cellular lineage and the closing off of a developmental pathway. Our studies identify three novel bivalent genes within cultured neurospheres and of these, two demonstrate significant loss of H3K4 Me3 (Fig. 1). Our studies also identified changes in H3K27 Me3 within the regulatory region of *Nkx2.2*, indicating the two marks examined are not uniformly affected. These results suggest that alcohol may impact cellular differentiation by disrupting the ability of precursor cells to activate and repress key genes in a developmentally appropriate manner. As a consequence, precursor cells would not be able to uniformly exit the stem cell state and initiate developmental patterning. Extending our *in vitro* observations into a clinical setting, postmortem and magnetic resonance imaging-based studies examining the brains of Fetal Alcohol Syndrome children have revealed ectopic, nodular accumulations of poorly differentiated neuronal and glial cells called heterotopias (Jones and Smith, 1975; Swayze et al., 1997). These abnormal structures are consistent with cortical dysplasia and are suggestive that alcohol may induce a persistent destabilization of neuronal differentiation. Whether cortical dysplasia is associated with altered levels of H3K4 Me3 and H3K27 Me3 is currently under investigation.

Alcohol Induced Epigenetic Changes and Transcription

It is interesting to note that while the majority of candidate regulatory regions examined in this study displayed significant changes in levels of H3K4 Me3 and/or H3K27 Me3, the transcription of very few of the genes examined were altered upon exposure to ethanol. A recent study by Hashimoto-Torii and colleagues examining global changes in gene transcription within ethanol-exposed cerebral cortices reported a similar observation, in that only 636 transcripts out of 39,000 examined were differentially expressed (268 up regulated and 368 down) (Hashimoto-Torii et al., 2011). In our study, twelve of the twenty one candidate genes exhibited alcohol induced reductions in the repressive H3K27 Me3 mark, yet none of these candidates demonstrated an increase in expression or became activated *de novo* in exposed cells. Unexpectedly, this drop in H3K27 Me3 levels was not associated with any dramatic changes in EZH2 localization. Of the differentially expressed genes identified, both *Nestin* and *Dlx2* displayed significant declines in the activating H3K4 Me3 mark, yet transcripts encoding these genes went up. Of all the candidates studied, only *Pax6* demonstrated both reduced H3K4 Me3 levels within its regulatory region and a correlative drop in mRNA levels. These observations raise more questions as to the functional nature of

the epigenetic changes resulting from ethanol exposure and suggest other post-translational histone modifications may have shifted to compensate for the loss of H3K4 and H3K27 trimethylation. While our data indicate the trimethyl state of H3K4 and H3K27 are affected, we have yet to address alterations in nucleosome positioning, polymerase II occupancy and changes to the mono and dimethylated states of these positions. Previous studies examining alcohol-exposed primary rat hepatocytes demonstrated an increase in H3K4 dimethylation (Pal-Bhadra et al., 2007). These observations suggest the trimethyl mark may be preferentially lost in favor of increasing the dimethyl state. Each of these questions will be the subject of future investigations and will work towards determining if the observed epigenetic changes are a coordinated response to alcohol teratogenesis or the byproduct of a generalized cellular response to stress.

In an effort to distinguish gene-specific epigenetic alterations from global changes, we examined select aspects of transposable element biology within alcohol treated neurospheres. Our data reveal global levels of the repressive H3K27 Me3 mark were depressed, yet the expression of the largest family of transposable elements, the *LINE1* family, exhibited a ~50% decrease in measured transcripts as compared to the control. This was surprising given the established role of H3K27 Me3 in suppressing transposable elements (Leeb et al., 2010). It is possible that alcohol exposed cells compensate for the loss of H3K27 Me3 by up-regulating *Dnmt1* and *Uhrf1* expression and increasing genomic methylation levels, which has been observed previously (Kaminen-Ahola et al., 2010). Similar disruptions in the epigenetic regulation of *LINE1* elements have been observed in the brains of chronic alcoholics (Ponomarev et al., 2012). Given the hypothesized role of *LINE1* elements in promoting somatic mosaicism, suppressed *LINE1* transcription may have a detrimental impact upon neural diversification and higher brain function (Muotri et al., 2005). This large-scale alteration in the developmental program regulating neurogenesis will likely lead to a myriad of central nervous system developmental pathways being affected.

Altered Epigenetic Programming and FASDs

While this study focused on the impacts of alcohol within a neuronal stem cell model of exposure, it is likely that other precursor cell types and developmental programs, including those executing formation of the skeletal system will exhibit similar alterations in epigenetic programming. Of the candidate genes examined in this study *Msx2*, and *Nkx2.1* have previously been associated with FASD birth defects (Godin et al., 2011; Rifas et al., 1997). Of note, *Ascl1* and *Twist1* regulate neural patterning and craniofacial development respectively and both of their promoter regions were altered by exposure to ethanol (Chen and Behringer, 1995; Guillemot et al., 1993). These data indicate that alcohol significantly impacts epigenetic processes regulating genes controlling neural patterning and facial development. Our observations indicate alterations to chromatin structure may represent a crucial component of alcohol teratogenesis and move towards better understanding the developmental origins of FASDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Alterations in Histone 3 Lysine 4 and Lysine 27 Trimethylation within ethanol exposed neurosphere stem cells. Levels in the relative enrichment of Histone 3 Lysine 4 Trimethylation (H3K4 Me3) and Histone 3 Lysine 27 Trimethylation (H3K27 Me3) were measure by ChIP for (A) eight candidates with established roles regulating neural stem cell biology (B) select homeobox genes with known roles regulating neural patterning and (C) a newly identified neurosphere stem cell bivalent domain. Neurosphere stem cells were treated with 320mg/dL ethanol for five days and extracts examined as outlined in the materials and methods. Statistical significance as measured using a paired students *t*-test at $p < 0.05$ is designated by a single *, $p < 0.01$ by two (**), and $p < 0.001$ by three (***). For these experiments, four independent cell culture experiments were conducted (N = 4). For each sample, background levels are represented by the IgG control. As a negative control, primers were used to examine the enrichment of an intergenic region of chromosome 6, which displayed minimal enrichment of H3K4 Me3 and H3K27 Me3.

Fig. 2. mRNA expression levels of candidate genes in ethanol exposed neurosphere stem cells exposed to control, 60mg/dL, 120mg/dL and 320mg/dL experimental treatments for five days. Quantitative RT-PCR measurements of mRNAs encoding (A) six candidate markers of various neural cell types and (B) six candidates from Fig. 1 were measured against the geometric mean of mean of *Gapdh*, *Hprt1* and *Pgk1* and graphed using the $\Delta\Delta CT$ method (Schmittgen, & Livak 2008). Although detectable, transcripts encoding *Vdr* were expressed at extremely low levels and exhibited wide variation. Graphs are representative of at least three independent cell culture experiments (N = 3). Statistical significance using ANOVA at $p < 0.05$ is designated by a single * and $p < 0.01$ by two (**). Results of Tukey's HSD analysis are represented by lower case letters. Samples not connected by the same letter are significantly different.

Fig. 3.

Analysis of the chromatin structure and transcriptional control of four families of transposable elements. (A) ChIP analysis of the *LINE1*, *IAP*, *MusD* and *Etn* families of transposable elements. Statistical significance as measured using a paired students *t*-test at $p < 0.05$ is designated by a single * and $p < 0.01$ by two (**). For these experiments, three independent cell culture experiments were conducted ($N = 3$). (B) Quantitative RT-PCR measurements of transcripts encoding the *LINE1*, *IAP*, *MusD* and *Etn* transposable elements in neurosphere stem cells exposed to control, 60mg/dL, 120mg/dL and 320mg/dL experimental treatments for five days. Measured CT values were normalized against the geometric mean of mean of *Gapdh*, *Hprt1* and *Pgk1*. Three independent primer sets measuring the second open reading frame (L1 ORF2), mid region (L1 Mid), and 5' untranslated region (L1 5' UTR) of the *LINE1* transcript were used. For the *IAP* transposable element two independent primers measuring the viral long terminal repeat (IAP LTR) and Gag (IAP Gag) coding region were used. Graphs are representative of at least three independent cell culture experiments ($N = 3$). Statistical significance using ANOVA at $p < 0.05$ is designated by a single * and $p < 0.01$ by two (**). Results of Tukey's HSD analysis

are represented by lower case letters. Samples not connected by the same letter are significantly different

Fig. 4.

Expression of Epigenetic Modifying Enzymes in Alcohol Treated Neurospheres.

Quantitative RT-PCR measurements of transcripts encoding several of the most prominently studied epigenetic modifying enzymes in neurosphere stem cells exposed to control, 60mg/dL, 120mg/dL and 320mg/dL experimental treatments for five days. Measured CT values were normalized against the geometric mean of mean of *Gapdh*, *Hprt1* and *Pgk1* and graphed using the $\Delta\Delta CT$ method (Schmittgen, & Livak 2008). Graphs are representative of at least three independent cell culture experiments (N = 3). Statistical significance using ANOVA at $p < 0.05$ is designated by a single * and $p < 0.01$ by two (**). Results of Tukey's

HSD analysis are represented by lower case letters. Samples not connected by the same letter are significantly different.

Fig. 5. Polycomb Complex Localization and Altered noncoding RNA Levels in Alcohol Treated Neurospheres. **(A)** Noncoding RNAs examined in our neural stem cell model. Using RT-PCR, all noncoding RNAs were detected within RNA extracts prepared from ES cells but only linc1354 was detected in primary neurospheres (N=3). **(B)** Quantitative RT-PCR measurements of linc1354 RNA levels in neurosphere stem cells exposed to control, 60mg/dL, 120mg/dL and 320mg/dL experimental treatments for five days. Measured CT values were normalized against the geometric mean of mean of *Gapdh*, *Hprt1* and *Pgk1* and graphed using the $\Delta\Delta CT$ method (Schmittgen, & Livak 2008). Using ANOVA the

calculated P value was 0.0022. Results of Tukey's HSD analysis are represented by lower case letters. Samples not connected by the same letter are significantly different. Graphs are representative of at least three independent cell culture experiments (N = 3). (C) ChIP analysis of candidate promoters examining enrichment of the *Polycomb Repressive Complex 2* methyltransferase EZH2. Neurosphere stem cells were treated with 320mg/dL ethanol for five days and extracts examined as outlined in the materials and methods. For these experiments, three independent cell culture experiments were conducted (N = 3).. The statistical significance of Dlx1 measurements using a paired students *t*-test was $p=0.0256$.