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Alcohol effects on the epigenome in the germline: role in the inheritance of alcohol-related pathology

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Abstract

Excessive alcohol exposure has severe health consequences, and clinical and animal studies have demonstrated that disruptions in the epigenome of somatic cells, such as those in brain, are an important factor in the development of alcohol-related pathologies, such as alcohol-use disorders (AUDs) and fetal alcohol spectrum disorders (FASDs). It is also well known that alcohol-related health problems are passed down across generations in human populations, but the complete mechanisms for this phenomenon are currently unknown. Recent studies in animal models have suggested that epigenetic factors are also responsible for the transmission of alcohol-related pathologies across generations. Alcohol exposure has been shown to induce changes in the epigenome of sperm of exposed male animals, and these epimutations are inherited in the offspring. This paper reviews evidence for multigenerational and transgenerational epigenetic inheritance of alcohol-related pathology through the germline. We also review the literature on the epigenetic effects of alcohol exposure on somatic cells in brain, and its contribution to AUDs and FASDs. We note gaps in knowledge in this field, such as the lack of clinical studies in human populations and the lack of data on epigenetic inheritance via the female germline, and we suggest future research directions.

Keywords

fetal alcohol; preconception alcohol; birth outcome; offspring anxiety behavior; offspring stress response; DNA methylation; germline transmission

1. Introduction

Epigenetics is the milieu of mechanisms that modify gene expression without changing the DNA sequence itself. These mechanisms may involve methylation of DNA, modifications of histones, and small noncoding RNAs. Epigenetics allows for adaptive changes in gene expression that can be quickly implemented but can have long-lasting effects. Much research

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has focused on the effects of environmental impacts on epigenetic machinery, such as the effects of stress (Franklin et al., 2010; Yao et al., 2014), environmental toxins (Anway, Cupp, Uzumcu, & Skinner, 2005), and drugs of abuse, including alcohol (Finegersh, Rompala, Martin, & Homanics, 2015; Yohn, Bartolomei, & Blendy, 2015) and other drugs (for a review see Walker, Cates, Heller, & Nestler, 2015). Recent evidence also suggests that epigenetic modifications can be carried through the germline and through multiple generations. This type of inheritance is termed “epigenetic inheritance” and stands as a novel mechanism for inheritance. This compelling phenomenon is the subject of much current research, although many questions remain unanswered as to the mechanisms for epigenetic inheritance and for transgenerational epigenetic inheritance. The focus of this paper is to review the latest research on the epigenetic effects of alcohol exposure on the germline and on epigenetic inheritance of alcohol-related pathology. This review also discusses the gaps in knowledge relating to the mechanisms for epigenetic inheritance of alcohol exposure-related pathologies.

2. Alcohol-related pathology

Alcohol-use disorders (AUDs) are a significant psychiatric problem in the United States and worldwide. AUDs are characterized by excessive drinking and abuse of alcohol that leads to adverse social or health consequences for the drinker (Agrawal, Heath, & Lynskey, 2011; APA, 2013). AUDs may involve alcohol dependence or addiction to alcohol, which involves neural adaptations to chronic alcohol use leading to aversive emotional and physical consequences when alcohol use is discontinued, thus maintaining the alcohol abuse to avoid negative consequences (Koob, 2013). AUDs are associated with increased risk for fatal accidents and many serious health problems including depression, stroke, sleep disorders, heart disease, liver disease, and cancer (Rehm et al., 2009; Rehm, Samokhvalov, & Shield, 2013; Shield, Gmel, Patra, & Rehm, 2012). It is estimated that as many as 17 million adults in the US may have AUDs (Kessler et al., 1997; Volpicelli, 2001).

When a pregnant woman abuses alcohol, her exposed children are at a high risk for developing fetal alcohol spectrum disorders (FASDs), which are developmental disorders which can be characterized by diverse symptoms, including delayed growth, craniofacial abnormalities, intellectual impairment, anxiety, depression, and social impairment (Bakoyiannis et al., 2014; Driscoll, Streissguth, & Riley, 1990; Kelly, Day, & Streissguth, 2000). The occurrence and severity of symptoms depend on the duration and developmental timing of alcohol exposure (Alfonso-Loeches & Guerri, 2011). The most severe form of the disorder is called fetal alcohol syndrome (FAS), which is characterized by facial dysmorphologies, impaired growth, and central nervous system dysfunction (Sokol, Delaney-Black, & Nordstrom, 2003). Children that do not meet the full criteria for FAS but show a spectrum of the disorder are included under the umbrella term FASD. Children with FASD suffer long-lasting intellectual, social, and emotional problems, which persist through adulthood. The risk for developing AUDs has been found to “run in families”, due to a combination of social and genetic components, and studies suggest epigenetic factors also play a role in the development and inheritance of alcohol-related health problems. However, the epigenetic mechanisms for heritability of AUDs and familial alcohol-related health defects are far from understood and are discussed in this review.

3. Epigenetics

Epigenetics is the study of changes in gene expression, cell fate, and potentially heritability without any changes in DNA sequence. It can be thought of broadly as a bridge between genotype and phenotype (Goldberg, Allis, & Bernstein, 2007). Epigenetics is known to be regulated by three mechanisms, which interact in complex ways, including 1) DNA methylation, 2) histone modifications, and 3) noncoding RNAs. DNA methylation occurs on the cytosine residue of CpG dinucleotides and is performed by DNA methyltransferases (DNMTs) through the transfer of 5-methylcytosine from the methyl donor S-adenosylmethionine (SAM) to the CpG. In eukaryotes, DNA methylation status is passed down during DNA replication to the daughter strand DNA by the enzyme DNMT1, which performs the “maintenance” methylation activities, ensuring the methylation status of the parent cells is preserved in the daughter cells, while DNMT3a and DNMT3b are “*de novo*” methylation enzymes, which establish new methylation patterns during embryogenesis (Holliday & Pugh, 1975; Okano, Bell, Haber, & Li, 1999; Riggs, 2002), although recent evidence suggests there is some overlap in the function of maintenance and *de novo* enzymes (Jones & Liang, 2009). Methylation of DNA is functionally associated with gene silencing and is for the most part limited to so-called “CpG islands”, which are areas rich in CpG dinucleotides and are typically located near the promoter regions of genes and the transcription start site. Methylated DNA at the promoter regions can inhibit transcription by recruiting gene-silencing repressive proteins to the methylated region, such as methyl-CpG-binding protein 2 (MeCP2) and methyl-binding-domain (MBD) proteins (Cedar & Bergman, 2009). In contrast, hypomethylated CpG islands and promoter regions are associated with transcriptionally active histone modifications (discussed further below) and increased accessibility of the DNA transcription factors and RNA polymerase II to the DNA (Zhou, Goren, & Bernstein, 2011). Methylation status is mitotically stable and relatively long lasting, and evidence suggests that methylation status can even be passed on from the germline to the offspring, as this review will discuss. Nonetheless, flexibility in methylation status is also possible, and DNA methylation can also be reversed passively by the loss of 5mC during successive cycles of DNA replication, or by the recently discovered active demethylation of DNA, which can occur by oxidation of 5-methylcytosine by ten-eleven translocation (TET) family enzymes (Kohli & Zhang, 2013). Recent studies also suggest oxidized 5-methylcytosine, 5-hydroxymethylcytosine (5-hmC), may play an important role as an epigenetic mark, although the functional role of 5-hmC is still under investigation (Branco, Ficz, & Reik, 2012).

Alongside changes in DNA methylation, epigenetic machinery also includes a variety of changes in histones, both in post-translational histone modifications and in histone variants (for review see Bannister & Kouzarides, 2011; Kouzarides, 2007; Zhou, Goren, et al., 2011). In eukaryotes, proteins called histones package DNA into units called nucleosomes, which allow for protection and organization of the DNA. The cell has several enzymes that enact numerous covalent modifications on residues of histone tails (for example, methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation), which have been theorized to serve as a “histone code” to allow for either increased transcription or silencing of genes (Strahl & Allis, 2000). Some examples of important modifications include methylation at

histone 3 lysine 9 (H3K9me3) and lysine 27 (H3K27me3), which are associated with heterochromatin and gene silencing. In contrast histone 3 lysine 4 methylation (H3K4me2 and H3K4me3), acetylation, and the histone variant H2A.Z are marks associated with euchromatin, active promoters, and increased activation of gene expression (Zhou, Goren, et al., 2011). Histone-modifying enzymes include histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases, and kinases that catalyze modifications on the histones, which are then interpreted by chromatin-modifying protein complexes which are able to modify chromatin structure, allowing for decreased or increased accessibility of the transcription machinery to the DNA (Henikoff, 2008; Kouzarides, 2007; Nakao, 2001; Ng & Bird, 1999). While DNA methylation is more permanent, histone modifications are more dynamic and transient, but it is important to stress that histone modifications and changes in DNA methylation are not separate entities, and that they work together to orchestrate transcriptional response and possibly affect epigenetic inheritance (Nakao, 2001; Ng & Bird, 1999).

An additional, a more recently identified mechanism of epigenetic modification includes modulation by noncoding RNAs (ncRNAs), RNAs that are transcribed from DNA but are not translated into protein (for reviews see Berezikov, 2011; Larriba & del Mazo, 2016; and Yan, 2014). Small ncRNAs, which are less than 200 nucleotides (nts) in length, include microRNAs (miRNAs), short interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs), which have been shown to silence gene expression by targeting mRNA transcripts, sometimes via histone and DNA methylation mechanisms, leading to their downregulation (Holoch & Moazed, 2015). Long non-coding (lncRNAs) (>200 nts in length) also participate in epigenetic-mediated gene silencing. Especially, long noncoding intergenic RNA (lincRNAs) are transcribed from regions close to genes that will be silenced, and have been discovered to serve as sequence guides to attract protein complexes to either increase DNA methylation or repressive histone modifications. Examples of the work of lincRNAs include important roles in inactivation of the X chromosome (Ng, Pullirsch, Leeb, & Wutz, 2007) and in genomic imprinting (Gabory, Jammes, & Dandolo, 2010). As ncRNAs participate in important developmental events that affect the primordial germ cells and can also be affected by environmental influences, they could participate, in theory, as an important mechanism in epigenetic inheritance (Larriba & del Mazo, 2016; Yan, 2014).

4. Epigenetic Effects of Alcohol Exposure on Somatic Cells

The effects of alcohol exposure on epigenetic machinery in somatic cells in the organism have been fairly well characterized in recent years. This review will focus on the effects of alcohol exposure on germline cells and evidence for epigenetic inheritance of alcohol-related pathologies. In this section, we will also cover studies on the epigenetic effects of alcohol exposure on somatic cells in humans and in animal models, and the way these epigenetic changes may contribute to the development and maintenance of AUDs and FASDs and other alcohol-related health problems. For comprehensive reviews of the epigenetic mechanisms in the development and maintenance of AUDs and addiction to alcohol, the reader is referred to outstanding reviews by Krishnan, Sakharkar, Teppen, Berkel, & Pandey, 2014; Moonat & Pandey, 2012; and Ponomarev, 2013. For comprehensive reviews of the epigenetic

mechanisms in FASD, the reader is referred to excellent reviews by Ramsay, 2010; and Ungerer, Knezovich, & Ramsay, 2013.

Epigenetic Effects of Alcohol Exposure in Adults

In animal models and in human populations, alcohol exposure during adulthood has been shown to affect DNA methylation, histone modifications, and ncRNAs in brain and other tissues, leading to disrupted gene expression and associated pathology (see Table 1 for a summary of studies). In many studies, chronic alcohol exposure has been associated with aberrant DNA methylation, in part by causing dysfunction in folic acid metabolism and deficits in the methyl donor SAM (Blasco et al., 2005; Hamid, Wani, & Kaur, 2009). In addition, alcohol exposure has also been shown to alter DNMT activity, which also leads to changes in DNA methylation (Garro, McBeth, Lima, & Lieber, 1991; Ponomarev, Wang, Zhang, Harris, & Mayfield, 2012; Zhang, Kusumo, Sakharkar, Pandey, & Guizzetti, 2014). In response to alcohol exposure, studies show either hypomethylation or hypermethylation of specific genes, with detrimental health consequences. Recent studies using human post mortem brain tissue and buccal swabs from either persons with AUDs or non-alcoholic controls have shown altered site-specific methylation of regions in long terminal repeat-(LTR) containing retrotransposons (Ponomarev et al., 2012) and in gene pathways involved in immune response, lipid metabolism, inflammation, and gastrointestinal disease (Hagerty, Bidwell, Harlaar, & Hutchinson, 2016) in persons with AUDs, linking alcohol-induced changes in methylation to some of the health issues that often co-occur with alcohol abuse. Examining single nucleotide polymorphisms (SNPs) on the prodynorphin gene, which are associated with alcohol dependence, Taqi and colleagues (2011) found differential methylation on three CPG-SNPs in the dorsolateral prefrontal cortex of post mortem samples from alcoholics, suggesting epigenetic and genetic mechanisms may interact to confer risk to developing alcohol dependence. Finally, aberrant DNA methylation status due to alcohol exposure has been found to be associated with many types of cancers, including liver, colorectal, breast, and upper respiratory cancers (Varela-Rey, Woodhoo, Martinez-Chantar, Mato, & Lu, 2013).

Changes in histone modifications and miRNAs due to adult alcohol exposure have also been implicated in the development of alcohol addiction and AUDs in animal models and human tissues. In a rodent model, acute ethanol exposure was associated with decreased HDAC activity and increased acetylation of H3 and H4 histones in the amygdala, whereas withdrawal was associated with increased HDAC activity and decreased acetylation of these histones in this brain region (Pandey, Ugale, Zhang, Tang, Prakash, 2008). Withdrawal-induced anxiety behavior, deficits in histone acetylation, and changes in neuropeptides were normalized by administration of an HDAC inhibitor, trichostatin-A, implicating increased amygdala HDAC activity and histone de-acetylation in alcohol withdrawal (Pandey et al., 2008; You, Zhang, Sakharkar, Teppen, & Pandey, 2014). Several other animal studies have also shown that HDAC inhibitors and knockdown of HDAC via siRNA ameliorate alcohol-induced anxiety and alcohol intoxication (Moonat, Sakharkar, Zhang, Tang, & Pandey, 2013; Sakharkar et al., 2014; Warnault, Darcq, Levine, Barak, & Ron, 2013), suggesting that HDAC inhibition has therapeutic potential for the treatment of AUDs. Studies using human post mortem tissue have also found differences in global and promoter-specific H3K4

trimethylation (an activation marker) in brain, indicating chromatin remodeling is associated with AUDs (Ponomarev et al., 2012; Zhou, Yuan, Mash, & Goldman, 2011). Finally, a small number of human and animal studies have noted altered expression of miRNAs as a consequence of chronic alcohol exposure, and suggested a role for these changes in neural plasticity underlying the development of AUDs (Lewohl et al., 2011; Pietrzykowski et al., 2008). (See also Miranda et al., 2010 for a review on the role of miRNAs in alcohol toxicity and in the development of alcohol abuse.)

Epigenetic Effects of Alcohol Exposure During Early Development

Alcohol exposure during the fetal period has been shown to have a dramatic effect on epigenetic programming, possibly leading to the development of FASDs (see Fig. 1 and Table 2). As also mentioned in Section 3 and Section 6, the genome of the developing embryo undergoes global changes in methylation in order to program cell development in an appropriate manner (Bao & Yan, 2012; Seisenberger et al., 2013). Thus, alcohol exposure during this time period is particularly risky, as it can interfere with normal epigenetic developmental programming. Both animal and human studies show aberrant DNA methylation patterns from exposure to alcohol *in utero*, because of mechanisms similar to those from adult exposure, such as disruption in folic acid metabolism and deficits in SAM as well as decreased DNMT activity (Garro et al., 1991; Ungerer et al., 2013; Zhang et al., 2014). Animal models have shown global DNA hypomethylation in the fetus (Garro et al., 1991), and also brain regional decreases in methyl-CpG-binding protein Mecp2 in prefrontal cortex and striatum (Kim et al., 2013) and hippocampus (Chen, Ozturk, & Zhou, 2013).

Numerous studies also show differential methylation on specific genes with functional consequences in gene expression and neural development (for a comprehensive review see Ungerer et al., 2013). Using *in vitro* models of chronic alcohol exposure, decreased DNA methylation in cultured fetal mouse neurons was found near the gene for the NMDA receptor subunit *NR2B*, a receptor involved in the development of AUDs (Marutha Ravindran & Ticku, 2004; Qiang et al., 2010). Changes in methylation coincided with increased expression of *NR2B*, demonstrating functional consequences (Marutha Ravindran & Ticku, 2004; Qiang et al., 2010). As the NMDA receptor plays a major role in neural development (Constantine-Paton, Cline, & Debski, 1990) and in learning and memory (Li & Tsien, 2009), it is possible that methylation changes in *NR2B* may play a role in the development of FASD pathology and cognitive symptoms, although further research using *in vivo* methods is needed to confirm this hypothesis. An *in vivo* study showed prenatal alcohol exposure altered the normal developmental methylation and hydroxymethylation gradient in the hippocampus, which was associated with reduced hippocampus size and developmentally delayed formation of mature neurons in the dentate gyrus, suggesting that altered developmental methylation patterns in hippocampus due to alcohol exposure may play a role in the neural and intellectual deficits seen in FASD (Chen et al., 2013). Another study utilizing whole embryo culture showed alcohol-induced changes in methylation on chromosomes 7, 10, and X, which correlated with neural tube defects. Specifically, increased methylation of genes known to play a role in metabolism, such as *Cyp4f13*, and decreased methylation of genes known to play a role in development (*Nlgn3*, *Elavl2*, *Sox21*, and *Sim1*) and the imprinted gene *Igf2r*, were observed in alcohol-exposed embryos (Liu,

Balaraman, Wang, Nephew, & Zhou, 2009). Other studies have also shown changes in methylation of imprinted genes including *Igf2* (Downing et al., 2011). Thus, alteration in methylation of these genes may play an important role in the development of neural dysfunction in FASD, as they play a key role in normal neural development. As people with FASD are more likely to suffer from anxiety disorders, studies in our lab focus on the role of epigenetic modifications in stress-axis disruption due to fetal alcohol exposure. Studies in our lab have shown that rats exposed to fetal alcohol show hypermethylation of the proopiomelanocortin (*Pomc*) gene promoter region in the hypothalamus, and also reduced expression of this gene. These changes correlate with reduced production of the peptide encoded by the *Pomc* gene, β -endorphin (BEP), a negative regulator of the stress axis, and increased stress response in alcohol-exposed animals (Bekdash, Zhang, & Sarkar, 2013; Govorko, Bekdash, Zhang, & Sarkar, 2012). Fetal alcohol-induced *Pomc* promoter hypermethylation, reduced *Pomc* expression, and hyper-stress response were normalized by gestational choline supplementation, a dietary source of methyl, suggesting that altered *Pomc* methylation may have been due in part to deficits in available methyl donors (Bekdash et al., 2013). In addition, fetal alcohol-induced decreases in *Pomc* expression also coincided with increased transcriptional repressor MeCP2 in *Pomc* cells in the hypothalamus. Also, lentiviral knockdown of MeCP2 normalized the decreases in *Pomc* and normalized the aberrant hormonal response to stress in fetal alcohol-exposed animals (Gangisetty, Bekdash, Maglakelidze, & Sarkar, 2014). Finally, DNA methylation is also differentially altered in human populations exposed to *in utero* alcohol, as a recent study of methylation status of buccal swabs from children with FASD showed hypermethylation in genes implicated in neurodevelopment and neurological diseases such as anxiety, epilepsy, and autism-spectrum disorders (Portales-Casamar et al., 2016).

In addition to alterations in methylation, data from animal models show fetal alcohol exposure also causes chromatin remodeling, including changes in histone acetylation and methylation, and changes in miRNA expression, which contribute to altered gene expression and the neurodevelopmental deficits in FASD (for review see Ungerer et al., 2013). In a study utilizing ethanol-exposed cultured mouse fetal neurospheres, it was found that alcohol exposure alters activating and repressive histone modifications (H3K4me3 and H3K27me3, respectively) enrichment at genes involved in neural precursor identity and neurogenesis, such as *nestin*, *Igf1*, *Dlx2*, *HoxA1*, and *Pax6*, with some of these genes also showing differences in transcription (Veazey, Carnahan, Muller, Miranda, Golding, 2013). Thus, alcohol-induced post-translational histone modifications may alter expression of key regulatory genes involved in brain development to produce the neural dysfunction in FASD. An *in vivo* study rats exposed to alcohol neonatally (third-trimester human equivalent) showed decreased CREB binding protein, a histone acetyltransferase, and reduced H3 and H4 acetylation in the developing cerebellum (Guo et al., 2011). The researchers noted that these epigenetic changes in cerebellum might underlie cerebellar dysfunction and motor impairment in FASD. In the previously mentioned studies from our lab, fetal alcohol-induced increases in hypothalamic *Pomc* promoter methylation and decreased *Pomc* expression coincided with changes in histone modifications in *Pomc* cells, including decreases in histone activation marks (H3K4me3, acetylated H3K9, phosphorylated H3S10), and increases in repressive marks (H3K9me2 and histone methyltransferases G9a and

Setdb1) (Bekdash et al., 2013). Treatment of fetal alcohol-exposed animals with HDAC inhibitor trichostatin-A also normalized *Pomc* expression and aberrant hormonal response to immune stress (Govorko et al., 2012). Thus, histone modifications appear to be crucial to some of the neurobiological defects produced with fetal alcohol exposure.

In addition, a few pioneering studies indicate fetal alcohol produces alterations in miRNAs that regulate neurodevelopment. Using cultured mouse fetal cortical neurospheres, high dose alcohol was found to suppress the expression of miR-21, miR-335, and miR-153, which were shown to synergistically regulate genes involved in neuronal maturation and proliferation, which are known to be aberrant in FASD (Sathyan, Golden, & Miranda, 2007). In an *in vivo* model, prenatal exposure to alcohol induced severe fetal teratogenesis and learning impairment in offspring, which was correlated with increased expression of several miRNAs, including miR-10a, miR-10b, miR-9, and decreased expression of miR-200a, miR-496, and miR-296 in brain (Wang et al., 2009). It is possible these miRNAs may play a role in the observed teratogenesis and learning deficits in the alcohol-exposed animals, but further studies are needed to confirm this mechanism. Finally, early gestational exposure to ethanol resulted in differential expression of miR138-2, miR290, and miR16-2 in hippocampus (Marjonen et al., 2015). It was noted by the researchers that miR290 has been connected with embryonic gene regulation (Houbaviy, Murray, & Sharp, 2003) and the pluripotent stem cell state (Tata, Tata, Kühl, & Sirbu, 2011), while miR138-2 is associated with dendritic spine size in the hippocampus (Siegel et al., 2009). These studies suggest miRNAs participate in the development of alcohol-induced neural defects, but more studies are needed on this topic to determine a role for ncRNAs in the development of FASDs.

5. Epigenetic Inheritance

Epigenetic inheritance is the phenomenon that occurs when so-called epimutations, or changes in gene expression that do not involve actual changes to the DNA sequence, are carried via the germline to the offspring, potentially through multiple generations. Epigenetic inheritance has been shown to occur from exposure of the parental or F0 organism to multiple environmental stimuli, including changes in diet, psychological stress, exposure to toxins, exposure to drugs of abuse, and exposure to alcohol, on which this review will focus. It is important to distinguish between “germline-dependent” from “germline-independent” epigenetic inheritance. Germline-dependent epigenetic inheritance occurs when the first generation organism (F0) is exposed to an environmental stimulus and his/her germ cells acquire epimutations that are then passed on to the next generation offspring (F1), affecting its germline and somatic cell epigenome in this generation in the absence of any external stimuli factors. In turn, these epimutations can then be transferred to the next generation (F2) through the germline, to alter F2’s somatic cells’ gene expression to express the same phenotype in the absence of external factors, and so on. In order for “transgenerational” inheritance to occur, the phenotype must be passed on from the F0 generation to the F3 generation along the female germline or from the F0 generation to the F2 generation along the male germline (Jirtle & Skinner, 2007; Skinner, 2008, 2011). This is because the germ cells of the F0 generation would be affected directly by exposure to the environmental factor (i.e., alcohol exposure), and thus any effects on the F1 generation would be due to the direct effects of alcohol on the germline. In the case of exposed females,

if the F0 female is pregnant, both her germ cells and the germ cells of the developing F1 embryo(s) would be exposed, so it is necessary for the epimutation to be transmitted to the F3 generation in order to meet the criteria for “transgenerational” epigenetic inheritance. Epigenetic inheritance that does not meet the criteria for “transgenerational” inheritance might be termed “multigenerational” inheritance instead (Skinner, 2008).

“Germline-independent” epigenetic inheritance is another phenomenon, and occurs because of behavioral parental factors when the effects of environmental insults (i.e., alcohol exposure) affect the uterine environment or the maternal behavior of the exposed F0 female, and thus induce epimutations in the F1 generation due to changes in the prenatal environment and/or maternal care, independent of any effect on the germline (Grossniklaus, Kelly, Ferguson-Smith, Pembrey, & Lindquist, 2013; Sarkar, 2016; Weaver, 2007). These epimutations may then be transmitted again through altered parental behavior by the adult F1 animals to the F2 offspring, and so on. A specific example of this effect was found in the epigenetic effects on the offspring of good versus negligent maternal care in rats. Neonatal rats that received high levels of licking and grooming from their mothers, an indicator of good maternal care, showed decreased methylation at a binding site in the promotor of the glucocorticoid receptor gene and increased expression of the gene in the hippocampal region of the brain, which was associated with resilience in response to stress (Weaver, 2007; Weaver et al., 2004). In addition, cross fostering of pups from high-licking and grooming dams to low-licking and grooming dams reversed this effect, demonstrating that this epigenetic effect was dependent on maternal care, and was thus germline-independent.

Mechanisms for Epigenetic Inheritance

Understanding the mechanisms of multigenerational or transgenerational epigenetic inheritance of alcohol-related disease is complex, for several reasons. First, as germline-dependent and germline-independent mechanisms exist, one must discriminate between these two mechanisms. In humans, epigenetic inheritance of alcohol-related disease in humans most likely involves both germline-dependent and germline-independent mechanisms of transmission, as having an AUD is known to impact the quality of maternal behavior, and social factors such as parental abuse and neglect and early life environment and adversity play an important role in developing AUDs in offspring later in life (Hill, Blow, Young, & Singer, 1994). Germline-dependent mechanisms for epigenetic inheritance of alcohol-related disease also exist based on human and animal studies. Notably, in animal models, the parental care effects can be eliminated by fostering offspring to un-exposed surrogate mothers, and thus, it can be determined whether the epimutations observed are due to germline-dependent or germline-independent mechanisms. This review will focus on germline-dependent mechanisms for epigenetic inheritance of alcohol-related health problems.

In addition, pinpointing specific epigenetic mechanisms for germline-dependent inheritance of alcohol-related disease has been difficult to date, and these mechanisms are still poorly understood. Theoretically, it would seem that transmission of epimutations through the germline are rare, as embryonic cells, including primordial germ cells, go through two waves of demethylation during early development, thus losing most epigenetic information

from the germ cells encoded in methylations (Bao & Yan, 2012; Seisenberger et al., 2013). Nonetheless, some sections of DNA, such as imprinted genes in the germline, do keep their methylation status inherited from one of the parents and escape the demethylation waves during early development. The methylation status in these regions is thus preserved and then transmitted to the offspring from the parent. A well-known example of imprinted genes is the *Igf2/H19* gene cluster (Nordin, Bergman, Halje, Engstrom, & Ward, 2014). Studies suggest that the number of imprinted genes might be fairly high, especially in brain, suggesting that genomic imprinting could be a common mechanism for epigenetic inheritance (Gregg et al., 2010). In addition, some non-imprinted genomic elements, such as IAPs, also escape the demethylation during development (Lane et al., 2003). Sperm cells also lose most of their histone epigenetic histone marks, as most histones are swapped for protamines during spermatogenesis. However, some histones in sperm are retained, which allows for an additional possible mechanism for germline-dependent epigenetic inheritance (Bench, Friz, Corzett, Morse, & Balhorn, 1996; Gatewood, Cook, Balhorn, Bradbury, & Schmid, 1987). It was also found that *C. elegans* embryos retained the repressive histone mark H3K27me from oocytes, indicating that epigenetic marks from the female germline might also be transmitted to the next generation (Gaydos, Wang, & Strome, 2014). In addition, recent studies suggest epigenetic inheritance may also involve transmission of epimutations through ncRNAs (Ashe et al., 2012; Larriba & del Mazo, 2016; Yan, 2014). Notably, alcohol exposure does affect epigenetic machinery in germline cells, and some studies have shown evidence for multigenerational and transgenerational epigenetic inheritance of alcohol-related health problems, although much more future research in this area is warranted. These studies are reviewed below.

6. Evidence for Epigenetic Inheritance of Alcohol-related Pathology

Human Studies: Multigenerational Effects

It is known that alcohol abuse and AUDs tend to be passed down across generations in human populations, although the mechanisms are hard to interpret in these observational studies. Having a parent or other relative with an AUD increases the risk for and the severity of alcohol abuse (Cotton, 1979; Kendler et al., 2015; Morean, Corbin, Sinha, & O'Malley, 2009; Sjoerds et al., 2013; Turner et al., 1993; Worobec et al., 1990), although environmental and social factors alongside genetic factors play a role in this relationship (Enoch & Goldman, 1999; Hill et al., 1994; Nieratschker, Batra, & Fallgatter, 2013). The heritability of alcohol dependence is estimated to be between 30%–70% as estimated from studies of twins, in which one twin was separated from the other and adopted, thus controlling the effects of similar environment (Agrawal & Lynskey, 2008). Several genetic factors including genes for alcohol metabolism, such as alcohol dehydrogenase, and genes for neuromodulators, including dopamine systems, serotonin systems, and opioid systems, have been found to be correlated to alcohol dependence (Demers, Bogdan, & Agrawal, 2014; Kreek, Nielsen, & LaForge, 2004).

It has been noted that genetic variants do not appear to explain all the heredity in complex disorders (such as AUDs) (Manolio et al., 2009), and perhaps epigenetic inheritance might be able to “fill in the blanks” to help better explain the inheritance of complex diseases

(Bohacek, Gapp, Saab, & Mansuy, 2013; Danchin et al., 2011). In addition to genetic variants, some studies have also opened the possibility of epigenetic factors for inherited transmission of alcohol-related health problems. It has long been noted that children born to fathers with AUDs show symptoms similar to children with FASD, including cognitive deficits, although they are not directly exposed to alcohol as is the case of children born to mothers who drank during pregnancy (Gabielli & Mednick, 1983; Lemoine, Harousseau, Borteyru, & Menuet, 2003). For women who drink prior to pregnancy, the risk is also increased for her children for lower birth weight (Nykjaer et al., 2014) and mental retardation (Roeleveld, Vingerhoets, Zielhuis, & Gabreëls, 1992). Data on possible epigenetic mechanisms for these phenomena in humans are lacking. In one recent study, heavy alcohol consumption has been shown to be correlated with a decrease in methylation of imprinted genes *H19* and *IG-DMR* in the sperm of men (Ouko et al., 2009). This novel study suggests a possible mechanism for the epigenetic transmission of alcohol-related health problems to the offspring via imprinted genes on sperm cells.

Human Studies: Transgenerational Effects

Studies investigating the possibility of transgenerational inheritance of alcohol-related health problems are also lacking. In one study, it was found that having a grandparent with major depressive disorder, which often co-occurred with substance abuse including alcohol abuse, greatly increased the risk of the individual having major depressive disorder himself or herself (Olino et al., 2008). Likewise with FASD, grandmothers of children with fetal alcohol syndrome or partial fetal alcohol syndrome were found to have a much greater rate of history of alcohol abuse, suggesting a generational phenomenon in FASDs (Kvigne, Leonardson, Borzelleca, & Welty, 2008). Of course, epigenetic inheritance is impossible to conclude in this case, as social factors were also likely at play, and the mothers drank during pregnancy, but the authors speculated that the risk for FASD seemed to be magnified with the incidence of grandparental alcohol abuse. These clinical studies hint at the possibility of multigenerational and transgenerational inheritance of psychiatric disorders and alcohol-related health problems, but they do not establish a mechanism for the inheritance of these disorders. Clearly, more clinical studies are needed to examine the family tree of persons with AUDs or FASDs to investigate a generational pattern for alcohol-related problems. In addition, incidences of other health problems, including stress-related and psychiatric problems in persons with a familial history of AUDs or FASDs might be studied. Finally, future studies could also examine the possibility and mechanisms of epigenetic inheritance of AUDs in humans.

Animal Studies: Multigenerational Effects

Although human studies of generational transfer of alcohol-related health problems is lacking, there are many studies showing multigenerational and a small number of studies showing transgenerational effects of alcohol exposure in animal models (for excellent reviews, see also Finegersh et al., 2015; Sarkar, 2016; and Yohn et al., 2015). Many studies have shown that paternal preconception alcohol exposure has detrimental effects on the F1 offspring behavior and neurobiology in mouse and rat models. Preconception paternal alcohol exposure results in reduced organ weight and an increase in the number of “runts” in litters in rat offspring (Abel, 1993b). F1 offspring also show neurobiological alterations,

including increased cortical thickening (Jamerson, Wulser, & Kimler, 2004), decreased cortical and striatal dopamine transporter expression (Kim et al., 2014), decreased expression of cortical and striatal MeCP2 (Kim et al., 2014), and decreased hypothalamic β -endorphin (Cicero et al., 1990). Paternal exposure to alcohol results in several behavioral abnormalities in the F1 offspring, including altered learning in the passive avoidance and radial arm maze tasks (Abel & Lee, 1988; Abel & Tan, 1988; Wozniak, Cicero, Kettinger, & Meyer, 1991). In addition, preconception paternal alcohol also results in behaviors resembling psychopathology in humans, including decreased grooming, decreased fear, increased aggression, decreased attention, increased impulsivity, increased hyperactivity, and increased anxiety (Abel, 1994; Kim et al., 2014; Liang et al., 2014; Meek, Myren, Sturm, & Burau, 2007). In addition, studies have also found hormonal alterations from paternal preconception alcohol in rats. Male mouse F1 offspring show decreased corticosterone response to stress, suggesting altered hypothalamic-pituitary-adrenal axes in these animals (Rompala, Finegersh, & Homanics, 2016), and also show reduced adult levels of testosterone (Abel & Lee, 1988). Finally, studies have shown paternal preconception increased sensitivity to amphetamine (Abel, 1993a) and decreased alcohol drinking, but increased sensitivity to alcohol (Finegersh & Homanics, 2014). Notably, the effects of preconception paternal alcohol exposure on F1 offspring were shown to be sexually dimorphic in many cases. These studies show, for the most part, that alcohol drinking in F0 males results in physiological and neurological abnormalities, disrupted learning, abnormal behavior, and increased sensitivity to drugs and alcohol in F1 offspring, findings which resemble deficits shown in human FASD populations and noted in children of fathers with AUDs.

While these studies examined the effects of paternal preconception alcohol exposure on offspring, studies in our lab have recently shown that maternal preconception exposure also negatively affects offspring health, leading to an increase in adult offspring vulnerability to stress (Jabbar et al., 2016). Specifically, maternal alcohol exposure 3 weeks prior to conception results in several defects in adult F1 offspring, including increased hormonal stress response to an immune stressor, increased hypothalamic corticotropin-releasing hormone, decreased hypothalamic β -endorphin, and altered methylation status on CpG islands in stress regulatory genes in various brain areas. Interestingly, F0 maternal behavior was not altered by preconception alcohol, and fostering F1 offspring from alcohol-treated dams to control dams did not change their enhanced stress response, suggesting the detrimental effects on the F1 offspring were not due to changes in F0 maternal behavior. In addition, the vulnerabilities in F1 offspring of preconception alcohol-treated dams were normalized by neonatal treatment with a DNMT blocker 5-azadeoxycytidine, suggesting the stress abnormalities observed in F1 offspring may have been inherited via epigenetic mechanisms, such as altered methylation at promoters of stress regulatory regions. Further studies of oocytes from preconception alcohol-treated dams are underway to determine a germline mechanism for the inheritance of stress vulnerabilities in F1 offspring.

In addition, future studies are needed to determine whether the effects of preconception maternal alcohol are transgenerational. In addition to preconception alcohol effects on offspring, recent rat studies have also shown that grandmaternal ethanol consumption during pregnancy has profound effects on behavior and endocrinology of second-generation

progeny. Exposure to ethanol *in utero* has been shown to cause profound metabolic, endocrine, and behavioral disruptions in the exposed F1 offspring, and recent studies from the Redei laboratory have also shown these effects are carried over into the unexposed F2 generation. Particularly, exposure to prenatal ethanol was shown to cause insulin resistance in both the F1 and F2 generations, but the phenotype was dependent on the sex of the exposed F1 parent and of the F2 offspring (Harper, Tunc-Ozcan, Graf, & Redei, 2014). In addition, grandmaternal alcohol consumption was shown to cause a fear memory deficit and altered expression of hippocampal thyroid hormone-regulated type 3 deiodinase (Dio3) and neurogranin (Nrgn) expression in the F1-exposed offspring and in the matrilinear F2 offspring, suggesting these deficits may have been carried over via the female germline (Tunc-Ozcan, Harper, Graf, & Redei, 2016). In both studies, thyroxine (T4) administration to the F0 mother during ethanol exposure alleviated the detrimental metabolic and behavioral effects on the F2 generation, suggesting that alcohol-disrupted thyroid function in the F0 female was in part responsible for the multigenerational effects of ethanol exposure.

Animal Studies: Transgenerational Effects

One of the first studies to examine the transgenerational effects of alcohol was done in the early 20th century using ethanol vapor-exposed male and female guinea pigs that were then bred with normal control animals over several generations (Stockard & Papanicolaou, 1918). The researchers found the effects of alcohol exposure affected offspring as far as the F4 generation, causing decreased weight, behavioral changes, increased mortality, and fertility problems in some portion of the descendants. The cause of this phenomenon was hypothesized to be damage to the germline.

Nearly a century later, studies in our lab investigated whether deficits in hypothalamic *Pomc* expression due to fetal alcohol exposure could be passed down to subsequent generations. It was found that F1-F3 male progeny of the male germline, but not female germline, did show consistent decreases in hypothalamic *Pomc* expression with concomitant increases in *Pomc* methylation (Govorko et al., 2012). Importantly, the hypermethylation of the *Pomc* gene in the hypothalamus was accompanied by a similar hypermethylation of *Pomc* in sperm cells in the F1-F3 males, demonstrating that the deficit in *Pomc* expression was carried from generation to generation via the male germline (see Fig. 2). Changes in methylation and expression of *Pomc* were accompanied by decreased production of the *Pomc*-encoded protein BEP, a negative regulator of the stress axis, and increased hypothalamic-pituitary-adrenal response to stress. These studies show that the negative effects of alcohol exposure on the stress axis can be transgenerationally inherited over several generations and, in the case of *Pomc* deficiency, are transmitted via methylation changes on the male germline.

7. Possible Mechanisms for Germline-dependent Epigenetic Inheritance of Alcohol-related Pathology

Knowledge about possible mechanisms of epigenetic inheritance of alcohol-related pathology is limited to date, but studies investigating alcohol's effects on the germline have offered promising directions for the research. Notably, alcohol exposure is known to have a direct detrimental effect on the male germline in humans (Emanuele & Emanuele, 1998;

Muthusami & Chinnaswamy, 2005). In order to investigate possible mechanisms for epigenetic germline-dependent inheritance, recent human and animal studies have focused investigation on alcohol-induced changes in methylation along the male germline. As mentioned previously, in human populations, heavy alcohol consumption has been shown to be correlated with a decrease in methylation of imprinted genes *H19* and *IG-DMR* in the sperm of men (Ouko et al., 2009). In animal models, it was also found that fetal alcohol exposure decreased methylation on the *H19* gene in the alcohol-exposed F1 generation, and that this decreased methylation pattern on *H19* was also observed in the brains of the F2 generation, demonstrating epigenetic inheritance of methylation changes in this imprinted gene (Stouder, Somm, & Paoloni-Giacobino, 2011). In another animal study, it was found that alcohol exposure in F0 sires decreased methylation on the *H19*- and *Peg3*-imprinted genes in sperm, and that similar decreases in methylation on *Peg3* were also found in the cerebral cortices of the F1 offspring (Liang et al., 2014). These changes in *Peg3* methylation in offspring brain were associated with aberrant circling behavior. These studies all offer evidence that alcohol-induced epigenetic transmission of pathology can be transmitted to the offspring, specifically via imprinted genes along the male germline. However, another study found paternal alcohol exposure was associated with decreased methylation at two *H19* binding sites in F1 offspring, but only decreased methylation at one CpG of the *H19* gene in the sperm of alcohol-exposed F0 sires (Knezovich & Ramsay, 2012). The authors suggested that the imprinted gene *H19* does seem to play a role in epigenetic transmission of paternal alcohol exposure effects to the offspring, but the mechanism may not be specifically via changes in methylation at this gene in sperm, but rather, different mechanisms such as sperm-borne RNA-mediated effects or histone modifications. Thus, further studies on the mechanisms of epigenetic inheritance involving the *H19* imprinted region are warranted.

Other studies have investigated the effects of alcohol on methylation of non-imprinted genes in the germline and its relation to epigenetic inheritance of alcohol-induced health problems. As discussed previously, our lab found increased methylation of the *Pomc* gene in sperm and hypothalamus of the F1-F3 male descendants of fetal alcohol exposed rats (see Fig. 2) (Govorko et al., 2012). This study shows that aberrant methylation due to alcohol can be passed down to offspring. The reason why methylation patterns on this gene along the male germline can be preserved and escape reprogramming during development is unknown. A possible explanation for this would be, since the phenotype is only passed down along the male germline and *Pomc* expression is regulated by the non-pairing region of the Y chromosome (Botbol et al., 2011), alcohol exposure leads to epigenetic modifications on the Y chromosome that are spared from reprogramming during development and are then passed down to subsequent generations (Sarkar, 2016). Future studies are needed to address this possibility.

Other studies have also found methylation changes in specific non-imprinted genes in the sperm of the alcohol-exposed F0 sires that were then transmitted to somatic brain cells of the F1 offspring generation. Finegersh and colleagues found that alcohol exposure in F0 males decreased methylation of the brain-derived neurotrophic factor (*BDNF*) promoter in sperm, a methylation change which was also present in the ventral tegmental area of the brain of F1 offspring (Finegersh & Homanics, 2014). This change in *BDNF* methylation was also accompanied by increased VTA *BDNF* expression in F1 offspring, as well as decreased

preference for and consumption of alcohol but increased sensitivity to the behavioral effects of alcohol. Thus, this study shows a functional change in offspring of sires exposed to alcohol, which may be due to an inherited change in methylation on the *BDNF* gene that was transmitted across the male germline. In another study, increased methylation of the dopamine transporter (DAT) gene was found in both the sperm of F0 alcohol-exposed sires and in the cortical and striatal regions of the brains of F1 offspring, and F1 offspring showed a decrease in DAT mRNA and protein in these brain regions (Kim et al., 2014). Alongside these methylation changes in DAT, decreases in expression of two regulators of methylation machinery, DNMT1 and MeCP2, were also found in the brains of F1 offspring, suggesting that changes in these regulators may play a role in the epigenetic inheritance of the methylation changes in DAT. In addition, the F1 offspring whose sires were exposed to alcohol were found to have increased hyperactivity, inattentiveness, and impulsive behaviors, resembling human ADHD symptoms. This study also shows another potential mechanism for the cross-generational inheritance of the behavioral disruptions observed in the offspring of fathers who drink heavily, specifically changes in methylation on the DAT gene on the male germline. Together, these studies provide evidence that alcohol-induced methylation changes on non-imprinted genes can be transmitted along the male germline across generations, and that these changes might mediate the multi-generational dysfunctional effects of alcohol on the offspring. Future studies are also needed to address the mechanisms required for epigenetic inheritance of specific methylation changes on these non-imprinted genes.

It is interesting to note that in all these cases, aberrant methylation of genes is observed in the germline and in somatic tissue of offspring. As discussed in Section 4, alcohol is known to affect methylation machinery, leading to severe health consequences, such as cancer (Varela-Rey et al., 2013). Particularly, alcohol has been shown to decrease activity of DNMT1 in somatic tissue (Ponomarev et al., 2012) and reduce cytosine methyltransferase mRNA in sperm (Bielawski, Zaher, Svinarich, & Abel, 2002). Alcohol is also known to reduce levels of the methyl donor SAM, leading to hypomethylation of somatic tissue (Lu & Mato, 2005). Alcohol's effects on these enzymes in the germline could potentially be a mechanism for the observed changes in methylation in sperm and for the epigenetic inheritance of these methylation changes. These mechanisms need to be investigated for future studies.

While these studies have shown evidence for alcohol-induced epigenetic inheritance by methylation changes along the male germline, it remains a likely possibility that other mechanisms exist, such as sperm-mediated epigenetic inheritance of histone modifications and ncRNAs. Alcohol is known to modify histones (Moonat & Pandey, 2012) and to change levels of several miRNAs (Miranda et al., 2010) in somatic cells, and it is possible these processes occur in germline cells as well and may play a role in epigenetic inheritance. Indeed, epigenetic inheritance via histone modifications and ncRNAs have been identified in other models (Ashe et al., 2012; Gaydos et al., 2014; Larriba & del Mazo, 2016; Yan, 2014). In *C. elegans*, the H3K27me mark can be transmitted via germ cells to subsequent generations (Gaydos et al., 2014). Also in *C. elegans*, Ashe and colleagues (2012) found that a piRNA-triggered mechanism could maintain epigenetic memory across many generations.

Similar mechanisms might be at play in epigenetic inheritance of alcohol-related pathologies also.

While many studies have focused on mechanisms of male germline-dependent inheritance of alcohol-related pathology, there are few studies to date that have investigated female germline-dependent epigenetic inheritance. Can alcohol exposure in females result in epimutations in oocytes that can be transmitted to future generations? Recent studies discussed in Section 6 showing that grandmaternal alcohol consumption caused behavioral and gene expression changes in the matrilinear F2 offspring suggest that some alcohol-induced functional changes may be carried over via the female germline (Tunc-Ozcan et al., 2016). Oocytes have been shown to be sensitive to the effects of alcohol (Cuthbertson 1983; Emanuele, Wezeman, & Emanuele, 2002). Also, as mentioned in the previous paragraph, histone marks in oocytes were found to be passed down to offspring in *C. elegans* (Gaydos et al., 2014), but whether this epigenetic inheritance occurs in mammals needs to be investigated. This question presents an open avenue for investigation, and is pertinent for understanding the epigenetic transmission of alcohol-related pathology.

8. Summary and Future Studies

In summary, animal and human studies demonstrate exposure to alcohol during early development and later in life leads to changes in the epigenome, including changes in DNA methylation, histone modifications, and ncRNAs, and these studies suggest these epigenetic changes play a role in the development of FASDs and AUDs. Future studies on the role of epigenetic processes in the development of alcohol-related pathology should build on these studies to determine precise mechanisms for the development of these pathologies and possible targets for treatment. In addition, animal and human studies show evidence that pathologies due to alcohol exposure can be inherited in offspring via epigenetic mechanisms. Multigenerational and transgenerational epigenetic inheritance of alcohol-related pathologies has been demonstrated in animal models, but the mechanisms for this inheritance are still under investigation. Animal studies have shown that a mechanism for epigenetic inheritance of alcohol-related pathologies occurs via methylation changes in imprinted and non-imprinted genes via the male germline, but more studies are needed to fully understand this mechanism. In addition, further studies are needed to assess the possibility of alternative germline mechanisms for multigenerational epigenetic inheritance, including the possibility of transference via histone modifications and ncRNAs, and inheritance via the female germline. Current animal studies are limited by the fact that most only go as far as the F1 generation to examine the effects of alcohol exposures on subsequent generations, and future studies might examine F2 and F3 generations for evidence of transgenerational epigenetic inheritance. Finally, studies on epigenetic inheritance of alcohol-related disorders in human populations are lacking, and future clinical studies could focus on descendants of persons with AUDs to better understand multigenerational and transgenerational inheritance of alcohol-related pathology. These future studies are crucial to a complete understanding of the phenomenon and mechanisms for epigenetic inheritance of alcohol-related health problems.

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Highlights

- Alcohol-related health problems are often passed down across generations in human populations.
- Recent studies in animal models have suggested that epigenetic factors are partially responsible for the transmission of alcohol-related pathologies across generations.
- This review summarizes evidence for multigenerational and transgenerational epigenetic inheritance of alcohol-related pathology through the germline.

Fig. 1.

Role of epigenetic processes in the development of FASD. *In vivo* and *in vitro* studies have shown evidence that early developmental exposure to alcohol alters DNA methylation (Bekdash et al., 2013; Chen et al., 2013; Downing et al., 2011; Garro et al., 1991; Govorko et al., 2012; Kim et al., 2013; Liu et al., 2009; Marutha Ravindran & Ticku, 2004; Qiang et al., 2010; Zhang et al., 2014) histone modifications (Bekdash et al., 2013; Guo et al., 2011; Veazey et al., 2013), and miRNAs (Marjonen et al., 2015; Sathyan et al., 2007; Wang et al., 2009). The genes targeted by these modifications are regulators of development, metabolism, neural maturation, learning and memory, motor coordination, and stress. These studies provide evidence that these epigenetic modifications are involved in mediating the development of FASD symptoms.

Alcohol Exposed Pregnant Female and Fetus

Fig. 2. Mechanisms for transgenerational inheritance of fetal alcohol-induced epigenetic marks on the *Pomc* gene and relation to stress axis disruption. Fetal alcohol exposure induces epigenetic modifications on the *Pomc* gene and hyper-stress response in male and female F1 offspring. These epigenetic changes and functional defects are carried on to the F2 and F3 generations via the male, but not female, germline (Govorko et al., 2012). Specifically, fetal alcohol exposure leads to increased repressive epigenetic marks on histones in *Pomc*-producing hypothalamic neurons including increased de-acetylation by HDACs. Fetal alcohol exposure also leads to increased levels of DNMTs and increased methylation of the *Pomc* promotor, leading to reduced hypothalamic expression of *Pomc*, reduced

hypothalamic production of the protein β -endorphin (β -EP), and increased stress response. Hypermethylation of the *Pomc* gene in the hypothalamus is accompanied by a similar hypermethylation of *Pomc* in sperm cells in the F1-F3 males.

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Table 1
Summary of the Epigenetic Effects of Alcohol in Adult Humans and Animal Models

Age	Model	ETOH BEC or Dose	Cell Type	Effects	Citation
Adult	Post-mortem human alcoholic, amygdala and frontal cortex	Not reported	Brain tissue	DNA hypomethylation and H3K4me3 enrichment at GC-rich regions and endogenous retroposons	Ponomarev et al., 2012
Adult	Post-mortem human alcoholic	Not Reported	Brain and buccal tissue	Differential methylation in more than 400 CpGs	Hagerty et al., 2016
Adult	Post-mortem human alcoholic	Not reported	Brain, frontal cortex	Differential methylation of prodynorphin gene SNPs in alcoholics	Taqi et al., 2011
Adult	Post-mortem human alcoholic	Not reported	Brain, frontal cortex	No differences in global methylation in alcoholics, differential methylation in specific promoters in alcoholics	Manzardo et al., 2012
Adult	Post-mortem human alcoholic	Not reported	Brain, cortex	Upregulation of 35 miRNAs in alcoholic brain	Lewohl et al., 2011
Adult	Post-mortem human alcoholic	Not reported	Brain, hippocampus	Overlapping H3K4me3 marks and gene expression changes between cocaine and alcohol users	Zhou et al., 2011
Adult	Human sperm	Not reported	Sperm	Decreased methylation in imprinted genes H19 and IG-DMR in men that consume alcohol	Ouko et al., 2009
Adult	Rat, acute and chronic ethanol exposure	Acute: 91 ± 3.2 mg% Chronic: 177 ± 11 mg%	Brain, amygdala	Acute alcohol: decrease in HDAC activity and increase in histone H3 and H4 acetylation; Chronic alcohol withdrawal: increase in HDAC activity and decrease in H3 and H4 acetylation	Pandey et al., 2008
Adult	Rat, Alcohol preferring and Non-preferring rats	$\sim 100 \pm 5$ mg%	Brain, amygdala	Acute alcohol decreased HDAC activity and HDAC2 protein, increased global and gene specific histone acetylation in amygdala	Moonat et al., 2013; Saktharkar et al., 2014
Adult and juvenile (30 days old)	Rat, intermittent for 2 weeks	Dose: 3 g/kg	Brain, prefrontal cortex	In juveniles, alcohol upregulates HAT activity and increases H3 and H4 acetylation and H3(K4) dimethylation in the promoters of cFos, Cdk5, and FosB genes	Pascual et al., 2012
Adult	Mice and rats	~ 100 mg%	Brain, nucleus accumbens	Increased DNMT1 protein and gene expression, decreased H4 acetylation	Warnault et al., 2013
Adult	Rat	Dose: 6 g/kg	Tissue from several organs	Increased H3K9 acetylation post- alcohol in liver, lung, spleen, testes, but no change in kidney, brain, heart, stomach, colorectum, pancreas, and vessels	Kim & Shukla, 2006
Adult	Rat	20 mM	Rat supratoptic nucleus and striatal neurons	Upregulation of miR-9	Pietrzykowski et al., 2008
Adult (F0 sires and F1 generation)	Rat, chronic alcohol effects on F0 and F1 generation	Dose: 1.1, 3.3 g/kg ETOH for 4 weeks	Sperm, cerebral cortex	Decreased methylation of H19 and Peg3 genes in F0 sperm, and decreased methylation of Peg3 genes in F1 cortex	Liang et al., 2014

Age	Model	ETOH BEC or Dose	Cell Type	Effects	Citation
Adult (F0 sires and F1 generation)	Mouse chronic alcohol	BEC: > 1.0 mg/mL	Sperm of F0 exposed males, somatic cells of F1 offspring	Decreased methylation at two H19 binding sites in F1 offspring; decreased methylation at one CpG of the H19 gene in sperm of F0 males	Knezovich & Ramsay, 2012
Adult (F0 sires and F1 generation)	Mouse chronic alcohol (5 weeks)	BEC: 147.1 ± 7.52 mg/dL	Sperm of F0 exposed males, brain of F1 offspring	Decreased methylation of BDNF promoter in F0 sperm and in VTA of F1 offspring	Finegersh & Homanics, 2014
Adult (F0 sires and F1 generation)	Mouse chronic alcohol (7 weeks)	0.5, 1, 2, 4 g/kg/day EtOH for 7 weeks	Sperm of F0 exposed males, brain of F1 offspring	Increased methylation of DAT in F0 sperm and in cortex and striatum of F1 offspring	Kim et al., 2014
Adult (F0 sires and F1 generation)	Rats chronic alcohol (9 weeks)	Dose: 6 g/kg 3 times/week BEC: 369.2 ± 30.0 mg/100 mL	Sperm of F0 exposed males	Decreased cytosine methyltransferase in sperm	Bielawski et al., 2002

Table 2

Summary of the Epigenetic Effects of Alcohol on Early Development

Age	Model	EtOH Dose or BEC	Cell Type	Effects	Citation
Fetal	Cultured rat fetal astrocytes	75 mM	Cultured astrocytes	Decreased DNMT activity, decreased methylation of tPA promoter, decreased DNMT3a protein	Zhang et al., 2014
Fetal	Cultured mouse fetal cortical neurons	75 mM, 5 days	Cultured neurons	Demethylation of NR2B gene	Marutha Ravindran & Ticku, 2004
Fetal	Cultured mouse fetal cortical neurons	75 mM, 5 days for chronic intermittent ethanol paradigm	Cultured neurons	Demethylation of NR2B gene	Qiang et al., 2010
Fetal	Mouse, cultured embryo	Dose: 88 mM	Cultured embryo	Altered methylation in many genes involved in metabolism, neural development, imprinting	Liu et al., 2009
Fetal	Mouse, cultured neurospheres	Dose: 13 mM, 26 mM, 70 mM	Cultured neurospheres	Altered activating and repressive histone marks (H3K4me3 and H3K27me3) on genes involved in neural precursor identity and neurogenesis	Veazey et al., 2013
Fetal	Cultured mouse fetal cortical neurospheres	13 mM or 70 mM EtOH	Cultured neurospheres	High dose suppressed miR-21, -335, -9, and -153; Low dose increased miR-335	Sathyan et al., 2007
Fetal	Mouse, Gestational day 9–11	Dose: 3 g/kg	Somatic embryo	DNA hypomethylation, lower methylase activity	Garro et al., 1991
Fetal	Mice, Gestational day 9	Dose: 5.8 g/kg EtOH	Somatic embryo	Subtle decrease in methylation of Igf2 locus with EtOH	Downing et al., 2011
Fetal	Mouse, Gestational day 6–15	BEC: 0.4 µg/mL EtOH	Brain	Upregulation of many miRNAs, including miR-10a, miR-10b, miR-9 with EtOH; Downregulation of many miRNAs, including miR-200a, miR-496, miR-296 with EtOH	Wang et al., 2009
Fetal	Mouse, Gestational Day 0.5–8.5	Dose: ~12 g/kg/day	Brain (hippocampus)	Altered miRNAs expression of miR138-2, miR290, miR16-2; Altered site specific methylation of several CpG islands	Marjonen et al., 2015
Fetal	Mouse and Rat, Gestational day 6–15	Dose: 0.5, 1, 2, 4 and 6 g/kg/day	Brain	Decreased MeCP2 in prefrontal cortex and striatum	Kim et al., 2013
Fetal	Rat, Gestational day 8–21	Dose: 5% w/v EtOH, 35% ethanol-derived calories	Brain (hippocampus and cortex)	Altered imprinting patterns and expression of Dio3	Sittig et al., 2011
Fetal	Mouse, Gestational day 7–16	BEC: 120–160 mg/dL	Brain (hippocampus)	Altered methylation program during development in hippocampus, decreased Mecp2 and Tet1	Chen et al., 2013

Age	Model	EtOH Dose or BEC	Cell Type	Effects	Citation
Fetal	Rats, Gestational day 7–21	Dose: 6.7% EtOH (v/v), BEC: ~110–120 mg/dL	Brain (hypothalamus) in Pomc cells	Increased MeCP2 protein and DNA binding to Pomc gene	Gangisetty et al., 2014
Neonatal	Rats, postnatal days 2–12	BEC: 30–80 mM	Brain (cerebellum)	Reduced CREB binding protein, reduced H3 and H4 acetylation with EtOH exposure	Guo et al., 2011
Fetal and preconception	Mouse; Fetal: gestational Day 0.5–8.5; Preconception: 4 days/week for 10 weeks	Dose: 10% EtOH (v/v), ~12 g/kg/day	Tail	Hypermethylation of the Agouti mutation (A ^{vy}) locus and transcriptional silencing of the gene	Kaminen-Ahola et al., 2010
Fetal (F1) exposure, F1–3 generations	Rats, Gestational day 7–21	Dose: 6.7% EtOH (v/v), BEC: ~110–120 mg/dL	Brain (hypothalamic neurons), sperm	Increased methylation of the Pomc gene promoter in hypothalamus and sperm cells in F1-F3 offspring	Govorko et al., 2012; Bekdash et al., 2013
Fetal (F1) exposure, F2 offspring	Mouse, Gestational day 10–18	Dose: 0.5 g/kg/day EtOH	Sperm, brain	Decrease in methylation of H19 gene in sperm of exposed F1 offspring, Decrease in methylation of H19 gene in brain of F2 offspring	Stouder et al., 2011
Human children	Human children with FASD and healthy control children	Not reported	Buccal epithelial cells	658 differentially methylated sites in FASD children compared to controls, overlapping with 95 different genes; increased methylation on genes relevant to neurodevelopment	Portales-Casamar et al., 2016