EPIGENETIC ALTERATION BY PRENATAL ALCOHOL EXPOSURE IN DEVELOPING MOUSE HIPPOCAMPUS AND CORTEX

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Fetal alcohol spectrum disorders (FASD) is the leading neurodevelopment deficit in children born to women who drink alcohol during pregnancy. The hippocampus and cortex are among brain regions vulnerable to alcohol-induced neurotoxicity, and are key regions underlying the cognitive impairment, learning and memory deficits shown in FASD individuals. Hippocampal and cortical neuronal differentiation and maturation are highly influenced by both intrinsic transcriptional signaling and extracellular cues. Epigenetic mechanisms, primarily DNA methylation and histone modifications, are hypothesized to be involved in regulating key neural development events, and are subject to alcohol exposure. Alcohol is shown to modify DNA methylation and histone modifications through altering methyl donor metabolisms. Recent studies in our laboratory have shown that alcohol disrupted genome-wide DNA methylation and delayed early embryonic development. However, how alcohol affects DNA methylation in fetal hippocampal and cortical development remains elusive, therefore, will be the theme of this study.

We reported that, in a dietary alcohol-intake model of FASD, prenatal alcohol exposure retarded the development of fetal hippocampus and cortex, accompanied by a

delayed cellular DNA methylation program. We identified a programed 5-methylcytosine (5mC) and 5-hydroxylmethylcytosine (5hmC) cellular and chromatic re-organization that was associated with neuronal differentiation and maturation spatiotemporally, and this process was hindered by prenatal alcohol exposure. Furthermore, we showed that alcohol disrupted locus-specific DNA methylation on neural specification genes and reduced neurogenic properties of neural stem cells, which might contribute to the aberration in neurogenesis of FASD individuals. The work of this dissertation suggested an important role of DNA methylation in neural development and elucidated a potential epigenetic mechanism in the alcohol teratogenesis.

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5-AZA	5-azacytidine
5'UTR	5' untranslated region
5CaC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ADH	Alcohol dehydrogenases
ALDH	Aldehyde dehydrogenases
BAC	Blood alcohol concentration
CA	Cornu ammonis
CNS	Central nervous system
CP	Cortical plate
DG	Dentate gyrus
DRG	Dorsal root ganglion
DMP	DNA methylation program
DNMTs	DNA methyltransferase
E	Embryonic day
ESCs	Embryonic stem cells
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorder
FLIM-FRET	Forster resonance energy transfer
HATs	Histone acetyltransferase
HDACs	Histone deacetylases
HMTs	Histone methyltransferase
HP1	Heterochromatin protein 1
HPA	Hypothalamic-pituitary-adrenal
IP	Intraperitoneal
LD	Liquid-diet
MBD1	Methyl-binding domain protein 1
MeCp2	Methyl-binding protein 2
ncRNA	Non-coding RNA
NE	Neuroepithelium
NPCs	Neural progenitor cells
NSCs	Neural stem cells
PCD	Programmed cell death
PcG	Polycomb group complex
PF	Pair-fed
PGCs	Primordial germ cells
PND	Postnatal day

RE	Repetitive elements
RNAi	RNA interference
SAMe	S-adenosylmethionine
SGZ	Subgranular zone
SP	Subplate
SVZ	Subventricular zone
TDG	Thymine-DNA glycosylase
TrxG	Trithorax group complex
TSSs	Transcriptional start sites
VZ	Ventricular zone

CHAPTER 1: INTRODUCTION

1.1 FASD clinical overview

Children born to women who drink alcohol during pregnancy suffer from various degrees of birth defects, including mental retardation, growth deficiencies, cranial-facial dysmorphologies and behavioral changes. These are categorized under a disease called Fetal Alcohol Syndrome (FAS). The term was first described in 1973 (Jones et al., 1973), where clinicians reported an association between maternal alcoholism and aberrant morphogenesis in the offspring. Till today, FAS remains the leading cause of non-genetic mental retardation in the western world, which occurs in 1-2 of 1000 newborns (May and Gossage, 2001, May et al., 2009). Clinical diagnosis of FAS is based on three main features (Stokowski, 2004, Chudley et al., 2005, Manning and Eugene Hoyme, 2007):

- Growth deficiency – growth retardation and reduced weight;
- Dysmorphic facial features – mid-facial hypoplasia, indistinct philtrum, thin upper lip, and short palpebral fissure;
- Central nervous system damages –structural, neurological, or functional impairments.

The degree of each of the key features of FAS can vary widely among individuals exposed to prenatal alcohol.

Approximately ten-times more children escape diagnosis owing to a lack of obvious dysmorphism (distinct facial dysmorphologies, growth retardation or behavior changes). These patients suffer from a slew of neurodevelopmental deficits and are now categorized into a broader class termed Fetal Alcohol Spectrum Disorders (FASD). Aside from primary abnormalities, some victims of fetal alcohol exposure do not present distinct phenotypes until later life when, typically, impaired cognitive plasticity and/or maladaptive behaviors emerge (Riley and McGee, 2005, Kodituwakku, 2007, Kully-Martens et al., 2012). On the other hand, FASD and related abnormalities have been identified in subjects without reported intrauterine alcohol exposure (Hegedus et al., 1984, Little and Sing, 1987).

Despite the knowledge on the adverse outcomes of maternal drinking, and despite

massive educational campaigns about the harms of alcohol consumption during pregnancy, the Centers for Disease Control and Prevention (CDC) reports that there are still greater than 50% of women in the United States of childbearing age consumed alcohol and that approximately 13% of these could be considered moderate or heavy drinkers (CDC, 2004). The estimated annual cost of FASD to the families and society reaches $1.4 million per individual and $6 billion per year in total in the United States (Lupton et al., 2004).

To date, no medications have been approved specifically to treat FASD. This is largely attributed to the lack of understanding of the mechanisms underlying complex FASD phenotypes. Alternatively, some of the children diagnosed with FASD can benefit from behavior and education therapy (O'Connor et al., 2006, Kable et al., 2007)

1.2 Maternal risk factors in FASD

Alcohol in the maternal bloodstream can cross the placenta to the fetus, and therefore exerts its teratogenic effects on the developing fetus. Due to wide varieties of factors that influence the likelihood of a fetus being affected by alcohol, it is still not certain how much prenatal alcohol exposure is necessary to cause FASD. In both human and animal studies, there were a great amount of variations in the traits or features of FASD produced by individual mothers, different species of laboratory animals, and different animal strains within a species (Streissguth et al., 1980, West and Goodlett, 1990, Thomas et al., 1996, Larkby and Day, 1997).

The genetic background of both the mother and fetus is one important factor that influences the effect of alcohol on the developing fetus. Once alcohol is absorbed, it is distributed through the mother's entire body. Alcohol then passes freely through the placenta from the mother's into the fetus' bloodstream. Alcohol metabolism in the mother, as well as other factors like nutrition, genetics and body weight, decide the rate of alcohol being eliminated in the system. For example, particular alleles for alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) allow for a faster alcohol metabolism, thereby reducing the risk of exposure to the fetus (McCarver et al., 1997,

Duester, 1998). Therefore, maternal genetic background is one important factor that contributes to the variation in FASD. At the same time, the fetal genetic background is another determinant of alcohol teratogenesis. For example, under embryonic culture conditions, in which maternal and intrauterine factors were eliminated, we showed that the C57BL/6N strain of mice displayed higher level of apoptosis and were severely growth-retarded after alcohol exposure, while the 129S6 strain was remarkably resistant to alcohol-induced growth deficits (Chen et al., 2011b).

Variation in maternal drinking pattern, that is, quantity, frequency pattern and duration of drinking during pregnancy is another key factor for the differential alcohol outcomes in fetus. Populations that consume alcohol in a binge pattern (five or more drinks in the span of 2 hours) are correlated with a greater incidence of FASD (Brady et al., 2013, May et al., 2013).

Another key factor is the developmental stage of the embryo or fetus at the time of alcohol exposure. For example, alcohol exposure during a relatively narrow temporal window on the seventh day of pregnancy in mice (first trimester equivalent in human) can induce craniofacial dysmorphology that models the craniofacial phenotype of FAS (Sulik et al., 1986; Sulik, 2005). Alcohol exposure during later stage causes significant behavioral and learning deficits, and these outcomes are improved if alcohol consumption is stopped or reduced during the first or second trimester (Berman and Hannigan, 2000, May et al., 2013).

Moreover, environmental factors related to nutrition and maternal care are also important risk modifiers in FASD (May and Gossage, 2011). Many mothers who drink alcohol during pregnancy also have poor nutrition and vitamin intake (Patten et al., 2013). Maternal nutrition interacts with alcohol metabolism. On one hand, alcohol affects how the placenta transfers important nutrients for fetal growth. For example, alcohol inhibits folate absorption (Wani et al., 2011), uptake (Biswas et al., 2012) and transportation to the fetus (Hutson et al., 2012). On the other hand, nutrients affect the rate of alcohol absorption and metabolism (Shankar et al., 2007). For example, several studies show that pregnant undernourished rats have impaired alcohol metabolism by

decreasing alcohol dehydrogenase 1 (ADH1) liver levels (He et al., 2002, He et al., 2004, Shankar et al., 2006).

Although animal studies with controlled animal genetic background and drinking pattern have elucidated potential risk factors underlying the alcohol-induced fetal damages, much work remains. More detailed and accurate human studies are needed to define what drinking patterns in pregnant women from specific population lead to FASD. Although alcohol affects different people in different ways, in general the appropriate message to deliver to the public is that women should avoid any alcohol drinking during pregnancy.

1.3 Outstanding Questions

The central nervous system (CNS) deficit is one of the main phenotypes shown in FASD individuals, including low IQ, learning deficit and behavior and emotional problems (Streissguth et al., 1989, Streissguth et al., 1990, Kodituwakku, 2007). The prenatal alcohol damage to the fetal brain structure and function is irreversible and can last till later age, affecting many aspects of life (Sowell et al., 2002a, Duquette et al., 2006, May et al., 2009). There is no therapeutic medicine for FASD so far. Understanding the mechanisms underlying alcohol' s teratogenic effects on brain development would help the development of potential therapeutic targets and protective therapies for FASD.

To date, investigations aimed at identifying mechanisms of alcohol-induced damage to the developing nervous system focused on cellular processes that regulate embryonic morphogenesis, for example, alcohol-induced cell death, oxidative stress, regulation of cell proliferation and survival, secretion of growth factors and production of neurotransmitters. However, progresses towards each pathway have been encumbered by lack of fundamental understanding of the common causations. This is possibly due to the complexity of FASD. First of all, FASD is probably not a single-gene disorder. We and

others have shown that prenatal alcohol induced changes in expression of groups of genes in the fetus, including homeobox genes, metabolic, cell programming and signaling, neurogenesis and synaptic genes (Hard et al., 2005, Toso et al., 2005, Toso et al., 2006, Green et al., 2007, Zhou et al., 2011c, Kleiber et al., 2012). Although some knockout studies have shown mutation in key neural developmental gene increased alcohol consumption (Anstee et al., 2013) or alcohol susceptibility in mutant animals (Ahlgren et al., 2002, Kietzman et al., 2014), no comprehensive causation can be drawn from these studies. Moreover, beside genetic factors, non-genetic factors, such as maternal nutritional status and stress have been shown to largely influence the alcohol teratology as well (Shankar et al., 2006, Shankar et al., 2007, Hellemans et al., 2008, Brocardo et al., 2011). These evidence led to the thought that the likely molecular mechanism, instead of acting on single pathways or genes, must be acting systematically to regulate different developmental processes during neural development.

Cellular differentiation from genetically identical cells (same DNA sequence) to functional diverse cells (different cell types) is a result of differences in gene activation and repression arising during development. Epigenetic mechanisms have the capacity of regulating gene expression without changes to the DNA sequence and is shown to regulate key developmental processes, including germ cell imprinting, stem cell maintenance and differentiation and plays a crucial role in early embryogenesis (Reik et al., 2001). Epigenetics is also a fundamental aspect of programmed fetal development, determining cell fate, pattern formation, terminal differentiation and maintenance of cellular memory in the central nervous system (Feng et al., 2007). Perturbation of epigenetic machinery is known to be associated with several neurodevelopmental and neurodegenerative diseases, for example, Rett syndrome (Amir et al., 1999, Meloni et al., 2000, Mellen et al., 2012) and schizophrenia (Zahir and Brown, 2011). Alcohol affects epigenetic mechanism through disrupting methyl-donor metabolism, thereby altering the key substrates needed to methylate DNA and histones (Halsted et al., 2002, Kruman and Fowler, 2014). Therefore, we and others hypothesize that epigenetic factors are among the important mechanisms of ethanol teratogenesis in the central nervous system.

Epigenetics regulates gene transcription by modifying 3D conformation and accessibility of DNA. For instance, methylated cytosines accumulated at densely packed X-chromosome mediate the process of X-chromosome inactivation (Chow et al., 2005). Recently, our laboratory demonstrated that alcohol altered the global and gene-specific DNA methylation in neural stem cells, and inhibited neural differentiation (Zhou et al., 2011a, Zhou et al., 2011c). The alcohol-induced phenotypes were similar to the effect of DNA methylation inhibitor, 5-azacytidine (5-AZA) (Zhou et al., 2011a). Alcohol altered the DNA methylation program during neural tube development, and correlated with dysregulation of development-related genes (Zhou et al., 2011b). Others have shown that supplement of methyl-donor at the time of alcohol exposure could mitigate the adverse effects of prenatal alcohol exposure on development in rats (Thomas et al., 2009). However, much remains illusive of how alcohol alters epigenetic machinery in the developing brain, and whether the altered epigenetics are responsible for the CNS malformation shown in FASD individuals, therefore, will be the theme of this study.

1.4 Prenatal alcohol exposure on hippocampus and cortex development

1.4.1 Effect of prenatal alcohol on CNS structure and function in FASD children

Studies using magnetic resonance imaging (MRI) have demonstrated alteration in many regions of the CNS of children with FASD. In general, FAS and FASD individuals revealed reduced overall brain size and disproportionate reductions in the size of specific brain structures (Mattson et al., 2001, Autti-Ramo et al., 2002, Treit et al., 2013).

One important brain region involved in FASD phenotypes (impaired cognitive functions and learning abilities) is the cerebral cortex. Individuals with FAS and FASD had significantly reduced white matter volume and density in the parietal and orbito-frontal cortex regions (Sowell et al., 2002a, Sowell et al., 2002b, Nardelli et al., 2011), reduced cortical thickness (Yang et al., 2012b) and cortical folding complexity (De Guio et al., 2013). The grey matter is also affected, but to a lesser degree (Sowell et al., 2002b). The frontal and parietal lobe abnormalities are consistent with executive functioning

deficits shown in FASD individuals such as poor planning, cognitive inflexibility, language processing and spatial memory problems (Rasmussen, 2005, Kable et al., 2007).

Another important brain region is the hippocampus. Children exposed to alcohol prenatally display reduced hippocampus volume and pronounced asymmetric hemisphere (right >left) (Spadoni et al., 2007, Willoughby et al., 2008). FASD individual performed poorly on tests involving visual-spatial and verbal memory and learning, and also had significant difficulties in recalling spatial locations and completing mazes (Kable et al., 2007, Willoughby et al., 2008). This indicates that prenatal ethanol exposure may lead to long-term impairments in hippocampal growth that could account for the deficits in various learning and memory functions.

Other alcohol-sensitive brain regions include the basal ganglia (Spadoni et al., 2007, Norman et al., 2009), the cerebellum (Bookstein et al., 2006), and the corpus callosum (Bookstein et al., 2002, Yang et al., 2012a). These regional damages shown in FASD children impair functions including spatial memory, cognitive processes, balance, coordination, attention and verbal learning abilities (Riley and McGee, 2005, Kodituwakku, 2007, Kully-Martens et al., 2012). The teratogenic consequences of alcohol on brain structure and function are not only shown in infant and childhood, but could also persist into adolescents and into adulthood (Streissguth et al., 1989, Hellemans et al., 2008).

1.4.2 Effect of prenatal alcohol on hippocampus and cortex development in animal models

Animal models with precisely controlled genetic background and alcohol administration provide tools to better understand the causal effect of alcohol exposure on specific cellular processes during brain development.

Animal studies using different alcohol paradigms revealed similar brain regional damages compared to human FASD phenotypes. In general, prenatal alcohol exposure

results in neuronal cell loss, altered neuronal morphology in both hippocampus and cortex and impairs cognitive and learning performance (Berman and Hannigan, 2000, Mattson et al., 2001). In the hippocampus, prenatal alcohol exposure in rodents (first and second trimester equivalent) significantly reduced the pyramidal cell numbers within CA1 and CA3 regions and granular cells (Barnes and Walker, 1981). Similar dose of 3rd trimester alcohol exposure resulted in reduction of dentate gyrus granule cell number, without significant changes to the pyramidal neuron population (Miller, 1993, Miller and Freedman, 1995, Miller, 1995). Prenatal alcohol exposure can also cause abnormal branching of mossy fibers and dendritic arborization of hippocampal pyramidal cells (West et al., 1981). Evidence also showed alcohol-induced changes in the neurochemical and electrophysiological properties of neurons. For example, prenatal alcohol exposure alters specific serotonin receptor subtypes distribution and expression (Zhou et al., 2001, Sari and Zhou, 2004, Zhou et al., 2005), reduces glutamate binding and receptors (Farr et al., 1988, Savage et al., 1991, Olney et al., 2002) and changes the expression of muscarinic cholinergic receptors (Costa and Guizzetti, 1999, Monk et al., 2012). Reduced synaptic plasticity has also been reported in rats prenatally exposed to alcohol, specifically, in the pyramidal cell and dentate granule cells (Zhou et al., 2012, Brady et al., 2013, Patten et al., 2013). Deficits in spatial learning and memory tasks, as well as behavioral alterations in rodents prenatally exposed to alcohol are consistent with the effects of hippocampal damages (Berman and Hannigan, 2000, Mooney and Varlinskaya, 2011a, Schneider et al., 2011).

In the cortex, prenatal alcohol exposure decreases the rate and distance of cortical neuronal migration (Miller, 1993), induces ectopia location of pyramidal neurons and reduced cellular density in cortical plate. Prenatal alcohol exposure delays the development of prefrontal cortex and cortical barrel field in neonatal rats (Medina et al., 2003, Margret et al., 2006) and decreases dendritic spines and synaptic plasticity in rodent prefrontal cortex (Cui et al., 2010, Kroener et al., 2012). Similarly, the animals exposed to prenatal alcohol showed degrees of social behavioral changes (Mooney and Varlinskaya, 2010, 2011b).

1.5 Mechanisms underlying alcohol-induced hippocampal and cortical malformation

Alcohol is considered a "dirty drug" to the central nervous system. There are many mechanisms reported so far for alcohol teratogenesis in the developing brain. The teratogenic effect of alcohol is integrated by both genetic and non-genetic factors (Ramsay, 2010). Maternal genetic variations in alcohol-metabolizing enzymes, for example, alcohol dehydrogenases (ADH), aldehyde dehydrogenases (ALDH) and cytochrome P450 2E1 (CYP2E1), can influence the susceptibility to alcohol-induced fetal growth deficits and teratogenesis. Fetal genotype has also been implicated in the risk for FASD in a twin study (Streissguth and Dehaene, 1993), in that monozygotic (MZ) twins were fully concordant for diagnosis, whereas in heterozygotic twins the concordance for diagnosis was seven out of eleven.

Alcohol affects many cellular processes critical for proper brain development. These processes include neurogenesis, neuronal migration, synaptogenesis, cell proliferation and survival (Goodlett et al., 2005). Potential mechanisms for alcohol's teratogenic effect include oxidative stress (Brocardo et al., 2011), reduced signaling by transcription factors (Ron and Messing, 2013), retinoic acid or growth factors (Deltour et al., 1996), disrupted cell-cell interactions (Ramanathan et al., 1996, Arevalo et al., 2008), impaired cell proliferation and apoptosis (Sulik, 2005). Disruption in the fetal hypothalamic-pituitary-adrenal (HPA) axis (which regulates the response of the body to acute and chronic stress) as a result of in utero alcohol exposure has also been reported (Gabriel et al., 1998, Weinberg et al., 2008). Several of these mechanisms may have direct roles in causing the cell death and growth retardation in the brain.

Non-genetic factors such as maternal nutrition, stress and substance of abuse, also influences the incidence of FASD through mechanism that is not fully understood. Possible mechanisms are raised that linking alcohol to alterations in one-carbon metabolism which is a target of alcohol, as well as epigenetic machineries that influence gene expression (explained in detail in the following text).

1.5.1 Neurogenesis in hippocampus and cortex

A major phenotype of FASD is the overall growth retardation of hippocampus and cortex, in particular, reduced size and volume of these structures. Both of these structures have highly organized cell types and cell layers, patterned neuronal migrations and axonal growth during development that once damaged, are hard to recover again. Hippocampus and cortex have strong neurogenesis capacities at embryonic stages, giving rise to the tremendous amount of neurons and glia cells acquired for higher level brain functions. This capacity diminishes as brain become mature. In adults, the neurogenesis becomes confined to two regions, the subventricular zone (SVZ) of cortex and subgranular zone (SGZ) of hippocampal dentate gyrus (DG) (Kintner, 2002).

The significant reduction of brain volume and brain size shown in FASD could be a result of massive neuronal loss, for example, programmed cell death (PCD), or alternatively, diminished ability in neurogenesis. The alcohol-induced cell death in cranial-face, hippocampus and cortex has been reported for a long time (Kotch and Sulik, 1992, Holownia et al., 1997, Smith, 1997, Ikonomidou et al., 2000, Chen et al., 2011b) (also see review (Goodlett and Horn, 2001)). At the same time, numerous studies have reported mechanisms of alcohol-induced alteration in neurogenesis (Crews and Nixon, 2003, Nixon et al., 2010).

Neurogenesis refers to the process that various types of neurons and glia being generated from neural stem cells (NSCs) and progenitor cells. Neural progenitor cells (NPCs) generate the various neuronal and glial subtypes in a precise order. NPCs first sequentially produce various types of neurons in the neurogenic phase and then produce astrocytes in the astrogenic phase. When NPCs produce a neuron, the cell first undergoes neuronal fate commitment and later neuronal migration, then acquires its unique features, such as synapses (Kintner, 2002, Gotz and Huttner, 2005).

NPCs in the neocortex first appear around embryonic day (E) 9.5–E10.5 in mouse. Peak of neurogenesis in cortex happens at E12.5-E14.5 and last till E18.5 right before birth in mice (Kintner, 2002, Tan and Shi, 2013). After birth, neurogenesis becomes confined to SVZ of cortex. In hippocampus, the neurogenesis begins at E15.5, peak from E17 to first postnatal week in mouse. Neurogenesis begins first in Cornu Ammonis (CA) neuroepithelium, then in dentate gyrus epithelium (Kintner, 2002, Tan and Shi, 2013). By the end of first postnatal week, most of the neural progenitor cells become confined to subgranular layer, and this stem cell pool will persist till adulthood (Gotz and Huttner, 2005).

Neurogenesis of neocortex begins in the ventricular zone (VZ), where neural progenitors cells proliferate to give rise to both neuronal precursor and glial precursors. Upon exiting the cell cycle, different precursor cells differentiate into glia or neurons accordingly. This is regulated by combination of intrinsic transcript factor expression and extracellular neurotropic signaling (see review (Tan and Shi, 2013)). The mammalian cortex has a laminar structure, and the development of the six layers of major projection neurons proceeds in an "inside-out" manner —neurons of the deepest layer are born first, and the more superficial neurons are born later in a sequential fashion, with the outer-most neurons born last (Bayer et al., 1991).

Similarly, the hippocampus pyramidal cells migrate from Cornu Ammonis (CA) epithelium to the CA1 and CA3 in an "inside-out" manner – innermost layer (VI) is formed the earliest, outer most layer (I) is formed the last. However, the hippocampal dentate gyrus granule cell migration is "outside-in" order – the outer most layer is formed earliest and composed of mature neurons; the inner most layer is composed of the youngest, differentiating progenitor cells (Altman and Bayer, 1990, Sampson et al., 1994, Grove and Tole, 1999) (Summarized in Figure 1).

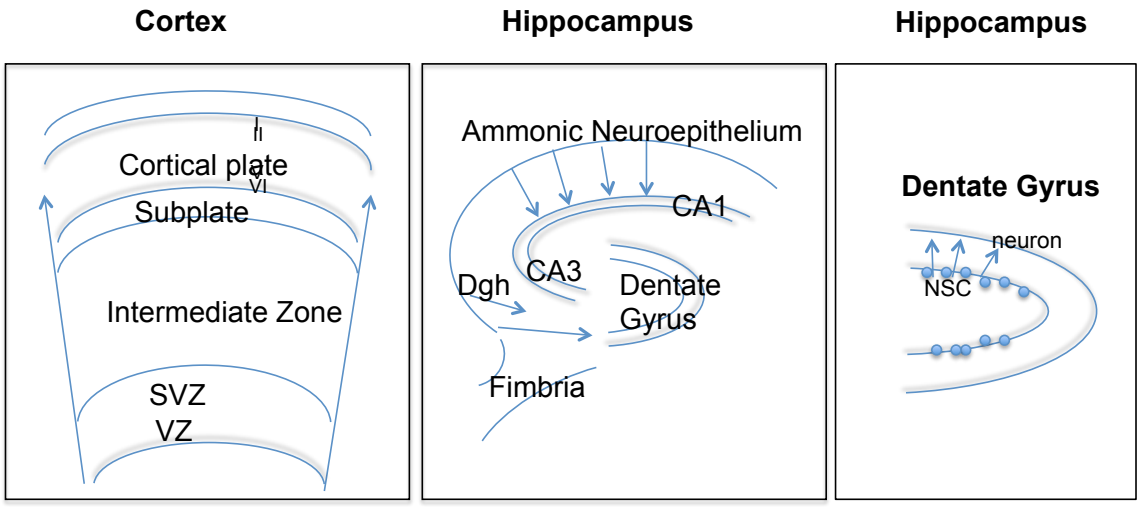

Figure 1. Schematic diagram of neuronal migration in cortex and hippocampus.

Arrows point to the direction of neuronal migration. NPCs/NSCs are generated in SVZ of cortex, NE of CA regions and SGZ of dentate gyrus. The six layers in the neocortex and hippocampus are formed in an "inside-out" manner – innermost layer (VI) is formed the earliest, outer most layer (I) is formed the last. Layers within dentate gyrus of hippocampus are formed in an "outside-in" manner –the outer most layer is formed earliest and composed of mature neurons; the inner most layer is composed of the youngest, differentiating progenitor cells. NPCs: neural progenitor cells; NSCs: neural stem cells; SVZ: subventricular zone; VZ: ventricular zone; SGZ: subgranular zone; NE: neuroepithelial layer; CA: Cornu ammonis.

1.5.2 Effect of alcohol on neurogenesis in hippocampus and cortex

Alcohol affects both embryonic neurogenesis and adult neurogenesis. Fetal exposure to alcohol decreases the neurogenesis in developing hippocampus and cortex (Gil-Mohapel et al., 2010); chronic alcohol drinking in adults or adolescents also affects the neurogenesis niches in hippocampus (Morris et al., 2010, Nixon et al., 2010, Anderson et al., 2012). Here we will focus on the effect of alcohol on peri-natal neurogenesis.

Prenatal alcohol exposure at differential development window has varied effects on embryonic neurogenesis. Specific cell types might be more sensitive at certain stages or even on certain days to alcohol exposure. Evidence on the effect of alcohol on neurogenesis can be categorized under four neuronal differential stages: 1) Neural stem cell/progenitor cell proliferation; 2) Neural specification; 3) Neuronal migration; 4) Synaptogenesis (reviewed below).

Alcohol exposure between early brain development stage in rat (Gestational day GD5 and GD11, first trimester equivalent in human) have prominent damages to the neural crest cells and neural progenitor cells, and induce significant apoptosis in the neural tube midline. The damages cause major neural tube defects and lead to facial dysmorphologies similar to the ones observed in children affected with FAS (Kotch and Sulik, 1992, Sulik, 2005).

Alcohol exposure at a later stage (GD11- GD21 in rats, second trimester equivalent in human) mostly affects cell differentiation, cell proliferation and neuronal migration in neocortex, hippocampus and main sensory nucleus (Miller, 1993, 1995). For example, in a rat model fed with alcohol containing diet from GD6 and GD21, the neocortical neuronal migration was delayed by ~2days, and neurons terminated their migration in ectopic locations (Miller, 1993). Moreover, even within a certain exposure window, the affected regions could be different. For example, in gestational day GD15 rats, neocortical cell differentiation peaks, but dentate gyrus neurogenesis has not yet

started. So the alcohol teratogenic effects may vary depending on the difference in development window in each brain region.

The last critical period of development (equivalent to 3rd trimester in human) occurs from GD18 to postnatal day (PND) 9 in rat, and from postnatal day (P) 1 to P7 in mouse. Collective studies in both rat and mouse indicated that during this period there was a marked increase in brain weight due in part to the proliferation of astroglial and oligodendroglial cells, as well as synaptogenesis and dendritic arborization (Kintner, 2002). Alcohol exposure at this time induced severe neuronal loss (Bonthius and West, 1990), dysregulation of glial generation (Holownia et al., 1997, Perez-Torrero et al., 1997), alterations in dendritic arborization and synaptogenesis (Cui et al., 2010, Zhou et al., 2012) and delayed myelination (Jacobson et al., 1979). Thus, peri-natal alcohol exposure can affect different brain areas depending on the proliferation, differentiation and migration pattern and neuronal types.

The mechanism underlying alcohol-induced deficits in neurogenesis is still not fully understood. Proposed mechanisms include disruptions in cellular signaling, interaction with extracellular cues and gene regulation (reference see below). At differential stages of neurogenesis, activation or repression of groups of intrinsic transcription factors are required at particular times and in particular cell types. These transcription factors include stem cell maintenance and proliferation genes, cell cycle genes, neural specification genes, neural patterning genes and synaptic-related genes. Besides the intrinsic cellular mechanism, extracellular cues, for example, growth factors and neurotrophic factors, are also critical in signaling the activation of transcription factors. It has been shown that prenatal alcohol reduces the level of growth factors (Resnicoff et al., 1994, Goodlett and Horn, 2001). Inhibition of such factors is likely to result in reduced cellular proliferation (Resnicoff et al., 1993, Srivastava et al., 2011). Alcohol also disrupts expression of critical transcription factor for morphogenesis including *Pax6, Tbx3* and *Notch* signaling. Moreover, prenatal alcohol exposure disrupts fetal genes that are critical for neural development, including proneural genes (*Neurogenin 1,2, Sox5, Bhlhe22*) (Zhou et al., 2011c), neural developmental genes

(*Notch, Dlx1, Wnt3a*) and synaptic-related genes (*Cannabinoid receptor 1 (Cnr1*), *Glutamate receptor (Grin2b*) and *Serotonin receptor 5A (Htr5a))* (El Shawa et al., 2013, Kleiber et al., 2013).

1.5.3 Role of neural specification genes in neurogenesis

Alcohol induces gene expression changes in a variety of biological processes in the developing nervous system. The sets of genes that are affected depend on the developmental timing at which alcohol is exposed (Kleiber et al., 2013). First trimester alcohol exposure induces gene expression changes in cell proliferation related-genes, while second trimester alcohol exposure affects cell migration and differentiation genes, and third trimester for cellular communication and neurotransmission genes (Kleiber et al., 2013).

Alcohol-induced cortical and hippocampal dysmorphologies are readily observable when exposed at early prenatal ages. Mice exposed to alcohol at E7 or E8 exhibit forebrain deficiencies including hypoplasia or aplasia and deficiencies in hippocampus (Sulik, 2005). Prenatal exposure of primates to alcohol during this period reduces the number of neurons in somatosensory-motor cortex and inhibits cortical neural stem cell proliferation (Miller, 2007). It is likely that disruption of early differentiation genes (cell proliferation and cell differentiation related genes) by alcohol is involved in the developmental malformation.

Genes of particular interest are a group of neural specification genes, including *Ascl1, Neurogenin 1 and 2*, and *Math1,2,3*. Activation of these genes in neural progenitor cells induces the neuronal specification and differentiation (Bertrand et al., 2002). Mutation in these genes leads neural progenitor cells to a non-neuronal fate (Skeath and Doe, 1996, Libert et al., 2008). Reduced neural specification and differentiation could produce defects in specific neural structures.

Prenatal alcohol has been shown induce imbalance between neurons and glia in cortex and hippocampus (Guerri et al., 2001, Miguel-Hidalgo et al., 2002, Uban et al., 2010). For example, alcohol reduces neuronal population, while triggers increase in gliogenesis in the adult motor cortex (Helfer et al., 2009). Considering neural specification genes' role in neuronal fate determination, these phenotypes might underlie dysregulation of neural specification genes in these regions. It has been reported that alcohol reduces *Neurogenin 1* expression in brain regions of zebrafish (unpublished data). We have shown previously that acute alcohol at neurulation stage altered gene expression of *Neurogenin 1* and *Neurogenin 2* (Zhou et al., 2011c). So far, no studies have looked into effect of alcohol exposure on expression of *Ascl1* in nervous system.

Ascl1 (Achaete-scute homolog 1, also known as Mash1) encodes a member of the basic helix-loop-helix (BHLH) family proteins. The protein activates transcription by binding to the E box (5'-CANNTG-3'). *Ascl1* is expressed during embryonic stages in ventricular zone of cortex and neuroepithelial layer in hippocampus (Battiste et al., 2007, Kim et al., 2008). Transcription of *Ascl1* is inhibited by *Hes1* (Libert et al., 2008). *Ascl1* is also regulated by notch signaling pathways (Libert et al., 2008).

Ascl1 plays a role in the neuronal commitment and differentiation (Pattyn et al., 2006, Kim et al., 2008). For example, in the chick neural tube, overexpression of *Ascl1* induced cells to stop cycling, exited earlier from ventricular zone (Nakada et al., 2004, Helms et al., 2005). Loss of *Ascl1* function resulted in loss or decrease of specific interneuron populations in the mouse spinal cord (Helms et al., 2005). In addition to these specific changes, there was a more general defect as cells stalled in the ventricular zone and different aspects of neuronal differentiation became uncoordinated (Casarosa et al., 1999, Horton et al., 1999). These phenotypes are similar to the alcohol-induced dysregulation of neurogenesis in cortex and hippocampus. Thus, it is likely that alteration in *Ascl1* expression is involved in the alcohol's teratogenic effect on developing nervous system.

1.6 Ethanol Teratogenesis and Epigenetics

1.6.1 Epigenetics and gene regulation

Many environmental factors are known to significantly alter the intrauterine environment and contribute to the dysregulation of the fetal growth processes. How these environmental challenges are recorded in active dividing cells and are translated to affect cellular and molecular processes during fetal development and in later life are not fully understood. Epigenetics offers an environmentally responsive plasticity in line with the fluctuant nature of transcriptional programs during cellular differentiation.

Epigenetics are heritable, and yet reversible, changes to the functionally diverse cells of a multicellular organism, which govern the compaction and folding of chromatin structure mainly by covalent modification on DNA and histones. Epigenetic mechanisms regulate gene expression at the level of transcription by modulating the accessibility of genes to the transcriptional machinery (Reik, 2007).

Epigenetic modifications include DNA methylation, histone modifications and microRNAs. There is a large interplay between DNA methylation, histone modification and a variety of chromatin modulating complexes. They together regulate chromatin structure, folding, and thus, transcription factor accessibility (Cedar and Bergman, 2009).

The covalent modifications on DNA and histones are produced by a specific group of enzymes, including DNA methyltransferase (DNMTs), histone methyltransferase (HMTs), histone acetyltransferase (HATs) and deacetylases (HDACs). These enzymes harbor a methylated-DNA or –histone binding domain, as well as a catalytic domain (Cheng, 1995, Zhang and Reinberg, 2001). Another group of proteins also have the capability to recognize DNA modification. They are the methyl-binding domain proteins (MBDs). The MBDs are capable of further recruiting co-repressor complexes that alter chromatin structure, thereby repressing transcription (Ballestar and Wolffe, 2001, Gu et al., 2011).

Epigenetics displays integration with the surrounding environment. One of the environmental factors is nutrition. Proper nutrient uptake and metabolism guarantees the

activities of enzymes involved in epigenetic modifications. For example, DNMTs, HATs and HDACs are regulated, in part, by the concentrations of their required substrates and cofactors, many of which are produced by nutrient metabolism (Lee and Workman, 2007). On the other hand, epigenetics changes can influence the expression of metabolism enzymes. Thus, the nutritional status is tightly integrated in the epigenome and transcriptional regulation. One good example of this integration is showed in the study utilizing *Agouti* mice (Cooney et al., 2002). The *Agouti* viable yellow (A^{vy}) mouse mutant contains a methylation-sensitive element within the A^{vy} locus, dictating coat color dynamics. In this way, coat color, ranging from yellow (unmethylated) to pseudoagouti (highly methylated), could be used as an indicator of methylation status (Duhl et al., 1994, Wolff et al., 1998). High methyl-donor maternal dietary supplement increases DNA methylation on the A^{vy}/a locus, which represses the gene expression and renders changes in the mouse coat color (Dolinoy, 2008).

Stress-epigenetic interplay has also been reported. It is shown that high levels of maternal care (measured by the extent of licking and grooming behavior) alters promoter methylation on glucocorticoid receptor gene in the offspring, as well as expression of the gene and the HPA response to stress (Weaver et al., 2005). Epigenetic interplay with stress not only happens in developing fetus but also in adult post-mitotic hippocampal neurons (Hunter et al., 2009).

Epigenetic mechanism regulates neural gene expression and plays an important role in the process of neurogenesis (Covic et al., 2010, Ma et al., 2010, Mateus-Pinheiro et al., 2011, Sun et al., 2011). Epigenetics regulates neural-developmental gene expression in the developing hippocampus and cerebellum (Feng et al., 2007, Szulwach et al., 2011), as well as expression of synapses related gene (Khare et al., 2012). Disruption of epigenetic modifiers leads to neural developmental disease, such as Rett syndrome, hyperactivity and psychiatric disorders in children (Portela and Esteller, 2010, Zahir and Brown, 2011).

1.6.2 Alcohol as a modifier of epigenetics

Alcohol can directly influence epigenetic marks through alterations of the one-carbon metabolic pathway (Figure 2). The synthesis of methionine allows the production of an active methyl donor, S-adenosylmethionine (SAMe), which is recruited by DNMTs for the transfer of a methyl group to the nucleotide cytosine. Dietary nutrients (methionine, folate and choline) all play substantial roles in the production of SAMe. Alcohol has been shown to affect each in an inter-dependent manner (Niculescu and Zeisel, 2002, Kruman and Fowler, 2014), allowing a biochemical pathway for alcohol to regulate DNA methylation. In return, DNA methylation can influence histone modifications and non-coding RNA (ncRNA), all of which are capable of independently altering genomic expression (Cedar and Bergman, 2009).

Alcohol depletes the methyl donor in the maternal and fetal system through interfering with nutrient uptake, metabolism and utilization (see review (Zakhari, 2013)). Besides interfering with the epigenetic substrates, alcohol also directly acts on the epigenetic enzymes (protein expression and activity) such as DNMTs and HDACs (see review (Zakhari, 2013)).

Alcohol's alteration of DNA methylation was exemplified by studies employing the agouti viable yellow (A^{vy}) mouse mutant as well. Prenatal exposure to alcohol increased the incidence of pseudoagouti animals, indicating that the biosensor was highly methylated and ectopic expression was silenced (Kaminen-Ahola et al., 2010). This is the first mechanistic evidence that alcohol induces phenotypic changes through epigenetic mechanisms.

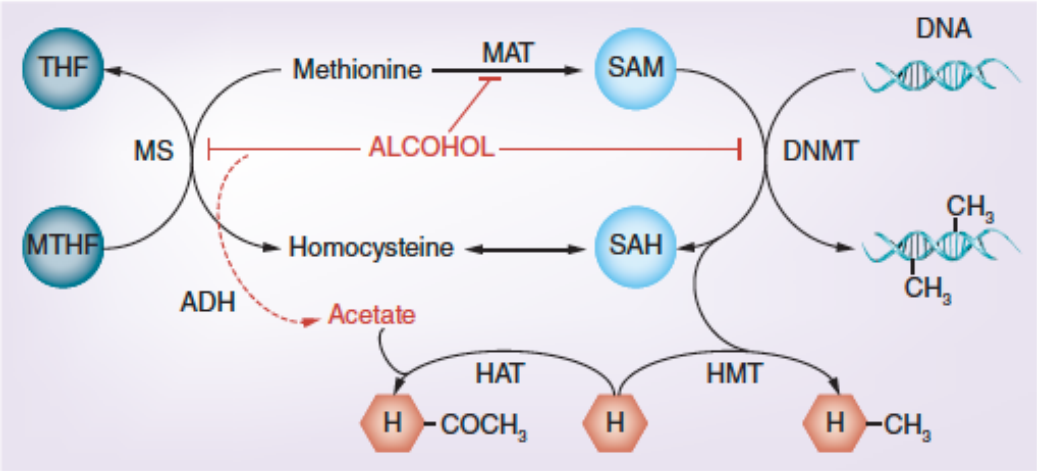

Figure 2. Alcohol and methyl metabolism.

DNA and histone methylation occurs mainly through the transfer of a methyl group to the substrate. SAM actively carries the methyl donor group. SAM is synthesized from methionine, which itself can be produced from the dietary intake of folate, choline and other methyl donors (only folate is shown here for simplicity). Alcohol can affect the methionine synthesis process by inhibiting metabolic enzymes, MAT and DNMTs, both through direct and indirect processes. This consequently induces a decrease in SAM production and hyperhomocysteinemia. In addition, acetate, a by-product of alcohol metabolism (indicated by a dashed arrow), is involved in the acetylation of histones.

DNMT: DNA methyl transferase; H: Histone; HAT: Histone acetyltransferase; HMT: Histone methyltransferase; MTHF: Methyltetrahydrofolate; SAH: S-adenosylhomocysteine; SAM: *S*-denosylmethionine; THF: Tetrahydrofolate.

(Figure published in (Resendiz et al., 2013))

It is now known that alcohol's effects on DNA methylation can be site-specific and bi-directionally, either increase or decrease DNA methylation. For example, alcohol decreases DNA methylation locus-specifically at imprinting control sites of the *H19* gene (Haycock and Ramsay, 2009, Ouko et al., 2009, Doshi et al., 2013) and the promoter of *POMC* gene (Govorko et al., 2012), as well as of the promoter of *Bdnf* gene (Moonat and Pandey, 2012). Alcohol altered DNA methylation bi-directionally on gene promoters in developing mouse embryos in a CpG content-associated way, rendering both hyper-methylated and hypo-methylated promoters (Liu et al., 2009). These studies indicate that fetal alcohol exposure has the capacity to alter developmentally significant regions of the genome.

1.6.3 Epigenetic machinery

1.6.3.1 DNA methylation

The cytosine nucleotide on DNA can be methylated into 5-methylcytosine (5mC) by enzymatic transfer of a methyl group from a methyl donor (e.g. SAMe) to the C5 position. The enzymes that catalyze this reaction belong to a family of DNA methyltransferses (DNMTs). Most of the methylated cytosine occurs adjacent to a guanine (CpG) in adult cells. However, in pluripotent cells and neuronal cells, there is detectable level of non-CpG methylation (Lister et al., 2013). The CpG are normally highly packed at promoter regions, called CpG islands.

The 5mC is abundant in all tissue types in animals and plants, and mostly accumulated in inactive regions of the genome, for example, the repetitive elements (RE) and transposons in plants and imprinting genes and X-chromosomes in mammals (Straussman et al., 2009). The accumulation of DNA methylation is associated with long-term silencing of endogenous mammalian genes (Chow et al., 2005). The transcriptional gene silencing is thought to be mediated through tightening the helix looping, therefore, blocking transcription factor binding, expelling DNA binding complexes, and recruiting repressive histone modifiers (Chow and Brown, 2003, Gu et al., 2011).

DNA methylation is essential during embryonic development. Disruption of global methylation patterns by mutation of *Dnmt* gene is lethal to mammals (Li et al., 1992). Even local and timely disruption of DNMT protein expression can cause genome instabilities and developmental abnormalities in mouse models (Bestor, 2000). Mutation in *DNMT* genes in human are linked to genetic disorders (Moarefi and Chedin, 2011, Zebisch et al., 2013) and neurodegenerative diseases (Klein et al., 2011). Furthermore, gain or loss of DNA methylation in somatic cells is linked to the onset of cancer (Kulis and Esteller, 2010). It is commonly known that inactivation of certain tumor-suppressor genes occurs as a consequence of hyper-methylation within the promoter regions (Das and Singal, 2004). In addition, DNA methylation alteration also contributes to dysregulation of microRNA expression that may play role in tumor suppression (Lopez-Serra and Esteller, 2012).

DNA methylation plays an important role in embryogenesis. There are two independent waves of DNA methylation reprogramming during development, where the DNA methylation gets totally erased and rewritten on a global scale (Li et al., 1993). Both zygotes and primordial germ cells (PGCs) go through a complete loss of 5mC, followed by orchestrated expression of de novo methylation enzymes DNMT3a and DNMT3b and maintenance methylation enzyme DNMT1 (Reik et al., 2001, Seisenberger et al., 2013). The organized DNA methylation reprogramming is required for proper embryogenesis (Reik et al., 2001, Seisenberger et al., 2013). However, the mechanism for the reprogramming remains elusive.

Beyond these early embryonic stages, DNA methylation plays a role in shaping and patterning the cellular properties. The 5mC is important for cellular differentiation (Chen et al., 2003, Jackson et al., 2004) and is required for proper neural tube development (Zhou et al., 2011b). Genome-wide analyses showed that DNA methylome landscape changed significantly upon cell differentiation (Meissner et al., 2008, Isagawa et al., 2011, Kaaij et al., 2013, Lister et al., 2013). New evidence showed that genomic pattern of 5mC dictated the property of the cells, such as glial cells and neurons (Lister et al., 2013, Kim et al., 2014).

Together, these studies have provided insight into the role of DNA methylation in embryogenesis and mammalian development. However, most of the genome-wide methylome studies of cell differentiation were conducted *in vitro* using stem cell culture, and the extracellular environment was shown to have a significant effect on the epigenetic profile (Zhu et al., 2013). Thus further studies are needed to elucidate the critical role of DNA methylation *in vivo*.

1.6.3.2 DNA "demethylation"

DNA demethylation is the process of removal of a methyl group from nucleotides in DNA. There are two proposed ways of demethylation – the passive demethylation and the active demethylation. Passive DNA demethylation refers to loss of 5mC during successive cycles of replication in the absence of functional DNA methyltransferases (Loenarz and Schofield, 2011). Active DNA demethylation occurs via direct enzymatic removal of a methyl group independently of DNA replication (Loenarz and Schofield, 2011).

The presence of the sixth base of nucleic acid, the 5-hydroxymethylcytosine (5hmC), was first detected in bacteria in 1950s (Wyatt and Cohen, 1953) and later found in mammals in 1970s (Penn et al., 1972). However, the significance of this novel cytosine was not recognized by that time. In 2009, two independent groups discovered that the ten-eleven translocation 1 (TET1) protein has the capability of catalyzing 5mC to 5hmC (Kriaucionis and Heintz, 2009, Tahiliani et al., 2009). Subsequently, TET2 and TET3 were found to have 5mC hydroxylase activity both *in vivo* and *in vitro* as well (Ito et al., 2010). TET1 and TET2 are highly expressed in embryonic stem (ES) cells, as well as primordial germ cells, while TET3 is present in male pronucleus and early zygotes (Hahn et al., 2013). Depletion of Tet1 leads to a significant decrease of 5hmC in ESCs (Koh et al., 2011). Depletion of Tet2 and Tet3 in the developing mouse cortex, by in utero electroporation of shRNA, causes a block in the differentiation of neural progenitor cells into neurons (Hahn et al., 2013).

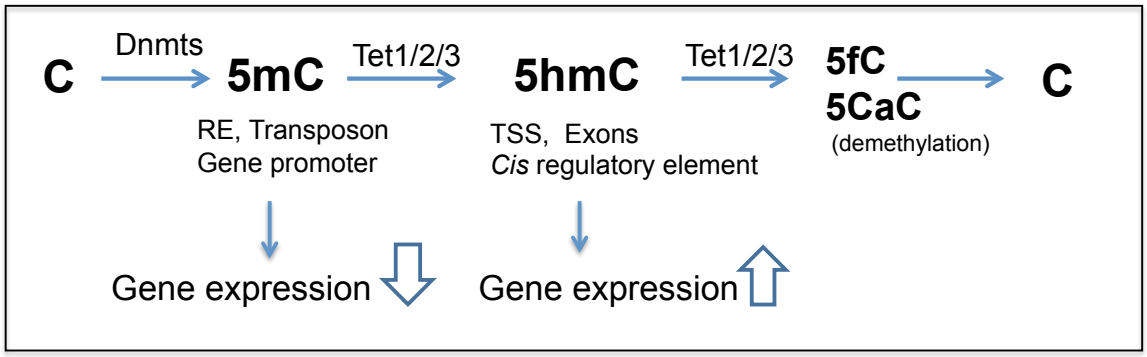

Figure 3. Dynamics of cytosine modification.

The DNMT transfers a methyl group to cytosine bases to form 5-methylcytosine (5mC). 5mC is found at CpG rich islands in the promoter regions of the gene. Canonically, CpG methylation is associated with silenced gene expression. The TET family enzymes can further bind 5mC and hydroxylate the methyl group into 5-hydroxylmethylcytosine (5hmC). The 5hmC can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5CaC) and excised to unmodified cytosine (C). C: Cytosine; DNMT: DNA methyl transferase; TET: ten-eleven translocation; TSS: Transcription start site.

Later on, it has been shown that 5hmC can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5CaC) by TET family proteins, which can be further excised to unmodified cytosine by thymine-DNA glycosylase (TDG) and the base excision repair (BER) pathway (Kohli and Zhang, 2013)(Figure 3). However, it is still not clear whether 5hmC has a functional role in the cellular processes, or is just an intermediate state of demethylation.

Several evidence have point to the possibility that 5hmC has a unique role in nervous system development, considering its unique genomic distribution and epigenetic interactions. First of all, though 5hmC only composes less than 1% of the cytosines in the genome (e.g. 0.4% in embryonic stem cells and 0.8% in mouse adult brain), the content of 5hmC changes profoundly from ESCs to early embryos to adult tissues (Li and Liu, 2011, Yu et al., 2012, Lister et al., 2013). The 5hmC is highly enriched in genomes of ESCs and mature neurons, e.g. purkinje neurons and granule cells (Szulwach et al., 2011, Wu et al., 2011). Moreover, 5hmC is enriched at active transcribed gene bodies, including CpG islands, 5'untranslated region (5'UTR), 3'UTR, exons and intragenic regions (Song et al., 2011, Wu et al., 2011). However, it is depleted at transcriptional start sites (TSSs) and intergenic regions (Ficz et al., 2011, Pastor et al., 2011, Szulwach et al., 2011). In addition, 5hmC is enriched in euchromatic regions, instead of heterochromatic regions (Ruzov et al., 2011, Szulwach et al., 2011), and depleted on the X-chromosome (Ficz et al., 2011).

Several reports have suggested 5hmC is a neuronal differentiation and age-dependent methylation mark. The 5hmC shows cell type–specific patterning and dynamics during mammalian brain development. The global level of 5hmC is highest in brain, followed by ESCs, and is very low in other tissues (Lister et al., 2013). In ES cells, the overall level of 5hmC decreases upon differentiation; however, in tissue-specific stem cells, such as neural stem/progenitor cells and hematopoietic stem cells, the 5hmC is acquired upon differentiation (Ruzov et al., 2011, Szulwach et al., 2011). In the differentiated tissues, the 5hmC accumulated in highly expressed genes for neuronal function and synaptogenesis. Kim et al. showed that the neurogenesis-related genes

Notch, Rgma and *Akt1* acquired 5hmC in the gene body and were up-regulated during differentiation, while pluripotency-related genes *Pou5f1* and *Zfp42* acquired 5mC in their promoters and were down-regulated during differentiation (Kim et al., 2014). Finally, Globish, et al. showed that 5hmC was correlated with neuronal function and not just brain localization (non-brain spinal cord exhibited high amounts of 5hmC (0.47%) and brain-localized pituitary gland tissue exhibited low 5hmC (0.06%)) (Globisch et al., 2010). These findings suggest that 5hmC might play a unique role in neural development. Further investigation is needed to understand what that role is and to address how 5hmC is regulated.

1.6.3.3 Chromatin remodeling

Chromatin remodeling refers to the dynamic modification of chromatin architecture to allow access of condensed genomic DNA to the regulatory transcription machinery proteins, and thereby control gene expression (Clapier and Cairns, 2009). Chromatin is present in two general states —the highly condensed heterochromatin associated with gene silencing, and loosely packed euchromatin associated with gene expression. The major components of chromatin are DNA and histone proteins. Two copies of each histone core proteins (H2A, H2B, H3 and H4) are assembled into an octamer that has DNA wrapped around it to form a nucleosome core. DNA and histones then can have multiple modifications, for example, histone 3 methylation at lysine 4 (H3K4me2/3), lysine 9 (H3K9me3) and lysine 27 (H3K27me3), histone 3 acetylation at lysine 9 (H3K9ac) and many others. The nucleosome controls the accessibility of DNA to the transcription machinery and chromatin remodeling factors (illustrated in Figure 4).

Chromatin remodeling is mediated by chromatin remodeling complexes. The complexes include enzymes that catalyze covalent histone modifications, enzymes that repackaging nucleosomes (ATP-dependent), as well as proteins that recruit other chromatin condensation proteins. One of the most obviously chromatin remodeling event is the formation of heterochromatin in association with gene silencing. A good example is

the formation of barr body in heterochromatic region of female somatic cells that contains condensed and inactive X-chromosome. Mechanistically, the heterochromatin-associated gene silencing is thought to be mediated by heterochromatin protein 1 (HP1), polycomb complexes and specific histone modifications and RNA interference (RNAi) (see review (van Steensel, 2011)). For example, HP1 mediates heterochromatization of euchromatin and has dosage-dependent effects on heterochromatin-induced gene silencing (Festenstein et al., 1999, Lomberk et al., 2006).

Chromatin remodeling plays an important role in neurogenesis. Chromatin modifiers, including polycomb group (PcG) and trithorax group (TrxG) complexes, are critical for cell fate determination, neuronal differentiation as well as synaptogenesis (see review (Covic et al., 2010)). For example, animal knockout studies showed that Ezh2, a member of polycomb complexes, is essential for stem cell maintenance and fate specification for neuron and oligodendrocytes (Sher et al., 2008, Yu et al., 2011). However, the molecular mechanism underlying how chromatin remodeling is involved in regulating steps of neurogenesis remain illusive.

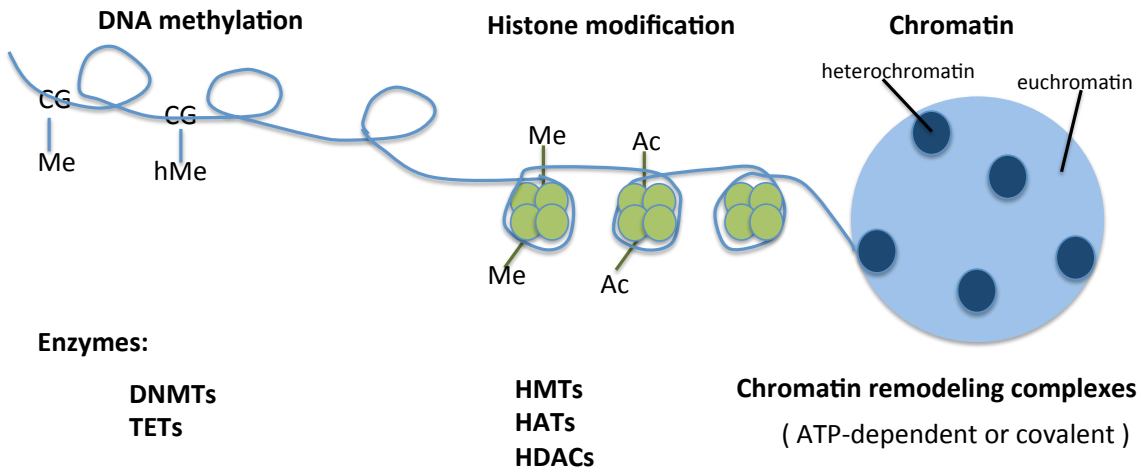

Figure 4. Schematic diagram of chromatin remodeling

DNA is wrapped around histones (in core octamer: H2A, H2B, H3 and H4) in chromatin. Several modifying complexes remodel chromatin and modify histones. Both DNA and histone tails undergo post translational modifications (Ac, acetylation; Me/hMe, methylation) that ultimately shape chromatin architecture (in euchromatic states or heterochomatic states) in a more or less favorable environment promoting or inhibiting transcription respectively. DNMT: DNA methyl transferase; TET: ten-eleven translocation methylcytosine dioxygenase; HAT: Histone acetyltransferase; HMT: Histone methyltransferase; HDACs: histone deacetylase.

1.6.4 Effect of prenatal alcohol on DNA methylation and histone modification in developing nervous system

Acute ethanol administration to pregnant mice at the first trimester resulted in hypomethylation of fetal DNA and reduced methylase activity (Garro et al., 1991). Alcohol administration through tightly controlled embryonic culture *in vitro* induced global DNA hypomethylation in the developing neural tube and correlated with reduced expression level of DNMT1 and methyl-binding protein 1 (MBD1) (Zhou et al., 2011b). Alcohol disruption of methylation was correlated with a developmental delay in many embryonic organs, including heart, limbs, cranioface and neural tube. The level of developmental dysmorphology in the embryos was similar to that of culturally exposure of 5-AZA, a potent DNMT inhibitor (Zhou et al., 2011b). Alcohol exposure to fetal rats during the first and second trimesters induced methylation changes in the hippocampus, as well a growth deficiency (Thomas et al., 2009, Monk et al., 2012). This deficiency was diminished after dietary supplement of choline, a methyl-donor (Thomas et al., 2009, Monk et al., 2012, Zeisel, 2012). Genome-widely, alcohol altered methylation profiles of group of neural developmental genes and neurological disease-related genes in embryonic mice (Zhou et al., 2011a).

Alcohol's effect on epigenetics is not limited to DNA methylation. Alcohol increased histone 3 acetylation, which is tied to gene up-regulation (Kim and Shukla, 2006). In a FASD rat model, histone 3 acetylation was decreased in the cerebellum as a consequence of a down-regulated histone acetyltransferase (Guo et al., 2011b). In hypothalamus, prenatal alcohol exposure led to decrease of hypothalamic level of H3K4me3, while increased H3K9me2 (Bekdash et al., 2013). Though these evidence were not exclusive for all the histone modifications and all the brain regions, they provided hints on the act of alcohol on the epigenetics of developing brain.

1.7 Animal models for alcohol teratogenesis

The use of animal models in the study of FASD is critical, because of the practical and ethical limitations of using human subjects for such studies. In addition, animal models provide controlled genetic background and controlled alcohol administration pattern that will help answer specific questions in alcohol teratology (Wilson and Cudd, 2011). For example, why do individuals respond differently to prenatal alcohol exposure? Does timing of exposure play a role in the heterogeneous effects? What is the role of environmental factors (such as maternal care and nutrition) in FASD? What are the alcohol-sensitive brain structures that are responsible for the cognitive and behavioral abnormalities?

A number of animal models have been used to explore the molecular mechanism underlying FASD. The rodent models are the most commonly used models, considering their similarity to human embryogenesis and neurodevelopmental timeline (except that the 3rd-trimester equivalent development in rodents is extra uterin (Dobbing and Sands, 1979, Ikonomidou et al., 2000). Other mammalian models such as sheep are also used. In addition, animal models that are different from human embryogenesis offer unique advantages in answering specific mechanistic questions. For example, zebrafish embryos are transparent, enabling direct visualization of nervous system development. Drosophila are fast reproducing and provide quick platform for genetic manipulation and screening (Marrs et al., 2010, McClure et al., 2011). These studies have provided significant genetic and molecular resources, such as genetic mapping, sequencing, and databases of single-nucleotide polymorphisms in the alcohol teratogenesis.

Rodent models provide better understanding towards the complexity of human drinking paradigms, because they mimic alcohol metabolism, placenta development, environmental factors, as well as cognitive and behavior consequences shown in FASD. There are several ways of administrating alcohol to the pregnant dams, including intraperitoneal (IP) injection, vapor chamber inhalation, dietary mixed intake, and voluntary two-bottle drinking (10% alcohol in water or water alone). Each has its own advantages and limitations for mimicking the FASD phenotypes.

The IP injection model and vapor chamber inhalation model ensure controlled blood alcohol concentration (BAC), which can eliminate the alcohol uptake differences (by both genetic and nutritional factors) between individuals. However, it is less similar to the human drinking situation. Thus, the dietary intake model and voluntary two-bottle drinking model are preferred in some translational studies. The voluntary two-bottle choice model better mimics the human drinking (voluntarily and sporadic), however, the level of BAC is often fluctuated cross days and among individuals (Kleiber et al., 2011).

In the dietary intake model, alcohol is provided to the dams in a specially formulated liquid-diet (LD), in which all the macro- and micro-nutrients are set to about the same level of pellet chow food (except some difference in the content of fat). However, there might still be differences between the metabolism and absorption between animals being fed with chow food or liquid diet, therefore, a liquid-diet pair-fed (PF) control group is necessarily introduced to the experiments. To control for caloric factors (alcohol itself contains calories that could be utilized in metabolism), the PF food is matched to the alcohol liquid diet food calories, by addition of the non-flavored polysaccharide maltodextrin. The PF group daily food intake is then matched to that of alcohol group, so that these two experiment groups received same calories and nutrition under the same setting (Anthony et al., 2010). Studies using this model have reported the relationship between ethanol exposure and specific FASD-related phenotypes. Offspring of maternal alcohol exposed rodents during 1st to 2nd trimester demonstrate distinct facial dysmorphology that parallels human dysmorphology (Sulik, 2005, Anthony et al., 2010) and correlate with CNS abnormalities (Parnell et al., 2006, Godin et al., 2010), as well as social behavioral changes (Fernandez et al., 1983).

However, this dietary intake model introduces certain level of stress as a result of food restriction. The alcohol-treated dams tend to drink less than normal (about 15% reduction in total food intake, unpublished data). To control for this restriction, the pair-fed group dams are fed with same volume of liquid diet daily to the alcohol group, resulting in a small level of under-nutrition states. Thus it is necessary to compare the PF

group phenotypes with the Chow phenotypes as well, as an indicator of nutritional effect. In a matter of fact, it has been shown that the PF groups demonstrated smaller pup weight at birth compared to Chow groups (Anthony et al., 2010).

Other forms of stress as environmental factors can be reduced to minimum in this animal model. For example, pregnant dams who received alcohol tend to perform poorly in maternal care. This would lead to increase stress level in the newborns at the time of the last neurodevelopment stage (3rd trimester equivalent in human). Pairing the newborns with surrogate moms who have not received alcohol during pregnancy can reduce this form of stress from poor maternal-care. In addition, gradually introducing alcohol to females before mating ensures the alcohol-induced stress to become minimum as well.

Another consideration of the use of animal model is the animal strain. It has been shown that the C57BL/6 mouse (B6) is acutely sensitive to both the physiological and the behavioral effects of neurodevelopmental ethanol exposure, while other mouse strains (129 and D2) are relatively repellent and protective towards alcohol (Chen et al., 2011b). The C57BL/6 strain of mouse has been successfully used to replicate a number of FASD-relevant phenotypes including impairments in cognitive function, activity levels, anxiety- and depression-related behaviors (Fernandez et al., 1983, Cronise et al., 2001, Carneiro et al., 2005). New high-throughput studies have also elicited fetal brain transcriptome alterations and specific neural gene expression changes in the B6 strain after alcohol exposure at three neurodevelopmental time points corresponding to human trimesters one, two and three (Kleiber et al., 2012, Kleiber et al., 2013, Mantha et al., 2014).

Taking into consideration of all the factors above, we chose to use the dietary intake model to achieve a chronic moderate level of alcohol exposure in the C57BL/6 strain mice.

1.8 Hypotheses and Objectives

This study seeks to extend our previous studies evaluating the epigenetic alteration in developing nervous system as a consequence of prenatal alcohol exposure. Previously, we have demonstrated that prenatal alcohol exposure induced developmental delay in many fetal organs, including developing neural tube, heart, limbs and craniofacе (Chen et al., 2011b, Zhou et al., 2011b). The retardation in neural tube development is correlated with delayed DNA methylation program, as well as associated epigenetic proteins such as DNMTs and MBDs (Zhou et al., 2011b). We have further identified groups of neurodevelopment related genes at the time of neurulation that display altered gene expression and/or DNA methylation at their promoter sites upon alcohol treatment (Zhou et al., 2011a, Zhou et al., 2011c). However, little is known about how alcohol affects the epigenetic program in the developing brain. Our objective is to identify the effect of alcohol on epigenetic program in specific stages of neurogenesis in developing hippocampus and cortex and its phenotypic consequences. It is hoped that this study will provide a better understanding of how alcohol alters epigenetic program in developing brain, and how the altered epigenetic program is involved in the teratology of FASD.

Specific Aim 1: To determine the effect of alcohol on the DNA methylation program (DMP) in developing mouse hippocampus and cortex. In preliminary studies, we have developed a dietary alcohol intake model to mimic the human drinking paradigm. We will use antibody-based detection methods to analyze DNA methylation and phenotypic markers *in vivo* to correlate with alcohol-induced dysmorphology.

Specific Aim 2: To determine the effect of alcohol on DNA methylation-associated chromatin remodeling during neuronal differentiation and maturation. In previous studies, we demonstrated that DNA methylation (5mC and 5hmC) was reorganized in the nucleus during neural differentiation. In this aim, we proposed that alcohol affect the transitioning of 5mC and 5hmC and their associated chromatin remodeling. We utilized confocal microcopy with fluorescent double immunostaining analysis (which is backed up by FLIM-FRET by my colleague) to illustrate the alcohol-induced alteration in chromatin remodeling.

Specific Aim 3: To determine the gene-specific DNA methylation alteration by alcohol in neural specification gene during neural differentiation. To investigate how alcohol alters gene-specific DNA methylation, we utilized a bisulfite-pyrosequencing platform to analyze site-specific DNA methylation level in a critical neural specification gene *Ascl1*. We also correlated the DNA methylation changes with gene expression changes of *Ascl1* gene using qPCR. This is critical in understanding the functional consequences of alcohol-induced DNA methylation alterations.

CHAPTER 2: AIM 1

DNA Methylation Program in Developing Hippocampus and Cortex and the Effect of Prenatal Alcohol Exposure

2.1 Background and Hypothesis

Epigenetic changes are believed to be among the earliest key regulators for cell fate and embryonic development. To support this premise, it is important to understand whether or not cell-wide epigenetic changes coordinate with the progression of development. We have demonstrated that DNA methylation is programmed when neural stem cells differentiate (Zhou et al., 2011a), as well as during early neural tube development (Zhou et al., 2011b). However, both studies were performed *in vitro*, and lacked certain degree of physiological complexity considering the dynamic nature of epigenetics. In this aim, I seek to understand what is the DNA methylation program in developing brain *in vivo*, and the effect of *in utero* alcohol exposure on the DNA methylation program during brain development, specifically in the hippocampus and cortex.

2.1.1 DNA methylation program

The term DNA methylation program (DMP) during development was first conveyed by our laboratory. DNA methylation as a modifiable and heritable modification has been considered a program since the beginning of life, from the fertilized egg to the blastocyte stage, including stages of total erasure and coordinated re-establishment. This orderly pattern is preserved over evolution and is considered a "memory" of cellular identities (Bird, 2002). At the same time, the DNA methylation is also progressing over the embryonic and postnatal development which has been hypothesized as a driving force in directing life events, including cell fate and tissue patterning. Thus, we have referred to the process of DNA methylation transformation in an orderly manner during tissue or organ development as *DNA methylation program (DMP)* (Zhou, 2012).

To date, how DNA methylation progresses (beyond blastocyte stages) during nervous system development and what role this DNA methylation plays is unclear. We have demonstrated for the first time a neurulation-stage DMP in which 5mC, DNMT1 and MBD1 exhibited distinct spatiotemporal patterns that coincided with neural differentiation in the neural tube (Zhou et al., 2011b). Notably, the neural progenitor cells acquire DNA methylation when proliferation ceases, begin their restriction (cell fate progression), and head for migration. How and if DNA methylation mediates tissue-specific differentiation and organ formation remain illusive, which is a major area of investigation.

2.1.2 Alcohol-induced neural tube development and DMP retardation

Previously, we have utilized an *in vitro* whole embryo culture system to identify the DMP alteration by alcohol exposure in the developing neural tube. Mouse embryos at embryonic (E) day 8 were dissected and cultured in an embryonic culture system for 48 hours in culture medium. Alcohol was added into the culture systems and was absorbed directly by the embryos. We have shown that binge-like alcohol exposure altered timely DNA methylation and retarded embryonic growth. The effect of alcohol on the DNA methylation was readily noticeable after 6 hours of exposure. We further demonstrated that direct inhibiting of DNA methylation with 5-AZA resulted in similar growth retardation (Zhou et al., 2011b).

2.1.3 Aim

Epigenetic regulation is important for neurogenesis in hippocampus and cortex, and alcohol (known as a methyl donor inhibitor) has been shown to disrupt the normal hippocampal and cortical development. Thus, in this aim, I seek to understand how alcohol affects DMP in the hippocampus and cortex and impacts its development.

2.2 Methods and Materials

2.2.1 Animals

To answer the question, we employed an *in vivo* mouse model to mimic human FASD, in which pregnant dams received alcohol as a part of dietary intake. We used a alcohol-sensitive strain of mice, C57BL/6 (B6), which has been shown to elicit cellular and behavioral phenotypes in the fetus upon alcohol treatment similar to human FASD.

All mice were used in accordance with National Institute of Health and Indiana University Animal Care and Use (IACUC) guidelines. The protocol was approved by the Laboratory Animal Resource Center (LARC) animal ethics committee of Indiana University (protocol ID: 10428). All efforts were made to assure minimal pain and discomfort. C57BL/6 (B6) (12-14 weeks old, 20g ±1) nulliparous female mice (Harlan, Inc., Indianapolis, IN) were used in the study. Mouse breeders were individually housed upon arrival and acclimated for at least one week before mating began. The mice were maintained on a 12 hrs light-dark cycle (light on: 22:00-10:00) and provided laboratory chow and water ad libitum.

2.2.2 Treatment Groups and Liquid Diet Administration

Mice were randomly assigned into three treatment groups: Chow, Pair-Fed (PF) and Alcohol (Alc). All alcohol treatments received 4% alcohol v/v in liquid diet [Purina Micro-Stabilized Alcohol Diet (PMI), Purina Mills Inc., Richmond, Indiana] as instructed by supplier with 4.4% w/v sucrose added (to introduce a sweet taste to promote alcohol drinking), and administered using a 35ml drinking tube (Dyets Inc, NY). The PF group was given the PMI diet mixture with equal caloric maltose dextran (MD) (Isocaloric diets) as a substitute for alcohol calories, and the volume of liquid diet intake was restricted to that of a matched dam from the alcohol group throughout all treatments. The Chow group maintained a standard chow diet and water ad libitum throughout gestation. The PF and Alc groups were pre-treated with liquid diet for 7 days before mating. Then females were bred with male breeders for a 2-hr period (10:00am to 12:00 noon). The presence of a vaginal plug at the end of the 2-hr mating was considered as indicative of

conceptus and that hour was designated as hour 0, and embryonic day (E) 0. All animals were mated daily over a period of no more than 3 weeks, at which time all animals were on ad libitum chow and water diets, until plugs were detected. On E5, pregnant dams in PF and Alc groups were placed on liquid diet, and started to receive 4% v/v alcohol (Alc group) or isocaloric liquid diet (PF group) as indicated above from E7 through the end of E16. On E17, dams were either euthanized for embryo harvesting or were returned to standard lab chow and water and allowed to give birth. Additional E16 embryos from Chow group were also harvested for stage comparison. The postnatal pups were all nursed at birth by surrogated Chow-fed dams until the days of tissue harvesting. E19 was designated as postnatal day (P) 0 regardless if birth took place. Following birth, litters were randomly culled to six pups/litter to decrease possible nutritional deficiencies due to within-litter competition. Pups were then weighed daily and observed for any gross malformations until sacrifice on P7 (E26). The E17 and P7 stage were used for the study for their critical morphological progression in cortex and hippocampal CA regions (at E17 and P7) and in dentate gyrus (at P7) development, and for their vulnerability to alcohol. Additional time point, P21, was also used to extend the observation after maturation.

2.2.3 Blood Alcohol Concentrations

An independent set of non-pregnant females (n=6) receiving 4% v/v alcohol drinking as in the above paradigm were used for blood alcohol concentration (BAC) analysis. Blood samples were collected through tail vein at 2 hrs or 6 hrs into the dark cycle on alternate days 2, 4 and 6 during treatment. 15 µl of blood was collected in heparinized tubes, and plasma was isolated through centrifugation and stored at −80°C prior to analysis with a Gas Chromatograph (GC, Agilent Technologies; model 6890). Each sample was analyzed in duplicate. (*Blood samples were harvest in collaboration with Nail Can Ozturk, and GC analysis were performed by Tammy Graves*)

2.2.4 Embryo Isolation and Tissue Preparation

Under deep CO2 euthanasia, embryos were harvested from dams at E17 by removal from the embryonic sack. Each embryo was either immersion-fixed in 20 ml of fixative prepared from 4% paraformaldyhyde (PFA) and stored at 4°C in the same fixative. For P7 group, pups were anaesthetized with CO2, and then perfused transcardially with 0.9% saline (100 ml) and 4% formaldehyde in phosphate buffer (0.2 M, pH 7.4). The brains were then removed, weighed, and post-fixed for at least 24 hrs at 4°C.

2.2.5 Immunocytochemistry Analysis

One Alc and either one PF or Chow brain were embedded in a single 10% gelatin block with careful rostrocaudal and dorsoventral alignments. Gelatin blocks were fixed with 4% PFA and sectioned in 40 μm thick coronal sections on a vibratome (Leica Microsystems; Buffalo Grove, IL). The section-pairs (Alc-PF or Alc-Chow) were processed equally in all immunocytochemical procedures. The section pairs were then cleared of endogenous peroxidases using a 10% H2O2 in phosphate buffered saline (PBS) for 10 min, and permeabilized with 1% TritonX-100 in PBS for 30 min before incubation with a primary antibody diluted in goat kit (1.5% goat serum, 0.1% TritonX-100 in PBS) for 18 hrs at room temperature. Antibodies used in this study were: 5mC (1:2000, mouse monoclonal; Eurogenetec, Fremont, CA), 5hmC (1:3000, rabbit monoclonal; Active Motif, Carlsbad, CA), TET1 (ten-eleven translocation, a 5hmC hydroxylation enzyme; 1:500, rabbit polyclonal; Millipore, Billerica, MA), MeCp2 (methyl CpG binding protein 2, a DNA methylation binding protein; 1:1000, rabbit monoclonal; Cell Signaling, Danvers, MA), NeuN (a marker for mature neuron; 1:500, cell signaling, Danvers, MA), Sox2 (a marker for neural progenitor cells; 1:500, Millipore, Billerica, MA) and Ki67 (a marker for proliferating cells; 1:1000, rabbit polyclonal; Abcam, Cambridge, MA). The section pairs were then incubated for 90 min in goat anti-rabbit IgG or goat anti-mouse secondary antibodies conjugated with biotin (Jackson ImmunoResearch, West Grove, PA) or fluorescent dyes (Invitrogen; Grand Island, NY), followed by Streptavidin-AP (1:500, Jackson ImmunoResearch, West

Grove, PA) for 90 min. The immunostaining was visualized by incubation of 0.05% 3'-3'-diaminobenzidine (DAB) and 0.003% H2O2 over minutes, followed by counterstained with methyl green. Fluorescent double staining was performed for co-localization studies (5hmC/ Sox2). Secondary antibodies conjugated with Alexa 488 or Alexa 550 were incubated for 90 min after primary antibodies, and sections were then washed with PBS and covered with Prolong-gold anti-fade mounting solution with nucleotide fluorescent dye 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Grand Island, NY). The fluorescent staining was photographed under fluorescent microscopy for cellular analysis (Leitz Orthoplan2 microscope; Ernst Leitz GMBH, Wetzlar, Germany) or confocal microscope (Olympus, Center valley, PA) for intra-nucleus analysis.

2.2.6 Densitometry Analysis

For density analysis, pictures were taken using a Leitz Orthoplan 2 microscope with a Spot RT color camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Bright-field images were taken with consistent setup and exposure time for each antibody staining. Immunostained images were converted to the 16-bit color format, and staining intensity was measured using Image J (National Institutes of Health, Bethesda, MD). Calibration was set based on 256 levels of the gray scale. Regions of interest (ROI) were picked by outlining the morphological areas at same level of brain section, e.g., whole hippocampus, cortex or dentate gyrus. For the measurement of subregions of cortex and hippocampus, a boxed region of equal dimensions from each cell layer was selected, and staining optical densities (OD) of the cells in ROI were compared among Chow, PF and Alc groups. Five brains from different dams were used from each treatment group and age. Statistical analyses were performed with one-way ANOVA followed by t-test using Prism software Version 4 (GraphPad Software Inc., San Diego, CA). All data were presented as Mean± SEM.

2.3 Results

2.3.1 Prenatal alcohol exposure delayed hippocampal and cortical development

The alcohol administration paradigm was summarized in Figure 5. In the PF and Alc groups, female mice were acclimated to liquid diet for 7 days before they were put on mating. Alcohol group started to receive alcohol from E7, and remain on alcohol diet until the end of E16. The pair-fed groups were fed with isocalaric liquid diet to the alcohol group from E7 to E16. Chow groups received pallet chow food throughout experiment.

Peak blood alcohol concentration (BAC) is commonly used in the study of FASD to access the level of alcohol that is effective in a system (a balance between alcohol absorption, metabolism and body weight). The blood alcohol concentration (BAC) in the current treatment paradigm peaked at ~120-160mg/dL (Figure 6) is similar to that of our previous study (Anthony et al., 2010). Under the treatment, there was no significant difference in dam body weight from the start of treatment (E7) to E14 among Chow, PF and Alc groups (One-way ANOVA, $P>0.05$) (Figure 7). However, dam body weight of the PF and Alc groups were lower than the Chow group at E15 and E16 (One-way ANOVA, $F[2,13]<0.005$), while there was no difference between PF and Alc groups (t-test, $P>0.05$) (Figure 7).

Dam daily food intake steadily increased throughout gestation stages (E7-E16) in PF and Alc groups (Figure 8). There was no difference in food intake between Alc and PF throughout gestation stages, because we controlled the liquid diet daily intake of PF to the same level of Alc groups. However, there was an about 15% reduction of daily liquid diet intake before and after alcohol treatment (Figure 8).

The embryos at E17 and pups at P7 were harvested for all three groups. There was a significant reduction of the embryo (E17) and pup (P7) whole body weight in Alc group comparing to PF and Chow ($P<0.005$), while no significant difference was noticeable between PF and Chow (Figure 9). Similarly, the embryos and pups of the alcohol group

displayed significantly smaller brain weight compare to that of PF and Chow ($P<0.005$) (Figure 10).

Alcohol delayed the maturation of hippocampus by approximately 1 day in mice, as indicated by comparison between E17 Alc to E16 Chow embryos. The hippocampus size was reduced significantly in the Alc group as compared to Chow and PF groups (Table 1a), and the lateral ventricle was expanded in the Alc group (Figure 11). The thickness of both the (undifferentiated) ammonic NE and the primary dentate NE layers was increased in alcohol group as compared to those of Chow and PF groups (Table 1c). The rate of proliferation was reduced by alcohol, as indicated by less Ki67+ cells (Table 1d). Outside the NE layer, there was a slight reduction in the ammonic cell migration, as well as reduced numbers of migrating cells in the intermediate zone (Figure 11A-C). The thickness of formed stratum pyramidale was thinner in alcohol groups as compared with PF and Chow groups (Table 1c).

In the developing dentate gyrus (DG) of Alc embryos at E17, the size of dentate gyrus was significantly reduced (Table 1b), the migration distance was shortened and the secondary and tertiary matrix was diminished (Figure 11A-C, dotted line). The number of NeuN+ granule cells that reached primordial DG was also reduced (Figure 11A-C, arrowhead) along with lower level of NeuN-im (Table 1e).

Though alcohol exposure was ceased at E16, at postnatal day 7 the DG was continuously affected by the prenatal alcohol exposure (Figure 15). The total number of NeuN+ cells was significantly reduced by 45.6% (t-test, $P<0.001$) in the Alc group compared to that of Chow group; no difference when comparing Chow to PF groups (one-way ANOVA: $F(2,18)=35.8452$, $P<0.001$) (Figure 12 M-O,T).

Similarly, alcohol delayed the cortical development by approximately 1 day, as compared the overall growth phenotypes between E17 Alc to E16 Chow embryos. The whole cortical thickness was significantly reduced in the Alc group as compared to Chow and PF (Appendix 1a). The cortical plate (CP) thickness was reduced in the Alc group (Appendix 1b), while the ventricular zone (VZ)/ Subventricular zone (SVZ) were expanded (Appendix1c)(also see figure in Appendix 2). The subplate (SP) neurons were the earliest matured neurons in developing cortex. We observed a significant decreased NeuN-immunointensity (-im) in the SP of the Alc groups at E17, indicating a reduction of the mature neuronal number ($P<0.05$) (Appendix 1d).

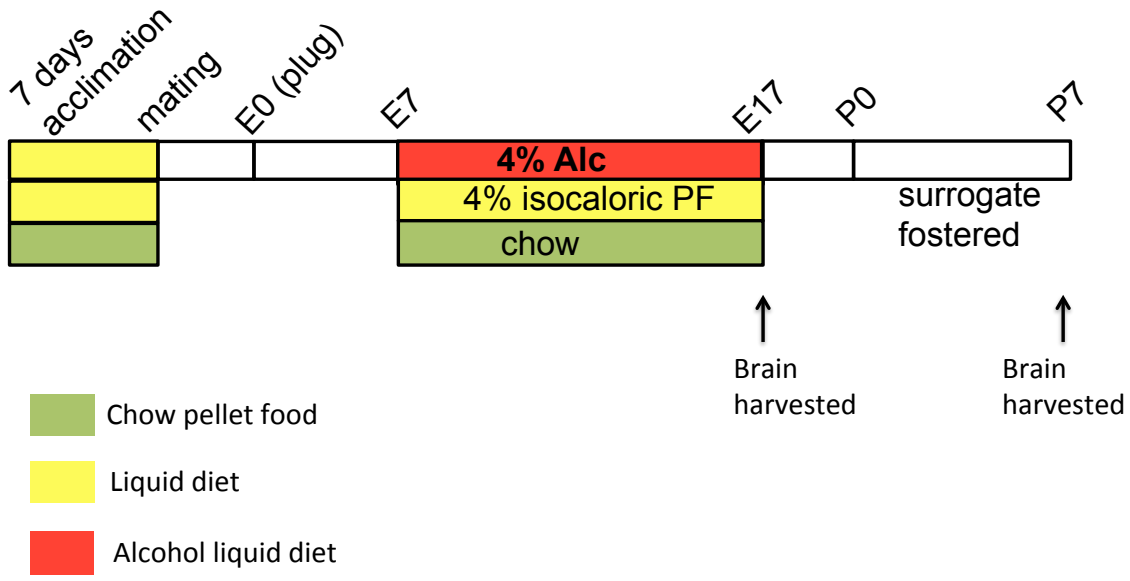

Figure 5: Dietary alcohol intake paradigm and experimental groups

Female animals were randomly assigned into three groups: Chow, PF and Alc. Animals received 7 days of acclimation to the liquid form of diet, then were put on mating. Upon detecting vaginal plugs, it was assigned as Embryonic day (E) 0. Dams started to receive treatments from E7. Alc group received 4% alcohol liquid diet. PF group received isocalaric 4% liquid diet. Chow group was remained on chow pellet food. At E17, embryonic brains were harvested from half of the dams of all three groups. The other half of dams were kept till they gave birth (pups assigned as postnatal day (P) 0). The Alc pups were surrogate fostered. All pup brains from all groups were harvested at P7.

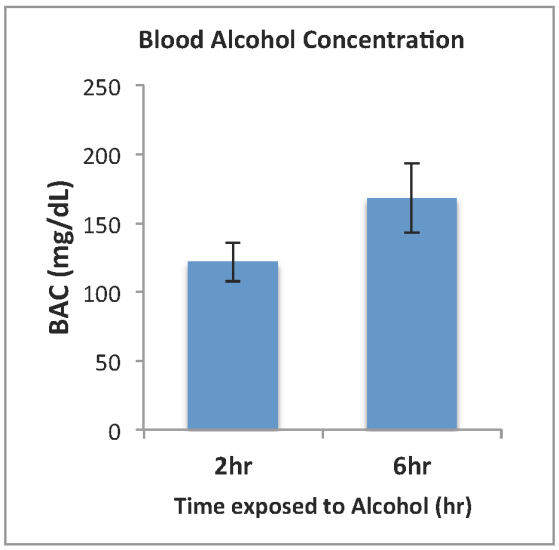

Figure 6: Measurements of Blood Alcohol Concentration.

Blood alcohol concentration (BAC) 2hrs and 6hrs after alcohol diet supply at beginning of the dark cycle. The BAC was collected from a group of females independent from the group of epigenetic and phenotypic studies (N=6). Tail blood was collected for measurement of BAC every other day for a total of 3 collections for each animal. All data were presented as Mean ± SEM.

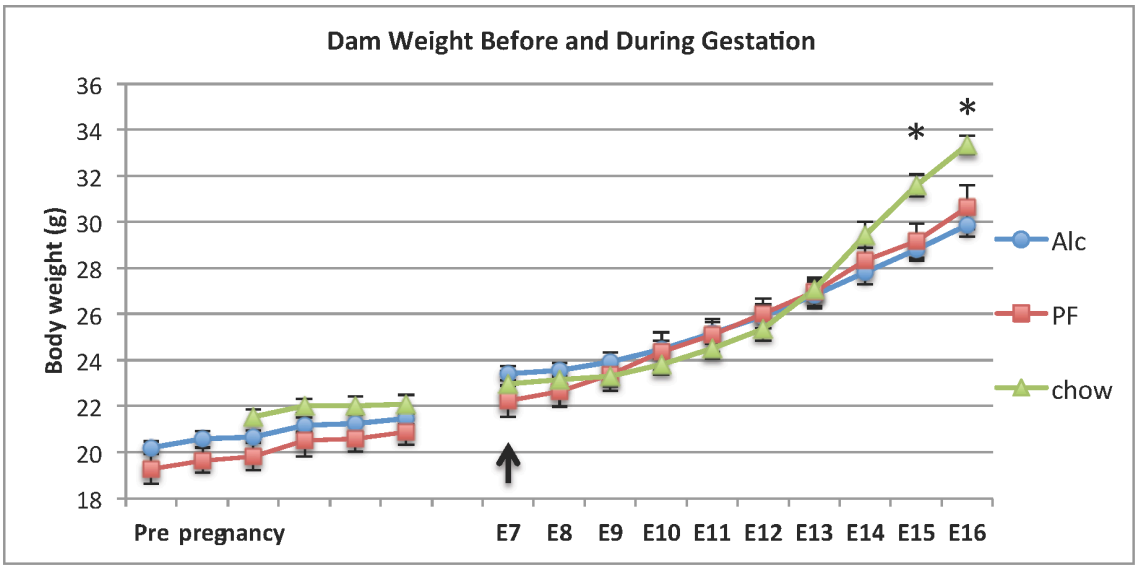

Figure 7: Dam weight before mating and during gestation 7-16 days.

Before mating, the Alc and PF groups were pre-treated with liquid diet for 7 days, while Chow group were on chow pallets diet throughout the time course. The treatment for 4%(v/v) alcohol or pair-fed liquid diet was administered from embryonic day (E) 7 to E16. Arrow: day treatments started. N= Chow (5), PF (5), Alc (7). *One-way ANOVA: $P<0.05$. All data were presented as Mean ± SEM.

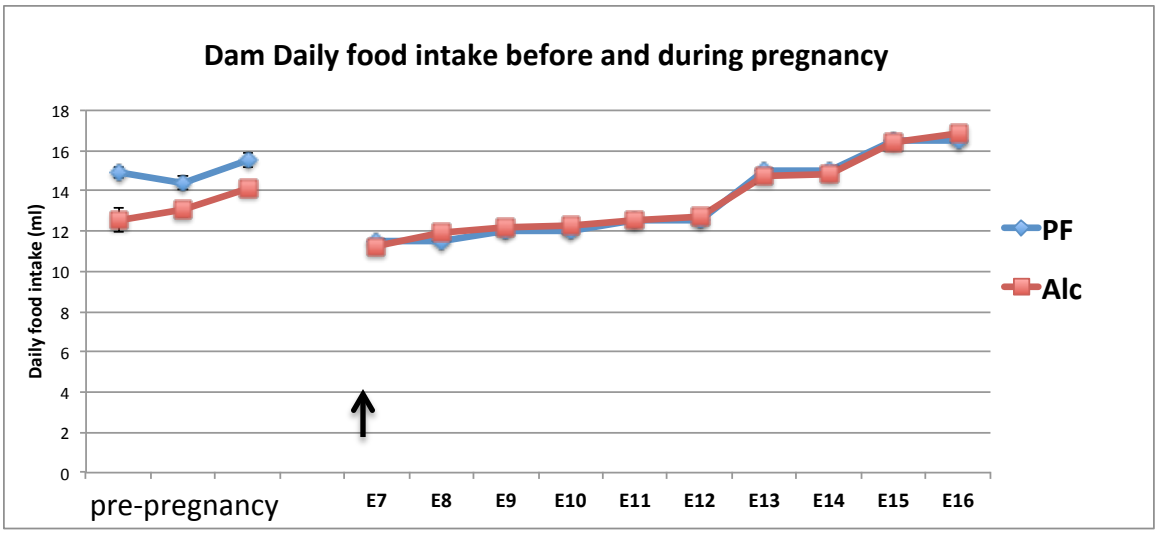

Figure 8. Dams daily liquid diet intake.

Animals were acclimated with liquid diet before mating (Pre-pregnancy). Dams received 4% alcohol liquid diet (Alc) or 4% isocalaric liquid diet (PF) from E7 to E16. Arrow: day treatments start. All data were presented as Mean ± SEM.

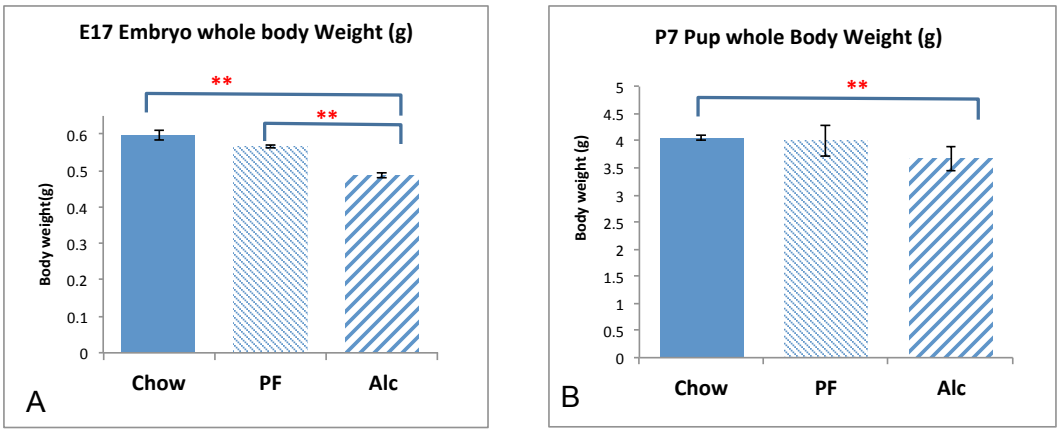

Figure 9. Embryonic and pups whole body weight at E17 (A) and P7 (B).

E17: N=Chow (5), PF (5), Alc (5); P7: N=Chow (5), PF (5), Alc (9). **Student t-test: P<0.005. All data were presented as Mean ± SEM.

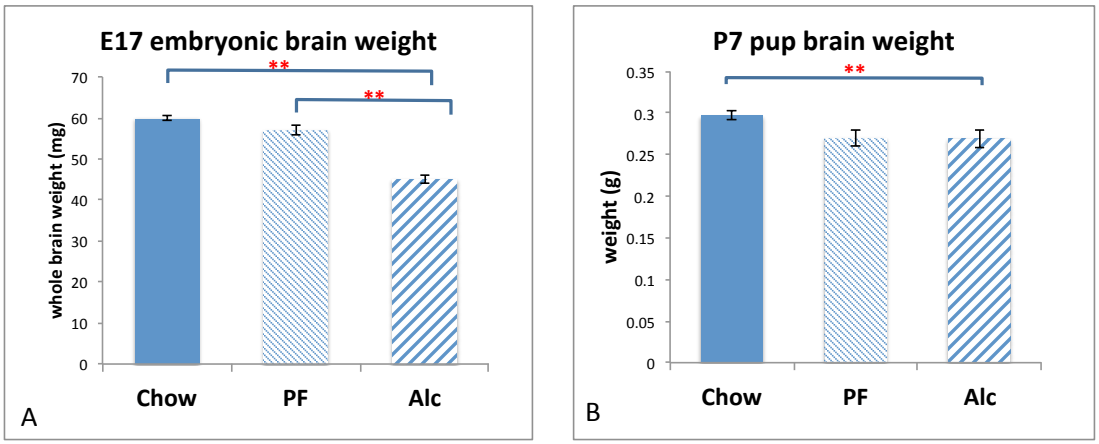

Figure 10. Embryos and pups whole brain weight at E17 (A) and P7 (B).

E17: N=Chow (5), PF (5), Alc (5); P7: N=Chow (5), PF (5), Alc (8). **Student t-test: P<0.005. All data were presented as Mean ± SEM.

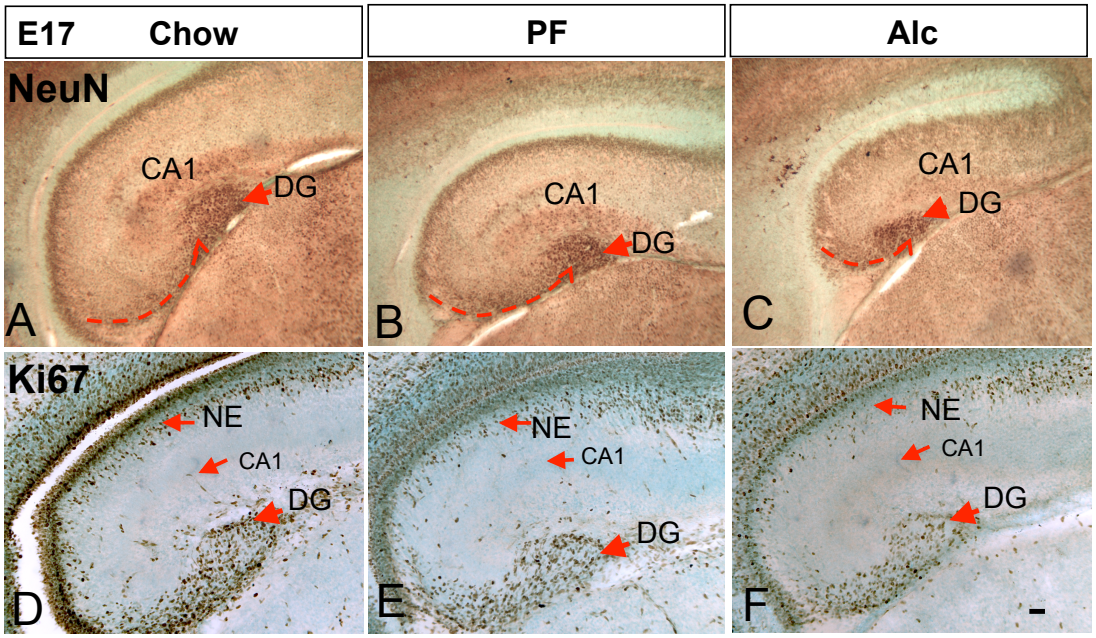

Figure 11. Alcohol reduced proliferation and maturation of hippocampal cells at E17. (Also see quantitation in Table 1d,e). Alcohol reduced NeuN+ cell number in NE, CA and DG (C, arrowhead) as compared to that of Chow (A) and PF (B), as well as reduced dentate granule cell migration distance (comparing A,B,C, dotted line). The proliferation marker Ki67-im was reduced in Alc groups in NE (F, crossed arrow) compared to Chow (D) and PF (E), and DG (comparing D,E,F, short arrows). Scale bar: all=100μm.

Table 1. Phenotypic measurements of E17 hippocampus in Chow, PF and Alc-treated mice

E17	Chow	PF	Alc
a. Hippocampus size (10^3µm)	392.3±16.0	387.4±22.0	280.0±20.0 ** #
b. DG size (10^3µm)	24.3±1.0	23.9±2.1	15.7±1.1 ** #
c. NE thickness (µm)	51.1±3.8	50.7±2.9	68.7±4.9* #
d. Ki67-im (in NE)	143.6±7.4	152.4±3.5	109.5±13.7* #
e. NeuN-im (in DG)	178.9±1.4	169.2±6.6	152.1±5.1* #

*P<0.05; **P<0.005 (Alc vs Chow); #P<0.05 (Alc vs PF); ^P<0.05 (PF vs Chow). N=4 each. Data presented as Mean±SEM. NE: neuroepithelial layer; DG: dentate gyrus.

2.3.2 Profile of DNA methylation program (DMP) in developing hippocampus and cortex

A patterned (temporal and spatial) epigenetic progression was evident in the developing hippocampal CA1 through CA4. A temporal increment of 5mC and 5hmC-immunostaining (im) was observed from E15 to E17 in hippocampal pyramidal layer, as well as neuroepithelial layer (Figure 12A, D).

There was also a spatial, cell-stage dependent gradation of DNA methylation in the developing hippocampus. At E17, NSCs in the ammonic neuroepithelium (NE) layer were composed of spindle-shaped self-renewal stem cells as indicated by the expression of neural stem cell marker Sox2 and proliferation marker Ki67 (Figure 11). These NE cells were either devoid or had only low levels of 5mC or 5hmC. As the NE cells committed to neural fate progression and begun differentiation (lost the Ki67 expression), they migrated out radially, presumably along glial processes through the intermediate zone, and finally settled in the stratum pyramidale (illustrated in Figure 12 G,H). These destined cells express neuronal marker NeuN and settled in an *inside-out* fashion (the early arrival differentiated cells settled in the inner layer, and the late arrival cells settled on the top of the earlier-arrived cells). The 5mC first appeared in neuroepithelial layer in Ki67- cells. The 5hmC appeared hours to a day behind the 5mC-im mark appeared (Figure 12C,F), and specifically appeared in cells that underwent migration (left proliferating zone). These migrating cells increased 5hmC as well as 5mC throughout their journey towards stratum pyramidale (Figure 12B,C,E,F). After settling in the CA primodium, these resident pyramidal neurons further matured by reducing 5mC methylation, and meanwhile went through a translocation of 5mC and 5hmC within the nucleus (see next stage).

At P7, the maturing pyramidal neurons were NeuN+ and expressed both 5mC and 5hmC (Figure 13G, H). However, there was a characteristic chromatic separation of the 5mC and 5hmC in the nucleus. The 5mC mark was preferentially clustered in the heterochromatic regions, which localized in the DAPI dense area, whereas 5hmC was more scattered in the euchromatic regions coinciding with the DAPI-sparse area (Figure

13G, H). Region wise, there was a general progression of maturation from CA1 towards CA4. There was also a progression of the above described 5mC and 5hmC in the same order.

Epigenetics also takes an orderly progression through the formation of dentate gyrus (DG). At E17, the primary dentate NEs which were located lateral to the ammonic CA in the hippocampal primodium (Figure 12G), just began to migrate medially to the Secondary Dentate Matrix (an intermediate regions within hippocampal primodium), and then further migrated to the target dentate regions forming a small granule cell cluster of angular dentate primodium (Figure 12G, dotted arrow). These NE exoduses first lost Sox2-im and Ki67-im by acquiring 5mC followed with 5hmC on the journey, and became granule cells expressing NeuN at the destined area.

Meanwhile, as the DG primodium continued its formation to P7, a new source of neural progenitor cells expressing Sox2 and Ki67 was established at the most inner layer, the subgranular layer (SGZ) (Figure 13 C,F, arrow). Very similar to CA, these new dentate progenitor cells launched their neural fate by acquiring first the 5mC, while 5hmC was absent or low. The 5hmC appeared when progenitor cells set off for migration outward (some inward-migrating cells became interneurons). This new wave of migrating cells increased 5hmC-im while decreased 5mC through the journey towards the outer dentate shell in both upper and lower limbs of DG. The 5hmC was closely associated with the maturation status of granule cells. Double fluorescent stainings showed co-localization of 5hmC with NeuN+ cells, but not Sox2+ cells in dentate gyrus (Figure 13A-F).

Similarly, the expression of 5hmC-converting enzyme TET1 was closely associated with 5hmC, but not with 5mC (Figure 15G, TET1 was expressed higher in the outer layer than in the inner layer). The DNA methylation binding protein MeCp2 followed similar expression gradation that high in the outer granule layer and low in the inner layer (Figure 15J).

The patterned epigenetic progression held true in the developing cortex as well (Appendix 2). The ventricular zone (VZ) of cortex at E17 was composed of Ki67+ proliferating neural stem cells (active dividing), as well as the cell nucleus of Tbr2+ radial glial cells (images not shown). Radial glial cells expressed GFAP (glial marker), and were capable of generating neurons, astrocytes, and oligodendrocytes. The subventricular zone (SVZ) composed of early-generated neuroblast cells that were committed to neuronal fates. In VZ, the Tbr2+ radial glial cell showed high level of 5mC-im and 5hmC-im, while the Ki67+ neural stem cells expressed none or low level of 5mC-im and 5hmC-im (images not shown). In SVZ, neuroblast cells started to gain first 5mC-im and then 5hmC-im, and continued to escalate when migrated to intermediate zone (IM) and cortical plate of cortex (CP). Noticeably, the earliest arrived, most matured neurons in subplate of cortex (SP) (compared to cortical plate neurons) expressed the highest level of 5mC-im and 5hmC-im (Appendix 2 E,F, arrowhead).

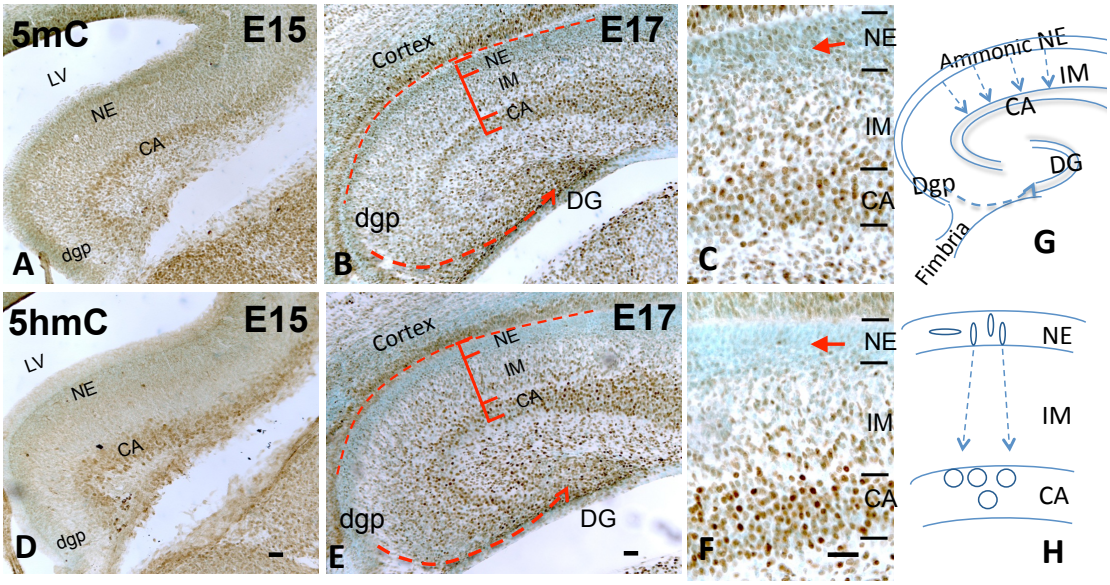

Figure 12. DNA methylation program of hippocampus during its early formation from E15 to E17. Cartoon (G, H) shows the differentiation processes where neuroepithelial (NE) cells migrate into intermediate zone (IM) and arrive in Cornus Ammonis (CA) to become pyramidal neurons; while dentate gyrus neuroepithelium (dgp) migrate through a long journey from lateral hippocampal primodium towards dentate gyrus (DG) and become granule cells. The DNA methylation formed an integral program in these differentiating cells. First, progenitor cells all acquire DNA methylation to begin their differentiation. The immunostaining (brown DAB coloration) shows that the 5mC appears ahead of 5hmC as shown in NE layer at E15 (A, D) and E17 (B, E) (enlargement, C, F, arrow). There is temporal increment of both methylation marks in CA (pyramidal layer) and developing dentate gyrus (DG) from E15 (A, D) to E17 (B, F). There is also a spatial increment of both 5mC-im and 5hmC-im from NE to IM and to CA at E17 (B, E; higher magnification in C, F, respectively). Moreover, the 5mC-im and 5hmC-im increase as the migration of granule cells from dentate neuroepithelium to the dentate primordial (B, E, dotted line). LV: lateral ventricle. Scale bar: A, B, D, E=100 μm; C, F=100μm.

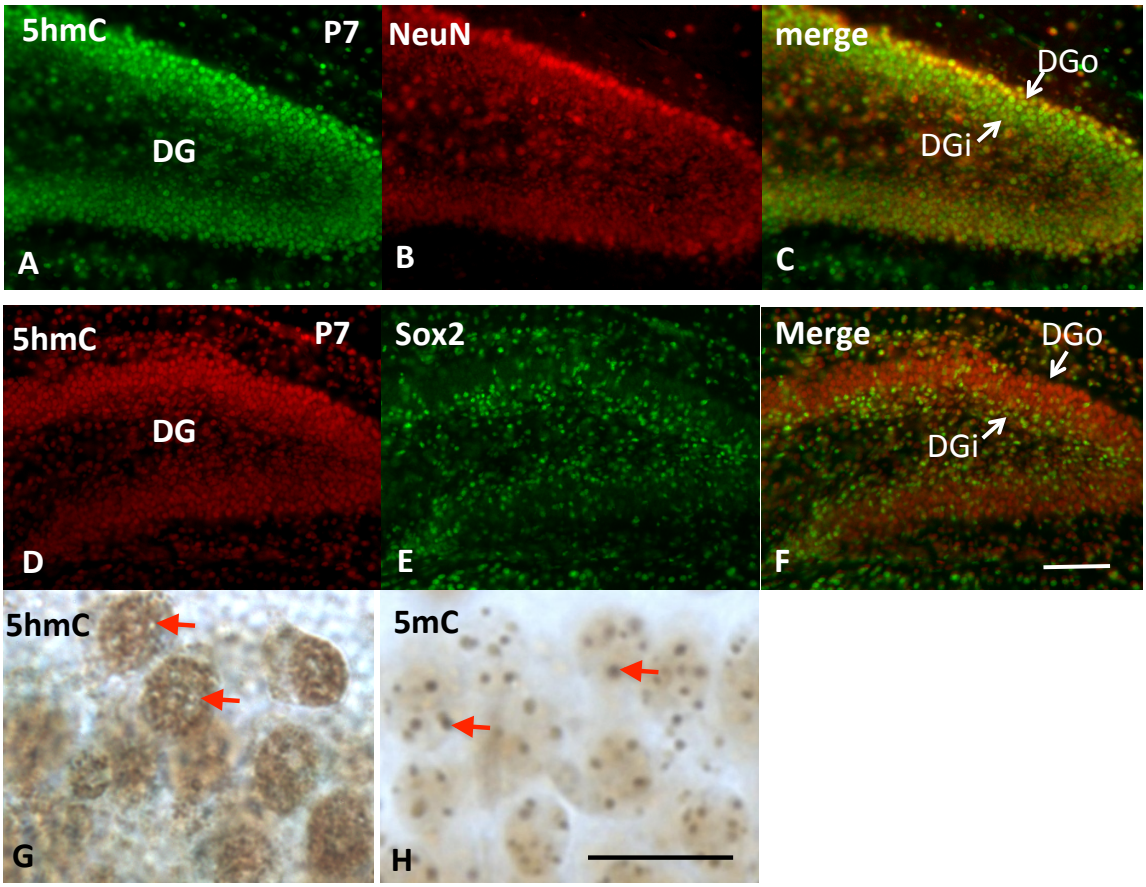

Figure 13. The association of 5hmC with neuronal maturation in P7 dentate gyrus and cortex. Neuronal maturation in postnatal dentate gyrus follows an *outside-in* pattern – neural progenitor cells were produced at inner granular layer (DGi); as cell mature, they migrate towards outer granular layer (DGo) to become mature neurons. The immune-fluorescent double staining reveals that the 5hmC accumulated in matured neuron as indicated by co-localization with NeuN (A,B,C), but not in neural progenitor cells marked with Sox2 (D,E,F) in P7 dentate gyrus. In the mature pyramidal cells (G and H), the 5mC and 5hmC become chromatic separated in the nucleus (bright field). The 5mC is packed in large granule (H, arrow) and co-localized with DAPI dense area (not shown) in chromatin, while 5hmC is distributed in DAPI-light area complementary to the 5mC+ area (G, arrow). DGo: dentate gyrus outer layer; DGi: dentate gyrus inner layer. Scale bar: A-F=100μm; G, H=50μm.

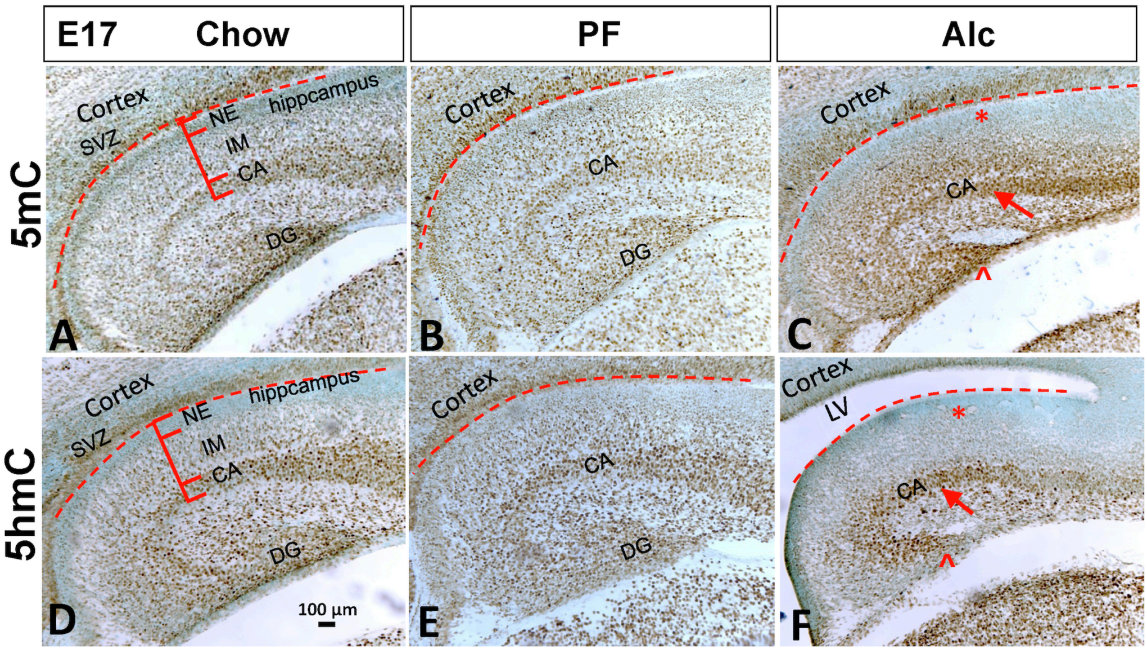

Figure 14. Alcohol altered DNA methylation while retarded the hippocampal formation at E17. Alcohol exposure reduced hippocampus size (C,F) as compared to that of Chow (A,D) and PF (B,E) (see quantitation in Table 1), increased lateral ventricle space, and diminished dentate gyrus size (C,F, arrowhead; also see Table 1). Alcohol exposure expanded NE thickness (see quantitation in Table 1), reduced both 5mC-im and 5hmC-im in NE (C, F, stars), while increased both 5mC-im and 5hmC-im in CA (C,F, arrow) (see quantitation in Table 2). There was no significant different between PF and Chow. Dotted line: separation of SVZ of cortex from NE of hippocampus. LV: lateral ventricle; SVZ: subventricular zone. NE: neuroepithelium; IM: intermedium zone; CA: Conus Ammonis; DG: dentate gyrus. Scale bar: all=100µm.

Table 2. DNA methylation immunostaining (im) intensity in E17 hippocampus.

	a. NE		
E17	**Chow**	**PF**	**Alc**
5mC-im	168.1±5.3	152.1±3.2^	150.9±6.9*
5hmC-im	116.9±6.4	127.2±3.6	84.4±5.4*#
	b. IM		
5mC-im	139.9±5.7	132.3±9.8	159.0±5.3*#
5hmC-im	113.52±5.7	134±5.2^	112.1±4.8#
	c. CA1		
5mC-im	157.8±6.4	151.8±11.7	173.7±1.8*
5hmC-im	144.7±2.6	163.6±3.6^	167.1±3.0**

*P<0.05, **P<0.005 (Alc vs Chow); #P<0.05 (Alc vs PF); ^P<0.05 (PF vs Chow). N=4 each. Data presented as Mean±SEM. NE: neuroepithelial layer; IM: intermediate zone.

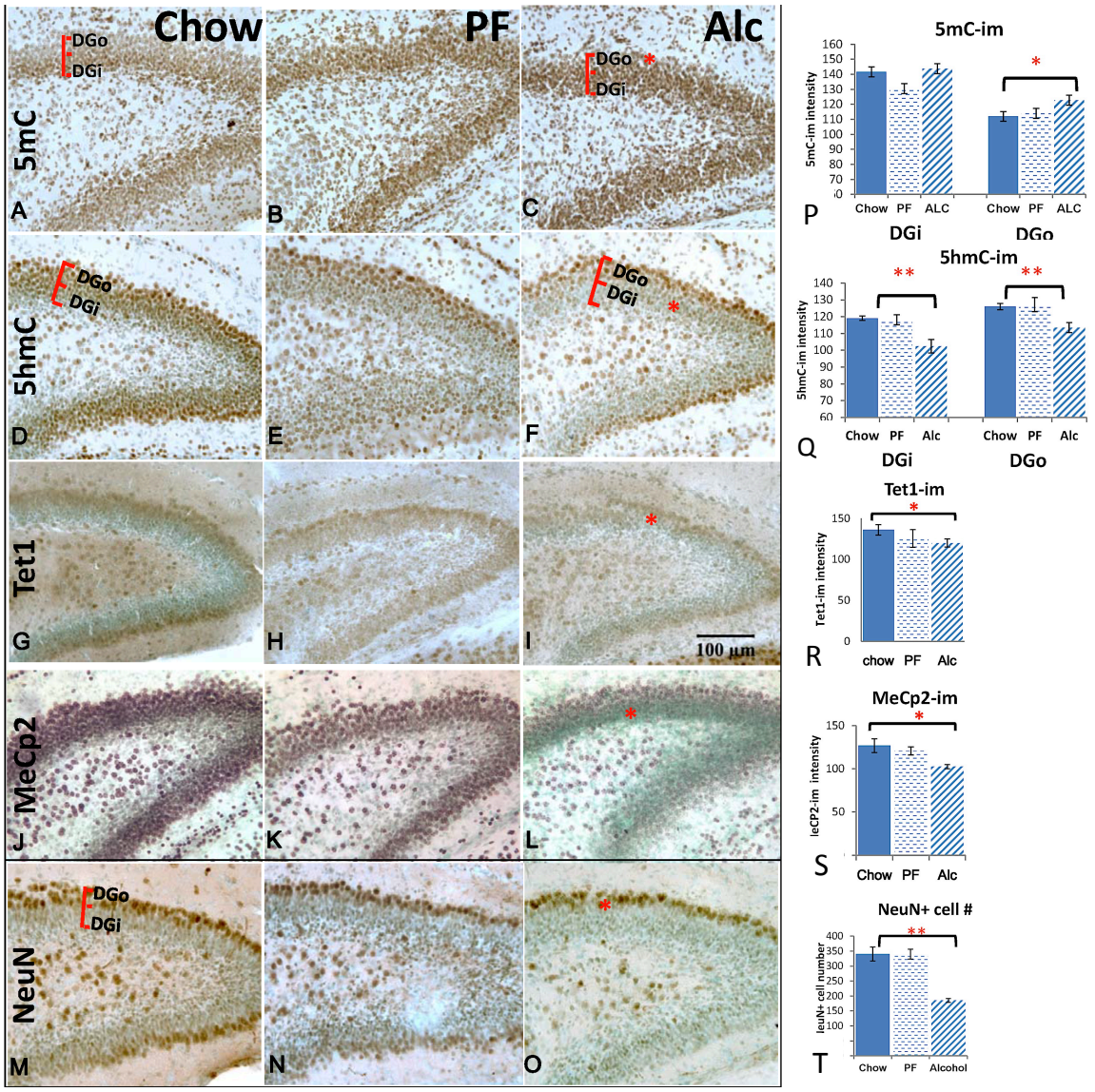

Figure 15. Neuronal maturation and DNA methylation in P7 dentate gyrus and the effect of alcohol. Immunostaining of DNA methylation marks: 5mC (A-C) and 5hmC (D-F), 5hmC-converting enzyme TET1 (G-I), DNA methylation binding protein MeCp2 (J-L) and mature neuronal marker NeuN (M-O). There is a methylation gradation correlating with the *Outside-In* pattern of neuronal maturation in DG (M), in which the 5mC-im decreases as granule cells mature (A: DGi>DGo), while 5hmC-im increases as granule cells mature (D: DGi<DGo). Alcohol exposure hindered the reduction of 5mC in DGo (C, star, also see quantitation in P) and the acquisition of 5hmC in both DGi and DGo (F, star, also see quantitation in Q), which is accompanied by delayed maturation of DG as indicated by decreased the NeuN+ cells (O, star, also see quantitation in T). There

is no significant difference between PF and Chow groups. The TET1 protein expression is closely associated with the expression of 5hmC (DGi<DGo), which is also reduced by Alc (I, see star, also see quantitation in R). The expression of MeCP2 (known to correlate with neuronal maturation) was also reduced by alcohol (L, star, also see quantitation in S). DGo: dentate gyrus outer layer; DGi: dentate gyrus inner layer. Scale bar: all=100μm.

2.3.3 Effect of alcohol exposure on DMP in the hippocampus and cortex

We noticed significant alteration in DMP after prenatal alcohol exposure in both hippocampus and cortex. First, in the (undifferentiated) hippocampal ammonic NE and the primary dentate NE layers at E17, there was a concomitant fall in the 5mC and 5hmC levels in Alc groups (Table 2a, Figure 14C). Outside the NE layer, in the migrating cells in intermediate zone (IM), the 5hmC-im was slightly reduced in Alc compared to PF groups (Table 2b). In the still developing CA1 region, the 5mC- and 5hmC-immunodensity were however higher in Alc groups, possibly due to pyramidal cells being more packed (reduced thickness of formed stratum pyramidale and reduced cell size) in the Alc groups (Table 2c).

The effect of alcohol exposure on DNA methylation in dentate gyrus continued till P7, even after ceasing of alcohol treatment after birth. In order to more closely analyze the methylation level in differentiating cells within the DG, the intensity of 5mC-im and 5hmC-im was measured separately in the inner granule layer (DGi) and the outer (shell of) granule layer (DGo) (Figure 15A-F). The 5mC-im was significantly higher (not entering the programmed reduction) in DGo (One-way ANOVA: $F(2,6)=5.26$, $P<0.05$) in the Alc group as compared with that of PF and Chow, while no difference was observed in DGi among treatments (One-way ANOVA, $F(2,9)=1.037$, $P>0.05$). The 5hmC-im was reduced significantly by alcohol in both DGo and DGi (One-way ANOVA: $F(2,6)=5.26$, $P<0.05$). Post-hoc analysis showed no difference between PF and Chow, however, the Alc group was significantly different from the other two (t-test, $P<0.005$).

Along with the alteration of 5mC and 5hmC, the TET1-im and MeCp2-im in DG were also altered upon alcohol treatment (Figure 15). The MeCp2-im was significantly reduced in alcohol treatment (one-way ANOVA: $F(2,12)=8.907$, $P<0.005$) (Figure 15J-L, see quantitation in S) followed by a Bonferroni post-hoc analysis determined that all three groups were significantly different from one another. TET1-im was also reduced in Alc group as compared to PF controls (t-test, $P<0.02$) (Figure 15G-I, see quantitation in R).

In the cortex at E17, alcohol increased both 5mC-im and 5hmC-im in the cortical plate (CP) (Appendix 2). In alcohol-treated cortex, the distance between subplate (SP) and cortical plate (CP) was reduced (more packed), accompanied by slightly increased 5mC-im and 5hmC-im (Appendix 2N). Alcohol significantly reduced the 5hmC-im in ventricular zone (VZ) of cortex (Appendix 2E,F arrow).

(Densitometry for the cortex part analyzed by Dr. Nail Can Ozturk)

In summary, alcohol altered the DNA methylation program in vivo, and correlated with a developmental delay in both hippocampus and cortex.

2.4 Discussion

2.4.1 Summary

In this aim, we demonstrated that there was a DNA methylation program (DMP) at the development window of hippocampal and cortical neurogenesis (see below). Chronic moderate prenatal alcohol altered this DNA methylation program in developing fetal hippocampus and cortex.

The DMP was precisely regulated in a temporal and spatial manner, along with the steps of neurogenesis. The multipotent neural progenitor cells (NPC) (located in SVZ and NE), possess extremely low DNA methylation marks until they ceased proliferation (lost Ki67 expression). Prior to embarking differentiation from a renewal state, the differentiating cells in NE acquired or drastically increase 5mC. The 5hmC escalated when cell migration begins in NE and continues its migration into intermediate zone (IZ). After settling in the final destination, the maturing neuronal nucleus kept gaining 5hmC while reduced 5mC (**illustrated in Figure 16**). The progression of the DMP is not synchronized to the age of the brain, but to the stage of differential state of each lineage of brain cells. In addition, the DMP not only progressed temporally, but also spatially as evidenced along the neuronal migration paths. This was supported by three different neurogenesis paths in both cortex and hippocampus (from ventricular zone to cortical plate in cortex, from neuroepithelium to CA in hippocampus and from subgranular layer to granular layer in dentate gyrus) (Figure 1). As such, each lineage of neuronal development occurred independently in its own temporal pace and spatial location followed the same DMP.

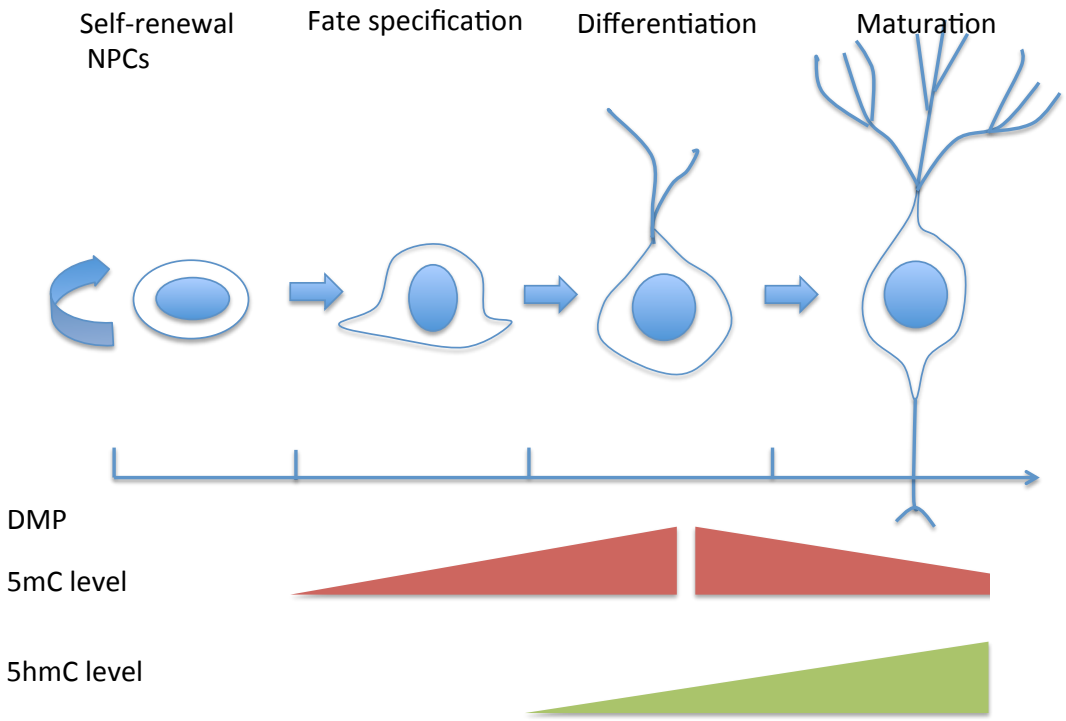

Figure 16. Schematic diagram of cellular DNA methylation program (DMP) during neurogenesis. Top panel shows cell state transition during neurogenesis, from proliferating neural progenitor cells (NPCs), to fate-determined neuroblasts, to differentiating and mature neurons. The according DMP is mapped in the bottom panel: cells gain 5mC at the beginning of cell specification, sequentially gain 5hmC at the beginning of cellular differentiation; both 5mC and 5hmC accumulated during neuronal differentiation and maturation; at later stage of neuronal maturation, cellular level of 5mC regresses, and 5mC become condensed to heterochromatic regions (see text for further details).

The functional aspect of the DMP is hinted by the coordinated translocation of the 5mC and 5hmC in association with the euchromatin and heterochromatin along the passage of the DMP. The 5mC started to appear as a fine granular DNA cluster in the nucleus, likely to silence the group of genes that maintain stem cell properties. The 5mC is translocated on the chromatin from sparsely distributed forms within nucleus in early differentiating cells to condensed granulated forms associated with heterochromatin. However, 5hmC is distributed mostly to euchromatin in mature neurons where neuronal specialization is settled and maintained. Increasing evidence indicates that 5hmC is sliding toward transcriptional activation or at least at bivalent states (Wu et al., 2011, Khare et al., 2012, Serandour et al., 2012). Besides the chromatic relocation, one more finding worthy of mentioning is that the maturing neuron gains the 5hmC mark, while reduces the 5mC mark, as shown from the migrating granule cells toward the settled granule layers. This is consistent with other reports that 5hmC accumulates in neurons during development and aging (Ruzov et al., 2011, Szulwach et al., 2011) and is supportive towards the role of 5hmC in neuronal maturation.

We further demonstrated chronic moderate alcohol exposure prior to and at the time of the peak neurogenesis in hippocampus and cortex led to significant alteration in DMP progress in the three neurogenesis lines described above. Alcohol exposure, through altering methyl donor metabolism, has been shown to affect global (Liu et al., 2009) and gene specific DNA methylation (Haycock and Ramsay, 2009). The 4% (v/v) alcohol we adapted reflected a moderate high chronic drinking paradigm in humans, with BAC reached 120-160mg/dL. Similar level of BAC during gestation stages in rat has been shown to induce spatial learning deficits and despair behaviors (Carneiro et al., 2005, Zink et al., 2011).

First, alcohol delays the acquisition of 5mC as well as 5hmC in neural progenitor cells in the NE. The embarking of DNA methylation (5mC and 5hmC) at the start of neural fate determination is likely associated with the silencing of stem cell maintaining genes, (e.g. *Pou5f1, Ddah2 reported being turned off during neuronal differentiation),* and the expression of neural differentiation genes (e.g. *Jag1* and *Tcf4* reported increased

during neuronal differentiation) (Cortese et al., 2011). Alcohol preventing the onset of gene methylation has been shown to retard the neural differentiation (Zhou et al., 2011a) or deviate towards glial properties (Schneider and d'Adda di Fagagna, 2012).

Another significant alteration is that in early maturing neurons (e.g. in the dentate granule layer), the intra-nucleus relocation of 5mC, as well as accumulation of 5hmC are hindered by alcohol exposure. Considering the diversification of 5mC and 5hmC during maturation in gene promoters and gene bodies (Flintoft, 2010, Jjingo et al., 2012), the functional results of alcohol exposure could be that it disrupts the recruitment of chromatin modifying enzymes, e.g. DNMT3, SIRT1, G9a (Lilja et al., 2013), and further alters associated gene transcription. The altered DNA methylation marks might also affect their binding protein to recruit transcription factors and other histone codes. Indeed, we have observed a reduced expression of MeCp2 in these early maturing neurons (Figure 15L).

Furthermore, we demonstrated a lasting effect of prenatal alcohol exposure on DNA methylation through newborn age (postnatal day 7 in mice). After alcohol exposure has been removed for the whole 3rd trimester period, we still observed significant DMP alteration in developing dentate gyrus. It might be explained by the biochemical inhibition of methyl-donor reactions, and/or the inhibition of de novo methyltransferase (DNMT3a) or the 5hmC-converting enzyme (TET1) from the previously exposed alcohol. These epigenetic discrepancies appear to line up after neurons are matured (e.g. pyramidal neurons at P21), when differentiation processes are completed. These results indicate that alcohol affected DNA methylation during active differentiation and its influence on methylation machinery is likely contributing to the dys-regulated developmental processes in the hippocampus and cortex as noted by many reports (Veazey et al., 2013, Elibol-Can et al., 2014, Mantha et al., 2014).

2.4.2 The role of DMP in neurogenesis and other epigenetic mechanisms

DNA methylation regulates gene expression by recruiting proteins involved in gene repression or by inhibiting the binding of transcription factors to DNA (Bird, 2002). During development, the pattern of DNA methylation in the genome changes as a result of a dynamic process involving both de novo DNA methylation and demethylation. As a consequence, differentiated cells develop a stable and unique DNA methylation pattern that regulates tissue-specific gene transcription (Smith and Meissner, 2013).

So far, there has been several evidence on DNA methylation pattern dynamics upon cellular differentiation reported. For example, in the process from pluripotent embryonic stem (ES) cells becoming multipotent stem cells, DNA methylation restricts the potential of multipotent stem cells to a particular lineage (mesodermal, endodermal, or ectodermal), yet still possesses the potential to differentiate into distinct somatic cell types with appropriate stimulation (Sorensen et al., 2010, Hupkes et al., 2011). This process was hypothesized to be regulated by pluripotent gene (*Nanog, Oct4, Sox2*) promoter hypermethylation in progenitor stem cell states (Barrand and Collas, 2010, Christophersen and Helin, 2010). Another example lies in the process from pluripotent/multipotent states to the lineage-committed states (differentiated cells, e.g. blood cells, muscle cells or neurons). Genome-scale maps of DNA methylation indicated that the pattern of DNA methylation differed between pluripotent cells and lineage-committed cells (Meissner et al., 2008, Kim et al., 2014). In addition, neurons and non-neuronal population in post-mitotic brain also possessed different DNA methylation profile (Iwamoto et al., 2011).

However, little has been known on DNA methylation dynamics at specific steps of neurogenesis, for example, at the time of neural fate specification or at the time of initiation of differentiation. We hypothesized that each of these steps was highly orchestrated with intrinsic transcription factors expression which were influenced by the DNA methylation dynamics. Indeed, we reported here, for the first time, a temporally and spatially regulated DNA methylation program (DMP) during neurogenesis, and that the

initiation of certain neurogenesis steps was accompanied by escalation or reorganization of particular DNA methylation marks in according cells.

Other epigenetic mechanisms (e.g. histone modifications) have been shown to be important in neurogenesis as well (Covic et al., 2010, Ma et al., 2010, Sun et al., 2011). Similar to DNA methylation, there is also a genome-wide chromatin state reorganization during cellular differentiation (Mikkelsen et al., 2007, Rando and Chang, 2009, Zhu et al., 2013). The enzymatic group of proteins responsible for chromatin remodeling, the Polycomb group (PcG) and Trithorax group (TrxG) proteins, are necessary and properly regulated during neurogenesis, and are shown to have crosstalk with DNA methylation at multiple steps of transcriptional regulation (see review (Jobe et al., 2012)).

However, less is known about what is the main player in the epigenetic cascade. For example, is the dynamic DNA methylation leading the remodeling of chromatic states, or the chromatic state first permits the DNA methylation modifiers (DNMTs and TETs) to according DNA sites thus allowing DNA methylation dynamics to happen. Evidence exists for both ways, and is largely dependent on the stage of the cells. Many lines of evidence suggest that DNA methylation regulates the timing of differentiation and maintenance of the cell-type identity, while histone modification provides the "bivalency" to the extracellular information input (Lunyak and Rosenfeld, 2008, Jobe et al., 2012). We demonstrated here, that the DNA methylation itself is an organized program, correlating with the course of neurogenesis. However, whether histone modification and chromatin structure parallel with DNA methylation program, and whether alcohol exerts its effect independently on each process or target both synergistically required further investigation. Thus, in the following aim, I will look into how alcohol exposure alters the chromatin structure in accordance to DNA methylation program.

2.4.3 Alcohol-induced delay of the DMP is accompanied by the developmental delay

Although alcohol affected the methylation program that correlated with a developmental delay in hippocampus *in vivo*, whether the aberrant DNA methylation program leads to the developmental delay remains to be seen. However, studies showed that ablation or overexpression of methylation machinery, e.g. DNMTs or TDG proteins, was embryonically lethal (Li et al., 1992, Biniszkiewicz et al., 2002, Cortazar et al., 2011). Mutations in methyl-binding proteins and imprinting genes are related to the onset of developmental deficits such as Rett syndrome, Angleman syndrome and Prader-Willi syndrome (Kishino et al., 1997, Amir et al., 1999, Ohta et al., 1999). Our previous studies showed that 5-AZA, a DNMT inhibitor, retarded the embryonic growth during early neurulation (Zhou et al., 2011b). Similarly, choline deficiency (lack of methyl donor) reduced global DNA methylation, and gene-specific methylation at the neuroepithelium in mice hippocampus, and impairs memory performance (Niculescu et al., 2006). On the other hand, supplementation of choline can improve the developmental deficits in hippocampus neural systems (Ryan et al., 2008, Thomas et al., 2009, Monk et al., 2012). Therefore, it is plausible that dysregulation of DNA methylation mediates the alcohol teratology in FASD model. Ongoing studies are investigating the DNA methylation changes and transcription at the gene level. Nevertheless, this study provides the first demonstration that the DNA methylation program is an upstream-orchestrated order during hippocampal and cortical neurogenesis, and alcohol disrupts the intricate order while retards the normal development.

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CHAPTER 3: AIM 2

Chromatin Remodeling During Neural Differentiation and Maturation and the Effect of Prenatal Alcohol Exposure

3.1 Background and Hypothesis

3.1.1 Chromatin remodeling in neurogenesis

Neurogenesis is a process characterized by regulated progression of cellular states, resulting from dynamic changes in gene expression programs. These changes in gene expression are initiated by rapidly changing environmental cues, which affect cellular states by altering chromatin structure and gene transcription. Both DNA methylation and histone modifications are capable of altering chromatin structures that may play a role in coordinating this highly regulated process.

It has been well recognized that changes in specific histone modifications is in close association with chromatin remodeling. For example, the H3K4me3 is found at virtually all active transcriptional start sites (TSS) (Schneider et al., 2004), while H3K27me3 enrichment occurs in genes that are transcriptionally repressed by polycomb proteins (the polycomb group proteins belongs to enzymatic protein families that produce the H3K27me3 modification to the histone) (Cao et al., 2002). Notably, the histone modifications have the capacity of providing a "bivalent" state for the transcription of genes. For example, in pluripotent ES cells, lineage-committed genes are in a transcriptionally poised state and show bivalent histone modifications: both active H3K4me3 and repressive H3K27me3 (Bernstein et al., 2006). On lineage commitment, genes controlling a distinct cell lineage maintain H3K4me3, remove H3K27me3, and become activated. Genes controlling other lineages maintain H3K27me3, gain H3K9me3 and 5mC, and become fully silenced (Mikkelsen et al., 2007).

However, the role of DNA methylation in chromatin remodeling still remains illusive. It is believed that DNA methylation-mediated chromatin remodeling is

associated with methyl-CpG-binding proteins and their downstream effector proteins that alter chromatin structure. For example, MeCp2 was shown to recruit HDACs as well as the histone H3 lysine (K) 9 methyltransferase, SUV39H1, to the promoter of *Bdnf* gene for active chromatin remodeling and subsequent silenced its expression (Martinowich et al., 2003). In addition, it was also shown that the DNA methyltransferase could directly interact with histone methyltransferase thus building a link between the DNA methylation and histone modifications. For example, the G9a enzyme (catalyzes H3K9 dimethylation) is responsible for recruiting DNMT3A and DNMT3B, thus producing *de novo* DNA methylation in ES cell (Tachibana et al., 2008).

Recently, the discovery of 5hmC added one more player to the chromatin remodeling machinery. The 5hmC and 5mC differed in their genomic locations: the 5hmC is enriched in the TSS and the gene body close to regions of H3K4me2; while 5mC is preferentially localized in the promoter CpG-rich regions of the gene, associated with H3K9me3 (Pastor et al., 2011, Lister et al., 2013). The 5hmC has been shown to interact with MBD3 and the NURD (Nucleosome Remodeling and Deacetylase) complexes that mediate chromatin remodeling (Yildirim et al., 2011).

However, critical questions about the significance of the two forms of DNA methylation (5mC and 5hmC) in neuronal lineage determination and neural development remain unanswered. For example, it is still unclear whether 5mC- and 5hmC-dependent changes in chromatin structure are associated with the cellular state transitions of neurogenesis, and whether the 5mC- and 5hmC- mediated chromatin remodeling has functional consequences that is altering gene transcription ability, and whether this machinery is subject to environmental input (e.g. alcohol) during critical stages of neurogenesis.

The major limitations for investigating these important questions have been 1) the heterogeneity of the cell population to analyze, and 2) the lack of use of *in vivo* models for the study of epigenetics. To date, most of such studies investigating chromatic protein interactions or DNA-histone interactions have been conducted *in vitro* with embryonic or neural stem cell cultures that culturally went through differentiation (Pastor et al., 2011,

Wu et al., 2011, Yildirim et al., 2011). Several *in vivo* studies (Szulwach et al., 2011, Mellen et al., 2012) isolated cell population from whole brain regions of cerebellum and hippocampus, however cannot represent the cell stage progression during neurogenesis. This limitation can be overcome by utilizing antibody-based detections for chromatic interactions during neurogenesis *in vivo.* Different stages of cells can be discriminated by their phenotypic markers as well as their brain-wise locations. We developed a quantitative analysis method measuring co-localization of individual epigenetic markers based on confocal images. It will facilitate the investigation of the dynamic nature of chromatin remodeling during neurogenesis.

3.1.2 Hypothesis

In the previous aim, we demonstrated that there was a programmed reorganization of DNA methylation (5mC and 5hmC) within chromatic regions along with neural differentiation, that is, 5mC become reorganized in DAPI-dense heterochromatic regions, whereas 5hmC become relocated to DAPI-light euchromatic regions in the nucleus (Figure 13). We hypothesize that the reorganization of DNA methylation plays a role on chromatin remodeling during neural differentiation and maturation. We further hypothesize that alcohol exposure at critical neural development window disrupts the programmed re-organization which could potentially alter the ability for neural gene transcription.

In this aim, we attempt to elucidate 1) the epigenetic program underlying chromatin remodeling (both DNA methylation and histone modification) during embryonic neurogenesis *in vivo*; and 2) how the development-regulated epigenetic program was affected by *in utero* alcohol exposure at critical period of neurogenesis. To answer these questions, we utilized antibody-based fluorescent double staining method. With the assistance of confocal microscopy, we investigated the co-localization of epigenetic marks (DNA methylation, histone modifications and chromatin states) in the nucleus along steps of neurogenesis in the cortex and hippocampal dentate gyrus. The co-localization was further confirmed with FLIM-FRET for physical interaction. We chose two stages of neurogenesis, from early neuronal differentiation stage (at E17) to early

maturation stage (P7). These two stages are marked by prominent changes in the 5mC and 5hmC distribution as we demonstrated in the previous aim (reorganization and regression of 5mC, while escalation of 5hmC). We further elucidated the epigenetic program in relation to the transcription machinery by co-localizing epigenetic marks with RNA polymerase II (*Pol*II).

3.2 Methods and Material

3.2.1 Immunohistochemistry

We used brain samples from the same groups of animals as illustrated in Aim 1. Chow, PF and Alc groups were processed the same as described in Aim 1 and were prepared and stained in parallel. For fluorescent double staining, samples were permeabilized with 1% TritonX-100 in PBS for 30 min followed by 2N HCL for 30 min, and then neutralized in Tris-HCl (PH 7.4) for 10 min. Sections were blocked using a goat kit containing 1.5% normal goat serum, 0.1% Triton X-100 in PBS for 1 hour, then incubated with primary antibodies. Antibodies used were against DNA methylation marks 5mC (1:2000, mouse monoclonal; Eurogenetec, Fremont, CA), 5hmC (1:3000, rabbit monoclonal; Active Motif, Carlsbad, CA; The specificity of the crucial anti-5hmC antibody is characterized by pre-absorption of anti-5hmC antibody with 5hmC (data not shown), MeCP2 (1:1000, rabbit monoclonal; Cell Signaling, Danvers, MA), MBD1 (1:500, Millipore, Billerica, MA), MBD3 (1:1000, Santa Cruz Biotechnology, Dallas, Texas); histone marks, trimethyl-H3K4 (H3K4me3, rabbit monoclonal, 1:1000, Millipore, Billerica, MA), trimethyl-H3K9 (H3K9me3, 1:500, Millipore, Billerica, MA), trimethyl-H3K27 (H3K27me3, 1:500, Millipore, Billerica, MA); and anti-serine-5 phosphorylated RNA polymerase II (*Pol*II) antibodies (rabbit, 1:1000; Abcam, Cambridge, MA). Primary antibody was diluted in PBS containing 0.1% Triton X-100 and 1.5% normal goat-serum. After three washes with PBS, samples were then incubated with secondary antibodies with fluorescent dye (Alexa 488, 546, or 633) for 1.5 hrs at room temperature. Slices were covered with anti-fade mounting solution with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen). Double-immunocytochemical staining was performed between 5mC and 5hmC, and the 5mC with MBD1, MeCP2, H3K9me3,

H3K4me2, or serine-5 phosphorylated *Pol*II; and the 5hmC with MBD3, H3K9me3, H3K4me2, or serine-5 phosphorylated *Pol*II.

3.2.2 Image acquisition

Confocal fluorescence images were obtained by an Olympus FV1000-MPE Confocal Microscope (Olympus America Inc., Center Valley, PA), mounted on an Olympus IX81 inverted microscope stand with a 60x water-immersed objective lens. Sequential excitation at 488 nm and 559 nm was provided by argon and diode lasers, respectively. Emissions were collected by spectral detectors in channels one and two with user-specified min and max wavelengths. A third channel, collecting fluorescence excited at 405nm was used for the detection of DAPI. Z-stack images were collected over a thickness of 4.5 μm with 0.3 μm step intervals. Laser intensities and gain values were adjusted to prevent saturation and to reduce the background noise. The same setting was used over the same pair of staining at different brain locations and ages. After sequential excitation, green and red fluorescence images of the same cell were saved and analyzed by Olympus Fluoview FV10-ASW software (Olympus Corporation 2003-2008).

3.2.3 Co-localization analysis

For visualization of co-localization of confocal images, different channels of images were overlaid at the same Z-plane. An overlay of green and red would give rise to yellow hotspots where the two molecules of interest were present in the same pixels. Quantification of co-localization was performed with intensity correlation coefficient-based method using an ImageJ plugin JACoP (http://rsb.info.nih.gov/ij/plugins/track/jacop.html). Methods were adapted from (Bolte and Cordelieres, 2006). Pearson's Coefficients (r) were collected for the area of interest in nuclei. The value of r could range from 1 to -1, with 1 denoting complete positive correlation, -1 for negative correlation, and 0 for no correlation. The value of M could vary from 0 to 1, with 0 corresponding to non-overlapping images and 1 reflecting 100% co-localization. Each individual cell was outlined by ImageJ selection tool and measured at the same threshold settings. Only the nuclei with +10% of the full diameter at section

were chosen for the analysis. For each pair of staining, ~20 cells per staining were measured respectively from 3 animals per age, and presented as Mean ± SEM. Statistical analyses legitimately evaluating co-localization adopted from McDonald and Dunn (McDonald and Dunn, 2013) were performed with ANOVA for groups or Student's T-test for pair comparisons.

3.2.4 Fluorescence Lifetime Imaging based Forster Resonance Energy Transfer (FLIM-FRET) analysis

The Immunocytochemistry procedure was done similar to the one described above. In brief, formalin fixed brain tissue section was used. The sections were washed in PBS 3 x 5 minutes, then permeabilized using Triton-X 1% in PBS for 30 minutes on a shaker. The first antibody (5mC or 5hmC) was labeled with Alexafluor 488 conjugated secondary antibody at 1:500 dilution in nomal goat serum, on a shaker for 90 minutes, washed for 3 (3x5 min;3x15 min; 4x30 min) hours. Tissue was then imaged and fluorescence lifetime was measured at the cortex and hypocampus area. The same tissue section was washed for 30 minutes on a shaker, and then incubated with a pair of primary antibody (MBD1, MBD3, or MeCP2) for 18 hours, and labeled with Alexa fluor 546 conjugated secondary antibody at 1:500 dilution for 90 minutes, and mounted in the imaging chamber for imaging. For each methylation (5hmC, 5mC)-protein (MBD1, MBD3) interaction 50 cells are used from ~8 cells /setion of hippocampal or cortical region of 4 P7 brains. Total, 400 cells were used for FLIM-FRET analysis. Methods for FLIM-FRET experiments can be found in Appendix 3.

(*This part of study was performed by Nur P. Damayanti from Dr. Joseph Irudayaraj lab in Purdue University)*

3.3 Results

3.3.1 Chromatin dynamics during neural differentiation

The 5mC and 5hmC were initially diffusely distributed in the nucleus in immature cortical plate neurons at E17 as indicated by confocal microscopy (Figure 17). The degree of co-localization was indicated by Pearson's Coefficient (r) analysis (r~1 means high co-localization, r~-1 means little to no co-localization) (see Methods). At E17, the 5mC and 5hmC showed high co-localization ($r=0.60\pm0.06$). As the immature cortical plate neurons progressed to differentiation and maturation at postnatal day (P) 7, the 5mC and 5hmC progressively decreased their co-localization ($r= 0.22\pm0.06$) and were finally found in complementary chromatin compartments and associated with varying chromatin states in the nucleus. Specifically, the 5mC-im distributed in 4',6'-diamidino-2-phenylindole (DAPI)-dense aggregates of heterochromatin regions (~0.5-1.5µm) is completely void of 5hmC-im. The 5hmC-im is solely distributed in fine, DAPI-sparse euchromatic regions (<0.3µm), which also contain small amount of 5mC (Figure 17). Chromatin remodeling is revealed by the co-localization analysis over a period of time. The mosaic distribution of the 5hmC and 5mC is also demonstrated by their association with DNA methylation binding proteins and histone codes associated with gene transcription states shown below.

3.3.2 Differential Binding Partners of 5mC and 5hmC

The 5mC and 5hmC are differentially associated with methyl-CpG binding proteins (MBDs) in neurons in the developing cortex and hippocampus. By confocal microscopy, double staining revealed a distinct partnership between MBDs and the 5mC and 5hmC. The 5mC preferentially co-localized with the MBD1 in both immature ($r= 0.70\pm0.03$) and mature cortical neurons ($r=0.82\pm0.03$). In the mature neurons, the MBD1 displayed heterochromatic aggregates that were co-localized with 5mC (Figure 18D,E,F). In contrast, the 5hmC is highly co-localized with MBD3 in cortex ($r=0.65\pm0.02$), and displayed euchromatic distribution co-localizing with 5hmC (Figure 18G,H,I).

In addition, the co-localization of 5mC and its binding partners evolved as the brain developed. The MeCp2 were initially partially co-localized with 5mC (r=0.28±0.02) and homogeneously distributed in DAPI-sparse regions in immature neurons at E17, however became highly co-localized with 5mC (r=0.82±0.01) and translocated to mostly heterochromatic aggregates in the cortex (Figure 19A-H). In contrast, the MeCp2 had little co-localization with 5hmC at both ages (r=0.30±0.02) in cortex (Figure 19I-P). The MeCp2 to 5mC co-localization was found in the large DAPI-dense aggregates as well as in the fine DAPI-sparse chromatin particles in the differentiated neurons (Figure 19E-H).

To better enumerate and validate the binding partners, DNA methylation-MBD interaction in real-time, Fluorescence Lifetime Imaging based Forster Resonance Energy Transfer (FLIM–FRET) approach was utilized for precise evaluation of the interactions. We demonstrated that there is low interaction between 5hmC and MBD1 in either hippocampus (FRET efficiency 2.98%) or cortex (FRET efficiency 1.11%) (Appendix 4A). In contrast, strong interaction between 5mC and MBD1 (FRET efficiency 8.57% and 9.65%) (Appendix 4B) and between 5hmC and MBD3 (FRET efficiency 7.68% and 8.40%) in both hippocampus and cortex (Appendix 4C) were noted. *(Data collected and analyzed by Nur P. Damayanti)*

3.3.3 Differential association with specific histone codes and transcription site of 5mC and 5hmC

The double-staining of DNA methylation with histone codes indicated that 5mC favorably co-localized with H3K9me3 (r=0.76±0.01) and with H3K27me3 (r=0.61±0.02), which were mostly located in the DAPI-dense aggregates (Figure 20A-F). On the contrary, 5hmC did not co-localize with H3K9me3 or H3K27me3. Instead, 5hmC co-localized with H3K4me2 (r=0.87±0.01) in the DAPI-sparse regions, while a lack of co-localization between 5mC with H3K4me2 was noted (Figure 20G-I). The divergent 5mC and 5hmC co-localization with histone codes was independent of the development stage.

The differential role of 5mC and 5hmC was summarized by their association with the *Pol*II transcription enzyme for transcription initiation (serine-5 phosphorylated *Pol*II) (Cho et al., 2001, Cheng and Sharp, 2003). A strong co-localization of 5hmC with *Pol*II was shown during immature neuronal stage (E17) ($r=0.75\pm0.01$) and slightly decreased in more matured (P7) neurons ($r=0.58\pm0.01$) as demonstrated in the cortex (Figure 21E-H,M-P). The co-localization between 5mC and *Pol*II was transient and dissociated as neuronal differentiation progressed at P7 ($r=0.17\pm0.01$) (Figure 21A-D, I-L).

3.3.4 The effect of alcohol

The effect of alcohol on the co-localization of 5mC and 5hmC with chromatin modifiers was indicated by the Pearson's coefficient value (Figure 22). Alcohol partially prevented the programmed separation of 5mC and 5hmC in P7 cortical neurons. In the alcohol-treated groups, the co-localization between 5mC and 5hmC were higher than normal ($r=0.32\pm0.08$ as compared to $r= 0.22\pm0.06$ in control), indicating a delayed chromatic separation of these two marks. In addition, alcohol significantly reduced the co-localization of 5hmC to H3K4me2 ($r=0.72\pm0.02$ as compared to $r=0.87\pm0.01$ in control) in maturing dentate granule cells (P7). Furthermore, alcohol significantly reduced the co-localization of MeCP2 with 5mC ($r=0.70\pm0.03$ as compared to $r=0.82\pm0.01$ in control) in mature cortical neurons, as well as MeCp2 with 5hmC ($r=0.19\pm0.02$ as compared to $r=0.30\pm0.02$ in control).

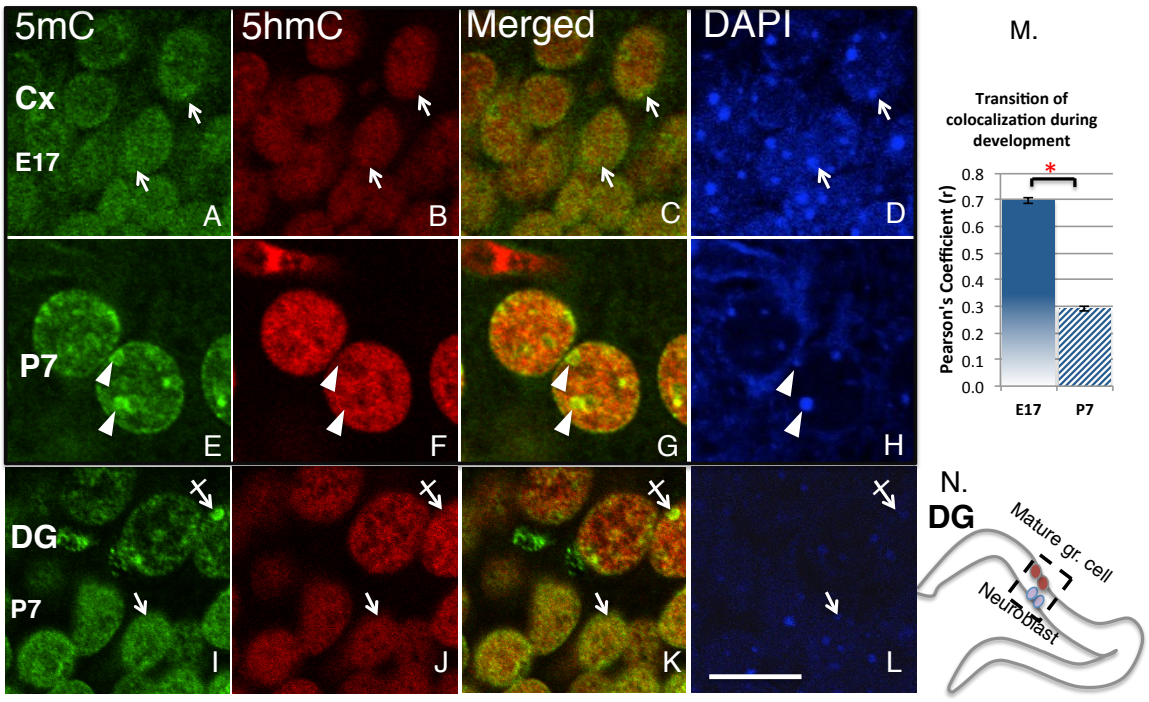

Figure 17. Chromatic re-organization of 5mC and 5hmC during neuronal maturation from young (E17) to mature (P7) in the cortex. In the developing cortex, both 5mC and 5hmC were diffusely distributed in undifferentiated cells and were highly co-localized at E17 (see quantitation in M). However, they became polarized into different compartments (C, F, G; arrows) as the neuron differentiated at P7. The 5mC became associated with DAPI-dense heterochromatic region while 5hmC with DAPI-light euchromatic region (E-H, arrowhead). Pearson's coefficient (r) was shown in chart m. **P<0.005. Similar remodeling was found in two stage of cells P7 dentate gyrus (DG; I-L), where undifferentiated (in inner layer, see N) and differentiated cells (in outer layer) are shown in different layer (see N). The 5mC and 5hmC were co-localized in younger future granule cells (I-L, arrows) but parted in different compartments (I-L; crossed arrow) in matured granule cells. Scale bar: A-L=10 um. All staining is within nuclei.

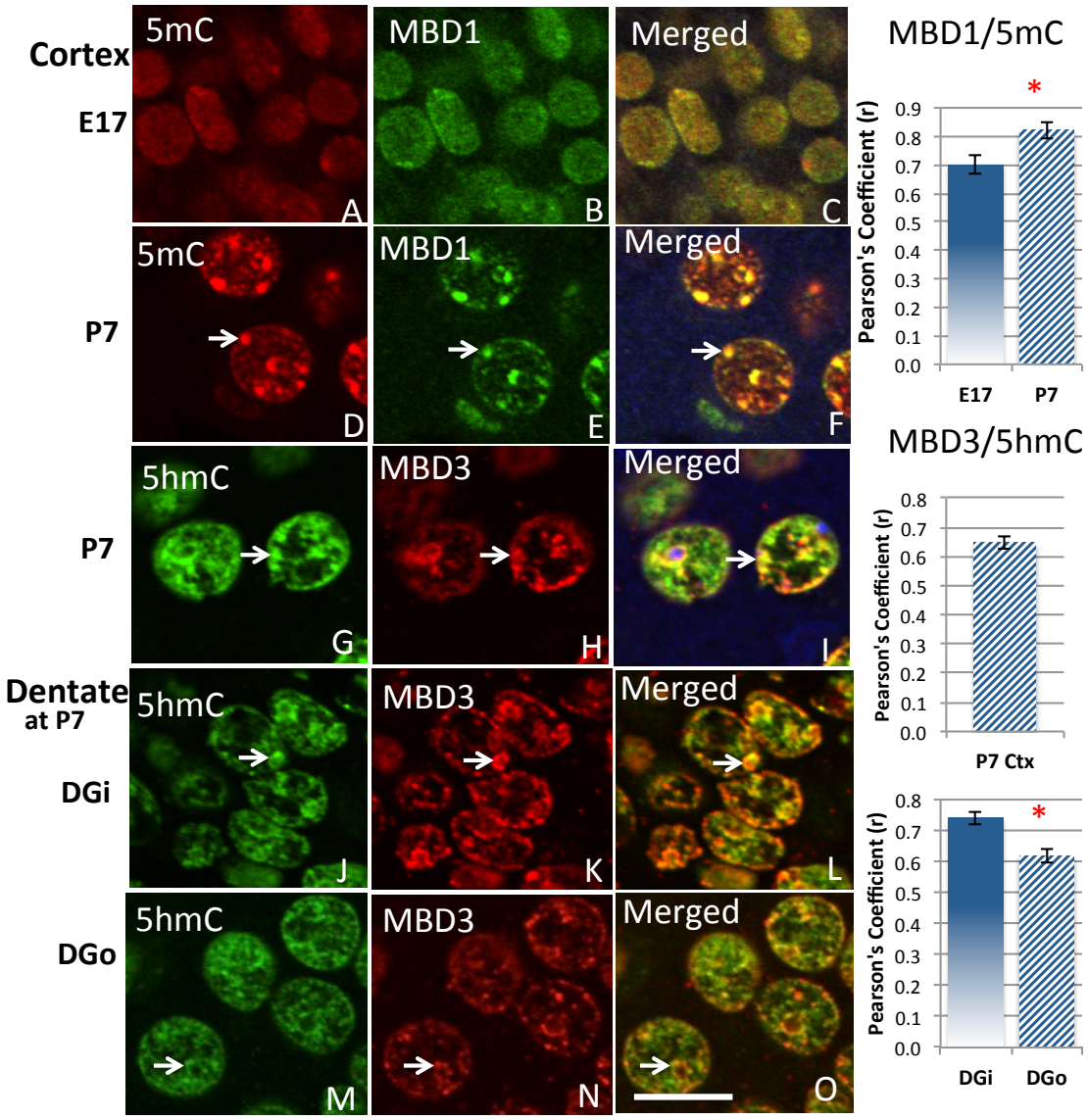

Figure 18. Differential DNA methylation binding protein partners and their co-translocation during chromatin remodeling during neuronal maturation. Two-color fluorescent confocal microscopy shows that the 5mC (red) predominantly co-localized (A, red) with MBD1 (B, green) initially in the homogeneous small punctate form at nuclei of young E17 neurons at layer IV of cortex (Ctx) (C). Their co-localization increased when cell became more differentiated at P7 and both co-localized in the heterochromatin aggregates (D-F, arrows). The 5hmC (G, green), on the other hand, co-localized with MBD3 (H, red) at both E17 and P7 (G-I, Pearson's Coefficient is shown in Q) in the cortex and in the dentate gyrus (R). They preferentially located to homogeneous euchromatic region (DAPI sparse) as cell matured (M-O). Scale bar: All=10um. The

Pearson's Coefficient analyses of co-localization are shown on P-R. DGi: Dentate gyrus inner layer; DGo: dentate gyrus outer layer.

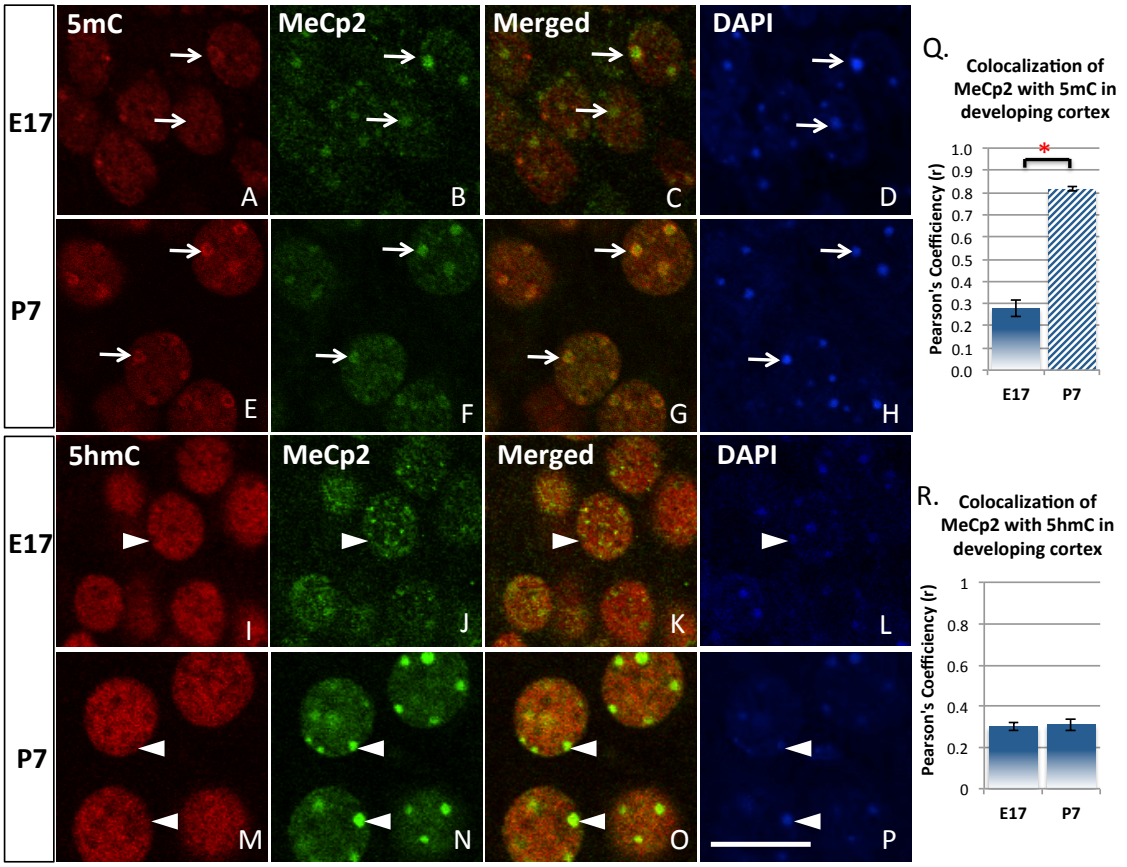

Figure 19. The transitional association of MeCP2 with 5mC and 5hmC during neuronal maturation. All images are from cortical neurons. Nuclear distribution of methyl-binding protein 2 (MeCP2) is homogeneous and mildly associated with 5mC (A-D) at E17 but greatly increased at P7 in cortex (E-H). Pearson's Coefficient (r) for co-localization was shown in the chart Q. In contrast, the MeCp2 is less co-localized with 5hmC at both E17 (I-L) and P7 (M-P). Pearson's coefficient for co-localization was shown in chart R. Scale bar: All=10 µm. It is shown that MeCP2 (J,N; arrowheads) are mostly distributed at DAPI-dense region with heterchromatin (L,P; arrowheads) where 5hmC is usually absent (I,M; arrowheads). Scale bar: all=10µm.

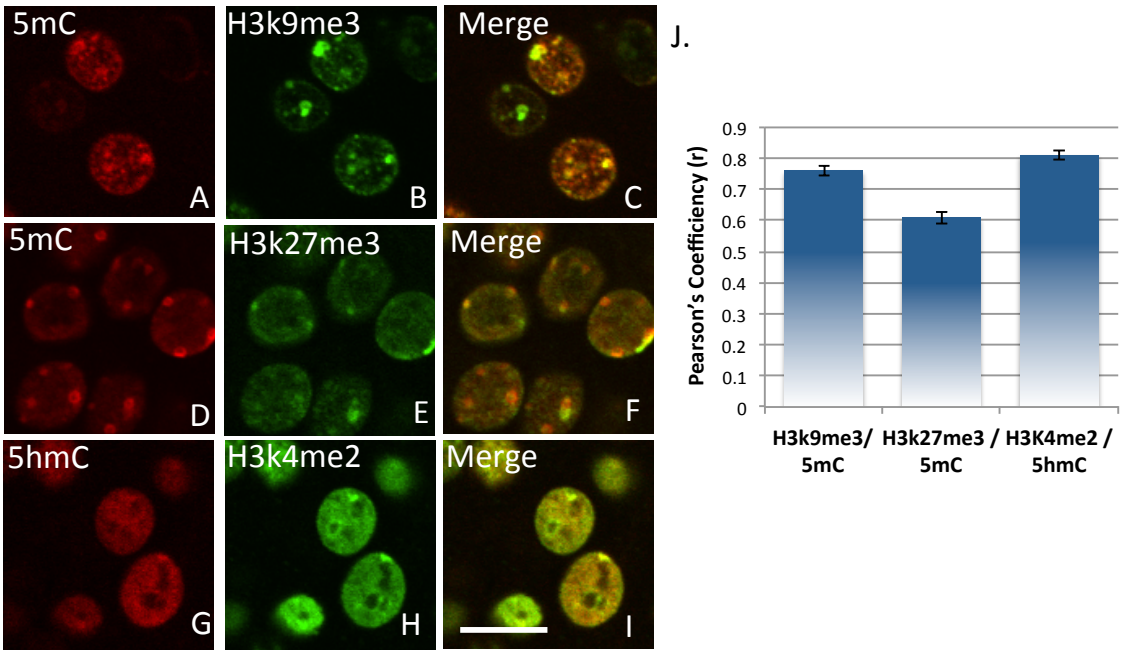

Figure 20. Differential co-localization of 5mC and 5hmC with histone marks in P7 cortical neurons. The 5mC (A and D, red) is closely co-localized with repressive histone marks H3K9me3 (B, green) (merged in C) and H3K27me3 (E, green) (merged in F). In contrast, the 5hmC (G, red) is highly co-localized with the activating histone mark, H3K4Me2 (H, green) as demonstrated in the merged image (I). Pearson's coefficient is shown in chart J.

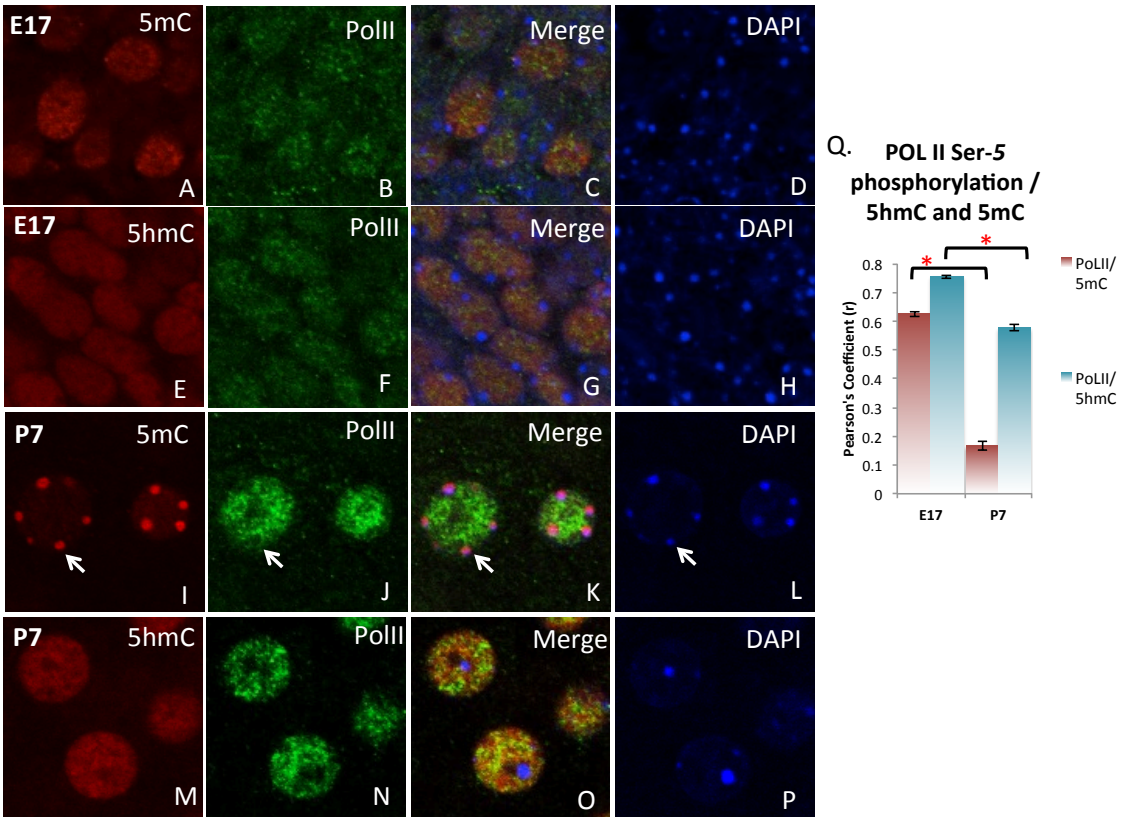

Figure 21. Differential co-localization of *Pol* II with 5hmC over 5mC during neuronal maturation in the cortex. At stage of early differentiating (E17), both 5mC (A-D) and 5hmC (E-H) are associated the *Pol*II. As neurons mature (P7), *Pol*II dissociates with 5mC (I-L, and Q), while specifically associates with 5hmC (M-P, and Q). The 5hmC (red) and *Pol*II (green) sites are all in the euchromatic regions exclusive from DAPI stained heterochromatic region (blue). Pearson's analysis of co-localization of DNA methylation marks and *Pol*II indicates that the co-localization between 5mC and *Pol*II diminished upon neuronal maturation (Q).

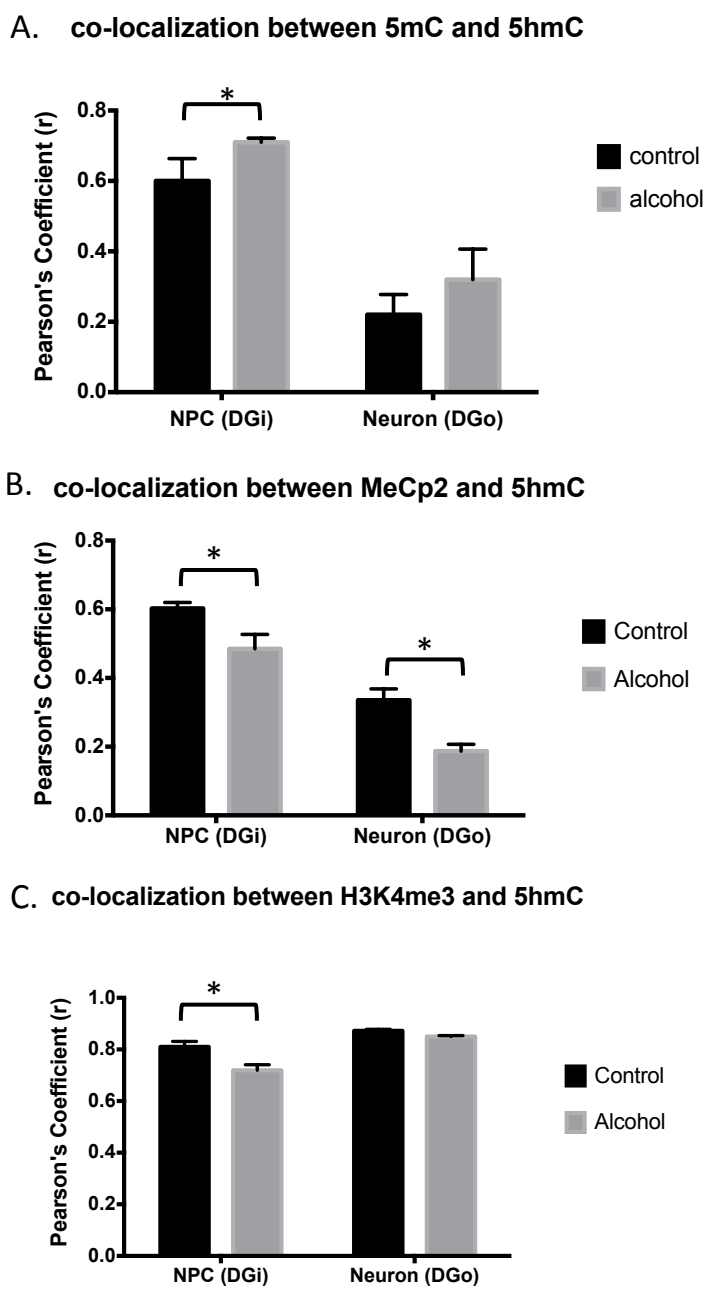

Figure 22. The effect of alcohol on nuclear co-localization between 5mC and 5hmC (A), MeCP2 and 5hmC (B), H3K4me3 and 5hmC (C) in the developing dentate gyrus. The X-axis label "NPC" represents immature neural progenitor cells in dentate gyrus inner layer (DGi); the lable "neurons" represent mature cells in dentate gyrus outer layer (DGo). The Y-axis shows Pearson's coefficient (r). All data presented as Mean ±SEM. N=5 each group. *P<0.05.

3.4 Discussion

3.4.1 Summary

In this study, we revealed dynamic chromatin remodeling that was associated with DNA methylation dynamics during neuronal differentiation and maturation, and that the process was subject to alcohol exposure. We demonstrated that 1) the two forms of DNA methylation (5mC and 5hmC) were initially diffusely distributed in the nuclei of immature cortical neurons, however, become spatially separated and reorganized into different chromatin compartments during neuronal maturation (5mC to heterochromatin, 5hmC to euchromatin), while associated with differential methyl-binding proteins; 2) 5hmC became preferentially associated with activating histone H3K4me2, while 5mC associated with repressive histone H3K9me3 and H3K37me3; and 3) RNA polymerase II (*Pol*II) preferentially co-localized to 5hmC sites during maturation. Alcohol partially prevented the programmed separation of 5mC and 5hmC in the cortical neurons, significantly reduced the co-localization of 5hmC to H3K4me2 in P7 dentate granule cells, and significantly reduced the co-localization of methyl-CpG-binding protein 2 (MeCP2) to 5mC, as well as to 5hmC.

We demonstrated here an integrated connection between DNA methylation, histone modifications, chromatin remodeling and furthermore, the transcriptional potential along neurogenesis. Though the epigenetic interplay has been proposed from many genome-wide studies and *in vitro* ES cell studies, this is the first evidence from *in vivo* brain at critical steps of neurogenesis showing the spatial and physical interaction between epigenetic players at the chromatin level. We further proposed that alcohol delaying this epigenetic reprograming would possibly alter the transcriptional ability of critical neural developmental genes.

3.4.2 The relationship between chromatin dynamics and transcriptional status

The chromatin landscape is modifiable by numerous posttranslational modifications to histone tails, as well as enzymatic chromatin modifiers. This landscape serves in part to regulate RNA polymerase II accessibility to specific DNA sequences (Barrero and Malik, 2013). The rate of *Pol*II recruitment is generally regarded as a measure of the transcriptional output for a given promoter and the rate-limiting step in transcription (Nevado et al., 1999). Specific histone codes and chromatin structure has been shown to influence the activity of RNA polymerase II (Adam et al., 2001, Gerber and Shilatifard, 2003, Spain and Govind, 2011). For example, H3K4me3 is shown to facilitate transcription initiation, while H3K27me3 is associated with the repression of transcription elongation (Guenther et al., 2007). We thus used antibody against RNA polymerase II-phosphorylated serine-5 (*Pol*II-ser5), which is mediating transcription initiation to access the transcriptional status in association with histone and DNA modification marks. We showed here that *Pol*II-ser5 become highly co-localized with euchromatic regions and 5hmC mark, while was absent of at heterochrmatic region where 5mC occupied (Figure 21). Our results indicated that chromatic states were associated with the recruitment of transcriptional initiation machinery, and this recruitment could possibly be a target of alcohol exposure. Further studies need to be performed on how alcohol affect the recruitment of RNA polymerase II machinery and affect the gene expression.

3.4.3 The functional correlation of 5hmC

The 5mC, through physically expelling transcription factor binding (Watt and Molloy, 1988) or recruiting repressive protein complexes (e.g. methyl-CpG-binding proteins) (Fan and Hutnick, 2005), is thought to mediate long-lasting silencing of associated genes (Bird, 2002). However, the role of 5hmC is less known. Recent genome-wide studies indicated that 5hmC played a role in ES cell maintenance, as well as neuronal maturation (Ficz et al., 2011, Pastor et al., 2011, Ruzov et al., 2011, Szulwach et al., 2011). Furthermore, it has been shown that the genes associated with 5hmC in the

gene bodies are actively transcribed in the mouse embryonic stem cells and mouse cerebellum (Guo et al., 2011a, Song et al., 2011, Wu et al., 2011, Yu et al., 2012). The 5hmC-accumulated genes are among groups of cell differentiation, cell communication, and synaptogenesis genes (Khare et al., 2012, Yu et al., 2012, Lister et al., 2013). More importantly, recent evidence suggests that the 5hmC marks a "bivalent" state of transcription: the 5hmC showed a high genomic correlation with bivalent domains that were labeled by both activating histone mark H3K4me3 and repressive mark H3K9me3 (Pastor et al., 2011, Yu et al., 2012). These evidence revealed that the 5mC and 5hmC have different roles in neurodevelopment.

Our results support the notion that 5mC and 5hmC have different roles in neurodevelopment. First, 5hmC and 5mC can be discriminated by differential chromatic locations, associated with differential transcriptional abilities. The functional diversity of the 5mC and 5hmC was further suggested in the current study by their nucleus affiliation with correspondent histone codes –5mC with the suppressive H3K9me3 and with H3K27me3; and 5hmC with H3K4me2.

Second, the 5mC and 5hmC can be discriminated via their binding partners. The 5mC preferentially binds to MeCP2 and MBD1, while 5hmC preferentially binds to MBD3 in mature neurons. Recent reports indicated a mixed view on the 5hmC-binding partners. We and others showed that 5hmC negatively correlated with MeCP2, but positively correlated with MBD3 (Yildirim et al., 2011, Hashimoto et al., 2012); while others argued that MeCP2 bind to 5hmC-labled genomic locations (Mellen et al., 2012). Most of these studies were conducted *in vitro* and mostly in ES cells. Given the dynamic nature of DNA methylation, the evolution of the respective binding partner is also likely dynamic, potentially resulting in differences between *in vitro* and *in vivo* interactions. Our observation suggested that there might be a dynamic developmental switch for the 5mC and 5hmC binding partners, and their roles at specific neurodevelopment stages might differ as well.

3.4.4 The role of 5mC and 5hmC transitioning in neurodevelopment

We demonstrated here, along with other groups, a transition of 5mC and 5hmC during neurodevelopment. First, 5mC and 5hmC appeared sequentially in neural progenitor cells. The temporal surge of 5mC and 5hmC is punctual (at very specific stage of neural differentiation) and step-wise (5mC first appear followed by 5hmC) throughout developing pre- and post-natal brain (see (Zhou et al., 2011b) and Aim 1).

Second, the 5hmC and 5mC are transitioning in their chromatic locations. At early differentiation stage, both 5mC and 5hmC are diffusely located within chromatin (show as small punctuates), at which time the DAPI-dense heterochromatin was readily formed (evident by the large DAPI aggregates, Figure 17D). At later neuronal maturation stage, the 5mC and 5hmC become spatially separated, and relocated into heterochromatic or euchromatic regions accordingly. This evident indicates that 5mC and 5hmC might have transient neurodevelopment-regulated roles at different stages of neurogenesis. For example, at early differentiation stages, progenitor cells hold the potential to become different cell lineages (e.g. neurons or glia, dopamine neurons or glutamate neurons) by time-dependent transcriptional ability of groups of differentiation genes (Gage, 2000). Upon receiving environmental signals (e.g. neurotropic factors), these genes become selectively expressed. This potential could be poised by maintaining both 5mC and 5hmC on a gene, thus providing a "bivalent" state. Upon differentiation and lineage determination, the functionally consistent-required genes (e.g. synaptic genes) would be marked with activating epigenetic markers; while those unnecessary genes (such as stem cell maintaining genes) would be permanently shut off by labeling with repressive markers. As we observed, in mature neurons, the 5mC and 5hmC become mutually exclusive, indicating their associated genes being labeled with different markers.

Furthermore, it has been shown that besides chromatic transitioning, there was also a dynamic reconfiguration of 5mC and 5hmC within gene promoter and gene bodies. When ES cells undergo differentiation towards neuronal lineage, actively transcribed gene acquired 5hmC near the TSS and within the gene bodies and lost 5mC at TSS, while

silenced genes acquired 5mC in their promoters (Kim et al., 2014). In addition, another event of DNA methylation reconfiguration was reported in maturing neurons, that CpG methylation (mCG) was switching to non-CpG methylation (mCH) that coincided with synaptogenesis (Lister et al., 2013). These evidence together point to a role of 5mC and 5hmC transitioning in neurodevelopment and in gene transcription regulation. Further studies are needed to elucidate how this transitioning is regulated.

3.4.5 The effect of alcohol on chromatin remodeling

In the last aim, we showed that alcohol delayed the acquisition of 5mC and 5hmC in neuroprogenitor cells; in this aim, we further showed that alcohol delayed the transition between 5mC and 5hmC and the associated chromatin remodeling during neural differentiation and maturation. Further investigation is needed on the mechanism underlying the alcohol-induced aberration in chromatin remodeling. For example, what is mediating the alcohol effect on chromatin structure, and how is the altered chromatin remodeling contributing to the developmental aberration in neurogenesis.

Alcohol acts on the chromatin structure probably through acting on enzymes that are required for chromatin remodeling. Alcohol disrupts steps of one-carbon metabolism (Kruman and Fowler, 2014), many intermediates of which provide donors for epigenetic enzymes, including DNA methyltranferase (DNMT), histone methyltransferase (HMT) and histone acetyltransferase (HAT). As a result, not only DNA methylation profile would be altered, the histone modification profile could be disrupted as well. It was shown that in a FASD rat model, histone 3 acetylation was decreased in the cerebellum as a consequence of a down-regulated HAT (Guo et al., 2011b). In the amygdala, acute alcohol decreased HDAC activity, thus increased level of histone 3 and 4 acetylation (Pandey et al., 2008). In hypothalamus, prenatal alcohol exposure lead to decrease of hypothalamic level of H3K4me3, while increased H3K9me2 (Bekdash et al., 2013). In alcohol-exposed adult rat, histone 3 acetylation on lysine 9 were significant altered in liver, lung and spleen tissues (Kim and Shukla, 2006). These changes in histone modification could possibly contribute to the chromatin structure changes.

In addition, alcohol directly affects the expression of enzymes involved in chromatin remodeling. Recent gene-expression microarray data indicated that after *in utero* alcohol exposure, the early embryos (at about E10) showed down-regulated expression of several genes involved in chromatin remodeling, including *Brwd1*, *Ilf3, Hist3h2a*, *Ube3a* (Zhou et al., 2011c, Downing et al., 2012). It is very likely that chromatin-remodeling complexes (such as the polycomb group and trithorax group proteins) are targets of alcohol. However, there were no studies reported so far on these subjects.

Another possible target of alcohol underlying chromatin remodeling is the methyl-binding protein. Alcohol modulates expression of methyl-binding proteins (MeCP2, MBD2 and MBD3) in murine embryonic fibroblasts (Mukhopadhyay et al., 2013). We reported before that alcohol decreased expression of MBD1 in association with decreased DNA methylation in developing neural tube (Zhou et al., 2011b). In the previous aim, we showed that prenatal alcohol reduced the MeCp2 expression in neural progenitor cells in dentate gyrus (Figure 15L). Studies in progress in our lab also suggested that MeCP2 and MBD1 protein expression were significantly altered in alcohol-treated mouse cortex. We demonstrated here that not only the protein expression of methyl-binding proteins, but also the co-localization of methyl-binding proteins to the methylated DNA (both 5mC and 5hmC) was altered by alcohol treatment. Since the binding of methyl-binding proteins to the methylated CpG sites is important for recruiting downstream chromatin repressor effectors, it is likely that alcohol disrupting either the expression or the localization of methyl-binding proteins in the developing cell nucleus contributes to the developmental aberration in steps of neurogenesis.

3.4.6 Proposed model of DNA methylation-associated chromatin remodeling during neuronal differentiation and maturation

Based on the results presented in this aim, I proposed a model in which DNA methylation mediated the chromatin remodeling process during neuronal differentiation and maturation (illustrated in Figure 23).

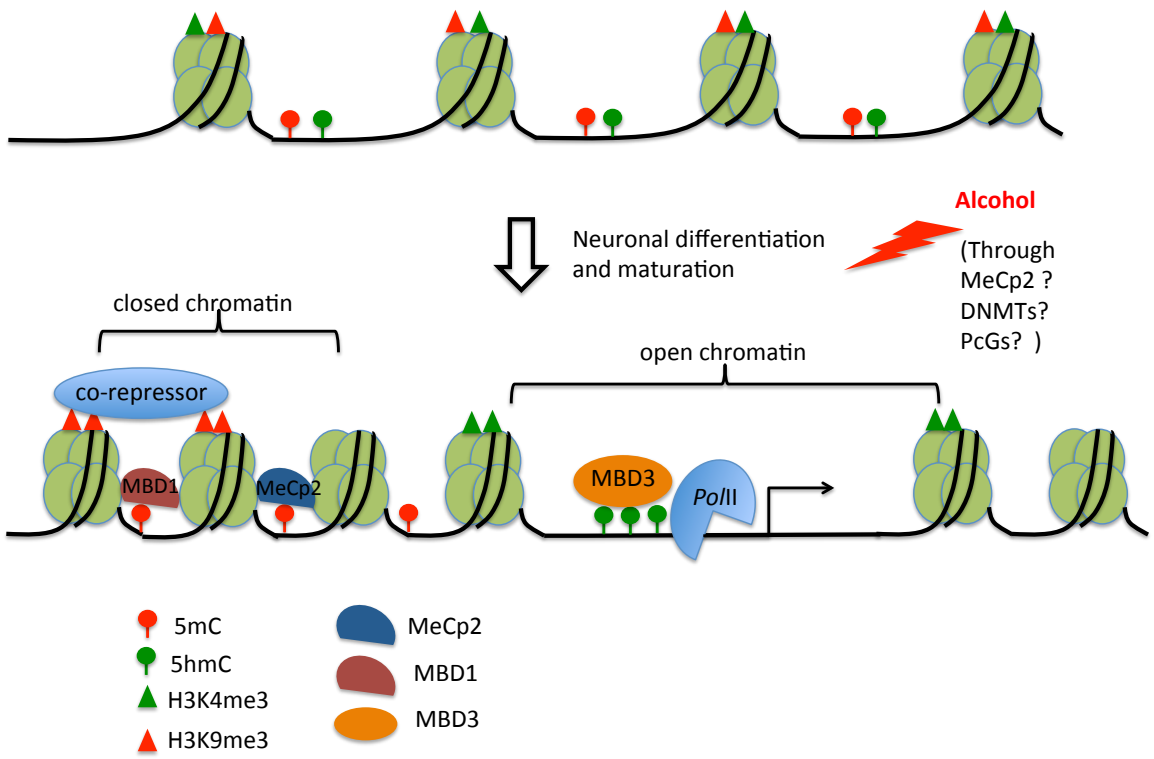

Figure 23. Proposed model of DNA methylation-associated chromatin remodeling during neuronal differentiation and maturation. In undifferentiated cells, the 5mC and 5hmC are "co-localized" (located in adjacent CpG sites) at regions of DNA, marked a "bivalent" state of transcription; upon neuronal differentiation and maturation, the 5mC and 5hmC become separated into different regions of the chromatin: the 5mC selectively recruits MBD1 and MeCp2, further recruit chromatic co-repressors and H3K9me3 modifications, and renders a closed chromatin; on the other hand, the 5hmC selectively recruits MBD3 and RNA polymerase II, co-localizes with H3K4me3, and opens the chromatin to facilitate gene transcription. Alcohol is shown to delay this chromatin remodeling process – alcohol delayed the re-organization of 5mC and 5hmC, hindered the MeCp2 binding to both 5mC and 5hmC, and impeded the association of H3K4me3 to 5hmC. The mechanism for alcohol-induced impairment in chromatin remodeling is not fully understood, possibly through disruption in MeCp2, DNMTs and polycomb group (PcG) proteins.

In summary, this is the first evidence reported so far on the effect of alcohol on DNA methylation-associated chromatin remodeling during neurogenesis. The functional consequence of this altered chromatin remodeling machinery contributing to the FASD phenotype is a subject required further investigation.

Acknowledgement:

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CHAPTER 4: AIM 3

Site-Specific DNA Methylation Alterations by Alcohol in Neural Specification Gene *Ascl1* during Neural Differentiation

4.1 Background and Hypothesis

4.1.1 Neural specification genes in neurogenesis

The neural stem cell differentiation is marked by cell changing its internal program from the self-renewal state to a committed fate. This transition is orchestrated with and guided by a global alteration of the transcriptome (Ulloa-Montoya et al., 2007, Wu et al., 2010, Nieto-Estevez et al., 2013). The genome of neural progenitor cells are maintained in a poised state, at which both activating and repressing transcriptional modulators co-influence the transcriptional potential of important development-related genes (Mikkelsen et al., 2007). These transcriptional modulators include (but not confined to) DNA methylation (Wu et al., 2011), histone modifications (Rando and Chang, 2009) and noncoding RNAs (Beisel and Paro, 2011). When differentiation is activated, specific cell-fate determination genes are up-regulated by activator markers, where repressive markers are lost (Bernstein et al., 2006). A poised state (or bivalent state) allows a quick transcriptional response to the extracellular and/or intrinsic stimulus. For example, a study showed that during early mammalian development, approximately 2,000 genes are bivalently marked by histone modifications and these marks progressively resolve towards the lineage-specific patterns characterizing each unique cell types as differentiation progresses (Rugg-Gunn et al., 2010).

A specific group of development-related genes are of particular interest during neural differentiation, that is the neural specification genes (also known as proneural genes). Among them are *neurogenins* 1,2,3 (*Neurog1-3*), *achaete-scute complex homologue 1* (*Ascl1*, also known as *Mash1*) and *Math1-5* (Bertrand et al., 2002). This

group of gene is known to promote cell cycle exit and neuronal differentiation when expressed in neural progenitor cells. Mutation in these genes expression deviates neuronal differentiation. For example, mutation in *Ascl1* gene expression delays differentiation in multiple neuronal cell lines and mutant mice (Pattyn et al., 2006, Huang et al., 2012, Krolewski et al., 2012). Another important role of this group of genes is that their expression drive the neural progenitors to differentiate into neuronal lineage, and suppress the glial fate in early developmental stage (Tomita et al., 2000, Sun et al., 2001, Kim et al., 2008). It has been reported that inhibiting transcription of *Ascl1* drives the cell toward an astrocyte fate in mouse subventricular zone (Prozorovski et al., 2008). Recent study using optogenetic technique suggested that the multipotency of neural stem cells is maintained by oscillating expression of neurogenic and gliogenic determination factors, and that the determination of neuronal fate is induced by sustained expression of *Ascl1* and other proneural factors (Imayoshi et al., 2013).

Alcohol impairs the cellular processes of neuronal differentiation. Studies using human and rodent neurosphere cultures demonstrated that treatment with alcohol reduced neurosphere migration, skews the developmental potential of neural progenitor cells (to become neuron or glial) and alters the neuronal differentiation program (Singh et al., 2009a, Roitbak et al., 2011, Vangipuram and Lyman, 2012). However, the molecular mechanism of neural specification genes in alcohol teratology is not fully understood.

4.1.2 Epigenetic control of neural specification genes

The transcription of neural specification genes is regulated by both genetic and epigenetic factors. The cell intrinsic program signals the proper transcription factor expression that drives the neural specification gene expression. For example, *Hes1* is a transcription repressor for *Ascl1*. Binding of *Hes1* complex to the TF-binding site on *Ascl1* promoter inhibits the expression of *Ascl1* (Ishibashi et al., 1995).

Epigenetic machinery also influences the transcription ability by altering accessibility of transcription factors to interact with target sequences through chromatin structure. For example, it was shown that the *Ascl1* gene was located in the nucleus periphery in ES cells associated with heterochromatin and repressive H3K9me3 marker, however, was relocated towards the interior of nucleus upon neural induction and associated with >100 fold up-regulation of transcription (Williams et al., 2006). In addition, epigenetic modifiers such as HDACs have been shown to interact with transcription factor binding. For example, a histone deacetylase, SIRT1, forms a complex with *Hes1* on the *Ascl1* promoter, deacetylates histones and recruits repressor proteins to inhibit transcription of *Ascl1* (Prozorovski et al., 2008).

Furthermore, epigenetic machinery holds the neural specification genes with bivalent markers and timely controls their expression during the course of neural differentiation. It was shown that the *Ascl1* and *Neurogenins* and are 'bivalently' marked with repressive marker H3K9me3 and activating marker H3K4me3 and are kept poised for activation in embryonic stem cells (ESCs), but the repression are removed upon differentiation into the neural lineage (Mikkelsen et al., 2007). Their expressions are tightly regulated during development (Bertrand et al., 2002, Kageyama et al., 2005).

4.1.3 Hypothesis

We have previously demonstrated that 1) the promoter DNA methylation pattern change significantly when stem cells undergo differentiation. Moderately methylated genes undergo either hyper- or hypo-methylation (Zhou et al., 2011a). This is expected because phenotypic specification of stem cells into neurons or other cell types involves permanent changes in gene expression patterns. This is also in accordance with other methylome studies (Cortese et al., 2011, Lister et al., 2013, Kim et al., 2014). 2) Exposing DRG neural stem cells to alcohol *in vitro* prevented this differentiation-associated diversification of gene methylation pattern, so that promoter methylation patterns of differentiating neurons became more closely resemble methylation patterns in immature stem cells (Zhou et al., 2011a). In other words, alcohol appeared to retard the

reorganization of promoter methylation that occurs during the maturation of neural stem cells. This notion was further proved from out last aim where we demonstrated that alcohol retarded the reorganization of chromatin *in vivo*.

In line with the methylation changes, we also reported according gene expression changes upon alcohol treatment at early neural development time-point. For example, we reported that alcohol disrupted neural specification genes (*e.g. Neurogenin1 and 2*) expression in developing neural tube (Zhou et al., 2011c). The developmental perturbations of neural specification genes may have long-term consequences for neuronal function. Therefore, in this aim, we seek to understand the epigenetic mechanism underlying alcohol-induced perturbation in the neural specification gene *Ascl1* expression. We hypothesize that alcohol disrupts gene-specific DNA methylation on *Ascl1* during neural differentiation and alters its expression, which might contribute to the aberration in neurogenesis.

4.2 Methods and Material

4.2.1 Neural stem cell culture

We utilized two NSCs that were previously established in our laboratory: an adult Dorsal Root Ganglia (DRG)-derived NSCs (Singh et al., 2009b, Singh et al., 2009c); and a striatum-derived astroglial progenitor stem cell line (STr) (Chiang et al., 1996).

The NSCs were maintained in free-floating neurosphere culture in Dulbecco's Modified Eagle Medium/F-12 Nutrient Mix (D-MEM/F-12) media containing N2 supplement (12 µl/ml; Invitrogen, Carlsbad, CA), and penicillin-streptomycin (12 µl/ml; Sigma, St Louis, MO) and grown in a humidified incubator at 37°C and 5% CO2. Media was supplemented with 10 ng/ml epidermal growth factor (EGF; Harlan Bioproducts for Science, Indianapolis, IN) and basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ) twice per week for maintenance of NSCs in neurosphere form. During the medium changes throughout the years, no passaging (trypsin digestion and cell transferring, except dividing into multiple flask) was performed. The multipotency and stability has been tested in these screened NSCs (Singh et al., 2009b).

For the differentiation study, NSCs were grown in absence of growth factors (FGF and EGF) to allow differentiation. In brief, the undifferentiated neurospheres were withdrawn from the aforementioned culture medium and equilibrated for 5 to 10 minutes in Neurobasal media (no supplements) before being plated into a 60 mm plastic Petri dish (for gene expression and DNA methylation analysis) or in 16-well chamber slides (for immunocytochemical analysis), both coated with poly-d-lysine and laminin (50 µg/ml each; Sigma). Neurospheres were allowed to differentiate for 3 days (for morphological, gene expression and DNA methylation analysis) or 7 days (for gene expression and DNA methylation analysis) in culture with differentiation media consisting of Neurobasal media supplemented with 10% fetal bovine serum, 1.2% B27, and 1.2% penicillin–streptomycin.

4.2.2 Neural stem cell treatments

The differentiating neurosphere cultures were randomly assigned to four groups: the control group (Cont), the alcohol group (Alc), the 5-aza-cytidine treated group (AZA) and the alcohol-treated plus SAMe supplement group (Alc+SAMe). The control group received no treatment throughout culture period. The alcohol group received 400 mg/dL alcohol exposure throughout the whole culture period (3days or 7days). We further divided the alcohol group into two treatment paradigm— a higher dose (longer exposure) receiving alcohol after 4 hours after initial plating (before cell attachment) or a lower dose (shorter exposure) receiving alcohol after 18hrs after plating (after cell attachment). Alcohol (95% ethanol, molecular grade, Sigma Inc.) was added directly to the culture dishes to reach a level of 400 mg/dL (88mM). This concentration was within the range of blood alcohol concentration of chronic alcoholics (320–620 mg/dL, 70–135 mM) (Perper et al., 1986, Adachi et al., 1991). To control for alcohol concentration due to alcohol metabolism and evaporation, culture dishes were maintained in closed chamber system over a bath of ethanol-water, with 95% ethanol added to both the media and bath at the desired concentration daily according to previously published method (Zhou et al., 2011a). The alcohol concentration within system has been report to maintain within 90% of the original concentration within 24 hours (Adickes et al., 1988). The SAMe supplement group received same alcohol treatment (400mg/dL, from 4hours after plating) with addition of daily SAMe supplementation (40uM, Sigma) at the time of alcohol treatment. The 5-AZA treatment was carried out as previously (Singh et al., 2009c). The 5-AZA (50 ng/ml; Sigma) was added to culture dishes or culture chambers from the beginning of differentiation (4 hours after plating) and added daily.

At the end of the differentiation period, the undifferentiated and differentiating cells were (i) collected by scraping all the cells from the petri-dish and snap-freezed with liquid nitrogen or immediately proceeded for mRNA and gDNA isolation, or (ii) washed with 0.1 M PBS and fixed with the 4% formaldehyde fixative in a chamber slide for immunocytochemical analysis of phenotypes.

4.2.3 Immunohistochemistry

The undifferentiated and differentiated neurosphere phenotypes were immunochemically analyzed with the neural stem cell culture. Antibodies used for the phenotypic markers include NeuN (mature neuronal marker, 1:250, Cell Signaling Inc., CA), Sox2 (multipotent stem cell marker, 1:250, Cell Signaling Inc., CA), MAP2 (microtubule associated protein 2, neuronal marker, 1:500, Abcam, Cambridge, UK). The above antibodies were commercially characterized for their titer as well as specificity, and screened through our initial staining in vivo with embryonic tissue for confirmation of their intranuclear staining and known distribution. There were N=4 wells for each immunostaining containing 5-10 neurospheres per well. Immunostaining procedures were performed following a routine procedure as previously established for stem cell cultures in our laboratory (Singh et al., 2009b, Singh et al., 2009c). In brief, endogenous peroxide was quenched with 3% H2O2 and 1% Triton X-100 in PBS was applied for 30 minutes to permeabilize the cell membranes. Non-specific binding was blocked using 1.5% normal serum (used to make secondary antibody) plus 0.1% Tx-100 in PBS. Primary antibodies (detailed above) were incubated for 18 hours at room temperature in the blocking buffer corresponding to the species of the secondary antibody. Biotinylated secondary antibodies (1:500, Jackson ImmunoResearch, West Grove, PA) were incubated for 90 minutes at room temperature followed by Streptavidin-conjugated-Peroxidase (1:500, Jackson ImmunoResearch, West Grove, PA) for 90 min. The immunostaining was visualized by incubation of 0.05% 3'-3'-diaminobenzidine (DAB) and 0.003% H_2O_2 over minutes, followed by counterstained with methyl green.

Fluorescent doublestaining was performed to analyze *Ascl1* expression in relationship to cellular phenotypes. Primary antibody against mouse *Ascl1* (1:250, mouse polyclonal, BD Biosciences, CA) was incubated in serum kit (containing 0.1% Triton X-100 and 1.5% normal goat-serum in PBS) for 18 hours, followed by 90 minutes incubation with fluorescent secondary antibody conjugated with Alexa 546 (1:500, Invitrogen). For double staining, Sox2 (1:250, rabbit polyclonal, Cell Signaling Inc., CA) or NeuN (1:250, rabbit polyclonal, Cell Signaling Inc., CA) primary antibodies were incubated afterwards to double with *Ascl1* expression, and labeled with Alexa 488 (1:500,

Invitrogen). Slices were covered with anti-fade mounting solution with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen) and stored in 4 ^{o}C in dark.

4.2.4 RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from each cell line using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). An on-column DNA digestion was performed during RNA purification using Qiagen RNase-Free DNase Set (Qiagen, Valencia, CA). The RNA quality and quantity was assessed using a NanoDrop spectrophotometer. 1ug of RNA were used from each cell line to convert into cDNA using ABI High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY). 100 ng of cDNA was used as a template for qRT-PCR in combination with TaqMan® Gene Expression Master Mix (Life Technologies, Grand Island, NY) and Taqman Gene-specific probes (Assay ID: Ascl1—Rn00574345_m1; 18S—Rn03928990_g1; GAPDH—Rn01775763_g1) on a StepOnePlus™ Real-Time PCR System (Life Technologies, Grand Island, NY). All reactions were incubated at 95°C for 3 min, followed by 40 cycles at 95°C for 15s, annealing at 60°C for 15s, and elongation at 72°C for 7min. We assayed a minimum of four biological replicates for each group; cycling reactions were performed in duplicate. The relative expression of each gene was calculated based on the ΔΔCt value, where the results were normalized to the average Ct value of 18S. We used one-way ANOVA to determine the significance.

4.2.5 gDNA isolation and bisulfite pyrosequencing

Genomic DNA was isolated from cells (Control, Alcohol, 5-AZA and Alcohol+SAMe groups at 3 day after differentiation; Control and Alcohol groups at 7 days after differentiation) using the DNeasy blood and tissue kit (Qiagen, Cat#69504). DNA was isolated according to the manufactures' instructions. Quantity of gDNA was measured by NanoDrop 1000 (Thermo Scientific, Waltham, MA), and stored in -20^{o}C. For bisulfite conversion, 500ng of gDNA was treated with EZ DNA Methylation-Gold kit (Zymo, Orange, CA, Cat#D5005) to convert unmethylated dCTPs to dUTPs.

Biotinylated primers for promoter and gene body of *Ascl1* gene (each covering 3 CpG sites) were used to amplify the bisulfite converted DNA. The primer sequences were listed in Table 3. The PCR reactions were performed using Pyromark PCR kit (Qiagen, Cat#978703) on an ABI 2720 Thermal Cycler (Applied Biosystem, CA). All PCR reactions were performed under the following condition: a denaturation step at 95°C for 10 minutes, followed by 45 cycles of polymerization (95C for 30 seconds, an annealing step at Tm for 30 seconds, and an extension step at 72°C for 60 seconds), and a final extension at 72C for 10 minutes. The annealing temperatures (Tm) for all primers were listed in Table 3. Some primers required additional $MgCl_2$ for efficient amplification, and the concentration for $MgCl_2$ used were listed in Table 3 as well. All PCR products were validated on 1.5% agarose gel for single band at appropriate size.

After PCR, the biotinylated strands were captured on Streptavidin-Sepharose beads (GE Healthcare, Cat #17-5113-01) and incubated with sequencing primers (Qiagen, listed in Table 3). Pyrosequencing was performed on PyroMark Q24 advanced system (Qiagen, Hilden, Germany), and followed manufacture's instruction. Non-CpG cytosines served as internal controls to verify efficient bisulfite DNA conversion, and unmethylated bisulfite-converted mouse control DNA and fully methylated bisulfite-converted DNA (CpG methyltransferase M.SssI-treated mouse gDNA, New England Lab, Cat #M0226S) were run as controls on each plate. All pyrosequencing runs were performed in technical duplicate from N=4 different samples for each treatment condition. Data were analyzed on PyroMark Q24 advanced software (Qiagen, Version 3.0).

4.2.6 Data analysis

Statistical tests were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., CA). Statistical significance from student t-tests was set at $P < 0.05$.

Table 3. *Ascl1* pyrosequencing primer design and PCR conditions

Assay Name	Qiagen Cat. No	Gene symbol	Amplicon length	No. of CpG	CpG location	Chromosome location
Rn_Ascl1_01	PM00574812	*Ascl1*	85	3	Exon 1	Chr7:24146373-24146458
Rn_Ascl1_02	PM00574819	*Ascl1*	138	3	downstream of TSS	Chr7:24147273-24147411

Assay Name	Primer	Tm (C)	MgCl2	Sequence to analyze	Sequence to analyze bisulfite converted
Rn_Ascl1_01	Biotinylated	50	4mM	CTCCGGGAGCATGTCCCCAACGGCGC	TTTYGGGAGTATGTTTTTAAYGGYGT
Rn_Ascl1_02	Biotinylated	52	4mM	CGGTACGCCTTCCACGT	YGGTAYGTTTTTTAYGT

4.3 Results

4.3.1 Two stem cells lines with contrast neurogenic properties

We used two distinct neural stem cell lines in this aim –the adult Dorsal Root Ganglia (DRG)-derived stem cells and Striatal (STr)-derived stem cells. Both DRG and STr stem cells showed multipotency towards differentiation in our *in vitro* culture system. The undifferentiated stem cells were aggregated as floating neurospheres, and expressed stem cell marker Oct3/4 and Sox2 (Appendix 5). After 3 days of differentiation in culture (in absence of FGFs and EGFs), neurospheres displayed a heterogeneous population of cell types—containing SOX2+ stem cells as well as NeuN+ and MAP2+ neurons (Figure 24).

The two neural stem cell lines displayed different neurogenic properties. At 3 days of differentiation, DRG cells expressed high level of ASCL1, and become mostly NeuN+ neurons (Figure 25A,C); the STr cells expressed low level of ASCL1 (Figure 25B, arrow) and displayed a low percentage of NeuN+ neurons (Figure 25D, arrow). ASCL1+ cells co-localized with NeuN+ cells and only partially co-localized with SOX2+ stem cells (Figure 26A,B).

We next analyzed quantitative *Ascl1* expression in both cell lines over differentiation time-points using real-time PCR. The *Ascl1* expression was consistently higher in the DRG cells as compared to that of STr cells (Figure 27). The expression of *Ascl1* increased over differentiation period from 0 day (undifferentiated) to 3 day and 7 days in both DRG and STr cells (Figure 27).

4.3.2 Alcohol retards neural differentiation in neurosphere culture

To investigate if alcohol induces phenotypic changes in neurosphere culture, the DRG cells were treated with 400mg/dL alcohol (binge alcohol level) from the beginning of differentiation (4 hours after initial plating) till the end of culture period. As

neurophere differentiated in culture, differentiating cells migrated out from the neurosphere core and lost stem cell marker SOX2, while gained neuronal marker NeuN. Alcohol increased the undifferentiated neurosphere core size (Figure 28A,B, dotted circle), and increased the stem cell marker SOX2 expression in culture (Figure 28A,B, also see quantitation in C). On the contrary, alcohol significantly reduced the percentage of NeuN+ cells in neurosphere culture ($P<0.05$), as well as the percentage of ASCL1+ cells ($P<0.05$) (Figure 28C).

4.3.3 Alcohol altered *Ascl1* expression in neurosphere culture

To investigate whether the alcohol-induced delay in neural differentiation was correlated with altered *Ascl1* expression, we performed quantitative RT-PCR analysis from cells of control and alcohol-treated DRG and STr neurospheres. Alcohol did not significantly alter *Ascl1* expression in DRG cells; however, it significantly reduced *Ascl1* expression in STr cells (Figure 29).

4.3.4 DNA methylation modifying agents altered *Ascl1* expression in neurosphere culture

To investigate whether the alcohol-induced alteration in *Ascl1* expression was mediated by DNA methylation, we applied DNA methylation-specific modifying agents (5-AZA—methyltransferase inhibitor and SAMe—methyl donor for methyl synthesis) to the neurosphere culture. 5-AZA significantly increased *Ascl1* expression in both DRG cells (~4 fold increase) and STr cells (~1.6 fold increase) at 3 day of differentiation (Figure 30). SAMe supplementation to alcohol exposure (400mg/dL) significantly decreased *Ascl1* expression in both DRG cells (~6.6 fold decrease) and STr cells (~2.8 fold decrease) compared to control (Figure 30).

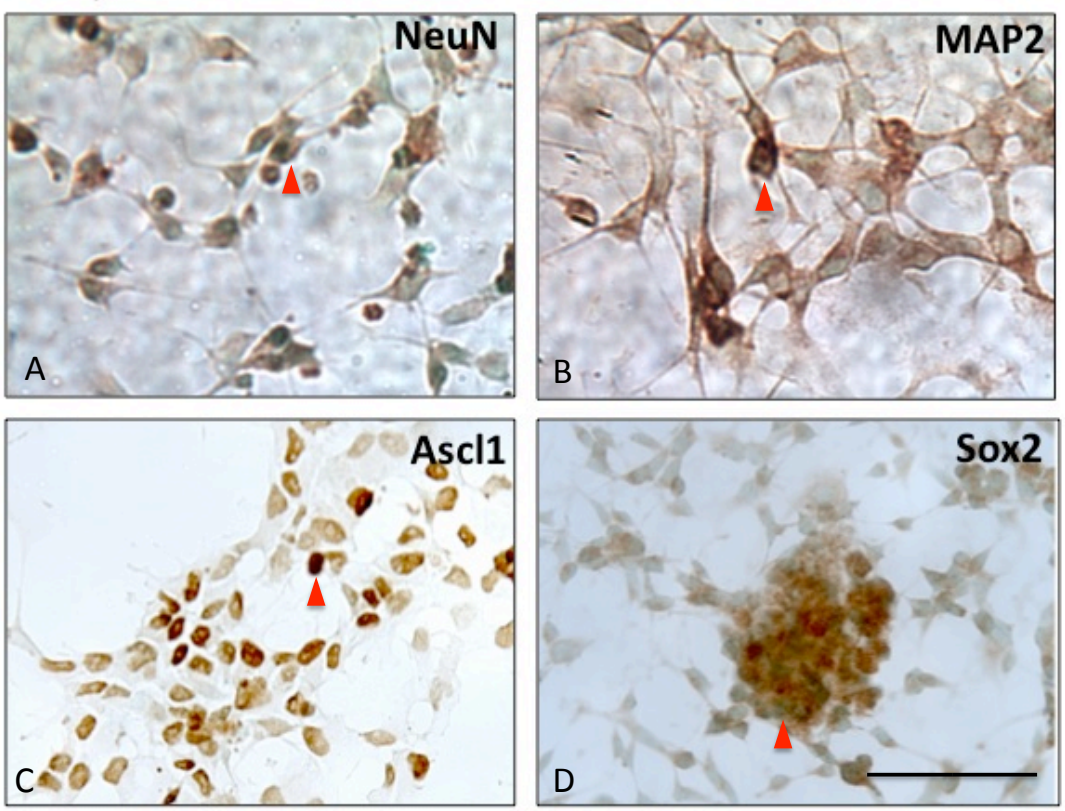

Figure 24. Multipotency of DRG neurospheres in culture. Immunostaining of phenotypic markers in DRG cells after 3 days of differentiation in culture. The DRG cells displayed heterogeneous cell population with NeuN+ (A) and MAP2+ (B) neurons, SOX2+ stem cells (D), and ASCL1+ neural progenitors (C) (arrowheads). Scale bar all=100um.

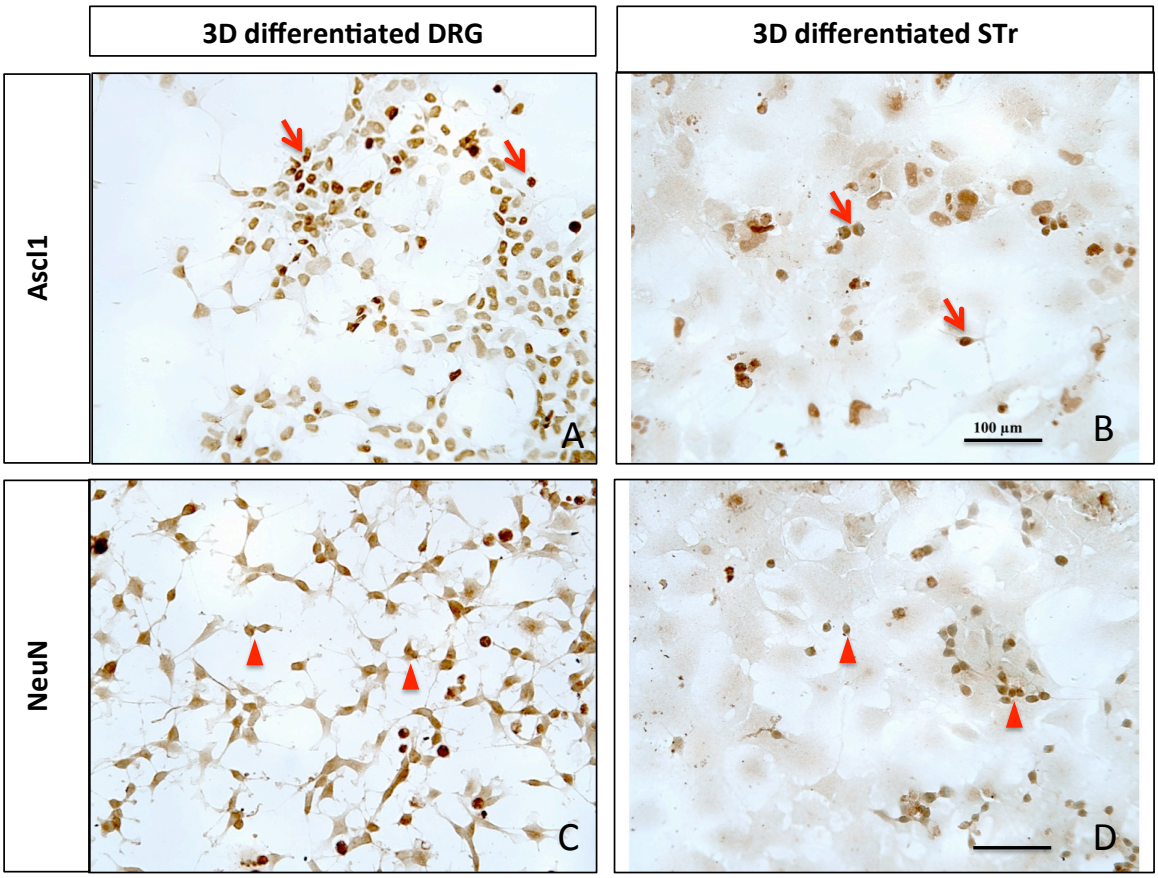

Figure 25. *In vitro* expression of ASCL1 and NeuN in 3-day (3D) differentiated DRG and STr cells. There are more ASCL1+ cells in DRG cells (A, arrows) than STr cells (B, arrows). There is higher percentage of NeuN+ neurons in the DRG cells (C, arrowhead) compared to the STr cells (D, arrowhead). Scale bar all=100um.

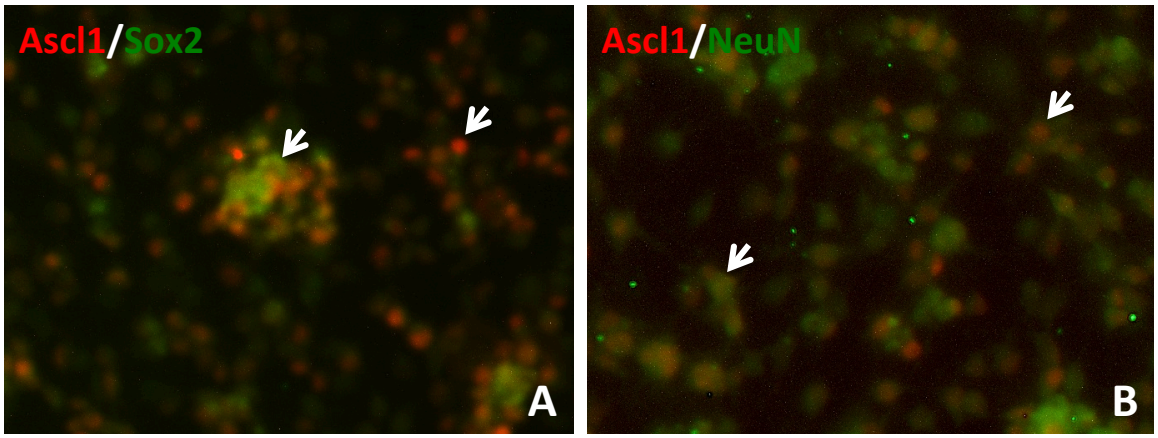

Figure 26. Fluorescent double staining of ASCL1 with phenotypic markers in 3-day differentiated DRG cells. ASCL1 (shown in red) is not co-localized with stem cell marker SOX2 (shown in green) (A, arrows), however, is largely co-localized with neuronal marker NeuN (shown in green) (B, arrows).

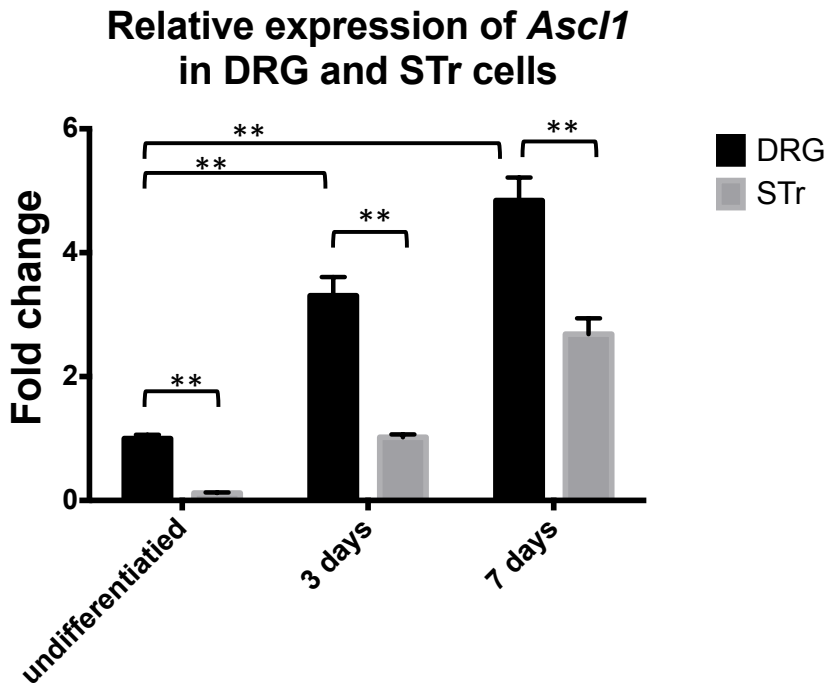

Figure 27. Timing analysis of *Ascl1* gene expression in both DRG cells and STr cells at different time points *in vitro.* At 0 day (undifferentiated), 3 days and 7 days in culture, total mRNA from both cells were extracted and mRNA level of *Ascl1* was analyzed by TaqMan quantitative PCR. Values were normalized against 18s RNA. Expression of *Ascl1* in DRG cells (N=4) was significantly higher than that of STr cells (N=4) at all time points. Expression of *Ascl1* increased significantly in both DRG and STr lines as cells differentiated *in vitro*. **P<0.005. All data were presented as Mean±SEM.

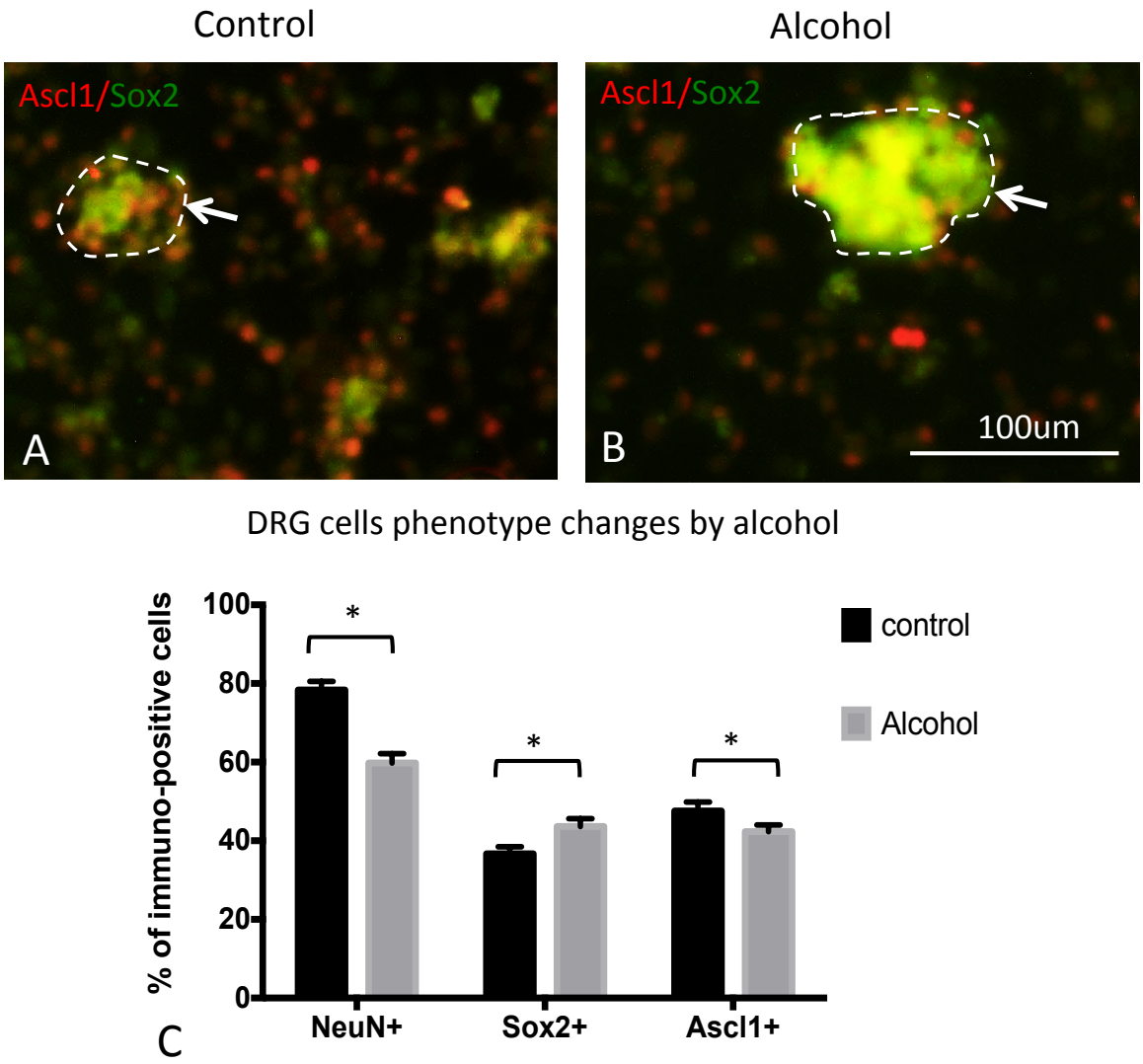

Figure 28. Alcohol retards neural differentiation in DRG neurosphere culture.

3-day cultured Control (A) and Alcohol-treated (B) DRG cells were fluorescent double-stained with antibodies against ASCL1 (red) and stem cell marker SOX2 (green) or mature neuronal marker NeuN. Alcohol increased the undifferentiated neurosphere size (reduced neurosphere migration)(A,B, dotted circle) and increased the SOX2+ expression and percentage of SOX2+ cells in culture (C). Alcohol reduced the percentage of NeuN+ cells in neurosphere culture, as well as the percentage of ASCL1+ cells in culture. N=8 each. *P<0.05. y-axis in C: the percentage of immune-positive cells out of total cells in culture. All data were presented as Mean±SEM

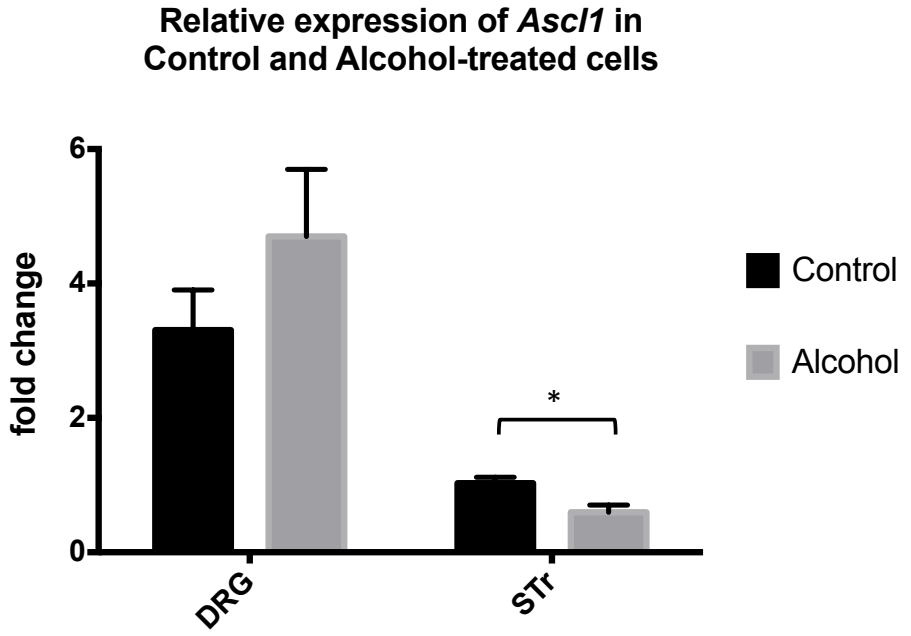

Figure 29. The effect of alcohol on *Ascl1* expression in DRG and STr cells.

Cells received 400mg/dL alcohol treatment (Alcohol) or no treatment (Control) from the beginning of differentiation (4hrs after plating) till the end of 3 days culture. Total mRNA from both cells (N=4 each) were extracted and mRNA level of *Ascl1* was analyzed by TaqMan quantitative PCR. Values were normalized against 18s RNA. *P<0.05. All data were presented as Mean±SEM

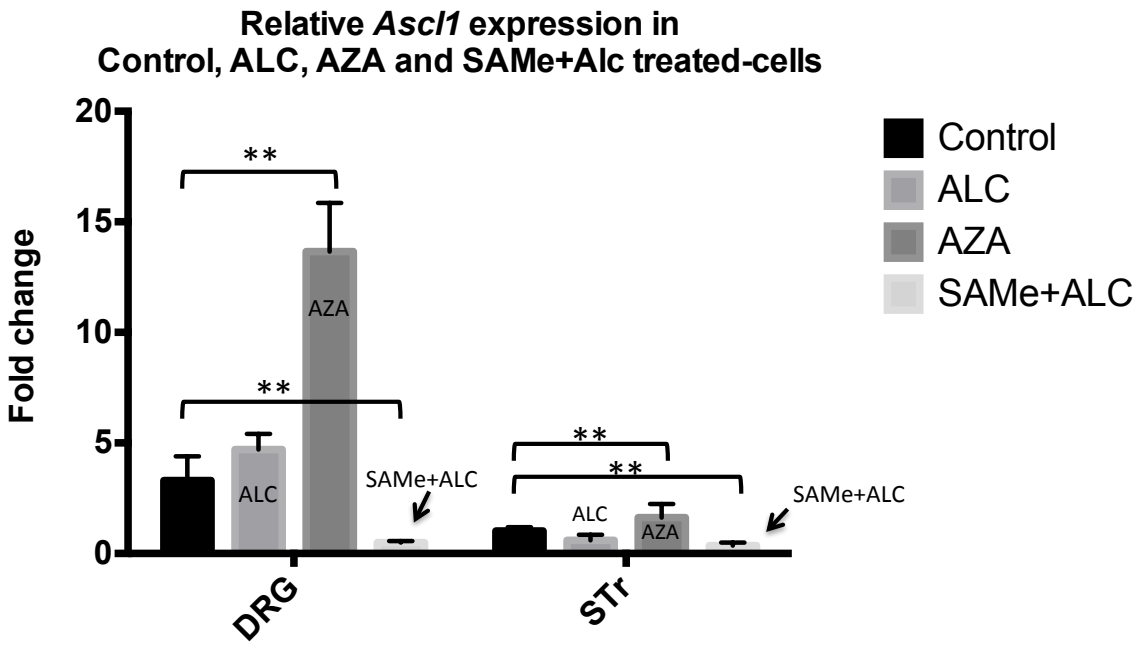

Figure 30. The effect of DNA methylation-modifying agents on *Ascl1* gene expression in DRG and STr cells. Both DRG and STr cells were treated with 400mg/dL alcohol (ALC), 100 ng/ml 5-AZA (AZA) or 40nM SAMe plus 400mg/dL alcohol (SAMe+ALC) throughout the 3-day culture periods. Total mRNA from both cells (N=4 each) were extracted and mRNA level of *Ascl1* was analyzed by TaqMan quantitative PCR. Values were normalized against 18s RNA. N=4 for each group. **P<0.005. All data were presented as Mean+SEM.

(Data produced by Dr. Chiao-ling Lo)

4.3.5 Alcohol altered *Ascl1* CpG-specific methylation

To investigate whether and how alcohol affects *Ascl1* gene-specific methylation, the sequencing-based method Bisulfite-Pyrosequencing was used to detect the ratio of methylated-cytosine (mC) to unmodified cytosine (C) at each CpG site. This method allowed assessing DNA methylation level at specific loci on *Ascl1* gene that are critical for transcriptional regulation.

We selected two PCR primers that amplify around 80-130bp fragments of bisulfite-treated DNA from *Ascl1* gene (one in exon, one around transcription start site). The primers were designed to avoid potential CpG methylation sites in order to minimize amplification biases for methylated or unmethylated DNA. We compared *Ascl1* sequence from our cells to USCS genome database to avoid potential C/T SNPs within the target region (SNPs could be generated over passages in stem cells). The two target regions on *Ascl1* covered by our primers are shown in Figure 31. Each primer covered 3 CpG sites.

We analyzed *Ascl1* CpG site-specific methylation from 3 days-differentiated DRG and STr cells. An example of CpG methylation analysis from Pyromark Q24 software was shown in figure 32. The methylation level of STr cells (25-45%) was significantly higher than that of DRG cells (1-4%) (Figure 33). Alcohol significantly decreased methylation at two CpG-sites at exon region in DRG cells (site 4 and 5), and 1 CpG site at TSS (site 6) in STr cells (Figure 33).

4.3.6 DNA methylation modifying agents altered *Ascl1* CpG-specific methylation

We next analyzed the effect of DNA methylation modifying agents on *Ascl1* CpG-specific methylation. In the DRG cells, 5-AZA treatment significantly reduced *Ascl1* CpG methylation in the exon region (site 4, 5 and 6) (Figure 34). The level of decrement is similar to that of alcohol treatment. In STr cells, 5-AZA treatment significantly decreased *Ascl1* methylation at both the exon region and the TSS region (site 2,3 and site 5, 6 respectively) (Figure 35). The SAMe supplementation replenished *Ascl1* methylation to approximately the control level at two CpG sites (Figure 35).

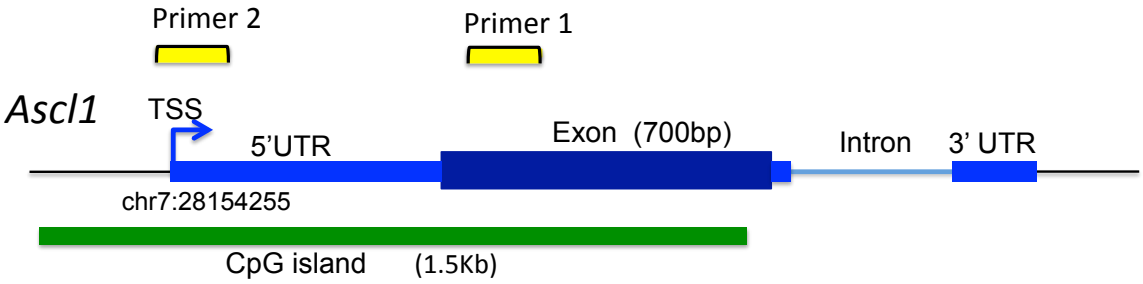

Figure 31. Schematic representation of the *Ascl1* gene structure and the two *Ascl1* site-specific primers. The *Ascl1* gene body is highlighted in blue; the CpG island is highlighted in green. TSS: transcriptional start site; 5'UTR: 5' un-translated region; 3' UTR: 3' un-translated region. Primer 1 and primer 2 each covers ~80-130bp regions, which are highlighted in yellow.

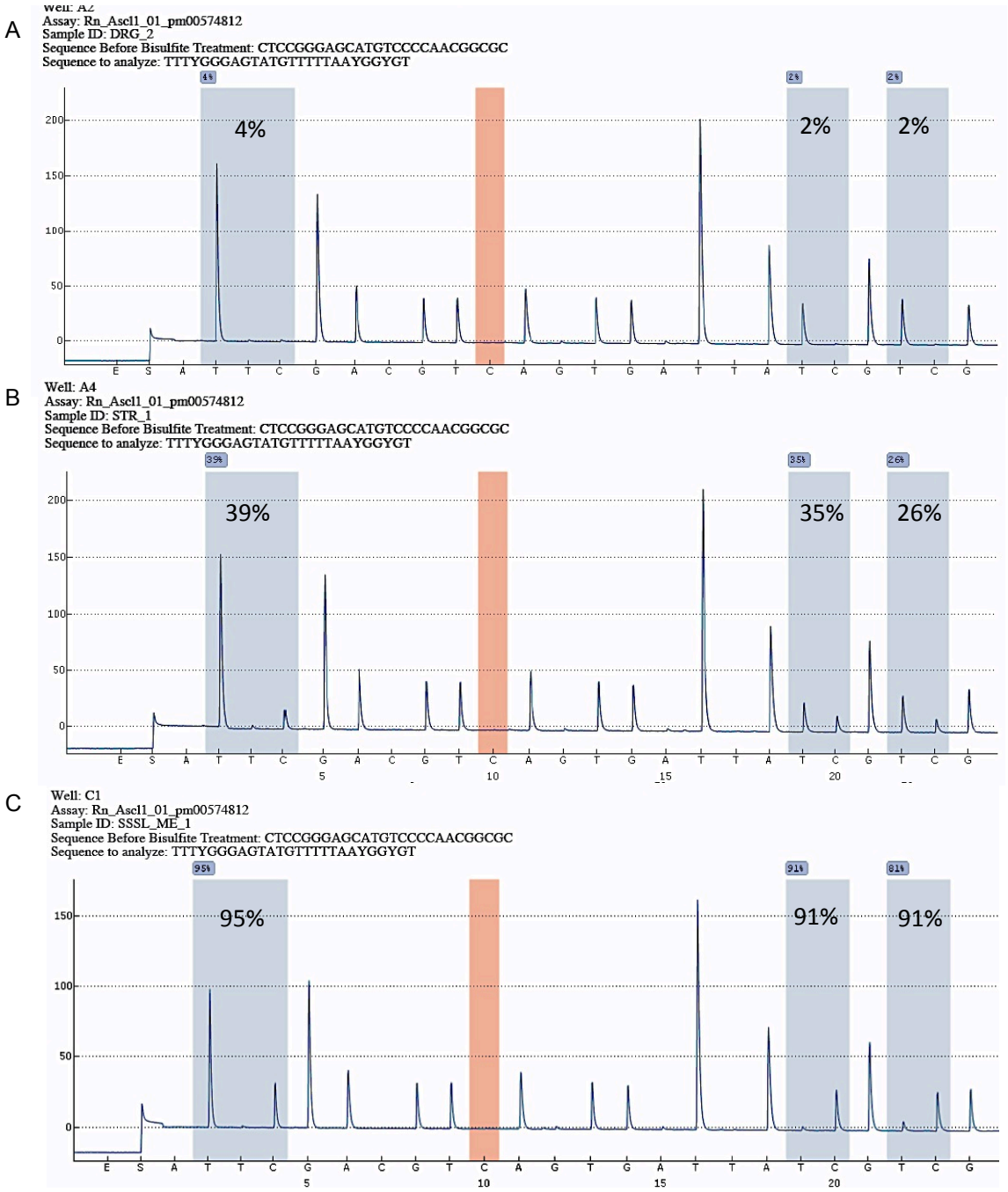

Figure 32. Examples of Pyrosequencing results for *Ascl1* gene.

The *y*-axis represents the signal intensity in arbitrary units (a.u.) while the *x*-axis shows the dispensation order. Panel (**A**) shows CpG methylation at *Ascl1* gene body region (3 CpG positions highlighted in blue) for DNA extracted from DRG cells. Panel (**B**) shows the same region analyzed for STr cells. Dispensations corresponding to the potentially methylated cytosine (C or T after bisulfite treatment) are highlighted in blue. The

percentage of methylation at individual CpG positions is shown as the percentage of methylation above the respective positions. An internal bisulfite-conversion control at a randomly picked non-CpG cytosine position was highlight in red (a complete bisulfite conversion renders <5% methylation at this position). Panel (**C**) shows CpG methylation at the same region from the same DNA treated with M.SssI (used as positive control).

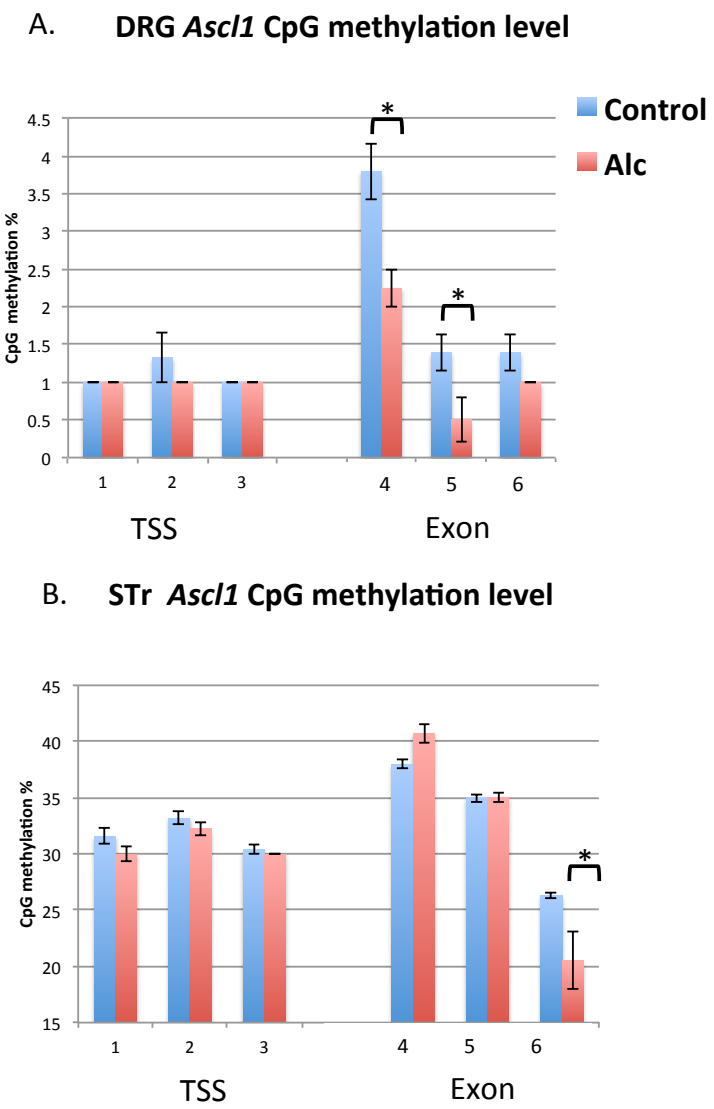

Figure 33. The effect of alcohol on *Ascl1* site-specific methylation in DRG and STr cells. Both DRG (panel A) and STr cells (panel B) received 400mg/dL alcohol (Alc) or no treatment (Control) from the beginning of differentiation (4hrs after initial plating) till the end of 3-day culture. gDNA were bisulfite-converted and analyzed for site-specific methylation using pyrosequencing. The *x*-axis represents individual CpG site: CpG sites 1-3 locate around TSS of *Ascl1* gene; CpG sites 4-6 locate on exon of *Ascl1* gene. The *y*-axis represents methylation level (% of methylated-cytosine at each CpG site). N=4 each group. *P<0.05. All data were presented as Mean±SEM.

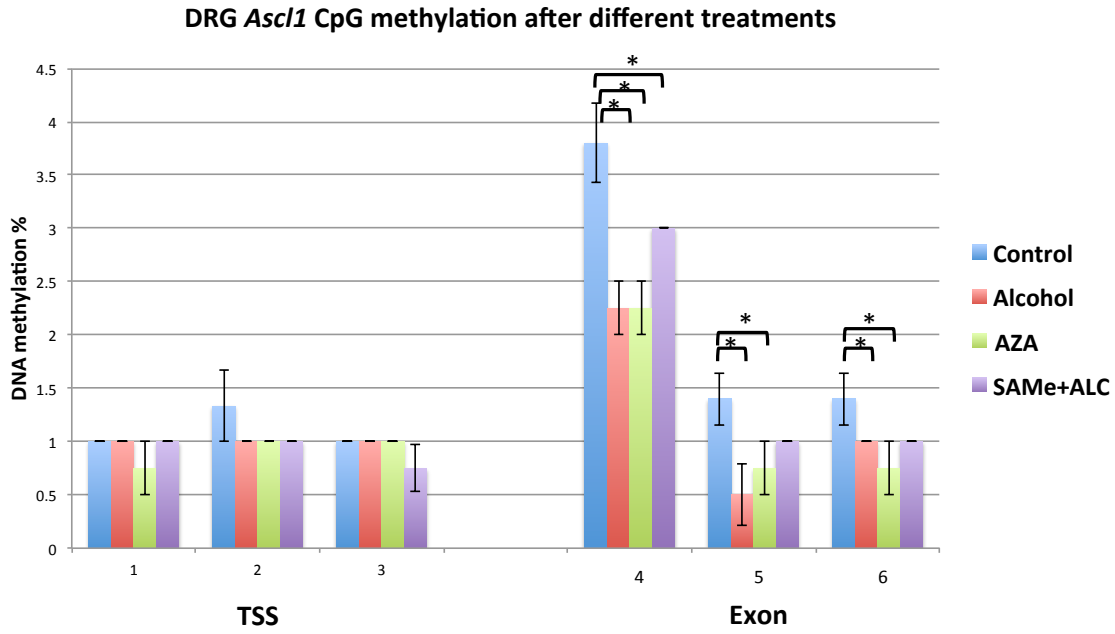

Figure 34. The effect of methylation-modifying agents on *Ascl1* site-specific methylation in DRG cells. The DRG cells were treated with alcohol, 5-AZA or SAMe plus alcohol (SAMe+ALC) from the beginning of differentiation (4hrs after initial plating) till the end of 3-day culture. The site-specific methylation of *Ascl1* gene (both TSS and exon region) was analyzed using pyrosequencing. N=4 each group. *P<0.05. All data were presented as Mean±SEM.

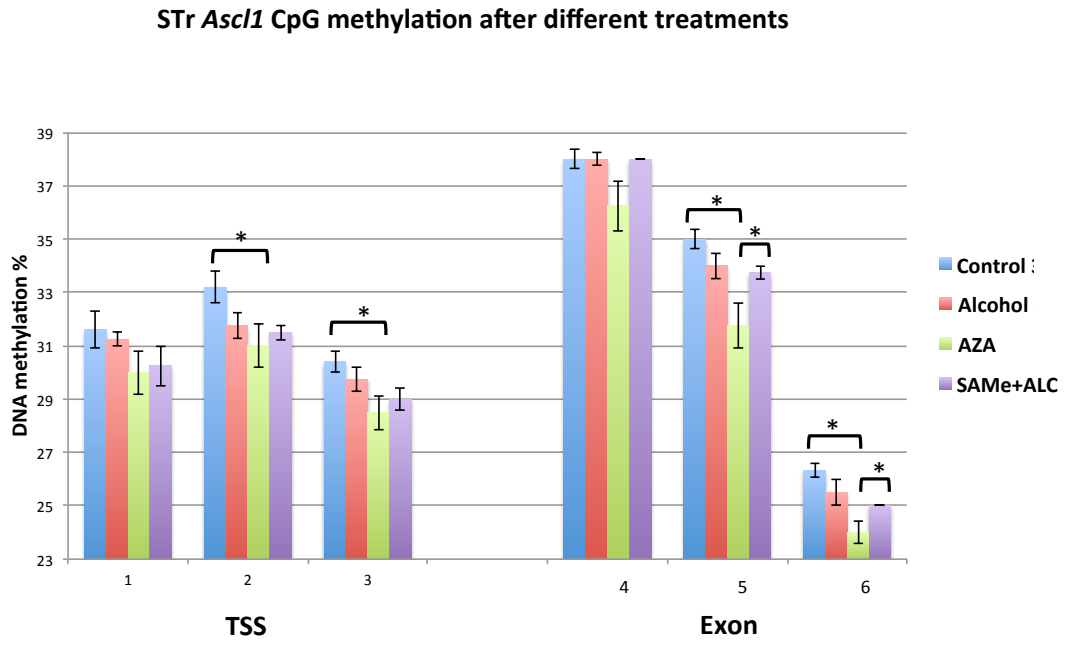

Figure 35. The effect of methylation-modifying agents on *Ascl1* site-specific methylation in STr cells. The STr cells were treated with alcohol, 5-AZA or SAMe plus alcohol (SAMe+ALC) from the beginning of differentiation (4hrs after initial plating) till the end of 3-day culture. The site-specific methylation of *Ascl1* gene (both TSS and exon region) was analyzed using pyrosequencing. N=4 each group. *P<0.05. All data were presented as Mean±SEM.

4.4 Discussion:

4.4.1 Summary

We demonstrated here that alcohol altered neural specification gene *Ascl1* expression and delayed neurosphere differentiation. Our data suggested that the alteration in *Ascl1* expression by alcohol might be a result of CpG site-specific methylation alteration of *Ascl1* gene.

4.4.2 *In vivo* versus *in vitro*

To illustrate the role of DNA methylation on *Ascl1* expression during neural differentiation, we employed a neural stem cell *in vitro* culture system, where isolated stem cells being grown in culture and treated with alcohol over differentiation period. Thus, we will discuss here to what degree can the *in vitro* system mimic the *in vivo* program, and the current knowledge on the role of *Ascl1* in neural differentiation in both systems.

In vivo, the *Ascl1* gene seems to have different contributions for neural differentiation depending on the cellular niche. In the SVZ, the *Ascl1* gene is highly expressed in neural progenitor cells, driving differentiation to neurons (Kim et al., 2008) (Also see Appendix 6). Conditional knockout of *Ascl1* by *in utero* delivery of Cre recombinase results in premature exit from cell cycle of a significant numbers of both ventricular zone (VZ) and SVZ progenitors (Kim et al., 2008). However, in the SGZ zone of dentate gyrus, neural progenitor cells are low in *Ascl1* expression, while having higher level of *Neurog1* expression (Kim et al., 2011). Overexpression of *Ascl1* in SGZ of dentate gyrus leads to a fate change that is driving cell differentiation towards oligodendrocytes (Jessberger et al., 2008). In addition, dorsal-ventral distribution seems to be important for the function of *Ascl1*. It was reported that in the telencephalon, *Ascl1* and *Neurog1/2* are expressed in a mutually exclusive manner in the dorsal and ventral germinal zones, giving rise to excitatory neurons and inhibitory interneurons respectively (Parras et al., 2002). Furthermore, *Ascl1* at early differentiation stage induced neuronal

fates, however, it also contributed to oligodendrocytes development at later stages (Battiste et al., 2007). These features point to the importance of cellular niche as well developmental timing as crucial factors determining cell fate.

In vitro neural stem cell/neurosphere cultures are often used to dissect out the cellular intrinsic properties from the environmental cues (cellular niches). Neural stem cells can be isolated from fetal brains and grown into free-floating neurospheres (stem cell aggregates) (Gage et al., 1995). Neurospheres contain a mixture of multipotent stem cells as well as lineage restricted progenitor populations (Bez et al., 2003). In the presence of growth factors (FGFs and EGFs), neurospheres hold their differentiation capacities into functional neurons, astrocytes and oligodendrocytes (Parmar et al., 2002). However, some intrinsic cellular properties of the stem cells might be changed since they were maintained in culture. For example, similar to *in vivo* studies, overexpression of *Ascl1* in cultured neural progenitors forces cell to exit cell cycle, and enter neuronal fate (Nakada et al., 2004, Berninger et al., 2007); inhibition of *Ascl1* expression enhances glia generation in dissociated retinal cell culture (Pollak et al., 2013). However, contrary to *in vivo* studies, cultured SGZ cells lost their capacities to further generate oligodendrocytes even overexpressed *Ascl1* (Jessberger et al., 2008).

We used two different neural stem cell lines in this aim. One cell line was isolated from adult rat Dorsal Root Ganglia (DRG) cells. After being isolated and cultured *in vitro* for about 10 years in our lab, the DRG stem cells were still capable of proliferation, expressing neuroepithelial, neuronal and glial markers (Singh et al., 2009b). These neural-crest derived stem cells possess sensory neuron properties, and expressed sensory neuronal markers vesicular glutamate transporter 2 (VGluT2), transient receptor potential vanilloid 1 (TrpV1) and the serotonin transporter (5-HTT) by 28 days in culture (Singh et al., 2009b). Another stem cell line was isolated from rat striatum (STr) (Chiang et al., 1996). The cultured STr cells mostly differentiated into GFAP+ astroglial cells.

We demonstrated that these two cell lines had distinct intrinsic cellular properties. The DRG cell expressed high level of *Ascl1* (Figure 25A) and the Ascl1+ cells were

mainly NeuN+ neuronal populations (Figure 25C and 26). The striatal stem cell, on the other hand, expressed low level of Ascl1 (Figure 25B), and contained less neuronal population (Figure 25D). Thus, whether the distinct cellular property is a result of differential *Ascl1* expression requires further investigation.

4.4.3 The role of DNA methylation in transcriptional regulation of *Ascl1*

DNA methylation and other epigenetic mechanism regulate neural gene transcription during neurodevelopment. Ongoing studies in our laboratory indicated that there was a negative association between gene-specific DNA methylation level and transcription level of *Ascl1* in neural stem cells. Our laboratory analyzed the whole *Ascl1* gene promoter and gene body regions (using a high-throughput platform MiSeq) and found that *Ascl1* gene was highly methylated (20~40%) in STr cells correlated with low gene transcription level, however in DRG cells, the *Ascl1* DNA methylation was very low to none (<5%), correlated with high gene transcription level (unpublished data). The *Ascl1* site-specific methylation level from pyrosequencing analyses was consistent with the above result.

It is worth mentioning that the bisulfite conversion method we utilized for DNA methylation analyses could not differentiate 5mC from 5hmC. After bisulfite treatment, all unmethylated cytosines (C) will be converted to uracil (U), and further sequenced as thymine (T) (C→ T); while methylated (methylated and hydroxylmethylated) cytosines will be protected and sequenced as C (mC/hmC →C). Thus, the total DNA methylation level at each CpG site will be represented as C/(T+C) ratio. In our case, the total methylation level would be a combination of both 5mC and 5hmC. However, it is reported that the genome-wide level of 5mC is about ~10 times higher than 5hmC in fetal brain (2.9% 5mC, 0.2% 5hmC)(Lister et al., 2013); even at 5hmC-enriched intragenic and enhancer regions, the 5hmC modification is about a quarter of modified cytosine residues (Kinney et al., 2011). Thus, the CpG methylation level from our analysis could approximately represent the level of 5mC.

The relationship between DNA methylation and transcription has been shown to be different depending on the location of the modification. For example, CpG methylation on the transcription factor (TF)-binding sites in promoters often impedes protein binding, therefore reduces transcription (Tate and Bird, 1993, Perini et al., 2005, Choy et al., 2010). Intragenic DNA methylation modulates alternative splicing important for tissue specification during neurodevelopment (Maunakea et al., 2013). Methylation surrounding the transcriptional start site (TSS) is tightly linked to transcriptional silencing by blocking transcription initiation or causing proximal polymerase pausing (Brookes and Pombo, 2009, Brenet et al., 2011).

Therefore in this aim, I further zoomed-in to analyze CpG site-specific methylation at two particular regions on the *Ascl1* gene. One is around the transcriptional start site (TSS) of *Ascl1* gene; another lies in the exon of *Ascl1* gene body. Each region covers three CpG sites. We showed here that, in accordance with the MiSeq data, both regions of *Ascl1* gene were highly methylated (30~40%) in STr cells, while were unmethylated (1~3%) in DRG cells (Figure 33). This result indicated that the innate DNA methylation level of neurodevelopment related gene was likely associated with the cellular intrinsic property (to become different cell fates). This notion was supported by several DNA methylome studies (Lister et al., 2013, Kim et al., 2014). They revealed differential global as well as neural-gene-specific methylation profiles in neuronal lineage cells and glial lineage cells.

We next asked whether the difference in *Ascl1* expression was mediated by DNA methylation changes? We treated both cell lines with DNA methylation modifying agents—5-AZA and SAMe, then analyzed *Ascl1* expression accordingly. It has been reported that 5-AZA treatments led to global DNA hypo-methylation and induced phenotypic changes in proliferating stem cells (Christman, 2002). For example, embryonic stem cells treated with 5-aza-2'-deoxycytidine (an analogue of 5-AZA) exited pluripotency towards endothelial cells (Banerjee and Bacanamwo, 2010) and cardiomyocytes fate (Yoon et al., 2006). Treating with 5-AZA alone was sufficient for activation of the expression of osteogenic genes (such as *Dlx5*) and drove the osteogenic

differentiation of mesenchymal stem cells (Lee et al., 2006, Zhou et al., 2009). We previously showed that 5-AZA treatments to our DRG cells reduced global DNA methylation, as well as decreased cell migration (Singh et al., 2009c). Similarly, supplementing methyl-donor SAMe was sufficient for altering methylome dynamics during myelination and correlated with alteration in myelination-related gene expression (Varela-Rey et al., 2014).

We showed here that 5-AZA treatment decreased *Ascl1* CpG site-specific methylation in both DRG (about 1-2% decrement per site) and STr cell lines (about 2% decrement per site). Furthermore, we observed over 4.3-fold increase in *Ascl1* expression in AZA treated DRG cells (Figure 33), and about 2-fold increase in STr cells. SAMe supplement replenished *Ascl1* methylation to the control level in DRG cells, correlated with decrease of *Ascl1* expression. These results suggested a potential role of DNA methylation in the transcriptional regulation of *Ascl1* gene.

4.4.2 The effect of alcohol on *Ascl1* methylation and neural differentiation

We employed the intrinsic neurogenic property of our previously established neural stem cell culture to investigate how alcohol exposure at the time of neural differentiation affects gene-specific DNA methylation in association with transcription. We demonstrated here that binge-like alcohol exposure (400mg/dL) delayed neural stem cell differentiation. This effect of alcohol on our DRG stem cells is similar to that of mouse embryonic stem cells (ESCs)(Arzumnayan et al., 2009, Ogony et al., 2013, Sanchez-Alvarez et al., 2013), as well as that of cultured NSC isolated from mouse telencephalon (Tateno et al., 2004) and hippocampal progenitor cells (Singh et al., 2009a).

We further demonstrated that alcohol decreased expression of *Ascl1* gene in DRG cells during neural differentiation in culture, and correlated with reduced neuronal cell (NeuN+) population. The alcohol-induced phenotype is similar to that of *Ascl1* mutant

neural progenitor culture. For example, neurosphere cultures from the lateral ventricular walls of *Ascl1* mutant mice produced less neurons than control while increased astrocytes population (Parras et al., 2004). Similarly, neurosphere culture from in *utero* alcohol-treated rat hippocampus or SVZ reduced neurogenic capacity, while induced astrogenic capacity (Santillano et al., 2005, Singh et al., 2009a, Roitbak et al., 2011).

As expected, alcohol also altered the methylation of *Ascl1*. We observed a relatively mild CpG methylation decrease by alcohol (<3% on average) in both cells; the level of decrement is similar to that of 5-AZA treatments. It is worth mentioning that neurospheres in our culture are a mixture of multipotent stem cells with lineage leading to neurons and glial cells. Therefore, the methylation alteration is based on an average of all cell types at the harvesting time-point. In addition, it is reported that DNA methylation changes might be exerted behind histone changes (to open the chromatin), due to low enzymatic accessibility to DNA sequences. Therefore, it would be of interest to analyze effect of alcohol on *Ascl1* methylation at a later time-point.

Altogether, our results suggested that the DNA methylation played a role in the transcriptional regulation of *Ascl1* gene, and that alcohol might exert its teratogenic effect on neural differentiation by altering *Ascl1* methylation and gene transcription **(see summary in Figure 36)**.

However, open questions remain for further investigation. For example, is DNA methylation alone or to what level an alteration in DNA methylation is sufficient to elicit a functional consequence. Our data showed distinct *Ascl1* methylation profiles in two distinct lineages of neural stem cells. The two cell lines held 40% difference in *Ascl1* methylation level, and showed 2-3 fold differences in *Ascl1* gene expression – DRG cells had low *Ascl1* methylation and high gene expression; while STr was the opposite. This is in accordance with evidence from genome-wide studies that there were cell-type specific DNA methylation profile in development-regulated genes (Lister et al., 2013, Kim et al., 2014). This suggested that there might be a certain level of epigenetic pre-disposition when cells committed to specific lineages in the nervous system.

Though the effect of environmental inputs (such as alcohol, AZA and SAMe) on *Ascl1* site-specific methylation was not prominent in this study (average <4% alteration), we do not exclude the possibility that other transcriptional regulatory regions might be more prominently affected by alcohol (such as TF-binding sites, enhancers, 3'UTRs).

For example, it was recently shown that SAMe deprivation led to significant de-methylation in promoter and enhancer regions where transcription factors bind (Smith and Meissner, 2013, Varela-Rey et al., 2014). Therefore, further investigations are needed to explore if and how methylation at TF-binding sites and promoters are involved.

Another question is if there were other epigenetic mechanisms regulating the transcription abilities. For example, the TFs for *Ascl1* expression (such as Hes1/2/3) could also be under regulation of DNA methylation— hypo-methylation on *Hes1* could induce *Hes1* expression, thus repress *Ascl1* expression (*Hes1* is a transcriptional repressor to *Ascl1*). In addition, it has been shown that, beyond DNA modifications, a histone modifier SIRT1 (a histone deacetylase) is required for the activation of *Ascl1* gene (Prozorovski et al., 2008, Zhang et al., 2011). Similarly, the let-7 microRNAs targeted *Ascl1* 3'UTR in neural progenitor cells and inhibited gene transcription and neural differentiation (Cimadamore et al., 2013). Thus, the regulation of *Ascl1* gene transcription is likely under combinatory control of DNA methylation, histone modification and microRNAs. (See figure 36 for summary)

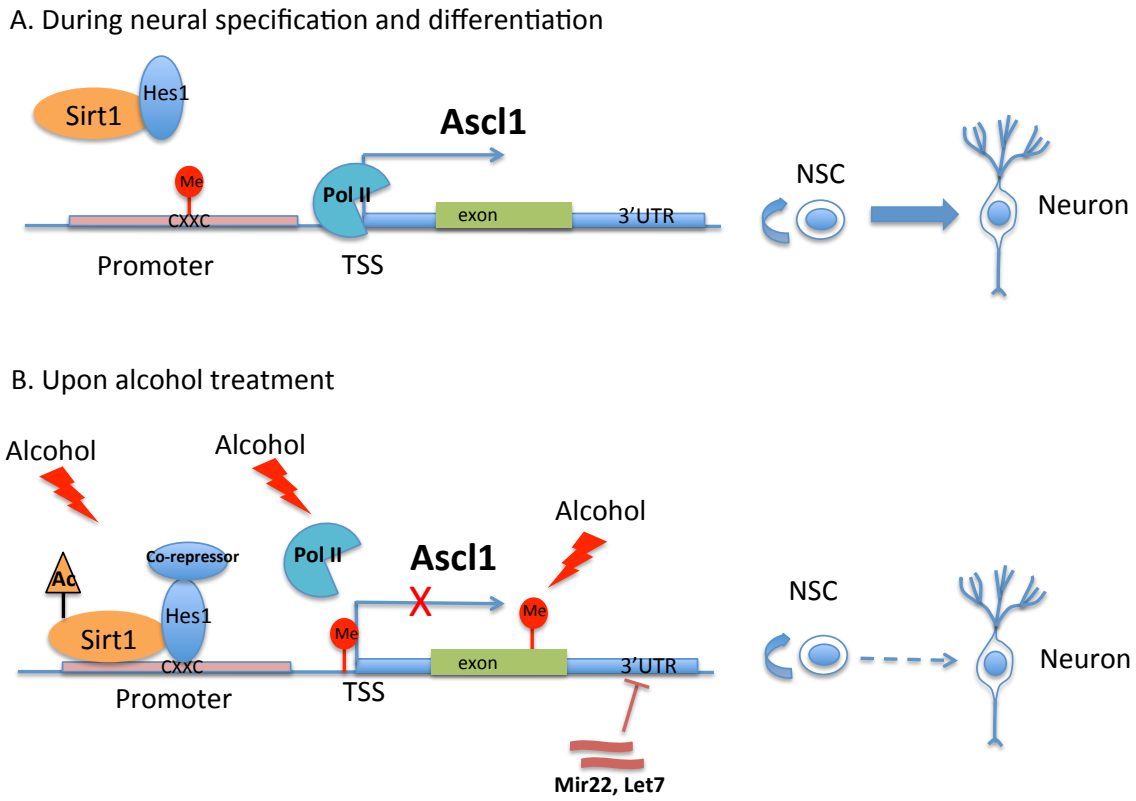

Figure 36. Schematic diagram of proposed epigenetic regulation of *Ascl1* transcription and the effect of alcohol.

Panel **(A)** During neural specification and differentiation, the DNA methylation (Me) were re-distributed from gene body to the promoter of *Ascl1*, impeding *Hes1* binding (transcription repressor) at promoter, while facilitating RNA polymerase II (*Pol* II) binding to TSS and *Ascl1* transcription. Therefore, neural stem cells were determined into neuronal fate. Panel **(B)** Alcohol delayed the re-distribution of DNA methylation from gene body to gene promoter. As a result, alcohol reduced *Pol* II binding to TSS. At the same time, *Hes1* complex (together with SIRT1 and co-repressor) was recruited to TF-binding site on promoter, inhibiting *Ascl1* transcription. In addition, microRNAs (*Mir22* and *Let 7*) might be involved in post-transcriptional regulation of *Ascl1* gene. Together, alcohol reduced the neurogenic capacity of neural stem cells.

3' UTR: 3' un-translated region; TSS: transcriptional start site; NSC: neural stem cell; *Pol*II: RNA polymerase II; Me: methylated cytosine; Ac: histone acetylation.

4.4.4 The role of neurogenesis niches in alcohol teratology

In this aim, we demonstrated that alcohol reduced neurogenic capacity of neural stem cells *in vitro*, possibly through reduced *Ascl1* gene expression. However, the mechanism of alcohol teratology on the *in vivo* neurogenic niches is more physiologically relevant to the onset of FASD. Within developmental niches, astrocytes and endothelial cells provide important signals to the intrinsic cellular programs, and are important for cell fate determination. Astrocytes in CNS stimulate neuronal differentiation through secretion of neurotropic factors such as Wnt3a (Song et al., 2002, Lie et al., 2005). Endothelial cells expand neurogenesis and activate *Notch* and *Hes1* to promote self-renewal (Shen et al., 2004, Calvo et al., 2011). Thus, it is critical to include these factors into consideration for the alcohol teratology. Several studies have demonstrated reduced neurogenic capacity in adult neurogenesis *in vivo* after prenatal alcohol exposure (Gil-Mohapel et al., 2010) (Miranda, 2012, Kajimoto et al., 2013). The epigenetic mechanisms underlying the alcohol-induced aberration in neurogenesis *in vivo* required further investigation.

Acknowledgement:

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CHAPTER 5. OVERALL DISCUSSION

5.1 Alcohol teratology in FASD

Maternal alcohol consumption is detrimental to the well-organized neurodevelopment processes in the fetus, and leads to varied degrees of physiological and neurological consequences, known as Fetal Alcohol Spectrum Disorders (FASD). To date, our understanding on how *in utero* alcohol exposure causes these persistent changes in physiological and cognitive functions is not fully understood. Recent transcriptome studies in animal models of FASD suggest that alcohol teratology is induced by gene expression perturbations at the time of alcohol exposure, which contributes to aberrant cellular physiology, neurodevelopment and FASD-related behaviors (Zhou et al., 2011c, Kleiber et al., 2013, Ron and Messing, 2013, Mantha et al., 2014). However, the molecular mechanisms underlying the long-lasting alteration on selected gene expression following prenatal alcohol exposure are still not known.

Many laboratories, including ours, have argued that these gene expression changes are accommodated by epigenetic mechanisms such as DNA methylation, histone modification and microRNAs (Haycock and Ramsay, 2009, Kobor and Weinberg, 2011, Zeisel, 2011, Laufer et al., 2013a, Resendiz et al., 2013). Our hypothesis was prompted by two key observations: 1) alcohol acts on one-carbon metabolism and alters key substrates needed to methylate DNA and histones (Kruman and Fowler, 2014) and 2) alcohol inhibits the activity and/or expression of DNA methyltransferases (DNMTs)(Perkins et al., 2013). Consequently, alcohol disrupts the methylome and histone modification dynamics, which ultimately leads to gene transcriptional changes (Zhou et al., 2011a, Bekdash et al., 2013, Laufer et al., 2013b, Varela-Rey et al., 2014). Our reasoning is further strengthened by the fact that maternal supplementation of a methyl-donor (such as choline) attenuates the effect of alcohol on gene expression (Ryan et al., 2008, Thomas et al., 2009).

Though epigenetic mechanisms have become intensively investigated in recent years. Different studies focused on different perspectives of epigenetic regulation in the teratology of FASD. For example, several laboratories have focused on genes critical for CNS developmental events, such as the epigenetic regulation of *Sonic hedgehog* (*Shh*) in neural tube closure (Ahlgren et al., 2002, Kietzman et al., 2014), the methylation of imprinting genes *H19* and *Igf2* in germline formation (Haycock and Ramsay, 2009, Ouko et al., 2009, Downing et al., 2011, Doshi et al., 2013), as well as DNA and histone methylation of the *POMC* gene in HPA axis formation (Bekdash et al., 2013). Other studies have entailed the use of more systematic approaches to examine the global "Epigenome" alterations (DNA methylome, histone methylome and transcriptome) caused by prenatal alcohol exposure (Zhou et al., 2011c, Kleiber et al., 2012, Perkins et al., 2013).

However, evidence is lacking as to which cellular processes (e.g. neural tube closure, neurogenesis, etc.) are directly or indirectly dysregulated by alcohol-induced epigenetic changes. Neurogenesis is of our particular interest because neural stem cells are critical targets for ethanol teratology. Collective evidence indicated that the prominent effect of alcohol was to delay neural stem cell differentiation and skew neurogenic potential toward other lineages (Miller, 1995, Camarillo and Miranda, 2008, Morris et al., 2010). Given that the progression of neurogenesis is guided by timely and spatially regulated gene expression, it is critical to understand how the epigenetic program is involved in this regulation, and how alcohol exposure at critical developmental time windows might disrupt this orchestrated program.

In this study, we focused on the key component of epigenetic regulation: DNA methylation. Our study revealed that aberrant DNA methylation is one possible mechanism for alcohol-induced dysmorphology in cortical and hippocampal development. In aim 1, we illustrated an organized DNA methylation program (DMP) during neurogenesis in hippocampal and cortical development. Our evidence suggested that the cellular DMP was neither random nor fixed, but spatiotemporally regulated and possibly associated with time-dependent gene expression. We demonstrated how prenatal alcohol exposure affected the DMP in the hippocampus and impacted its development. In

aim 2, we further demonstrated an organized 5mC/5hmC-mediated chromatin remodeling during neural differentiation and maturation. Our evidence that there was a chromatic shift between 5mC and 5hmC during neural differentiation suggested that there might be equilibrations between methylation and demethylation at development-regulated genes that "prime" the genes for either activation or repression upon lineage determination. We reported that alcohol delayed the transition between 5mC and 5hmC and their associated chromatin remodeling. In aim 3, we focused on the epigenetic regulation of neural specification gene *Ascl1* expression in neural differentiation, and the effect of alcohol of CpG-specific methylation and transcription of *Ascl1* gene. We suggested that the altered neurogenic ability in alcohol-treated neural stem cells might be a result of aberration of DNA methylation on neural specification gene.

All together, our results suggested a crucial role of DNA methylation in the regulation of neurogenesis, and a potential role of DNA methylation as a mediator for alcohol teratology. It is plausible that disrupting critical gene expression through alteration of DNA methylation from *in utero* alcohol exposure might contribute to the developmental dysregulation and cognitive dysfunction shown in FASD individuals.

5.2 Methodology considerations

The picture for the mechanisms of alcohol on neurodevelopment has been fuzzy. This is largely due to alcohol being a "dirty drug" affecting many genetic and epigenetic pathways. Unlike in the case of genetic disorders where a direct causal relationship can be proved by single or multiple genes mutations in a certain pathway, the teratogenic effect of alcohol is rather spread, and poses on complexes of cellular processes, from cell proliferation, cell cycle regulation to cell differentiation and cell apoptosis. Thus the field of alcohol research has lacked a cohesive hypothesis for the overreaching alcohol teratology.

Haycock et al. suggested in 2009 that the wide range of morphological and

physiological abnormalities that have been associated with *in utero* alcohol exposure suggested a high degree of "causal fan out" from the primary insults at the molecular and cellular levels to the defects observed at the clinical level (Haycock, 2009). By reviewing current evidence of the effect of alcohol on epigenetics, they proposed that epigenetic alterations in epigenetic programming might underlie the teratogenic consequences of alcohol exposure prior to conception, as well as after conception, during pre-implantation and gastrulation. Singh and colleagues argued that the study for alcohol teratology requires rather a systematic methodology, than a reductionist approach (Laufer et al., 2013a), since a 1.2-fold increase in many genes of a concert pathway can have a potentially greater physiological impact than a 20-fold increase in a single gene in complex diseases.

For the past 5 years, our laboratory has investigated the role of the DNA methylation program in the etiology of alcohol (Resendiz et al., 2013). We employed cellular approaches, as well as genome-wide analyses, in combination with cellular physiology to understand the correlation between DNA methylation aberration and alcohol-induced dysmorphology. One limitation for this methodology is that it is a correlative study, rather than a causation study. Therefore, in the last part of this study, we seek to understand the mechanisms of DNA methylation in regulating neural development through manipulation of DNA methylation by methylation-specific drugs (such as 5-AZA and SAMe). For example, we showed that inhibiting global DNA methylation by applying 5-AZA inhibited neural differentiation in neural stem cells, and delayed neural growth in mouse embryos. At the same time we have to take into considerations that these drugs are neither specific to genes nor to DNA methylation. For example, the SAMe supplementation increases both DNA methylation, as well as histone methylation (Teperino et al., 2010); HDAC inhibitors (such as TSA) not only alter histone modifications, but also alter DNA methylation (Dokmanovic et al., 2007); 5-AZA though acts specifically on DNA methyltransferases, could also causes DNA damages (Palii et al., 2008). These factors make the interpretation for the specific role of DNA methylation in alcohol teratology difficult. New technologies and methodologies are required to understand the specific role of epigenetic modification in mediating the effect

of alcohol. The Keck Foundation has been supporting the team from our laboratory and Purdue University to develop such technologies. The goal is to establish a gene-specific methylation-manipulating construct, by recognizing specific methylated sequence on DNA and recruiting genetically engineered enzymes to manipulate the site-specific DNA methylation *in vitro*. This method will further the current understanding of a causal role of DNA methylation in relation to gene transcription.

5.3 Implication in alcohol-related neurodevelopmental and neurodegenerative diseases etiology and therapeutics

Alcohol exposure as an environmental insult during gestational period causes immediate harm on the developing fetus. Increasing evidence suggested that continuous environmental insults might lead to increased epigenetic abnormalities and increase the incidence of late-onset neurological diseases (Shukla et al., 2008). The primary seeding of epigenetic errors from prenatal alcohol exposure, and secondary cumulative epigenetic insults via an abrasive environment (e.g., childhood abuse and stress) may result in a potential manifestation of neurological abnormalities later in life, such as mood disorders and schizophrenia (Resendiz et al., 2013). Moreover, epigenetic errors on imprinting genes (such as *H19, Igf2*) carried in the germlines may influence the future generations.

The chronic use of alcohol by pregnant women is commonly accompanied with mal-nutrition. This is partly due to women lacking proper nutritional intake, as well as the ability of alcohol to have an inhibitory effect on micronutrient metabolism and uptake. Well-known nutrients like folic acid, choline and B vitamins are key components of the epigenetic methyl-making pathway. Diets high in these methyl-donating nutrients can rapidly alter gene expression, especially during early development when the epigenome is first being established (Wolff et al., 1998). The importance of mother's diet in shaping the epigenome of her offspring is manifested in the case of *Agouti* mice. The mouse *agouti* gene is normally methylated, coding for a brown fur color. When mice that are prone to obesity and diabetes are fed a high-fat diet, the agouti gene becomes

unmethylated and mice fur color changes from brown to yellow (Dolinoy, 2008). In other words, genetically identical mice showed different phenotypes because they had an "epigenetic mutation". In deed, low systematic level of methyl-donor is related to the onset of several neurodegenerative disorders, such as neuropathy (Varela-Rey et al., 2014) and Alzheimer's disease (Smith et al., 2012).

Therefore, great efforts have been put into utilizing nutrition as a therapeutic approach for prevention, treatment and alleviation of epigenetic-related disorders. For example, maternal choline supplementation during 1st to 2nd trimester, or third trimester alone attenuated alcohol-induced fetal hippocampal dysmorphologies and learning deficits (Ryan et al., 2008, Thomas et al., 2009, Monk et al., 2012). Furthermore, gestational folic acid supplementation reduced the incidence of neural tube defects (Hewitt et al., 2011). A combined supplementation of B12, choline, folic acid and betaine to pregnant dams prevented the adverse effect of Bisphenol A (BPA, a compound used to make polycarbonate plastic) on phenotypes in the offspring (Dolinoy et al., 2007). Our laboratory is currently investigating the effect of SAMe supplementation throughout 1st and 2nd trimester on alcohol-induced dysmorphologies in mice fetal brains *in vivo*. These investigations will shed light on the mechanisms of epigenetic medicine.

CHAPTER 6: FUTURE DIRECTIONS

While the results presented here are compelling, further investigations are needed to understand the alcohol-induced, DNA methylation-mediated dysregulation in brain development. For example, in the *in vitro* setting of neural stem cell culture, it is crucial to explore the effect of alcohol exposure on CpG methylation of other regions of *Ascl1* genes (such as transcription binding sites and enhancer regions) and correlated *Ascl1* gene expression. Also, since neural specification gene does not work alone, they work in concert with upstream or downstream signaling cascade (such as *Hes1* and *Notch* signaling), as well as other compensative neural specification genes (such as *Neurogenins* and *Maths)*, it is also important to understand how these signaling are concerted under the regulation of DNA methylation. In addition, since the effect of DNA methylation on transcriptional alteration might be following that of histone modifications, it is of interest to investigate beyond our current time-point, for example, to extend the culture duration from 3 days to 7 days. Finally, for a further global understanding on the etiology of alcohol on brain development, it is crucial to extend our current *in vitro* settings to an *in vivo* setting. For example, it would be interesting to investigate how 5-AZA administration to pregnant dams damage the organized DNA methylation program *in vivo*, at a cellular and molecular level. Since 5-AZA has a certain level of toxicity to the biological system, there have been very few studies reported on the *in vivo* effect of 5-AZA in a mouse model. However, a novel DNA methylation inhibitor, zebularine, has been shown to be less toxic (Stresemann et al., 2006) and could be used as an alternative for 5-AZA for the *in vivo* studies. Simultaneously, we are investigating the effect of maternal nutrient supplementation on counteracting the adverse effect of alcohol on brain development. These experiments together will further the current understanding on the role of DNA methylation in mediating alcohol teratology.

APPENDICES

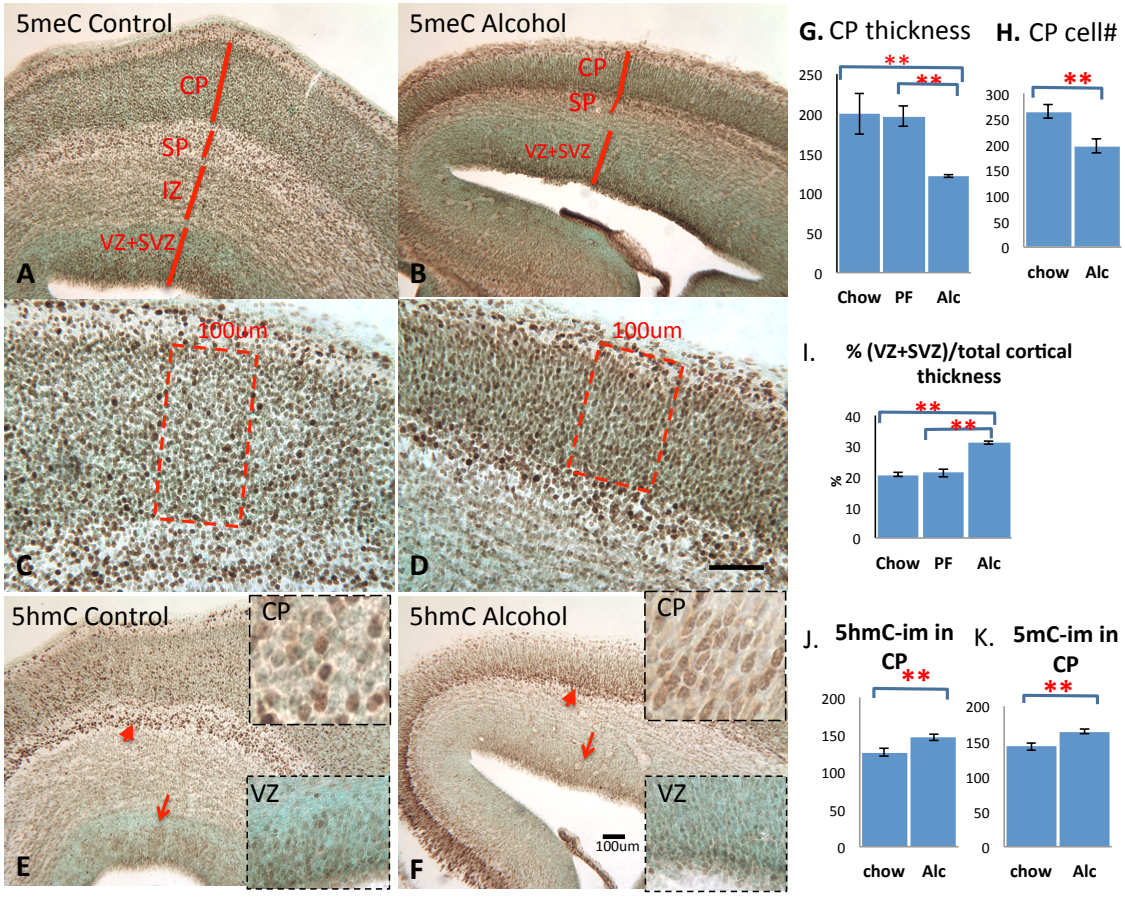
Appendix 1. Phenotypic measurements of E17 cortex in Chow, PF and Alc-treated mice

E17 Embryos	Chow	PF	Alc
a. Neocortex Thickness (µm)	688 ± 20	663±25	495±6.5$^{**\#\#}$
b. CP Thickness (µm)	199±9	189±12.9	121 ±3.7$^{**\#}$
c. (VZ+SVZ)%	20.67±0.56	21.22±1.24	30.97±0.55$^{**\#\#}$
d. NeuN-im (in SP)	194±12.5	188± 33.5	104±23$^{*\#}$

*P<0.05; **P<0.005 (Alc vs Chow); $^{\#}$P<0.05; $^{\#\#}$P<0.005 (Alc vs PF); N=4 each.

SP: subplate layer; CP: cortical plate; VZ: ventricular zone; SVZ: subventricular zone

Data presented as Mean±SEM.



Appendix 2. Alcohol delayed cortex development at E17 and altered DNA methylation program. Alcohol reduced cortical thickness (A,B) and cortical plate (CP) thickness (A,B,G)(also see quantitation in Table 2). The cell number per area in cortical plate was reduced (boxed region in C,D; also see quantitation in H). At the same time, the VZ+SVZ thickness was proportionally increased (A,B, also see quantitation in I). Alcohol altered DNA methylation (5mC shown in A-D; 5hmC shown in E,F) in cortical plate (also see quantitation in J,K). Alcohol increased 5hmC-im in subplate (E,F, arrowhead) and reduced 5hmC-im in VZ (E,F, arrow). Boxes in E,F showed enlarged region of cortical plate (CP) and ventricular zone (VZ) respectively. Scale bar: A,B,E,F=100μm; C,D=100μm. ** P<0.005. All data were presented as Mean ± SEM.

Appendix 3: Fluorescence Lifetime Imaging based Forster Resonance Energy

The following method provided by Nur P. Damayanti from Purdue University

The Immunocytochemistry procedure was done similar to the one described above. In brief, formalin fixed brain tissue section was used. The sections were washed in PBS 3 x 5 minutes, then permeabilized using Triton-X 1% in PBS for 30 minutes on a shaker. The first antibody (5mC or 5hmC) was labeled with Alexafluor 488 conjugated secondary antibody at 1:500 dilution in nomal goat serum, on a shaker for 90 minutes, washed for 3 (3x5 min;3x15 min; 4x30 min) hours. Tissue was then imaged and fluorescence lifetime was measured at the cortex and hypocampus area. The same tissue section was washed for 30 minutes on a shaker, and then incubated with a pair of primary antibody (MBD1, MBD3, or MeCP2) for 18 hours, and labeled with Alexa fluor 546 conjugated secondary antibody at 1:500 dilution for 90 minutes, and mounted in the imaging chamber for imaging. For each demethylation (5hmc, 5mc) / protein (MBD1, MBD3) interaction 50 cells are used from ~8 cells /setion of hippocampal or cortical region of 4 P7 brains. Total, 400 cells were used for FLIM-FRET analysis.

FLIM-FRET experiment was performed by measuring the fluorescence lifetime of donor in the absence and the presence of acceptor using *Microtime 200* (Picoquant, GmbH, Berlin Germany). Fluorescence lifetime is defined as the time in which fluorescence intensity of a fluorophore is decreased to 1/e of its initial intensity. The source of excitation is a picosecond pulsing diode laser at 467 nm excitation wavelength for the donor (Alexa fluor 488) and 530 nm for acceptor (Alexa fluor 546). The laser repetition rate was 40 mHz, and the laser power was ~3 mW. The laser was focused on the sample using an apochromatic 60x water immersion objective with1.2 NA. Off-focus fluorescence was rejected with a 50 mm pinhole for efficient collection of the emission. Emitted fluorescence was collected using the same objective and separated from excitation beam using dichroic mirror. Band pass filter 480-520 nm for Alexa fluor 488 , and 570-645 nm was used to ensure that the collected photons came from the donor fluorophore only. Fluorescence emission was collected using two single photon avalanche photodiodes using (SPAD, SPCM-AQR-14, Perkin-Elmer). Each photon was tagged with a time stamp that identifies its arrival time in the detector after the laser pulse, using time correlated single photon counting (TCSPC) in the Time Tagged Time

Resolved Single Photon Mode (TTTR) and the FLIM image and the decay curve were obtained. Details of instrumentation can be found in (Varghese, 2008, Chen et al., 2012).

Fluorescence lifetime was obtained from the multiexponential reconvolution fitting with the appropriate instrument response function (IRF), following (Lakowicz, Chen et al., 2011a):

$$I(t) = \sum_{i=1}^{n} \alpha_1 \exp\left(-\frac{t}{t_i}\right)$$

Time bin was applied to remove the background and threshold was set for 500 photon per pixel. A minimum of 25 different cells from 4-5 separate experiments were fitted for efficiency calculations.

FRET efficiency (Vidi et al., 2008) was calculated by:

$$\text{Efficiency} = 1 - \frac{\tau a}{\tau b}$$

where :

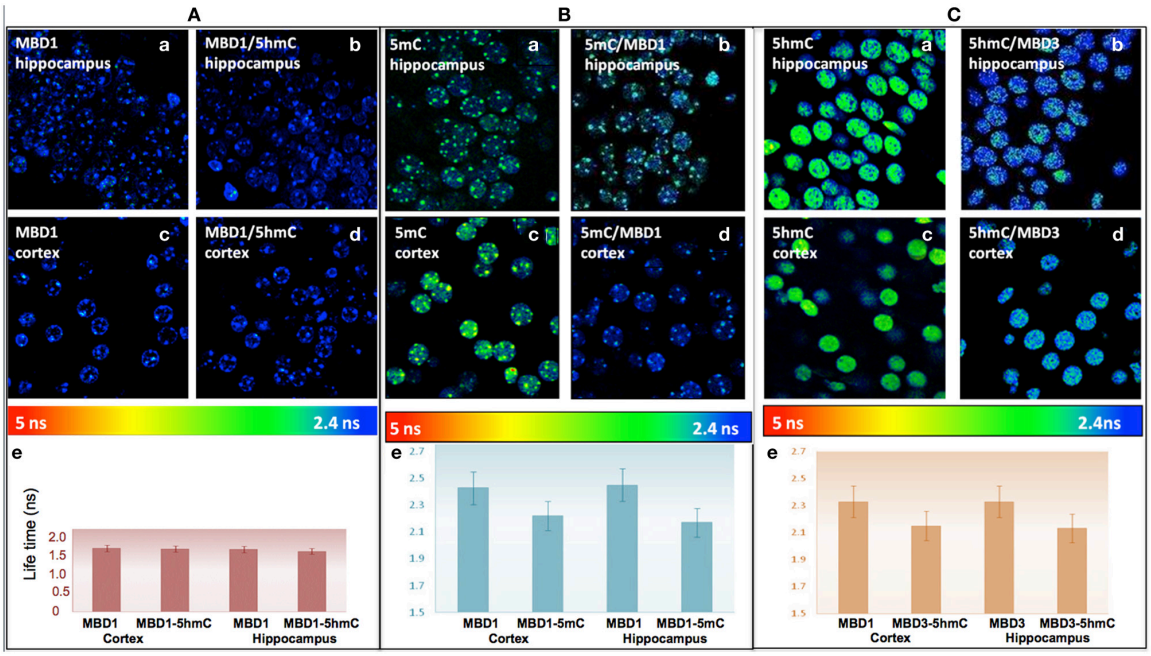
τa = Fluorescence lifetime of donor with the absence of acceptor

τa= Fluorescence lifetime of donor with the presence of acceptor

Efficiency above 5% is considered as significan and vice versa (Ruttinger et al., 2006).

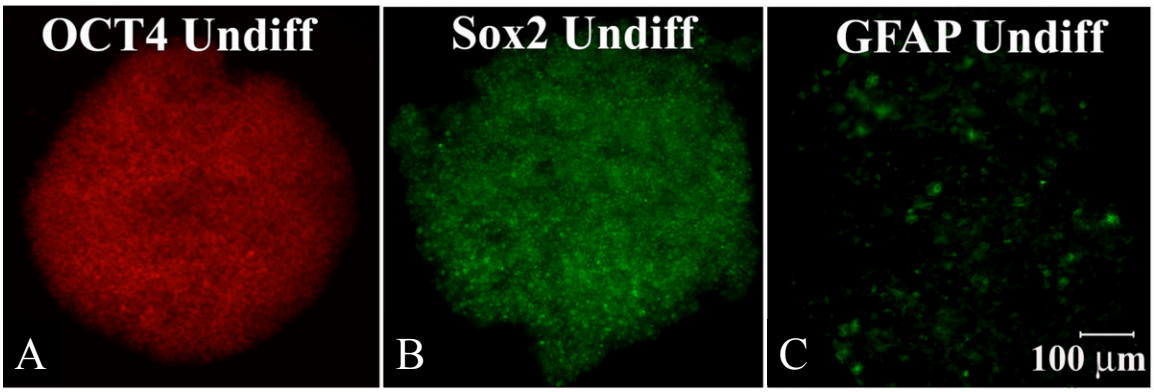
Both the positive and negative controls were also performed by FLIM- FRET to validate our findings. For positive control, we demonstrated that the goat anti-mouse IgG Alexa 488 and donkey anti-goat Alexa 546 IgG (depicting co-localization of the two fluorophores, acceptor and donor,) were within the FRET distance denoting co-localization of the two marks. For negative control, the primary mouse anti-5mC and the secondary anti-mouse Alexa488 donor (blue channel) does not show any binding proximity to the anti-rabbit Alexa 633 or anti-rabbit Alexa 546 (Acceptor red channel).

Appendix 4. Results for FLIM-FRET analyses in hippocampus and cortex.



There is no significant FRET between MBD1 and 5hmC in the hippocampus **(a)** MBD1 **(b)** MBD1/5hmC, and in cortex **(c)** MBD1 **(d)** MBD1/5hmC. There is no significant change ($P > 0.05$) (FRET efficiency <3%) of the lifetime of donor in the cortex and hippocampus. Average lifetime **(e)** of MBD1 was 1.53 ns, and MBD1/5hmC was 1.51 ns in cortex, and MBD1 was 1.5 ns and MBD1/5hmC was 1.47 ns in the hippocampus. **(B)** There is a significant FRET between 5mC and MBD1 in hippocampus: **(a)** 5mC **(b)** MBD1/5mC, and in cortex **(c)** 5mC **(d)** 5mC/MBD1. There was a significant decrease ($P < 0.05$) (FRET efficiency >5%) in the lifetime of donor in the cortex and hippocampus. Average lifetime **(e)** of 5mC was 2.45 ns and 5mC/MBD1 was 2.17 ns in cortex, 5mC was 2.42 ns and 5mC/MBD1 was 2.22 ns in the hippocampus. **(C)** There is a physical interaction between 5hmC and MBD3 in hippocampus (**a**, 5hmC; **b**, MBD3/5hmC) and in cortex (**c**, 5hmC; **d**, 5hmC/MBD3). There is a significant decrease ($P < 0.05$) (FRET efficiency >5%) of lifetime donor in cortex and hippocampus. Average lifetime **(e)** of 5hmC was 2.45 ns, and 5hmC/MBD3 was 2.17 ns in cortex; and 5hmC was 2.42 ns, and 5mC/MBD3 was 2.22 ns in the hippocampus.

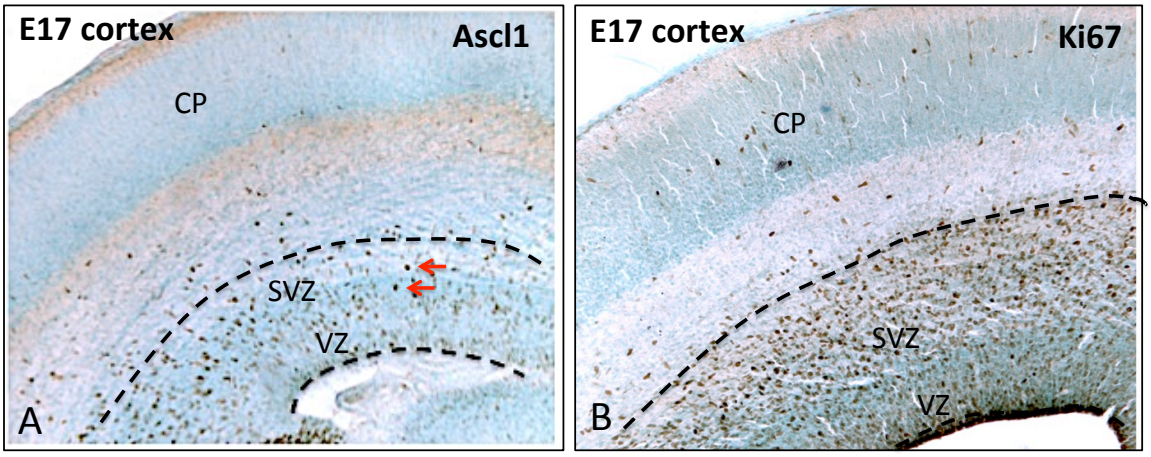
Appendix 5. Multipotency of undifferentiated neural stem cells.



The undifferentiated DRG stem cells were aggregated as floating neurospheres, and expressed stem cell marker Oct3/4 and Sox2. Scale bar: all=100um.

Data published in (Singh et al., 2009c).

Appendix 6. ASCL1 expression *in vivo* in E17 mouse cortex.



The ASCL1 is highly expressed in subventricular zone (SVZ) and ventricular zone (VZ) of cortex at E17 (A, arrows), where proliferating neural progenitor cells located (Ki67+) (B). The ASCL1 is not expressed (or lower expressed) in cortical plate (CP), where cells already differentiated and began to mature into neurons (Ki67-).

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2. Chen Y.; Ozturk N.; Zhou FC. DNA Methylation Program in Developing Hippocampus and its Alteration by Alcohol. ***PLoS One*** 2013;8(3):e60503
3. Resendiz M.; Chen Y.; Ozturk NC; Zhou FC. Epigenetic Medicine and Fetal Alcohol Spectrum Disorders. ***Future Medicine*** **2013 Feb;5(1):73-86.**
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CONFERENCE ABSTRACT AND PRESENTATIONS:

1. Y. Chen; NC Ozturk; FC Zhou. DNA methylation program during brain neurogenesis and differentiation and the effect of alcohol exposure. Poster presentation. Research Society on Alcoholism, Orlando, FL, 2013
2. Y. Chen; NC Ozturk; FC Zhou. Alcohol alters cellular DNA methylation program in growth retarded cortex and hippocampus. Poster presentation. Research Society on Alcoholism, San Francisco, CA, 2012
3. FC Zhou; Y. Balaraman; Y. Chen; Y. Liu; K. Nephew. DNA methylation diversification signals neural stem cell differentiation. Poster presentation. Society for Neuroscience, Washington DC, 2012.
4. Y. Chen; A. Love; FC Zhou. Cellular DNA methylation program during neurulation and its alteration by alcohol exposure. Poster presentation. Research Society on Alcoholism, Atlanta, GA, 2011
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