MIR-153 AND MIR-335, ETHANOL SENSITIVE MICRORNAS, CONTROL NSC/NPC MATURATION DURING FETAL BRAIN DEVELOPMENT

A Dissertation

by

PAI-CHI TSAI

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Chair of Committee,	Rajesh Miranda
Committee Members,	Farida Sohrabji
	Robert Chapkin
	Jane Welsh
Head of Department,	William Griffith

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ABSTRACT

Maternal alcohol consumption during pregnancy, especially during the first and second trimester, can cause the wide range of severe birth defects classified as fetal alcohol spectrum disorder (FASD). FASD is a wide spectrum disease characterized by delayed fetal growth, facial abnormalities, and cognitive and behavioral deficits of the central nervous system. The cost of FASD to the U.S. healthcare system is estimated at more than $6 billion annually, suggesting the problem of maternal consumption is increasing through years.

Previous research by Sathyan and his colleague has shown that only few miRNAs, miR-9, miR-21, miR-153, and miR-335, were able to mediate ethanol's teratogenic effects through regulation neural stem/progenitor cell (NSC/NPC). However, the mechanisms whereby miRNAs mediate fetal neural stem cell (NSC) vulnerability and contribute to ethanol's teratology are complex and still unclear. Therefore, the goal of my dissertation is to discover novel pathways of two miRNAs, miR-153 and miR-335, as key modulators of ethanol. Data from the miR-153 study identified NFIA (nuclear factor-1A) and its paralog, NFIB, as direct targets of miR-153 *ex vivo* as well as *in vivo*, and miR-153 overexpression prevented neuronal differentiation. MiR-153 functional analysis in neurospheres suggested that overexpression of this microRNA prevented, and partly reversed ethanol's teratogenic effects on miR-153 target transcripts. This antagonistic effect was also found in the pharmacology studies using varenicline, a FDA-approved drug as a partial nicotinic acetylcholine receptor agonist, which increased miR-153 expression. These data

collectively suggested a role for miR-153 in preventing NSCs/NPCs differentiation, and showed that direct or pharmacological manipulation of miRNAs in an *ex vivo* model have the potential capability to prevent or even reverse ethanol's effects on fetal brain development. Data from miR-335 research presented show that miR-335 regulates NSCs/NPCs markers DCX, NeuroD1, and c-Kit, and that miR-335 dysregulation results in neuronal premature-maturation via increasing asymmetric cell divisions, driving neuronal early differentiation through inducing stem cell genes DCX and NeuroD1 in the ventricular zone (VZ) of the developing cortex.

In sum, my data conclude that miR-335 and miR-153 act as molecular brakes to prevent NSCs/NPCs early maturation by regulating cell differentiation genes during the second trimester, and ethanol leads to organizational defects in the developing cerebral cortex through driving premature-maturation. In addition, preliminary data from miRNA functional studies indicate that misregulation of miRNA-regulated genes by ethanol exposure can be prevented or reversed in the presence of microRNAs or specific pharmaceuticals. This evidence explores the application of candidate miRNAs, as well as other medical drugs, as potential therapeutics to overcome ethanol's teratology.

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CHAPTER I

INTRODUCTION

General background

In all organisms that replicate by sexual reproduction, fertilization results in the mixing of parental genetic information and the transformation of male and female germ cells into a totipotent stem cell that has the capacity to regenerate an entire organism. In mammals, this stem cell divides to create a mass of similarly totipotent stem cells that are collectively termed the morula. Mammalian pregnancy is a unique evolutionary adaptation to sexual reproduction in that it facilitates a protracted and protected period of fetal development, which facilitates the transformation of the morula into highly complex organisms. This extended period of protected development facilitated the evolution of the brain to favor increased size and complexity, which in turn supported the acquisition of increasingly complex mammalian skills like tool building, language, mathematics and the writing of PhD dissertations. As with non mammals, much of mammalian development appears to be genetically programmed (Watson and Crick, 1953; Gluecksohn-Waelsch, 1961) so that progeny of one mammalian species look and behave like their parents rather than like adults of another species of mammals, though the starting material, the morula exhibits gross morphological similarity across all mammals, and indeed, across all vertebrates.

It was previously believed that the mammalian uterus and placenta was an impervious barrier to the environment and that the fetus developed within this

environment, protected from all external factors. However, following the thalidomide disaster of the 1960s (see below) it became increasingly apparent and accepted that the developing embryo could be highly vulnerable to environmental agents, increasing the risk for developmental defects and adverse consequences that last for the lifetime of an the organism. Therefore, maternal activity has become an important focus for scientific research, since maternal health, dietary habits and a variety of toxins accumulated within the environment directly influence fetal development through the placental barrier. Agents in the maternal fetal environment that adversely affect fetal development are often termed, '*teratogens*', and the effects of these agents on the developing fetus is the subject of the field of biomedical study termed '*Teratology*'. The medical, legal, economic and sociological costs of developmental defects are enormous. Therefore understanding the actions of teratogen and mechanisms underlying developmental defects is an important research endeavor.

As mentioned above, mammalian pregnancy evolved to support the development of increasingly complex nervous systems. Therefore, the formation of the brain, is one of the primary foci of fetal development. The developing brain begins from a single epithelial layer, the neuroectoderm. During the first trimester of pregnancy, this layer invaginates into the developing embryo. At the dorsal boundary of this invagination, the neuoectoderm comes together to form a tube-like structure, the neural tube, containing the stem cells of the germinal epithelium of the brain and spinal cord, surrounding an elongated lumen.. The lumen becomes the brain's developing ventricular system, while the layer of neuroectoderm becomes the ventricular zone (VZ), the location for the stem

cells that will form the future brain and spinal cord (Gage, 2000; Paspala et al., 2011). Neural tube defects including spina-bifida and anencephaly are caused by failure of neural tube closure. While genetic factors may result in neural tube defects (Detrait et al., 2005; Bassuk and Kibar, 2009), substantial evidence implicate maternal dietary deficiencies including deficiencies in folic acid (Emery, 1977; Molloy et al., 2009), and other environmental factors (Graham and Ferm, 1985; Campbell et al., 1986) as causative agents. This evidence suggests that even the earliest stages of brain development are vulnerable to the maternal environment.

These proliferative cells in the VZ will either directly or indirectly give rise to all of the intrinsic cells (neurons, astrocytes and oligodendrocytes) of the developing central nervous system. Most neurons of the mature brain are generated from the end of the first trimester through the second trimester (Bystron et al., 2008). Approximately 2,500 new neurons are born in the human brain every minute during this significant period (Noback, 2005) because of an enormous rate of stem/progenitor cell (NSCs/NPCs) proliferation (Caviness et al., 1995). Because this unique and extensive proliferative drive of NSCs occurs only during this one period of fetal development and will never be recapitulated at any subsequent period in the life of the organism, we term this a 'critical period for neurogenesis'. Our laboratory has focused on the hypothesis that the *critical period for neurogenesis* is a particularly vulnerable period, where the developing brain is more susceptible to environmental teratogens. Because of the enormous rate of proliferation, perturbations in the fetal-maternal environment, that result in even small alternations in

NSC biology are likely to be amplified, resulting in large brain defects, and severe and permanent consequences for adult brain function.

In my dissertation, I will focus on fetal neural effects of alcohol (ethanol or ethyl alcohol) and my work to understand teratogenic mechanisms that are triggered by alcohol. Alcohol has been widely consumed by humans since antiquity (McGovern et al., 2004). Alcohol has rewarding properties and acts to stimulate neural circuitry within mammalian brains that mediate feelings of pleasure in response to rewarding stimuli (Colombo et al., 2002; Colombo et al., 2005; Stefano et al., 2007). Other non-human mammals including primates, elephants, pigs and some rodents also exhibit preference for diets that contain alcohol (Carlson and Lydic, 1976; Williams et al., 2009), and can become intoxicated following alcohol consumption, suggesting that alcohol activates common mammalian reward pathways. However, alcohol is also a strong fetal teratogen. Maternal alcohol consumption during pregnancy is a known causative factor for fetal developmental defects including, intrauterine growth retardation, microencephaly craniofacial malformations, cardiac abnormalities and skeletal defects (Jones and Smith, 1973; Chudley et al., 2005; Hoyme et al., 2005). However, the fetal brain is a particularly vulnerable target of alcohol, and fetal exposure to alcohol is the leading non-genetic cause of mental retardation in the United States (Abel and Sokol, 1987; West et al., 1998). Collectively, the fetal defects caused by maternal alcohol consumption have been termed the 'fetal alcohol spectrum disorder' (FASD), and the severe end of the FASD continuum has been termed the 'fetal alcohol syndrome' or FAS. However, the mechanisms of FASD are complicated and not yet fully understood.

My dissertation will focus on my work describing a novel pathway for teratogenesis, i.e., the involvement of a class of non-protein-coding RNA molecules. Non-coding RNAs (ncRNA) account for approximately 92-98% of genomic output and in the last decade have been found to control genes in myriad ways, from chromosome modification of DNA and RNA, to protein regulation (Lee et al., 1993; Zhao et al., 2008; Bartel, 2009). They are evolutionally diversified but functionally conserved between species (Lee et al., 2007). MicroRNAs (miRNAs), one of the novel classes of ncRNAs, have attracted great interest, particularly because these 20-22 small nucleotide molecules have long been understood to play important roles in development and diseases through regulating the post-transcription/translation of protein-coding genes (Pasquinelli et al., 2000). MicroRNAs are also important for the renewal and maturation of stem cells (Yi and Fuchs, 2011). In 2007, we first provided the evidence revealing that miRNAs mediate ethanol's teratogenic effects (Sathyan et al., 2007). Two hundred and eighteen mouse miRNAs were screened for ethanol sensitivity in fetal NSCs/NPCs and only a few, miR-9,-21,-153,-335, were significantly decreased by ethanol exposure. Recent studies have expanded the list of ethanol-sensitive miRNAs (Wang et al., 2009; Guo et al., 2012), further supporting the importance of miRNAs in mediating ethanol's effects. However, the mechanisms whereby miRNAs mediate fetal NSC vulnerability and contribute to teratology associated with maternal drug abuse are still poorly understood. Therefore, this dissertation will focus on my studies which provide evidence that miRNAs mediate ethanol's teratologenic effects on the growth and maturation of fetal NSCs/NPCs during brain development.

Teratology: the history and the emerging evidence

As mentioned earlier, a teratogen is a drug or other substance capable of interfering with the development of an embryo fetus, leading to congenital malformations. Teratology is the scientific discipline that deals with the causes and consequences of congenital malformations (Warkany, 1971). While chromosomal abnormalities can and do result in developmental defects, and genetic variation can increase the susceptibility of the developing organism, the primary etiological factors in teratology are thought to be environmental; dietary deficiencies, drugs of abuse and pharmaceutical agents, chemical by products of industrial activity, toxins, and viruses can all be teratogenic (Warkany, 1971; Lancaster, 2011). In the early 1940s, several animal studies demonstrated that in addition to genetic disorders, vitamin deficiencies like lack of folic acid, and various chemicals such as diethylstilbestrol (des Portes et al., 1998) could also cause birth defects (Warkany, 1971). In addition, the ophthalmologist, Gregg reported in 1941 (Gregg, 1941) a case of a mother who contracted German measles (Rubella) in early pregnancy which resulted in congenital cataracts. This discovery marked the first time when environmental agents and viruses were speculated to be potential teratogens like other infective agents that cause fetal infections.

Until the 1960s at least, many researchers still did not consider that any drug ingested by a pregnant woman could pass across the placental barrier and influence the developing fetus, until the thalidomide tragedy revealed that assumption to be untrue in the early 1960s.

Thalidomide is a derivative of glutamic acid named α-phthalimidoglutarimide that consists of two rings with different chemical structures (Franks et al., 2004). The ring on the left side of the molecule phthalimide is believed to be responsible for teratogenic effects, while the ring on the right, called glutarimide, is thought to have sedative properties (Franks et al., 2004). Thalidomide was made in 1954, first released into the market and used as a sedative or hypnotic to treat anxiety, nausea, and morning sickness in pregnant women in 1957 in West Germany (Knapp and Lenz, 1963). Shortly after this drug was introduced, approximately 5000-7000 children in Germany were born with limb defects (Bren, 2001). In the early 1960s, more than 10,000 infants in 46 countries were born with malformations such as phocomelia (malformation of limbs) and amelia (the absence of limbs), and only 40-50% of these children survived (Bren, 2001). The cause of this birth defect crisis was initially unknown; however, after mass reports of findings showed that the incidence of phocomelia and amelia were associated with the maternal use of thalidomide (Webb, 1963), the drug was immediately withdrawn and banned in 1961. Because of the thalidomide disaster, the study of teratology had drawn more attention to the general public and researchers, and emerged as a legitimate field of scientific study. The modern study of teratology integrates diverse fields of developmental biology, embryology, genetics, epigenetics and molecular and cellular biology.

In the United States, alcohol was not identified as a teratogen till the 1970s, following the discovery of fetal alcohol syndrome by Dr. David W. Smith and Dr. Kenneth Lyons Jones (Jones et al., 1973), though an until-then, largely ignored study in France, had

documented earlier, the teratogenic effects of alcohol in several alcoholic mothers (Lemoine et al., 1968).

Principles and classifications of teratogens

As the *in utero* vulnerability of the developing embryo became more recognized, the emerging challenge was ‘how to define different forms of teratology and classify the possible mechanisms by which teratology occurred'. In 1959, embryologist James Wilson identified *the six principles of teratology* in his *Environment and Birth Defects* (Wilson, 1973). These principles elucidate common features to identify teratogenic agents and their effects on developing embryos and remain important basic tenets in the study of teratology today:

1. Teratogenic susceptibility depends on the way in which genes of the developing fetus interact with teratogens.

2. Teratogens cause different degrees of abnormalities during development based on the time period of exposure. There are critical periods of fetal development, where the fetus is more susceptible to teratogens because particular organs are developing during those periods.

3. Abnormal developmental events are initiated through specific mechanisms based on teratogenic exposure in cells and tissues.

4. The nature of the teratogens as well as the route and the degree of exposure determine the teratogenic effects on the developing fetus.

5. There are four major classifications of deviant development including malformation, growth retardation, functional defects and death.

6. The severity of altered development increase in frequency and in degree from the no observable adverse effect level (NOAEL) to a dose producing 100% Lethality (LD100).

Today, various forms of teratogens have been discovered and scientists classify congenital genetic defects and environment agents as two major categories known to be teratogenic in mammals. It is estimated that 10% of all human malformations are caused by known environmental factors and another 10% by genetic or chromosomal defects. The remaining 80% are caused presumably by intricate interactions between environmental agents and genetics (Sadler and Langman, 1990).

Congenital genetic defects

Congenital genetic defects usually result from genetic and chromosomal abnormalities that lead to teratology due to errors in genetic information. For example, Down syndrome was identified in 1959 with the presence of all or part of an extra 21st chromosome that causes errors of chromosome numbers (Down, 1995; Arron et al., 2006), with prevalence of 1 in 700 infants (Besser et al., 2007); Klinefelter's syndrome is another case of chromosomal abnormality and identified as a 47,XXY aneuploidy in 1942 (Klinefelter, 1986) with prevalence of 1 in 600 live males (Bojesen et al., 2003). One of the more controversial chromosomal abnormalities involves males that are 47,XYY aneuploidy, identified in 1961 by cytogeneticist Avery Sandberg (Sandberg et

al., 1961), with prevalence of 1 in 1000 males (Milunsky and Milunsky, 2010). Wolf-Hirschhorn syndrome is a chromosome 4p deletion syndrome discovered in 1961 (Hirschhorn et al., 1965), while Cri du chat syndrome is a chromosome 5p deletion syndrome identified in 1963 (Lejeune et al., 1963). Angelman syndrome was identified in 1965 with deletion or inactivation of genes on the maternally inherited chromosome 15 (Williams et al., 1995); Rett syndrome is a neurodevelopmental disorder identified in 1966 with genetic mutations in MeCP2, a transcriptional repressor (Xq28) (Rett, 1992). The prevalence of Rett syndrome is about one in every 10,000-22,000 female live births by age 12 years (Kozinetz et al., 1993). Silver-Russell syndrome, originally described by Silver and colleagues in 1953 and, soon afterwards, by Russell in 1954, is a growth disorder with prevalence of 1 in 50,000 to 100,000 births (McDowell and Sproles, 1973). These listed congenital malformations are examples of disorders based on genetic defects.

Environmental factors

The complex biology involved in fetal development creates numerous ways for environmental agents to generate teratology. Therefore, environmental agents such as chemicals, drugs, alcohol, and nutrition can alter signaling molecules that control fetal development. They can also disrupt or modify the expression of genes in an epigenetic manner. In addition, teratogenic exposure to a fetus, even at a relative low dose during vulnerable developmental windows, can contribute to a lasting impact later in life. Years of research have suggested a wide range of different chemicals and environmental

factors that are suspected or known to be teratogenic in mammals. A selected list of well known teratogens includes:

Alcohol: alcohol consumption during pregnancy can cause abnormal fetal development and fetal alcohol spectrum disorder. Even relatively low levels of alcohol exposure or single high peaks of exposure at critical times during pregnancy can impair fetal development and result in different deficits. FASD is a congenital syndrome characterized by delayed fetal growth, facial abnormalities, and dysfunction of the central nervous system. It is known that alcohol is especially toxic during the first trimester, as this is the time period when organgenesis begins (Streissguth, 1997; Kelly et al., 2009).

Narcotics: narcotics includes drugs such as heroin, morphine, and methadone with morphine-like effects. Women who use narcotics during pregnancy are at high risk to give birth to infants with growth problems such as slit-like ventricles with smaller lateral ventricles or neonatal death (Kaltenbach and Finnegan, 1989; Ornoy, 2002).

Amphetamines: the use of this drug may result in high risks of early placental separation, fatal growth problems, and induce embryonic death (Monissey and Mottet, 1981).

Lead: lead is the most well-known brain toxicant. Prenatal lead exposure in a high dose during the first trimester period can produce serious neurological damage, including cerebral palsy and mental retardation (David et al., 1976).

Methylmercury: methylmercury is a developmental neurotoxicant that causes fetal brain injury, such as cerebral palsy and growth retardation (Myers and Davidson, 1998; Trasande et al., 2006).

Polychlorinated biphenols (PCBs): prenatal PCBs exposure in the developing brain results in IQ loss and defects in learning and behavior. Exposure at a high dose can produce serious neurological damage, such as cerebral palsy and mental retardation (Grandjean et al., 2001).

Tobacco smoke: tobacco smoke can be harmful to brain development at certain times and doses of exposure. Studies of the effects of nicotine on fetal development have confirmed that nicotine, by itself, is able to cause fetal brain damage, as evidenced by cell loss, synaptic abnormalities, and behavioral defects (Roy and Sabherwal, 1998).

Bisphenol-A: bisohenol-A is an environmental hormone in many plastics found in many households. Some animal studies have indicated that bisphenol-A is associated with cancer, endocrine-related health conditions, and a chromosome defect associated with Down syndrome (Hunt et al., 2003; Leranth et al., 2008).

Alcohol's intoxication

Alcohol, also known as ethanol, is an organic compound composed of carbon, oxygen, and hydrogen, and is very soluble in water. It is a depressant of the central nervous system, and the degree of impairment to the function of the central nervous system is directly proportional to the concentration of alcohol in the blood (Garriott, 1996). Alcohol is rapidly absorbed into the blood, and spread out throughout the body

after ingestion. Because of this feature, alcohol has been found to influence the central nervous system in small concentrations. For examples, alcohol consumption in low concentrations reduces inhibitions. As the blood alcohol concentration (BAC) increases, a person decreases response to stimuli remarkably, starts to have trouble walking, and slur in talking (Table 1-1). With the BAC in very high concentration, usually greater than 0.35 grams/100 milliliters of blood (equivalent to 0.35 grams/210 liters of breath), a person can be in deep unconsciousness and die (Table 1-1). In addition, base on the drinking habits, researchers also classify these into different categories. Men or women who drink moderately with drinking levels no more than 4 or 3 drinks on any single day and no more than 14 or 7 drinks per week, are defined as low-risk drinkers (Ockene et al., 1999). On the other hand, heavy or at-risk drinkers refer to people who drink more than the single-day or the weekly amounts listed above, and they usually have higher tendency to have alcohol dependence or alcohol abuse problems. Besides, binge drinking means that people drink too much within two hours, resulting in BAC level at or above 0.08g/dL (Ockene et al., 1999). Long term binge drinking can cause health problems that result from damage of the liver or other organs.

Fetal alcohol syndrome: impact of maternal drinking on developing fetuses

Ethanol, one of the strong fetal teratogens, causes fetal alcohol syndrome by maternal consumption of alcohol during pregnancy. Fetal alcohol syndrome was not well characterized until in 1968 by the pediatrician Paul LeMoine (Lemoine et al., 1968), and the diagnosis did not receive widespread attention until 1973 by David Smith and Ken

Table 1-1. Different stages of alcohol intoxication. The table is modified from (Garriott and Aguayo, 2008)

BAC (g/100 ml of blood or g/210 L of breath)	Feature of Stages	Clinical symptoms
0.03 - 0.12	Euphoria	Mild euphoria, sociability, talkativeness; Self-confidence increases; Decreased attention, judgment and control; Beginning of sensory-motor impairment; Finer performance tests impairment
0.09 - 0.25	Excitement	Unstable emotion; Loss of critical judgment; Loss function of perception, memory and comprehension; Reaction time increases; Visual acuity decreses; Sensory-motor incoordination; Drowsiness
0.18 - 0.30	Confusion	Disorientation; dizziness; Emotional exaggeration; Vision disturbance; Pain threshold increases; Muscular incoordination; Speak indistinctly
0.25 - 0.40	Stupor	General inertia; beginning of motor functional loss; Decreased response to stimuli remarkably; Muscular incoordination begins; Unable to stand or walk; Vomiting; incontinence; Impaired consciousness; Insensibility
0.35 - 0.50	Coma	Unconsciousness; Loss of reflexes; Low body temperature; Incontinence; Impairment of circulation and respiration; Possible death with BAC 0.45+

Jones, who suggested specific criteria for its diagnosis (Jones et al., 1973). Today, fetal alcohol syndrome has been known to reflect the most severe malformation due to alcohol's effects during gestation (Calhoun and Warren, 2007). Major deficits of fetal alcohol syndrome include intrauterine and postnatal growth retardation, central nervous system dysfunction, and craniofacial abnormalities, such as the presence of a thin vermilion line, smooth philtrum, and small palpebral fissures (Jones et al., 1973; Astley and Clarren, 1996; Lemoine et al., 2003). According to the report from Centers for Disease Control and Prevention (CDC), 0.5 to 2.0 cases of FAS occur per 1,000 live births and the rates increase to 10-15 per 1,000 in at-risk groups, such as the United States foster care population (May and Gossage, 2001), and the prevalence of FASD may be as high as 5% of the population of school-aged children in the US (May et al., 2009), and ~20% of the population in other parts of the world including South Africa and nations of Eastern Europe (May et al., 2008; May et al., 2009). The lifetime cost for one individual with FAS in 2002 was estimated to be $2 million, and the estimated annual cost to the United States for FAS alone is over $4 billion, as of 2004 (May and Gossage, 2001). Amendah and colleagues in a 2011 report also demonstrated that incurred health costs were nine times higher for a child with identified FAS than for children without deficits attributable to FAS (Amendah et al., 2011).

Fetal alcohol spectrum disorder

Fetal alcohol spectrum disorder refers to the range of birth defects that result from gestational alcohol exposure during pregnancy. Given the wide range of factors,

including the doses of maternal alcohol consumption, timing of exposure, genetic components, and the nutritional status of the mother during pregnancy, the severity of the impairments reveals a wide gamut of physical and behavior outcomes classified as fetal alcohol spectrum disorder (Lemoine et al., 2003; Sokol et al., 2003; Bertrand et al., 2005). The primary key feature of FASD diagnosis is central nervous system damage, including structural brain abnormalities, such as agenesis of the corpus callosum and cerebellar hypoplasia (Sulik and Johnston, 1983; Feldman et al., 2012); neuronal and functional defects, such as loss of inter-hemispheric communicating tracts (Wozniak et al., 2011); the presence of neuronal heterotopias (Clarren et al., 1978); developmental delay; and learning and memory problems (Jones, 1975; Abel et al., 1983; Astley, 2004; Chudley et al., 2005). FASD is a non-diagnostic umbrella term that includes fetal alcohol syndrome, as well as other conditions based on partial expressions of FAS, including fetal alcohol effect (FAE), partial fetal alcohol syndrome (pFAS), Alcohol-related neurodevelopmental disorder (ARND), and Alcohol-related birth defects (ARBD). The abnormal phenotypes that exhibit the less complete or partial expression of FAS with confirmed maternal alcohol exposure were initially classified as the term "suspected fetal alcohol effects" (FAE) in 1978 (Clarren and Smith, 1978). This term subsequently was jettisoned from official terminology because of a lack of precise criteria and a common misuse (Aase et al., 1995). pFAS, previously known as Atypical FAS in the 1997, is a term suggested by the Institute of Medicine (IOM) to indicate a confirmed history of prenatal alcohol exposure, but without a full complement of growth deficiencies and facial features of FAS (Stratton et al., 1996; Astley, 2004). The terms

ARBD and ARND, suggested by IOM, were added to include physical or behavioral disorders that change over time but are linked to clinical or animal studies by maternal alcohol consumption (Chudley et al., 2005; Hoyme et al., 2005). They also include a complex pattern of behavioral and cognitive abnormalities that are observed but cannot be explained.

Although The Center for Disease Control and Prevention acknowledges a wide range of estimates for FASD, the prevalence of FASD is difficult to establish. Based on the report from May and Gossage in 2001, the prevalence of FAS was to be 0.5 to 2 cases in 1000 births (May and Gossage, 2001). Therefore, the prevalence of FASD was estimated higher, approximately 1 per 100 (1%) live births (Sampson et al., 1997). The recent epidemiologic study even reveals that the prevalence of FASD has escalated to 2-5% of younger school children in the US and Western Europe (Mays and Thompson, 2009). Compared to the annual cost with $4 billion to the U.S. of FAS alone (May and Gossage, 2001), the overall cost of FASD is expected to increase in coming years. A recent study indicates that the annual cost of FASD to the U.S. healthcare system is estimated at more than $6 billion, suggesting the problem of maternal consumption is increasing (Wattendorf and Muenke, 2005).

Ethanol exposure causes neuronal defects during vulnerable periods of fetal brain development

As a strong fetal teratogen, alcohol can alter signaling components that control brain development. Maternal alcohol exposure even at low doses during particularly

vulnerable developmental windows in fetuses and young children can contribute to a lasting impact on how the brain functions later in life. Alcohol can cause severe behavioral and anatomical defects depending on the timing of exposure, and this reflects the underlying periods of susceptibility of critical developmental programs. Prenatal alcohol exposure during the first trimester leads to a 12 percent greater likelihood that the child will have a smaller-than-normal head and exhibit neural tube defects (Sulik and Johnston, 1983; Feldman et al., 2012). Second trimester-equivalent prenatal alcohol exposure has been shown to alter cortical neurogenesis (Miller, 1989b; Miller and Nowakowski, 1991; Miller, 1996), enlarge the layer of the sub ventricular zone (Kotkoskie and Norton, 1989), disrupt cortical organization (Kotkoskie and Norton, 1989) and lamination patterns specifically in the emerging cortical plate (Kotkoskie and Norton, 1989). Ethanol exposure during the third trimester appears to alter structure of different hippocampal regions and cause loss of developing neurons in CA1 of the hippocampus and dentate gyrus (Bonthius and West, 1991; Klintsova et al., 2007).

Alcohol-induced disruption on neuronal maturation as one potential mechanism that causes CNS deficits

Prenatal alcohol exposure has been shown to cause various CNS defects, including decreased thickness within the ventricular zone, enlargement of the subventricular zone (Miller, 1989b), and disruption of the laminar formation of the emerging cortical plate (Kotkoskie and Norton, 1989). These deficits indicate the consequences of ethanol's impact on cell proliferation and migration, both important for proper embryonic

development. Therefore, understanding the molecular mechanisms of signaling pathways that control cell proliferation, differentiation, and migration provide the clues on the cellular level to determine which components are susceptible to ethanol's teratology.

Neural development: A specific focus on the development of the cerebral cortex

Since the developing cerebral cortex is an important target of ethanol, and since the major period of cortical growth is initiated during the period of neurogenesis at the end of the first trimester and the beginning of the second trimester, my research focused on modeling this particular period of cortical development. Subsequent discussion will focus on the developing cerebral cortex, and on the second trimester developmental period. Early neural stem cells reside in the ventricular zone (VZ) of the developing cortex, and most neocortical neurons, including all projection neurons, are generated within the VZ of the dorsal aspect of the lateral ventricle. A second and smaller population of cortical interneurons is generated from a ventrally located germinal zone, the lateral ganglionic eminence (LGE) and migrates tangentially into the developing cerebral cortex. During rodent cortical neurogenesis, which begins during the second half of the gestational period (gestational day (GD) 11 in the mouse), the VZ lining the cerebral ventricles generates progenitors for neurons of the cerebral cortex. In the VZ, neural progenitors divide at the apical surface and undergo interkinetic migration during G1 and G2 phases of their cycle (Spear and Erickson, 2012). Symmetric division at this apical surface results in expansion of the neural stem cell pool whereas asymmetric cell

division results in the formation of daughter cells that are more mature. These more differentiated daughter cells, the products of asymmetric division, migrate past the basal surface of the VZ to progressively form the subventricular zone (SVZ) where neural stem/progenitor cells (NSCs/NPCs) do not exhibit interkinetic migration (Willardsen and Link, 2011). The post-mitotic cells will then migrate out by somal translocation or radial locomotion towards the neocortex for mature differentiation, following tracks provided by the radial glial cells (RGC), the primary scaffolding molecule used by most future cortical cells to migrate into the cortex. Neurons are ordered by birth date in such a way that older neurons remain in deep layers, and younger neurons migrate through the deep layers to attain a superficial position (Dehay and Kennedy, 2007). The first neurons to be generated establish the preplate; their axons, as well as ingrowing axons from the thalamus, establish the intermediate zone (Klintsova et al., 2007). Neurons of cortical layers II–VI establish the cortical plate, which splits the preplate into the marginal zone, or future layer I, and the subplate (Spear and Erickson, 2012), a transient population of neurons. At the end, six cortical layers are visible overlying the white matter, and the subplate has largely disappeared. Thus, this process is arranged as an “inside-to-outside” pattern to establish the lamination of cortical development (Zecevic and Rakic, 2001; Shipp, 2007).

Once these newly born neurons reach their destinations, particular genes will be switched on to differentiate and mature these neurons by inducing growth of axons, dendrites, and synapses. Recent studies have shown that several mechanisms are involved in this complex process. For examples, signals such as components of the

cytoskeleton (LIS1, DCX, FILAMIN1, and CDK5/P35), neuron-glia binding proteins (ASTN1, and INTEGRIN), or transcription factors (NeuroD1 and NFI genes) are also critical to the proper formation of the cortical layers. Cyclin-dependent kinase 5 (Cdk5) affects the cytoskeleton organization and the properties of migrating neurons through phosphorylation of cytoskeletal components such as neurofilaments (Dhavan and Tsai, 2001). Previous studies have also indicated that Cdk5 and its activator p35, are responsible for cortical layer formation (Ohshima et al., 2001). Doublecortin (DCX), a cytoplasmic protein that binds to microtubules, propels neuronal migration by forming a meshwork around the nucleus of the cell (Deuel et al., 2006). Genetic mutations of DCX cause abnormal neuronal migration during fetal brain development and disrupt the cortical lamination, resulting in epilepsy, mental retardation, and heterotopias (Manent et al., 2009). Astrotactin1 (ASTN1) is a neuronal adhesion protein required for migration of young postmitotic neuroblasts in cortical regions of the developing brain (Fink et al., 1997). Axonal guidance by short- or long-range secreted molecular cues is another important mechanism that drives neuron maturation. For example, SLIT family are secreted proteins that control midline repulsion by signaling through roundabout receptors (Robo) (Liu et al., 2004); Ephrins (A +B) are membrane-anchored proteins that have both repellent and attractive functions via signaling through their receptors: EphA, EphB (Flanagan and Vanderhaeghen, 1998); Other components such as Netrins, Semaphorins and their corresponding receptors are also important for axon guidance (Dickson and Keleman, 2002; Kullander and Klein, 2002). NeuroD1 is one of the basic helix-loop-helix (bHLH) transcription factors that regulate downstream genes that

determine cell fate and cell survival/differentiation, particularly of progenitor cells during neurogenesis (Tapscott and Weintraub, 1991; Fode et al., 1998; Ma et al., 1998). Recent evidence has revealed that NeuroD1 acts as a cell survival and maturation regulator during the late stages of neurogenesis (Lee, 1997; Miyata et al., 1999; Kim et al., 2001; Kim, 2012). The Nuclear Factor One transcription factor family, including NFIA, NFIB, NFIC and NFIX, has been shown to be highly expressed in the developing mouse brain (Plachez et al., 2008). The NFI gene family regulates maturation of neurons in the spinal cord, basilar pons and hippocampus by controlling the differentiation of progenitor cells into neurons or glia (Plachez et al., 2008; Mason et al., 2009). NFIA, coordinated with Sox9, plays a crucial role in the onset of gliogenesis (Kang et al., 2012), while NFIB is essential for normal brain development (Plachez et al., 2008; Kumbasar et al., 2009; Piper et al., 2009). Genetic mutations of NFIB ($NFIB^{-/-}$) in mice show several neural defects, such as enlarged lateral ventricles, agenesis of the corpus callosum, and disruption of the hippocampus formation (Plachez et al., 2008; Kumbasar et al., 2009; Piper et al., 2009).

Neural stem cells are targets of ethanol

During fetal brain development, the tail-period of the first trimester and the second trimester, the time periods when neurogenesis proceeds, are especially vulnerable to prenatal exposure because neural stem cells and progenitor cells from the ventricular zone and subventricular zone proliferate rapidly to generate most of the neurons to form the adult brain (Sulik, 2005; Bystron et al., 2008). Because of the rapidity with which neural stem cells proliferate and generate new neurons, even small alternations in the

renewal and maturation capacity of these cells during susceptible periods would be predicted to amplify ethanol's influence in fetal brain development. Previous studies, including some form our laboratory using in vivo models and mouse fetal neurosphere cultures to model the second trimester fetal neuroepithelium, have established that ethanol exposure during this time results in altered fetal neuron stem and progenitor cell proliferation and maturation (Miller, 1993; Camarillo and Miranda, 2008), consequently leading to brain abnormalities in the neocortex and the hippocampus (Miller, 1993). This increase in NSCs/NPCs proliferation was accompanied by the reduction of specific stem cell/progenitor markers such as ABCG2, CD117, CD133 and Sca-1 (Santillano et al., 2005). Therefore, we hypothesized that fetal alcohol exposure promotes neuron stem/progenitor cell proliferation by depleting NSCs/NPCs pools in the developing brain. The decreased capacity of the stem cell population results in early neuronal maturation and differentiation, which is consistent with our observation that ethanol-treated differentiating neuroblasts increased cell migration on a permissive extracellular matrix substrate (Camarillo and Miranda, 2008). These data propose a possible mechanism to explain why ethanol exposure can lead to the formation of heterotopias in fetal brains of FAS children (Clarren et al., 1978). However, which components mediate alcohol's affects on NSCs/NPCs pre-maturation during fetal brain development were not well understood, until recent evidence revealed that microRNAs, one class of ncRNAs (non-coding RNAs), might intermediate alcohol's teratology in developing brains (Sathyan et al., 2007; Wang et al., 2009; Guo et al., 2012).

MicroRNAs: tiny regulators with great potential in biology

MicroRNAs, 20-25 base pairs in length, are miniscule regulators that are processed from hairpin structures and can regulate downstream genes either by transcriptional or translational repression (He and Hannon, 2004; Brodersen et al., 2008). The first small non-coding RNA, lin-4, was identified by Victor Ambros's laboratory in 1993 by regulating lin-14 gene that controls larval development in *C. elegans* (Lee et al., 1993). Their findings not only explored a new field in biology but also provided a future clue to understand the mysterious 98% of the human genome that doesn't direct the production of proteins but instead exercises control over those protein-coding mRNAs.

However, these miRNAs were not recognized as functional and evolutionally conserved regulators until the early 2000s (Tanzer and Stadler, 2004; Lee et al., 2007). According to recent human genome studies, now there are over 2000 miRNAs that may target approximately 60% of mammalian genes (Bentwich et al., 2005; Griffiths-Jones et al., 2008; Friedman et al., 2009). These small non-coding molecules are usually transcribed by RNA polymerase II (Pol II), either from intergentic, intronic or polycistronic regions of the genomes (Lee et al., 2004), and then processed through several steps to become the mature form of miRNAs. The first miRNA precursors, pri-miRNAs, which consist of 5' cap and poly A tail, are processed in the nucleus by a microprocessor complex that consists of a nuclear protein known as DiGeorge syndrome critical region 8 (DGCR8) and a class 2 RNase III enzyme known as Drosha that cuts short RNA molecules (Gregory et al., 2006). Pri-miRNAs are processed by the Drosha complex, to form a truncated, ~70 nucleotide stem-loop product with a di-nucleotide

overhang at the 3' end, called a pre-miRNA. Pre-miRNA hairpins are recognized and transported from nucleus to cytoplasm by a nucleocytoplasmic protein, Exportin-5 (Lund et al., 2004). Cytoplasmic pre-miRNAs are further processed by the RNase III enzyme Dicer into short dsRNAs (miRNA/miRNA* duplex) and only one strand of miRNA is incorporated into the RNA-induced silencing complex where the miRNA and its mRNA target interact (Hammond and Wood, 2011). These functional activated RISC assembled miRNAs can control gene expression by either cleavage of target mRNAs or by translational repression (Kusenda et al., 2006; Bartel, 2009). Emerging studies also suggest that miRNAs can enhance mRNA translation rather than repression through 5'UTR binding (Jopling et al., 2005; Orom et al., 2008), suggesting multiple functions of miRNA regulation.

MicroRNA regulation in the nervous system

MicroRNAs have been found to be expressed in precise regional and temporal patterns in developing and adult brains, indicating their widespread and diverse regulation (Fiore et al., 2011). These neural miRNAs are involved at various stages, including neural stem cell maintenance and differentiation, synaptic development and plasticity, and neurological diseases in mammals.

A key strategy by targeting miRNA biogenesis factor DICER1 has demonstrated that several miRNAs are specifically associated with neural development because a wide range of neural defects was observed in Dicer1 knock-out animals (Kanellopoulou et al., 2005). To further study the impact of specific miRNAs in the developing nervous system,

scientists focused on the expression profiling of miRNAs during the different stages of neural development *in vitro* (from NSCs to mature neurons or glia) and *in vivo* (during embryonic and postnatal development). Several reports have shown that the expression level of several miRNAs, including miR-9, miR-124, miR-125, miR-128, and some of the let-7 family members, are elevated during neurogenesis (Krichevsky et al., 2003; Sempere et al., 2004; Rybak et al., 2008). Later studies further suggested that these miRNAs are critical regulators to promote NSCs/NPCs maturation into virtually all types of neurons (Cheng et al., 2009; Yoo et al., 2009; Zhao et al., 2010).

Brain enriched miRNAs in CNS regulation

Let-7, one of the well-studied miRNAs, has been found to participate in a differentiation pathway mediated by the tripartite motif-containing protein 32 (TRIM32) which results in the repression of the NSC renewal factor c-myc (Schwamborn et al., 2009; Melton et al., 2010) and consequent differentiation and loss of renewal capacity of NSCs. In the mouse telencephalon, TRIM32 is strongly expressed in differentiating neurons in the ventricular zone at E12.5 and focally in cortical layers at E18.5. TRIM32 knock-down experiments demonstrated that this gene participates in NSC differentiation by repressing cell proliferation (Schwamborn et al., 2009), and binds to RISC-associated let-7 to repress c-Myc. Another study also suggested that let-7b regulates neural stem cell proliferation and differentiation by targeting the stem cell regulator TLX and the cell cycle regulator cyclin D1 (Zhao et al., 2010). TLX is an orphan nuclear receptor that has known to maintain neural stem cells in an undifferentiated state through recruiting

histone deacetylases to repress downstream genes, the cyclin-dependent kinase inhibitor p21, and the tumor suppressor gene PTEN (Sun et al., 2007).

MiR-124, the most abundant microRNA in neuronal cells, induces neurogenesis by suppressing small C-terminal domain phosphatase 1 (SCP1), one component of repressor element 1-silencing transcription factor transcription repressor complex (Visvanathan et al., 2007). It also targets Polypyrimidine tract-binding protein 1 (PTBP1), a repressor of neuron-specific splicing (Makeyev et al., 2007). REST complex associated with cAMP response element-binding (CREB) are crucial transcriptional regulators of neural genes and neuronal fate determinants (Wu and Xie, 2006), while PTBP1, expressed high levels in non-neuronal cells but downregulated in the nervous system, is a RNA-binding protein that binds to pyrimidine-rich sequences in pre-mRNAs and inhibits the splicing of nearby neuron-specific alternative exons (Sharma et al., 2005). In the developing neural tube, miR-124 acts as a determinant of neuronal differentiation. It regulates laminin gamma 1 (LAMC1) and integrin beta1 (ITGB1), two extracellular matrix genes that are highly expressed in neural progenitors but repressed upon neuronal differentiation (Cao et al., 2007). A recent study in an adult mammalian brain also suggested that miR-124 determines NSCs fates by controlling SRY-box transcription factor (*S*ox9) in subventricular zone (SVZ) neuroblasts. Sox9 overexpression in SVZ cells decreased the production of neurons, whereas Sox9 knockdown leads to generate neurons (Cheng et al., 2009).

Besides determining the fate of neuron stem cells, several microRNAs have also been found to involve in regulation of synaptic development, maintenance, and plasticity. For

instance, several miRNAs such as miR-124a, miR-9 and miR-132, regulate synaptic plasticity by targeting CREB, one major gene that is important for long term potential (LTP) and memory (Wu and Xie, 2006), while some other miRNAs participate in modulating axon guidance and dendrite growth (miR-124, miR-132, miR-134, miR-137, miR-485), synapse formation (let-7, miR-125b, miR-132, miR-137, miR-138 and miR-485), and synaptic functions (miR-1, miR-132, miR-134, miR-153, miR-219, miR-485) (Cohen et al., 2011; Fiore et al., 2011). MiR-132 mediates the regulation of dendritic growth by targeting Rac family GTPases (Wayman et al., 2008). MiR-134 modulates synaptic plasticity and memory formation by regulating cAMP response element-binding protein (CREB) expression (Gao et al., 2010). CREB, a cellular transcription factor, has been found to regulate dendritic growth, activity-regulated dendritic refinement, synaptogenesis, and synaptic plasticity in mouse hippocampus neurons. Abnormal up-regulation of miR-134 would negatively regulate synaptic plasticity and induce plasticity impairments (Gao et al., 2010). MiR-485 controls dendritic spine number and synapse formation in an activity-dependent homeostatic manner by targeting the presynaptic protein SV2A in the mouse hippocampus. In the experiments of microRNA gain function, miR-485 overexpression reduced spontaneous synaptic responses and transmitter release by reducing hippocampus dendritic spine density and suppressing spine formation (Cohen et al., 2011).

Ethanol-sensitive miRNAs in regulating CNS development

Pertinent to the focus of my dissertation, the ethanol-sensitive miRNA miR-153 regulates SNAP-25, the critical core SNARE component, to modulate vesicle exocytosis and synaptic activity (Wei et al., 2013). Overexpression of miR-153 in a zebrafish model results in reduction of SNAP-25 expression that leads to decreased neuronal secretion, decreased neuronal growth, and defects of embryonic movement (Wei et al., 2013). Another ethanol-sensitive miR-9 has also been discovered to maintain neural stem cell self-renewal, and controls stem cell migration in the midbrain-hindbrain boundary (MHB) by targeting Her5 and Her9 (anti-neurogenic genes), in a zebrafish model (Leucht et al., 2008). Moreover, other studies in mouse models also demonstrated that miR-9 promotes neuron stem cells differentiation by controlling TLX in a feedback regulatory loop (Zhao et al., 2009), and controls Cajal-Retzius cell differentiation through targeting forkhead box protein G1 (FOXG1) in the medial pallium (Shibata et al., 2008).

MiRNAs intermediates ethanol's effects on neuron stem cell maturation

Since many miRNAs are critical to regulate different developmental stages in the nervous system by controlling hundred of RNA transcripts, it is reasonable to expect that ethanol may interfere with neural development through miRNA disruption. Previous studies have shown that ethanol-induced alternation of miRNA expression in cortical neurons is highly associated with the regulation of neural stem cell proliferation and differentiation (Sathyan et al., 2007; Pietrzykowski et al., 2008; Tang et al., 2008).

Ethanol's teratology on NSCs/NPCs maturation is complex and usually involves in dysregulation of large gene networks, further suggesting that miRNAs can mediate ethanol's effects. While the population of ethanol-sensitive miRNAs is small, the change of expression level in individual miRNA that controls hundreds of genes results in the amplification at the cellular and system level.

In 2007, the first evidence displaying that miRNAs mediate ethanol's teratogenic effects was established in our laboratory using an *ex vivo* mouse fetal neurosphere model (Sathyan et al., 2007), indicating that only few microRNAs (4 out of 218), miR-9, miR-21, miR-153, and miR-335, were significantly suppressed under the exposure of ethanol. In this study, miR-21 was identified as an anti-apoptotic factor, while miR-335 was found to be an anti-proliferative miRNA. However, concurrent knocking down miR-21 and miR-335 prevented cell death, suggesting that miR-335 was an antagonist to miR-21. Results from miR-335 knockdown neurosphere samples in the mouse genome microarray analysis following with RT-PCR validation by Dr. Sathyan revealed that suppression of miR-335 promotes NSCs/NPCs maturation by up-regulating stem cell and progenitor markers, such as DCX, c-Kit, PDGFrA, and NeuroD1. Recent studies also demonstrated that these ethanol-sensitive miRNAs control NSCs/NPCs maturation in different mechanisms during cortical development. MiR-153 acts as a pro-apoptotic miRNA by suppressing anti-apoptotic genes such as Bcl-2 (Xu et al., 2010) and controls synaptic transmission and neural movement through regulating Snap-25 in early zebrafish embryos (Wei et al., 2013). In addition, Tal and colleague discovered that developmental ethanol exposure in a zebrafish model results in decreased miR-153; in

turn, dysregulation of miR-153 leads to neurobehavioral impairment (Tal et al., 2012). MiR-9 controls NSCs/NPCs differentiation through targeting TLX gene (Shi et al., 2010). A recent study from Pappalardo-Carter et al. further showed that suppression of miR-9 function by alcohol exposure during fetal development recapitulates phenotypes associated with prenatal alcohol exposure (Pappalardo-Carter et al., 2013). These data collectively suggest that concurrent suppression of these microRNAs by ethanol exposure results in neural deficits in CNS, and potentially mediates ethanol's effects through driving early cell proliferation and differentiation of NSCs/NPCs. Recent studies also expand the list of ethanol-sensitive microRNAs in CNS, and suggest the temporal and spatial specificity of ethanol's effects on miRNAs during fetal brain development (Wang et al., 2009; Guo et al., 2012). For example, Wang and colleague in a 2009 report showed that ethanol exposure between the tail of first trimester to the middle second trimester affected only 3% changes (1.8% suppressed and 1.2% overexpressed) of miRNAs expression in mouse whole brain RNA samples (Wang et al., 2009). However, the molecular mechanisms of ethanol sensitive microRNAs on determining NSCs/NPCs maturation are still unclear and need to be elucidated.

Central hypothesis

Base on the information above as well as the preliminary data established from our laboratory, we therefore hypothesized that **miR-153 and miR-335 control NSC/NPC population maturation by regulating cell proliferation and differentiation genes**

during fetal brain development. To address the central hypothesis, the following specific aims were addressed.

Specific aim 1 (Chapter II): to identify signaling network genes as miR-153 targets and to observe if miR-153 can prevent or reverse ethanol's teratology in mouse NSCs/NPCs

To screen potential candidate genes regulated by miR-153, mRNA microarray analysis following with gene ontology analysis was performed to identify miR-153 suppressed genes. To further identify the direct targets of miR-153, 3'UTR luciferase reporter analysis in neurosphere cultures was utilized. Additionally, an *in vivo* model using ultrasound-guided *in utero electroporation* of control or miR-153 expression vector was established to confirm the regulation of miR-153 on its direct target genes in mouse fetal brains during development. Functional analysis using miR-153 and pharmacology study of FDA-approved varenicline in ethanol-treated neuropheres were utilized to evaluate whether these molecules can be potential therapeutics as functional antagonist to ameliorate ethanol's effects.

Specific aim 2 (Chapter III): to identify the direct targets of miR-335 and to observe behaviors of NSCs/NPCs population using transgenic mice during fetal brain development

Data from preliminary microarray and q-PCR analysis have indicated that miR-335 prevents NSC/NPC maturation by regulating neuron stem/progenitor cell markers

PDGFra, CD117, DCX, and NeuroD1. It is particular interesting to identify the direct targets regulated by miR-335, which provides more convincing information to understand how ethanol disrupts the balance of NSC/NPC population through the impact of miR-335 regulatory loops. I utilized 3'UTR luciferase construct reporter assay (*ex vivo*) and *in utero electroporation* of miR-335 mimetics in fetal mouse brains (*in vivo*), to validate the direct targets of miR-335. Beside, by evaluating miR-335 expression in different mouse demyelination models including EAE and cuprizone treated animals, the relationship between miR-335 and genes involved in the regulation of the oligodendrocytes was determined. Finally, generating miR-335 transgenic mouse fetuses provided the strong evidence to observe the behaviors of NSCs/NPCs population in the developing cortex during fetal brain development.

Summary

My dissertation focuses on the role of miRNAs as key modulators of ethanol that affects NSCs/NPCs proliferation and differentiation. Ethanol exposure during second trimester pregnancy may disrupt the maintenance of stem cell and progenitors available for normal development, resulting in irreversible damage to the developing brain. MiRNAs as targets of ethanol may be key regulators to explore the mechanisms of alcohol induced developmental defects, because individual microRNA has the capability to regulate hundreds of genes, corresponding to the complex misregulation of gene networks caused by ethanol. To understand the molecular mechanisms of miR-335 regulated NSCs/NPCs markers, I used bioinformatic tools and studied the 3'-

untranslated regions (3'UTR) of identified miRNA target transcripts in an ex vivo model to determine if these mRNAs were direct targets of miR-335. These targets were also validated in the mouse fetal brain injection model, and the miR-335 knock-out transgenic mice also suggested early differentiation of NSCs/NPCs and increased migration by inducing miR-335 target genes such as DCX and NeuroD1, indicating the potential mechanism by depleting and promoting early maturation of NSCs/NPCs. To understand how miR-153 controls NSCs/NPCs maintenance, I induced this developmentally important microRNA and looked at mRNA expression via a genome scale microarray to find out potential candidate targets, and validated the direct targets of miR-153 both in the neurosphere model and in the mouse ultrasound-guided injected model. These two studies provide the information of miRNA-gene networks in understanding the complexity of gene regulation during fetal brain development, and might also present as therapeutic candidates to prevent early developmental disruption that subsequently causes irreversible defects by alcohol.

CHAPTER II

MIR-153 TARGETS THE NUCLEAR FACTOR-1 FAMILY AND PROTECTS AGAINST TERATOGENIC EFFECTS OF ETHANOL EXPOSURE IN FETAL NEURAL STEM CELLS

Overview

Ethanol exposure during pregnancy is an established cause of birth defects including neurodevelopmental defects. Most adult neurons are produced during the second trimester-equivalent period. The fetal neural stem cells (NSCs) that generate these neurons are an important but poorly understood target for teratogenesis. A cohort of miRNAs including miR-153 may serve as mediators of teratogenesis. We previously showed that ethanol decreased, while nicotine increased, miR-153 expression in NSCs. To understand the role of miR-153 in the etiology of teratology, we first screened fetal cortical NSCs cultured *ex vivo*, by microarray and quantitative RT-PCR analyses to identify cell-signaling mRNAs and gene networks as important miR-153 targets. Moreover, miR-153 overexpression prevented neuronal differentiation without altering neuroepithelial cell survival or proliferation. Analysis of 3'UTRs and *in utero* overexpression of pre-miR-153 in fetal mouse brain identified Nfia (nuclear factor-1A) and its paralog, Nfib, as direct targets of miR-153. *In utero* ethanol exposure resulted in a predicted expansion of Nfia and Nfib expression in the fetal telencephalon. In turn, miR-153-overexpression prevented, and partly reversed the effects of ethanol exposure on miR-153 target transcripts. Varenicline, a partial nicotinic acetylcholine receptor

agonist that, like nicotine, induces miR-153 expression, also prevented and reversed the effects of ethanol exposure. These data collectively provide evidence for a role for miR-153 in preventing premature NSC differentiation. Moreover, they provide the first evidence in a preclinical model that direct or pharmacological manipulation of miRNAs have the potential to prevent or even reverse effects of a teratogen like ethanol on fetal development.

Introduction

Neural stem cells (NSCs), within the human fetal ventricular zone (VZ), generate most neurons of the adult human brain during a restricted developmental window encompassing the end of the first trimester, through the second trimester of pregnancy(Bystron et al., 2008). In rodent models, this neurogenic-equivalent period encompasses the second-half of pregnancy (Takahashi et al., 1995; Noctor et al., 2004). The rate of drug abuse among pregnant women is also highest during these trimesters (SAMHSA, 2009). Therefore, the peak period for maternal consumption of drugs like alcohol, a causative factor in the etiology of the fetal alcohol syndrome (FAS,(Jones et al., 1973)), coincides with, and is therefore likely to interfere with the critical period for fetal neurogenesis. In rodent models, maternal ethanol exposure during this neurogenic period has been shown to result in fetal brain growth deficits (Kotkoskie and Norton, 1989; Miller and Nowakowski, 1991; Sudheendran et al., 2013). Importantly, these deficits were not due to NSC death, but rather due to NSC depletion by altered

programming and premature maturation (Santillano et al., 2005; Prock and Miranda, 2007; Tingling et al., 2013).

A question that arises is, "what molecular mechanisms mediate fetal NSC vulnerability and contribute to teratology associated with maternal drug abuse? "MicroRNAs (miRNAs), a class of small non coding RNAs that regulate the translation of networks of protein-coding genes, have long been known to control development, (Pasquinelli et al., 2000).Therefore, we hypothesized that miRNAs would mediate the effects of teratogens on the growth and maturation of fetal NSCs.

MiR-153, a brain-enriched (Sempere et al., 2004), evolutionarily conserved miRNA located within the PTPRN2 gene locus, is a candidate mediator of teratogenesis. We identified miR-153 as one of a small cohort of miRNAs that were significantly decreased in fetal NSCs, following ethanol exposure (Sathyan et al., 2007). In that, and subsequent studies (Pappalardo-Carter et al., 2013), we also showed that the suppression of ethanol-sensitive miRNAs individually and collectively, explained some of the teratogenic effects of ethanol. Recently, developmental ethanol exposure was shown to also result in decreased miR-153 in a zebrafish model, and dysregulation of miR-153 in that model in turn resulted in neurobehavioral impairment (Tal et al., 2012). Moreover, nicotine also influenced miR-153 expression (Balaraman et al., 2012), suggesting that miR-153 is a target for other teratogenic agents and drugs of abuse.

In the following series of experiments, we identified cell-signaling gene networks as important targets of miR-153 in fetal cortical NSCs. We further identified the Nfia (nuclear factor-1A) and its paralog, Nfib as direct targets of miR-153, and showed that

these and other miR-153 target transcripts were also up-regulated following ethanol exposure. Importantly, we present evidence showing that miR-153 prevents, and even partly reverses the effects of ethanol exposure. Moreover, varenicline, a partial nicotinic acetyl choline receptor (nAChR) agonist (Mihalak et al., 2006), prevented and reversed the effects of ethanol on miR-153 target transcripts. Collectively, these data provide evidence for the efficacy of miRNA-mediated mechanisms in preventing and even reversing effects of teratogen exposure in fetal NSCs.

Materials and methods

Isolation and expansion of mouse cortical neural precursors

Timed pregnant mice (C57BL6/SJ, Harlan Laboratories) were housed in AAALAC accredited facilities at Texas A&M Health Science Center. All animal procedures were approved by the University Laboratory Animal Care Committee. Acutely dissociated cortical neural stem cells and neural progenitor cells from the dorsal telencephalic vesicles, corresponding to the region of the future isocortex, of gestational day 12.5 mouse fetuses (GD0 was defined as the day the dams were sperm-positive), were maintained and expanded using non-adherent culture methods by growing those cells as free-floating spherical aggregates known as neurospheres, to model the period of neuroblast precursor expansion during neurogenesis in the rodent. This model system was first introduced in the 90s (Reynolds and Weiss, 1992) and is still commonly used in the laboratory (Santillano et al., 2005; Camarillo et al., 2007; Prock and Miranda, 2007). Briefly, approximately 1~2 million neural precursor cells isolated from the cortical

region from fetuses of both sexes were combined and cultured in serum-free mitogenic media DMEM/F12 (#11330-032; Life Technologies, CA), 20ng/ml bFGF (basic fibroblast growth factor; #354060, BD Biosciences, CA), 20ng/ml hEGF (human epidermal growth factor;#53003-018, Life Technologies, CA), 1% ITS-X (insulin-transferrin-selenium-X; #51500- 056 Life Technologies, CA), 0.15ng/ml LIF (leukemia inhibitory factor;#L200 Alomone Labs), 0.85Us/ml heparin (#15077-019, Life Technologies, CA), and 20nM progesterone (# P6149, Sigma, MO). Neurospheres were mechanically dissociated into single cells with medium changed every two or three days. These cultures maintain stem cell markers and renewal characters(Santillano et al., 2005; Tingling et al., 2013). To assess the differentiation capacity of control or miR-153 overexpressing NSC populations, transfected neurospheres were cultured on laminin-coated (0.5mg/ml) glass coverslips (#16004-342; VWR, PA) in 6-well plates for 48 hours, in a mitogen-withdrawal paradigm (+bFGF/-EGF/-LIF) that results in stereotypic transformation of NSCs into early migratory bipolar cells and expression of neuronal markers (Camarillo et al., 2007; Camarillo and Miranda, 2008). Cultures were fixed in ice-cold methanol for immunofluorescence analysis.

Neurosphere culture treatment paradigm

Ethanol Exposure: neurosphere cultures containing ~3 million cells were assigned to either a vehicle control, or an ethanol-treated group (320 mg/dl, 70mM) for 5 days, to bracket a period equivalent to the peak neurogenic period in the mouse. The ethanol concentration in culture medium was determined by an alcohol analyzer (Analox

instruments, MA). The particular dose of ethanol was chosen to reflect levels within ranges attainable during episodes in chronic alcoholics (Adachi et al., 1991), and is expected to reflect levels attained by a fetus following prenatal exposure (Gottesfeld et al., 1990). Four independent samples from either control or ethanol- treated groups were collected for further analysis.

Varenicline exposure: Varenicline tartrate (Tocris Bioscience, Bristol, UK) at 1.0μmwas administered alone or in combination with 320mg/dl ethanol according to previous protocols established in our laboratory (Balaraman et al., 2012), and four independent samples from each group were collected for analysis.

MiR-153 mimetic exposure: the human genome encodes two copies of miR-153 (mir-153-1 and miR153-2), whereas the mouse genome encodes a single copy of this miRNA (Mandemakers et al., 2013). The reported experiments utilized mouse (*mmu*)-miR-153 as an experimental model. Furthermore, we focused on a strategy of miR-153 overexpression rather than repression, as a means to prevent and reverse ethanol's effects on NSCs. Neural progenitors were exposed to *mmu*-miR-153 mimetic (miRNASelect™ pEGP-mmu-mir-153 Expression Vector, Cell Biolabs, CA) or control (miRNASelect™ pEGP-miR Null Control Vector,Cell Biolabs, CA), either alone or in combination with ethanol at 320mg/dl. The miRNA expression construct also encoded a green fluorescent protein (GFP)/puromycin resistance fusion protein for monitoring transfection efficiency and as a selection marker. For cell transfection experiments, neurospheres were collected and trypsinized into a single cell suspension. Cell density was determined by a *Countess*® Automated Cell Counter (Life Technologies, CA). A

10µl cell suspension containing 3~4 million cells was transfected by electroporation with 7µg of either control or miR-153 precursor clone using a NEON electroporator with transfection kit (settings 1200V, 20ms, 2 pulses, Life Technologies, CA). Six independent replicate experiments were harvested for further analysis.

MiRNA and mRNA isolation, mouse whole genome microarray analysis

MiRNA and mRNA were extracted from harvested cells using a *mir*Vana™ miRNA Isolation Kit (#AM1560; Life Technologies, CA) according to the manufacturer's protocol. Purified miRNA and mRNA samples were quantified by NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, MA). For mRNA microarray analysis, 1.0µg of purified mRNA samples from either control or miR-153 mimetic-treated groups, mixed with 50pg RNA spike-in control, were used to generate biotin labeled cRNA samples by a linear amplification method using Ambion's MessageAmp™ II-Biotin Enhanced Single Round aRNA Amplification Kits (#Am1791; Life Technologies, CA). 10µg labeled cRNAsamples were fragmented at 94^{o}C for 20minutes, mixed with hybridization buffer (Applied Microarray, Tempe AZ) and hybridized with CodeLink whole genome arrays (Applied Microarray, Tempe AZ). Post-hybridization processing and secondary-labelingwithCy™5-conjugated to Streptavidin were performed according to the manufacturer's instructions (Applied Microarray). Microarrays were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA).A total of 12 microarrays (six in each condition) were used to

assess changes in the transcriptome. Array images were processed using CodeLink™ software (Applied Microarray, AZ), and global median normalization was used to generate normalized expression values. The data were analyzed further, using GeneSifter analysis edition (GSAE, PerkinElmer-Geospiza, Seattle WA).

Real-time PCR validation of miRNA and mRNA

MiRNA expression of control and miR-153 mimetic-treated samples was validated before performing mouse whole genome microarray analysis. Briefly, 25ng of purified total RNA was used to generate cDNA, using a Universal cDNA Synthesis Kit according to the manufacturer's protocol (#203300; Exiqon, Denmark). cDNA samples were diluted 80x and 4µl was used as aPCR reaction template in a 10µl PCR reaction. PCR reactions were run in triplicate on an Applied Biosystems 7900HT real-time PCR instrument (Applied Biosystems, CA) using a SYBR green-based real-time PCR reaction kit (#203450; Exiqon, Denmark) with miR-153 (#204338; Exiqon, Denmark) and U6 snRNA primer sets(#203907; Exiqon, Vedbaek, Denmark). U6 snRNA was used as a normalization control. Real-time RT-PCR data of miRNA expression were quantified using the SDS 2.4 software package (Applied Biosystems, CA). Transfection with the miR-153 mimetic resulted in a ~30-fold induction of miR-153expression relative to control groups, without a statistically significant change in another ethanol-sensitive miRNA, miR-21 (data not shown).RT-PCR of mRNA was performed to evaluate expression of candidate miR-153 target transcripts that were identified in the mouse whole genome microarray analysis. Total RNA (500ng) was used to generate first

strand cDNA using qScriptcDNA Supermix kit (#95048-100; Quanta Biosciences, MD). Real-time PCR was performed on a 7900HT Real-Time PCR System(Life Technologies, CA) using the PerfeCTa SYBRGreen SuperMix with ROX kit (#95053-500; Quanta Biosciences, Gaithersburg, MD). 2.0µl of cDNA was used as the template in a reaction volume of 10µl. RNA expression was quantified by calculating the difference between the cycle threshold of the mRNA of interest, and the reference gene (18s mRNA) for each sample. Specificity of the amplification was evaluated by thermal stability analysis of the amplicon. Individual and reference gene primer sets (Integrated DNA Technologies, Coralville, Iowa) are listed in Table 2-1.

3'UTR analysis of miR-153 candidate genes

Plasmids containing 3'UTR firefly/Renilla Duo-Luciferase reporter luciferase constructs (miTarget™ 3'UTR miRNA Targets, GeneCopoeia, Rockville, MD) were amplified and purified using a EndoFree plasmid Maxi kit (#12362; Qiagen, Germantown, MD) according to manufacturer's protocol. Purified plasmids were quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA).150ng of 3'UTR construct (Matn2 (#MmiT031425; GeneCopoeia), Vegfa (#MmiT024368; GeneCopoeia), Nfia (#MmiT054793; GeneCopoeia), Nfib (#MmiT027729; GeneCopoeia), pmiR153-Luc (a positive control for miR-153 translation repression activity, #LR-0064; Signosis, Sunnyvale, CA), 100ng of either control or miR-153 mimetic expression plasmids (Cell Biolabs, CA), and 10µm of control or targeted morpholino antisense, anti-3'UTR oligonucleotides (Gene Tools,

Table 2-1. List of real time RT-PCR primer sequences.

Gene Name	Forward Primer	Reverse Primer
Akt1	AGTCCCCACTCAACAACTTCT	GAAGGTGCGCTCAATGACTG
Arl2bp	TGCAGAGAAACTTCATGGACA	ATGTTGAAGCCTGGGATACG
Ccl2	TGCTACTCATTCACCAGCAA	GTCTGGACCCATTCCTTCTT
Ccl7	GATCCCCAAGAGGAATCTCA	CTTCTTGGCTCCTAGGTTGG
Cxcl1	CATCCAGAGCTTGAAGGTGTT	AAGGGAGCTTCAGGGTCAAG
Ddit4	ACTCTTCCTTGGTCCCTGGT	GCTGCTCGGAGCTGTAGAGT
Fbxo2	CTGTCGGAGAACGAAGATGT	TTCCAGTAGACCGAGTCCTG
Foxj2	CTGACATTTCCCGGAAGAGA	TTCTCCACTCCCTGGAACAC
Hdac8	AGCCAAGAAGGTGATGAGGA	ACCCTCCAGACCAGTTGATG
Matn2	GCAGCACGGTCAAGAACGA	GAGGGCATATTGGATGGCAAG
Mid1	AGCCTGTGGAGTCCATCAAC	GGTCTCACTGGGAGAGTTGG
Mkln1	GGCCATCAAATGGTCATTGACG	CAGAGGAATCCAAGTAACGGC
Mtap1s	CCATCGCCCTTCGAGTTGTT	GCCTTGCTAAGCGCAGAAAG
Nf1a	TTCCAACGTCACCCATCATCC	CAGCATCAGGACAGACAAGTT
Nf1b	CTCAGTGAGAAGCCCGAAATC	CAGTCACGGTAAGCACAAAGT
Nf1c	CTCACCCACGAGTAGCAGC	TTCTTCACCGGGGATGAGATG
Rab13	CAAAGCCTACGACCACCTCT	TATGTCCACGGTTCGGATCT
Vegfa	TGGATGTCTACCAGCGAAGC	ACAAGGCTCACAGTGATTTT
18S	ATGGCCGTTCTTAGTTGGTG	CGCTGAGCCAGTCAGTGTAG

Philomath, OR), to protect predicted miR-153 binding sites in 3'UTRs of target mRNA transcripts), were co-transfected into 20,000 cells, using a NEON electroporator according to manufacturer's protocol (as outlined above). At 24 hours after transfection, luciferase signals were determined by Dual-Glo® luciferase assay (#E2920; Promega, Madison, WI), and quantified using the Synergy-2 multi-mode plate reader (BioTek, Winooski,VT). Firefly luciferase intensity from each sample was normalized to the internal control, Renilla luciferase.

Ultrasound-guided in utero electroporation

Ultrasound guided micro-injection procedures were adapted from our previously published protocol (Tingling et al., 2013). GD13.5 timed pregnant mice were anesthetized with 4% isoflurane and 0.5% oxygen, then positioned on a heated mouse platform (Integrated Rail System, Visual Sonics, Toronto, Canada) to monitor temperature, respiration, and heart rate. Maternal core body temperature was maintained at 33-35^{o}C and maternal heart rate was monitored with adjustments to the level of anesthesia made to maintain a constant heart rate of ~450 beats/minute. Following hair removal (Nair® hair remover), the skin was sterilized with ethanol (80% v/v) followed by Betadine®. A midline incision was made through the skin and peritoneal wall, and one uterine horn was gently exteriorized through the incision, and carefully drawn through a Parafilm® flap in the bottom of a sterilized petridish. The uterine horn was completely immersed in warmed ultrasound gel (Ecogel, Eco-Med, Mississauga, Canada). The brain and lateral ventricle of each fetus was located by ultrasound imaging

(VEVO2100, Visual Sonics, Toronto, Canada). Once the fetal orientation was determined, 5μg of control or miR-153 plasmid was injected with a micropipette, under ultrasound guidance, through the uterine wall, into the fetal lateral ventricle. Fetuses of either sex were used for microinjection experiments. A forceps-type electrode connected to a BTX ECM 630 electroporator (4 pulses, each at 35V and 950msec duration, Harvard Apparatus, Holliston, MA) was used to electroporate the plasmid into the fetal telencephalon. Following electroporation, the uterine horn was reinserted into the peritoneal cavity and the abdominal wall and skin were closed with sutures. Mice were sacrificed and the injected embryos were collected after 48 hours post-transfection. Collected embryos were fixed in 4% buffered paraformaldehyde, cyro-protected with 30% sucrose, and cryo-sectioned at 20μm.

In utero ethanol exposure model

Timed pregnant mice were exposed to ethanol (3 gm/kg) or an equivalent volume of water by intragastric gavage twice daily between GD12.5 and 14.5, bracketing the first half of the second trimester-equivalent period of cerebral cortical neurogenesis (Takahashi et al., 1995), according to our previously published protocols (Bake et al., 2012; Sudheendran et al., 2013; Tingling et al., 2013). The dose of ethanol results in a peak blood alcohol content of 117 mg/dl (Bake et al., 2012) that can be attained in humans following binge episodes of ethanol consumption (Table 1-1), which are particularly damaging to the fetus (Maier and West, 2001). At the end of the exposure period, one fetus from each dam (four control and four ethanol exposed in total of either

sex) was cryo-sectioned and subjected to immunofluorescence analysis.

Immunofluorescence analysis

Mouse tissue sections from either microRNA-injected or ethanol treated fetuses were incubated in a blocking solution (10% normal serum, 0.6% TrionX-100 in PBS) at room temperature for 1 hour. Sections were incubated with primary antibody overnight at 4°C in appropriate dilution (anti-GFP to visualize co-expression of miR-153 (1:800; #ab13970; Abcam, MA), anti-Nfib (1:100, #HPA003956; Sigma-Aldrich, MO), anti-Nfia (1:100, #AP14133b, Abgent, CA), anti-Hdac8 (1:400; #ab39664, Abcam, MA), anti-DCX (1:800; #ab18723, Abcam, MA) and anti-CD24 (1:300; #ab64065, Abcam, MA)). Slides were washed in PBS three times, and Alexa-Fluor conjugated secondary antibodies were used in order to visualize. (#A11001, A11032, A11037; Life Technologies, CA; #SC-362261, SC-362271; Santa Cruz Biotechnology, CA). Cultured cells were also immuno-labeled with ant-GFP antibody. Cells and tissues were mounted with fluorescence mounting media containing DAPI (#H-1200; Vector Laboratories, CA) and photographed using an Olympus microscope.

Cell proliferation assay

DNA synthesis was measured using the Click-iT® EdU cell proliferation assay (Life Technologies, #C-10352) according to our published protocol (Tingling et al., 2013). Briefly, 400,000 cells were treated with 1.5 μg of the scrambled control-GFP or pre-miR-153-GFP expression vector followed by incubation with 10 μM EdU (5-ethynyl-2′-

deoxyuridine) for 16 hours to monitor DNA synthesis. Cells were fixed, washed, and Incorporated EdU was detected by covalently binding Alexa Fluor® 555-azide. The percent of EdU-labeled cells was then quantified with an Olympus fluorescence microscope.

Apoptosis assay

Apoptosis was detected by a Homogeneous Caspase Assay (#03005372001, Roche Applied Science, IN). Briefly, cell aliquots (4.0×10^5 cells) were treated with 1.5 μg of the scrambled control-GFP or pre-miR-153-GFP expression vector for 12 hours. Staurosporine (2 μM for 2 hours)-treated neurosphere cultures were used as a positive control and U937 cells treated with camptothecin (4μg/ml for 4 hours) were used as positive control. Caspase activity was measured by adding the diluted caspase substrate (DEVD-R110) at 37°C for 2 hours. Cleavage of the caspase substrate and release of the fluorescent dye, Rhodamine 110, was determined at λ_{max} = 521nm with the fluorescence microplate reader (Tecan Infinite M200, Switzerland).

Data analyses

Sample sizes from each group ranged from 4 to 6 independent replicates. Scholl analysis of neurite length was conducted using ImageJ. Data were analyzed with the Chi-squared test. Median-normalized mRNA microarray data were analyzed by T-tests with Benjamin and Hochberg correction for multiple comparisons(α=0.05), using GeneSifter analysis edition (GSEA, Perkin-Elmer/Geospiza, WA), with a fold-change

threshold set at ±1.3-fold. Gene ontology analysis was conducted and z-scores computed for over-representation analysis. Data from all other experiments were analyzed by multivariate (MANOVA, Pillai's trace statistic) or univariate (ANOVA) analysis of variance. MANOVAs were followed by *post hoc* univariate ANOVA, and planned comparisons were further computed with the Fisher's least significant difference (f-LSD) test (SPSS v20, IBM). Single comparison experiments were evaluated with T-tests with the statistical significance set at $p<0.05$. Data are expressed as mean ±SEM.

Results

Identification of miR-153 regulated candidate genes

Our previous studies indicated that teratogens like ethanol disrupted NSC maintenance in part, by suppressing specific miRNAs that regulate stem cell or progenitor makers (Sathyan et al., 2007). To better understand how miRNAs like miR-153 may mediate teratogenesis, we first needed to identify miR-153 targets in fetal NSCs/NPCs. We therefore overexpressed miR-153in fetal NSCs for 24 hours in a transient transfection assay (see Figure 2-1a for map of expression construct and Figure 2-1b for GFP expression in NSCs cultured on a laminin substrate after 24 hours), and performed microarray analysis to identify potential target genes. Overexpression of pre-miR-153 resulted in a ~32-fold induction of miR-153 compared to the transfection control ($t_{(10)}$=-8.33, $p<8.27e-06$, Figure 2-1c). Transcript profiles were assessed from RNA samples (6 independent replicates in each condition, microarray data submitted to NCBI-GEO (Accession #GSE49684)). Data analysis showed that miR-153 over-

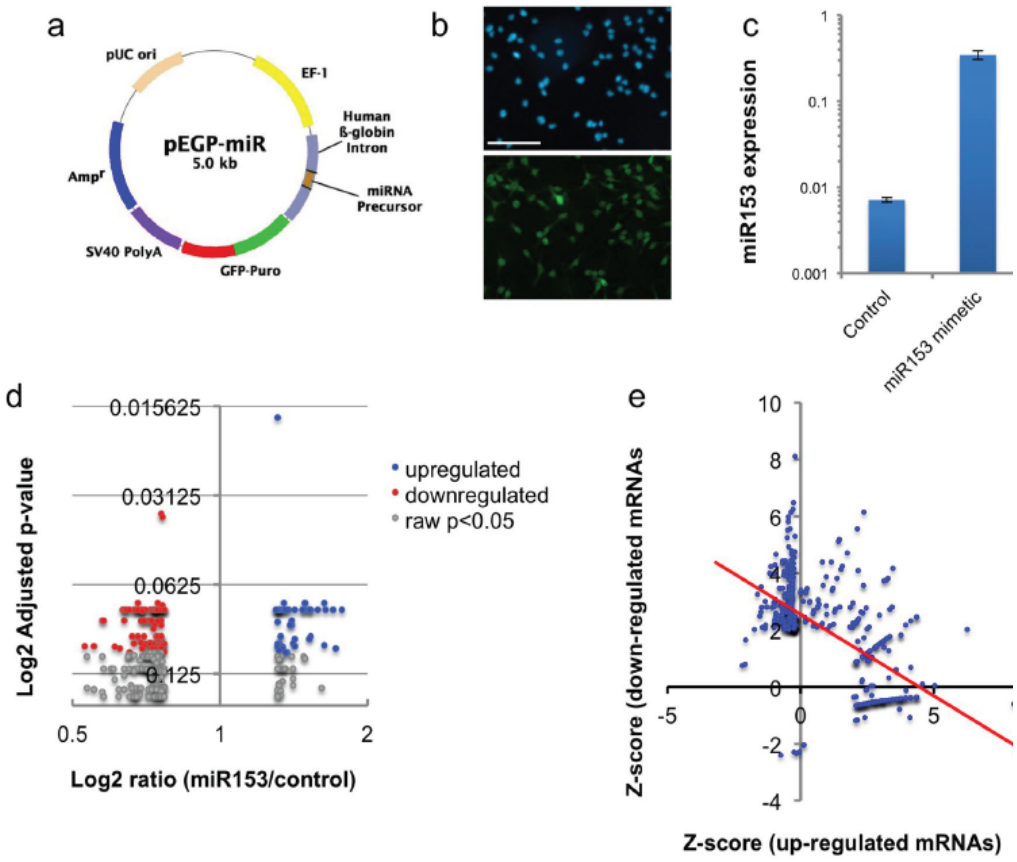


Figure 2-1. Identification and gene ontology classification of mRNAs that are down-regulated following miR-153 overexpression. (a) Schematic structure of the pre-miR-153/GFP-puromycin expression vector. (b) Sample photomicrographs documenting transfection efficiency. Upper panel depicts DAPI stained nuclei and lower panel depicts GFP immunofluorescence. Cells were cultured for 24-hours before visualization of GFP labeling. (c) Bar graph shows that transfection of neurosphere-derived cells with pre-miR-153 expression vector results in a 30-fold increase in miR-153 expression compared to transfection with vector control. Data from six independent replicates (n=6) are expressed as mean +/- SEM. (d) Volcano-plot illustrates relationship between $\log_2$(mRNA expression ratio) and $\log_2$(FDR-corrected p-value), in miR-153 overexpressing cultures compared to controls. Filled red and blue circles indicate mRNA transcripts that are suppressed or induced respectively by more than 1.3-fold following miR-153 overexpression, at an FDR (Benjamini and Hochberg)-adjusted $p<0.1$. Gray circles indicate genes that reached statistical significance at a raw $p<0.05$. Data summarized from 6 independent replicate experiments.(e) The plot shows the correlation of gene ontology analysis of mRNA transcripts that were suppressed and induced following miR-153 overexpression. The X-axis depicts the z-score of ontology analysis of mRNAs induced by miR-153. The Y-axis indicates the z-score of ontology analysis of mRNAs suppressed by miR-153. The red line is the regression line that indicates a negative linear relationship between ontology classes of miR-153 suppressed and induced mRNA transcripts.

expression resulted in statistically significant down-regulation of 133 genes (at a false discovery rate α=0.1, of a total of 860 transcripts down-regulated by ≥1.3-fold, Figure 2-1d, Table 2-2). Ontology analysis showed that there was a negative correlation between ontologies associated with down-regulated and up-regulated transcripts (Pearson's r=-0.592, p<0.1E-10, Figure 2-1e), indicating that the functions of the down-regulated, including presumably conventionally 3'UTR-targeted mRNAs, did not overlap with the functions of the up-regulated genes. Transcripts that were down-regulated following miR-153 overexpression were significantly overrepresented in ontological categories related to synaptic transmission and G-protein coupled receptor (GPCR) signaling (Table 2-3). Moreover, pathway analysis (Kyoto Encyclopedia of Genes and Genomes, KEGG) identified "Neuroactive ligand-receptor interaction" as a significantly suppressed pathway. These bioinformatics analyses suggest that in NSCs, miR-153 suppresses pathways that are important for the function of differentiated neurons.

Interestingly, miRNAs are also known to induce gene expression by binding to alternate target sites outside 3'UTRs, including promoter regions (Place et al., 2008). *In silico* analysis (MirWalk, (Dweep et al., 2011)) of the top six significantly induced RNA transcripts, Patl2, Dhrs13, Rps25, Rai14, Foxo3, and EG665934/GM7854 (FDR-corrected, α=0.02), predicted that two, Rai14 and Rps25, each contained a miR-153 target site with a seed region of 9nt in length (p<0.037) within the presumptive promoter, a 2kb upstream gene flanking region. The sixth target, EG665934/GM7854, a candidate long noncoding RNA (lncRNA), is predicted (RNA hybrid, (Kruger and Rehmsmeier, 2006)) to contain a miR-153 target site with a 10nt seed region. The possibility that these

Table 2-2. Sample miR-153 candidate gene targets from microarray analysis.

The table represents 10 candidate genes down regulated in miR-153 overexpression with adjusted (Benjamini and Hochberg correction for multiple comparisons) P value <0.1 and four additional genes chosen for validation with a raw p-value <0.05. "Gene Identifier" exhibits gene accession number from GenBank and "Gene ID" represents Hugo Gene Nomenclature.

Gene ID	Gene Identifier	Ratio	p-value	adj. p-value
Aplp2	NM_009691	1.47	0.0007	0.07
Fbxo2	NM_176848	1.34	0.0006	0.07
Rab13	NM_026677	1.5	0.003	0.07
Nfic	NM_026756	1.36	0.002	0.07
Cxcl1	NM_008176	2.71	0.007	0.08
Hdac8	AK011332	1.74	0.007	0.08
Arl2bp	NM_024191	1.43	0.006	0.08
Matn2	NM_016762	1.32	0.01	0.09
Ddit4	NM_029083	1.89	0.01	0.10
Ccl2	NM_011333	1.87	0.02	0.10
Vegfa	NM_009505	1.67	0.03	0.12
Mid1	CF536475	1.57	0.03	0.12
Mtap1s	NM_173013	1.63	0.05	0.14
Ccl7	NM_013654	1.61	0.05	0.14

Table 2-3. List of major gene ontologies (GO) and KEGG pathways down-regulated by miR-153 with a Z-score >2.0.

Gene Ontology	GO ID	Number of regulated Genes	Number of Genes in Set	%	Z-score
regulation of inhibitory postsynaptic membrane potential	GO:0060080	3	10	30.00	10.08
membrane hyperpolarization	GO:0060081	3	16	18.75	7.83
negative regulation of synaptic transmission	GO:0050805	3	32	9.38	5.28
G-protein signaling, coupled to cAMP nucleotide second messenger	GO:0007188	4	93	4.30	3.65
regulation of synaptic transmission	GO:0050804	5	172	2.91	2.97
regulation of synaptic plasticity	GO:0048167	3	78	3.85	2.9
second-messenger-mediated signaling	GO:0019932	5	201	2.49	2.56
transmission of nerve impulse	GO:0019226	8	436	1.83	2.29
ion transport	GO:0006811	13	845	1.54	2.26
G-protein coupled receptor protein signaling pathway	GO:0007186	16	1160	1.38	2.06
KEGG Pathway					
Neuroactive ligand-receptor interaction	map04080	9	287	3.135889	4.43
Metabolism of xenobiotics by cytochrome P450	map00980	3	62	4.83871	3.5

are functional sites remains to be determined. However, these data do suggest the possibility that some of the induced transcripts may be direct targets of miR-153 as well.

Nineteen genes were selected for further assessment. These included 10 candidate miR-153-repressed transcripts that reached an FDR-corrected $p<0.1$ (Aplp2, Arl2bp, Ccl2, Cxcl1, Ddit4, Fbxo2, Hdac8, Matn2, Nfic, Rab13), and 4 with an un-corrected $p<0.05$ (Ccl7, Mid1, Mtap1s, Vegfa). Three genes, Akt1, Foxj2, and Mkln, were chosen as validation controls that were not statistically significantly altered by miR-153 overexpression. Additionally, because the microarray screen identified Nfic as a candidate target for miR-153, we also assessed other members of the nuclear factor family, Nfia and Nfib. The Nfia family is a particularly important target because of their implicated role in suppressing NSC self-renewal (Namihira et al., 2009; Piper et al., 2010), promoting gliogenesis (Deneen et al., 2006; Barry et al., 2008), and neuronal differentiation and migration (Wang et al., 2010). Moreover, Nfia haplo-insufficiency is associated with brain malformations including agenesis of the corpus callosum, ventriculomegaly, and Chiari-type-1 malformations (Lu et al., 2007), features associated with fetal alcohol exposure. None of the validation control mRNAs were altered following miR153 over-expression. Among the genes that met the adjusted-$p<0.1$ criteria, Arl2bp ($t_{(10)}=3.09$, $p<0.01$), Ccl2 ($t_{(10)}=3.14$, $p<0.01$), Ddit4 ($t_{(10)}=2.70$, $p<0.02$), Fbxo2 ($t_{(10)}=2.48$, $p<0.03$), Hdac8 ($t_{(10)}=3.66$, $p<0.004$) and importantly, Nfia, Nfib and Nfic ($t_{(10)}=2.67$, $p<0.024$; $t_{(10)}=5.49$, $p<0.001$; $t_{(10)}=5.28$, $p<0.001$, respectively, Figure 2-2a.i), were significantly decreased following miR-153 overexpression in the validation

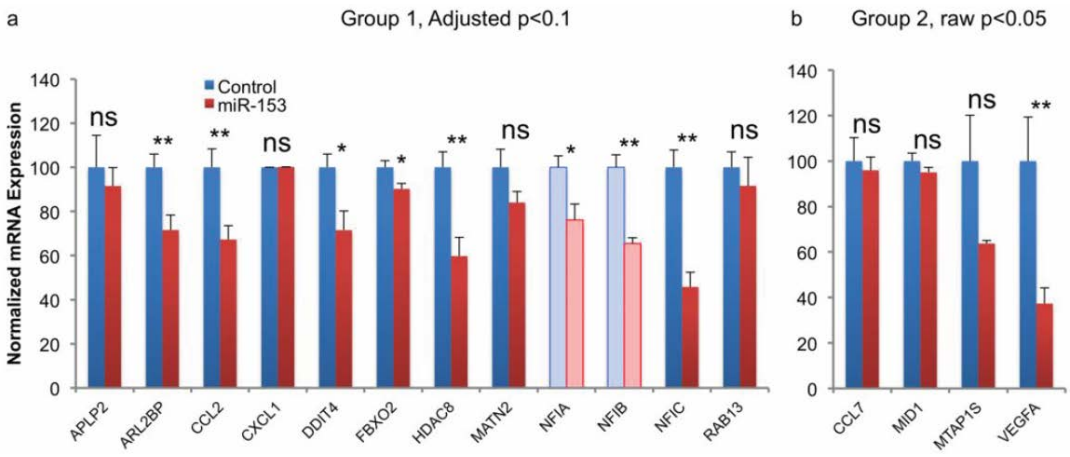


Figure 2-2. RT-PCR validation of candidate miR-153-regulated mRNAs.(a and b) Bar graphs depict the real time RT-PCR quantification of mRNAs in vector control and miR-153 overexpression conditions for candidate mRNA transcripts identified from the microarray experiment that achieved the adjusted p-value cutoff of 0.1 (a) and raw p-value of 0.05 (b) respectively. Y-axis indicates normalized mRNA expression (expressed as 1/2ΔCt relative to 18s RNA). Data were expressed as mean +/- SEM and quantified from 6 independent replicates (*=p<0.001; **=p<0.05).

experiment. Among the cohort of genes that exceeded the 'raw' $p<0.05$ criterion, only Vegfa ($t_{(10)}=3.07$, $p<0.012$, Figure 2-2a.ii) was significantly reduced following miR-153 exposure in the validation sample.

Following transfection with control-GFP or miR-153-GFP expression vectors, neurosphere-derived cells were cultured for an additional 48 hours on a laminin substrate in a mitogen-withdrawal, differentiation paradigm that has previously been shown to result in transformation of NSCs into early migratory neurons (Camarillo et al., 2007; Camarillo and Miranda, 2008; Tingling et al., 2013). Control NSCs exhibited morphological transformation into bipolar and multipolar cells with elongated processes (Figure 2-3a.i-iii), whereas miR-153-GFP transfected cells exhibited deficient morphological transformation (Figure 2-3b.i-iv). Scholl analysis (Figure 2-3c) indicated that differentiating cells overexpressing miR-153 had significantly shorter neurites compared to cells transfected with the control plasmid (Chi-square, $p<3.8e-46$). However, miR-153 overexpression did not result in significant apoptosis (Figure 2-3d) or change in cell proliferation (Figure 2-3e).

Identification of 3'UTR regulatory motifs in miR-153 targeted transcript

We utilized bioinformatic tools (Targetscan, www.targetscan.org) to perform an *in silico* analysis 3'UTRs.Nfia and Nfib each contained several predicted miR-153 binding sites in their 3'UTRs with high aggregate P_{CT}s (probability of conserved targeting (Friedman et al., 2009)) of 0.99 and 0.96 respectively indicating that miR-153

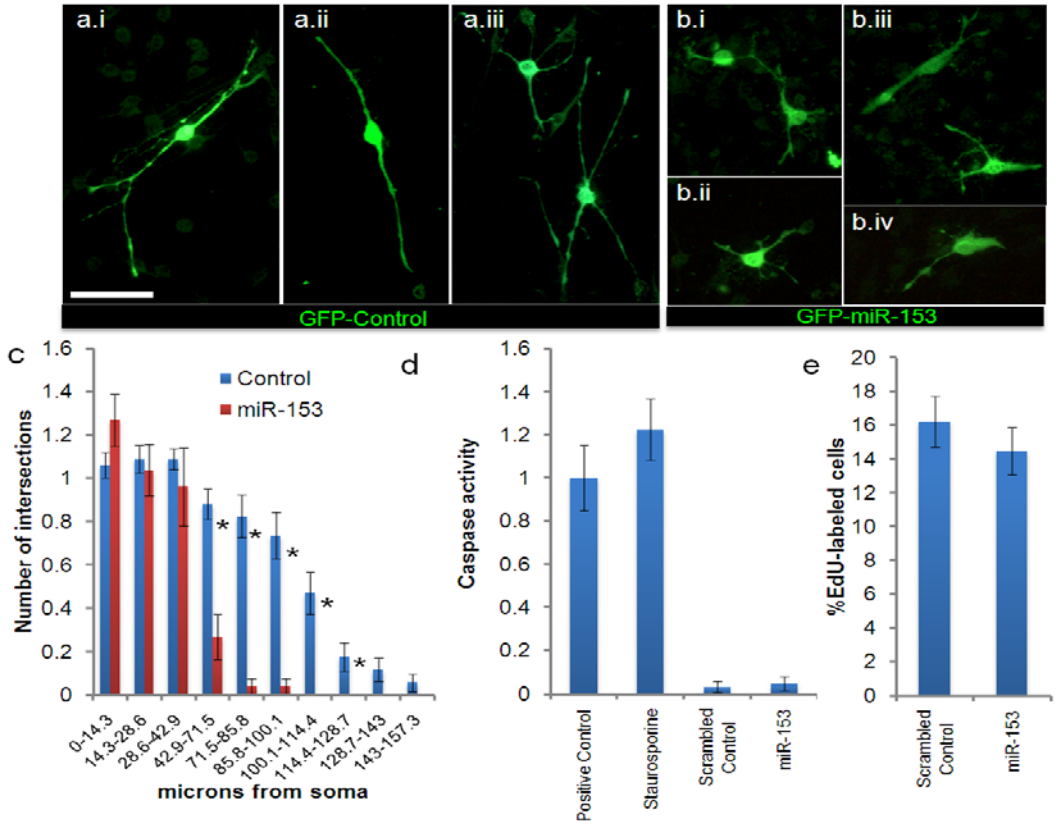


Figure 2-3. Effects of miR-153 overexpression on differentiation, apoptosis and cell proliferation. (a,b) Photomicrographs depicting GFP immunofluorescent cells cultured under mitogen-withdrawal-induced differentiation conditions on a laminin substrate, 48 hours after transfection with GFP control vector (a.i-a.iii) or with GFP-premiR-153 (b.i-b.iv). MiR-153 overexpressing cells exhibited deficient morphological transformation compared to control cells. (c) Bar graph depicts Scholl analysis of neurite length expressed as a number of intersections (Y-axis) as a function of distance from soma (X-axis) per cell. MiR-153 overexpressing cells have a shorter neurites compared to controls. Data based on analysis of 27 control and 16 miR-153-overexpressing cells from 3 independent transfection assays. Photomicrographs were obtained from all four quadrants of each culture dish, and cells whose processes showed no overlap with those of an adjacent cell were selected for analysis. (d) Pan-caspase activity was measured as an estimate of apoptosis. Caspase activity was high in U937 cells treated with camptothecin (4μg/ml for 4 hours) and Staurosporine (2 μM for two hours)-treated neurosphere cultured cells and low in neurosphere cultures transfected with control or pre-miR-153 expressing vectors. There was no statistically significant difference between the latter two conditions (n=6). (e) EdU incorporation into DNA was used as a marker for cell proliferation. The percentage of labeled cells was not significantly different indicating that cell proliferation was not altered by miR-153 overexposure. Data based on three independent transfection experiments. One photomicrograph was obtained from each quadrant of the culture well (three culture wells per sample) and labeled and total number of cells counted.

interactions with Nfia/Nfib 3'UTRs are likely to be evolutionarily conserved. Matn2 and Vegfa 3'UTRs were assessed as controls. Matn2 was not validated as a miR-153 target in our earlier miR-153 overexpression study, scored a lower P_{CT} of 0.78,and is predicted to contain a 3'UTR miR-153 target site in primates, but not rodents. Vegfa 3'UTR contained no predicted miR-153 binding sites, though VEGFa mRNA was significantly reduced following miR-153 transfection. Moreover, Vegfa is the most highly expressed cytokine in proliferating NSCs, and its expression levels decrease significantly with neural differentiation (Camarillo et al., 2007), suggesting that this cytokine is important for NSCs. Luciferase-reporter constructs (Figure 2-4a) containing murine 3'UTRs of Nfia, Nfib, the negative controls, Matn2 and Vegfa, and as a positive control, Luc153, were each transfected into single cell suspensions derived from neurosphere cultures. Some cell aliquots, were also co-transfected with pre-miR-153 or control expression vectors. Additionally, antisense morpholino oligonucleotides can be used to protect 3'UTR target sites from miRNAs, by interfering with miRNA-regulated translation (Choi et al., 2007). We therefore co-transfected either scrambled or antisense morpholino oligonucleotides (Table 2-4, Figure 2-4b, 2-5a) into some samples to mask predicted miR-153 binding sites. At the end of 24 hours, luciferase activity, normalized to Renilla luciferase, was determined. Because of their length, the 3'UTRregion within the Nfia and Nfib transcripts were each fragmented into three parts and cloned into separate constructs downstream from the luciferase reporter (Figure 2-4b, 2-5a).

MiR-153 overexpression resulted in a 60% reduction in luciferase activity from the co-transfected luciferase expression construct with miR153 binding sites in the 3'UTR

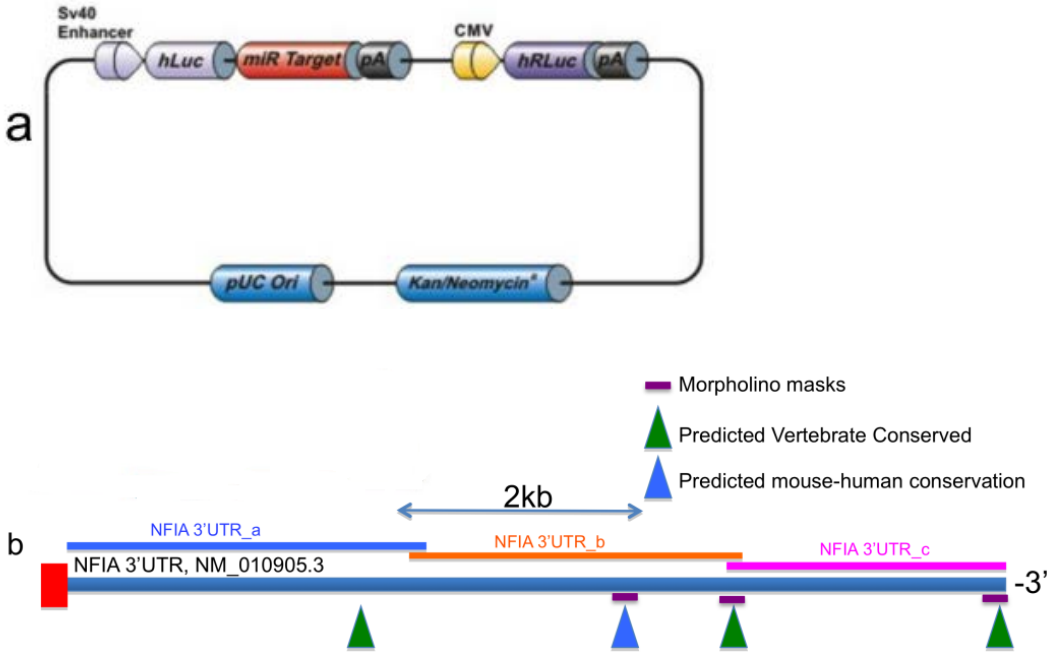


Figure 2-4. Identification of Nfia 3'UTR as a direct target of miR-153. (a) Schematic structure of the luciferase reporter constructs containing murine Nfia 3'UTR fragments. (b) Schematic of the full length of Nfia 3'UTR depicting the location of the three Nfia 3'UTR fragments (Nfia 3'UTR_a, b, c) that were cloned into luciferase reporters for studies. Green triangles indicate the predicted miR-153 binding sites that are conserved among vertebrates, whereas the blue triangle illustrates the predicted miR-153 binding site that shares conservation between mouse and human. Purple bars represent the morpholinos used to protect the predicted miR-153 binding sites. (c) We assayed firefly activityrelative to RLuc luciferase activity in NSCs 24 h after transfection with the Luc_miR153 binding site reporter construct as the positive control and transfected control or miR-153 mimetics. Bar graphs represent luciferase activity normalized to the mean activity of samples transfected with the miR-153 control vector. The X-axis depicts treatment conditions. Y axis indicates normalized luciferase activity. Data were expressed as mean +/- SEM. n=5. (d)(e)(f) NSCs were transfected with luciferase reporter constructs containing different fragments of Nfia 3'UTR, (d) 3'UTR_a, (e) 3'UTR_b, (f) 3'UTR_c, with control or miR-153 mimetics for 24 hours. Additional control or antisense morpholinos, (e) mask_i, mask_ii, and (f) mask_iii, used to protect the miR-153 binding sites were co-transfected along with other constructs as indicated on the X-axis. Bars are normalized to the relative firefly units of samples treated with the transfected control. Data were expressed as mean +/- SEM. n=5.

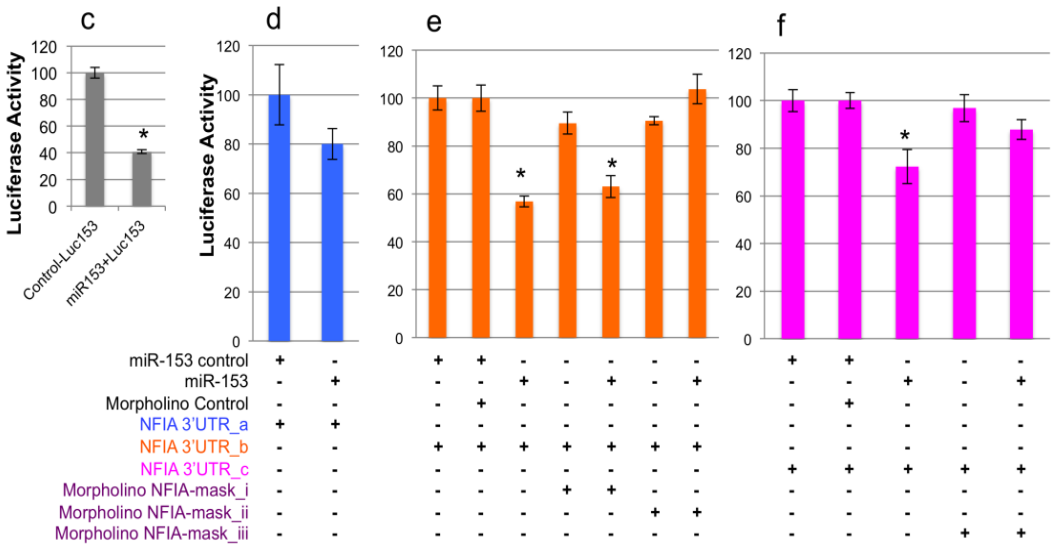


Figure 2-4 continued.

Table 2-4. Sequences of morpholino oligonucleotides.

Control	5’- GTGTAACACGTCTATACGCCCA-3’
NFIA-mask_i	5’- GTTTGCATATTCTTGTGGGCTGACT -3’
NFIA-mask_ii	5’- TAGTGCATAGTTCTCATGCAGGTAA -3’
NFIA-mask_iii	5’- TTGCATAGGAAATGCAGTACTTGCT -3’
NFIB-mask_i	5’- TTGCATAGGAAATATATCCGCTTCC -3’

(Luc153, Figure 2-4c, p<6.64E-07).In the case of Nfia, miR-153 targeting of Luc-Nfia-3'UTR_a did not significantly repress luciferase activity (Figure 2-4d). However miR-153 did repress luciferase activity from the Luc-Nfia-3'UTR_b and from the Luc-Nfia-3'UTR_cconstructs (overall ANOVAs, $F_{(6,28)}$=17.23, p<3.07e-08, and $F_{(4,18)}$=4.59, p<0.01 respectively, Figure 2-4e,f). Masking morpholinos, Nfia_mask_ii and Nfia_mask_iii, but not Nfia_mask_i, were able to completely protect against miR-153-mediated translation repression. Collectively, these data indicate that two out of four predicted miR-153 binding sites localized near the end of the Nfia-3'UTR (Nfia/NM_010905.$3_{7285-7306}$ and Nfia/NM_010905.$3_{9451-9469}$) mediated miR-153 translation repression in fetal neuroepithelial cells. Similarly, Nfib was a direct target of miR-153. Translation of Luc-Nfib-3'UTR_a (Figure 2-5b) or Luc-Nfib-3'UTR_b (Figure 2-5c) was not repressed by miR-153 overexpression. However, translation of Luc-Nfib-3'UTR_c (Figure 2-5d) was repressed by miR-153 over-expression (overall ANOVA, $F_{(4,18)}$=4.6, p<0.01). The Nfib-mask_i morpholino protected the second, more distally located predicted miR-153 binding site (Nfib/NM_008687.$5_{6559-6566}$), located near the 3'-end of the 3'UTR, and prevented translation repression. *In silico* analysis (Mfold, http://mfold.rna.albany.edu/, (Zuker, 2003)) of the folding of the Nfia 3'UTR indicates that miR-153 target site Nfia/NM_010905.$3_{77285-7306}$ localizes to a complex of branched stem-loop structures, whereas Nfia/NM_010905.$3_{9451-9469}$ localized to a predicted linearized portion of the 3'UTR. However, both sites are positioned proximate to the 3' and 5' termini of the 3'UTR (Figure 2-6a). Analysis of Nfib-3'UTR folding showed that the Nfib/NM_08689.$5_{6559-6566}$ site, like Nfia/NM_010905.$3_{9451-9469}$, was

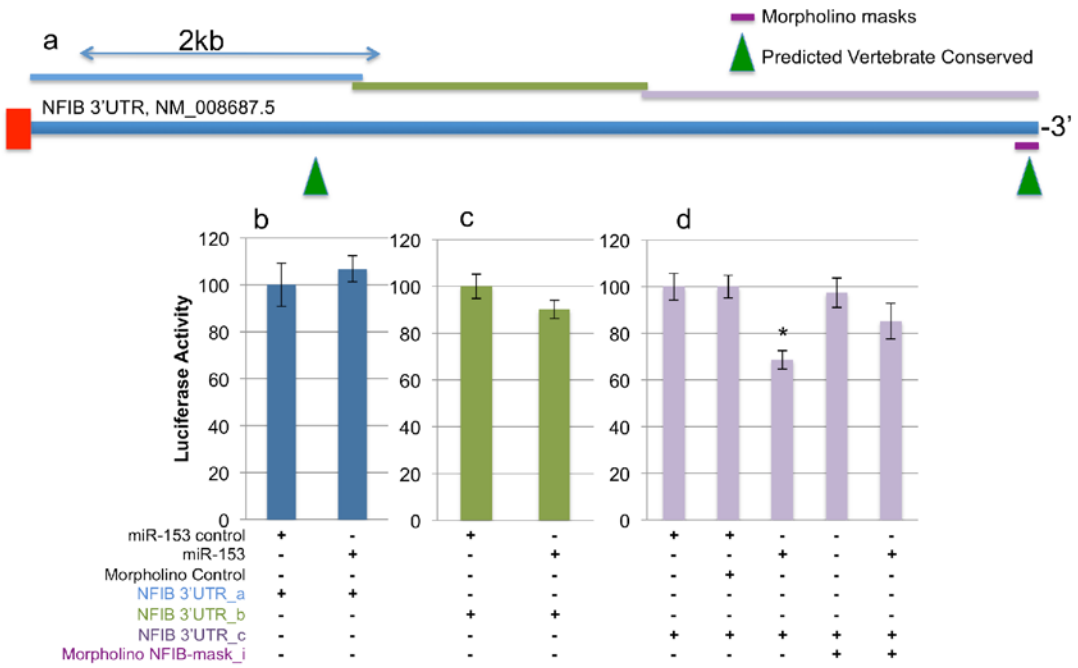


Figure 2-5. Nfib is a direct target of miR-153. (a) Schematic showsthe full length Nfib 3'UTR in relation to the three 3'UTR fragments (Nfib 3'UTR_a in blue, 3'UTR_b in orange, 3'UTR_c in pink) that were cloned into luciferase constructs for studies. Green triangles indicate the predicted miR-153 binding sites that are conserved among vertebrates. Purple bars represent the morpholinos used to mask the miR-153 binding sites. (b)(c)(d) Firefly activityrelative to RLuc luciferase activity is determined in NSCs 24 h after transfection with luciferase reporter constructs containing different fragments of Nfib 3'UTR, (b)3'UTR_a ,(c) 3'UTR_b, (d) 3'UTR_c, with control or miR-153 mimetics. Additional control or antisense morpholinos, (d) Nfib-mask_i, used to protect the predicted miR-153 binding sites inNfib 3'UTR were co-transfected into same samples as indicated. Data were normalized to the samples treated with the transfected control. The X-axis depicts treatment conditions. Y-axis indicates normalized luciferase activity. Data were expressed as mean +/- SEM. n=5.

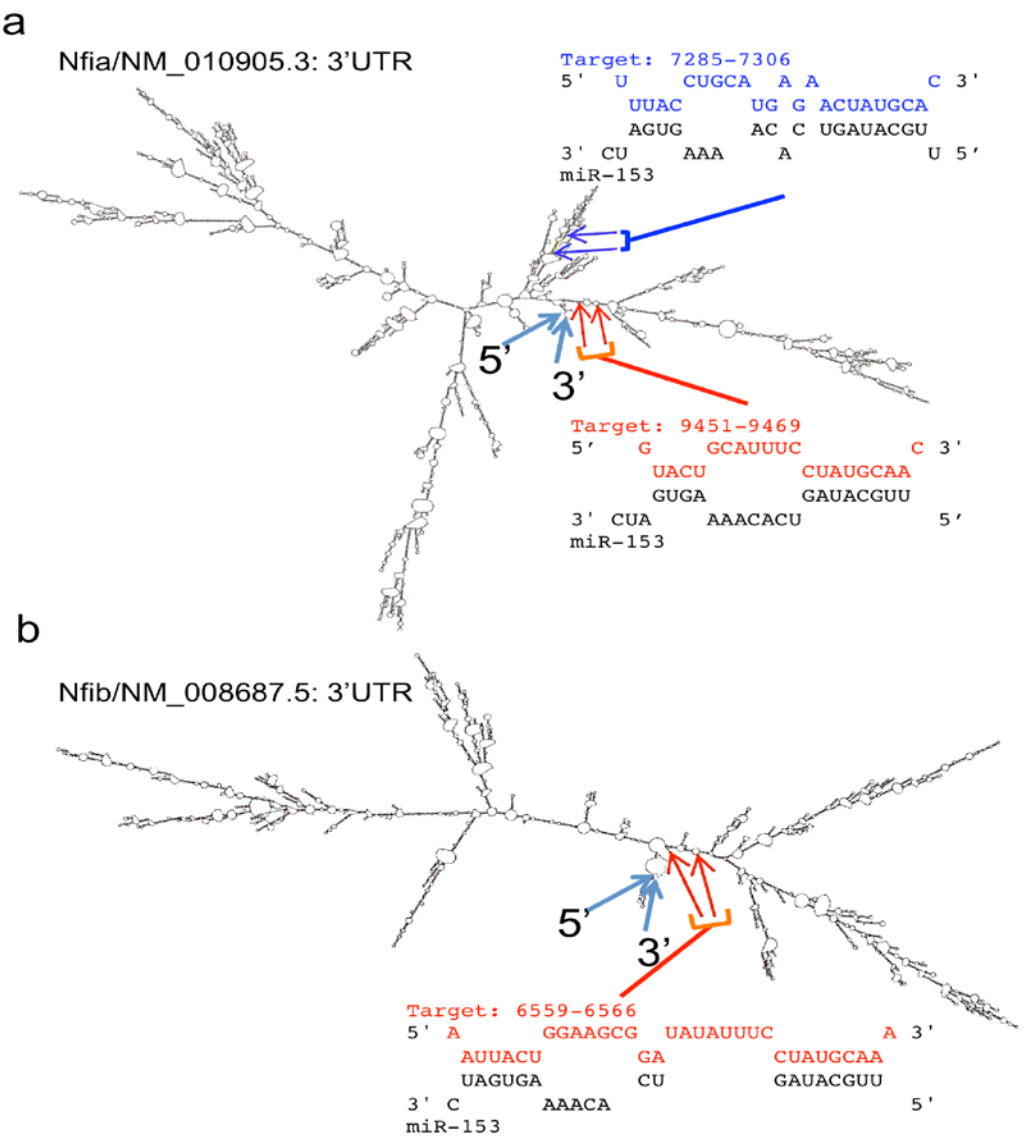


Figure 2-6. *In silico* analysis of the RNA folding structure of Nfia and Nfib. (a)(b) Predicted secondary structure conformation of the 3'UTR of (a)Nfia and (b)Nfib. Locations of the 5' and 3'ends of the 3'UTR sequences are marked with blue arrows. (a) Two miR-153 binding sites, target_7285 and target_9451, validated from luciferase assay above are shown on Nfia 3'UTR. Target_7285 is located close to complex stem loop structures, while target_9451 localizes to a linear portion, of the Nfia 3'UTR. MiR-153 sequences are shown in black while the matching binding site sequences are illustrated in blue (target_7285) and red (target_9451). (b) One miR-153 binding site, target_6559, validated from luciferase analysis is located on the linear portion of Nfib 3'UTR and is labeled in red, whereas miR-153 sequence is shown in black.

located within a region predicted to assume a more linearized structure, in close physical proximity to the 5' and 3'-ends of the 3'UTR (Figure 2-6b). These data indicate that miR-153 binding sites within Nfia and Nfib 3'UTRs are positioned to influence translation activity within the open reading frame. Neither Matn2 nor Vegfa 3'UTRs exhibited evidence for regulation by miR-153 (Figure 2-7), indicating that these were not direct miR-153 targets in NSCs.

MiR-153 modulates Nfia and Nfib expression in mouse fetal brains

Evidence for translational regulation by miR-153 acting at 3'UTRs suggested that Nfia and Nfib were direct miR-153 targets. We therefore further tested this association in an *in vivo* model. Pre-miR-153-GFP or control-GFP expression constructs were delivered to the telencephalic wall of GD13.5 fetuses by intrauterine injection under ultrasound guidance (Figure 2-8a) followed by electroporation. After 48 hours (GD15.5), fetuses were euthanized, fixed, and cryo-sectioned. The presence of anti-GFP immunofluorescence was used as a localization marker for cellular overexpression of miR-153.Following *in utero* electroporation with the control-GFP vector, GFP-immuno-reactivity was localized to the cytoplasm of cortical cells that also expressed nuclear Nfia and Nfib (e.g., Figure 2-8b-d) immuno-reactivity, indicating that the expression vector did not non-specifically interfere with target gene expression. In contrast, following *in utero* electroporation of the pre-miR-153/GFP construct, GFP immunopositive cells showed little-to-no immunofluorescence for either Nfia or Nfib,

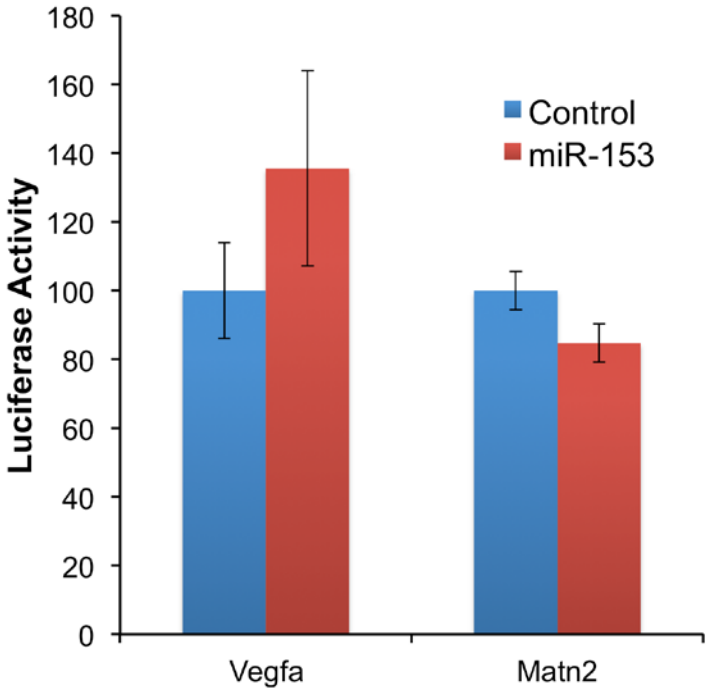


Figure 2-7. Matn2 and Vegfa are not direct targets of miR-153. Firefly activityrelative to RLuc luciferase activity was measured in NSCs 24 hours after transfection with control or miR-153 mimetics and the luciferase construct containing 3'UTR of Matn2 or Vegfa.Bars are normalized to therelative firefly units of samples treated with the transfected control. The X-axis depicts treatment conditions (control or miR-153). Y-axis indicates normalized luciferaseactivity. Data were expressed as mean +/- SEM. n=5.

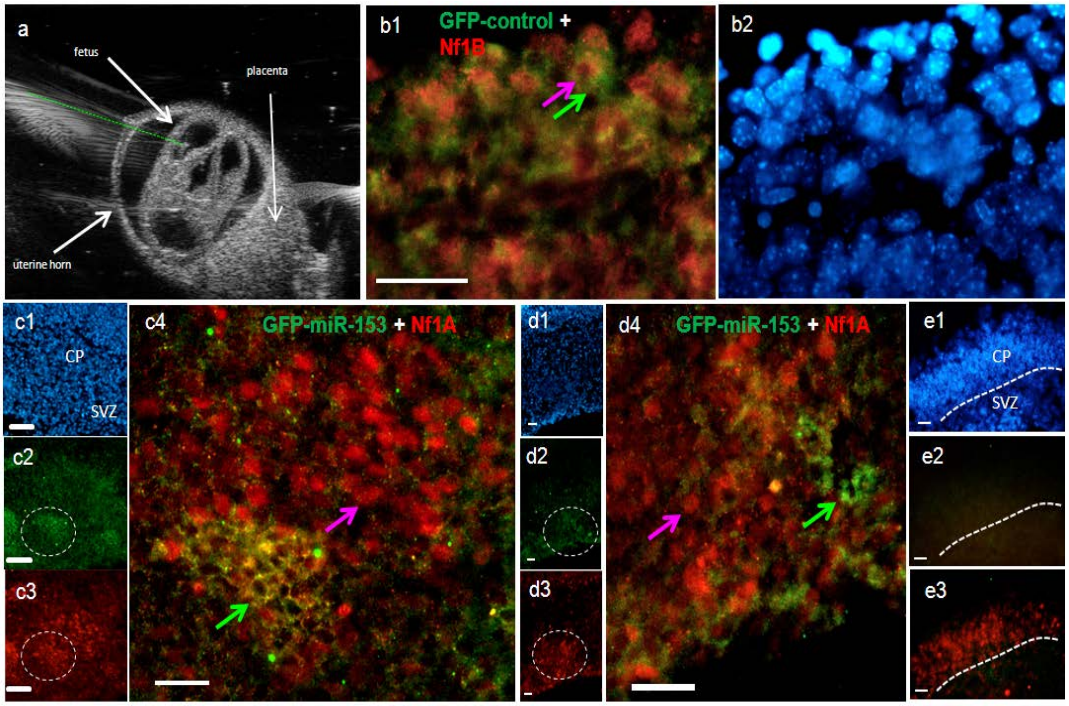


Figure 2-8. MiR-153 regulates Nfia and Nfib expression in fetal brains. (a) Photo-micrograph depicts ultrasound-guided trans-uterine insertion of a micro-capillary pipette (dashed green line)into the lateral ventricle in a GD13 fetal brain. Following in utero electroporation of control-GFP or miR-153/GFP vectors, fetuses were maintained for an additional period of 48 hours, before being analyzed at GD15.5. Double immunohistochemistry of anti-GFP with anti-Nfia or anti-Nfib in control-GFP (b) or Pre-miR-153-GFP (c-i) transfected GD15.5 mouse frozen sections. (b1,2) Photomicrograph shows (b1) control-GFP (green) localizes to the cytoplasm of nuclearNfib-labeled neurons of the cortical plate and (b2) DAPI-counterstained nuclei (c-e) c1-e1, c2-e2 and c3-e3 show low magnification images of the same sections counterstained with DAPI (c1-e1) to visualize nuclei or immuno-fluorescently labeled for GFP (c2-e2) to as a marker for miR-153 overexpression, or Nfia (c3-e3). (c4, d4) High magnification photomicrographs showing that GFP expression from the pre-miR-153/GFP construct does not co-localize with nuclear immuno-labeling for Nfia (c4, d4). (f-h) f1-h1, f2-h2 and f3-h3 show low magnification images of the same sections counterstained with DAPI (f1-h1) to visualize nuclei or immuno-fluorescently labeled for GFP (f2-h2) to as a marker for miR-153 overexpression, or Nfib (f3-h3). (f4, and g4) High magnification photomicrographs showing that GFP expression from the pre-miR-153/GFP construct does not co-localize with nuclear immuno-labeling for Nfib (f4,g4). Dotted circles indicate regions depicted in high magnification images. Dotted squares depict regions of cortical plate with disrupted expression of Nfib overlying strong GFP expression in the ventricular/sub-ventricular zones. Pink arrows show nucleiimmuno-stained for Nfia or Nfib, while green arrows indicate strong cytoplasmic miR-153-GFP immuno-staining.VZ: ventricular zone; SVZ: subventricular zone; CP: cortical plate. Scale bar, 25 μm.

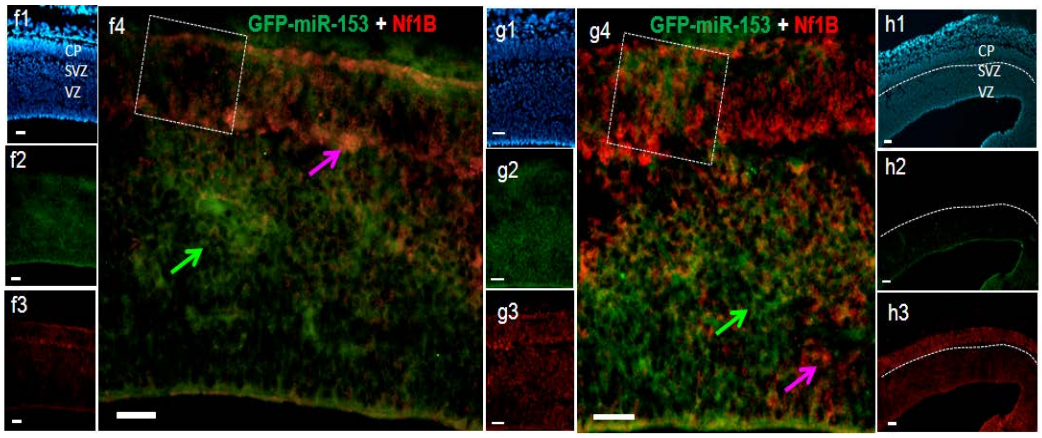


Figure 2-8 continued.

whereas adjacent GFP-negative cells exhibited strong nuclear localization of Nfia and Nfib immunoreactivity (Figures 2-8c1-4, d1-4, f1-4 and g1-4). In adjacent regions of brain tissue that exhibited little-to-no GFP immunofluorescence, the expression and laminar organization of Nfia and Nfib were undisturbed (Figures 2-8e1-3, h1-3). However, in regions where there was strong GFP immunofluorescence localized within the ventricular and sub-ventricular zones, expression of Nfia and Nfib in the overlying cortical plate was also disrupted (e.g., Figure 2-8f4), though GFP itself was not localized to the cortical plate. Finally, expression of miR-153/GFP in groups of ventricular zone cells appeared to result in compensatory Nfia and Nfib up-regulation in adjacent GFP-negative cells (e.g., Figure 2-8d4, g4, green vs. purple arrows), suggesting the presence of adaptive communication mechanisms between adjacent neural progenitors. These data show that *in vivo* overexpression of miR-153 results in disrupted translation from target Nfia and Nfib mRNA transcripts.

HDAC8 lacks a predicted miR-153 target site within its 3'UTR, but we examined its expression following miR153 overexpression because it is the earliest type-1 HDAC to be expressed during neurogenesis in the fetal murine telencephalon (Murko et al., 2010) and is implicated in the etiology of the Wilson-Turner X-linked (Harakalova et al., 2012) and Cornelia de Lange (Deardorff et al., 2012) syndromes, both of which are characterized by cognitive impairment. HDAC8-immunoreactivity was localized predominantly to the cytoplasm of cells within the VZ and SVZ, consistent with previously observed cytoplasmic localization in neural (Takase et al., 2013) and non-neural (Waltregny et al., 2004) tissues. Although its mRNA transcript was suppressed in

neurosphere cultures following miR-153 overexpression (Figure 2-2), *in vivo* expression of HDAC8-immunoreactivity was not altered by miR-153 overexpression (Figure 2-9a.i-iv). This discrepancy between mRNA and protein expression is consistent with recent literature which suggests that class I HDAC family members are stabilized by post-translational modification (Citro et al., 2013).

To further assess the role of miR-153 in neural differentiation, we examined the expression of a marker for early migrating and differentiating neurons (des Portes et al., 1998), doublecortin (DCX), which is not predicted to contain a miR-153 binding site in its 3'UTR, and is therefore unlikely to be a direct miR-153 target. Overexpression of miR-153/GFP within the SVZ coincided with loss of expression of DCX-like immunofluorescence (Figure 2-9c.i-iv *vs.*b.i-iv), indicating that miR-153 overexpression results in a loss of neuronal differentiation, consistent with predictions from *in vitro* experiments. We previously showed that ethanol exposure resulted in decreased expression of CD24, the neuronal lineage commitment marker, both *in vivo*, and in neurosphere cultures (Tingling et al., 2013). However, miR153 overexpression did not result in altered expression of CD24-immunofluorescence (Figure 2-9e.i-iv *vs.*2-9d.i-iv), indicating no effect on this ethanol-sensitive marker for neuronal-lineage committed neural precursors.

Ethanol disrupts the expression pattern of Nfia and Nfib in mouse fetal brains

We previously showed that ethanol exposure resulted in decreased miR-153 expression in NSCs (Sathyan et al., 2007; Balaraman et al., 2012). Since Nfia and Nfib

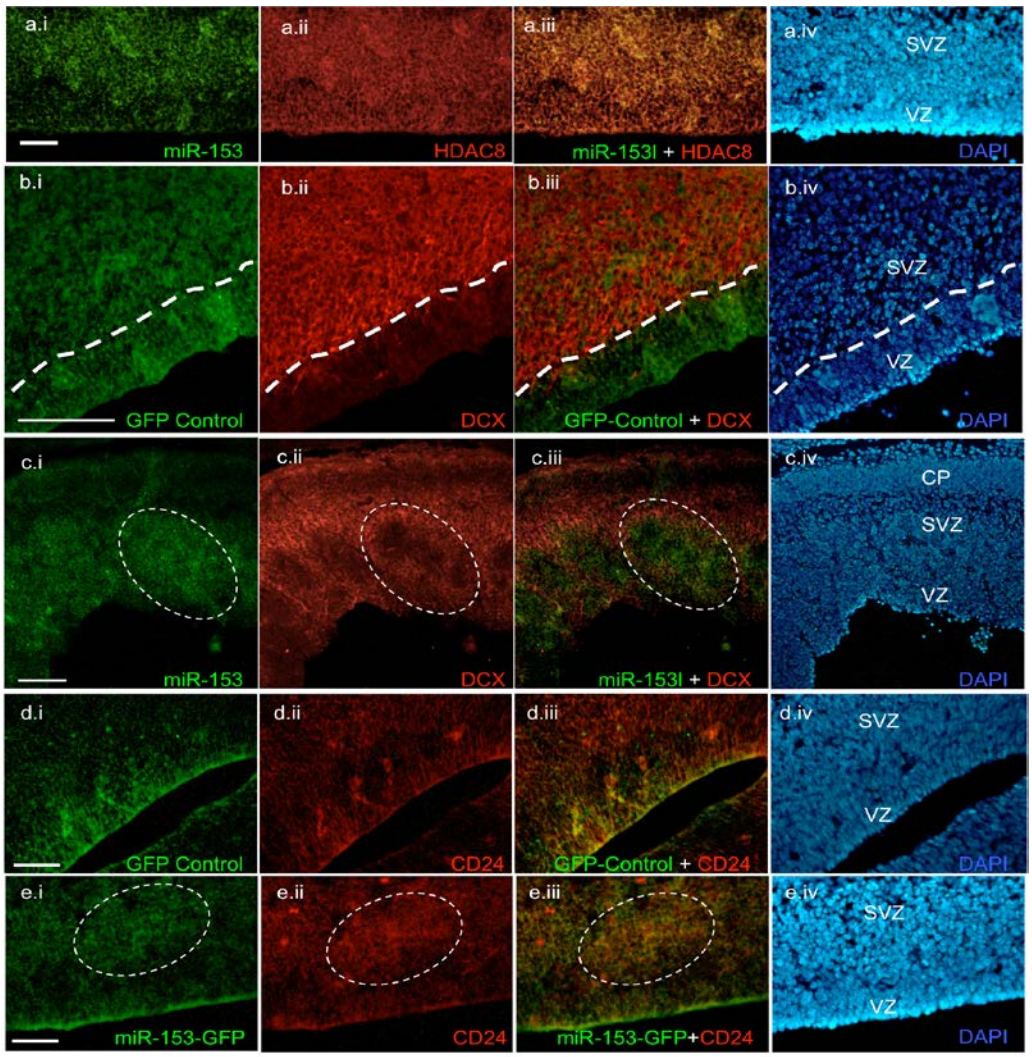


Figure 2-9. Relationship between miR-153 overexpression and the expression of the indirect miR-153 target, Hdac8, the neuronal differentiation marker DCX, and the neuronal lineage stem cell marker, CD24 in the cerebral cortical VZ and SVZ. In each row, panel 'i' depicts miR-153-GFP or control GFP expression, panel 'ii' depicts immunofluorescence for Hdac8, DCX or CD24, pame; 'iii' depicts combined immunofluorescence and panel 'iv' depicts DAPI labeling of cell nuclei. (a.i-a.iv) MiR-153 overexpression does not result in a loss of Hdac8 expression in the VZ or SVZ. (b-c) DCX-immunofluorescence is localized to the SVZ, but not VZ (b.ii and c.ii). Control-GFP overexpression (b.i-b.iv) does not alter DCX expression in the SVZ, however, miR-153 overexpression (c.i-c.iv, circled areas) results in loss of DCX expression in the SVZ. (d-e) CD24-immunofluorescence localizes to VZ and SVZ in GFP-control (d.i-d.iv) and following miR-153-overexpression (e.i-e.iv). miR-153 overexpression does not result in a loss of CD24 immunofluorescence (white circles). VZ: ventricular zone; SVZ: subventricular zone; CP: cortical plate. Scale bar, 50μm

were direct targets of miR-153, we hypothesized that exposure to ethanol in an *in vivo* model would result in an increased expression of these factors. Gestational day 12.5 (GD 12.5) pregnant mice were divided into a control and an ethanol-exposed group. Binge-like bolus of ethanol (3 g⁄kg b.wt) administration, twice daily, between GD12.5 and GD14.5, was performed by intragastric gavage on pregnant female mice as ethanol treated groups, to model ethanol exposure during the second-trimester equivalent period of neurogenesis. Control animals received saline at the same time intervals. Intragastric gavage of ethanol treated animals with this dose of ethanol resulted in a mean peak maternal blood ethanol concentrations (BEC) of 117 mg⁄dl, representing the level of binge-like intoxication attainable in nonalcoholic human populations. *In utero* ethanol exposure between GD12.5 and 14.5, i.e., during the first half of the second trimester equivalent period of cortical neurogenesis (Takahashi et al., 1995) results in an overall thinning of the cortical plate (Figure 2-10a *vs.*2-10b) accompanied by ventriculomegaly as previously described (Sudheendran et al., 2013). Immunofluorescence analysis showed that while control fetuses expressed Nfia and Nfib mainly within the cortical plate, ethanol-exposed fetuses exhibited a broader expansion of Nfia and Nfib immunoreactivity throughout the extent of the dorsal telencephalic wall, including within the ventricular zone (Figure 2-10c-j).

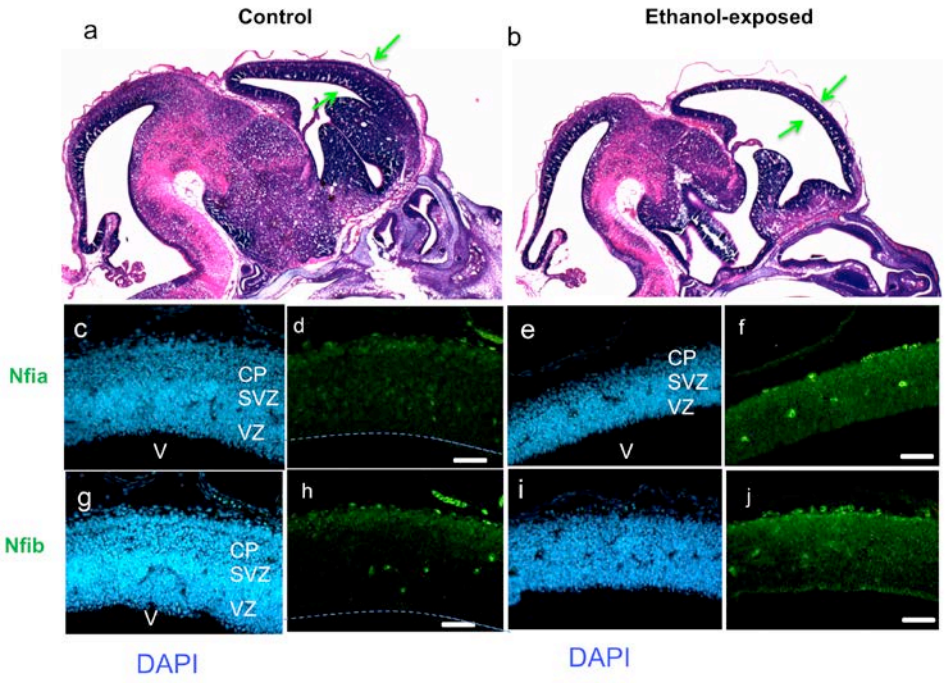


Figure 2-10. *In utero* ethanol exposure results in expanded Nfia and Nfib immuno-labeling in fetal brains. (a,b) Hematoxylin and eosin (H&E) staining of (a) control and (b) ethanol exposure mouse fetal brains. Ethanol exposure resulted in increased ventricular space and thinner dorsal telencephalon compared to control animals. The arrows indicate the thickness of the cortical plate.(c-j) DAPI-stained sections (c,e,g,i) and their corresponding immuno-fluorescence forNfia (d,f)or Nfib (h,j) in tissue sections obtained from control (left panel, c,d,g,h) and ethanol-exposed (right panel, e,f,i,j) fetal brains.Immuno-label for Nfia and Nfib are mainly localized in the developing cortical plate in control animals, but is spread through the VZ and SVZ in ethanol-exposed animals. Data obtained from four fetuses in each condition, each obtained from a separate pregnant dam. CP: cortical plate; SVZ: subventricular zone; VZ: ventricular zone; V: ventricular. Scale bar, 50 μm.

MiR-153 prevents and partly reverses effects of ethanol exposure on mRNA transcript levels

We next tested the prediction that because of its effect on miR-153, ethanol exposure would generally result in increased expression of miR-153-sensitive mRNA transcripts. We were also interested in examining the extent to which miR-153 could prevent or even reverse the effects of ethanol on NSCs. Neurosphere cultures were exposed to control medium or to ethanol, at a concentration (320mg/dl) that was previously shown to suppress miR-153 (Sathyan et al., 2007; Balaraman et al., 2012), and was within the range of blood alcohol content achievable in alcoholics (Adachi et al., 1991). To assess the capacity of miR-153 to *prevent* ethanol's effects (prevention paradigm), some ethanol-treated cultures were concurrently exposed to miR-153 by transient transfection. To assess the capacity of miR-153 to *reverse* the effects of prior ethanol exposure (reversal paradigm), other ethanol-treated cultures were exposed to miR-153 for 48 hours following a five-day period of ethanol exposure. Our data (Figure 2-11) showed a global (MANOVA, Pillai's Trace Statistic, $F_{(36,56)}=2.786$, $p<0.001$), as well as a transcript-specific effect of treatment on mRNA expression(Nfia (ANOVA, $F_{(4,19)}=41.555$, $p<0.001$), Nfib ($F_{(4,19)}=16.609$, $p<0.001$), Nfic ($F_{(4,19)}=13.722$, $p<0.001$), Ddit4($F_{(4,19)}=5.313$, $p<0.004$), Hdac8($F_{(4,19)}=17.715$, $p<0.001$), and Arl2bp ($F_{(4,19)}=14.9$, $p<0.001$)). Post-hoc analyses showed that ethanol exposure did result in increased expression of Nfia ($p<0.00003$), Nfib ($p<0.045$), Nfic ($p<0.0007$),Arl2bp ($p<0.0002$),Ddit4 ($p<0.044$), and Hdac8 ($p<0.0004$), and that simultaneous overexpression of miR-153 prevented the inductive effect of ethanol on these mRNA

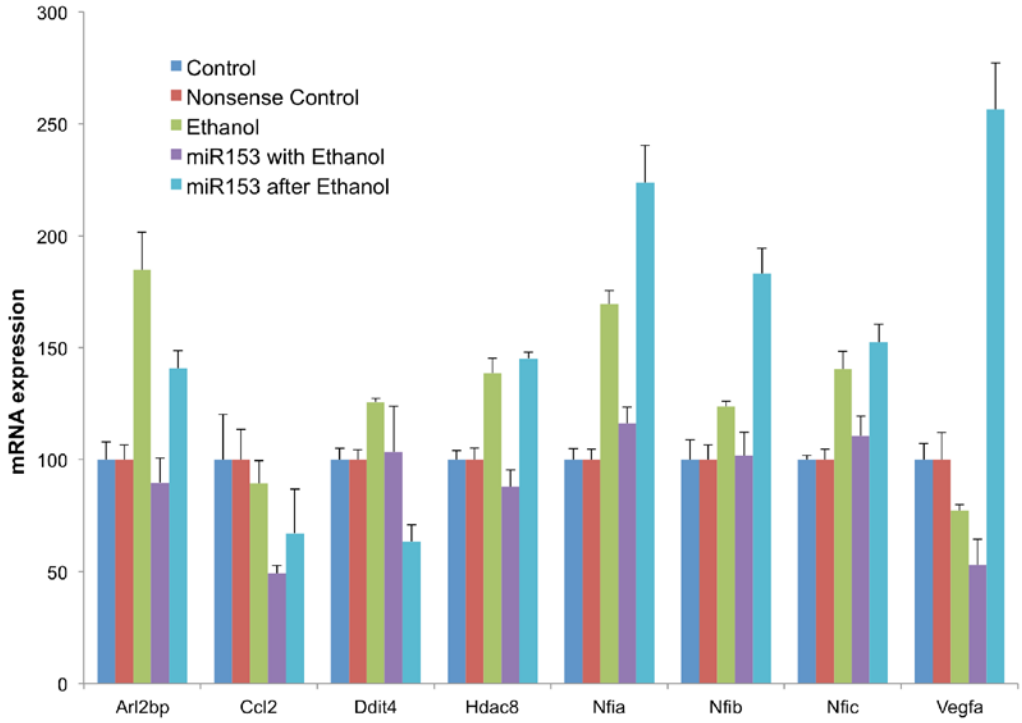


Figure 2-11. MiR-153 prevents and partly reverses ethanol's effectson miR-153-regulated gene transcripts. Bar graphs represent real-time RT-PCR analysis for mRNA expression of miR-153 sensitive genes in control neurosphere cultures (untreated or transfection control), ethanol (320 mg/dl) alone, miR-153-overexpression with ethanol exposure (prevention paradigm), or miR-153-overexpressionfor 48 hours after 5-days of ethanol exposure (reversal paradigm), of NSCs. Y-axis indicates normalized mRNA expression (normalized to 18s) relative to control samples. Data were expressed as mean +/- SEM. n=4 independent experiments.

transcripts (cultures administered miR-153 along with ethanol were not significantly different from controls). The increased expression of Nfia and Nfib mRNA following ethanol exposure validated the results from the *in vivo* exposure paradigm (Figure 2-10) and indicate that ethanol directly influences Nfia/b expression in fetal neuroepithelial cells, rather than indirectly, *via* altered maternal-fetal physiology.

Overexpression of miR-153 *after* an episode of ethanol exposure (the reversal paradigm), on the other hand, resulted in varied outcomes. In the case of Ddit4 and Arl2bp, overexpression of miR-153 after ethanol exposure, resulted in the predicted reversal of transcript expression to control levels ($p<0.0002$ and $p<0.027$ relative to ethanol exposure respectively). Nfic and Hdac8 mRNA expression was not reversed by subsequent miR-153 overexpression. However, overexpression of miR-153 after ethanol exposure resulted in a surprising and significant additional increase in Nfia, Nfib and Vegfa mRNA transcript levels relative to ethanol exposure alone ($p<0.0004$, $p<0.0002$, and $p<0.1E-09$, respectively). These data show that simultaneous exposure to miR-153 prevents ethanol induction of most miR-153 responsive transcripts, while sequential overexpression of miR-153 after ethanol exposure results in reversal of ethanol effects on some transcripts. However, sequential exposure to miR-153 following ethanol also unexpectedly resulted in an induction of transcripts (Nfia and Nfib) that are direct miR-153 targets.

The partial nicotinic agonist, varenicline, induces expression of miR-153

We previously reported that nicotine, acting at nAChRs, induced miR-153 expression and prevented the ethanol-mediated suppression of this miRNA in NSCs (Balaraman et al., 2012). We therefore re-tested the effect of nicotine on miR-153. Additionally, we tested the effect of varenicline, a partial nAChR agonist (Mihalak et al., 2006) and a Food and Drug Administration (FDA)-approved agent for smoking cessation (Hartmann-Boyce et al., 2013), because of recent evidence showing that it is also an effective treatment for alcohol use disorders (Steensland et al., 2007; Litten et al., 2013). Both nicotine and varenicline (each at 1.0μM for 5 days) resulted in a statistically significant induction of miR-153 expression in neurosphere cultures ($F_{(2,15)}$=5.621, $p<0.015$, Figure 2-12).

Varenicline decreases expression of miR-153-dependent mRNAs and prevents and reverses effects of ethanol

Based on the above data, we hypothesized that varenicline would prevent and reverse the effects of ethanol exposure on miR-153-regulated mRNAs. In the next series of experiments, we exposed neurosphere cultures to either control medium, to varenicline alone, to the 'prevention paradigm' (varenicline together with ethanol for 5 days), or to the 'reversal paradigm'(varenicline for 48 hours subsequent to a 5-day episode of ethanol exposure), and examined the regulation of miR-153 target gene transcripts. In all conditions, varenicline was administered at 1.0μM, and ethanol at a concentration of 320mg/dl. There was an overall significant effect (MANOVA, Pillai's Trace Statistic,

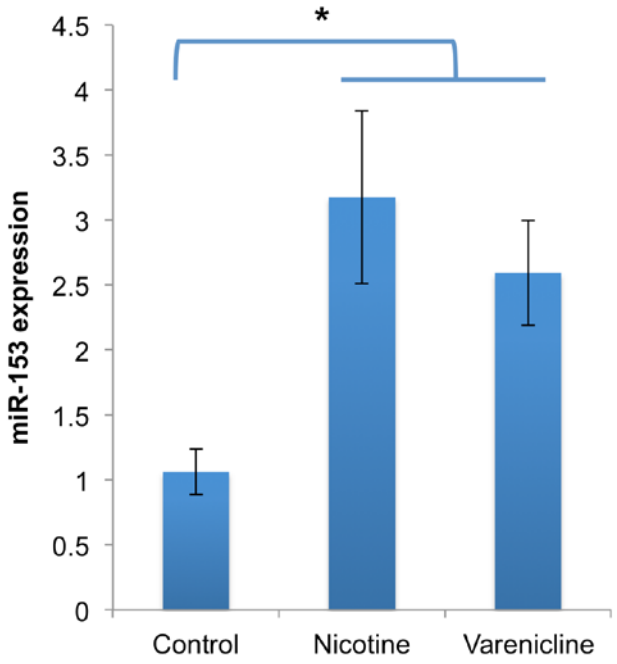


Figure 2-12. Nicotine and the nAChR partial agonist varenicline induce miR-153 expression. Bar graph depicts real-time RT-PCR expression of miR-153 in control, nicotine and varenicline-exposed neurosphere cultures. MiR-153 expression is significantly induced in nicotine and varenicline treated groups. Y-axis indicates normalized miR-153 expression (normalized to U6) relative to control samples. Data were expressed as mean +/- SEM. n=4 independent replicates.

$F_{(27,24)}$=2.36, p<0.018), as well as a transcript-specific effect of nicotinic activation on miR-153-regulated transcripts (ANOVAs, $F_{(3,14)Nfia}$=13.51, p<0.0002; $F_{(3,14)Nfib}$=6.946, p<0.004;$F_{(3,14)Nfic}$=16.1, p<8.15E-05; $F_{(3,14)Ddit4}$ =21.8,p<1.5E-05; $F_{(3,14)Hdac8}$=21.18, p<1.8E-05; $F_{(3,14)Arl2bp}$=3.55, p<0.04; $F_{(3,14)Vegfa}$=5.031, p<0.014; and $F_{(3,14)Ccl2}$=10.24, p<0.0008). Consistent with our finding that varenicline induced miR-153, we found that this partial nAChR agonist decreased expression of miR-153-target mRNA transcripts (*post hoc* fLSD relative to control**,** p_{Nfia}<0.0002, p_{Nfib}<0.003, p_{Nfic}<0.0001, p_{Ddit4}<0.008, p_{Hdac8}<0.0002, p_{Arl2bp}<0.037,p_{Vegfa}<0.02, and p_{Ccl2}<0.046, Figure 2-13). Moreover, in the 'prevention paradigm' (the presence of varenicline), ethanol exposure did not result in increased mRNA transcript expression (*post hoc* fLSD-relative to control, p_{Nfia}<0.0003, p_{Nfib}<0.003, p_{Nfic}<9.82E-05, p_{Ddit4}<1.8E-06, p_{Hdac8}<9.14E-06, p_{Arl2bp}<0.02, p_{Vegfa}<0.01, p_{Ccl2}<0.02, Figure 2-13). Finally, in the 'reversal paradigm', varenicline exposure subsequent to ethanol exposure prevented the ethanol-induced increase in all miR-153 target transcripts (Nfia, Nfib, Nfic, Hdac8, Arl2bp, and Vegfa, all p=n.s. (not significantly different), or Ddit4, decreased, p<0.0008, relative to controls). Ccl2 represented an exception. Varenicline exposure (like miR-153) was unable to reverse the increase in Ccl2 expression due to previous ethanol exposure (p<0.006 relative to controls). These data show that varenicline mimics the effects of miR-153, and like miR-153, behaves as a functional antagonist to ethanol. Moreover, varenicline is able to both prevent and largely reverse the effects of ethanol exposure.

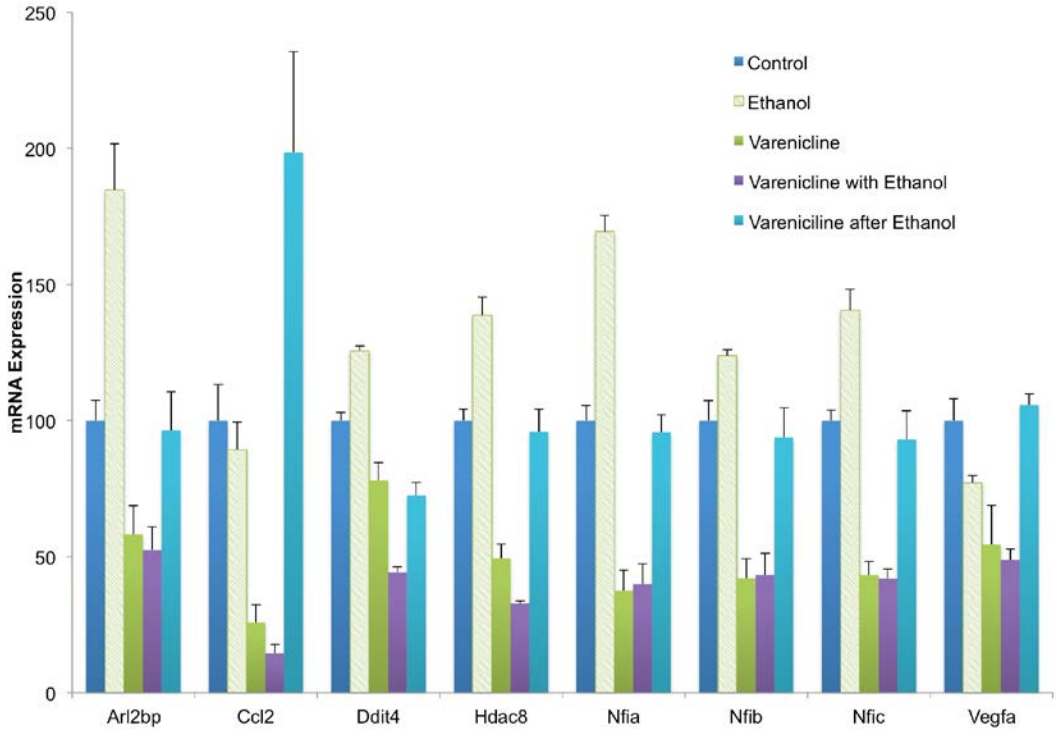


Figure 2-13. Varenicline prevents and reverses the effects of ethanol on miR-153 target gene expression. Bar graph depicts real-time RT-PCR analysis of mRNA expression of miR-153 regulated genes in control neurospheres, or neurospheres treated with varenicline (1μM) alone, varenicline in combination with ethanol (prevention paradigm), and varenicline treatment for 48 hours following5 days of ethanol exposure. The striped bars show reference ethanol exposure data from figure 9.Y-axis indicates normalized mRNA expression (normalized to 18S) relative to control samples. Data were expressed as mean +/- SEM. n=4 independent replicates.

Discussion

Teratogens like ethanol are an established cause of birth defects. We focused on the vulnerability of NSCs, based on a hypothesis that even small environment-induced perturbations in the biology of NSCs are likely to be amplified during the processes of NSC renewal and maturation, with significant consequences for brain development and maturation. Fetal NSCs produce neurons during the second trimester-equivalent period, before switching to gliogenesis during the third trimester-equivalent period (Miller and Gauthier, 2007). During this neurogenic period, ethanol exposure interferes with neuronal lineage commitment (Tingling et al., 2013), resulting in depletion of NSCs (Santillano et al., 2005; Tingling et al., 2013). Ethanol-sensitive miRNAs like miR-153 may mediate ethanol's teratogenic effects. For example, ethanol's effects in neurosphere cultures were mimicked by loss of miRNAs, including miR-153 (Sathyan et al., 2007), and in a zebrafish model, developmental miR-153 knockdown was shown to mimic ethanol's effects on both craniofacial development and behavior (Tal et al., 2012). The question that arises is, "what miR-153-regulated transcriptome networks explain NSC vulnerability to teratogens?" Microarray and gene ontology analysis shows that miR-153 overexpression represses cell-signaling pathways including GPCR pathways, which control cell morphology, polarity, migration, proliferation and differentiation (Kobayashi et al., 2010; Callihan et al., 2011), functions that are relevant to NSC maturation. Collectively, regulated ontology categories were broadly associated with mature neural function, suggesting that miR-153 served as a repressor of neuronal differentiation in NSCs. These implicated ontology categories are consistent with our observations that

miR-153-overexpressing cells exhibit deficient morphological transformation when cultured in a mitogen-withdrawal differentiation paradigm. We also observed that miR-153 overexpression within the SVZ coincided with loss of DCX immuno-labeling, without loss of CD24 expression, suggesting that miR-153 does not interfere with neuronal lineage commitment of VZ and SVZ precursors, but does interfere with subsequent neuronal differentiation. MiR-153 overexpression did not however alter cell survival or proliferation. These data are collectively consistent with recent evidence that miR-153 prevents the development and maturation of motor neurons (Wei et al., 2013), and serves as a translational repressor for synaptic and signaling proteins that are important for neuronal maturation and function (Doxakis, 2010; Long et al., 2012; Wei et al., 2013). While current analysis focused on miR-153-repressed transcripts, potential direct effects on induced transcripts cannot be discounted. MiRNA interactions with promoter regions of mRNA coding genes reportedly result in increased gene transcription (Place et al., 2008), and presumptive binding sites on lncRNAs serve as a mechanism for regulating miRNA function (Hansen et al., 2013). Therefore, potential interactions of miR-153 with induced mRNAs and lncRNAs need further assessment.

The effects of miR-153 are likely to be mediated by a large network of directly and indirectly regulated genes. This network includes the nuclear factor-1 family, Nfia, Nfib, and Nfic,that were all suppressed by miR-153 in neurosphere cultures. Moreover, *in utero* overexpression of pre-miR-153 coincided with loss of nuclear Nfia/Nfib immunofluorescence, and miR-153 overexpression in the ventricular zone (VZ) resulted in disrupted Nfia/Nfib expression in the overlying cortical plate, suggesting that miR-

153 regulates the Nf1 family in the developing brain. Our data collectively show that members of the Nf1 family are specific, direct targets of miR-153. Both Nfia and Nfib possess extended 3'UTRs, which are predicted to assume complex, branched stem-loop structures, and are likely, as with other long 3'UTRs (Irier et al., 2009), to serve as a focus for regulated, context-dependent translation. In each of these transcripts, experimental analysis showed the presence of specific, active miR-153 binding sites that, based on the predicted folding of the 3'UTRs, are in close proximity to both 5' and 3'-termini of the 3'UTRs, positioning these sites to influence translation. Interestingly, other equally strong predicted 3'UTR target sites were found to be non-functional, perhaps because they are masked in NSCs. However, a role for these cryptic sites at other stages of neuronal differentiation cannot be discounted.

Since developmental exposure to ethanol suppressed miR-153 expression, we predicted that *in utero* ethanol exposure would result in increased expression of Nfia and Nfib. Our data from both *in vivo* and neurosphere models shows that this is indeed the case. *In vivo*, in control animals, at GD15, Nfia and Nfib expression were mainly localized to the emerging cortical plate consistent with the published literature (Plachez et al., 2008). However, following episodes of ethanol exposure, Nfia and Nfib-like immunofluorescence could be observed throughout the sub-ventricular and ventricular zones in addition to the cortical plate. This dysregulated pattern of expression is certainly interpretable as indicating general exposure-induced delay in fetal development. However, these data were validated in the neurosphere culture model where ethanol

exposure resulted in increased expression of Nf1 family transcripts, supporting a direct relationship between exposure and target gene expression.

While little is known about mR-153, an extensive literature documents the developmental role of the Nf1 family. During the neurogenesis period, Nfia and Nfib are dynamically expressed in differentiating cortical plate neurons (Plachez et al., 2008). Nfia expression in neuronal lineage-committed precursors and in young neurons promotes their neural differentiation, but decreases self-renewal capacity of the remaining NSCs and causes them to switch from neurogenesis to gliogenesis (Deneen et al., 2006; Barry et al., 2008; Namihira et al., 2009; Piper et al., 2010; Wang et al., 2010). Ethanol is a complex teratogen, and its effects are likely to be broader than those mediated by a single miRNA and its target mRNA transcripts. For example, miR-153 did not influence proliferation in cultured neuroepithelial cells and did not result in altered expression of CD24+ neuronal lineage committed precursors. In contrast ethanol did induce neuroepithelial proliferation (Santillano et al., 2005), an effect mediated by miR-335 (Sathyan et al., 2007), and did result in loss of the CD24+ population (Tingling et al., 2013). However, since the Nf1 family serves as a feedback inhibitor for NSC self-renewal (Piper et al., 2010), these data advance an explanation for the observed loss of NSCs (Santillano et al., 2005; Tingling et al., 2013), following ethanol exposure.

An important question is, “Can we progress beyond the diagnosis of teratology towards developing intervention strategies to prevent or even reverse fetal growth defects?” Education strategies aimed at limiting maternal alcohol consumption have been modestly successful at best, since the incidence of FAS in the United States has

remained constant (0.05-0.2% of live births (Bertrand et al., 2005)) over the 40-year period since the syndrome was first described, while in countries like South Africa the prevalence rate is reportedly as high as 9% (May et al., 2013). Therefore alternate approaches, including biomedical interventional approaches designed to directly influence fetal development, need further investigation. In this context, the published literature implicating miRNAs like miR-153 as mediators of teratogenesis (Sathyan et al., 2007; Tal et al., 2012) is important, because miRNAs can be manipulated to promote neuro-protection *after* the onset of neuro-trauma (Selvamani et al., 2012). We therefore asked whether simultaneous overexpression of miR-153 *prevented*, and sequential overexpression *reversed* ethanol's effects on target genes. Our data indicate that simultaneous overexpression of miR-153 effectively prevents ethanol-mediated induction of miR-153 target genes. Moreover, miR-153 overexpression following ethanol exposure did reverse the effect of ethanol on some genes, i.e., Arl2bp and Ddit4. Surprisingly, and contrary to our prediction, miR153 overexpression following ethanol exposure resulted in significantly increased transcript levels of Nfia and Nfib, even though these transcripts are direct targets of miR-153. The mechanism for this up-regulation is unknown. It is possible that the post-ethanol inductive effect of miR-153 on Nfia and Nfib is indirect, i.e., due to repression of an intermediate regulatory factor. However, collectively, these data provide the first promising evidence that miRNA manipulation successfully prevents, and even partially reverses a teratogen's effects on NSCs.

We next assessed the possibility that pharmacological interventions could prevent the inductive effects of ethanol on miR-153 target genes. We replicated our previously published observations (Balaraman et al., 2012) that low-dose nicotine exposure (at 1μM) resulted in increased miR-153, and moreover, found that low-dose exposure to the partial nAChR agonist, varenicline also induced miR-153 expression. Exposure to varenicline along with ethanol prevented the ethanol induction of miR-153 target mRNAs. Importantly, varenicline administration *subsequent to* ethanol exposure, reversed the ethanol-induction of nearly all of the assessed miR-153 regulated transcripts, including importantly, Nfia/b/c. Varenicline, is not only a clinically effective, FDA-approved, smoking-cessation agent (Hartmann-Boyce et al., 2013), but has been found to be effective in reducing alcohol craving in preclinical (Steensland et al., 2007) and human studies (Litten et al., 2013). Therefore, an agent that provides benefit to the mother by mitigating drug-seeking behavior, also effectively mimics miR-153 in preventing and reversing the effect of a teratogen on fetal NSCs.

Our data collectively show, in pre-clinical models, the translational potential for miRNAs, as a means to therapeutically intervene in fetal development. Pharmacological approaches to miRNA manipulation, particularly those involving nicotinic receptor activation are certainly controversial, in light of the long-documented teratogenic effects of fetal nicotine exposure (Nishimura and Nakai, 1958). However, successful pharmacological interventions to reverse developmental defects are likely to entail using agents that are themselves teratogenic. Precedence for using a teratogen to mitigate developmental defects comes from data showing that the alkaloid teratogen and

hedgehog pathway antagonist, cyclopamine, which causes craniofacial defects in fetal mice (Lipinski et al., 2008), was nevertheless able, at sub-teratogenic doses, to partly rescue craniofacial defects due to genetic ablation of the transcription factor, SP8 (Kasberg et al., 2013). With careful attention to factors like dose and timing of exposure, pharmacological manipulation of miRNAs and their target gene networks may well be a feasible approach to prevent and perhaps even reverse fetal damage following teratogen exposure.

CHAPTER III

MIR-335, ETHANOL SENSITIVE MICRORNA, PREVENTS NSC/NPC MATURATION BY REGULATING CELL PROLIFERATION AND DIFFERENTIATION GENES

Overview

MiR335, one of the ethanol sensitive microRNAs, is an evolutionarily mammal conserved intronic microRNA. In this study, we reveal that miR335 acts as a molecular brake to prevent neuron stem cell maturation and potentially mediates ethanol's teratology. *In situ hybridization* of miR-335 expression in different demyelination mouse models suggests that miR-335 is not highly involved in the regulation of the central nervous system during later developmental stages. 3'UTR analysis demonstrates that miR-335 targets fetal stem/progenitor cell (NSCs/NPCs) markers DCX, NeuroD1, and c-Kit; these results are further supported by the *in utero injection* mouse model. In addition, we first generate miR-335 transgenic mice, and, surprisingly, constitutive deletion of miR-335 results in homozygous lethality, suggesting the importance of this microRNA during early fetal development. MiR-335$^{(+/-)}$ mutants exhibit neuronal pre-maturation through increasing asymmetric cell divisions, driving neuron early differentiation by inducing stem cell genes DCX and NeuroD1 in the developing cortex. These data collectively provide strong evidence for a role of miR-335 in regulating NSC/NPC markers, preventing NSC/NPC pre-maturation, and potentially mediating the effects of alcohol through promoting NSC/NPC proliferation and differentiation.

Introduction

Maternal alcohol consumption during pregnancy causes permanent birth defects, including fetal craniofacial, cardiovascular, skeletal, and neurological deficits collectively termed the fetal alcohol spectrum disorder (FASD) (Jones and Smith, 1973; Lemoine et al., 2003; Sokol et al., 2003). Because of a various range of effects due to alcohol exposure, FASD includes several subtypes such as fetal alcohol syndrome, partial fetal alcohol syndrome (pFAS), alcohol-related neurodevelopmental disorder (ARND), alcohol-related birth defects (ARBD), and fetal alcohol effect (FAE) (Riley et al., 2011). There are several key features of FASD, such as growth retardation, facial deficits, damage of the nervous system, and cognitive and behavioral malfunctions in childhood (Jones and Smith, 1973; Lemoine et al., 2003; Sokol et al., 2003). Central nervous system (CNS) damage is the primary feature of any FASD diagnosis. For example, ethanol exposure during pregnancy causes a variety of fetal brain defects, such as microencephaly, the loss of the corpus callosum, and heterotopias formation (Jones and Smith, 1973; Clarren and Smith, 1978) that eventually lead to structural, functional, and neurological impairments. However, the molecular mechanisms of FASD in CNS underlying ethanol teratology are ultimately complicated and varied because of disruption of large gene networks. The end of the first trimester and the second trimester-equivalent period are especially susceptible to ethanol teratology, because neuronal stem/progenitor cells (NSCs/NPCs) generate most of neurons to form the mature brain during this time period (Bystron et al., 2008). Therefore, fetal ethanol exposure during this vulnerable time window can disrupt the NSCs/NPCs maintenance

and lead to the smaller brain size by decreasing the cell proliferation rate within the ventricular zone (VZ) (Miller, 1989a; Miller and Nowakowski, 1991). In addition, alcohol exposure also disrupts the emerging cortical plate's laminar organization (Kotkoskie and Norton, 1988) and results in the formation of subpial heterotopias found in the brains of FASD children via promotion of abnormal neuronal migration (Mooney et al., 2004).

Previous evidence in a mouse fetal NSCs/NPCs model from our laboratory has showed that dysregulation of ethanol-sensitive miRNAs, including miR-335, affected NSCs/NPCs maintenance by promoting NSCs/NPCs proliferation (Sathyan et al., 2007). These results suggested that miR-335 could potentially mediate ethanol's effects on stem cell maturation. As with miR-153, miR-335 is an evolutionarily mammal conserved intronic microRNA that is located within the second intron of a maternally imprinted gene, mesoderm specific transcripts (MEST). Disruption of the MEST locus has been associated with a fetal growth retardation syndrome named the Russell-Silver Syndrome (Kobayashi et al., 1997) that results from an imprinting error inherited from the mother. In addition, overexpression of miR-335 results in inhibition of cell proliferation and migration in human mesenchymal stem cells (hMSCs) (Tome et al., 2011), and causes reduction of the capacity of mouse embryonic stem cell self-renewal through promoting cell differentiation by targeting cell self-renewal factors Oct4 and pRb on the post-transcriptional level (Schoeftner et al., 2012). These reports collectively suggested that miR-335 is critical for stem cell maturation during development. Mouse mRNA microarray screening using an *ex vivo* neurosphere model from our laboratory has shown

that miR-335 controls the expansion of NSCs/NPCs by potentially regulating stem cell markers. However, the role of miR-335 that alters the fates of NSCs/NPCs is still unclear. Here we hypothesized that miR-335, a mammalian-specific miRNA, regulates NSCs/NPCs maintenance by targeting stem cell markers DCX, NeuroD1 and c-Kit[1].

Materials and methods

Isolation and culturing of mouse cortical neural precursors

All animal procedures were approved by the University Laboratory Animal Care Committee. C57BL/6 timed pregnant mice obtained from Harlan Laboratories (Houston, TX) were housed in Texas A&M Health Science Center. Cortical neuroepithelial tissues from the dorsal telecephalic vesicles of mouse fetuses at gestational day 12.5 were isolated (GD 0 was defined as the day the dams were sperm-positive), and the adjacent meningeal tissues such as hippocampus and the underlying striatum tissue precursors were removed carefully. About 1~2 millions of neural precursors isolated from the cortical tissues were cultured in the constituted medium, including serum-free mitogenic media DMEM/F12 (#11330-032; Life Technologies, CA), 20 ng/ml bFGF (basic fibroblast growth factor; #354060, BD Biosciences, CA), 20 ng/ml hEGF (human epidermal growth factor; #53003-018, Life Technologies, CA), 20 nM progesterone (# P6149, Sigma, MO), 1% ITS-X (insulin-transferrin-selenium-X; #51500- 056 Life Technologies, CA), 0.15 ng/ml LIF (leukemia inhibitory factor; #L200 Alomone Labs),

[1]These genes were selected from a microarray analysis of miR335 responsive genes, conducted by Dr. Pratheesh Sathyan, in part fulfillment of the requirements for his PhD dissertation.

0.85 Us/ml heparin (#15077-019, Life Technologies, CA). Neural progenitors were cultured as non-adherent neurosphere aggregates following with previously published protocols (Santillano et al., 2005; Camarillo et al., 2007; Prock and Miranda, 2007). Neurospheres were mechanically dissociated into single cells every two or three days to prevent large aggregates.

Generation of a miR-335 mouse knockout model

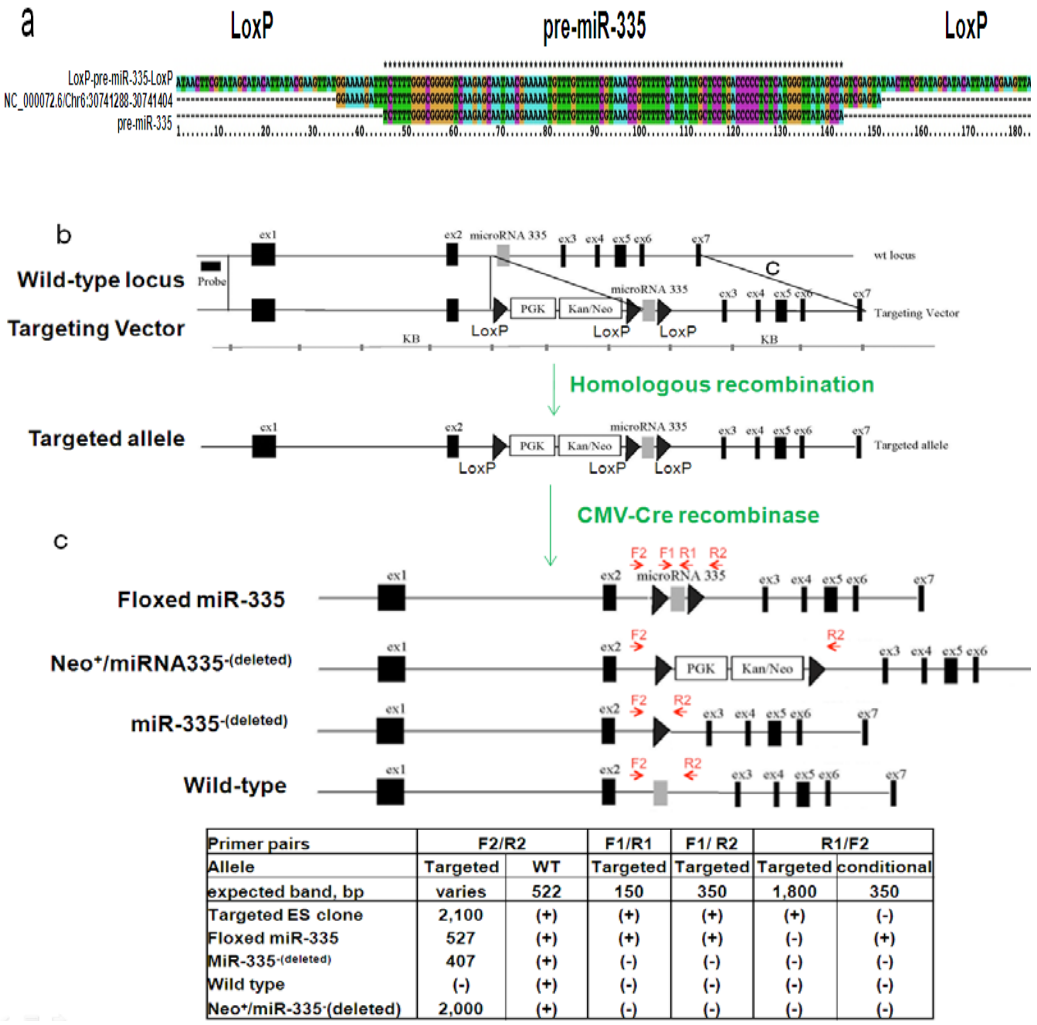
The miR-335 conditional knockout construct was generated at the Texas Institute for Genomic Medicine (TIGM) at Texas A&M University. All animal procedures were approved by the University Laboratory Animal Care Committee.

Generation of mice containing a floxed miR-335 allele: a double-stranded, 194bp DNA fragment with engineered cloning sites and two flanking loxP sites, bracketing a 116nt MEST intronic DNA fragment overlapping the 98nt pre-miR335 region of mouse MEST locus (HindIII-LoxP-pre-miR335-LoxP-Sal1), was synthesized commercially and sub-cloned into pBS (Invitrogen). A LoxP -PGK promoter - Neomycin transferase - polyA cassette was additionally cloned into the targeting vector. For the 5' arm, a 3955bp PCR product was amplified using primers 5'-CAGTTTTGTCCGCACCTCTA, and 5'-TGGCACCTATCTCCAAATGC, and 129S5/SvEvBrd genomic DNA, subcloned into PCR-Script (Stratagene) and then cloned into the targeting vector. For the 3' arm, a 2962 bp PCR product was amplified using primers 5'-CCCAGACTTGATATTCAGTGTT and 5'-TTTTGGAGAAGGA GAGGACG and 129/SvEv genomic DNA, subcloned into PCR-Script and then cloned into the targeting

vector (Figure 3-1b). All vector components were sequence-verified. The targeting vector was electroporated into 129S5/SvEvBrd ES cells (Lex2 line provided by Lexicon Pharmaceuticals, Inc.). A total of 200 clones were screened by Southern blot analysis using a probe to the 5' end of the targeting construct and BamHI enzyme to digest genomic DNA (wild type band: 13 kbp; targeted band: 9 kbp). Four targeted clones were identified: D12, H1, E2 and F12 (Figure 3-1d). The identity of the clones was further confirmed by 3' and 5' long distance PCR followed by sequencing of the products and Taqman-based transgene copy number analysis. As a result, 3 clones (H1, E2 and F12) were confirmed as carrying the correct mutation. ES cell clones H1 and F12 were injected into blastocysts isolated from 3.5dpc albino C57BL/6 donors, several high % male chimeras were obtained and bred with albino C57BL/6 females. The resulting germline mice were confirmed by genotyping with primers 5'-GCATACATTATACGAAGTTATGGAAAA and 5'-GCCAACAGTACTGGATT GAAA (wild type band: none, targeted band: 350 bp). Germ-line mice were obtained from both clones, but only the progeny from clone H1 was used in further studies. The resulting mice were in a mixed C57BL/6J x 129/SvEv genetic background.

Removal of the selection cassette and production of the miRN335 floxed and null alleles: the B6.C-Tg(CMV-cre)1Cgn/J mouse line carrying Cre recombinase under control of weak CMV promoter was obtained from Jackson Laboratories (Bar Harbor, Maine). A low level of Cre expression in this allele resulted in incomplete excision of the floxed cassettes and produces several genetic outcomes (Figure 3-1c). The heterozygous mice carrying the targeted mutation were mated with the latter and all

offspring was genotyped according to the schema shown in Figure 1 using these primers: F2: 5'-CCATTCCCAAATTCATGCAC, R2: 5'-GCCAACAGTA CTGGATTGAAA, F1: 5'-GCATACATTATACGAAGTTATGGAAAA, and R1: 5'-TGCTA TACGAAGTTATA CTCGACTGG. Only mice carrying the deleted and floxed miRN335 alleles were retained for further studies. Following a series of matings, two breading pairs were established for each allele. After several litters have been produced, and genotyped, no omozygous mice were recovered for either the deleted or the floxed miR335 allele (Figure 3-1c). These data indicate that constitutive deletion of the pre-miR335 locus results in homozygous lethality. In the case of the floxed miR-335 allele, because of the size constraints of the intronic region containing miR-335, it is likely that LoxP sites were positioned too close to the miRNA and interfered with posttranscriptional miRNA processing. To further assess the source of embryo lethality, deleted miR335 heterozygous mice pairs were timed mated, embryos flushed from the uterine horns at GD3.5 and genotyped. In this analysis we identified one instance of a homozygous miR335 deletion mutant (Figure 3-1e, g). No homozygous mutant mice were obtained at any later assessed time points (Figure 3-1f, g). This suggests that the miR335 homozygous deletion mutant is embryonically lethal prior to implantation. Heterozygous miR335 knockout mice bred well with the average litter size of 7 and were used for subsequent analyses, and compared to wild-type littermates from the same genetic background.



Primer pairs	F2/R2		F1/R1	F1/ R2	R1/F2	
Allele	Targeted	WT	Targeted	Targeted	Targeted	conditional
expected band, bp	varies	522	150	350	1,800	350
Targeted ES clone	2,100	(+)	(+)	(+)	(+)	(-)
Floxed miR-335	527	(+)	(+)	(+)	(-)	(+)
MiR-335-(deleted)	407	(+)	(-)	(-)	(-)	(-)
Wild type	(-)	(+)	(-)	(-)	(-)	(-)
Neo+/miR-335-(deleted)	2,000	(+)	(-)	(-)	(-)	(-)

Figure 3-1. Experimental designs to obtain floxed and deleted miR-335 alleles. (a) The schematic shows the sequence alignment of LoxP-miR335-LoxP, pre-miR335 loci on chromosome 6, and pre-miR335 (b) The schematic illustrates that LoxP-miR335- LoxP cassettes were cloned into the targeting vector and floxed miR335 allele was obtained via homologous recombination in order to create conditional transgenic mice by Lox-Cre recombination system. (c) Mouse line carrying Cre-recombinase under control of weak CMV promoter was used to cross with floxed miR335 allele to create various genetic outcomes. Two pairs of primers were used for genotyping and only mice with floxed or deleted miR-335 alleles were kept. Primer sets: (Mest screen) F2: ccattcccaaattcatgcac; (Mest screen) R2: gccaacagtactggattgaaa; (L-miR335-L) F1: gcatacattatacgaagttatggaaaa; (L-miR335-L) R1: tgctatacgaagttatactcgactgg. PGK: phosphoglycerate kinase I promoter; Kan/Neo: kanamycin/neomycin resistance gene; ex: exon. (d) The Southern blot shows the target clones containing Loxp sites (D12, H1) identified by BamHI enzyme digestion after homologous recombination. (e)(f) The images depict PCR results of genotypes of offspring of deleted miR-335 alleles using specific primer sets at ED3.5 (e) and ED 10.5 (f). The potential deleted miR-335 homozygous allele was found with more 407 bp PCR products at ED3.5, while no homozygous mutants were observed at ED10.5. (g) The pie chart illustrates the percentage of miR-335 genotypes. Fourteen percent of miR-335 homozygous alleles were identified at ED 3.5, while only one percent of miR-335 homozygous alleles were found among 142 fetuses from ED3.5 to P1 stages.

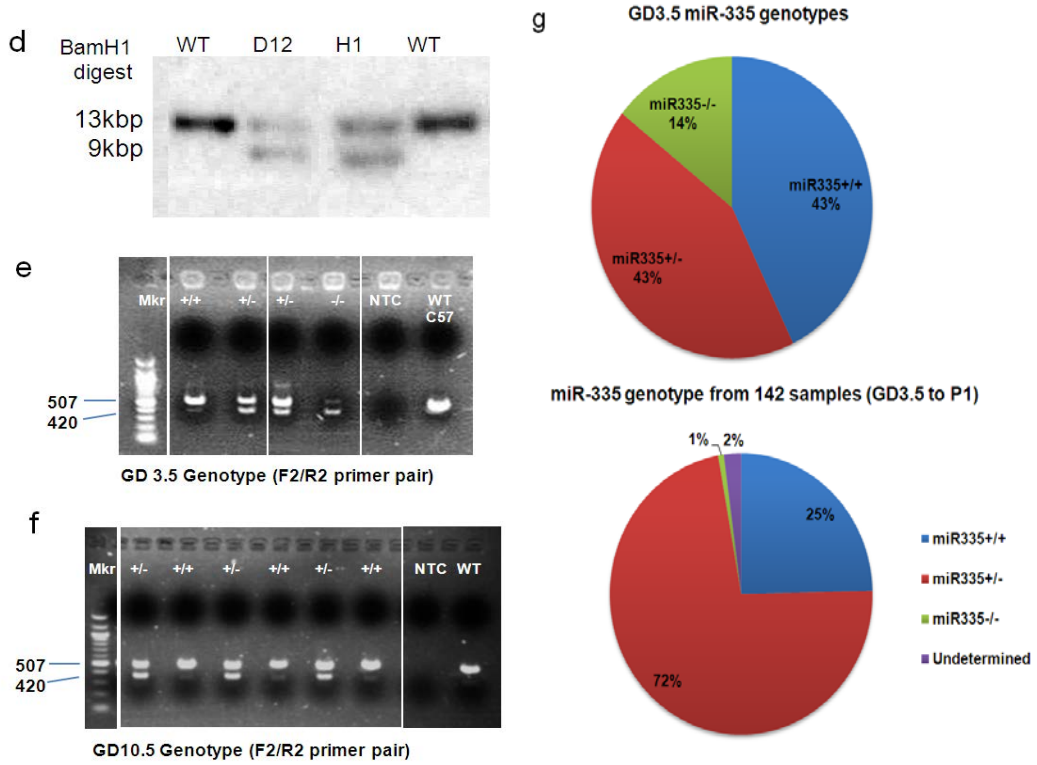


Figure 3-1 continued.

3'UTR analysis of miR-335 candidate genes

3'UTR luciferase constructs obtained from GeneCopoeia (GeneCopoeia, MD) were amplified in bacteria following with extracting and purifying using the EndoFree plasmid Maxi kit (#12362; Qiagen, CA) according to manufacturer's protocol. Purified plasmids were quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, MA). Neural precursors were grown to assigned density, around 1~2 million cells, before performing transfection. Briefly, 100 ng of *mmu*-miR-335 mimetic (miRNASelect™ pEGP-mmu-mir-335 Expression Vector, Cell Biolabs, CA) or control (miRNASelect™ pEGP-miR Null Control Vector, Cell Biolabs, CA), 150 ng of individual 3'UTR clone (Dcx (#MmiT029004; GeneCopoeia, MD), CD117/c-Kit (#MmiT032441; GeneCopoeia, MD), Pdgfra (#MmiT026421; GeneCopoeia, MD), NeuroD1(#MmiT029761; GeneCopoeia, MD)) in combination with 10 um of control or designed morpholinos (used as binding site protectors of miR-335 candidate genes) obtained from Gene Tools (Gene Tools, OR) were co- transfected into 20,000 cells respectively using a NEON electroporator (Life Technology, CA) according to manufacturer's suggestion. Transfected samples were seeded into a 96 well plate format and kept in an incubator at 37^0C. To determine the luciferase intensity, Dual-Glo® luciferase assay kits (#E2920; Promega, WI) were performed after 24 hours post-transfection, and luciferase signal was determined using the Synergy 2 multi-mode microplate reader (BioTek, VT). Relative firefly luciferase intensity from each sample set was normalized to internal control Renilla luciferase, respectively.

In situ hybridization

To determine the expression of miR-335 in cortical regions during fetal development, or in different demyelination models, LNA-modified, biotin labeled miR-335 (#38160-03; Exiqon, Denmark), U6 (#99002-03; Exiqon, Denmark), and scramble control probes (#99004-03; Exiqon, Denmark) were utilized in situ hybridization. Paraffin-fixed mouse developmental brain tissue slides (GD 9 to GD 17) obtained from Folio Bioscience (Columbus, OH)), and brain sections obtained from demyelination models such as the experimental autoimmune encephalomyelitis (EAE) treated model (Prepared by Dr. Stephen Crocker's laboratory) and cuprizone treated model (Prepared by Dr. Jianrong Li's laboratory) were utilized to study the miR-335 expression. In the EAE model, C57BL/6 mice between 8 to 10 weeks of age were immunized with 3 mg/ml myelin oligodendrocyte glycoprotein peptides (MOG_{35-55}, The Scripps Research Institute Peptide Synthesis Core Facility, La Jolla, CA) to induce EAE. Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO) containing *Mycobacterium tuberculosis* (200 ng/ml; Difco, Detroit, MI) was utilized to emulsify MOG_{35-55} peptides, and a subcutaneous injection containing 100 µl of emulsion was performed in the region of the thigh of each hind leg (these mice are hereafter referred to as EAE groups). On the other hand, control mice were injected with an equivalent volume of CFA solution that did not contain MOG peptides. 500 ng of pertussis toxin (islet activating protein, i.p.; List Biological Laboratories, Inc., Campbell, CA) was utilized for animal administration at the time of immunization and again 2 days later. These animals were evaluated on a daily clinical signs of EAE. In addition, cuprizone and vehicle control treated animals

were also utilized for this study. In brief, 5 week 0.2% cuprizone administration were performed to evaluate the overall health of C57BL/6 mice. Cuprizone treated mice lost approximately 10% of their body weight during the first week of intoxication, following by a gradual weight gain during the next 4 weeks. Even though the time-dependent increase in weight, cuprizone-fed mice weighed significantly less than control mice over the course of 5 weeks intoxication. For the *in situ hybridization*, slides were cleared with Histo-Clear (National Diagnostics, Atlanta, GA) to remove paraffin and rehydrated through alcohol grades and PBS**.** Tissue sections were then incubated in pre-hybridization blocking cocktail including 50% formamide, 1 M NaCl, 1x Denharts, 0.5mg/ml yeast tRNA, and 0.3% Triton x-100 at 52^{o} C for 2 hours and subsequently hybridized at 52^{o} C overnight (>16 hours) in a hybridization chamber with hybridization solution (including biotin-labeled oligonucleotide probes diluted to a concentration of 25ng/ml in pre-hybridization solution). A series of wash steps after hybridization was performed, including 5X SSC wash for 5 minutes, following by washing in 50% formamide, 1xSSC and 0.1% Tween 20 mixture at 52^{o} C for 30 minutes, 0.2XSSC wash for 30 minutes, and then 3 washes in PBS (each for 5 minutes), to remove non-specific binding of probes. Rhodamin-avidin conjugated antibody (1:500 dilution in PBS; #A-2002, Vector Laboratories, CA) was utilized to visualize the microRNA expression, and slides were preserved in the mounting media containing DAPI (#H-1200; Vector Laboratories, CA) and photographed using an Olympus microscope.

MiRNA and mRNA isolation

MiRNA and mRNA were extracted from harvested cells or desire tissues using *mir*Vana™ miRNA Isolation Kit (#AM1560; Life Technologies, CA) or RecoverAll Total Nucleic Acid Isolation Kit (#AM1975; Life Technologies, CA) according to the manufacturer's protocol. Purified miRNA and mRNA samples were quantified by NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, MA) for further analysis.

Real-time PCR of miRNA and mRNA

MiR-335 expression of wild type and Timp1KO neurosphere cultures was evaluated to determine if Timp1 controls miR-335 expression in mouse fetal brain cultures. Briefly, 25ng of purified total RNA was used to generate cDNA using the Universal Synthesis Kit according to manufacturer's protocol (#203300; Exiqon, Denmark). cDNA samples were diluted 80x and 4µl used as a PCR reaction template, in a total 10µl PCR reaction of each sample. PCR reactions were performed with miR-335 (#204151; Exiqon, Denmark) and U6snRNA primer sets (#203907; Exiqon, Vedbaek, Denmark) using the SYBR green-based real-time PCR reaction kit (#203450; Exiqon, Denmark) on an Applied Biosystems 7900HT real-time PCR instrument (Applied Biosystems, CA). U6snRNA was used as a normalization control. RT-PCR was performed to evaluate mRNA expression of MEST gene in tissues of wild type and miR-335 mutants. 500 ng total RNA was used to generate first strand cDNA using qScript cDNA Supermix kit (#95048-100; Quanta Biosciences, MD). Real-time PCR was performed on a 7900HT

Real-Time PCR System (Life Technologies, CA) using the PerfeCTa SYBR Green SuperMix with ROX kit (#95053-500; Quanta Biosciences, Gaithersburg, MD) with MEST (Forward primer: ACATCCCGG TGCTTCTTCT; Reverse primer: AGCGATACAGGATCGGAGGT) and 18S primers (Forward primer: ATGGCCGTTCTTAGTTGGTG; Reverse primer: CGCTGAGCCAGTCAGTGTAG). 18S mRNA was used as the reference gene to normalize RNA expression. Real-time RT-PCR data of mRNA and miRNA expression were quantified using the SDS 2.4 software package (Applied Biosystems, CA). Real-time RT-PCR data of both miRNA and mRNA expression were quantified using the SDS 2.4 software package (Applied Biosystems, CA).

Immunohistochemistry

To determine the regulation between miR-335 and its target genes during fetal brain development, paraffin-embedded mouse brain tissue slides obtained from fetuses that were intrauterine injection with scramble control-GFP or Pre-miR-335-GFP expression vector followed by *in utero electroporation*[2], or from fetuses generated from miR-335 transgenic mice at different developmental stages by TIGM, were rehydrated by graded alcohol, rinsed in PBS, and then incubated in a blocking solution containing 10% normal serum, 0.6% TrionX-100 to minimize non-specific antibody binding at room temperature for 1 hour. After blocking, slides were incubated with primary antibody in

[2] These embryos injected with scramble control-GFP or Pre-miR-335-GFP following with *in utero electroporation* were generously prepared by Dr. Shubha Tole's laboratory.

appropriate dilution (anti-GFP (1:800; # ab13970; Abcam, MA), anti-DCX (1:1000, # ab18723; Abcam, MA), anti-NeuroD1 (1:200, # ab16508, Abgent, CA), anti-Pdgfra (1:100, # ab61219**;** Abcam, MA)) at 4^0C overnight, and then visualized by Alexa-fluor conjugated secondary antibodies (# A11001, A11032, A-11037; Life technologies, CA) after 1 hour incubation at room temperture. Following washes in PBS and slides were mounted with fluorescence mounting media containing DAPI (#H-1200; Vector Laboratories, CA) and photographed using an Olympus microscope.

Statistical analyses

Sample sizes from each group ranged from 4 to 6 in order to reach statistic significance. Data were analyzed by multivariate or univariate Analysis of Variance (MANOVA or ANOVA), followed by the Fisher's least significant difference test (SPSS v20, IBM). Single comparisons were assessed using a t-test with the statistical significance set at $p< 0.05$. Data were expressed as mean $\pm$ standard error of the mean.

Results

Detection of miR-335 expression in mouse demyelination models

Evidence from our laboratory has established that miR-335 might determine the fate of oligodendrocyte precursors (OPC) by regulating specific stem cell markers such as PDGFRα (platelet-derived growth factor receptor α), PADI2 (a negative regulator of myelin basic protein (Musse et al., 2008; Musse et al., 2009)) and Myt1 (Myelin transcription factor 1, negative regulator that prevents oligodendrocyte precursors

differentiation (Gogate et al., 1994)). Oligodendrocytes are the major cells of myelination in the central nervous system, and experimental demyelination stimulate oligodendrocyte precursors to proliferate and differentiate in order to remyelinate (Franklin and Ffrench-Constant, 2008). Therefore, to understand if miR-335 is involved in OPC regulation in later stages during cortical development, we evaluated the miR-335 expression by observing different mouse demyelinated brain tissue sections obtained from the experimental autoimmune encephalomyelitis (EAE) or the cuprizone-treated mouse model. *In situ hybridization* of these sections using locked nucleic acid (LNA) based oligonucleotides suggested that miR-335 expression was absent in the ventricular and the subventricular zone of postnatal day 7 (P7) brain sections obtained from control, EAE, and cuprizone-treated mice (Figure 3-2). Evaluation of miR-335 expression at P7 is particularly interesting because this is when neurite outgrowth, myelination, and synaptic pruning become relevant (Dangata and Kaufman, 1997). The results of abolished miR-335 expression in these slides of later developmental stages, together with previous findings that high miR-335 expression was observed in non mitotic cells of the developing ventricular zone during the second trimester-equivalent period (data not shown), suggested that this microRNA acts as a fetal brain developmental regulator during peak periods of neurogenesis but becomes quiescent after the end of second trimester. We also evaluated the miR-335 expression on brain tissue slides obtained from TIMP metallopeptidase inhibitor 1 (Timp1) KO mice. Timp1 is a tissue inhibitor of metalloproteinases, known to be involved in the degradation of the extracellular matrix.

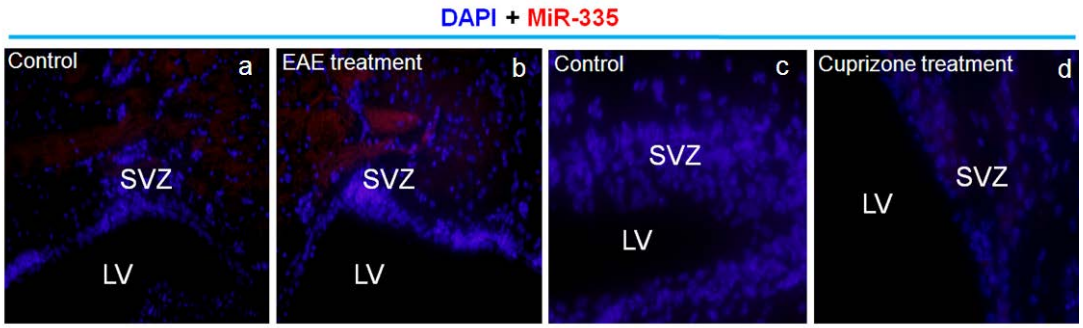


Figure 3-2. MiR-335 expression is near abolished in demyelinated mouse cortical regions at postnatal day seven (P7). *In situ hybridization* of miR-335 using LNA antisense probes on sections obtained from control (a)(c), experimental autoimmune encephalomyelitis-treated (b), and cuprizone-treated mouse brains. MiR-335 expression was labeled in red fluorescence and DAPI was stained in blue. Intensive red fluorescence in (a) and (b) indicates auto-fluorescent cells. The results suggest that miR-335 expression is absent in the ventricular and the subventricular zone at this developmental stage.

A recent study also demonstrated that astrocytic Timp1 is responsible for promotion of oligodendrocyte precursors and enhancement of CNS myelination (Moore et al., 2011).

Results from *in situ hybridization* of miR-335 expression in wild-type and Timp1 KO mouse brain slides at postnatal day 0 (P0) suggested that miR-335 expression was nearly abolished in the ventricular zone and subventricular zone of wild-type mice brains (Figure 3-3a), while its expression was retained in those regions of Timp1KO mouse Whitney U test, $p<0.0001$, Figure 3-3d). These data show that Timp1 expression in fetal neural tissues in turn controls the expression of miR-335. However, at P7, miR-335 brains (Figure 3-3b-c), and these data were also supported by RT-PCR results of miR-335 expression from wild-type and Timp1KO mouse neurosphere cultures at P0 (Mann– expression was almost absent in the rostral migratory streak, dentate gyrus, and subgranular layer of both wild-type and Timp1 KO mouse brain tissue slides (Figure 3-3e-h), consistent with those results observed from demyelinated samples. These data show that the loss of TIMP1 interferes with developmental timing and delays the disappearance of miR-335.

MiR-335 targets cell proliferation and differentiation genes DCX, NeuroD1 and c-Kit

Data from microarray screening followed by RT-PCR validation in mouse neurosphere cultures in our laboratory have showed that miR-335 prevented NSCs/NPCs maturation by regulating cell proliferation and differentiation genes, including NeuroD1 (neurogenic differentiation factor), DCX (Doublecortin; neuronal migration regulatory

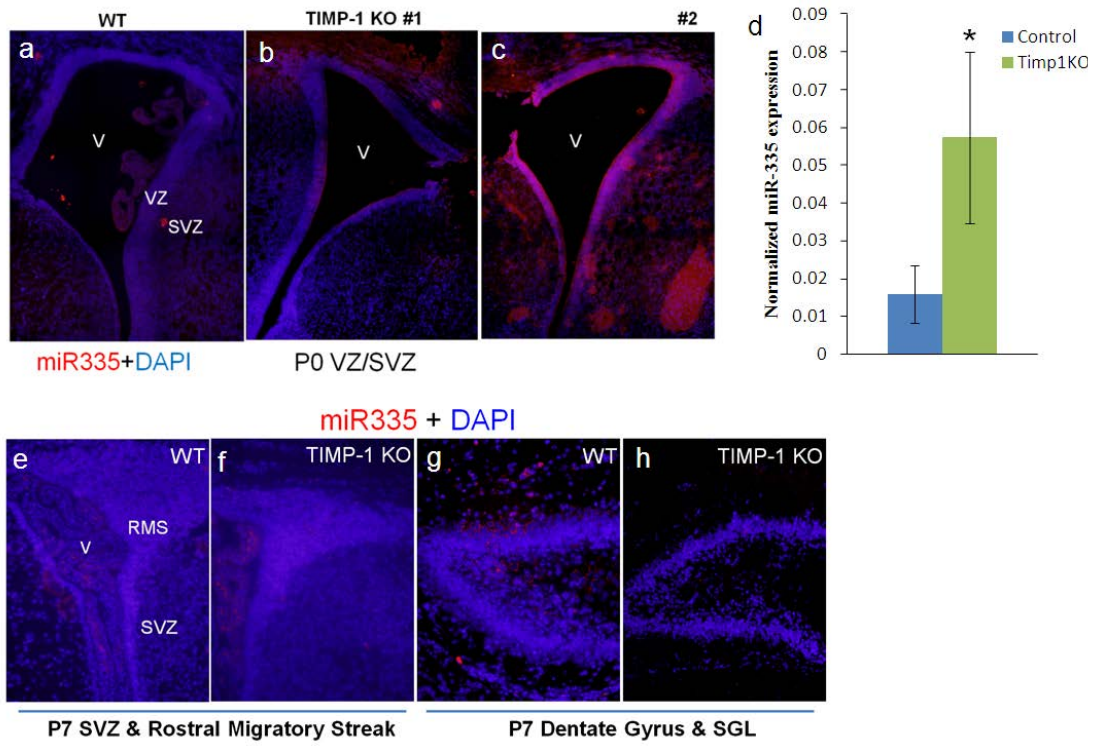


Figure 3-3. MiR-335 expression is likely to be induced in Timp1 KO mouse brain sections. *In situ hybridization* of miR-335 expression on sections obtained from wild-type and Timp1 KO brain tissues at P0 (a)(b)(c) and P7 (e)(f)(g)(h). MiR-335 expression was labeled in red fluorescence and DAPI was stained blue. (d) RT-PCR results of miR-335 expression (normalized to U6) from wild-type and Timp1 KO neurosphere cultures. Neuropshere cultures were first maintained in the constituted medium containing DMEM/F12, EGF, FGF-2, B27, and heparin for seven days, and then they were differentiated into astrocytes, neurons, and oligodendrocytes (respectively) in another medium, including DMEM in 1% FBS and N1 supplement for another seven days. Cells were collected and total RNA containing miRNAs were extracted for this study. Results from *in situ hybridization* and RT-PCR suggested that miR-335 expression level was increased in Timp1KO samples. In contrast, miR-335 expression was near absent on wild-type and Timp1KO mouse brain sections at postnatal day seven.

gene), PDGFRα (regulator of neural stem cell maturation (Erlandsson et al., 2001; Erlandsson et al., 2006)), HES5 (maintenance of neural stem cells (Ohtsuka et al., 2001)), Npdc1 (Neural proliferation, differentiation, and control gene 1 (Yang et al., 2006)). To understand the genes that determine NSCs/NPCs maintenance are the direct targets of miR-335, we first performed *in silico* analysis using miRwalk (Heidelberg, Germany), and the results suggested DCX, NeuroD1, c-Kit, and PDGFRα as potential targets of miR-335. To further confirm this prediction, luciferase constructs containing murine 3'UTR of DCX, NeuroD1, c-Kit, and PDGFRα, in combination with scramble control-GFP or pre-miR-335-GFP expression vector, were co-transfected into neurosphere cultures. The luciferase signals were determined using a microplate reader after 24 hours post-transfection, and the normalized luciferase intensity was quantified by the ratio of Firefly to Renilla luciferase signal. Because of the length of 3'UTR, the regions within the DCX transcript were fragmented into three parts and cloned into the downstream of the luciferase reporter construct. MiR-335 over-expression resulted in a significant reduction of luciferase activity of the luciferase constructs containing 3'UTR of NeuroD1 (95% reduction; ANOVA; $F_{(5,33)}$=9.065, P<1.69e-05; Figure 3-4a), c-Kit (45% reduction; ANOVA; $F_{(5,32)}$=2.939, P<0.03; Figure 3-4a) and DCX_c (30% reduction; ANOVA; $F_{(7,42)}$=21.621, P<4.94e-12, Figure 3-4b), whereas the luciferase activity of the construct containing PDGFRα 3'UTR was further induced in the presence of miR-335 mimetic (Figure 3-4a). These data suggested that DCX, NeuroD1, c-Kit, but not PDGFRα, are the direct targets of miR-335. To further investigate the binding site regions of miR-335 target genes, antisense morpholino oligonucleotides that have

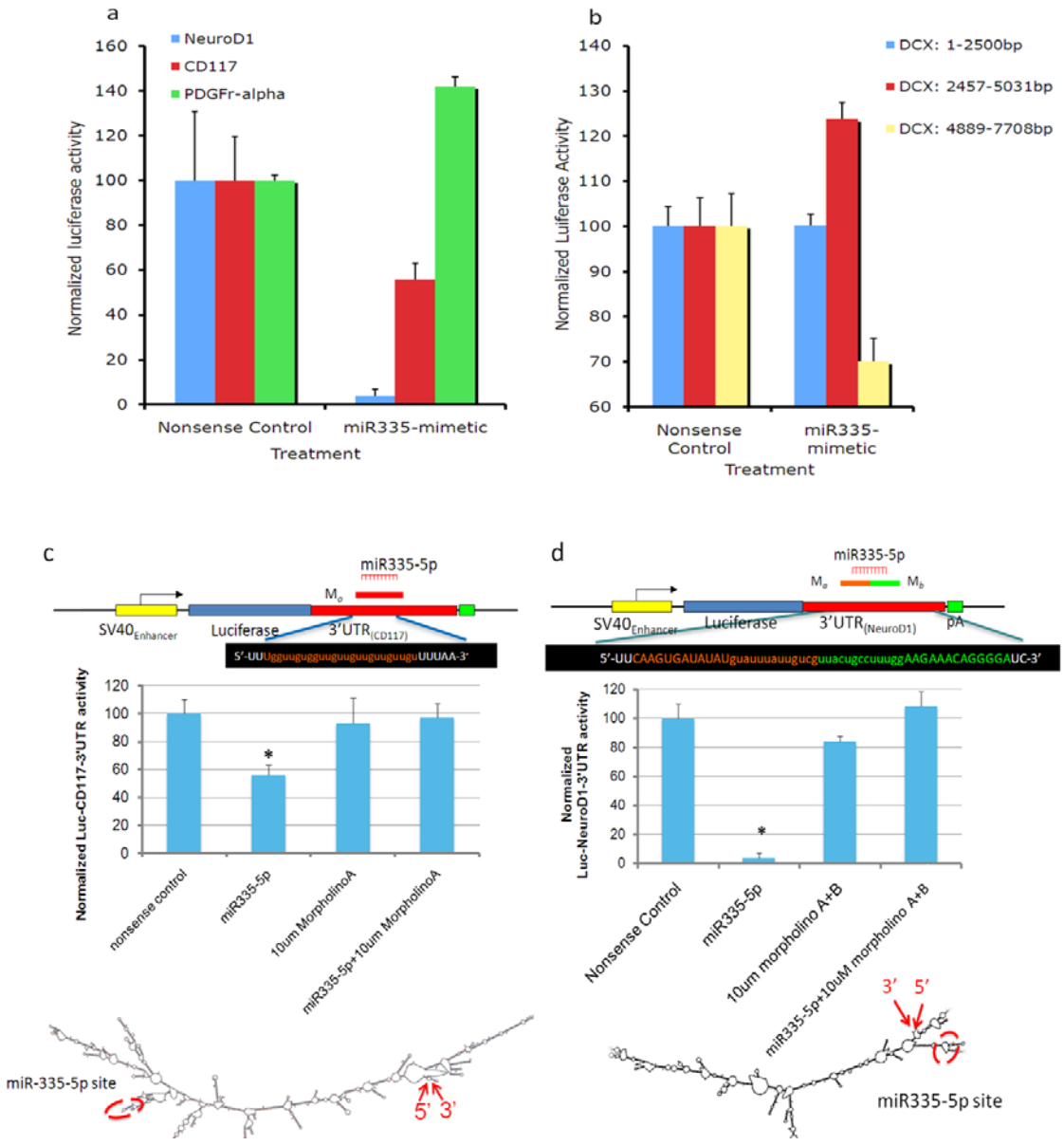


Figure 3-4. 3'UTR analysis of potential target gene of miR-335. Bar graphs show that luciferase constructs containing 3'UTR of genes of interest, (a)(d)NeuroD1, (a)(c)Cd117, (a)PDGFRa, (b)three fragment parts of DCX_a,b,c, were co-transfected with scramble control or miR-335 mimetic, as well as designed morpholinos (c)(d)(e) into some neurophere aliquots to evaluate the luciferase activity. Relative luciferase intensity was normalized to Renilla luciferase (RLuc). Morpholinos (Ma(red), Mb(green)) were used as miR335 binding site protectors to recover the luciferase activity. Binding site sequences of 3'UTR corresponding to morpholinos are shown in red and green. *In silico* analyses of these 3'UTRs indicated the structures of miR-335 binding sites (c)(d)(e). 5' and 3' depict 5' and 3' end of RNA structures.

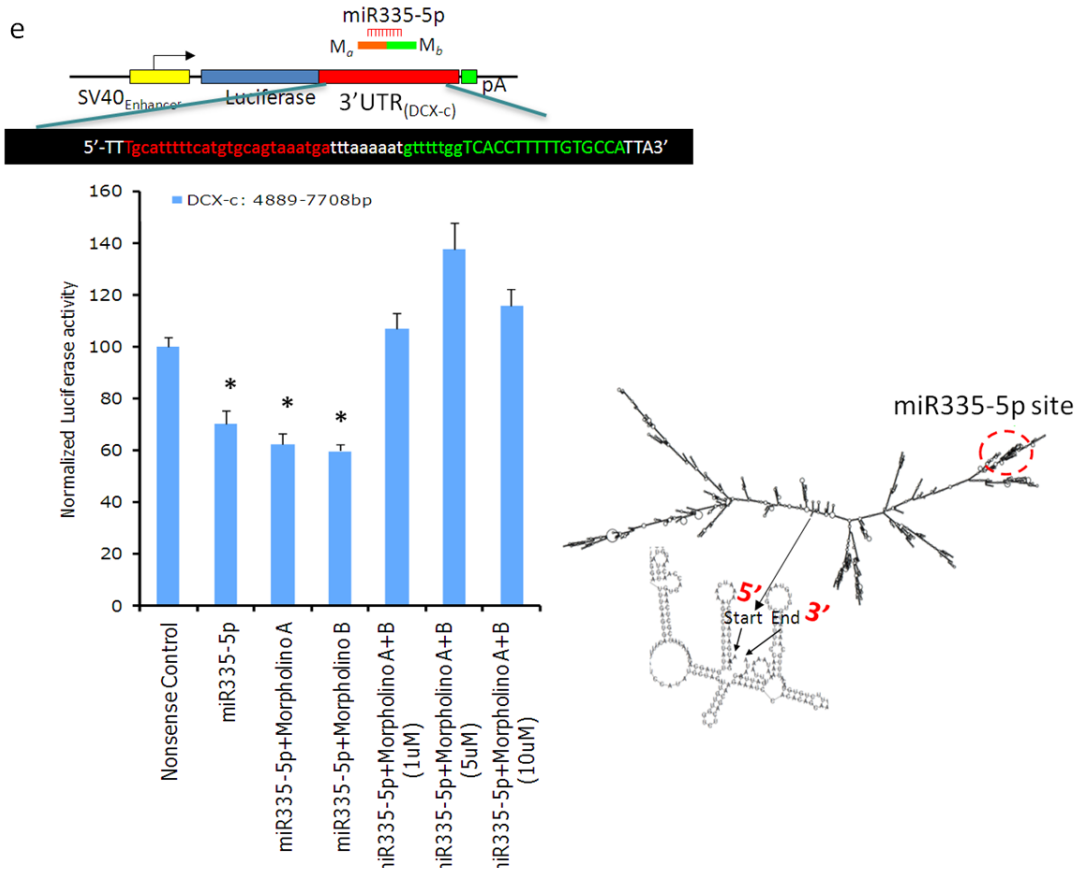


Figure 3-4 continued.

sequences complementary to the predicted miR-335 target sites on 3'UTR of individual gene were used to protect 3'UTR target sites. Scramble or antisense morpholino oligonucleotides were co-transfected into some aliquots to mask predicted miR-153 binding sites (Figure 3-4c-e) and the normalized luciferase intensity was evaluated. The data demonstrate that the sequences 852-875 on 3'UTR of c-Kit/NM_021099.2 (Figure 3-4c), the sequences 131-156 on 3'UTR of NeuroD1/ NM_010894.2 (Figure 3-4d), and the sequences 7181-7204 on 3'UTR of DCX/NM_010025.2 (Figure 3-4e), are the binding sites of miR-335. *In silico* analysis (mfold web server; SUNY at Albany, NY) of 3'UTR transcripts of NeuroD1, c-Kit, as well as DCX, indicated that the binding sites of miR-335 on these RNAs were localized to a complex of branched stem-loop structures, indicating the common feature of miR-335 regulated transcripts (Figure 3-4c-e).

MiR-335 regulates NSCs/NPCs markers DCX and NeuroD1 in mouse fetal brains

Results from 3'UTR analysis have identified NeuroD1, c-KIT, and DCX as the direct targets of miR-335. To further confirm this association between miR-335 and its target genes *in vivo*, we co-operated with Dr. Shubha Tole's laboratory and utilized *in utero electroporation* to study miR-335 regulation in the developing neocortex of mice. Scramble control-GFP or pre-miR-335-GFP expression vector was delivered into the lateral ventricular region of ED13.5 mouse fetal brains following with *in utero electroporation*. After 24 or 48 hours post-transfection, injected fetuses were euthanized, fixed, embedded, and processed for immunohistochemistry. Immunofluorescence of anti-GFP was used as a marker to localize cellular expression of scramble control or

miR-335 mimetic. Immuno-reactivity of control-GFP showed that GFP expression was co-localized with NeuroD1 or PDGFRα immuno-reactivity in the cytoplasm of cortical cells, respectively (Figure 3-5e,h), suggesting that the control vector did not disrupt target gene expression. In contrast, following *in utero electroporation* of the pre-miR-335-GFP construct after 24 or 48 hours, GFP immune-positive cells showed little-to-no immunofluorescence for either NeuroD1 or DCX, whereas adjacent GFP-negative cells exhibited strong cellular immunoactivity of NeuroD1 and DCX (Figure 3-5b-d, f-g). In addition, within the ventricular and sub-ventricular zones where there was strong miR-335-GFP immunofluorescence, expression of NeuroD1 and DCX in the overlying cortical plate was also effected (Figure 3-5d,g). Immuno-label for PDGFRα, however, was co-localized with miR-335-GFP immunoactivity and the expression was further induced in the subventricular zone (SVZ) where miR-335-GFP was presented (Figure 3-5i-k), confirming that PDGFRα is an indirect target of miR-335 but involved in miR-335 regulatory loops. These data collectively suggest that miR-335 overexpression suppresses the translation of DCX and NeuroD1, but not PDGFRα, in mouse-developing cortical neurons.

MiR-335 is crucial for early embryonic development

Data from *in vitro* 3'UTR analysis and *in vivo* mouse injection model have indicated that specific NSCs/NPCs markers DCX, c-Kit, and NeuroD1, which control stem cell maturation, are regulated by miR-335. To further investigate the effects of miR-335 in

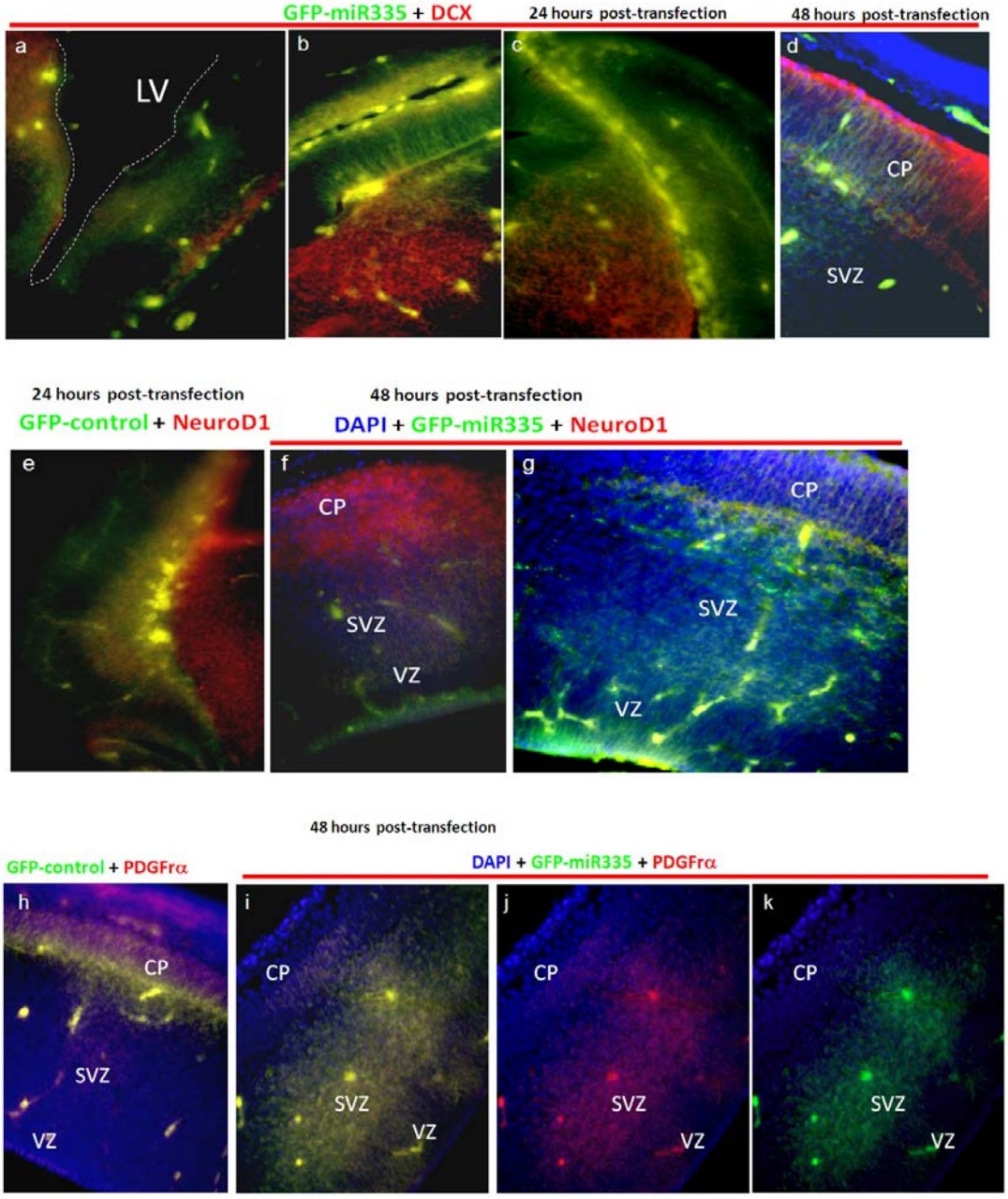


Figure 3-5. MiR-153 regulates DCX and NeuroD1 expression in fetal brains. Photo-micrographs illustrate double immunohistochemistry of anti-GFP with anti-DCX, anti-NeuroD1, or anti-PDGFRa in control-GFP (e)(h) or Pre-miR-335-GFP (b-d; f-g; i-k) transfected mouse brain sections at ED14 (24 hours post transfection) or ED15 (48 hours post-transfection). Photomicrograph depicts (e,h) control-GFP (green) localizes to the cytoplasm of NeuroD1 and PDGFRa-labeled neurons of the cortical plate. Photomicrographs show that GFP expression from the pre-miR-335/GFP construct does not co-localize with immuno-labeling for DCX (a-d) or NeuroD1 (f-g), but co-localize with PDGFRa (i-k) immunoactivity after 24 or 48 hours post-transfection. Nucleus was counterstained by DAPI. VZ: ventricular zone; SVZ: subventricular zone; CP: cortical plate.

determining NSCs/NPCs commitment, we co-operated with Dr. Andrei Golovko from Texas A&M Institute for Genomic Medicine (TIGM) to create miR-335 conditional knock-out mice (Figure 3-1), and only the mice carrying deleted and floxed miR-335 alleles validated by PCR were kept (Figure 3-1e,f). Surprisingly, the floxed allele failed to produce homozygous offspring because of embryonic lethality in floxed miR-335 alleles. One possible explanation is that the LoxP sites are positioned too close to the miRNA so that they might interfere with the post-transcriptional processing. Therefore, we decided to collect deleted miR-335 alleles for later studies. Among 142 different timed matings, the genotypes of these mouse offspring on PCR gels suggested that only one mouse fetus collected at ED 3.5 was potentially homozygous (miR-335$^{-/-}$) (Figure 3-1g), even though the PCR products of this embryo seemed to contaminate with maternal tissues (Figure 3-1e). These data collectively suggest that miR-335 is a developmental regulator during embryogenesis and constitutive deletion of this microRNA results in embryonic lethality at earlier stages.

MiR-335$^{(+/-)}$ mutants show no morphology differences and do not exhibit disruption in the expression of its parent gene, MEST

Since miR-335$^{(-/-)}$ mutants are embryonic lethal, we next examined whether miR-335 heterozygous (miR-335$^{(+/-)}$) mice would show any deficits that affect cortical neuron stem cell maintenance during fetal brain development. MiR-335$^{(+/-)}$ and wild-type litters from ED 12.5 (Figure 3-6a) and ED 14.5 (Figure 3-6b) showed no difference in morphology, such as body size, from observation. In addition, we further examined the

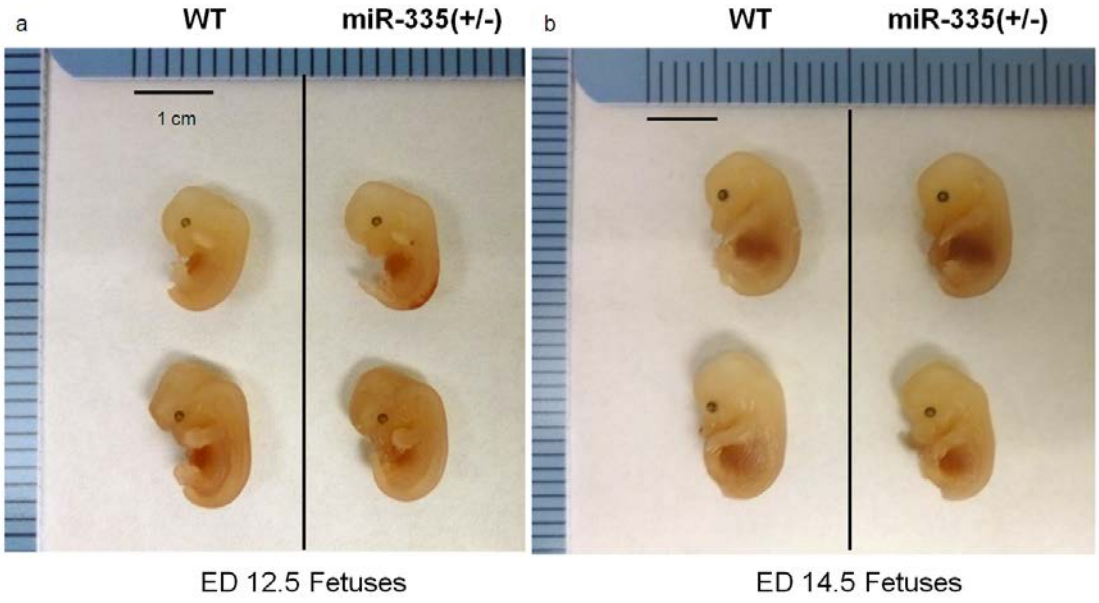


Figure 3-6. MiR-335$^{(+/-)}$ transgenic mice are not morphologically different from wild-type animals. Photographs illustrate morphological observation of (a) ED 12.5 and (b) ED 14.5 mouse fetuses suggest no differences in the body size between wild-type and miR-335 heterozygous mice.

gene expression of miR-335's parent gene, mesoderm specific transcript (MEST), to observe if deletion of the miR-335 loci, which is located within the intronic region of MEST, would disrupt MEST transcripts. Immunohistochemistry analysis of MEST indicated the presence of MEST expression in mesoderm tissues such as placenta (Figure 3-7b, e) and somites (Figure 3-7c, f) from wild-type and miR-335$^{(+/-)}$ tissue slides at ED10.5 and ED 12.5. These data were also supported by the RT-PCR results, showing that there were no significant differences of MEST mRNA expression between wild-type and miR-335$^{(+/-)}$ mutants from the whole brain, the head, and the trunk extracts at ED 14.5 (Figure 3-7g). These data collectively conclude that the removal of miR-335 loci within MEST doesn't disrupt its parent gene expression. Next, we examined miR-335 expression and see if genetic deletion of miR-335 loci would suppress the expression level of this microRNA. Surprisingly, miR-335$^{(+/-)}$ mutants did not show suppression of miR-335; instead, the fold change of miR-335 expression relative to MEST gene expression was significantly induced in brains and head regions (Figure 3-7h) of miR-335 mutants, when comparing to the change in wild-types. These data potentially suggested a globally compensatory mechanism in order to overcome the consecutive genetic miR-335 suppression in miR-335$^{(+/-)}$ alleles.

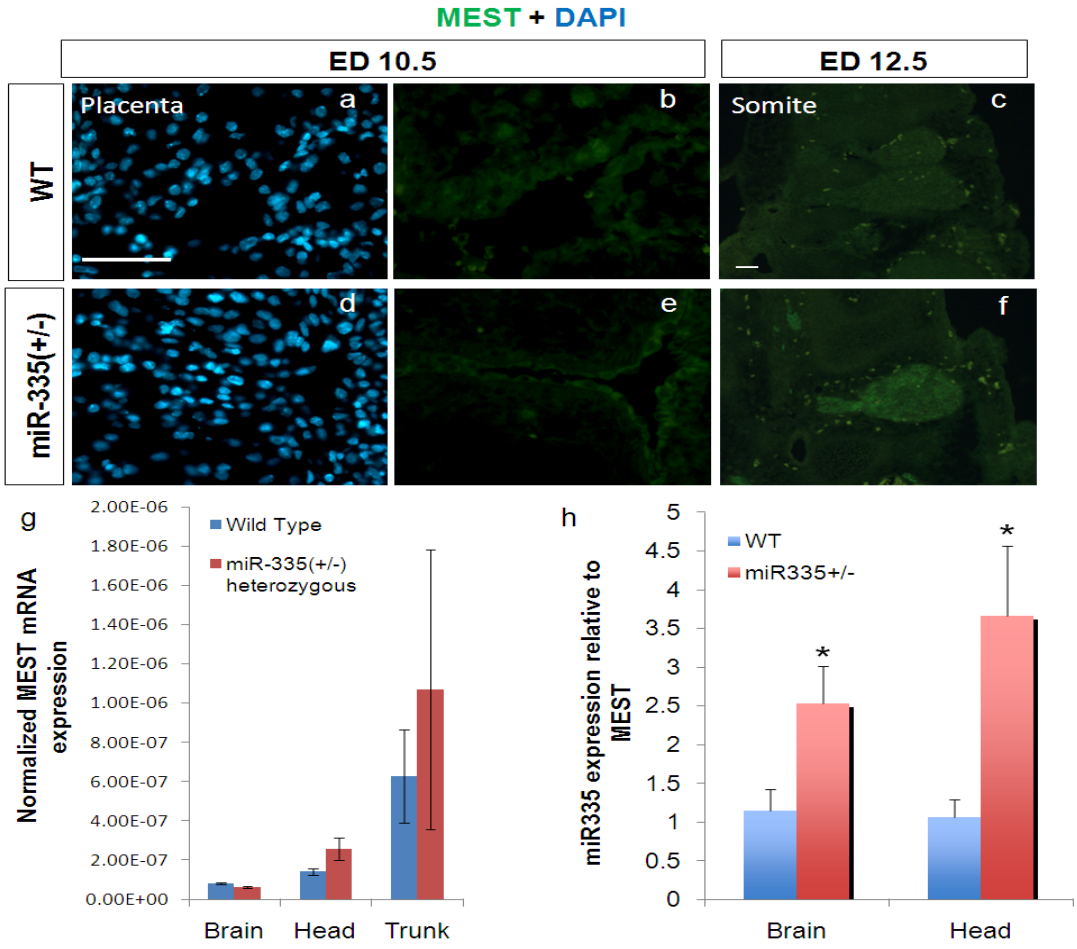


Figure 3-7. Deleted miR-335 allele does not disrupt its parent gene, MEST expression, but results in global compensation of miR-335 expression that is further induced. Photo-micographs illustrate immonuhistochemistry of MEST expression (green) on placenta sections of ED 10.5(b)(e) and tissue sections of ED 12.5 (c)(f), obtained from wild-type and miR-335$^{(+/-)}$ mutants. (a)(d) Nuclei were counterstained by DAPI. (g) Bar graphs show RT-PCR analysis of MEST transcripts (normalized to 18S) in brain, head, and trunk total RNA extracts from wild-type and miR-335$^{(+/-)}$ mice. (h) Bar graphs illustrate the ratio of miR-335 expression relative to MEST mRNA expression in brain and head RNA extracts from wild-type and miR-335$^{(+/-)}$ mice. Scale bar: 50 um.

MiR-335$^{(+/-)}$ mutants exhibited premature maturation of NSCs/NPCs in developing cortex

Even though we observed miR-335's unexpected induction in overall regions of the head of miR-335$^{(+/-)}$ alleles from the global view, we further investigated if miR-335 regulates NSCs/NPCs maturation in a local manner within the cortical layers. During

fetal brain development, proliferative stem cells in ventricular zone (VZ) undergoes mitosis give rise to either two identical daughter cells by symmetric divisions, or cells undergo asymmetric divisions that produce two distinct daughter cells with one copy of the original stem cell and a second daughter cell programmed to differentiate into a non-stem cell fate (Morrison and Kimble, 2006).

Evidence from our laboratory has established that ethanol not only promoted early neuroepithelial cell proliferation through depleting the numbers of cells expressing NSCs/NPCs markers (Santillano et al., 2005; Tingling et al., 2013); indeed, ethanol also promoted asymmetrical cell division, as well as early differentiation of NSC population (Santillano et al., 2005; Camarillo and Miranda, 2008). Hence, to further confirm whether this mechanism is associated with miR-335 dysregulation under ethanol exposure (Sathyan et al., 2007), we next analyzed the behavior of proliferative neuronal precursors in VZ of fetal brain tissue slides at GD 10.5, to determine whether down-regulation of miR-335 promotes NSCs/NPCs pre-maturation during brain development. Interestingly, we discovered a significantly higher frequency of proliferative progenitors underwent asymmetric mitosis below 15 degree cell divisions in miR-335$^{(+/-)}$ mutant mice (23.74±5.36% vs 7.69±2.88% in wild-type, ANOVA *post hoc* LSD, $p<7.1E-03$;

Figure 3-8d-f,g), whereas more symmetric divisions of stem cells above 75 degree were observed in wild-type mice (43.04±6.98% vs 23.32±2.88% in miR-335 mutants, ANOVA *post hoc* LSD, p<1.4E-03; Figure 3-8a-c,g). Cumulative frequency analysis of wild-type and miR-335$^{(+/-)}$ samples further suggested significantly higher percentages (49.05±4.90% vs 30.83±3.22% in wild-type; ANONVA, $F_{(5,24)}$=5.23, p<2.1E-03; Figure 3-8h) of neuronal precursors underwent asymmetric cell divisions in miR-335$^{(+/-)}$ fetuses. These data demonstrate that miR-335 acts as a molecular brake to maintain NSCs/NPCs population, and loss function of this microRNA results in premature-maturation of NSCs/NPCs through driving more asymmetric divisions in VZ.

Data from *in vitro* neurosphere cultures and *in vivo* injected mice have suggested the miR-335 regulation in determining NSCs/NPCs fates by targeting stem cell marker DCX and NeuroD1. To further confirm these results, we observed NSCs/NPCs behaviors by tracking these stem cell markers at different developmental stages in mouse tissue slides obtained from wild-type and miR-335$^{(+/-)}$ mice during the second and third trimester-equivalent period. Immunohistochemistry data on ED10.5 and ED 12.5 mouse brain tissue slides indicated that immunoactivity of DCX is mainly located within the preplate region or the cortical plate respectively, and is slightly induced in miR-335$^{(+/-)}$ tissue sections (Figure 3-9d-f), when comparing to wild-type slides (Figure 3-9a-c). However, immune-label for NeuroD1 was localized throughout the VZ to PP/CP and showed no significant differences between wild-type and miR-335$^{(+/-)}$ samples at these stages (Figure 3-10a-e, g-k). Interestingly, data obtained from ED 14.5 miR-335$^{(+/-)}$ mouse tissue slides exhibited a broader expansion of DCX and NeuroD1 immunoreactivity

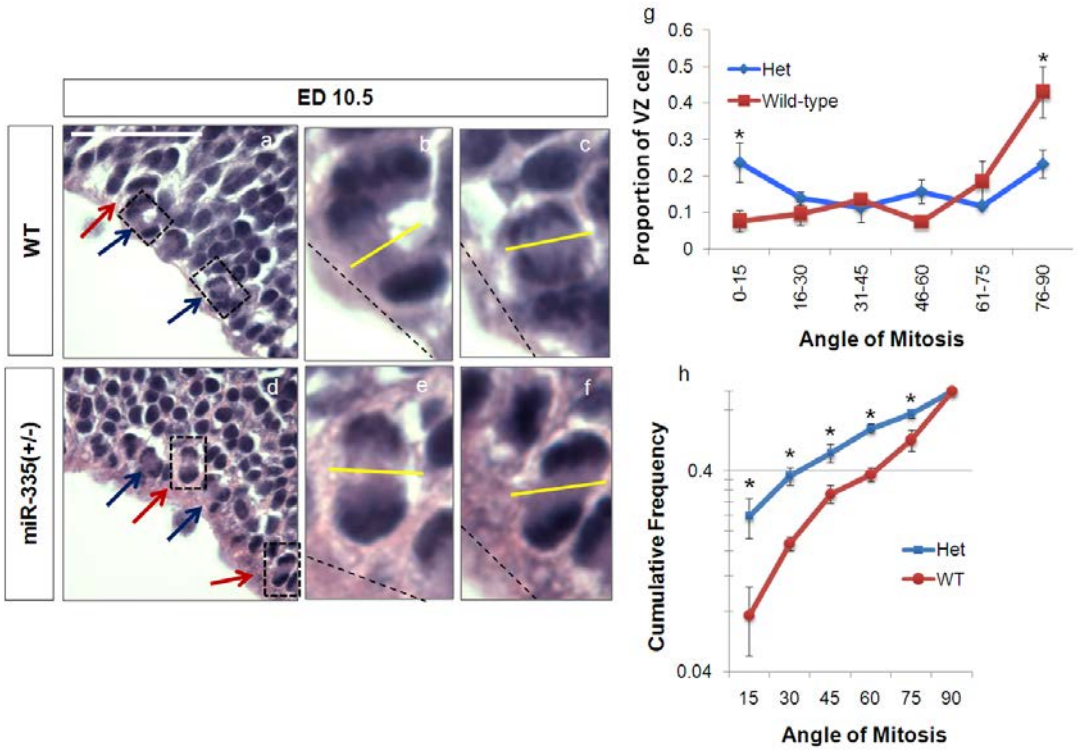


Figure 3-8. Lost function of miR-335 promotes proliferative neuronal precursors undergoing asymmetric cell divisions in the mouse ventricular zone at ED 10.5. Photo-micrographs show hematoxylin and eosin stain of wild-type and miR-335$^{(+/-)}$ (d-f) mouse brain slides. Blue arrows indicate mitotic cells under symmetric divisions, and red arrows suggest cells undergoing asymmetric divisions. High magnification photomicrographs of dotted rectangles show the examples of symmetric cell divisions (b)(c) or asymmetric divisions (e)(f) in the ventricular zone. Dotted lines indicate basal membrane lines, and yellow lines show cell division planes. The mitosis angles are determined by the crossing of cell division planes and membrane lines. Scale bar: 50 um. Line graphs illustrate the proportion of progenitor cells of VZ (g) and the cumulative frequency of those mitotic cells (h), in different mitotic angles. Red solid squares: miR-335 heterozygous mice; Blue solid circles: wild-type mice.

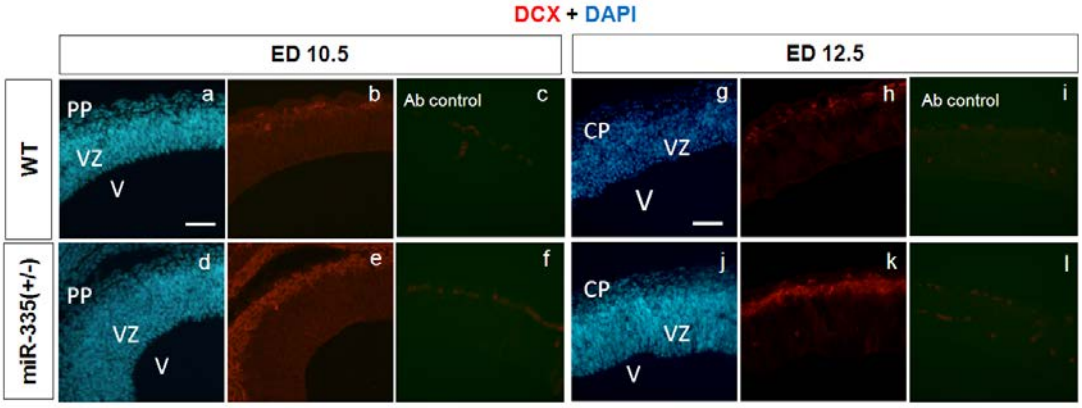
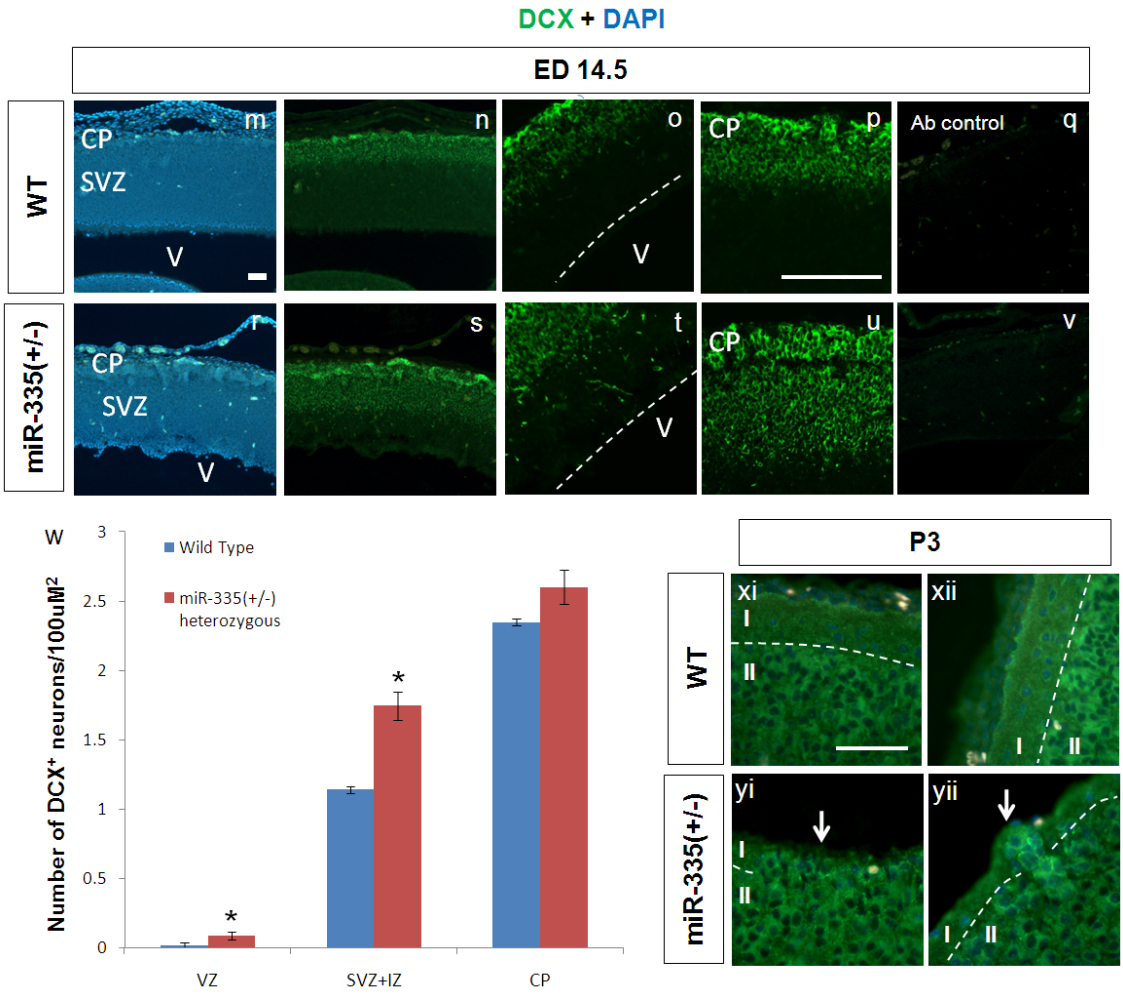


Figure 3-9. MiR-335$^{(+/-)}$ fetuses exhibit early neuronal differentiation through promoting DCX expression in mouse neocortex. Photo-micrographs illustrate immunoreactivity of DCX on mouse brain slides of ED10.5 obtained from wild-type (a-b) and miR-335$^{(+/-)}$ mice (d-e), of ED12.5 from wild-type (g-h) and miR-335$^{(+/-)}$ mice (j-k), of ED14.5 from wild-type(m-p) and miR-335$^{(+/-)}$ mice (r-u), and of postnatal day3 from wild-type (xi-xii) and miR-335$^{(+/-)}$ mutants (yi-yii). Immunoactivity of DCX expression is mainly presented in the preplate/cortical plate and is slightly induced in miR-335$^{(+/-)}$ mice (b,e), when comparing to wild-type animals (h,k), at ED10.5 and ED12.5. At ED14.5, Immune-label for DCX is mainly localized in the developing cortical plate and the subventricular zone close to the cortical plate in wild-type mice (n-p), but is spread through the SVZ and VZ in miR-335$^{(+/-)}$ mutants (s-u). At P3, immunoactivity of DCX is localized in the layer II of the cortex in wild-type mice (xi-xii), while immune-label for DCX is spread through the layer I and these DCX^{+} neurons form heterotopias and displaced neuronal aggregates (arrows in yi-yii) in miR-335$^{(+/-)}$ mice. Antibody control of ED10.5 (c,f), of ED12.5(i,l), of ED14.5(q,v). Nucleus was counterstained by DAPI. VZ: ventricular zone; SVZ: subventricular zone; CP: cortical plate. Scale bar: 50 um. (w) Bar graphs depict distribution of DCX immuno-positive neurons throughout the cortical regions in wild-type and miR-335$^{(+/-)}$ at ED14.5. X axis depicts different regions of the cortex. Y axis indicates numbers of DCX^{+} neurons in the same area unit.



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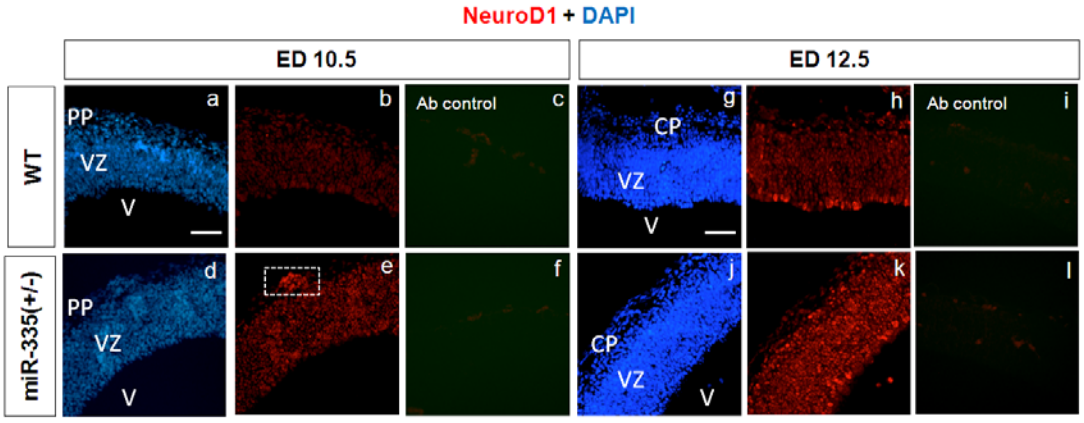

Figure 3-10. Dysregulation of miR-335 drives NSCs/NPCs pre-maturation by promoting NeuroD1 expression in mouse neocortex. Photomicrographs show immune-label of NeuroD1 on mouse brain sections of ED10.5 obtained from wild-type (a-b) and miR-335$^{(+/-)}$ mice (d-e), of ED12.5 from wild-type (g-h) and miR-335$^{(+/-)}$ mice (j-k), of ED14.5 from wild-type (m-p) and miR-335$^{(+/-)}$ mice (r-u). At ED10.5 and ED12.5, immune-label for NeuroD1 is located through VZ, SVZ, and PP/CP and is no obvious difference between wild-type (b,h) and miR-335$^{(+/-)}$ mice (e,k). However, evidence of heterotopias in the preplate is discovered in miR-335$^{(+/-)}$ mice (the dotted rectangle in e). At ED14.5, Immunoactivity of NeuroD1 is mainly localized in the developing cortical plate, the subventricular zone close to the cortical plate, and the resting neurons in the VZ in wild-type mice (m-q), but is spread through the SVZ and CP in miR-335$^{(+/-)}$ mutants (s-w). Nucleus was counterstained by DAPI. Antibody control of ED10.5 (c,f), of ED12.5(i,l), of ED14.5(q,v), used as negative control. VZ: ventricular zone; SVZ: subventricular zone; CP: cortical plate. Scale bar: 50 um. (w) Bar graphs illustrate numbers of NeuroD1 immuno-positive cortical neurons throughout developing cortical regions in wild-type and miR-335$^{(+/-)}$ mice at ED14.5. X axis depicts different regions of the cortex. Y axis indicates numbers of NeuroD1 immunoactive neurons in the same area unit.

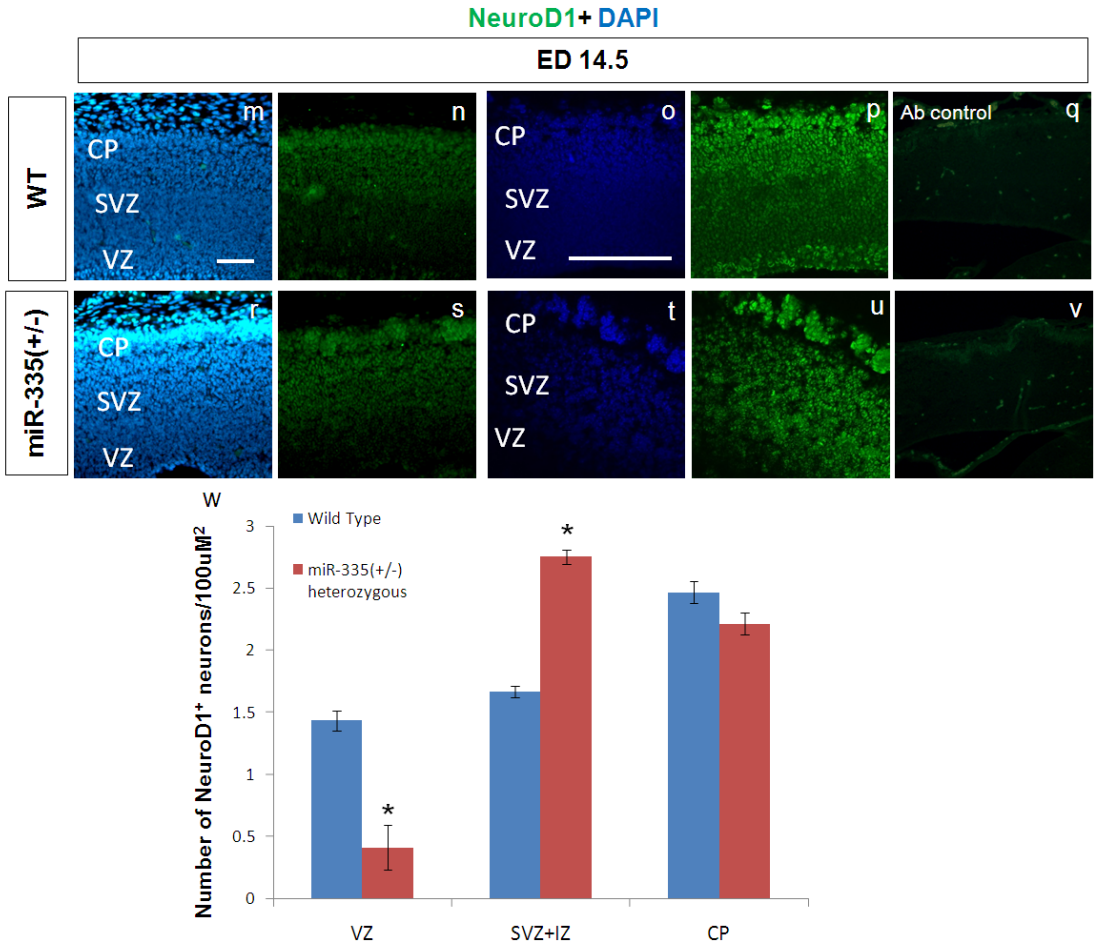

Figure 3-10 continued.

throughout the extent of the subventricular zone (SVZ) (Figure 3-9s-u; Figure 3-10s-w), while wild-type fetuses expressed NeuroD1 and DCX mainly within the cortical plate and the SVZ regions close to CP (Figure 3-9m-p; Figure 3-10m-q). Additionally, neuronal counting analysis of ED14.5 tissue slides also suggested that significant difference of NeuroD1 and DCX positive neuronal distribution in global between wild-type and miR-335$^{(+/-)}$ was observed (ANOVA Pillai's Trace, $F_{(4,24)}$=5, p<4.5E-03). Moreover, more DCX (Figure 3-9w,) and NeuroD1 (Figure 3-10y) immune- positive neurons were found within the SVZ in miR-335$^{(+/-)}$ animals than those in wild-type mice under the same area unit. Moreover, fewer NeuroD1^{+} neuronal precursors from miR-335$^{(+/-)}$ fetuses were observed in the basal VZ, within the layer of resting neurons (Figure 3-10y), providing potential evidence of NSCs/NPCs depletion, while more DCX^{+} neurons in miR-335$^{(+/-)}$ mutants were detected in the apical VZ where the mitotic neurons locate (Figure 3-9w), suggesting early neuronal differentiation.

These data collectively suggest that dysregulation of miR-335 leads to NSCs/NPCs premature-maturation by promoting cell proliferation and differentiation marker DCX and NeuroD1. At postnatal day 3 (P3) stage, we observed DCX immunoactivity with stronger expression in the cortical layer I and II of miR-335$^{(+/-)}$ mouse brain slides (Figure 3-9 xi-xii), while DCX^{+} neurons were located only in the cortical layer II (Figure 3-9 yi-yii). Unexpectedly, we also discovered the formation of heterotopias and displaced neuronal aggregates of DCX^{+} neurons in miR-335$^{(+/-)}$ mutants (Figure 3-9 xi-xii), and these results cohere with the evidence of subpial heterotopias found in FASD children brains. These results in combination with other *in vitro* and *in vivo* data above

collectively conclude that miR-335 acts as a molecular brake to prevent NSCs/NPCs pre-maturation by regulating stem cell markers DCX and NeuroD1 during fetal brain development, and disruption of this microRNA results in NSCs/NPCs pre-maturation, increases abnormal neuronal migration, and eventually disrupts lamination and causes the formation of heterotopias. In addition, miR-335$^{(+/-)}$ mutants seemed to express higher immunoactivity of NeuroD1 in specific organs, such as the dorsal root ganglions (DRG) (Figure 3-11a,d), the cartilages (Figure 3-11b,e), and the lung (Figure 3-11c,f) on ED14.5 tissue slides, as well as in the entorhinal cortex (Figure 3-11h,k) and the prefrontal cortex (Figure 3-11i,l), but not in the CA region (Figure 3-11g,j), on P3 slides. These data collectively suggest the diverse regulation potentially contributed by miR-335 at different embryonic stages during fetal development.

Discussion

Ethanol is a strong teratogen that causes different stereotype of fetal developmental deficits. The gestational period, especially during the tail of first trimester and second trimester when neurogenesis begins, is a specific time period of vulnerability because of rapid generation of future neurons from NSCs/NPCs to form a mature brain (Caviness and Takahashi, 1995). Hence, the enormous rate of cell proliferation and differentiation during this time period also amplifies the effects of early disruption from any environmental teratogen such as alcohol, which then leads to irreversible birth defects. Previous studies have shown that prenatal alcohol exposure interferes with neuronal lineage commitment (Tingling et al., 2013), resulting in NSCs/NPCs depletion

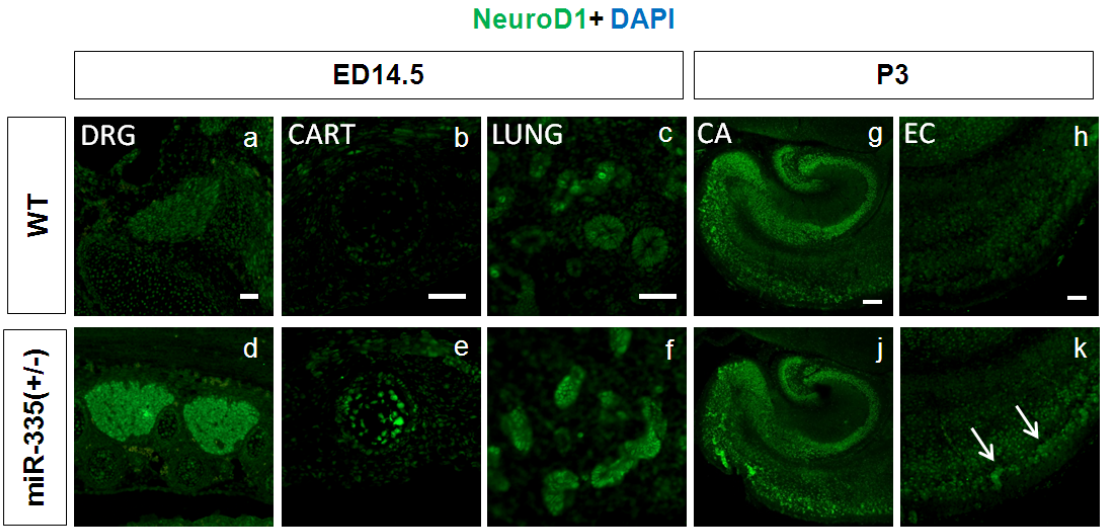

Figure 3-11. MiR-335 may potentially involve in embryonic development in different organs through regulating transcription factor like NeuroD1. Photo-micrographs show immune-label for NeuroD1 on wild-type (a-c, g-i) and miR-335$^{(+/-)}$ (d-f, j-l) mouse tissue sections of ED 14.5 and P3. At ED 14.5, immunoactivity of NeuroD1 is likely to be induced in miR-335$^{(+/-)}$ samples in different organs such as the DRG (d), CART (e) and lung (f). At P3, immune-label for NeuroD1 is prone to induce in EC (arrows in k), but not in CA (j), in miR-335$^{(+/-)}$ mice. Scale bar: 50 um. DRG: dorsal root ganglion; CART: Cartilage; CA: hippocampus regions; EC: entorhinal cortex.

(Santillano et al., 2005; Tingling et al., 2013), and ethanol's effects have been associated with dysregulation of miRNAs, including miR-335, that mediate ethanol's teratology (Sathyan et al., 2007). However, how miR-335 mediates fetal NSC vulnerability and contributes to teratology is still unclear. In this study, we report that miR-335, one of the ethanol-sensitive miRNAs, controls NSCs/ NPCs maintenance by modulating cell proliferation and differentiation genes DCX, NeuroD1, c-Kit, and that miR-335 lost-of-function results in early differentiation of NSCs/NPCs. Temporal and spatial observation of miR-335 expression in mouse brain tissue slides suggests that this miRNA is highly expressed close to the basal region of the VZ, containing resting NSCs/NPCs during the early period of cortical plate neurogenesis (data not shown), but is near abolished after the end of the second trimester-equivalent period. These data cohere with the results from *in situ hybridization* of miR-335 expression in brain sections obtained from different demyelination models at P7, or slides obtained from Timp1 KO mouse at P0. These data collectively suggest a hypothesis that miR335 controls the initial period of NSC/NPC maturation, and may not be associated with neuronal regulation in later phases such as the time periods of oligogenesis and myelination. This idea is supported by a recent study showing that miR-335 anticipates in mouse embryonic stem cell self-renewal and cell cycle control by regulating Oct4-pRb complex (Schoeftner et al., 2012), and elevated levels of miR-335 increases cell growth and inhibits cell cycle arrest in the G0/G1 phase in meningioma cells (Shi et al., 2012).

Data from *in vitro* 3'UTR and *in utero electroporation* analysis suggest that NeuroD1, DCX, and c-Kit, but not PDGFRα, are direct targets of miR-335. NeuroD1 has been

found involved in several cell differentiation pathways such as fate determination of early retinal ganglion cells (Cherry et al., 2011), formation of inner ear sensory neurons (Evsen et al., 2013), adult neurogenesis, and survival of neuronal progenitors (Kuwabara et al., 2009). DCX is a brain-specific gene and is also a differentiation marker of fetal neurons and migrating neural progenitors (Gleeson et al., 1998). Genetic mutation in the DCX gene loci have been found to cause neural heterotopias and mental retardation (Sakamoto et al., 2000; Manent et al., 2009). C-Kit is the receptor of the cytokine stem cell factor (SCF) and plays an essential role in the regulation of cell survival and proliferation, hematopoiesis, stem cell maintenance, and primary sensory neuron development (James et al., 2004; Zhang and Sieber-Blum, 2009). These studies illustrate the importance of miR-335 targeted genes that determine the fate of NSCs/NPCs by regulating cell proliferation and differentiation. We also discover PDGFRα as a non-direct target of miR-335, and, surprisingly, this gene turns out to be further induced when miR-335 is overexpressed. One possible mechanism may be the induction of PDGFRα's ligand, PDGFα, which is the predicted target of miR-335-3p (the antisense of miR-335), and overexpression of miR-335 results in blocking miR-335-3p regulation on PDGFα gene and eventually increases the gene expression of its receptor.

In addition, *in vivo* data from our miR-335$^{(+/-)}$ transgenic mice further provide convincing evidence that lost of function of miR-335 results in induction of stem cell proliferation and differentiation markers DCX and NeuroD1, driving pre-maturation of NSCs/NPCs population into differentiating neurons through increasing asymmetric divisions, promoting early migration of neuronal progenitors out of the VZ and SVZ

during the second trimester, and eventually causing the formation of heterotopias or mis-migrated neuron aggregates shown in the laminar layer I and II of the fetal brain in later developmental stages. These data are consistent with the appearance of subpital heterotopias that have been found in the brains of FASD children (Mooney et al., 2004), and therefore suggest that miR-335 intermediates ethanol's teratology by regulating NSCs/NPCs behaviors through driving cell proliferation and differentiation during early stages of fetal brain development. Other evidence such as induction of NeuroD1 expression in other organs, i.e. DRG, the lung, and cartilages at ED 14.5, or EC and PFC at P3 stage of miR-335$^{(+/-)}$ mutants, suggests that miR-335 not only affects central nervous system (CNS) development, but might act as a key embryonic regulator that contributes to fetal development in different aspects. For example, DRG, developing in the embryo from neural crest cells that are derived from the surface ectoderm, is a nodule on a dorsal root of the spine containing cell bodies of nerve cells neurons that sense and respond to many environmental and proprioceptive signals (Currie and Scott, 1992)**.** A recent study has suggested that abnormal up-regulation of NeuroD1 promotes DRG progenitors precociously differentiating into sensory neurons in early development but eventually causes deficits of sensory neurons at later stages because of reduced cell proliferation and abnormal cell death (Hu et al., 2011). Cartilage is a flexible connective tissue that is composed of specialized cells called chondroblasts, which produce a large amount of extracellular matrix. During embryogenesis, the skeletal system is derived from the mesoderm germ layer and the cartilage is formed from a condensed mesenchyme tissue, which then differentiates into chondroblasts that form the

extracellular matrix. A recent study has suggested that late gestational chronic binge alcohol exposure reduces growth and causes functional impairments of the fetal skeletal system (Sawant et al., 2013), which might relate to disruption of chondroblasts during development. Entorhinal cortex has been found to play important roles in memory formation and consolidation (Ekstrom et al., 2003; Yang and Raine, 2009). Early induction of NeuroD1 in this cortex region may also indicate the evidence of early neuronal maturation. These data collectively suggest that in addition to CNS regulation, miR-335 may also potentially influence other developmental regulatory pathways and therefore contributes to ethanol's teratology.

According to these results, we propose a potential mechanism that environmental teratogens like ethanol, disrupt the homeostasis of fetal miRNA that results in mis-regulation of NSCs/NPCs, and consequentially leads to irreversible CNS disease susceptibility later in life. The key primary defect of ethanol's effects on fetal development is the CNS damage that causes mental retardation and other psychiatric disorders via maternal alcohol abuse (Abel and Sokol, 1986; Livingston and Lyall, 1986; Burd et al., 2003; Suzuki, 2004). Severe defects in CNS have been associated with the disruption of NSCs/NPCs growth regulatory through miRNAs (Sathyan et al., 2007; Wang et al., 2009; Guo et al., 2012). In this study, we demonstrate that miR-335, one of the ethanol-sensitive microRNAs, controls NSCs/NPCs maturation by regulating stem cell markers NeuroD1, DCX and c-Kit. In addition, dysregulation of miR-335 during the critical period of neurogenesis promotes NSCs/NPCs early proliferation and differentiation, leads to early neuronal migration, and eventually causes the formation of

heterotopias or displaced neuronal aggregates associated with FASD patients. In sum, the results conclude that fetal miR-335 not only is an important developmental modulator that maintains stem/ progenitor cell population, but also functions as an ethanol antagonist that might be potential therapeutics to prevent or reverse alcohol's effects during fetal brain development. Future *in vivo* studies of miR-335 administration in ethanol-treated animals might explore the application of microRNAs in teratology medication**.**

CHAPTER IV

SUMMARY AND DISCUSSION

Discussion

My dissertation explored role of fetal cerebral cortical development and its vulnerability to environmental teratogens such as ethanol using multiple model systems, such as *ex-vivo* neurosphere culture model, ultrasound guided *in utero electroporation* of fetal brains, and the transgenic mouse model, to illustrate ethanol's teratology in altering neuron stem/progenitor cell (NSCs/NPCs) commitment. The end of the first trimester through the second trimester of fetal development, the time period of neurogenesis when most neurons are generated to form adult brains, constituted a critical period of vulnerability, as an enormous rate of cell proliferation and maturation also amplifies the early disruption of external influence. Previous reports from our laboratory have demonstrated that ethanol did not kill neuroepithelial cells obtained from the second trimester; instead, ethanol induced cell proliferation. This induction of cell proliferation was accompanied by the loss of cells expressing NSCs/NPCs markers such as Abcg2, Sca-1, c-kit, CD24 and CD113 (Santillano et al., 2005; Camarillo et al., 2007; Tingling et al., 2013). These results suggested a mechanism that ethanol drives neurospheres pre-maturation by depleting the reserve stem cell pool of future neurons during this critical period. Therefore, we utilized the established neurosphere cultures obtained from mouse fetal dorsal telencephalic vesicles in the second trimester as source of multipotent neural precursors that have a similar gene expression pattern to embryonic stem cells (Reynolds

and Weiss, 1992; Ramalho-Santos et al., 2002; Santillano et al., 2005; Camarillo et al., 2007). Moreover, maternal ethanol administration of pregnant mice during the second trimester-equivalent period enriched the study (Sudheendran et al., 2013), as this process helped to elucidate how ethanol affects NSCs/NPCs maturation in fetal brains of mammals through maternal alcohol consumption.

Not until recent decades have microRNAs been identified as minute regulators that control different biological processes, from development to various diseases (Brennecke et al., 2003; Lim et al., 2005). These non-coding small RNA transcripts, about 18~25 nucleotides in length, have been discovered to have the capacity to regulate hundreds of genes by destabilizing mRNA transcripts or repressing translation (Lim et al., 2005). Therefore, it is reasonable to hypothesize, that altered expression of some miRNAs, and consequent mis-regulation of large gene networks, might account for the developmental effects of ethanol. The report in 2007 from our laboratory first showed that four miRNAs, miR-9, miR-21, miR-153, miR-335, were significantly suppressed in *ex-vivo* neurosphere cultures following ethanol exposure (Sathyan et al., 2007). Recent studies also suggested that microRNAs are important for the renewal, proliferation, migration, and maturation of stem cells (Reinhart et al., 2000; Houbaviy et al., 2003; Landgraf et al., 2007; Yi and Fuchs, 2011). Therefore, miRNA misregulation in neural epithelial cells via ethanol can have a profound impact on future cortical development through altering the fates of NSCs/NPCs, and depletion of the stem cell pool available for future development. However, less is understood about regulation of ethanol-sensitive miRNAs in NSCs/NPCs maintenance. Therefore, in the chapter II and III of this dissertation, I

focused on the functional study of two intronic miRNAs (miR-153 and miR-335) to determine their roles in NSCs/NPCs maturation.

MiR-153 controls NSCs/NPCs maturation by targeting fetal transcription factors Nfia and Nfib

MiR-153, an intronic microRNA, is encoded within gene loci that also encode two of the major type 1 diabetes autoantigens: islet-associated protein (IA)-2 and IA-2β. Mutation on these genes results in impaired secretion of hormones and neurotransmitters (Kim et al., 2009). A recent study in a zebrafish model indicated that down-regulation of miR-153 in embryos resulted in neurobehavioral impairments and skeletal developmental defects (Tal et al., 2012). Another report in 2013 also suggested that miR-153 is involved in SNAP-25 regulation, controlling motor neuron development, neurosecretion, synaptic activity, and movement in zebrafish (Wei et al., 2013). These data indicate the important role of miR-153 in CNS development, but less is known about this miRNA in NSCs/NPCs regulation. Therefore, in chapter III, I present a functional study of miR-153. Results from microarray analyses suggested that miR-153 regulates cell proliferation and differentiation genes such as VEGFa, Arl2bp, Ccl2, Ddit4, Nfia, Nfib, Nfic. In addition, ontology analysis also suggested that miR-153 is related to synaptic transmission and G-protein coupled receptor signaling, both of which indicate the importance of miR-153 regulation for differentiating neurons. Among these miR-153 sensitive genes, Nfi genes are particularly interesting, as they are a family of CCAAT box element-binding site-specific transcription factors that are expressed at

high levels in the developing and postnatal mouse brain (Chaudhry et al., 1997; Gronostajski, 2000). Moreover, the published research showed that knock-out of Nfia and Nfib genes in the mouse model resulted in agenesis of the corpus callosum (Lucenteforte et al., 2012) and enlarged lateral ventricles (Shu et al., 2003; Steele-Perkins et al., 2005). In my dissertation, I present the first evidence that both Nfia and Nfib are the direct targets of miR-153, based on my analysis of their 3'UTRs. Furthermore, this result was also confirmed in an *in vivo* model using ultrasound-guided *in utero electroporation* to overexpress miR-153 in the fetal telencephalon. Immunofluorescence analysis of tissue slides obtained from *in utero* ethanol exposed fetuses during the second trimester-equivalent period also showed that the immunoreactivity of Nfia and Nfib was disrupted following ethanol exposure, and this misregulation results in widespread gene expression of Nfia and Nfib in SVZ and CP, an outcome that is consistent with the suppression of miR-153. I also first demonstrated that dysregulation of miR-153 by ethanol exposure that leads to abnormal induction of miR-153-regulated genes can be prevented or partially reversed through exogenous addition of miR-153, indicating that miRNAs can be potential therapeutics to ameliorate alcohol's effects. In a pharmacology study I found that the nicotine agonist verenciline, like nicotine, could induce the expression of miR-153. Moreover, verenciline prevented the ethanol-mediated induction of miR-153 sensitive genes in NSCs/NPCs. These results advance exploration into a new field of pharmaceutical application of drugs or microRNAs to prevent or ameliorate the alcohol-induced developmental deficits.

MiR-335 prevents NSCs/NPCs maturation by regulating stem cell differentiation factors, DCX, NeuroD1 and c-Kit

MiR-335 is an intronic miRNA located on the second intron of a maternally imprinted gene, MEST/Peg1. Disruption of the MEST locus is associated with a fetal growth retardation disease called Russell-Silver Syndrome (Kobayashi et al., 1997). Therefore, it suggested that miR-335 might be critical in fetal development as well. Previous microarray data from our laboratory have indicated that miR-335 targets both neuronal and oligodendrocyte markers, such as PDGFRα, CNTF, DCX, and NeuroD1, thereby positioning itself as a regulator that prevents NSCs/NPCs maturation. This result is coherent with the *in situ hybridization* data and reveals that miR-335 is preferentially expressed in cortical SVZ during early stages but nearly abolished after tail of the second trimester of fetal brain development. Results from my 3'UTR studies further indicated that DCX, NeuroD1, as well as c-Kit, are the direct targets of miR-335, and this result is further supported by the ultrasound-guided mouse injection model, as well as the miR-335 transgenic mouse model. I first established the model system of ultrasound-guided *in utero electroporation* that extends more flexibility and accuracy to monitor the injection sites of mouse fetuses and minimizes the potential damage of fetal brains during the injection, relative to the traditional *in utero* injection model. In addition, I also generated miR-335 transgenic mice that provide stronger evidence by observing genotype and phenotype changes to directly study the effects of miR-335 on cortical neuron development. Surprisingly, miR-335$^{(-/-)}$ homozygous mutants are embryonic-lethal, which suggests that miR-335 acts as a developmental regulator crucial to fetuses,

just as its parent gene. I also present convincing evidence that the premature-maturation was observed in miR-335$^{(+/-)}$ mice. That is, more mitotic neuronal precursors undergo asymmetric cell divisions in the ventricular zone (VZ) of miR-335$^{(+/-)}$ mutants at ED 10.5, when comparing to wild-type mice. Moreover, I discovered that dysregulation of miR-335 was accompanied by abnormal induction of DCX and NeuroD1 during the second trimester, and this would be predicted to eventually lead to faulty migration, the formation of heterotopias or displaced neuronal aggregates of these future cortical neurons. Indeed we observed evidence for over-migration defects in the miR335+/- mice, consistent with these predictions. From my results we conclude that ethanol-sensitive miR-335 acts as a switch to prevent premature-maturation of NSCs/NPCs, and dysregulation of this miRNA results in increased neuronal migration and differentiation by promoting NSCs/NPCs early maturation. These findings are also supported by a recent study that indicated increased miR-335 levels efficiently reduced mouse embryonic stem cell self-renewal potential and promoted cell differentiation by targets Oct4 and pRb on the post-transcriptional level (Schoeftner et al., 2012).

Future direction: mechanisms of ethanol on miRNA regulation

In my dissertation, I discovered the downstream signaling target genes of miR-153 and miR-335 that mediate ethanol's effects on NSC/NPC maturation. However, there is little known about ethanol's effects on miRNAs regulation. Two recent studies have suggested that $GABA_A$ receptors ($GABA_AR$) (Sathyan et al., 2007) and nicotinic acetylcholine receptors (nAChRs) (Balaraman et al., 2012) may be mediators of ethanol

on miRNA expression. In addition, bio-informatic analysis also suggests that some ethanol-sensitive miRNAs, including miR-335 and miR-153, are localized within the chromosomal loci that are susceptible to epigenetic modification. Recent evidence further suggested that ethanol changes methylation processing in differentiating NSCs/NPCs (Zhou et al., 2011), indicating a potential mechanism of epigenetic regulation. MiR-335, the mammalian-specific miRNA, is an intronic miRNA within the MEST/Peg1 imprinted gene locus. The structure analysis of MEST indicates that this gene contains CpG islands that are associated with genomic methlyation (Sathyan et al., 2007), and hyper-methylation of this gene in human leads to Russell–Silver syndrome (Kagami et al., 2007). MiR-153, another intronic miRNA, has been found that the expression of this miRNA is controlled by cytosine methylation on CpG islands of upstream promoter regions in human kidney cells (Bao et al., 2012). Therefore, these reports revealed that brain effects of these two miRNAs may be also susceptible to epigenetic modification during fetal development, and ethanol may potentially affect miRNA regulation through an epigenetic mechanism. The mechanism remains to be determined to understand how ethanol alters miRNA processing and generation.

Conclusion

My research provides evidence on the role of ethanol-sensitive microRNAs, miR-335, and miR-153, as master molecular switches to determine the fates of NSCs/NPCs by regulating cell proliferation and differentiation genes. Each miRNA controls NSCs/NPCs by regulating different biological processes and genes, yet both are

sensitive to ethanol. These data speak to both the diversity and complexity of microRNA regulation required to maintain biological processes, as well as the complex actions of a teratogen like ethanol. Collectively in my dissertation, my data leads to the conclusion that miR-335 and miR-153 act as molecular brakes to prevent maturation of neural stem/progenitor cells during the second trimester, and ethanol causes organizational defects in the developing cerebral cortex through promoting premature-maturation, thereby depleting NSCs/NPCs populations. Interestingly, preliminary data from my study suggest that misregulation of miRNA-regulated genes by ethanol exposure can be prevented or reversed in the presence of microRNAs or specific pharmaceuticals. Therefore, identification of candidate miRNAs, medical drugs, as well as nutrition supplements, can be considered as potential therapeutics to reverse or ameliorate the teratology of ethanol.

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