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The BAF (BRG1/BRM-Associated Factor) chromatin-remodeling complex exhibits ethanol sensitivity in fetal neural progenitor cells and regulates transcription at the miR-9-2 encoding gene locus

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ABSTRACT

Fetal alcohol spectrum disorders are a leading cause of intellectual disability worldwide. Previous studies have shown that developmental ethanol exposure results in loss of microRNAs (miRNAs), including miR-9, and loss of these miRNAs, in turn, mediates some of ethanol's teratogenic effects in the developing brain. We previously found that ethanol increased methylation at the miR-9-2 encoding gene locus in mouse fetal neural stem cells (NSC), advancing a mechanism for epigenetic silencing of this locus and consequently, miR-9 loss in NSCs. Therefore, we assessed the role of the BAF (BRG1/BRM-Associated Factor) complex, which disassembles nucleosomes to facilitate access to chromatin, as an epigenetic mediator of ethanol's effects on miR-9. Chromatin immunoprecipitation and DNAse I-hypersensitivity analyses showed that the BAF complex was associated with both transcriptionally accessible and heterochromatic regions of the miR-9-2 locus, and that disintegration of the BAF complex by combined knockdown of BAF170 and BAF155 resulted in a significant decrease in miR-9. We hypothesized that ethanol exposure would result in loss of BAF-complex function at the miR-9-2 locus. However, ethanol exposure significantly increased mRNA transcripts for maturation-associated BAF-complex members BAF170, SS18, ARID2, BAF60a, BRM/BAF190b, and BAF53b. Ethanol also significantly increased BAF-complex binding within an intron containing a CpG island and in the terminal exon encoding precursor (pre)-miR-9-2. These data suggest that the BAF complex may adaptively respond to ethanol exposure to protect against a complete loss of miR-9-2 in fetal NSCs. Chromatin remodeling factors may adapt to the presence of a teratogen, to maintain transcription of critical miRNA regulatory pathways.

Introduction

Fetal alcohol spectrum disorders (FASDs) are a leading cause of intellectual disability in the US and worldwide, with an estimated global prevalence of ~2.3% (Roozen et al., 2016). Despite long-term evidence that alcohol is teratogenic (Jones, Smith, Ulleland, & Streissguth, 1973; Lemoine, Harouseau, Borteryu, & Menuet, 1968), FASDs remain difficult to prevent in the US, due in part to the combined prevalence of unplanned pregnancies (Finer & Zolna, 2016) and patterns of heavy alcohol consumption, particularly binge-type consumption, among women of child-bearing age (Tan, Denny, Cheal, Sniezek, & Kanny, 2015). Such socio-cultural influences increase the risk for inadvertent prenatal alcohol exposure, particularly during the first and second trimesters, when fetal neural stem cells (NSCs) produce most of the neurons of the adult brain (Bystron, Blakemore, & Rakic, 2008). The worldwide persistence of FASD is a challenging public health problem and emphasizes the need to identify aspects of alcohol teratogenicity that can be mitigated by early intervention.

In rodent models, alcohol exposure during the first trimester-equivalent period was shown to reduce neurogenesis (Miller, 1989) and decrease the thickness of the NSC-enriched fetal cortical ventricular neuroepithelium (Sudheendran, Bake, Miranda,

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& Larin, 2013), resulting in persistent brain growth deficits (Maier, Miller, & West, 1999). However, surprisingly, research in *ex vivo* rodent models of fetal NSCs showed that ethanol exposure did not result in cell death (Prock & Miranda, 2007; Santillano et al., 2005), but rather NSC loss, due to increased proliferation associated with premature and aberrant maturation (Camarillo & Miranda, 2008; Miller & Nowakowski, 1991; Santillano et al., 2005; Tingling et al., 2013). We further determined that many effects of ethanol on fetal NSCs and early embryogenesis were mediated by repression of a class of non-protein-coding regulatory microRNAs (miRNAs; Pappalardo-Carter et al., 2013; Sathyan, Golden, & Miranda, 2007; Tsai et al., 2014). miRNA, miR-9 in particular, is an important regulator of brain segmentation (Leucht et al., 2008) and neuronal maturation (Shibata, Kurokawa, Nakao, Ohmura, & Aizawa, 2008; Shibata, Nakao, Kiyonari, Abe, & Aizawa, 2011), and is suppressed by ethanol during development in both mouse and zebrafish neural tissues (Pappalardo-Carter et al., 2013; Sathyan et al., 2007; Tal et al., 2012), but is induced by ethanol in the adult mouse brain (Pietrzykowski et al., 2008). Moreover, loss of miR-9 in a zebrafish embryo recapitulated craniofacial (Pappalardo-Carter et al., 2013) and behavioral (Tal et al., 2012) defects associated with prenatal ethanol exposure. However, the mechanisms whereby prenatal alcohol exposure (PAE) alters miRNAs in neural stem cells are unclear.

PAE is known to broadly interfere with epigenetic programming in fetal NSCs (Liu, Balaraman, Wang, Nephew, & Zhou, 2009; Veazey, Carnahan, Muller, Miranda, & Golding, 2013; Veazey, Parnell, Miranda, & Golding, 2015; Zhou et al., 2011). Moreover, epigenetic alterations persist, and can be assayed in more differentiated cells and tissues obtained from FASD children (Laufer et al., 2015; Masemola, van der Merwe, Lombard, Viljoen, & Ramsay, 2015; Portales-Casamar et al., 2016). In an initial study, we found evidence that ethanol exposure resulted in increased methylation in fetal mouse neuroepithelial cells, specifically at the miR-9-2 gene locus, and not the miR-9-1 and miR-9-3 gene loci (Pappalardo-Carter et al., 2013). These data suggested that epigenetic silencing of miRNA genes might mediate ethanol effects. In this study, we therefore assessed the effects of ethanol on methylation across the murine chromosome 13 locus encoding the primary miRNA transcript, pri-miR-9-2.

Mechanisms that facilitate chromatin access through nucleosome disassembly are a potentially important but uninvestigated epigenetic mediator of PAE effects. The SWI/SNF (Switch/Sucrose Non-Fermentable)/BAF (BRG1/BRM-Associated Factor) heteromeric chromatin remodeling complex, a combinatorial assembly of ~10–15 different proteins assembled from 29 different gene products, causes ATP-dependent disassembly of nucleosomes by dissociating histones from DNA, to facilitate chromatin remodeling and transcriptional activation (reviewed in Kadoch, Copeland, and Keilhack (2016)). The BAF complex controls neural stem cell maturation (Lessard et al., 2007; Yoo, Staahl, Chen, & Crabtree, 2009), and whereas component members change through the process of neural differentiation to promote neuron-specific chromatin remodeling (Vogel-Ciernia & Wood, 2014), the core catalytic subunits of the BAF complex, BRG1 (SMARCA4/BAF190A) and BRM (SMARCA2/BAF190B), remain constant. In humans, BRG1, BRM, and other BAF-complex mutations result in significant intellectual disability, craniofacial abnormalities, and growth deficits (Kosho & Okamoto, 2014; Van Houdt, Nowakowska, Sousa, van Schaik, Seuntjens, Avonce, & et al, 2012), and mice heterozygous for a BRG1 null mutation exhibit exencephaly (Bultman et al., 2000), all of which are phenotypes that have been associated with FASD.

A previous study showed that ethanol increased methylation of SMARCA2 (Zhou et al., 2011) in NSCs, suggesting that the BAF complex itself is an epigenetic target of ethanol. Moreover, in adult invertebrate and rodent vertebrate models, the BAF complex (Mathies et al., 2015) and miR-9 (Pietrzykowski et al., 2008), respectively, have both been shown to mediate acute tolerance to alcohol, suggesting a mechanistic linkage between miR-9 and the BAF complex. Therefore, in the current study, we investigated the effect of ethanol on the BAF complex in fetal NSCs, and the impact of the BAF complex on the gene locus encoding one of the miR-9 genes, miR-9-2, which had been previously identified (Pappalardo-Carter et al., 2013) as an epigenetic target of ethanol.

Methods

Ex vivo fetal mouse neurosphere cultures

Neuroepithelial cells from the dorsal telencephalic vesicle, corresponding to the regional precursor of the murine iso-cortex were microdissected from gestational day (GD) 12.5 mouse fetuses as previously published (Prock & Miranda, 2007; Santillano et al., 2005; Sathyan et al., 2007; Tsai et al., 2014). All procedures were approved by the Texas A&M University Committee on Animal Care (IACUC). Cells were maintained as non-adherent cultures in defined culture medium, resulting in the formation of neurospheres, an *ex vivo* cell culture model system of the fetal neural stem cell niche (Miranda, Santillano, Camarillo, & Dohrman, 2008). For all experiments, total cell counts were determined using a hemocytometer. Dispersed neuroepithelial precursors were established in culture at an initial density of 10^6 cells in T-25 flasks containing serum-free mitogenic media. Cells were allowed to proliferate as neurospheres until cultures achieved a density of 2×10^6 cells per T-25 flask, then randomly assigned to control (0 mg/dL) or ethanol-treatment groups (120 mg/dL [26 mM] and 320 mg/dL [70 mM]), capped tightly with phenolic caps, and sealed with Parafilm® to limit the loss of ethanol. Ethanol concentrations in culture media were monitored by gas chromatography as previously published (Prock & Miranda, 2007; Santillano et al., 2005). Cultures were treated with ethanol for 5 days to mimic exposure through the period of neurogenesis and medium was changed every 2 days.

Differentiation paradigm

We previously showed that neurospheres cultured on a laminin substrate and maintained in the absence of bFGF became adherent and underwent terminal differentiation into migratory bipolar cells that expressed markers of neuronal identity (Camarillo & Miranda, 2008). Neurosphere cultures were suspended in media without bFGF, but supplemented with BDNF (25 ng/mL), and plated on laminin-coated (1.2 mg/mL, Thermo Fisher Scientific; Waltham, MA) 12-well plates. Culture medium was replaced every day. Photomicrographs were obtained every 24 h over a 3-day period.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was used to examine mRNA levels of the BAF-complex subunits. Three intron-spanning PCR primer pairs were obtained for each gene of interest using the NCBI Primer-BLAST program (Ye et al., 2012). Primer sequences were assessed for dimerization potential using IDT Oligo Analyzer 3.1 (Integrated DNA Technologies, Coralville, IA), and gene specificity and localization were assessed via the UCSC genome browser (Kent et al., 2002) *in silico* PCR tool. Primers were then tested in duplicates using 250 ng of cDNA harvested from GD12.5 murine neural stem cells, and thermal stability curves were assessed for evidence of a single amplicon. Selected amplicons were verified by sequencing (Texas A&M University Gene Technologies Lab) and compared to GenBank reference