MICRORNA FUNCTION IN DEVELOPING SPINAL CORD MOTOR NEURONS

by

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

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ABSTRACT

During central nervous system (CNS) development, neural progenitor cells undergo dramatic changes in gene expression to differentiate into diverse types of neurons. One of the fundamental challenges in neurobiology is to understand the molecular mechanisms that drive this drastic and thorough transformation of the gene expression profile. Previous research suggests that transcription factors are the primary regulators of gene expression changes during neurogenesis. However, recent studies have shown that microRNAs (miRNAs) are also important components of the gene regulatory networks that direct neuronal cell fate. The extent to which miRNAs collaborate with transcription factors in the gene network that determines neuronal identity remains unclear.

Previous work in the Lee laboratory has shown that two LIM-homeodomain factors, LIM homeobox 3 (Lhx3) and Islet-1 (Isl1), form a transcription complex that is a potent driver of spinal motor neuron fate specification. Work in our laboratory and others have shown that the Isl1-Lhx3 complex directly upregulates genes that promote motor neuron characteristics. To determine whether miRNAs are also upregulated by Isl1-Lhx3, I performed a miRNA array in Isl1-Lhx3-induced mouse embryonic stem cells (Isl1-Lhx3 ESCs). This experiment showed that miR-218 is uniquely and highly upregulated during Isl1-Lhx3 ESC motor neuron differentiation. The fact that miR-218 is highly induced during Isl1-Lhx3-directed motor neurogenesis in Isl1-Lhx3 ESCs led us to investigate whether miR-218 expression is directly controlled by the Isl1-Lhx3 complex. The analysis of our chromatin immunoprecipitation deep sequencing (ChIP-seq) data from Isl1-Lhx3 ESCs uncovered potential Isl1-Lhx3-bound ChIP-seq peaks near both miR-218-1 and miR-218-2 genes. To validate these ChIP-seq peaks, we performed ChIP experiments in Isl1-Lhx3 ESCs and embryonic mouse spinal cord. We found that the ChIP-seq peak regions near both miR-218 genomic loci are occupied by the Isl1-Lhx3 complex in Isl1-Lhx3 ESCs and the developing spinal cord. Altogether, these results strongly suggest that miR-218 is directly upregulated by Isl1-Lhx3 during spinal motor neuron differentiation.

The robust expression of miR-218 in Isl1-Lhx3 ESC-derived motor neurons led me to test whether miR-218 is upregulated in motor neurons in vivo. I performed in situ hybridization analyses in developing mouse and chick spinal cords, which showed that

miR-218 is exclusively expressed in motor neurons throughout embryonic spinal cord development. Additionally, miR-218 expression began at the onset of motor neuron differentiation and endogenous miR-218 activity was sufficient to repress the expression of synthetic miR-218 target mRNAs specifically in spinal motor neurons.

To examine whether miR-218 is important for motor neuron development, I designed a miR-218 sponge and a 2'O methyl RNA antisense inhibitor and performed loss-of-function studies. Using in ovo electroporation, I found that inhibition of miR-218 resulted in a small, but significant (10%) reduction of motor neurons in embryonic spinal cord. Additionally, we generated mouse ESC lines, which express either miR-218 sponge or scramble sponge in a doxycycline-dependent manner, and found that miR-218 was essential for the generation of motor neurons from ESCs.

In order to understand the function of a miRNA, it is important to identify authentic target mRNAs. To determine direct miR-218 targets, I collaborated with the Goodman laboratory to perform RISC-trap screens in HEK293T cells. The RISC-trap experiments identified numerous novel miR-218 target mRNAs as well as previously known miR-218 targets. Remarkably, some of the miR-218 targets have well established roles in progenitor cell maintenance or interneuron differentiation in the developing spinal cord. Using target 3'UTR reporter assays both in vitro and in vivo, I further validated five miR-218 target mRNAs: TEA Domain Family Member 1 (Tead1), Solute Carrier Family 6 Member 1 (SLC6A1), B-Cell CLL/Lymphoma 11A (BCL11A), LIM homeodomain 1 (Lhx1), and Forkhead box protein 2 (Foxp2).

Next, to test whether misexpression of miR-218 inhibits the expression of the newly identified miR-218 target mRNAs, as well as interneuron or progenitor fates, I designed a miR-218 overexpression construct and performed in ovo electroporation. Overexpression of miR-218 in the developing chick neural tube significantly decreased interneurons, but did not have a significant effect on the number of neural progenitors or motor neurons. Additionally, we generated mouse ESC lines that constitutively express miR-218 or a control miRNA. When we directed these miRNA-expressing ESCs to differentiate into interneurons, miR-218 repressed the expression of interneuron markers, while it did not affect the expression of a broad neuronal marker. Together, these gain-of-function experiments validated RISC-trap target the interneuron genes, and provided

strong evidence that miR-218 downregulates the expression of genes that promote interneuron programs.

Our data show that miR-218 is upregulated by the Isl1-Lhx3 complex and is important for the generation of motor neurons in vitro and in vivo. Our data also demonstrate that miR-218 downregulates target mRNAs that are important for interneuron differentiation. However, these experiments did not directly assess whether miR-218 activity is important for motor neuron fate specification downstream of the Isl1-Lhx3 complex. Previous work in the Lee laboratory has shown that electroporation of Isl1-Lhx3 generates ectopic motor neurons in the dorsal spinal cord. To assess whether miR-218, which is induced by Isl1-Lhx3, is important in the gene regulatory network that determines motor neuron fate, I performed co-electroporation experiments with Isl1 and Lhx3 and either miR-218 sponge or scramble sponge inhibitor. These experiments revealed that inhibition of miR-218 activity significantly reduces the ability of the Isl1-Lhx3 complex to generate ectopic motor neurons at the expense of interneurons. These data support our major finding that miR-218 is essential for motor neuron differentiation downstream of Isl1-Lhx3, and provide further evidence that miR-218 functions to establish motor neuron identity by repressing the expression of genes that promote interneuron characteristics.

In addition to investigating the role of miR-218 in motor neuron development, I also identified other miRNAs that are upregulated during motor neuron differentiation. Further analysis of the activity of multiple motor neuron miRNA candidates revealed that numerous miRNAs may have dynamic spatiotemporal expression pattern in the developing spinal cord. In particular, miR-153 was identified as a promising motor neuron miRNA candidate, and further investigation of miR-153 expression suggests that miR-153 may play a role in spinal cord neurogenesis. Additionally, previous work in the Lee laboratory show that miR-218 and miR-153 co-regulate an axon guidance factor roundabout 2 (Robo2). I performed a Robo2 3'UTR sensor analysis and found that endogenous miR-218 and miR-153 combinatorially regulate Robo2 expression in motor neurons. This result suggests that combinatorial function of miRNAs is important to effectively repress target mRNAs.

CHAPTER 1

THESIS INTRODUCTION

Over a century ago, Ramón y Cajal observed that strikingly different neuronal cell types arise during early stages of vertebrate development (Ramon y Cajal 1899). Since this discovery, researchers have been determined to understand the mechanisms that produce the remarkable diversity of neurons in the nervous system. Understanding how distinct neuronal cell types are established is an essential aim in biology because neuronal cellular diversity is inexorably linked to the circuits that form the basis of vertebrate behavior.

Recent advances in molecular, genetic, informatics, and electrophysiological methods have allowed researchers to examine complex profiles that determines neuronal identity. The Lee laboratory has been a leader in the field of applying genomic and transcriptome profiling techniques to describe the complex gene networks that control neurogenesis in the central nervous system. By performing genome-wide screens to determine the chromatin binding sites of transcription factors and the expression of mRNAs during motor neuron differentiation in vivo and in vitro, the Lee laboratory has played a particularly important role in refining the molecular profile of developing spinal motor neurons (Lee et al. 2008; 2012; 2013). My dissertation work extends this investigation to the role of miRNAs in determining spinal cord motor neuron fate.

An overview of spinal cord neurogenesis

Nervous system development requires precise spatiotemporal regulation of genes that control tissue patterning and the formation of distinct neuronal cell types. Extracellular signals, known as morphogens, are essential factors that drive the initial patterning of embryonic tissue and neuronal subtypes. One of the most extensively studied examples of morphogen patterned neuronal tissue is the developing vertebrate spinal cord (Ulloa and Briscoe 2007; Stifani 2014), where four morphogen signaling molecules are required for patterning neural tube tissue: bone morphogenetic proteins (BMPs), wingless-type MMTV integration site (Wnt), retinoic acid (RA), and sonic hedgehog (Shh). These morphogens are upregulated in discrete tissue locations shortly after the neuroepithelial

tissue folds to form the neural tube. Wnt and BMP are derived from the neural tube roof plate, RA is upregulated in the mesoderm and somites and Shh is upregulated in the notochord and ventral floor plate (Liem et al. 1997; Briscoe et al. 1999; Novitch et al. 2003; Ulloa and Briscoe 2007). From these locations, morphogens are secreted to produce extracellular signaling gradients that control the spatiotemporal pattern of cell specification in the neural tube in a concentration-dependent manner (Figure 1.1 A).

Along the dorsoventral axis of the developing neural tube, the opposing dorsal BMP-Wnt and ventral Shh morphogen gradients are essential to induce or repress the expression of specific genes in discrete dorsal (pd1-6) and ventral (p0-p3, pMN) progenitor domains (Figure 1.1 A) (Caspary and Anderson 2003; Helms and Johnson 2003; Ulloa and Briscoe 2007; Dessaud et al. 2008). These domains are primarily defined by the expression of homeodomain and basic helix-loop-helix (bHLH) transcription factors, which bind directly to DNA to control the expression of determinant genes that promote specific postmitotic motor neuron (MN) and interneuron fates (V0-V3, dl1-dl6) (Figure 1.1 A) (Alaynick et al. 2011). For example, in the ventral spinal cord, graded concentrations of Shh induce the expression of the transcription factors such as NK6 homeobox 1 (Nkx6.1), NK6 homeobox 2 (Nkx6.2), Oligodendrocyte transcription factor 2 (Olig2), and NK2 homeobox 2 (Nxk2.2) and also represses the expression of transcription factors such as Developing brain homeobox 1 (Dbx1), Developing brain homeobox 2 (Dbx2), Iroquois-class homeodomain protein (Irx3), and Paired box protein 6 (Pax6) (Figure 1.1 A) (Briscoe et al. 1999; Jessell 2000; Lee and Pfaff 2001; Muhr et al. 2001; Novitch et al. 2001). The selective expression of these transcription factors in discrete ventral progenitor domains leads to the upregulation of genes that drive the differentiation of postmitotic motor neurons (MN) and interneuron subtypes (V0-V3) (Figure 1.1 A).

One of the striking features of both ventral and dorsal fate-specifying transcription factors is their precise spatial expression pattern in the spinal cord (Alaynick et al. 2011). Some of this precision can be explained by transcription factor regulation of the spatial boundaries via cross-repressive interactions in neighboring domains (Figure 1.1 B) (Ericson et al. 1997; Briscoe et al. 2000; Jessell 2000; Novitch et al. 2001; Glasgow 2005; Lee et al. 2008). This cross-repression hypothesis is supported by

evidence that overexpressing or repressing domain-specific transcription factors leads to altered progenitor boundary patterns and the misexpression of postmitotic neuronal cell types (Ericson et al. 1997; Briscoe et al. 2000). These data suggest that a morphogen-induced transcription factor expression "code" is largely responsible for patterning neurogenesis in the spinal cord.

Although this transcription factor code has been considered a prominent mechanism that controls neuronal subtype differentiation, it is evident that additional mechanisms are required to establish the wide variety of neurons generated during development. For example, many progenitor and postmitotic fate-specifying transcription factors overlap in the developing spinal cord without known cross-repressive functions (Alaynick et al. 2011). Additionally, many transcription factors are crucial for cell-specific phenotypes in the spinal cord are also expressed and important in different developmental and functional contexts. In particular, Olig2 is required for motor neuron differentiation (Mizuguchi et al. 2001; Novitch et al. 2001), but is also essential for oligodendrocyte and glia differentiation in the spinal cord (Zhou et al. 2001; Zhou and Anderson 2002). Another example is the LIM homeodomain transcription factor Islet-1 (Isl1). Isl1 is known to be essential for the generation of motor neurons (Pfaff et al. 1996; Lee and Pfaff 2003), but it also functions to promote the development of spinal interneuron populations (Pfaff et al. 1996), dorsal root ganglia sensory neurons (Sun et al. 2008), developing heart muscle (Ahlgren et al. 1997), and pancreas islet cells (Sun et al. 2007). Although it is clear that transcription factors are essential to establish postmitotic neuron fate, it is also important to investigate additional mechanisms that maintain the accuracy of neuronal cell fate decisions.

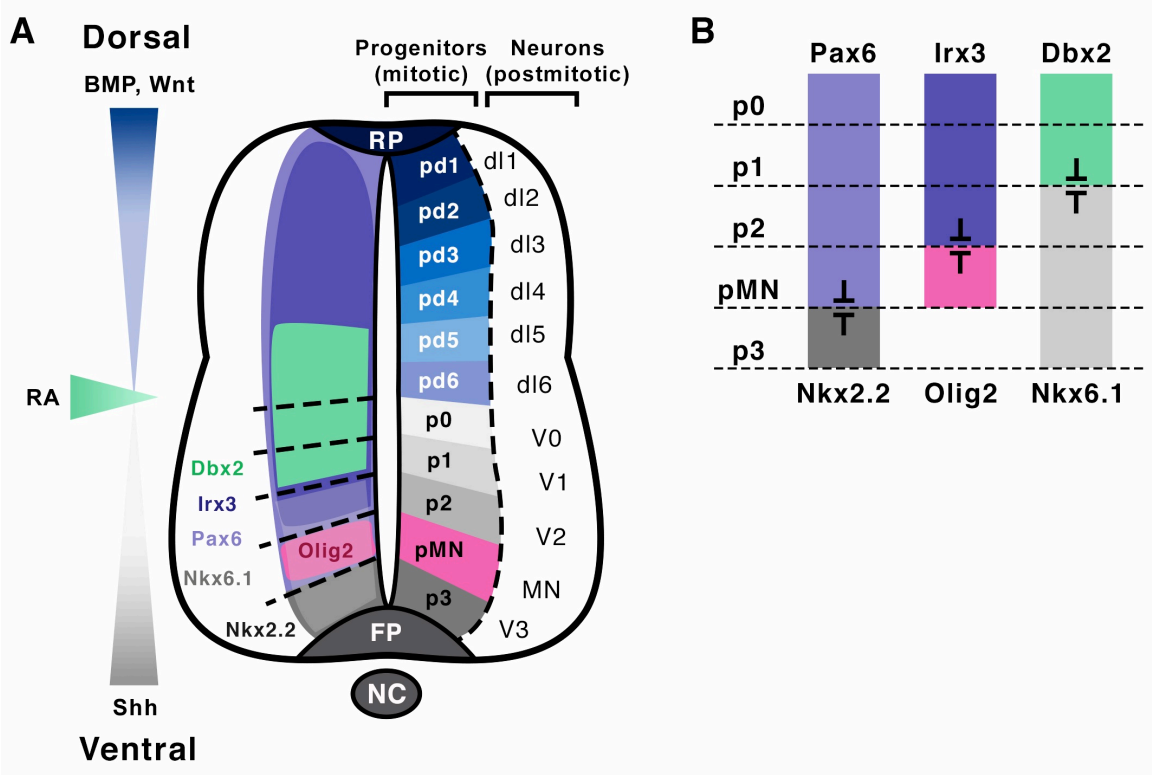

Figure 1.1. Schematic model of spinal cord neurogenesis
(A) Illustration of a transverse section of the developing neural tube representing mouse embryonic day 10.5 and chick Hamburger Hamilton stage 18. The morphogen gradients – bone morphogenetic protein (BMP) and wingless-type MMTV integration site (Wnt) from the roof plate (RP), retinoic acid (RA) from somites, and sonic hedgehog (Shh) from the notochord (NC) and floor plate (FP) – determine the patterning of progenitor tissue in the developing spinal cord. These morphogen gradients regulate the expression of transcription factors in discrete progenitor (p0-p3, pMN, dI1-dI6) and postmitotic domains (V0-V3, MN, dI1-dI6). The progenitor zone expression patterns of selected Shh repressed transcription factors (Pax6, Irx3, Dbx2) and Shh induced transcription factors (Nkx2.2, Olig2, Nkx6.1) are shown. Paired box protein 6 (Pax6); Iroquois-class homeodomain protein (Irx3); Developing brain homeobox 2 (Dbx2); NK2 homeobox 2 (Nxk2.2); Oligodendrocyte transcription factor 2 (Olig2); NK6 homeobox 1 (Nkx6.1).
(B) Schematic model of transcription factor cross-repression in the ventral spinal cord during neurogenesis. Some of the morphogen-regulated transcription factors are expressed in distinct regions of the developing spinal cord and function to repress the expression of transcription factors in neighboring domains. The cross-repressive interactions between these transcription factors is important to establish the position of discrete progenitor domains and proper neuronal subtype specification.

*Figure adapted from Briscoe et al. 2000; Jessell 2000; Kicheva et al. 2014.

An overview of spinal cord motor neuron development

Spinal cord motor neurons are the terminal links in the neuron network that control movement in the nervous system. Motor neuron cell bodies are in the ventral spinal cord and send axon projections to the periphery that synapse directly onto muscles and visceral targets. The majority of motor neurons are characterized by their cholinergic neurotransmitter phenotype and motor neuron subtypes are further categorized by the muscle or tissue groups that they innervate (Kanning et al. 2010; Stifani 2014).

The patterning of spinal motor neuron subtypes occurs in stereotypical locations along the rostrocaudal axis, and is primarily regulated by the expression of homeobox (Hox) genes (Figure 1.2 A). These Hox genes control the selective expression of transcription factors in distinct motor neuron subtypes, termed columns (Dasen et al. 2003; 2005; 2008; Shah et al. 2004; Stifani 2014). The four main columns are the medial motor column (MMC), the lateral motor column (LMC), the hypaxial motor column (HMC), and the preganglionic column (PGC). Each of these motor neuron columns are identified by their gene expression profile and their axonal projection pattern, where MMC neurons innervate dorsal body wall muscles, HMC neurons innervate respiratory and ventral body wall muscles, PGC neurons innervate sympathetic ganglia, and LMC neurons innervate limb muscles (Figure 1.2 A, B) (Gutman et al. 1993; Landmesser 2001; Kanning et al. 2010). Motor neurons have been extensively studied as a model for neuron fate specification and the expression of reliable, subtype-specific markers are used to study the molecular pathways that determine different motor neuron column locations and identities (Figure 1.2 B) (Stifani 2014). Although the mechanisms that guide motor neuron column patterning are relatively well understood, less is known about the developmental mechanisms that underlie the initial transition from neural progenitors to postmitotic motor neurons, and the maintence of motor neuron characteristics.

All motor neurons arise from the motor neuron progenitor domain (pMN) in the ventral spinal cord, which is primarily defined by the expression of bHLH transcription factor Olig2 (Figures 1.1 A and 1.2 C) (Novitch et al. 2001). Olig2 is required for spinal motor neurogenesis and upregulates the expression of key transcription factors, such as neurogenin 2 (Ngn2), LIM homeobox 3 (Lhx3), and Islet-1 (Isl1), which initiate motor neuron differentiation (Mizuguchi et al. 2001; Thaler et al. 2002; Lee and Pfaff 2003;

Novitch et al. 2003; Lee 2004; Lee 2005; Lee et al. 2008). In particular, Isl1 and Lhx3 are known to form a transcription complex that directly binds and upregulates genes that are essential to promote motor neuron development, such as homeobox gene 9 (Hb9) and cholinergic pathway genes (Figure 1.2 C) (Lee and Pfaff 2003; Lee et al. 2012; Mazzoni et al. 2013; Cho et al. 2014). Although these transcription factors are crucial regulators of motor neuron differentiation, there is evidence of plasticity within the system. Inhibition of Isl1 or Hb9 in motor neurons leads to the aberrant upregulation of interneuron genes and Olig2-lineage cells produce ventral interneurons in addition to motor neurons (Pfaff et al. 1996; Arber et al. 1999; Thaler et al. 1999; Dessaud et al. 2007; Lee et al. 2008; Song et al. 2009; Chen et al. 2011). These data support the hypothesis that additional mechanisms beyond transcription factor regulation are required to ensure the development of motor neuron phenotypes.

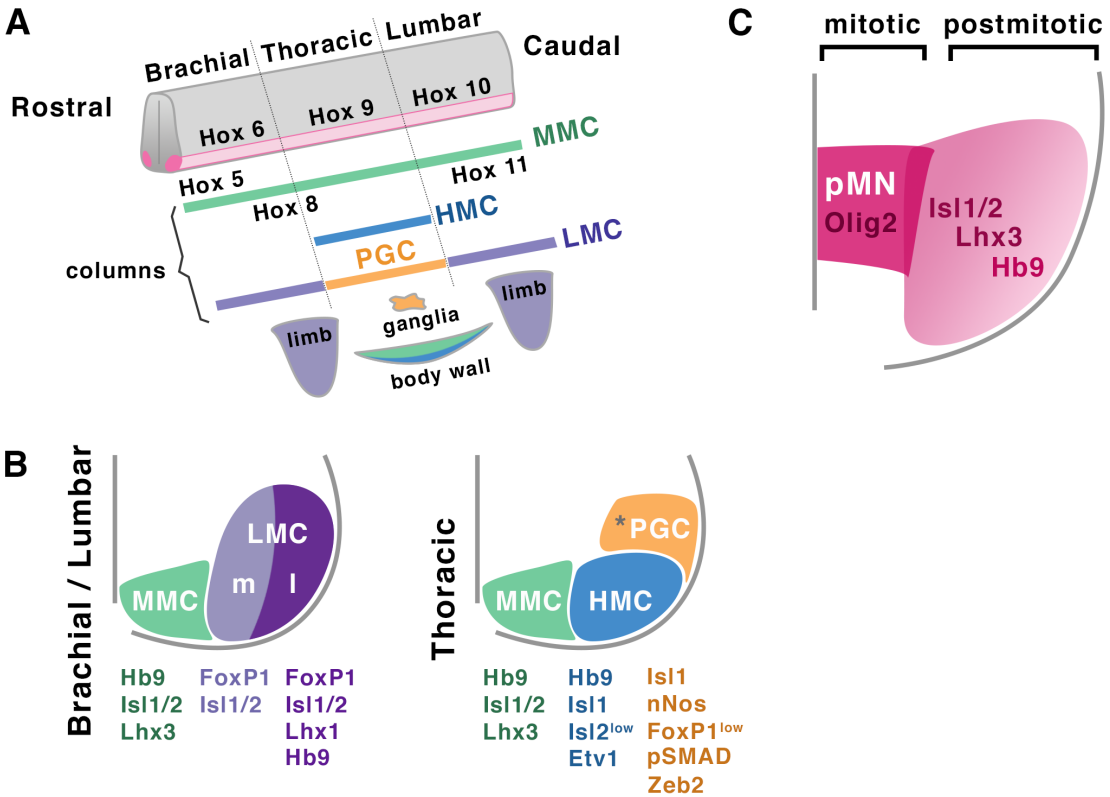

Figure 1.2. Schematic representation of motor neuron development
(A) Schematic model of rostrocaudal patterning of motor neuron spinal cord development. Along the rostrocaudal axis, the patterning of motor neuron subtypes (columns) is controlled by the spatiotemporal expression of homeobox (Hox) genes. The four main columns are the medial motor column (MMC), the lateral motor column (LMC), the hypaxial motor column (HMC), and the preganglionic column (PGC). Each of these motor neuron columns are identified by their gene expression profile and their axon projection pattern, where MMC neurons innervate dorsal body wall muscles, HMC neurons innervate respiratory and ventral body wall muscles, PGC neurons innervate sympathetic ganglia, and LMC neurons innervate limb muscles.
(B) Illustrations of transverse sections of one half of the ventral embryonic spinal cord showing stereotypical developmental motor neuron column locations and molecular markers in the brachial/lumbar (A) and thoracic (B) spinal cord. This illustration represents mouse embryonic day 13.5 and chick Hamburger Hamilton stage 28. *PGC neurons in chick are positioned in the medial, not lateral spinal cord in chick spinal cord. Homeobox gene 9 (Hb9); Islet-1 and Islet-2 (Isl1/2); LIM homeobox 3 (Lhx3); Forkhead box protein 1 (FoxP1); LIM homeobox 1 (Lhx1); Ets variant 1 (Etv1); Neuronal nitric oxide synthase 1 (nNos); Phospho-mothers against decapentaplegic (pSMAD); Zinc finger E-Box binding homeobox 2 (Zeb2).
(C) Illustrations of transverse sections of one half of the ventral embryonic spinal cord during the initial stages of differentiation, representing mouse embryonic day 10.5 and embryonic chick Hamburger Hamilton stage 18. Motor neuron progenitors (pMN) are defined by the expression of oligodendrocyte transcription factor 2 (Olig2). As pMN cells migrate laterally and exit the cell cycle, they express transcription factors Islet-1 and Islet-2 (Isl1/2), LIM homeobox 3 (Lhx3), and homeobox gene 9 (Hb9).

*Figure adapted from Tsuchida et al. 1994; Dasen et al. 2003; 2005; 2008; Stifani 2014.

Chicken Embryo: a model system for studying spinal cord development

Chicken (Gallus gallus) embryos have a long, distinguished history in studies of embryonic spinal cord development. From Aristotle in 300 BC to Rita Levi-Montalcini in the 20th century, the ease of maintence, visualization, and manipulation of the chick embryo has produced some of the most important scientific advances in vertebrate developmental biology. In particular, the concept of competence – defining a stage at which developing tissue is first able to respond to inductive cues – was first identified in chick (Waddington 1940). Additionally, grafting experiments using the developing chick spinal cord revealed the importance of growth factors in nerve and limb development (Levi-Montalcini and Cohen 1956).

Today, with technological advances in microscopy and molecular biology tools, such as in ovo electroporation, chick embryos continue to be an exceptional model organism to study spinal cord development. Gene transfer via spinal cord in ovo electroporation, the method of injecting plasmid DNA into the developing neural tube and applying a short square wave pulse, has been important tool for developmental neurobiology (Figure 1.3 A) (Momose et al. 1999; Nakamura and Funahashi 2001). This

method allows for the misexpression of genes at the onset of chick neural tube development, providing a fast and inexpensive paradigm for loss-of-function and gain-of-function experiments in the developing spinal cord. Importantly, in ovo electroporation of DNA or RNA constructs in the neural tube results in the transfection of progenitor cells in only one half of the neural tube, allowing for a perfect developmental control when comparing electroporated versus unelectroporated sides of the spinal cord (Figure 1.3 B). Additionally, in ovo electroporation allows for precision in assessing different developmental time points. The development of chick embryos is easily assessed by cutting a window in the eggshell to monitor the morphological development of the embryo before and after electroporation.

Another advantage of using chick embryos as a model for spinal cord development is their rapid development. Chick embryo development is temperature dependent and growth of the embryo only requires 48-56 hours until reaching a stage where the neural tube closes and the embryos can be electroporated. After electroporation, eggs are placed back into the incubator and the developmental progression of postmitotic neuronal populations can be studied within a week. Because spinal cord development is highly conserved among vertebrates, the molecular mechanisms in the chick neural tube are often recapitulated in other vertebrates (Briscoe et al. 2000; Jessell 2000; Stern 2005). Altogether, chick embryos are a fundamental model system for teasing out the molecular mechanisms of neurogenesis in the developing spinal cord.

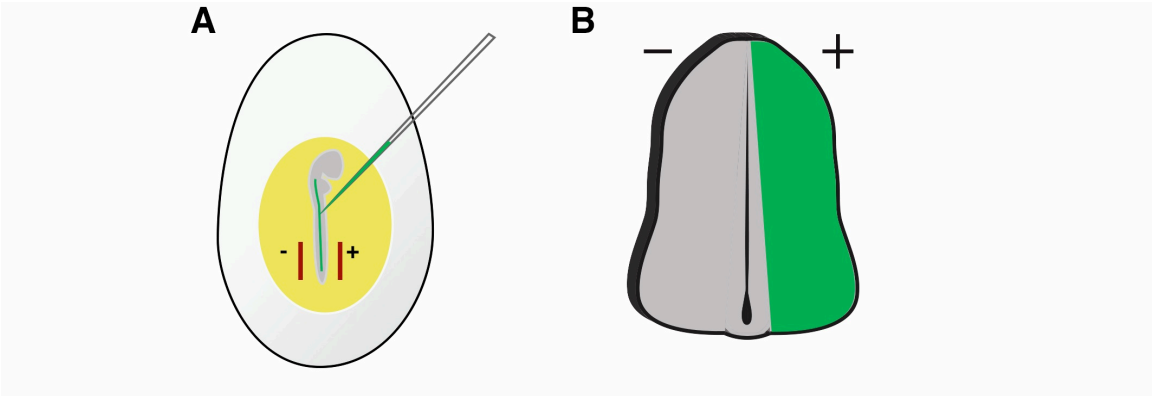

Figure 1.3. Schematic model of in ovo electroporation
(A) Schematic model of in ovo electroporation. DNA or RNA expression constructs are injected into the neural tube lumen of chick embryos at Hamburger Hamilton stages 11-13. A square wave pulse is then immediately applied to the chick embryo to transfect progenitor cells on one half of the developing neural tube.
(B) Illustration of a transverse section of electroporated embryonic chick spinal cord at Hamburger Hamilton stage 25. Only one half of the developing neural tube becomes electroporated (green).

Embryonic stem cells: a model system for studying motor neuron differentiation

Embryonic stem cells (ESCs) were first isolated and cultured from developing mouse embryos in 1981 (Evans and Kaufman 1981; Martin 1981). Further advances in culturing methods allowed for scientists to reliably induce ESCs to differentiate into specific neuronal cell types such as dopaminergic and motor neuron cell lineages (Renoncourt et al. 1998; Wichterle et al. 2002; Lee et al. 2007). The ability to control the differentiation of mouse ESCs in vitro provides essential tool to study the molecular mechanisms that trigger the differentiation of ESCs into specific postmitotic neurons. The first experiments that directed ESC differentiation toward a neuronal phenotype used retinoic acid (RA), which was known to be essential for neurogenesis (Wilkinson et al. 1987; Bain et al. 1996). Further characterization of genes upregulated in RA-induced ESC neurons revealed that the differentiated ESCs resembled a heterogeneous population of interneurons and few motor neurons (Renoncourt et al. 1998).

Interestingly, the ability to induce neuronal markers in RA-differentiated ESCs coincided with a boom in our understanding of the molecular pathways that drive motor neuron specification in the developing spinal cord (Briscoe et al. 1999). Using clues from studies of embryonic spinal cord development, researchers tested whether the sequential application of the morphogens RA and Shh could induce motor neuron differentiation in ESCs. Remarkably, RA and Shh-treated ESCs recapitulated the gene expression observed in ventral spinal cord development, in that different concentrations of Shh could reliably induce motor neuron versus ventral interneuron phenotypes. Additionally, ESC-derived motor neurons could be isolated in vitro and implanted into the developing chick neural tube where they formed synapses with target muscles (Wichterle et al. 2002). This groundbreaking study highlighted how ESCs could be differentiated into specific neuronal cell types using extracellular signaling molecules and provides an essential tool for studying motor neuron differentiation in vitro.

Another key advance was the development of methods to generate transgenic ESCs and inducible transgenic ESCs. In particular, the creation of a doxycycline (Dox) inducible ESC line was a major step in creating robust tools to manipulate ESC gene expression (Iacovino et al. 2011). The transfection efficiency of ESCs is notoriously low and therefore, a method with which the expression of an inserted DNA construct can be precisely and strongly upregulated by Dox was a crucial technological advance. In the context of studying motor neuron differentiation in ESCs, the Dox-inducible system provided a method to test whether the regulation of transcription factors could improve the efficacy of ESC motor neuron differentiation.

Although extracellular signaling molecules RA and Shh induces motor neuron differentiation in ESCs, the percentage of ESCs that express motor neuron markers using this method is relatively low (Wichterle et al. 2002; Lee et al. 2012). The Lee laboratory and independent groups have shown that the co-expression of Isl1 and Lhx3 enhances motor neuron differentiation in numerous contexts (Thaler et al. 2002; Lee et al. 2008; 2009; Son et al. 2011; Hester et al. 2011). To test whether the upregulation of the Isl1-Lhx3 transcription factor complex could improve ESC motor neuron differentiation, the Lee laboratory generated a Dox-inducible ESC line to express an Isl1-Lhx3 fusion protein (Figure 1.4) (Lee et al. 2012). The motor neuron differentiation of Isl1-Lhx3 ESCs showed a ~37% increase in upregulating motor neuron markers compared to the morphogens alone, and when co-cultured with myotubes, the Isl1-Lhx3 ESCs formed neuromuscular synapses (Lee et al. 2012). These results established Isl1-Lhx3 ESCs as a model for studying the molecular mechanisms that govern motor neuron differentiation.

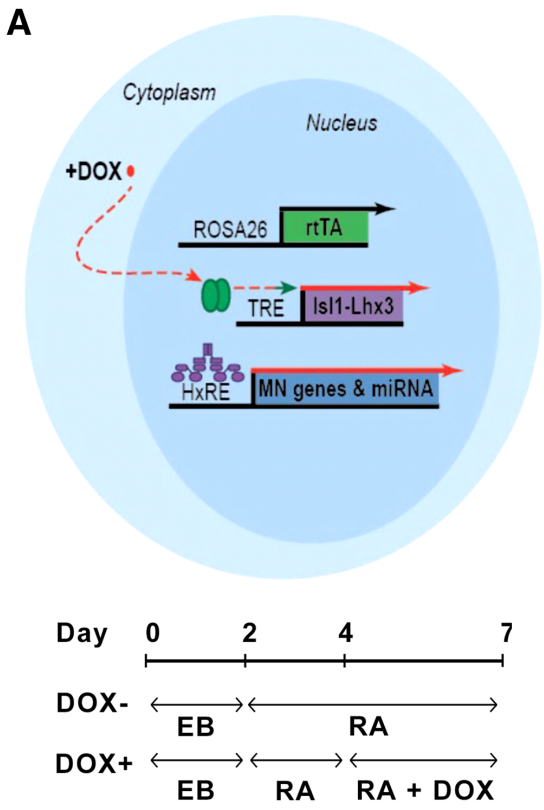

Figure 1.4. Schematic model of Isl1-Lhx3 ESC differentiation.
Schematic model of the doxycycline-inducible Isl1-Lhx3 embryonic stem cell line (Isl1-Lhx3 ESC) and the experimental protocol to differentiate Isl1-Lhx3 ESCs into motor neurons. The treatment of doxycycline (DOX) induces the expression of Isl1-Lhx3 fusion protein, which is controlled by tetracycline response element promoter (TRE). Then, Isl1-Lhx3 forms a hexamer transcription complex with endogenous nuclear LIM interacting protein (NLI) and upregulates its direct target genes that have hexamer response elements (HxRE), such as motor neuron (MN) genes and miRNAs (Lee et al. 2012; 2013). EB, embryoid bodies; RA, retinoic acid.

Clinical relevance of studying motor neuron development

In addition to providing insights into the molecular mechanisms that regulate neurogenesis, motor neuron development is also relevant to clinical research. Motor neurons are required for movement, breathing and swallowing and therefore, the loss of motor neurons due to disease or injury often results in debilitating symptoms or death (Rowland and Shneider 2001; McDonald and Sadowsky 2002; Lunn and Wang 2008). By uncovering the molecular mechanisms that support spinal motor neuron formation, it brings researchers another step closer to identifying innovative ways to prevent their loss

or degeneration due to disease or injury. Thus, our study may contribute to the development of novel therapies for a wide variety of spinal cord injuries and diseases that result from impaired motor function such as pediatric motor neuron diseases, amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease), spinal muscular atrophy (SMA) and post-polio syndrome.

In particular, the molecular mechanisms that govern motor neuron development are relevant to the neurobiology of motor neuron disease and nerve injury where a desired treatment is the generation of new motor neurons from stem cells. The potential for stem cells to be used for cell replacement therapy in humans is an exciting goal of biomedical research. Recent studies have demonstrated that human stem cells can be cultured into spinal motor neurons (Li et al. 2005; Karumbayaram et al. 2009; Hester et al. 2011), and can form neuromuscular junctions when transplanted into chicks and rats (Lee et al. 2007; Su et al. 2013). These human stem cell motor neuron differentiation studies relied on knowledge gained from motor neuron differentiation in animal models and mouse ESCs. Future research to refine our understanding of motor neuron development networks in model systems has the potential improve the differentiation methods that may be used for future human stem cell transplant studies.

Overview of miRNA biogenesis and target recognition

Since their discovery in c. elegans in 1993, miRNAs have emerged as crucial regulators of many biological processes (Wightman et al. 1993; He and Hannon 2004). miRNAs are small noncoding RNA molecules that bind to target mRNAs and prevent translation or trigger degradation of their target transcripts. Canonical miRNA biogenesis begins in the nucleus where pri-miRNAs are transcribed as independent genes (intergenic) by polymerase II or within a host gene (intragenic) by polymerase III (Figure 1.5 A). The pri-miRNA stem loop structure is then cleaved by RNA-binding protein, Drosha, to form an approximately 70 nucleotide long pre-miRNA (Figure 1.5 A) Next, pre-miRNAs are exported to the cytoplasm by Exportin5, where the enzyme Dicer severs the stem loop to generate a pair of ~23 nucleotide long mature miRNAs. A mature miRNA strand is then incorporated into a miRNA-induced silencing complex (miRISC) to mediate postranscriptional repression or degradation of mRNA targets in the cytoplasm

(Figure1.5 A) (Krol et al. 2010). Within miRISC, a mature miRNA is directly bound to Argonaute (Ago), which stabalizes the miRNA-mRNA interaction, and other complex proteins, such as GW182, promote deadenylation and degradation of the mRNA (Figure 1.5 A).

The target selection of miRNAs is dependent on complementary nueclotide matches in specific regions of a miRNA (seed region) and a corresponding mRNA target (Figure 1.5 B). The matching nucleotiedes on a mRNA target are termed miRNA response elements (MREs). Cannonical mRNA targeting requires MREs with continous seed region base-pair matches in nucleotide postitions 2 to 7 at the 5' end of the miRNA (Figure 1.5 B) (Bartel 2009). However, additional pairing of nucleotides at miRNA positions 12-17 can enhance targeting, and mismatches between miRNA nucleotide positions 5-6 form a pivot MRE that can still function as an effective target recognition site (Figure 1.5 C) (Grimson et al. 2007; Chi et al. 2012)

The dominant theory used to predicit miRNA-mRNA interactions is based on the assumption that mRNAs are primarily targeted via MREs that are located within their 3' untranslated regions (UTRs) (Filipowicz et al. 2008; Bartel 2009). However, recent methods to determine direct miRNA-mRNA target interactions have found that the 5'UTRs, coding sequences (CDS) and 3'UTRs of mRNA targets that contain MREs can reliably function as miRNA binding sites (Chi et al. 2009; Hafner et al. 2010; Cambronne et al. 2012). These results suggest that the current methods used to predict miRNA targets based solely on analysis of 3'UTR sequences may exclude numerous potential targets. Overall, since their discovery twenty years ago, miRNAs have been shown to be essential posttrasncriptional repressors that shape the gene expression profiles in many biological contexts (Bartel 2009). However, the characterization of miRNA function is still in its infancy, and future research is needed to improve our understanding of miRNA biogenesis and miRNA-mRNA target interactions.

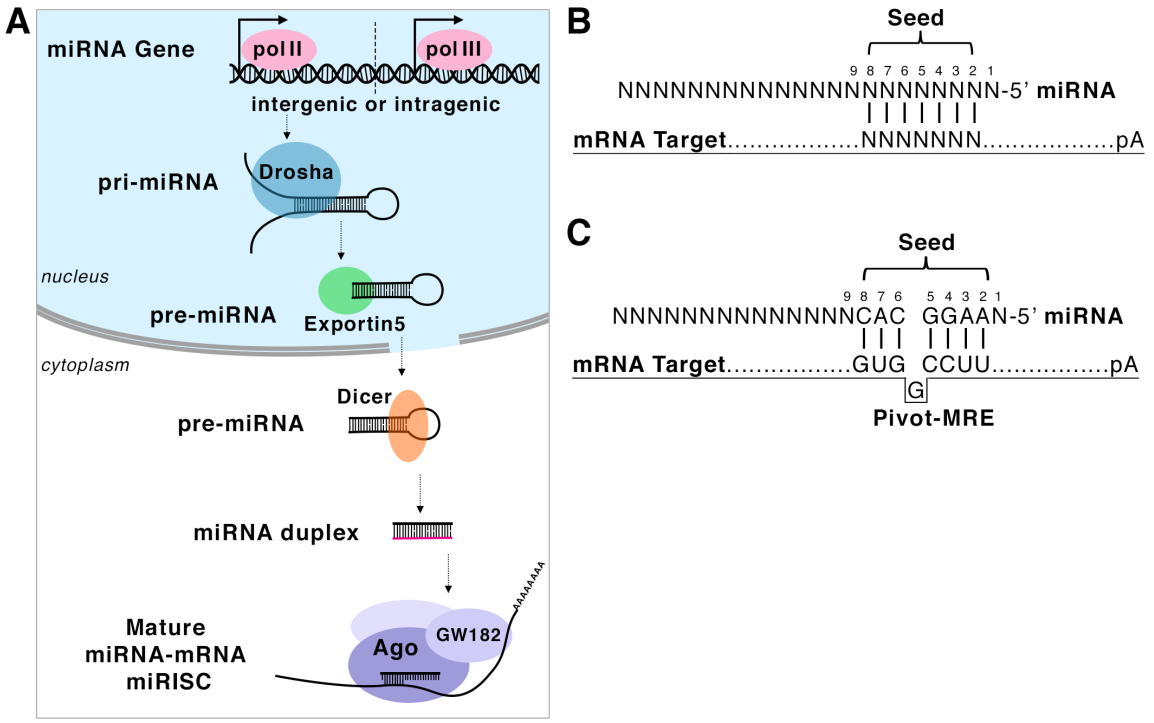

Figure 1.5. Overview of miRNA biogenesis and target mRNA regulation
(A) Schematic model of miRNA biogenesis. miRNAs are transcribed from genomic DNA as an intergenic gene by Polymerase II (pol II) or an intragenic gene, within another gene intron or exon, by Polymerase III (pol III) to form pri-miRNAs. pri-miRNAs are processed by Drosha to form a pre-miRNA, which is then exported to the cytoplasm by Exportin5. The pre-miRNA is then cleaved by Dicer to form a 21-22 nucleotide duplex of two mature miRNA strands. One mature miRNA is then bound to Argonaut (Ago) protein within the miRNA induced silencing complex (miRISC). Within miRISC, miRNAs guide the complex to bind directly to mRNA targets that contain complementary nucleotide sequences. Other miRISC proteins, such as GW182, mediate destabilization and degradation of the mRNA target.
(B) Illustration of a canonical 7-mer miRNA response element (MRE). The mRNA target MRE contains 7 complementary nucleotides to the miRNA seed region, miRNA nucleotide positions 2-8.
(C) Illustration of a pivot miRNA response element (MRE). The mRNA target MRE contains at least 6 complementary nucleotides in the seed region and either a C or G "pivot" nucleotide between miRNA nucleotide positions 5-6.

*Figure adapted from Grimson et al. 2007; Bartel et al. 2009; Chi et al. 2012.

The role of miRNAs during neurogenesis

miRNA regulation of neural stem cells and neurogenesis is a rapidly growing field of research. miRNAs are required for neuronal differentiation in the cortex (Makeyev et al. 2007; De Pietri Tonelli et al. 2008; Davis et al. 2008; Tuncdemir et al. 2014), promote neurogenesis in vitro (Yoo et al. 2011; Victor et al. 2014), and are also important to regulate the transition between neural progenitor proliferation and neurogenesis (Shi et al.

2010; Zhao et al. 2010; Brett et al. 2011; Rago et al. 2014). Of particular interest to my thesis work, recent studies have reported that miR-124, miR-9 and miR-17-3p play important roles in spinal cord neurogenesis. miR-124 is one of the best characterized neurogenic miRNAs and within the developing spinal cord, miR-124 supports neuronal differentiation by repressing the expression of neural progenitor genes (Cao et al. 2007; Visvanathan et al. 2007; Yoo et al. 2009). Another important neurogenic miRNA, miR-9, fine-tunes the specification of motor neuron subtypes (Otaegi et al. 2011a; Luxenhofer et al. 2014). And finally, miR-17-3p regulates neural progenitor patterning in the ventral spinal cord via repression of pMN marker Olig2 (Chen et al. 2011). These experiments suggest that miRNAs play an important role in influencing neuronal fate specification in the spinal cord. However, important questions remain concerning how miRNAs are regulated within these gene regulatory networks and whether additional miRNAs can direct the specification neuronal phenotypes.

Overview of miRNA-218

My thesis work uncovered a novel role for miR-218 in spinal cord motor neuron development. miR-218 has previously been described as a tumor-suppressor that is down regulated in cancerous tissues including glioblastoma , medulloblastoma, and breast cancer (Tu et al. 2013; Venkataraman et al. 2013; Gao and Jin 2014; Mathew et al. 2014). Within the context of cancer research, miR-218 represses numerous targets that promote proliferation and stem cell maintenance and miR-218 is considered a primary miRNA involved in glioblastoma (Tu et al. 2013; Gao and Jin 2014). These previously identified functions of miR-218 are interesting in the context of neuronal differentiation, where miRNAs have been shown to repress cell cycle regulators to stimulate neurogenesis (Dill et al. 2012; Peng et al. 2012; Bersten et al. 2014).

Additionally, there are studies that suggest that miR-218 plays a role in heart development, brain function, and there is one report of miR-218 expression in developing chick spinal cord motor neurons. During zebrafish heart development, miR-218 has been shown to repress the expression of roundabout 1 and 2, (Robo1, Robo2) and T-box gene 5 (Tbx5) to regulate heart field migration and heart tube morphogenesis (Fish et al. 2011; Chiavacci et al. 2012). Also, recent miRNA array studies in the developing brain have

shown that miR-218 is enriched in mouse and human brain (Sempere et al. 2004), and also the hippocampus (Kaalund et al. 2014) and hypothalamus (Sangiao-Alvarellos et al. 2014). Finally, miR-218 expression in chick spinal motor neurons was established using whole embryo chick in situ hybridization to screen the expression of 135 miRNAs, but they did not assess miRNA function (Darnell et al. 2006).

Complications in studying miRNAs

Although miRNAs are essential for numerous biological processes, there are many challenges in studying miRNA function. One of the primary complications is the fact that miRNAs can direct the repression of target mRNAs with as little as six complementary base-pairs in the seed region (Figure 1.5 B) (Grimson et al. 2007). This makes for an astonishingly large number of potential targets for a single miRNA, thus determining authentic miRNA-target interactions has been a tremendous challenge. Bioinformatic algorithms, such as miRANDA, miRBase, TargetScan, PicTar, have been used to predict the presence of conserved MREs within potential target 3'UTRs (Lewis et al. 2003; Bartel 2009). However, these prediction methods do not determine direct target interactions, ignore cellular context, and neglect potential MREs that are non-canonical or located within the coding regions of target mRNAs. To overcome some of these challenges, new immunoprecipitation techniques have been developed to reliably detect miRNA targets in an unbiased manner. These methods use the expression of a miRNA followed by immunoprecipitation (IP) for Ago protein, isolation of co-IPed target mRNAs, and cDNA or deep sequencing analysis (Chi et al. 2009; Hafner et al. 2010; Cambronne et al. 2012). The development of these transcriptome-wide screens has greatly improved our ability to identify direct miRNA-mRNA target interactions and unbaised miRNA regulatory networks.

Another complication in studying miRNAs is the lack of obvious phenotypes in miRNA knockout models. There have been many reports where knocking out miRNA loci in flies, worms, and vertebrates results in no or subtle phenotypes (Liu et al. 2005; Miska et al. 2007; Xiao et al. 2007). Subtle phenotypes are often attributed to gene network compensation, a lack of meaningful situations to assess potential consequences, and the fact that effective target repression may require the action of multiple different

miRNAs (Bartel 2009). Although this challenge is difficult to overcome, alternative experiments that assess individual miRNA action and targets in acute regulatory contexts can provide essential clues about miRNA function.

Thesis Overview

The goal of this thesis was to determine the role of miRNAs in spinal cord motor neuron development. Using a miRNA array screen, I identified miR-218 as the most promising motor neuron miRNA candidate and, in collaboration with others, I performed an extensive characterization of miR-218 regulation, expression, activity, function, and direct targets in the developing chick spinal cord and mouse ESCs. I uncovered a novel role for miR-218 as an essential regulator of motor neuron fate specification downstream of the Isl1-Lhx3 transcription factor complex and I show that miR-218 directly represses numerous target mRNAs that promote interneuron fates (Chapter 2). Additionally, I examined the expression and activity of multiple miRNAs in the developing spinal cord and describe numerous miRNAs that may play important roles during spinal cord development (Chapter 3).

CHAPTER 2

miR-218 is Essential to Establish Motor Neuron Fate as a Downstream Effector of Isl1-Lhx3

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ABSTRACT

While microRNAs have emerged as an important component of gene regulatory networks, how microRNAs collaborate with transcription factors in the gene network that determines neuronal cell fate remains unclear. Here we show that in the developing spinal cord, the expression of miR-218 is directly upregulated by the Isl1-Lhx3 complex, which drives motor neuron fate. Inhibition of miR-218 suppresses the generation of motor neurons in chick neural tube and mouse embryonic stem cells, suggesting that miR-218 plays a crucial role in motor neuron differentiation. Our unbiased RISC-trap screens, in vivo reporter assays, and expression studies revealed that miR-218 directly represses transcripts that promote developmental programs for interneurons and neural progenitors. In addition, miR-218 activity is required for Isl1-Lhx3 to effectively induce motor neurons and suppress interneuron fates. Together, our studies uncovered an essential role for miR-218 as a downstream effector of the Isl1-Lhx3 complex in establishing motor neuron identity.

MATERIALS AND METHODS

DNA and RNA Constructs

Mammalian expression constructs for Isl1, Lhx3, Isl1-Lhx3, and GFP were previously described (Lee et al. 2008; 2012). The generation of the miRNA sensor plasmid was previously described (Cao et al. 2007). miRNA sensor MREs were cloned into the 3'UTR of sensor d4EGFP by multimerization of the following oligos: miR-218 MRE forward 5'- CGC GTA CAT GGT TAG ATC AAG CAC AAG, reverse 5'- CGC GCT TGT GCT TGA TCT AAC CAT GTA; miR-218* MRE forward 5'- CGC GCT TGT GCT TGA TCT AAC CAT GTA, reverse 5'- CGC GC AAC ATG GTT AGA TCA AGC ACA AA.

RISC-trap target miR-218 MRE containing 3'UTRs were amplified from HEK293T cDNA and cloned into the 3'UTR of sensor d4EGFP or luciferase reporter using the following primers: Tead1 forward 5'- GGG AGA GCT GTC TGG TTC, reverse 5'- GG CTC TGG GAA GGC TTC TTT; SLC6A1 forward 5'- GTG CCC TGT AGC TCC TTA GC, reverse 5'- GGG AAG TGG GAC CAT GAG AC; BCL11A forward 5'- CAA AAG CCC TGG AAC GCA AT, reverse 5'- ACA GGC AGA GTC AAG TGC T; Lhx1 forward 5'- CAG ATT TGC AGG GCT TTC GG, reverse 5'- TGC ACT GGA GGT CAC ACA AG; FoxP2 forward 5'- TTT CTG CAT CTG CTT TGC GT, reverse 5'- ACA ACT GTG CCA CGA ATC CA. Target 3'UTR luciferase mutant reporters were generated using overlap extension PCR by combining the previously described flanking primer sets and the following internal primers to mutate the miR-218 MREs: Tead1 forward 5'- TTC CAA GCT AGC AAA ATA CTG G, reverse 5'- TTT TGC TAG CTT GGA AAG GA; SLC6A1 forward 5'-ACA ATA TGC TAG CTA ATA TTC TGA GG, reverse 5'- GAA TAT TAG CTA GCA TAT TGT AGA GAA A; BCL11A forward 5'- TAT AGC TAG CAC GTG GTA CTA TTT GC, reverse 5'- CGT GCT AGC TAT AAA TCA TAT TAT TTT C; Lhx1 forward 5'- GTA TTG CTA GCT TAA TTA TTC TAT TTG G, reverse 5'- TAA TTA AGC TAG CAA TAC TGT AAA GGT G; FoxP2 forward 5'-TGT TGC TAG CTC AGT TTA AAA TTT, reverse 5'- CTG AGC TAG CAA CAT CTG TTT ATG.

Sponge inhibitor constructs were generated by multimerizing 10x bulge sponge sequences that were ordered using GeneArt synthetic gene assembly (Life Technologies)

and cloning the 40X sponge sequences into the 3'UTR of a CMV-LacZ reporter. The following sequences were ordered as synthetic genes: miR-218 bulge sponge 10X – TAG ACA ACA TGG TTT GGG AAG CAC AAT AAT CAA CAT GGT TTG GGA AGC ACA ATA ATC AAC ATG GTT TGG GAA GCA CAA TAA TCA ACAT GGT TTG GGA AGC ACA ATA ATC AAC ATG GTT TGG GAA GCA CAA TAA TCA ACA TGG TTT GGG AAG CAC AAT AAT CAA CAT GGT TTG GGA AGC ACA ATA ATC AAC ATG GTT TGG GAA GCA CAA TAA TCA ACA TGG TTT GGG AAG CAC AAT AAT CAA CAT GGT TTG GGA AGC ACA ATA ATA CTA; Scramble miR-218 bulge sponge 10x – TAG AGA CTA CTA TAC GAG TAA CAG ATA ATG ACT ACT ATA CGA GTA ACA GAT AAT GAC TAC TAT ACG AGT AAC AGA TAA TGA CTA CTA TAC GAG TAA CAG ATA ATG ACT ACT ATA CGA GTA ACA GAT AAT GAC TAC TAT ACG AGT AAC AGA TAA TGA CTA CTA TAC GAG TAA CAG ATA ATG ACT ACT ATA CGA GTA ACA GAT AAT GAC TAC TAT ACG AGT AAC AGA TAA GAC TAC TAT ACG AGT AAC AGA TAA TAC TA. The anti-2'Ome RNA constructs were ordered from Integrated DNA Technologies (IDT) with the following sequences: Anti-miR-218 – mAmCmA mUmGmG mUmUmA mGmAmU mCmAmA mGmCmA mCmAmA mA; Anti-miR-67 – mUmCmU mAmCmU mCmUmU mUmCmU mAmGmGm AmGmGm UmUmGm UmGmA.

The miR-218 and miR-Control expression constructs were generated by annealing and cloning the following oligos into the EFU6-300 hairpin vector: miR-218 fwd 5' – GAT CCA CAT GGT TAG ATC AAG CAC AAT TCA AGA GAT TGT GCT TGA TCT AAC CAT GTT TTT TA; miR-218 rev 5' – AGC TTA AAA AAC ATG GTT AGA TCA AGC ACA ATC TCT TGA ATT GTG CTT GAT CTA ACC ATG TG; miR-Ctrl fwd 5' – GAT CCC CGG CTT ACG CGT TCT CGT CTT CTC TTG AAA GAC GAG AAC GCG TAA GCC GGT TTT TA; miR-Ctrl rev 5' – AGC TTA AAA ACC GGC TTA CGC GTT CTC GTC TTC TCT TGA AAG ACG AGA ACG CGT AAG CCG GG.

Isl1-Lhx3 ESC miRNA Array and Small RNA Quantitative RT-PCR

The generation and differentiation of Isl1-Lhx3 ESCs was previously described (Lee et al. 2012). The miRNA array assays were performed with TaqMan® Array Rodent

MicroRNA Card A (Life Technologies). The miRNA Array analyzes 380 miRNAs and contains five endogenous controls and one negative control assay. RNA extraction and cDNA amplification for TaqMan® miRNA array, miRNA and pri-miRNA qRT-PCR assays were performed according to manufacturer's instructions. (http://www.lifetechnologies.com/us/en/website-overview/ab-welcome.html).

In Ovo Electroporation and Immunohistochemistry

Expression constructs were injected into the lumens of chick embryonic spinal cords at Hamburger Hamilton stages 12-14 (Hamburger and Hamilton 1951). Electroporation was performed using a square wave electroporator (BTX) as previously described (Nakamura and Funahashi 2001). Incubated chicks were harvested and analyzed at Hamburger Hamilton stages 17-30, fixed in 4% paraformaldehyde, and cryosectioned at 12 um. Immunohistochemistry was performed using 0.1% Fish gelatin (Sigma) blocking buffer with overnight incubation at 4 degrees Celsius, using the following primary antibodies: mouse anti-Hb9/MNR2 (DSHB, 5C10), rabbit anti-Pax2 (Zymed), rabbit anti-Lhx1/Lim1-2 (Tsuchida et al. 1994), rabbit anti-FoxP2 (Abcam), rabbit anti-Lhx3 (Tsuchida et al. 1994), rabbit anti-Olig2 (Lee et al. 2012), rabbit anti-Ngn2 (Zhou and Anderson 2002), chicken anti-GFP (AVES). Quantification of chick electroporation data where n = number of embryos included in the analyses, with 2-3 sections quantified per embryo. The methods used for statistical analyses are listed within each figure legend.

RISC-trap, RNA Extraction and Quantitative RT-PCR

RISC-trap experiments and data analyses were performed as previously described (Cambronne et al. 2012), except that reads for each gene were counted by HTSeq (Simon.Huber.2013_HTSeq – A Python framework to work with high-throughput sequencing data_BioRxiv002824). For independent miR-218 and miR-181 RISC-trap assays, transfections and immunoprecipitations were performed as previously described (Cambronne et al. 2012) and Maxima H Minus (Thermo Scientific) was used for reverse transcription. The levels of mRNA were determined with quantitative RT-PCR using SYBR-Green kit (Invitrogen) and Mx3000P (Stratagene).

The following primers were used for RT-PCR: Cyclophilin A forward, 5'- GAT

GCC AGG ACC TGT ATG CT, reverse 5'- GTC TCC TTC GAG CTG TTT GC; Tead1 forward 5'- CAC CTG CAT CCT CTT GCT CA, reverse 5'- GAG AAG CCC ACT GGG ATG AC; SLC6A1 forward 5' – TGT TCT TCC GTG GAG TGA CG, reverse 5'- GAC GAA TCC TGC GAA CAT GC; BCL11A forward 5' – GGG AGC ACG CCC CAT ATT AG, reverse 5'- GCA CAG GCA TAG TTG CAC AG; Lhx1 forward 5' – TCA TCC CCT GGG CTC TAC TT, reverse 5'- GGT ACC GAA ACA CCG GAA GA; FoxP2 forward 5' – AGT GCA AGA CGA GAC AGC TC, reverse 5'- CGG TCA TCC AAT GCG TGT TC; GLCE forward 5'- CGT GCC TTA ACA ATG TGG CTG TCC, reverse 5'- TGC TGT TGC AAT GTG GAA GGC AGT; RFT1 forward 5'- TCA GAA GCA GGA GGA CGT TG, reverse 5'- AGC ATG GTC CCT CCG TAG AT.

Luciferase Reporter Assays

HEK293T cells were cultured in DMEM media supplemented with 10% fetal bovine serum. Cells were plated in 48-well plate and incubated for 24 hr and transient transfections were performed using Lipofectamine 2000 (Invitrogen). An actin-β-galactosidase plasmid was cotransfected for normalization of transfection efficiency and 20 nM of miRIDIAN microRNA mimics (Dharmacon) were used. Cells were harvested 24 hr after transfection. Cell extracts were assayed for luciferase activity and the values were normalized with β-galactosidase activity, vector and miR-181 treated reporter relative luciferase units. All transfections were repeated independently at least three times. Data are represented as the mean of triplicate values obtained from representative experiments. Error bars represent standard deviation.

Quantification of Pixel Intensity

In ovo electroporation of miRNA sensor plasmids (1.2 μg/μl) was performed as described (Thaler et al. 2002). Unsaturated images were acquired on a Zeiss Axio Imager.Z2 microscope, maintaining the same exposure time ratio of GFP and RFP for each section. Pixel intensity was determined using an ImageJ analysis script, which was developed by Dr. Greg Scott, using selections on an 8-bit image stack that contains unadjusted RFP, GFP and DAPI channel images. To use the ImageJ script, the 8-bit stack must contain the RFP, GFP and DAPI channels in the proper order where RFP = channel 1, GFP =

channel 2, and DAPI = channel 3, respectively. Using ImageJ software, open an image stack and select the DAPI channel image. Create a selection using the free-hand selection tool to outline the desired area for pixel intensity measurement. Next, click on the "Plugins" in the toolbar, select "Macros" and then "Run" from the dropdown menus. Select the ImjageJ script text shown below (saved as .txt file). Double click on this program text file and it will automatically use the area that was selected in the DAPI panel to define the pixel selection mask in the RFP image. Next, the program will measure the RFP pixel intensity within the selected area, excluding background with a min/max threshold of 20/255. Next, the program applies the same area selection mask to the GFP channel and measures pixel intensity, excluding background with a min/max threshold of 20/255. The program then calculates the average GFP divided by RFP pixel intensity ratio and provides the output with sample number, area measured, GFP/RFP pixel intensity, as well as minimum and maximum GFP/RFP pixel intensity ratios. The final text in the script closes the remaining open images.

```
//CH1 = RFP, CH2 = GFP, CH3
= DAPI
//Create selection in DAPI
channel before use of this
//8-bit conversion
run("8-bit");
//Grab Titles/Slice Names
parent=getTitle();
setSlice(1);
setMetadata("Label",
"channel1");
imc1=getInfo("slice.label");
setSlice(2);
setMetadata("Label",
"channel2");
imc2=getInfo("slice.label");
setSlice(3);
setMetadata("Label",
"channel3");
imc3=getInfo("slice.label");
//Clear Data Outside DAPI
region selection
run("Create Mask");
mask1=getTitle();
imageCalculator("AND stack",
parent,mask1);
selectImage(mask1);
run("Close");
selectImage(parent);
//run("Clear Outside", "stack");
//Create mask from threshold of
RFP slice
run("Select None");

setSlice(1);
setThreshold(20, 255);
run("Create Selection");
slxn=getInfo("selection.name")
run("Create Mask");
mask2=getTitle();
//Mask image stack with RFP
slice mask
imageCalculator("AND stack",
parent,mask2);
selectImage(parent);
resetThreshold();
run("Select None");
//Delete DAPI channel
setSlice(3);
run("Delete Slice");
//Split channels
run("Stack to Images");
selectImage(imc1);
//divide GFP by RFP
imageCalculator("Divide create
32-bit", imc2,imc1);
quotient=getTitle();
selectImage(quotient);
run("Measure");
//CLOSE REMAINING
IMAgES
selectImage(mask2);
run("Close");
selectImage(imc1);
run("Close");
selectImage(imc2);
run("Close");
selectImage(quotient);
run("Close");

//END OF SCRIPT.  SCRAPS
BELOW

//imc1=getInfo("slice.label");
//print(imc1);
//run("Stack to Images");
//selectimage(imc1);
//joe=getTitle();
//print(joe);
//imid1 = getImageID();
//selectimage();
//run("Create Mask");
//run("Stack to Images");
//setSlice(4);
//run("Delete Sl
```

In Situ Hybridization Assay

For in situ hybridization analysis, embryos were harvested at indicated stage, fixed in 4% formaldehyde, embedded in OCT, and cryosectioned at 18 μm. Locked nucleic acid (LNA)-modified miR-218 or miR-218* oligonucleotide probe (Exiqon) was labeled with digoxigenin according to the suppliers protocol (Roche) and used for in situ hybridization as described (Kloosterman et al. 2006).

Gene Ontology and MRE Analyses

The Gene Ontology (GO) functional analysis was carried out with the online tool DAVID using the default configurations (Huang et al. 2007). For the MRE-directed search analyses, the 3'UTR, coding sequences (CDS), 5'UTR or the whole transcripts were directly searched by the miR218 MREs, including 8mer (AAGCACAA), 7mer (AAGCACA), 6mer (AGCACA) and 7mer pivots (AAGgCACA or AAGcCACA).

Mouse Models

Generation of $Olig2^{Cre/+}$(Dessaud et al. 2007; Chen and Wichterle 2012), $Dicer^{flox/flox}$ (Harfe et al. 2005) and the $Slit2^{-/-}$ and $Slit3^{-/-}$ (Nguyen-Ba-Charvet et al. 2002; Yuan et al. 2003; Long et al. 2004) mice were previously reported.

ESC Culture, Generation, and Differentiation of ESCs

The A172LoxP ES cell line was maintained in an undifferentiated state on 0.1% gelatin-coated dishes in the ES cell growth medium that consisted of knockout DMEM, 10% FBS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and recombinant leukemia inhibitory factor (LIF) (1,000 units/mL, Chemicon). GFP-miR218 and GFP-scramble were inserted into Tet-uninducible p2Lox vector. GFP-miR-218 sponge inhibitor and GFP-scramble sponge inhibitor constructs were inserted into Tet-inducible plasmid p2Lox.Then these constructs were cotransfected with pSALK-Cre into ESC lines by electroporation. Stable transfectants were isolated by selection with neomycin (G418, 400 μg/mL) for 7 days. Dox-dependent induction of GFP-miR218 sponge inhibitor and GFP-miR-scramble sponge inhibitor was monitored by western blotting and immunohistochemical analyses using α-GFP antibody. For motor neuronal

differentiation assays, GFP-miR218 sponge inhibitor and GFP-miR-scramble sponge inhibitor ESCs were trypsinized and grown in the ESC growth medium without LIF in suspension as cell aggregates for 2 days. The ESC embryoid bodies (EBs) were treated with all-trans RA (0.5 μM) and a Shh agonist Purmorphamine (1 μM, Calbiochem) for 2 days. Then, RA and Purm-treated EBs were cultured without or with doxycycline (2 μg/mL) in the presence of RA and Purmorphamine for another 2 days. For neuronal differentiation of GFP-miR218 and GFP-scramble ESC, cell aggregates were treated with all-trans RA (0.5 μM) for 4 days. Immunohistochemistry was performed using the following antibodies: guinea pig anti-Hb9 (home-made), mouse anti-TuJ1 (Covance), rabbit anti-FoxP2 (Abcam) and rabbit anti-Pax2 (Zymed).

Chromatin immunoprecipitation (ChIP) assays

ChIP was performed as described previously (Joshi et al. 2009; Lee et al. 2013) in Isl1-Lhx3 ESCs and mouse embryonic spinal cord cells. Isl1-Lhx3 ESCs were cultured on 0.1% gelatin-coated dishes in the ESC growth media lacking LIF in the presence or absence of doxycycline (2 μg/mL), which induces the expression of Flag-tagged Isl1-Lhx3, for 1 d. The spinal cords were micro-dissected from E12.5 mouse embryos and cells were dissociated and subject to ChIP assays. Cells were washed with PBS buffer, fixed by 1% formaldehyde for 10 min at room temperature, and quenched by 125 mM glycine. Cells were washed with Buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5) and Buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Hepes, pH 6.5) sequentially. Then, cells were lysed with lysis buffer (0.5% SDS, 5 mM EDTA, 50 mM Tris·HCl, pH 8.0, protease inhibitor mixture) and were subjected to sonication for DNA shearing. Next, cell lysates were diluted 1:10 in ChIP buffer (0.5% Triton X-100, 2 mM EDTA, 100 mM NaCl, 50 mM Tris·HCl, pH 8.0, protease inhibitor mixture) and, for immunoclearing, were incubated with IgG and protein A agarose beads for 1 h at 4 °C. Supernatant was collected after quick spin and incubated with anti-Flag antibody (Sigma) and protein A agarose beads to precipitate Flag-Isl1-Lhx3/chromatin complex overnight at 4 °C. After pull-down of Flag-Isl1-Lhx3/chromatin/antibody complex with protein A agarose beads, the beads were washed with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl, pH 8.0, 150 mM NaCl), TSE II (same

components as in TSE I except 500 mM NaCl), and Buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris·HCl, pH 8.0) sequentially for 10 min at each step. Then the beads were washed with Tris-EDTA (TE) buffer three times. Flag-Isl1-Lhx3/chromatin complexes were eluted in elution buffer (1% SDS, 1 mM EDTA, 0.1 M NaHCO3, 50 mM Tris·HCl, pH 8.0) and decross-linked by incubating at 65 °C overnight. Eluate was incubated at 50 °C for more than 2 h with Proteinase K. Next, DNA was purified with Phenol/chloroform. Immunoprecipitation were performed using anti-IgG, anti-Isl1 and anti-Lhx3 antibodies for mouse embryonic spinal cord. The following primers were used for ChIP PCR: miR-218-1 Peak A forward, 5'- ATA TAA AAC CCA TTA ATC CAA GCC, reverse, 5'- AAG GGT AAA TCT AAG CTT CAA GGT; miR-218-2 Peak A forward, 5'- AGA GCA GTG ACC TCC AAT GAT TTA, reverse, 5'- TGC TCT GTC TCT TCT CTC TGA CTG; miR-218-2 Peak B forward, 5'- GCT ATT CTA TGG GAA ATG GCT TGG, reverse, 5'- GCT GTA CAT CCT TCT GGA GAG AGT.

INTRODUCTION

Throughout development of the central nervous system (CNS), a vast number of neuronal types are produced with striking precision. Understanding the intricate gene regulatory networks, which establish the unique identity of each neuronal cell type and eventually lead to the great cellular complexity in the CNS, is an important topic in neurobiology.

In the developing spinal cord, neuronal cell fate specification is initiated by the integration of morphogen gradients that direct the patterning of progenitor domains, each of which gives rise to a specific neuronal type (Jessell 2000; Lee and Pfaff 2001; Helms and Johnson 2003). The boundaries of the progenitor domains are sharpened by cross-repressive interactions between transcription factors that are expressed in neighboring progenitor domains (Briscoe et al. 2000; Muhr et al. 2001; Novitch et al. 2001; Lee and Pfaff 2001). As progenitor cells exit the cell cycle, transcription factors that promote the differentiation of distinct interneuron types and motor neurons are upregulated (Helms and Johnson 2003; Lee and Pfaff 2003). Two LIM-homeodomain factors, LIM homeobox 3 (Lhx3) and Islet-1 (Isl1) are co-expressed in differentiating motor neurons,

while Lhx3, but not Isl1, is expressed in newly born V2 interneurons (Tsuchida et al. 1994; Sharma et al. 1998; Thaler et al. 1999). Isl1 and Lhx3 form a hexameric Isl1-Lhx3 transcription complex with nuclear LIM interactor (NLI) (Figure 2.1 A) (Thaler et al. 2002). The co-expression of Isl1 and Lhx3, along with neurogenic factors, triggers the generation of motor neurons in various cellular contexts, such as the dorsal spinal cord, embryonic stem cells (ESCs), and induced pluripotent stem cells (Thaler et al. 2002; Lee and Pfaff 2003; Lee et al. 2008; 2012; Lee et al. 2009; Hester et al. 2011; Son et al. 2011; Mazzoni et al. 2013; Cho et al. 2014). This potent activity of Isl1-Lhx3 that drives motor neuron fate specification is partly attributed to the fact that Isl1-Lhx3 directly binds and robustly upregulates a wide range of terminal differentiation genes, including a battery of cholinergic pathway genes that enable cholinergic neurotransmission (Lee et al. 2012; 2013; Mazzoni et al. 2013; Cho et al. 2014). Another critical factor is that the Isl1-Lhx3 complex inhibits the acquisition of non-motor neuron fates, as evident from the observation that Isl1-Lhx3 suppresses the interneuron programs in ESCs that are directed to differentiate into neurons (Lee et al. 2012). However, the mechanisms by which Isl1-Lhx3 represses interneuron differentiation or progenitor fate remain unknown.

MicroRNAs (miRNAs) are small RNA molecules that bind to target mRNAs and prevent translation or trigger degradation of their target transcripts (Bartel 2009). A growing body of research has established that miRNAs serve as a crucial constituent of gene regulatory networks. Recent studies of miRNAs in the developing spinal cord have revealed that miR-124 and miR-17-3p play a role in neuronal differentiation and progenitor domain patterning, respectively (Cao et al. 2007; Visvanathan et al. 2007; Chen et al. 2011), and that miR-9 is involved in fine-tuning the differentiation of motor neuron subtypes (Otaegi et al. 2011a; Luxenhofer et al. 2014). However, what remains unclear is how miRNAs are interconnected with cell fate-specifying transcription factors in the regulatory networks that determine neuronal cell fates in CNS development.

In this study, we investigated the role of miRNAs in the gene networks that specify motor neuron fate. We found that a single miRNA, miR-218, is highly and directly upregulated by Isl1-Lhx3 at the onset of motor neuron differentiation and that miR-218 is specifically expressed in motor neurons throughout spinal cord development. We also found that miR-218 is essential for the generation of motor neurons both in vitro

and in vivo. Our RISC-trap screen revealed many direct miR-218 target transcripts whose primary function is to promote interneuron or neural progenitor characteristics in the spinal cord. Together, our results suggest that miR-218 functions as a crucial downstream effector of the Isl1-Lhx3 complex in establishing motor neuron identity by downregulating genes that promote non-motor neuron fates. Our study highlights an intricate gene regulatory network in which cell fate-specifying transcription factors cooperate with downstream miRNAs to define the gene expression profile for an appropriate cell fate.

RESULTS

miR-218 is highly upregulated during motor neuron differentiation

To identify miRNAs that play a role in promoting motor neuron cell fate, we took advantage of Isl1-Lhx3 ESCs, which are a robust model of motor neuron differentiation. Isl1-Lhx3 ESCs express an Isl1-Lhx3 fusion protein upon doxycycline (Dox) treatment, which forms the Isl1-Lhx3 hexamer complex with endogenous NLI (Figure 2.1 A,B) (Lee et al. 2012; 2013). Upon the treatment of Dox and retinoic acid (RA) following the formation of embryoid bodies (EBs), Isl1-Lhx3 ESCs differentiate into motor neurons, which express numerous motor neuron markers and form neuromuscular junctions with myotubes (Lee et al. 2012). To systemically monitor the expression pattern of miRNAs during motor neuron differentiation in Isl1-Lhx3 ESCs in an unbiased manner, we determined the expression profiles of miRNAs in RA-treated Isl1-Lhx3 ESCs, incubated either with or without Dox (i.e. expression of Isl1-Lhx3), using a TaqMan miRNA array. Pairwise comparison of miRNA arrays revealed that 18 miRNAs are induced more than 3 fold (Figure 2.1 C). Interestingly, a single miRNA, miR-218, showed a remarkable ~ 71 fold induction, while the next highest induced miRNA, miR-382, was upregulated by ~ 9 fold. Additionally, miR-218 was the fifth highest expressed miRNA in Dox-treated conditions, indicating that miR-218 is abundantly present in embryonic motor neurons (Figure 2.1 C). The expression levels of other miRNAs, which are implicated in the development of motor neurons, such as miR-9 (Otaegi et al. 2011a; Luxenhofer et al. 2014), miR-124 (Cao et al. 2007; Visvanathan et al. 2007), and the miR-17-92 cluster were unaltered between Dox-treated versus Dox-untreated conditions (Chen et al. 2011).

The relative level of miR-218 was 16-fold higher than miR-9 in the motor neuron differentiation conditions, supporting a prominent role for miR-218 in motor neurons (Supplementary Data 1). To validate the miRNA array results, we performed independent TaqMan quantitative PCR (qPCR) analyses and confirmed the robust upregulation of miR-218 in Dox-treated conditions (Figure 2.1 D). These results suggest that miR-218 may play a role in motor neuron cell fate specification downstream of the Isl1-Lhx3 complex.

Isl1-Lhx3 directly binds and upregulates miR-218-1 and miR-218-2 genes

miR-218 is an evolutionarily conserved miRNA that is encoded in the introns of Slit2 and Slit3 genes, which produce miRNA precursor hairpins pri-miR-218-1 and pri-miR-218-2, respectively (Figure 2.2 A-C). To test whether the Isl1-Lhx3 complex directly regulates the expression of miR-218 genes, we analyzed the genome-wide binding pattern of Isl1-Lhx3 using three independent chromatin immunoprecipitation deep sequencing (ChIP-seq) datasets (Lee et al. 2013; Mazzoni et al. 2013). Interestingly, Isl1-Lhx3-bound ChIP-seq peaks were found in the introns of both Slit2 and Slit3 genes (Figures 2.3 A). Our ChIP analyses in Isl1-Lhx3 ESCs further confirmed that Isl1-Lhx3 binds to the ChIP-seq peaks in Slit2 and Slit3 genes (Figure 2.3 B). Next, the ChIP assays in E12.5 mouse spinal cord using anti-Isl1 and anti-Lhx3 antibodies revealed that both Isl1 and Lhx3 are recruited to the ChIP-seq peaks in Slit2 and Slit3 genes in vivo (Figure 2.3 C). The binding of Isl1-Lhx3 to miR-218-1 and miR-218-2 loci suggests that a remarkable upregulation of mature miR-218 by Isl1-Lhx3 is due to the induction of both miR-218 genes. Indeed, both miR-218-1 and miR-218-2 pri-miRNAs were markedly upregulated during Isl1-Lhx3-directed motor neuron differentiation of ESCs (Figure 2.3 D).

Next, to test whether the Isl1-Lhx3-mediated induction of miR-218 genes is a direct outcome of recruitment of Isl1-Lhx3 to the genes, which triggers transcriptional activation of the miR-218 genes, or an indirect result of motor neuron differentiation, we monitored the miR-218 levels in ESCs cultured in monolayer. Under this condition, Isl1-Lhx3 expression does not induce motor neuron differentiation (data not shown). Interestingly, however, Isl1-Lhx3 expression still led to a drastic upregulation of miR-218 within 48 hours of Dox treatment (Figure 2.3 E), indicating that Isl1-Lhx3-mediated

induction of miR-218 is not dependent on motor neuron differentiation. Together, our data demonstrate that the Isl1-Lhx3 complex directly upregulates both miR-218-1 and miR-218-2 genes during motor neuron differentiation (Figure 2.3 F).

miR-218 is specifically expressed in motor neurons and induced by Isl1-Lhx3 in vivo

The robust upregulation of miR-218 in ESC-derived motor neurons prompted us to investigate the in vivo expression pattern of miR-218 in developing embryos. In situ hybridization analyses revealed that miR-218 is specifically expressed in motor neurons during motor neuron cell fate specification (Figure 2.4 A-D). miR-218 expression is barely detected in the ventral neural tube at chick Hamburger Hamilton stage 17 (st.17) and mouse embryonic day 9.5 (E9.5), stages in which few postmitotic motor neurons are present, but the expression is specifically induced in motor neurons by chick Hamburger Hamilton stage 20 (st.20) and mouse embryonic day 10.5 (E10.5) (Figure 2.4 C,D). Additionally, miR-218 maintains its motor neuron-specific expression pattern in the spinal cord throughout mouse embryonic development (Figure 2.4 D).

The specific and robust upregulation of miR-218 in newly born motor neurons during spinal cord development, along with a marked and direct induction of miR-218 by Isl1-Lhx3 during motor neurogenesis of ESCs, point to the possibility that miR-218 functions downstream of the Isl1-Lhx3 complex in developing motor neurons. To test this possibility in vivo, we misexpressed Isl1-Lhx3 in the chick neural tube and monitored the expression patterns of miR-218 using in situ hybridization analyses. Isl1-Lhx3 ectopically induced the expression of miR-218 in the dorsal spinal cord, in a pattern that closely overlaps with the formation of ectopic $Hb9^{+}$ motor neurons (Figure 2.4 E,F). These results suggest that miR-218 is expressed downstream of the Isl1-Lhx3 complex in the course of motor neuron differentiation.

miR-218 is specifically active in motor neurons

To determine where endogenous miR-218 actively suppresses target gene expression in vivo, we used miRNA sensor plasmids, in which a cytomegalovirus/chicken β actin (CAG) promoter drives the expression of a destabilized nuclear GFP with a half-life of 4 hours (d4EGFP) that is linked with complete complementary miRNA response elements

(MREs). The miRNA sensor plasmids also have another CAG promoter directing the expression of monomeric nuclear RFP (mRFPn) (Figure 2.5 A,B) (Cao et al. 2007). To assess the endogenous activity of miR-218 in embryonic spinal cord, we electroporated chick neural tubes with either miRNA sensor vector or miR-218 sensor, in which miR-218 MREs are inserted into the 3' untranslated region (3'UTR) of the d4EGFP. We then monitored the expression levels of GFP compared to RFP, which labels all transfected cells, three days after electroporation. In ovo electroporation of miRNA sensor vector resulted in the expression of GFP and RFP in similar ratios throughout the spinal cord (Figure 2.5 B,E). In contrast, the miR-218 sensor showed a drastic downregulation of GFP specifically in motor neurons, compared to interneurons (Figure 2.5 C,E), indicating that endogenous miR-218 actively represses target genes with miR-218 MREs in developing motor neurons.

Both strands of a miRNA precursor hairpin can be expressed and actively repress mRNA targets (Yoo et al. 2009). To test whether the complementary, miR-218-3p "star" strand is also active in motor neurons, we generated a miR-218-star sensor. The chick spinal cord electroporated with the miR-218-star sensor did not exhibit any regional differences between motor neurons and interneurons in GFP/RFP pixel intensity (Figure 2.5 D,E), indicating that the complementary miR-218-star strand is not functional in the developing spinal cord. Consistently, in situ hybridization analyses with a miR-218-star probe did not develop signal in the spinal cord of chicken and mouse embryos (data not shown). Together, these results demonstrate that miR-218, but not miR-218-star, is selectively expressed and actively represses its target genes with miR-218-MRE in motor neurons within the developing spinal cord.

miR-218 is important for the generation of spinal cord motor neurons

Next, to inhibit the action of endogenous miR-218, we designed miRNA bulge sponge inhibitors that function as stable and competitive miRNA inhibitors both in vitro and in vivo (Ebert et al. 2007; Otaegi et al. 2011b; Kluiver et al. 2012). The miR-218 sponge inhibitor has 40 repeats of bulged miR-218 MRE sequences that are cloned into the 3'UTR of a CMV-promoter driven LacZ gene, whereas the control scrambled sponge has 40 repeats of scrambled (scrm) bulge miR-218 MRE sequences (Figure 2.6 A). To

enhance the inhibition of endogenous miR-218, we combined the miR-218 sponge inhibitor with 2'-O-methylated (2'Ome) antisense RNA inhibitors, which function as specific miRNA inhibitors in vivo (Schratt et al. 2006). Co-electroporation of the miR-218 sponge inhibitor with the 2'Ome-miR-218-inhibitor resulted in a significant 10% reduction of motor neuron generation in the developing spinal cord compared to the control condition (Figures 2.6 B,C), although this miR-218 loss-of-function condition only partially suppressed the action of miR-218 as analyzed by miRNA sensor plasmids (data not shown). The miR-218 loss-of-function condition did not decrease the number of $Pax2^+$ or $Lhx1^+$ spinal interneurons or $Olig2^+$ motor neuron progenitors (Figures 2.6 D-I), suggesting that the inhibitory effect of miR-218 blockade on neuronal fate specification is specific to motor neurons. In addition, there was no difference in the apoptotic cell death between miR-218 inhibition and control conditions, determined by immunohistochemical analyses with activated-Caspase3 antibody (data not shown). This indicates that the loss of motor neurons upon miR-218 inhibition is not due to increased cell death. Together, our data provide in vivo evidence that miR-218 plays an important role in the specification of motor neurons.

miR-218 is essential for the generation of motor neurons from ESCs

To further investigate whether miR-218 is important for motor neuron specification, we sought a cellular context in which miR-218 might be inhibited more efficiently. ESCs differentiate into motor neurons when embryoid bodies are formed and treated with retinoic acid and a sonic hedgehog agonist (Wichterle et al. 2002). This method recapitulates the in vivo differentiation process, and allows for motor neuron differentiation to progress in a relatively synchronized fashion through neuroepithelial, spinal neural progenitor and eventually motor neuron fates. We first tested whether miR-218 was upregulated in this model of motor neuron differentiation. The level of miR-218 expression was low in ESCs and was low in embryoid bodies but miR-218 was upregulated ~ 27 fold when ESCs acquire motor neuron characteristics, including the expression of Isl1, Lhx3 and Hb9 (Figure 2.7 A, data not shown). These results are consistent with the motor neuron-specific expression of miR-218 in the developing spinal

cord (Figure 2.4) and the upregulation of miR-218 during Isl1-Lhx3-directed motor neuron differentiation in ESCs (Figure 2.1).

To test whether a robust upregulation of miR-218 is important for the specification of motor neuron fate, we took advantage of the ESC-derived motor neuron differentiation paradigm, which enables the inhibition of miR-218 action at a time when miR-218 begins to be induced in newly born motor neurons (Wichterle et al. 2002). To control the precise timing of miR-218 inhibition, we generated a mouse ESC line, in which Dox induces the expression of miR-218 sponge inhibitor linked to the GFP gene (Figure 2.7 B). In parallel, we also created a control ESC line that expresses scrambled sponge inhibitor in a Dox-dependent manner (Figure 2.7 B). In the absence of Dox, both ESC lines exhibited effective motor neuron differentiation, as determined by the expression of motor neuron markers including Hb9 (Figure 2.7 C). The miR-218 sponge inhibitor, which is induced by Dox in miR-218 sponge ESCs, strongly suppressed the generation of $Hb9^{+}$ motor neurons, compared to the scrambled control inhibitor (Figures 2.7 C,D). Furthermore, a majority of $Hb9^{+}$ motor neurons produced under miR-218 inhibition condition lacked GFP expression (Figures 2.7 C,E), indicating that miR-218 sponge inhibitor-expressing cells are resistant to motor neuron differentiation. Together, our data demonstrate that a high level of miR-218 activity is critical for the specification of motor neuron fate at the onset of motor neurogenesis.

Mouse knockout models express miR-218

To assess whether miR-218 was important for mouse spinal cord development, we generated two potential miRNA knockout mouse models. The first motor neuron miRNA mouse model was previously described and uses $Olig2^{Cre}$ to drive the deletion of Dicer, an enzyme that is essential for canonical miRNA biogenesis, in the motor neuron progenitor domain. Motor neuron analyses in these mice revealed a significant loss of lateral motor column (LMC) motor neurons, which was attributed to the loss of miR-9 expression (Chen and Wichterle 2012). To test whether these motor neuron miRNA knockout mice also lacked miR-218 expression, we performed in situ hybridization for miR-218 in $Olig2^{Cre}$ $Dicer^{flox/flox}$ knockout mice and $Dicer^{flox/flox}$ littermate controls. These results show that miR-218 expression is decreased, but not absent in the $Olig2^{Cre}$

Dicer$^{flox/flox}$ miRNA knockout mouse (Figure 2.8 A). Next, we examined the miR-218 expression in Slit2 and Slit3 knockout mice. Slit2 and Slit3 are the host genes for miR-218-1 and miR-218-2, respectively (Figure 2.3). Our results show that miR-218 is strongly expressed in both single and double Slit2 and Slit3 knockout mice (Figure 2.8 B, data not shown). From these data, we conclude that these mouse models are not suitable for robust miR-218 loss-of-function analyses.

Identification of direct miR-218 target mRNAs

To understand the mechanisms by which miR-218 contributes to the specification of the motor neuron identity, it is important to determine miR-218 target mRNAs that directly bind to miR-218 in a genome-wide manner. To this end, we employed a RISC-trap assay, which effectively detects the interactions between miRNA and its target transcripts, even when the target mRNAs are present at low abundance (Cambronne et al. 2012). miRNAs regulate target mRNAs via a miRNA-induced silencing complex (miRISC), in which a mature miRNA directly binds to Argonaute that interacts with GW182, leading to destabilization of the mRNA transcripts (Figure 2.9 A). The RISC-trap assay utilizes a dominant negative form of GW182 (dnGW182), which is incorporated into miRISC and stabilizes the RISC-miRNA-mRNA intereaction. We expressed Flag-tagged dnGW182 along with miR-218 in HEK293T cells, where many targets of neuronal miRNAs were successfully identified in this cell line (Cambronne et al. 2012). In this condition, dnGW182-incorporated RISC is preferentially loaded with miR-218 and reduces degradation of target transcripts. We then immunopurified RISC using anti-Flag antibodies and isolated co-purified mRNAs, which were then subject to sequencing on a HiSeq platform. To identify miR-218-specific target transcripts, the RISC-trap datasets for miR-218 were compared against previously generated RISC-trap datasets of three miRNAs, miR-181, miR-124 and miR-132, by applying statistical methods for cross-comparison of datasets that allow for strong signal-to-noise isolation of transcripts (Cambronne et al. 2012). Significantly enriched transcripts for miR-218 were determined with pairwise comparison with each of three miRNAs using an ANOVA (FDR < .05) and a stringent four-fold enrichment cutoff. This analysis revealed a high confidence list of 1178 target mRNAs, which satisfied the criteria for the significant enrichment in at least

one pairwise comparison (Figure 2.9 B, Supplementary Data 2). Our list of miR-218 targets included many previously identified miR-218 target transcripts, such as Rictor, CDK6, and GLCE (Figures 2.9 B and Figure 2.10) (Prudnikova et al. 2012; Venkataraman et al. 2013), validating our RISC-trap analyses. In addition, the list revealed many novel miR-218 target mRNAs with high fold enrichments (Figures 2.9 B, Supplementary Data 2).

Direct MRE search analyses using our high confidence miR-218 RISC-trap target list revealed that the majority of targets contained expected miR-218 binding motifs (Figure 2.9 C). The RISC-trap target mRNAs contained an average of ~1.7 miR-218 7mer MREs per target. The majority of MREs were located in both the 3'UTR and the open reading frame (ORF) (Figure 2.9 D). The equal distribution of miR-218 7-mer MREs in the 3'UTR and ORF suggest that de novo searches for miR-218 targets that utilize only 3'UTR sequence information may neglect numerous potential targets with miR-218 MREs in the target transcript ORF.

To assess the functional significance of the miR-218 target transcripts, we performed gene ontology (GO) term and cluster anlayses. These analyses revealed that cell cycle and DNA stress response GO clusters were highly enriched for miR-218 targets (Figure 2.9 E, Supplementary Data 3), consistent with the previous reports that miR-218 functions as a tumor supressor miRNA (Liu et al. 2012; Tu et al. 2013; Venkataraman et al. 2013). Interestingly, the analyses also uncovered significant GO term clusters including transcription regulation and neuron development related genes (Figure 2.9 E, Supplementary Data 3), suggesting that miR-218 targets that were identified in the RISC-trap contain a significant number of mRNAs that are relevant to neuronal differentiation and development.

miR-218 target genes that are important for neural progenitors and interneurons

A subset of miR-218 target genes identified in the RISC-trap screen and GO analyses are particularly relevant in the context of motor neuron development due to their expression pattern and previously reported functions in the developing spinal cord. These target mRNAs include genes that are important for the differentiation and function of spinal interneurons, such as Lhx1, BCL11A, SLC6A1, FoxP2, Pou4f1, Prdm13, Sox21, and

Bmpr1b, as well as genes that play roles in spinal neural progenitors, including Tead1, FoxP2 and Sox21 (Figures 2.11 and 2.12 A) (Wine-Lee 2004; Sandberg et al. 2005; Pillai et al. 2007; Cao et al. 2008; Morikawa et al. 2009; John et al. 2012; Rousso et al. 2012; Zou et al. 2012; Hanotel et al. 2014). These genes are either expressed at a very low abundance in newly born motor neurons or are downregulated as progenitors differentiate into motor neurons (Figures 2.11 and 2.12 A). Thus, their expression pattern is roughly complementary with miR-218 expression during motor neurogenesis in the developing spinal cord. The functional relevance and the presence of evolutionarily conserved miR-218 MREs on a subset of miR-218 target candidates led us to further validate five target mRNAs: Tead1, SLC6A1, BCL11A, Lhx1, and FoxP2.

We performed independent RISC-trap experiments with miR-218 and miR-181 in HEK293T cells, and quantified the enrichment levels of the selected targets using qRT-PCR analyses (Figure 2.12 B). The differential enrichment pattern of miRNA target transcripts in this RISC-trap assay was highly correlated with the RISC-trap/RNA-seq datasets (Figure 2.13, Supplementary Data 2). Tead1 and SLC6A1 were strongly enriched and BCL11A, Lhx1 and FoxP2 were also substantially enriched in the miR-218 RISC-trap samples, compared to the miR-181 RISC-trap samples (Figure 2.12 B). Conversely, RFT1, a miR-181 target mRNA identified in the RISC-trap screen, was enriched in miR-181 RISC-trap over miR-218 RISC-trap (Figure 2.12 B).

Each of the Tead1, SLC6A1, BCL11A, Lhx1, and FoxP2 mRNAs has at least two putative miR-218 MREs in the 3'UTR region (Figures 2.13 and 2.14). To test whether miR-218 regulates the expression of target transcripts via a miR-218 MRE, we constructed luciferase reporters, in which the 3'UTR region of each target containing one evolutionarily conserved miR-218 MRE is inserted between a luciferase gene and poly adenylation sequences (Figures 2.14 and 2.15 A,B). We then transfected luciferase reporters with either miR-218 or miR-181 in HEK293T cells, and monitored the luciferase expression levels. The expression of miR-218 led to significant repression in all five luciferase reporters linked with the 3'UTR of miR-218 targets, compared to miR-181 expression (Figure 2.15 D), suggesting that a miR-218 MRE in the target mRNAs 3'UTR triggers the target gene suppression. Additionally, we constructed five luciferase reporters in which the 3'UTR miR-218 MRE was mutated (Figure 2.15 C). Transfection

of these mutated reporters with miR-218 or miR-181 in HEK293T cells showed that the miR-218 mediated repression was abolished, indicating that the miR-218 MRE is necessary for regulation of these 3'UTR target reporters (Figure 2.15 D).

Together, our results suggest that miR-218 inhibits the expression of Tead1, SLC6A1, BCL11A, Lhx1, and FoxP2 by directly binding to their 3'UTR regions.

Endogenous miR-218 represses target genes in motor neurons via 3'UTR MREs

To test whether endogenous miR-218 in motor neurons controls each target mRNA via 3'UTR-dependent downregulation in vivo, we generated miRNA sensor plasmids in which the partial 3'UTR of Tead1, SLC6A1, BCL11A, Lhx1, or FoxP2 is cloned downstream of d4EGFP, and monitored the expression ratios of GFP and RFP in motor neurons and interneurons in the developing chick spinal cord three days after electroporation (Figure 2.16 A). The control miRNA sensor showed the same GFP/RFP ratio in interneurons and motor neurons (Figure 2.16 B,H). In contrast, the miRNA sensors with the 3'UTR of Tead1, SLC6A1, BCL11A, Lhx1, or FoxP2 transcripts showed varying degree of downregulation of GFP in motor neurons compared to interneurons. The 3'UTRs of Tead1, SLC6A1, and BCL11A directed ~ 45% downregulation of GFP in motor neurons, whereas the 3'UTRs of Lhx1 and FoxP2 led to 20% and 10% knockdown of GFP, respectively (Figure 2.16 C-H). These data suggest that endogenous miR-218 in developing motor neurons is capable of suppressing the expression of Tead1, SLC6A1, BCL11A, Lhx1, and FoxP2 via their 3'UTRs containing miR-218 MREs.

The partial 3'UTRs of each target gene were sufficient to respond to miR-218 in our luciferase and miRNA sensor analyses, showing that each miR-218 target transcript has at least one functional miR-218 binding site. However, it is notable that each of the selected miR-218 targets contains multiple 218 MREs throughout the gene body (Figures 2.13 and 2.14). For example, the FoxP2 gene has three different miR-218 MREs in the 3'UTR, while the 3'UTR region that we tested contains only one miR-218 MRE (Figure 2.14). Thus, miR-218-directed suppression of the target genes in our reporter assays likely represents only a fraction of responsiveness of gene repression to miR-218 for each gene.

miR-218 suppresses spinal cord interneuron differentiation

Our RISC-trap data led us to hypothesize that miR-218 suppresses spinal interneuron fate in developing spinal cord. To test this hypothesis, we misexpressed miR-218 in the dorsal spinal cord of chick embryos using in ovo electroporation of miR-218 expression construct (Figure 2.17 A,B). miR-218 did not trigger ectopic generation of motor neurons in the dorsal neural tube (Figure 2.17 B), suggesting that miR-218 alone is not sufficient to drive motor neuron differentiation. Interestingly, however, miR-218 expression resulted in a substantial reduction of $Lhx1^+$, $Pax2^+$, or $FoxP2^+$ interneurons, whereas it did not make a significant change in the number of $Ngn2^+$ cells or $Olig2^+$ motor neuron progenitors (Figure 2.17 C,D). These results are consistent with our finding that Lhx1 and FoxP2 are miR-218 targets. In addition, sequence analyses uncovered that Pax2 has an evolutionarily conserved miR-218 MRE in the 3'UTR (Figure 2.18 A,B). To further test the action of miR-218 in spinal interneuron differentiation, we generated ESC lines, in which either miR-218 or miR-control is constitutively expressed, and differentiated the ESCs to spinal interneurons by treating embryoid bodies with retinoic acid (RA) (Figure 2.19 A, B). miR-218-expressing ESCs failed to differentiate into $Pax2^+$ or $FoxP2^+$ interneurons, while miR-control ESCs readily express Pax2 and FoxP2 (Figure 2.19 C). Similarly to the spinal cord, miR-218 failed to trigger motor neuron generation and there was no difference in overall neuronal differentiation between miR-218 and miR-Ctrl ESCs, as determine by broad neuronal marker Tuj1 (Figure 2.19 C, data not shown). Together, our data show that miR-218 selectively inhibits spinal interneuron differentiation, but miR-218 alone is not sufficient to induce motor neuron fate.

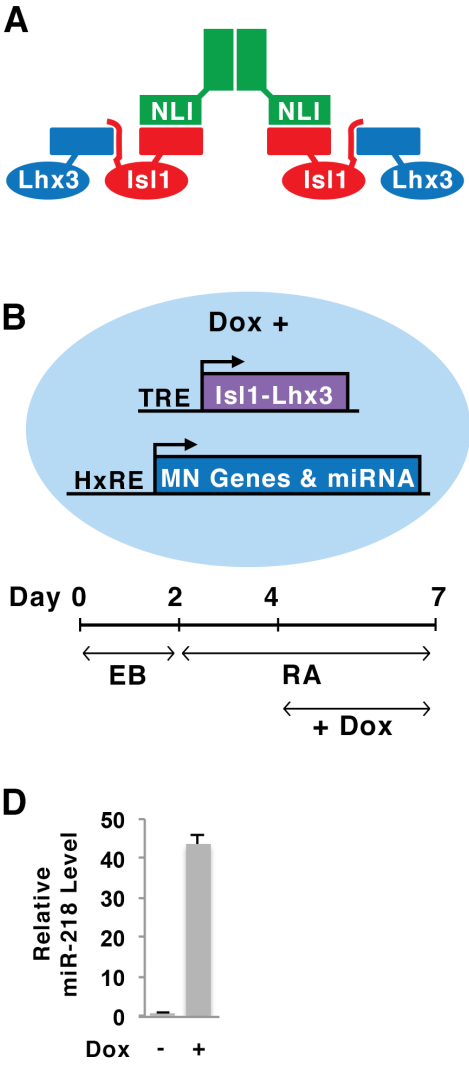
Isl1-Lhx3 generates ectopic motor neurons at the expense of interneurons

Given our finding that miR-218 is upregulated by the Isl1-Lhx3 complex and directly targets a number of genes controlling the specification of spinal interneurons, we hypothesized that miR-218 is a critical downstream effector of the Isl1-Lhx3 complex in suppressing non-motor neuron genes, thus ensuring the proper acquisition of motor neuron cell identity. To determine whether the Isl1-Lhx3 complex represses the differentiation of interneurons in vivo, we misexpressed Isl1-Lhx3 in the chick neural

tube using in ovo electroporation and performed immunohistochemical analyses with Lhx1 and Pax2 antibodies, each of which label multiple types of interneurons in the spinal cord (Pillai et al. 2007; Huang et al. 2008). Isl1-Lhx3 substantially suppressed the formation of $Lhx1^+$ and $Pax2^+$ interneurons in the developing spinal cord, compared to the unelectroporated control side (Figure 2.20 A-D). Additionally, in situ hybridization and immunohistochemistry analyses revealed that the ectopic miR-218/Hb9-expressing cells in the dorsal neural tube are mutually exclusive with $Pax2^+$ cells (Figure 2.20 E). These results suggest that Isl1-Lhx3 directs a complete fate transition from interneurons to motor neurons by strongly suppressing interneuron differentiation in the dorsal spinal cord.

miR-218 is required for efficient generation of motor neurons by Isl1-Lhx3

Next, to determine whether miR-218 is required for Isl1-Lhx3 to effectively trigger motor neuron differentiation, we electroporated Isl1-Lhx3 with either miR-218 sponge inhibitor or scrambled sponge control, and assessed the formation of ectopic motor neurons. Inhibition of miR-218 action suppressed Isl1-Lhx3-directed differentiation of motor neurons by ~ 50% (Figure 2.21 A,B). Likewise, the miR-218 sponge inhibitor reduced the efficiency of motor neuron production by the co-expression of Isl1 and Lhx3 by ~35% (Figure 2.21 C,D), while significantly increasing the proportion of Lhx1-expressing cells among ectopic Lhx3-electroporated cells (Figure 2.22 A,B). These results demonstrate that the activity of miR-218, which is induced by Isl1-Lhx3, to suppress interneuron fate is crucial for Isl1-Lhx3 to effectively establish motor neuron identity.



C

microRNA	Fold Change	P-Value	Dox+ Exp. Rank
miR-218	70.76	0.006	5
miR-382	8.89	0.034	87
miR-203	6.57	0.045	142
miR-200a	4.30	0.043	114
miR-376c	4.15	0.047	62
miR-411	3.28	0.048	34
miR-26b	3.27	0.044	54
miR-28	3.27	0.049	110
miR-495	3.24	0.046	82
miR-106b	3.23	0.047	32
miR-224	3.23	0.049	166
miR-34b-3p	3.22	0.049	120
miR-539	3.20	0.050	56
miR-29a	3.20	0.049	111
miR-362-3p	3.18	0.046	222
miR-379	3.06	0.021	72
miR-145	3.05	0.022	132
miR-350	3.03	0.025	210

Figure 2.1. Identification of miRNAs upregulated by Isl1-Lhx3 during motor neuron differentiation
(A) Illustration of the Isl1-Lhx3 hexamer transcription complex, consisting of two NLI, two Isl1 and two Lhx3 proteins.
(B) Schematic model of Isl1-Lhx3 embryonic stem cell line (Isl1-Lhx3 ESC) and the experimental design to differentiate Isl1-Lhx3 ESCs. The treatment of doxycycline (Dox) induces the expression of Isl1-Lhx3, which is controlled by tetracycline response element (TRE). Then, Isl1-Lhx3 binds endogenous NLI to form a hexamer transcription complex that upregulates its direct target genes that have hexamer response element (HxRE), such as motor neuron (MN) genes and miRNAs (Lee et al. 2012). EB, embryoid bodies; RA, retinoic acid.
(C) A list of miRNAs that exhibit a significant induction by Dox treatment (> 3-fold, $p < 0.05$), as determined by TaqMan miRNA arrays. Expression rank (Exp. Rank) describes the rank of relative expression levels of each miRNA in Dox-treated conditions.
(D) Isl1-Lhx3 ESC motor neuron differentiation triggered the expression of mature miR-218, as determined by qPCR using TaqMan probes. Error bars represent the standard deviation; n = 2, biological duplicates.

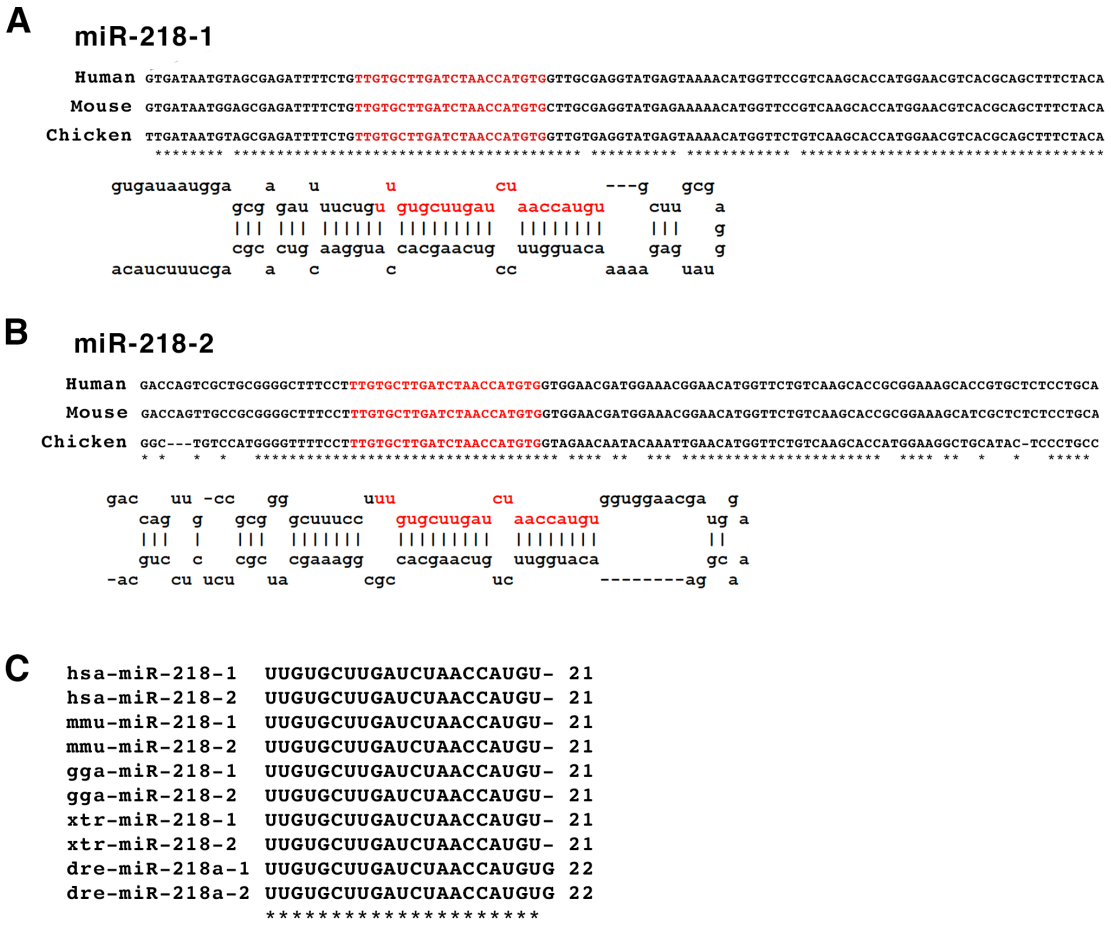


Figure 2.2. miR-218 is a highly conserved miRNA.
(A-B) Illustrations of the conservation of miR-218-1 and miR-218-2 gene and hairpin structures.
(C) Sequence alignment shows that the mature miR-218 sequence is highly conserved in multiple species.

*Figure provided by Dr. Stacey Glasgow, a former Lee lab member

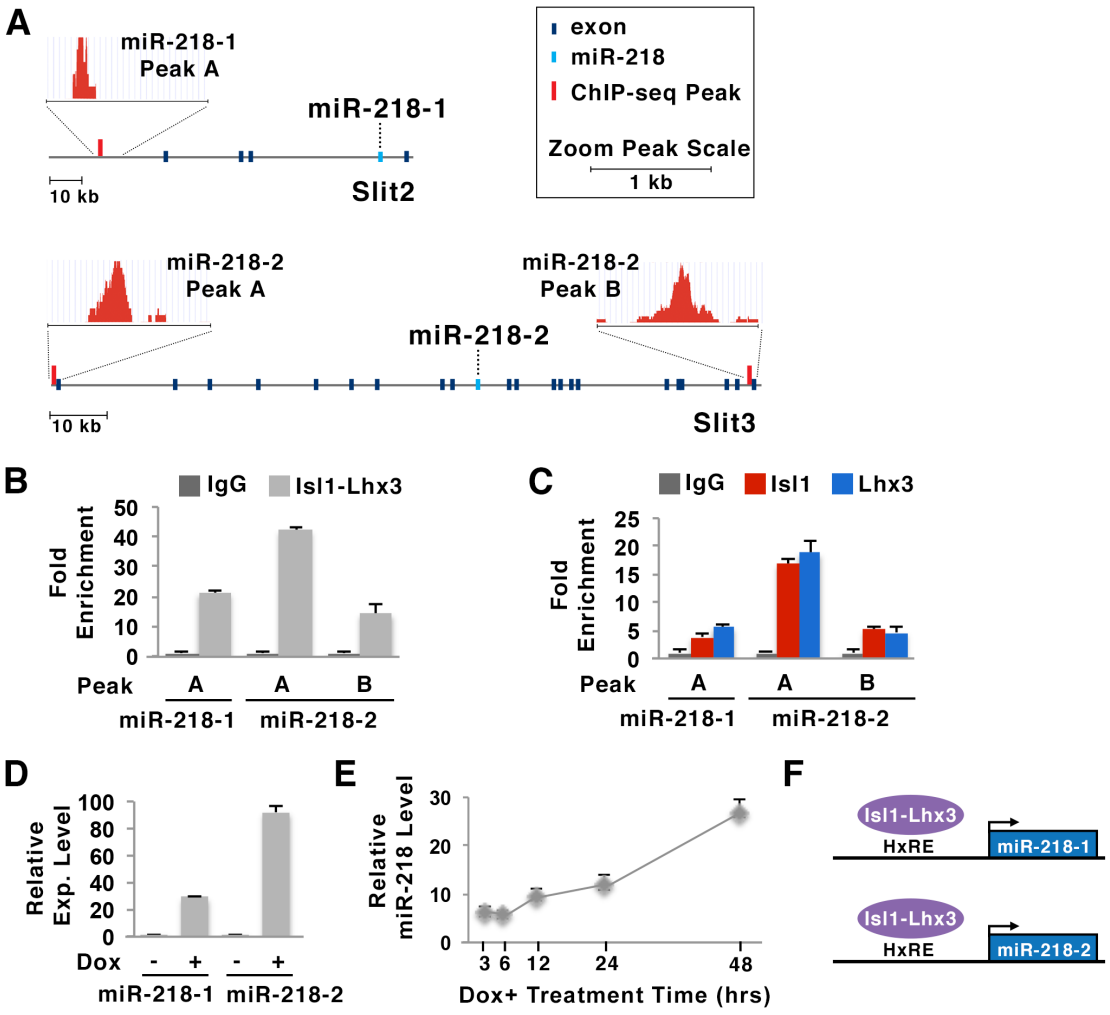


Figure 2.3. Isl1-Lhx3 directly binds genomic loci near miR-218-1 and miR-218-2 genes
(A) Isl1-Lhx3-bound ChIP-seq peaks were identified near miR-218-1 and miR-218-2 genes, within the introns of Slit2 and Slit3, respectively.
(B) Isl1-Lhx3-bound to three Isl1-Lhx3 ChIP-seq peak regions near miR-218-1 and miR-218-2 genes in Isl1-Lhx3 ESCs. Error bars represent the standard deviation; n = 3, technical triplicates.
(C) Both Isl1 and Lhx3 were recruited to three Isl1-Lhx3-bound ChIP-seq peaks near miR-218-1 and miR-218-2 genes in mouse E12.5. Error bars represent the standard deviation; n = 3, technical triplicates.
(D) Isl1-Lhx3 induced the expression of pri-miR-218-1 and pri-miR-218-2 in Isl1-Lhx3-ESC-derived MNs, as determined by qPCR using TaqMan probes. Exp, expression; Error bars represent the standard deviation; n = 2, biological duplicates.
(E) Isl1-Lhx3 strongly upregulated miR-218 expression within 48 hours in Isl1-Lhx3-ESCs cultured in monolayer without retinoic acid (RA), as determined by qPCR using TaqMan probes. Error bars represent the standard deviation; n = 3, technical triplicates.
(F) Schematic model showing that the Isl1-Lhx3 complex binds to hexamer response element (HxRE) near miR-218-1 and miR-218-2 genes and triggers the expression of miR-218 genes in differentiating motor neurons.

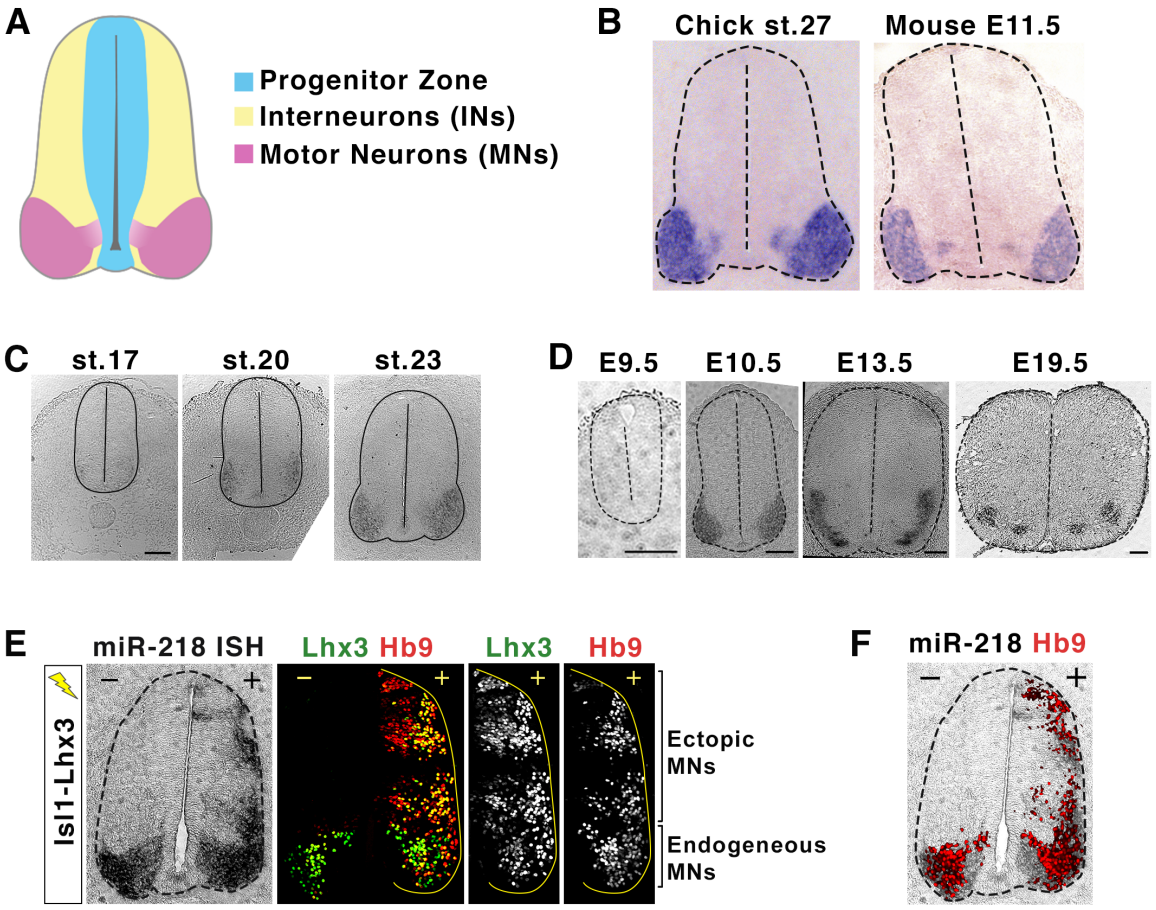


Figure 2.4. miR-218 is expressed in embryonic motor neurons and upregulated by Isl1-Lhx3 in vivo
(A) Illustration of the developing spinal cord. Stereotypical locations of neural progenitor cells, interneurons (INs), and motor neurons (MNs) in mouse E11.5 and chick Hamburger Hamilton stage 27 embryos are shown.
(B) miR-218 is highly expressed in the developing motor neurons of mouse and chick embryos, as shown by in situ hybridization with a probe detecting mature miR-218.
(C-D) miR-218 is induced at the onset of motor neuron differentiation in chick (C) and mouse (D) and continues to be expressed in motor neurons throughout mouse embryonic development (D), as shown by in situ hybridization with a probe detecting mature miR-218. Scale bars represent 100 μm.
(E-F) Analyses using serial sections from the same chicken embryo electroporated with Isl1-Lhx3. In situ hybridization for miR-218 and immunohistochemical analyses with Lhx3 and Hb9 antibodies reveals that the expression of miR-218 was highly induced by Isl1-Lhx3 in the dorsal spinal cord, where $Hb9^{+}$ ectopic motor neurons are formed. +, electroporated side; -, unelectroporated control side.

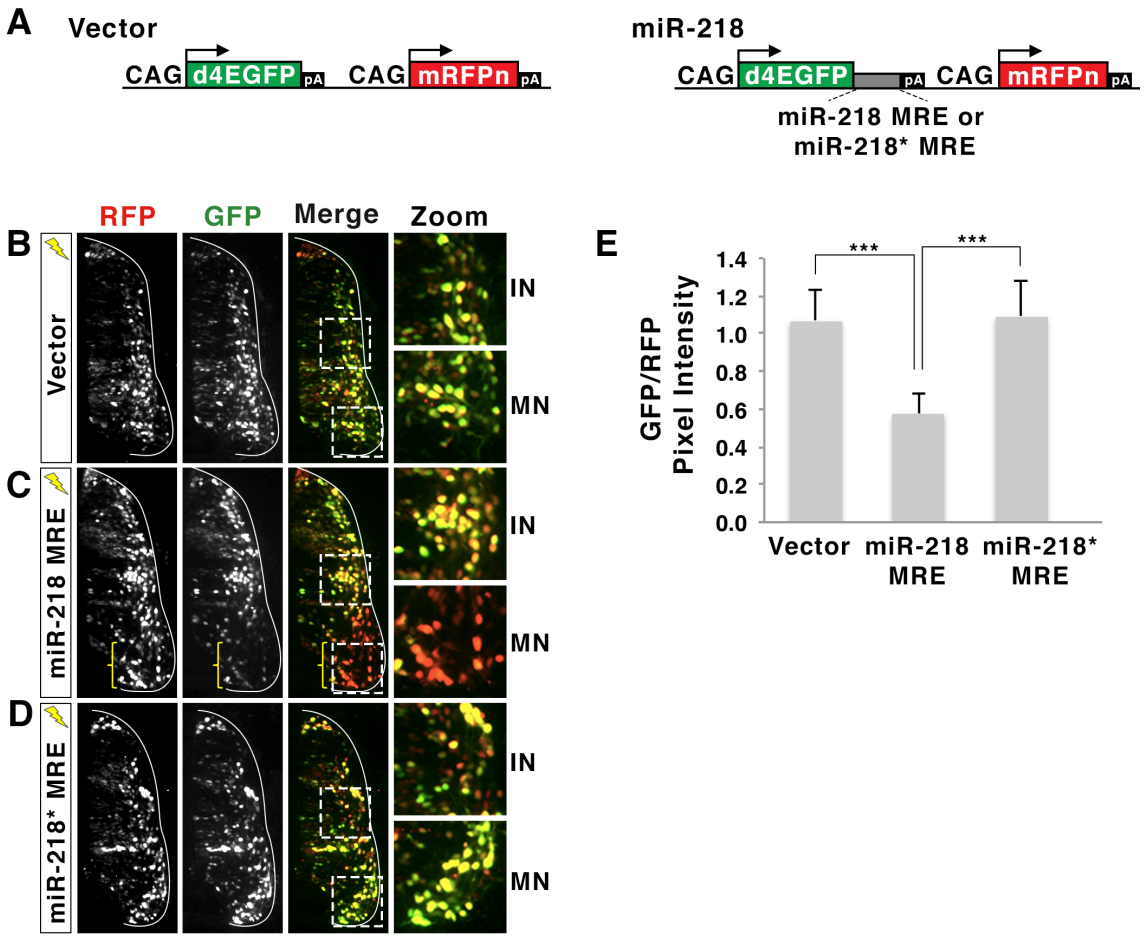


Figure 2.5. miR-218 is active in developing spinal cord motor neurons

(A) Illustrations of miRNA sensor plasmids. The multimerized miRNA response element (MRE) for miR-218 or miR-218* was inserted between the destabilized GFP (d4EGFP) gene and polyA (pA) sequences. The expression of both d4EGFP and mononuclear RFP (mRFPn) is driven by two separate, ubiquitously active CAG promoters.

(B-D) The expression pattern of GFP and RFP in the chick spinal cord electroporated with miRNA sensor vector (B), miR-218-5p MRE sensor (C) or miR-218-3p (*) MRE sensor (D). Only the electroporated half of the spinal cord is shown. GFP expression is regulated by endogenous miRNA that binds to the MREs present in 3'UTR of the GFP gene, while RFP expression depicts the electroporated cells. The areas of interneuron (IN) and motor neuron (MN) are magnified. The miR-218 MRE sensor shows drastically down regulated GFP expression in motor neuron area (bracket in C), indicating that endogenous miR-218 in motor neurons suppresses the expression of GFP.

(E) Quantification of relative pixel intensity of GFP/RFP in motor neurons, as quantified using ImageJ program. Error bars represent the standard deviation; ***$p < 0.0001$ in two-tailed Student's t-test; $n = 5$ embryos.

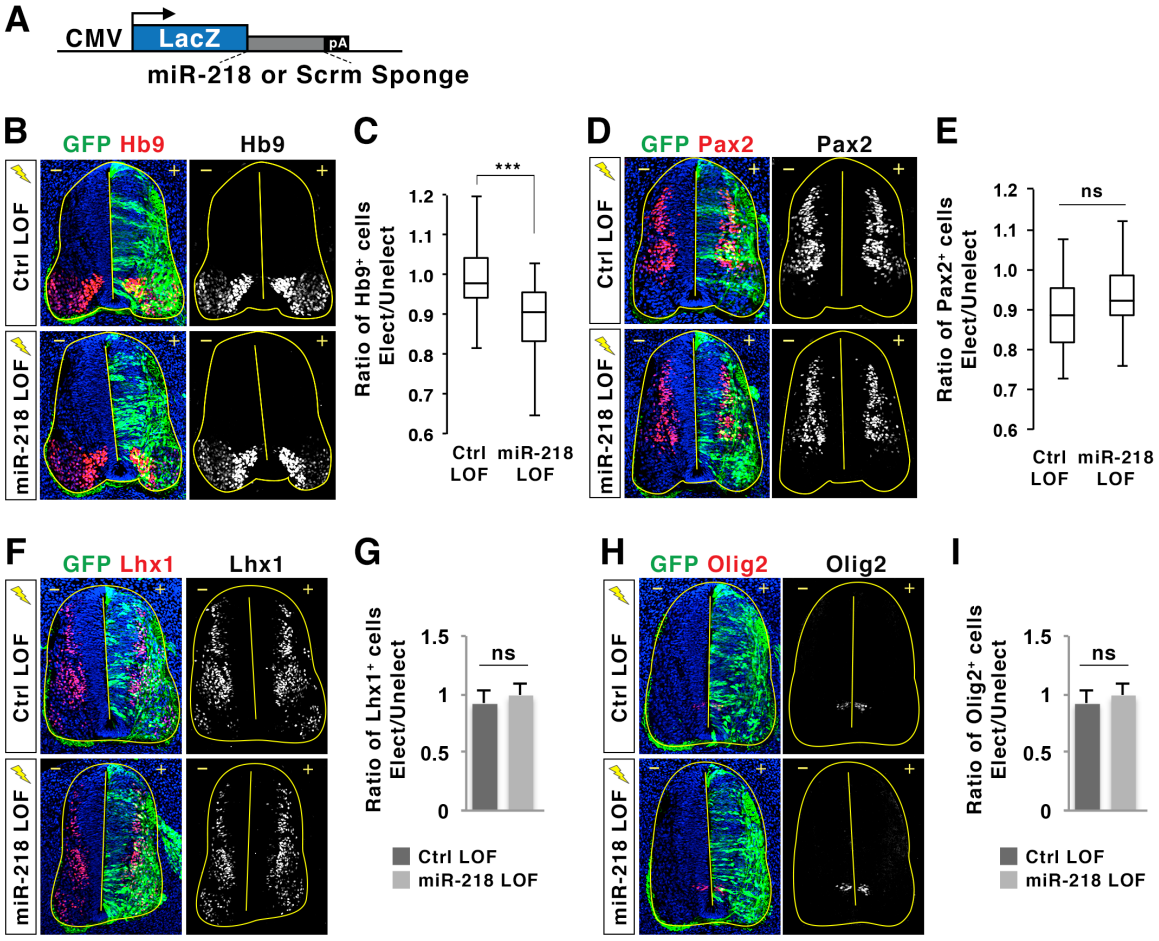


Figure 2.6. miR-218 is important for the generation of spinal cord motor neurons
(A) Illustration of bulge sponge inhibitor constructs, which have either 40 repeats of bulged miR-218 MRE or control scrambled (Scrm) sequences in the 3'UTR of a CMV-promoter driven LacZ gene.
(B,D,F,H) Loss of function (LOF) analyses in chicks electroporated with control (Ctrl) LOF conditions (scrambled sponge inhibitor, 2'Ome-inhibitor control, and CMV-GFP reporter) and miR-218 LOF conditions (miR-218 sponge inhibitor, miR-218 2'Ome-inhibitor, and CMV-GFP reporter). Hb9 antibody labels motor neurons (B), Pax2 antibody labels a broad population of interneurons (D), Lhx1 antibody labels a broad population of interneurons and LMCl motor neurons (F), and Olig2 labels motor neuron progenitors (H) in immunohistochemical analyses. +, electroporated side; -, unelectroporated control side.
(**C,E,G,I**) The effect of LOF conditions was quantified by the ratio of $Hb9^+$ cells, $Pax2^+$ cells, $Lhx1^+$ cells, and $Olig2^+$ cells on the electroporated (elect) side over the unelectroporated (unelect) side. Error bars represent the standard deviation; ***$p < 0.0001$ in two-tailed Student's t-test; ns, not significant; n = 18-21 embryos for Hb9 (C) and Pax2 (E), n=12-13 embryos for Lhx1 (G), and n=6-8 embryos for Olig2 (I).

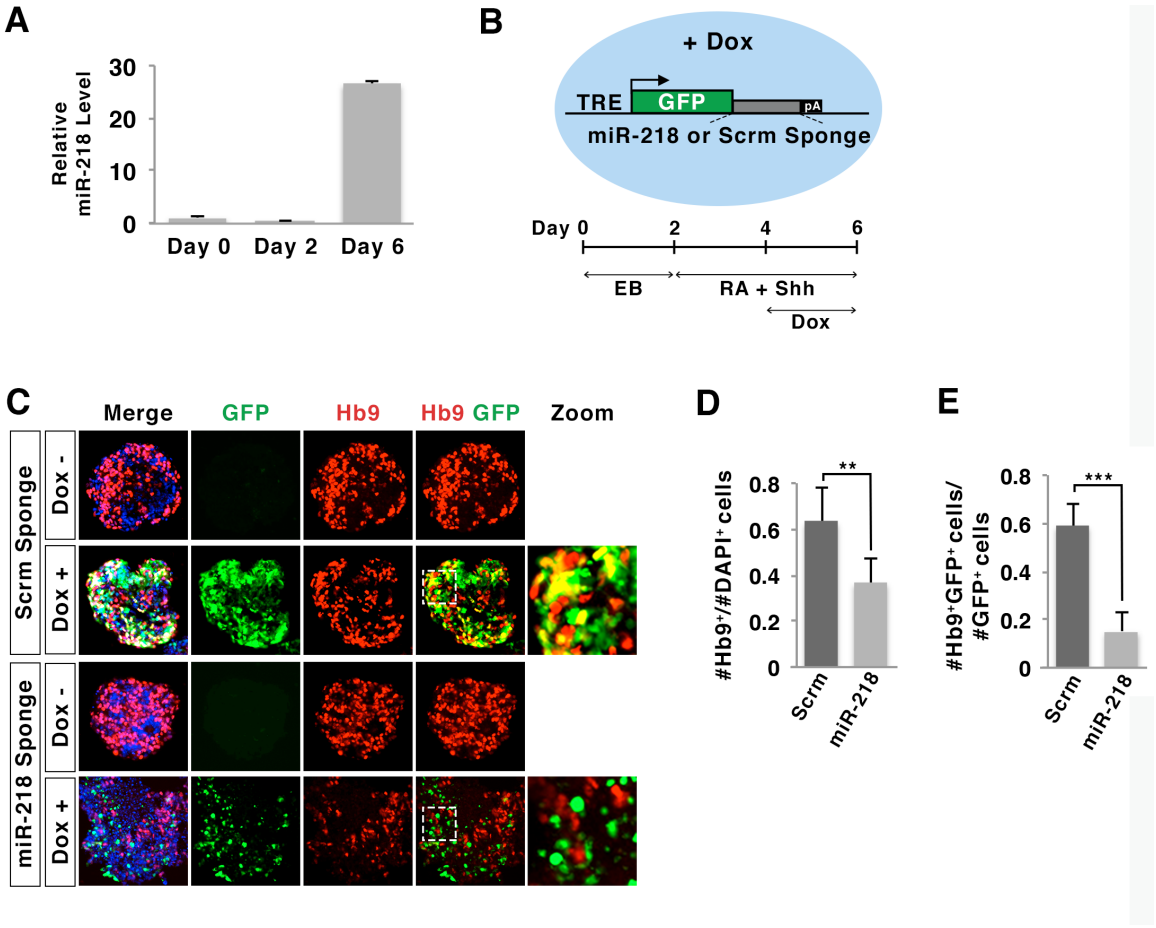


Figure 2.7. miR-218 is essential for the generation of motor neurons from ESCs

(A) The qPCR analysis using miR-218 TaqMan probe revealed that miR-218 expression is low in ESCs cultured in monolayer (day 0) or in embryoid bodies (day 2), but is highly induced in ESC-derived motor neurons (day 6). Error bars represent the standard deviation.

(B) Illustration of doxycycline (Dox)-inducible sponge mouse ESC lines, in which Dox induces the expression of either miR-218 sponge inhibitor or scrambled (Scrm) sponge inhibitor, and the experimental design to differentiate ESCs to motor neurons. TRE, tetracycline response element; EB, embryoid body; RA, retinoic acid; Shh, a sonic hedgehog agonist Purmorphamine.

(C) Immunohistochemical analyses in Dox-inducible sponge ESC-derived motor neurons at differentiation day 6. Hb9 antibody labels motor neurons, and GFP labels the cells in which the expression of Scramble (Scrm) or miR-218 sponge inhibitor is induced by Dox.

(D-E) The effect of Dox-inducible sponge inhibitors was quantified by the ratio of $Hb9^+$cells over all cells ($DAPI^+$) (D), or by the ratio of Hb9 and GFP double-positive motor neurons over the total number of GFP^+ cells (E). Error bars represent the standard deviation; $^{**}p < 0.001$ and $^{***}p < 0.0001$ in two-tailed Student's t-test; n = 15 embryoid bodies.

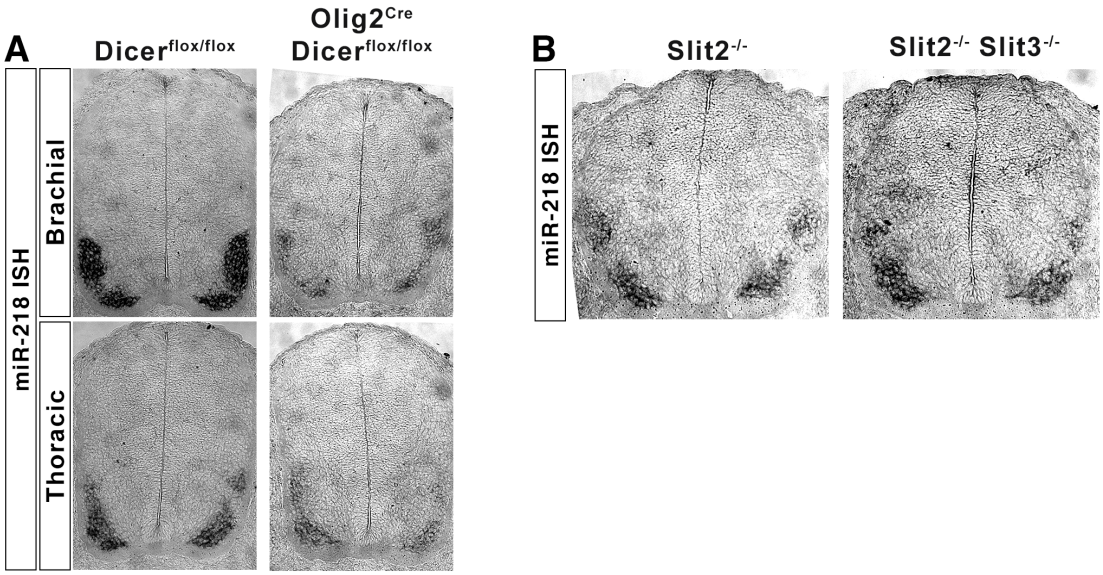
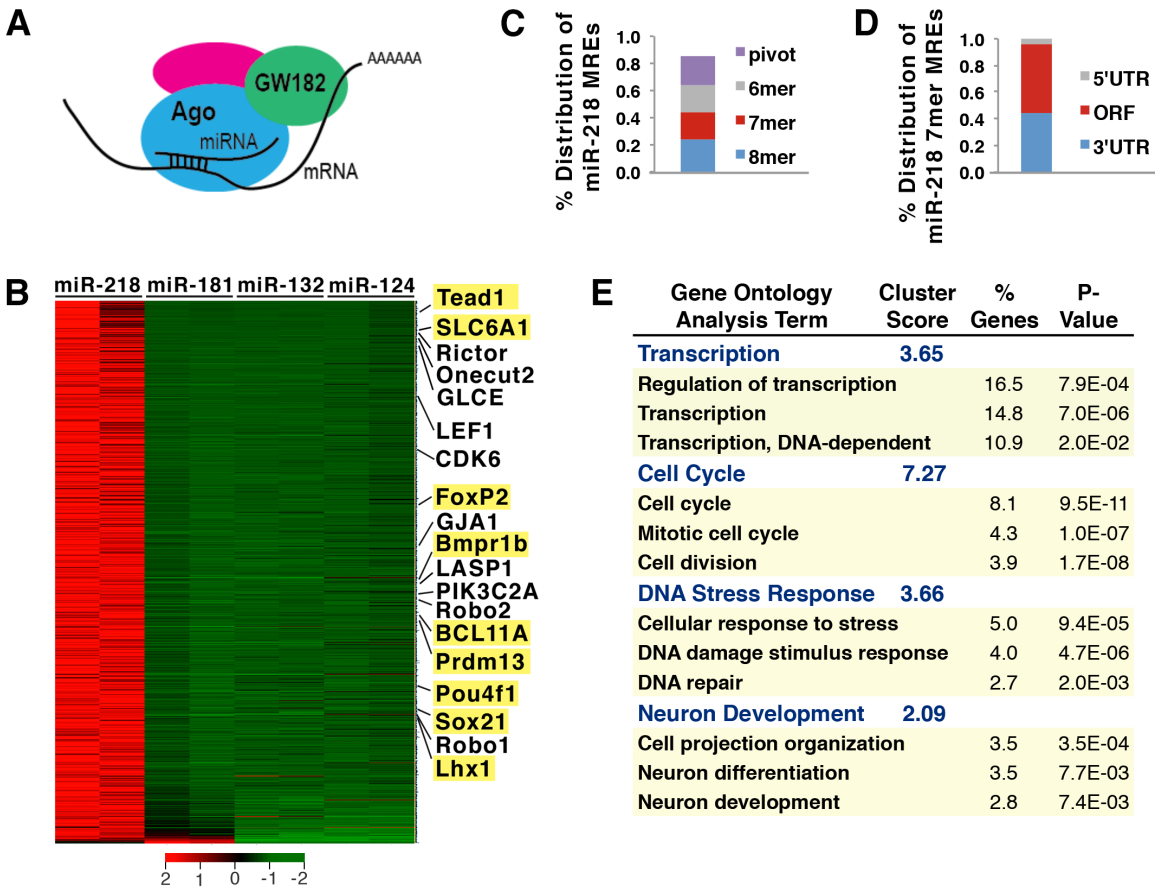


Figure 2.8. Mouse knockout models still express miR-218
(A) In situ hybridization for mature miR-218 in Olig2$^{Cre/+}$, Dicer$^{flox/flox}$ and Dicer$^{flox/flox}$ littermate control in mouse spinal cords at embryonic day 12.5 (E12.5).
(B) In situ hybridization for mature miR-218 in Slit2$^{-/-}$ single knockout and Slit2$^{-/-}$, Slit3$^{-/-}$ double knockout mouse spinal cords at embryonic day 13.5 (E13.5). Slit2 and Slit3 are the host genes for intronic miR-218-1 and miR-218-2, respectively.



E

Gene Ontology Analysis Term	Cluster Score	% Genes	P-Value
Transcription	**3.65**		
Regulation of transcription		16.5	7.9E-04
Transcription		14.8	7.0E-06
Transcription, DNA-dependent		10.9	2.0E-02
Cell Cycle	**7.27**		
Cell cycle		8.1	9.5E-11
Mitotic cell cycle		4.3	1.0E-07
Cell division		3.9	1.7E-08
DNA Stress Response	**3.66**		
Cellular response to stress		5.0	9.4E-05
DNA damage stimulus response		4.0	4.7E-06
DNA repair		2.7	2.0E-03
Neuron Development	**2.09**		
Cell projection organization		3.5	3.5E-04
Neuron differentiation		3.5	7.7E-03
Neuron development		2.8	7.4E-03

Figure 2.9. Identification of direct miR-218 target mRNAs using RISC-trap screen

(A) Illustration of the miRNA induced silencing complex (miRISC).

(B) RISC-trap screens identified direct target genes for miR-218, when analyzed against RISC-trap screens for miR-181, miR-132, miR-124 (Cambronne et al., 2012). All identified miR-218 targets are sorted in a heatmap by fold-change and biological replicates, compared to miR-181 RISC-trap (FDR <0.05, fold enrichment ≥ 4). Previously published miR-218 targets identified in the RISC-trap screen are labeled and selected previously unknown miR-218 targets are highlighted in yellow. Scale bar represents Z-score of row.

(C,D) Analyses for miR-218 MREs in 1178 genes targeted by miR-218. (B) Percent distribution of miR-218 MREs was classified by the inclusion of at least 1 MRE motif in the order of 8mer > 7mer > 6mer > pivot and each transcript is counted only once. (C) Percent distribution of miR-218 7-mer MRE motifs per target in the 5' untranslated region (5'UTR), open reading frame (ORF), and 3' untranslated region (3'UTR).

(E) Gene ontology cluster analysis. Table represents biological process terms from each significantly enriched cluster. % genes, percent genes out of 781 genes that were classified with biological process term.

Published miR-218 Target	RISC-trap Fold Change (218 vs. 181)	References
RICTOR	17.83	Uesugi et al. 2011; Venkataraman et al. 2013
ONECUT2	17.5	Simion et al. 2010
GLCE	14.41	Prudnikova et al. 2012
LEF1	8.41	Liu et al. 2012; Tu et al. 2013
CDK6	6.68	Venkataraman et al. 2013
GJA1	5.39	Alajez et al. 2011
LASP1	5.23	Chiyomaru et al. 2012
PIK3C2A	5.12	Mathew et al. 2014
Robo2	5.09	Fish et al. 2011
Robo1	4.27	Fish et al. 2011; Alajez et al. 2011
Cav2	3.52	Yamasaki et al. 2013
Birc5	2.5	Alajez et al. 2011
IKBKB	2.18	Song et al. 2010
BMI1	2.11	Tu et al. 2013; Venkataraman et al. 2013
HMGB1	1.79	Mathew et al. 2014
DTL	1.72	Liu et al. 2013

Figure 2.10. miR-218 RISC-trap screen validates previously published miR-218 targets
Table of previously published miR-218 targets that were identified in the miR-218 RISC-trap screen. RISC-trap Fold Change for miR-218 vs. miR-181 target mRNAs and corresponding references are shown. All targets were significantly enriched in the miR-218 vs. miR-181 RISC-trap analyses with $p < 0.05$.

miR-218 RISC-trap Target mRNA	Spinal Cord Expression	Function	Reference
Tead1	Progenitor Zone	**Transcription Factor** Neural progenitor proliferation	Cao et al. 2008
SLC6A1 (GAT1)	Interneurons	**GABA Transporter** GABAergic interneuron neurotransmission	Jursky et al. 1999 Chen et al. 2004
BCL11A (Ctip1)	Interneurons	**Transcription Factor** Neuronal morphogenesis and sensory circuit formation	Li et al. 2006 John et al. 2012
Lhx1	Interneurons	**Transcription Factor** Inhibitory interneuron differentiation	Pillai et al. 2007 Huang et al. 2008 Brohl et al. 2008
FoxP2	Progenitor Zone V1 Interneurons	**Transcription Factor** Neurogenesis	Morikawa et al. 2009 Rousso et al. 2012
Prdm13	Interneurons	**Histone Methyltransferase** Inhibitory interneuron differentiation	Kinameri et al. 2008 Chang et al. 2013 Hanotel et al. 2014
Sox21	Progenitor Zone V2a Interneurons	**Transcription Factor** Neurogenesis	Uchikawa et al. 1999 Sandberg et al. 2005
Pou4f1 (Brn3a)	Interneurons Dorsal Root Ganglia	**Transcription Factor** Sensory and interneuron differentiation	Müller et al. 2002 Eng et al. 2007 Zou et al. 2012
BMPR1b	Interneurons	**Transcription Factor** Dorsal interneuron differentiation	Wine-Lee et al. 2004

Figure 2.11. Expression and function of selected miR-218 RISC-trap targets in the spinal cord
A subset of miR-218 targets identified in the RISC-trap screen and GO analyses play important roles in the developing spinal cord. Tead1, FoxP2 and Sox21 are known to be important for neurogenesis in the progenitor zone, while Lhx1, BCL11A, SLC6A1, Foxp2, Pou4f1, Prdm13, Sox21, and BMPR1b play crucial roles in spinal interneuron function and differentiation. TEA Domain Family Member 1 (Tead1); Solute Carrier Family 6 Member 1 (SLC6A1); B-Cell CLL/Lymphoma 11A (BCL11A); LIM homeodomain 1 (Lhx1); Forkhead box P2 (Foxp2); PR Domain Containing 13 (Prdm13); Sex Determining Region Y Box 21 (Sox21); POU Class 4 Homeobox (Pou4f1); Bone Morphogenetic Protein Receptor Type 1B (BMPR1b).

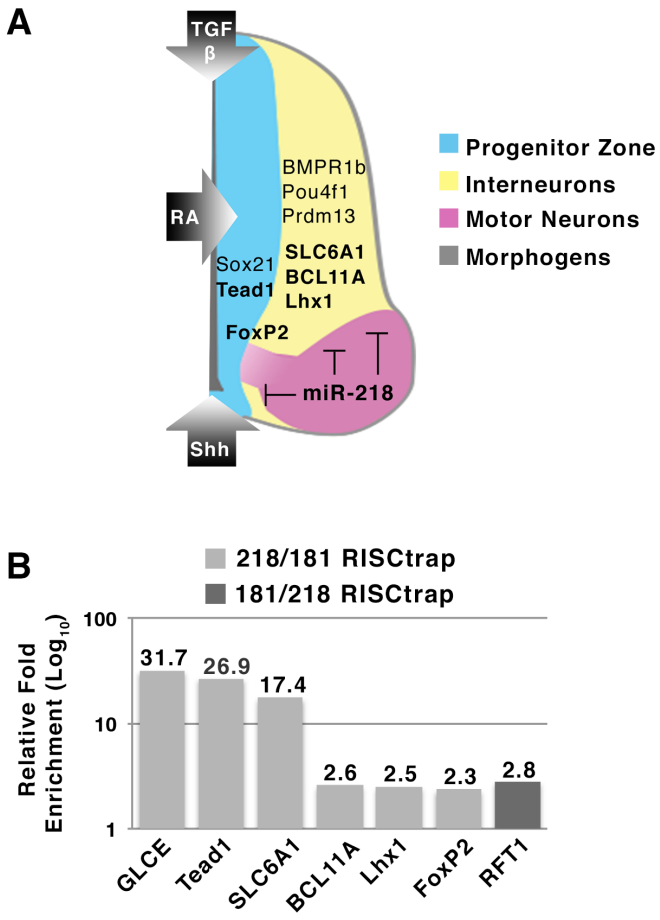


Figure 2.12. Model of miR-218 RISC-trap target regulation in the developing spinal cord
(A) Schematic model of the developing spinal cord expression patterns of selected RISC-trap miR-218 target mRNAs representing mouse E11.5 and chick Hamburger Hamilton stage 27. Dorsoventral patterning of the developing spinal cord requires progenitor cells to integrate gradients of morphogens, such as sonic hedgehog (Shh), retinoic acid (RA), and transforming growth factor β (TGFβ). These morphogens are essential to induce the expression of the selected miR-218 RISC-trap targets in the spinal cord: Tead1, SLC6A1, BCL11A, Lhx1, FoxP2, Sox21, Prdm13, Pou4f1 and BMPR1b. The expression of these selected targets is largely excluded from developing motor neurons and our model suggests a role for miR-218 in repressing the expression of these genes, that promote neural progenitor maintence and interneuron differentiation, in motor neurons.
(B) Independent RISC-trap experiments with qRT-PCR analyses validated selected miR-218 target genes enriched against miR-181 RISC-trap. RFT1 is a miR-181 target mRNA identified in the RISC-trap screen. The qPCR results were shown as relative fold change in Log_{10} scale between miR-218 and miR-181 RISC-trap experiments.

miR-218 Target mRNA	RISC-trap Fold Change	# 218 MREs
TEAD1	vs. miR-181: 37.3 vs. miR-132: 32.2 vs. miR-124: 14.5	Human - 10 Mouse - 7 *Chicken - 2
SLC6A1 (GAT1)	vs. miR-181: 18.3 vs. miR-132: 13.7 vs. miR-124: 17.9	Human - 4 Mouse - 4 *Chicken - 2
BCL11A (Ctip1)	vs. miR-181: 4.8 vs. miR-132: 6.8 vs. miR-124: 5.8	Human - 6 Mouse - 4 Chicken - 6
Lhx1	vs. miR-181: 4.2 vs. miR-132: 4.9 vs. miR-124: 4.3	Human - 2 Mouse - 1 Chicken - 1
FoxP2	vs. miR-181: 5.9 vs. miR-132: 4.9 vs. miR-124: 4.8	Human - 4 Mouse - 6 Chicken - 4
Prdm13	vs. miR-181: 4.3 vs. miR-132: 5.9 vs. miR-124: 6.5	Human - 1 Mouse - 1 *Chicken: no seq
Sox21	vs. miR-181: 4.3 vs. miR-132: 5.9 vs. miR-124: 6.5	Human - 1 Mouse - 1 *Chicken: no seq
Pou4f1 (Brn3a)	vs. miR-181: 4.4 vs. miR-132: 4.8 vs. miR-124: 5.4	Human - 2 Mouse - 3 *Chicken: no seq
BMPR1b	vs. miR-181: 5.2 vs. miR-132: 5.3 vs. miR-124: 4.8	Human - 2 Mouse - 2 *Chicken - 1

*Only partial or provisional sequence available

Figure 2.13. Selected miR-218 RISC-trap target mRNAs
RISC-trap fold change shows enrichment folds in miR-218 RISC-trap against RISC-trap screens with miR-181, miR-132, or miR-124. The number of miR-218 MRE shows the total number of miR-218 MRE in each gene from human, mouse and chicken. Note that, for some genes, the full-length sequences of chicken gene are unavailable.

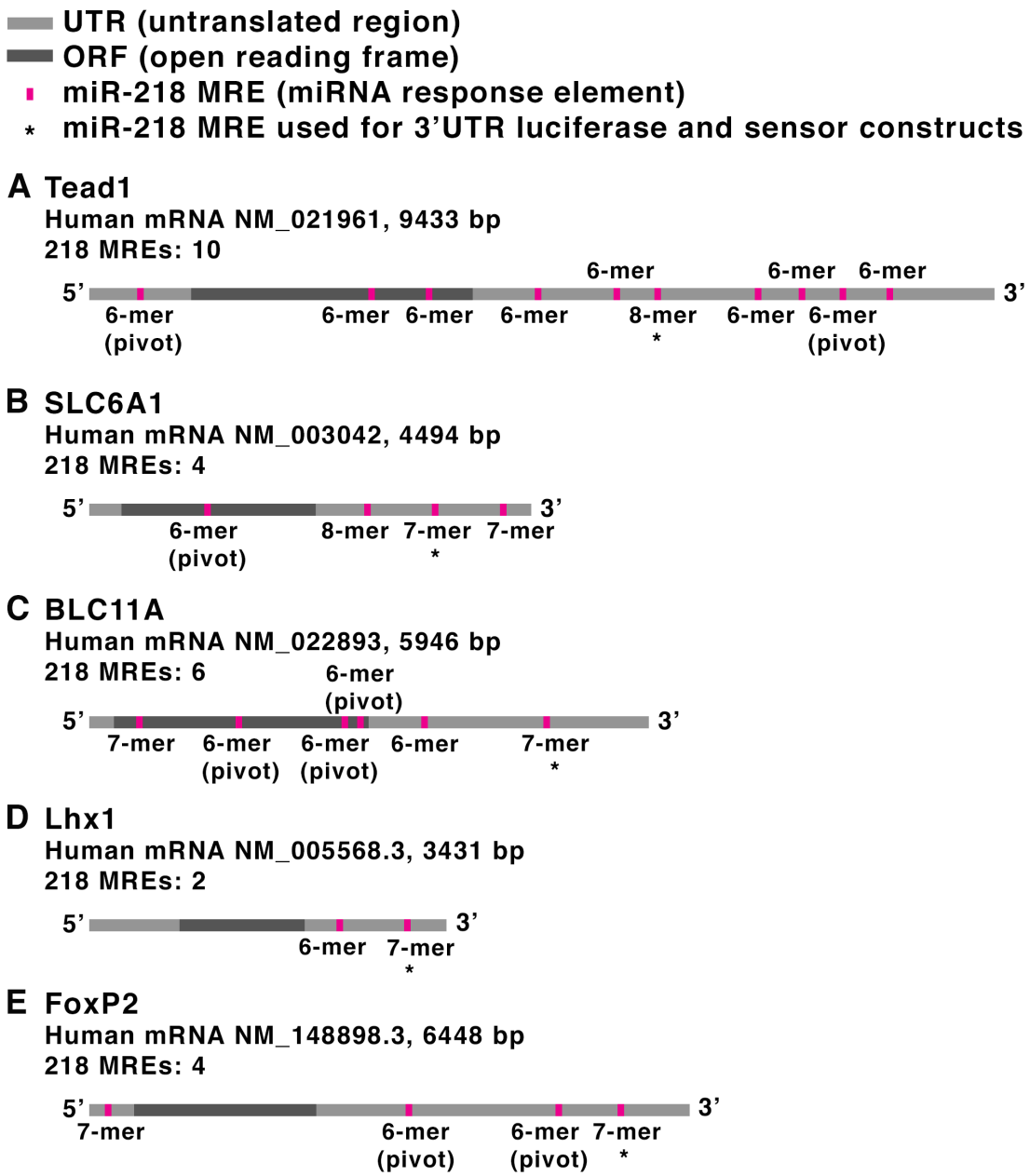


Figure 2.14. miR-218 MRE distribution on the selected miR-218 RISC-trap target mRNAs
(A-E) Illustrations showing the relative distribution of miR-218 MREs on the selected miR-218 RISC-trap Target mRNAs. The miR-218 MRE used for the 3'UTR sensor constructs was the highest conserved miR-218 MRE in 3'UTR of each gene identified by TargetScan. The 6-mer pivot MREs contain 6 matching nucleotides and either a C or G bulge in MRE positions 5-6 (Chi et al., 2012).

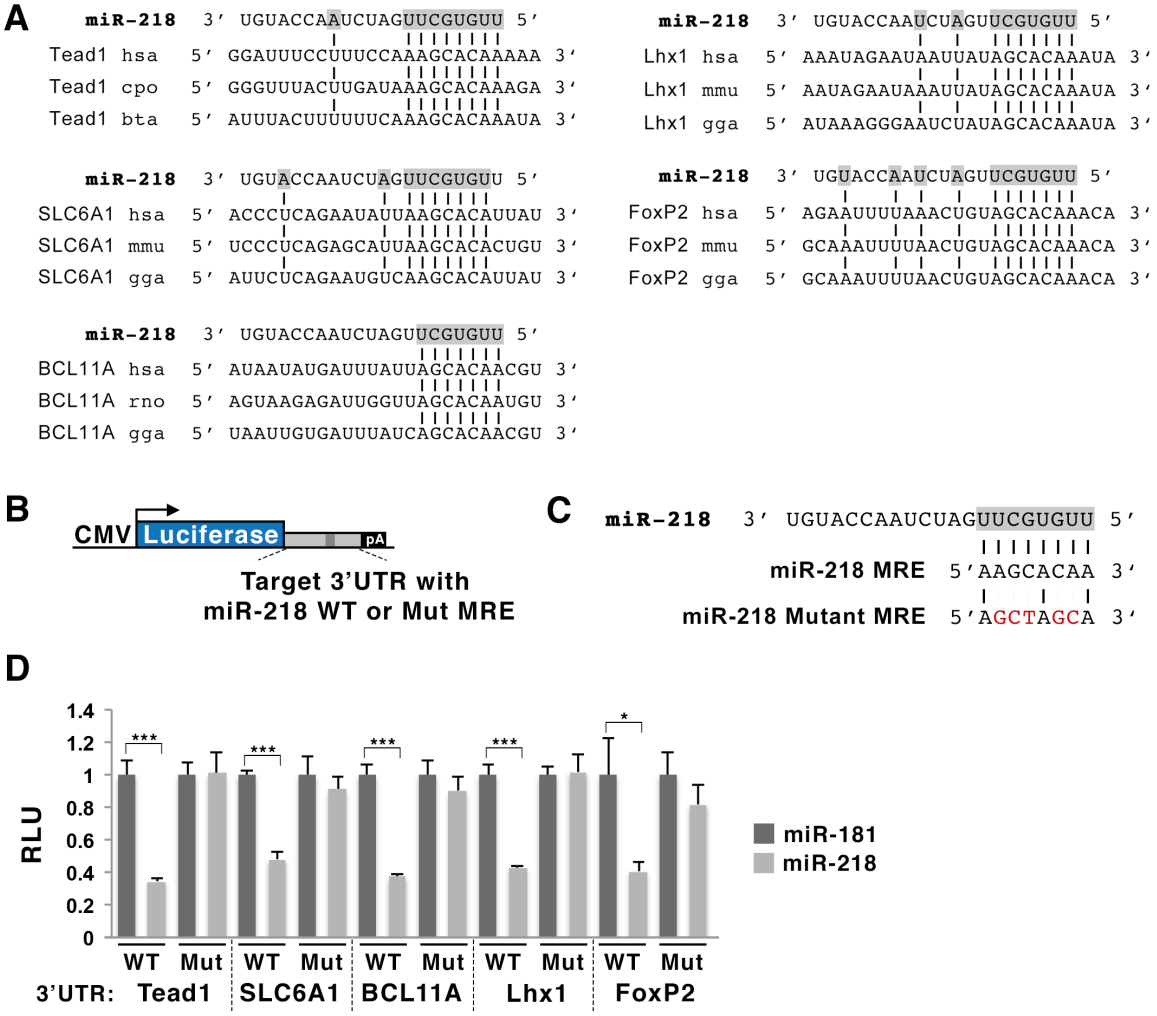


Figure 2.15. Tead1, SLC6A1, BCL11A, Lhx1 and FoxP2 target 3'UTR's containing miR-218 MREs are sufficient for miR-218 regulation in vitro

(A) Evolutionarily conserved miR218-MREs in the 3'UTRs of selected RISC-trap targets as identified by TargetScan.

(B) Illustration of the miRNA target luciferase reporters in which the partial 3'UTR of miR-218 targets is cloned downstream luciferase gene.

(C) Illustration of the miR-218 wild type and mutated MREs within the 3'UTRs of the miRNA target luciferase reporters.

(D) Luciferase assays using luciferase reporters linked with the 3'UTR of miR-218 targets in HEK293T cells. miR-218 inhibits the 3'UTRs in a miR-218 MRE-dependent manner. Each reporter was transfected with either miR-218 or miR-181. WT, luciferase reporters linked to the wild-type 3'UTR sequences of each gene; Mut, luciferase reporters linked to the 3'UTR, in which miR-218 MRE is mutated to eliminate the binding of miR-218. RLU, relative luciferase unit. Error bars represent the standard deviation; *$p < 0.05$ and ***$p < 0.0001$ in two-tailed Student's t-test.

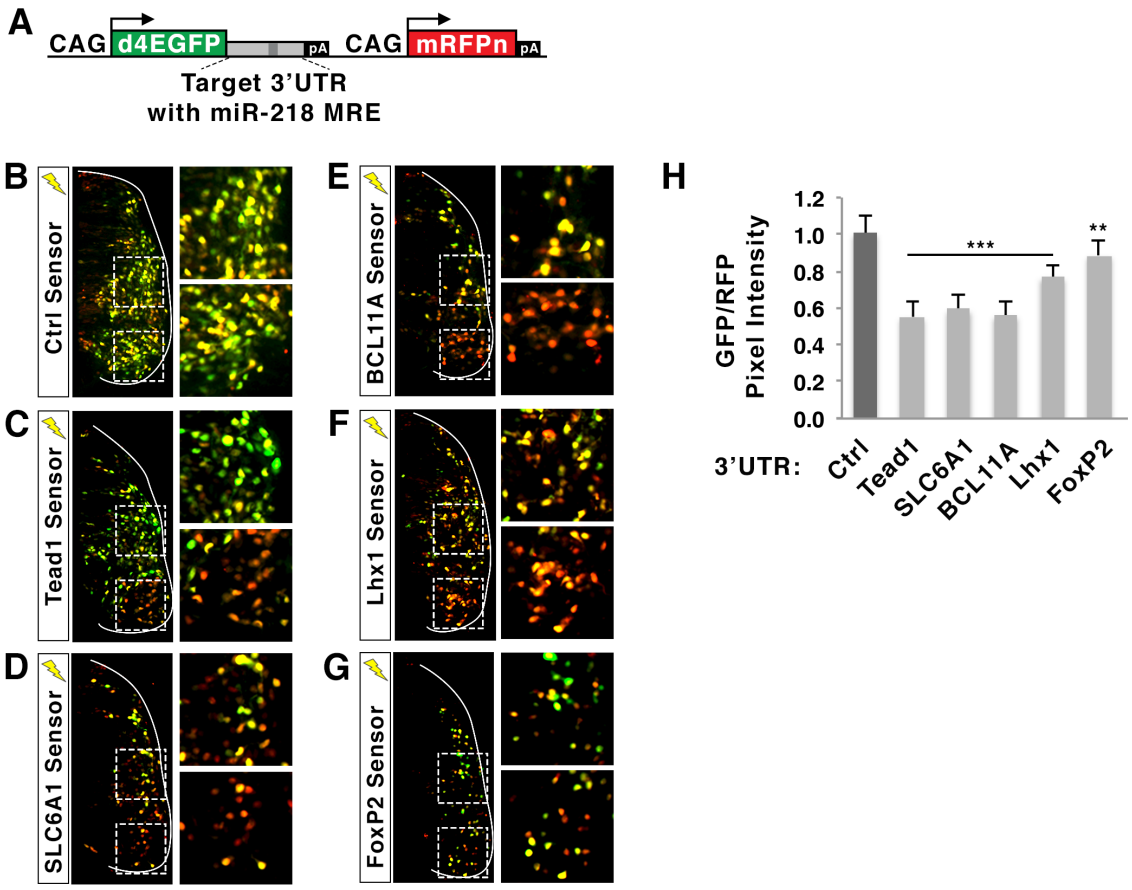


Figure 2.16. Tead1, SLC6A1, BCL11A, Lhx1 and FoxP2 target 3'UTR's containing miR-218 MREs are sufficient for miR-218 regulation in vivo

(A) Illustration of miRNA sensor plasmids, in which the 3'UTR of miR-218 targets is cloned downstream of the d4EGFP gene. The expression of both GFP and RFP is driven by two separate, ubiquitously active CAG promoters.

(B-G) The in vivo miRNA sensor analyses in the developing chick spinal cord electroporated with each miRNA sensor as indicated. Only the electroporated side of the spinal cord is shown. GFP expression is regulated by the 3'UTR of miR-218 target genes containing a miR-218 MRE, while RFP is ubiquitously expressed in all electroporated cells. Interneuron and motor neuron regions are magnified. The miRNA sensors show significant downregulation of GFP in motor neuron area compared to interneuron area.

(H) Quantification of relative pixel intensity of GFP/RFP in motor neurons, as quantified using ImageJ program. Error bars represent the standard deviation; ***$p < 0.0001$ and **$p < 0.005$ in two-tailed Student's t-test; n = 6-8 embryos.

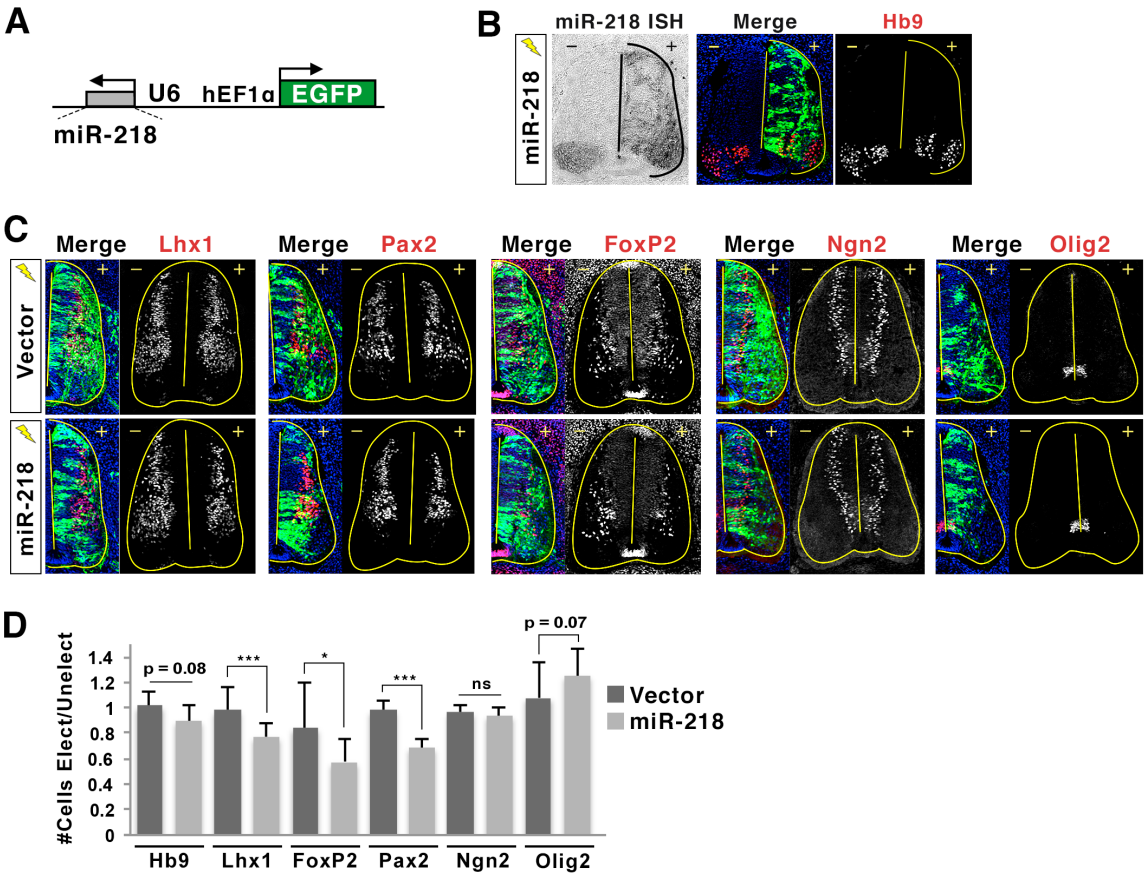


Figure 2.17. miR-218 represses differentiation of spinal interneurons
(A) Illustration of miR-218 expression construct, in which the miR-218 sequence is cloned into the hairpin structure of the EFU6 shRNA plasmid. The expression of miR-218 is driven by the ubiquitously active U6 promoter and the EGFP gene is regulated by a separate, ubiquitously active hEF1α promoter.
(B) Electroporation of the miR-218 construct results in robust and overlapping expression of miR-218 and GFP, as determined by *in situ* hybridization (ISH) with miR-218. miR-218 expression did not trigger ectopic formation of $Hb9^+$ motor neurons. +, electroporated side; -, unelectroporated control side.
(C,D) Immunohistochemical analyses in chicks electroporated with miR-218 expression construct or vector. miR-218 expression led to the reduction of $Lhx1^+$, $Pax2^+$ and $FoxP2^+$ interneurons, while it did not make a significant change in the number of $Ngn2^+$ differentiating neurons, $Olig2^+$ motor neuron progenitors, or $Hb9^+$ motor neurons. +, electroporated side; -, unelectroporated control side. Error bars represent the standard deviation; ***$p < 0.0001$ and *$p < 0.05$ in two-tailed Student's t-test; ns, not significant; n = 6-8 embryos.

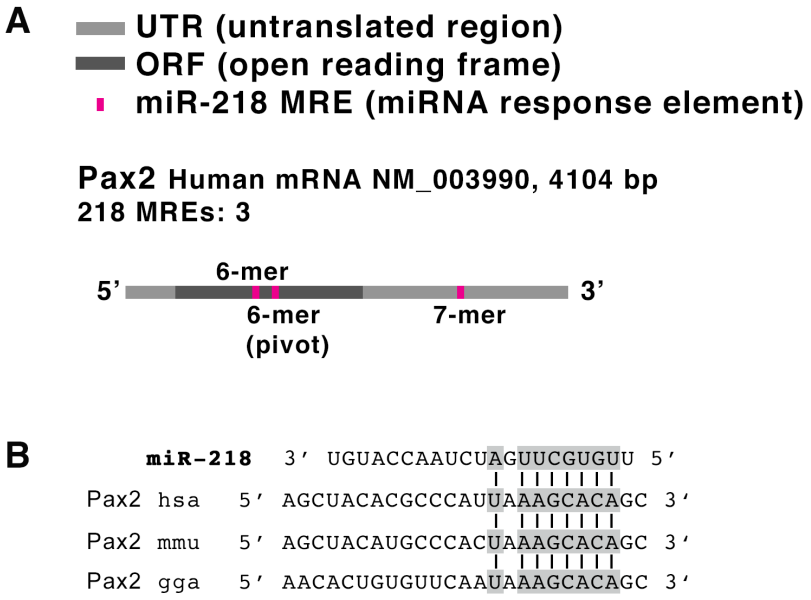


Figure 2.18. Pax2 is a potential target of miR-218
(A) Illustration showing the relative distribution of miR-218 MREs on Pax2 human mRNA.
(B) The Pax2 3'UTR miR-218 MRE is highly conserved in human, mouse and chicken, as identified by TargetScan.

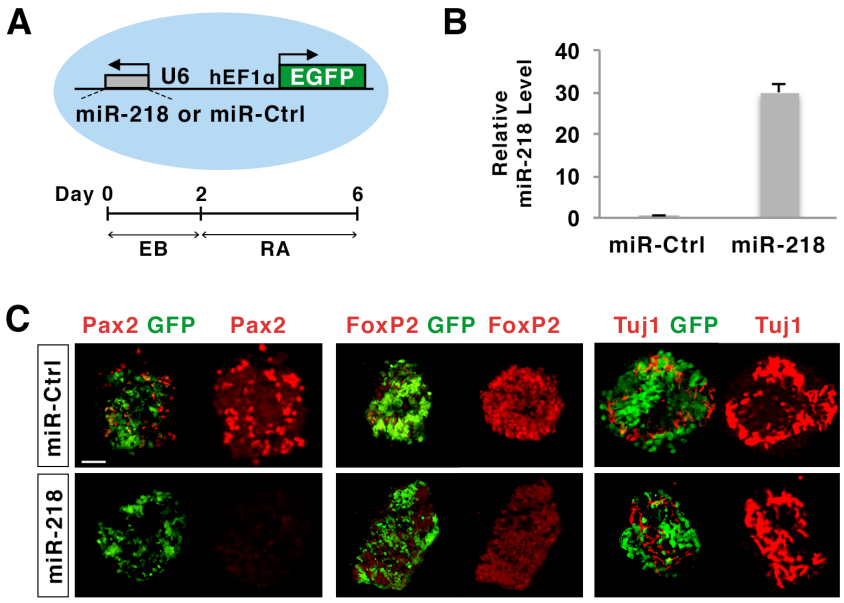


Figure 2.19. miR-218 expression represses interneuron differentiation in ESCs
(A) Illustration of miR-218 and miR-control (Ctrl) ESC lines, and the protocol to differentiate miRNA ESCs into $Pax2^+$ and $FoxP2^+$ spinal interneurons. In these ESCs, the expression of miR-218 or miR-Ctrl and EGFP are constitutively driven by U6 promoter and hEF1α promoter, respectively. EB, embryoid body; RA, retinoic acid.
(B) The miR-218 expression construct used to generate miR-218 ESCs triggers robust expression of miR-218 compared to the miR-Ctrl construct in HEK293T cells, as determined by qPCR analysis using miR-218 TaqMan probe. Error bars represent the standard deviation.
(C) Immunohistochemical analyses of miRNA ESCs at interneuron differentiation day 6. miR-218 effectively inhibited the generation of $Pax2^+$ and $FoxP2^+$ interneurons and had no effect on $Tuj1^+$ neurons. Scale bar represents 50 μM.

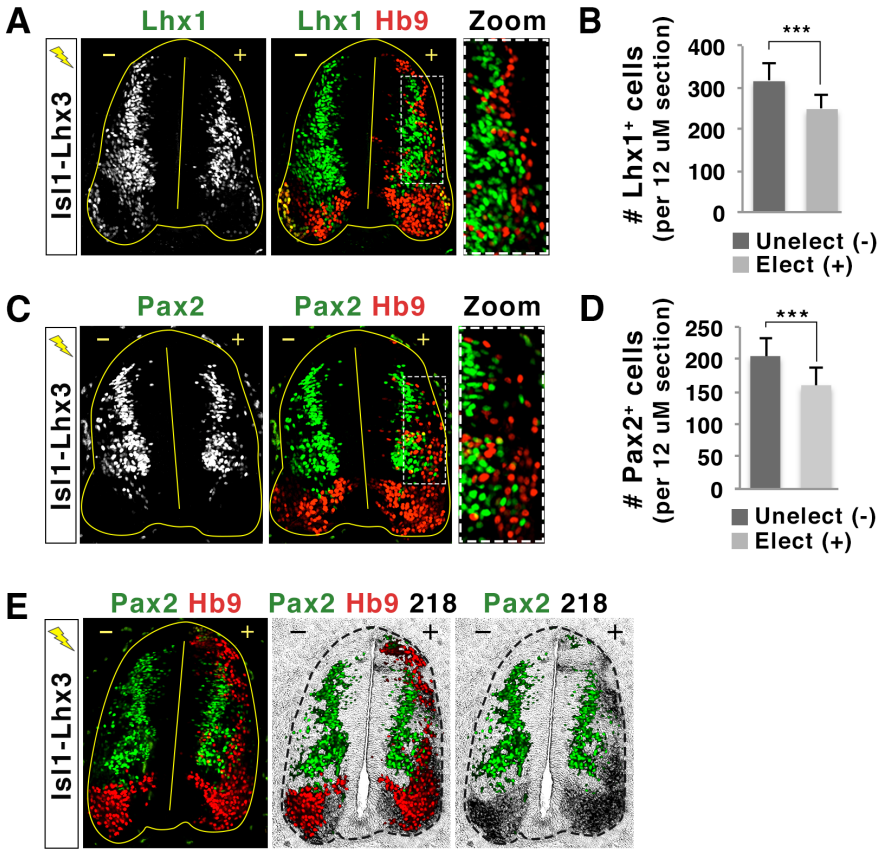


Figure 2.20. Isl1-Lhx3 generates ectopic motor neurons at the expense of interneurons
(A,C) Immunohistochemical analyses in the chick neural tube electroporated with Isl1-Lhx3. Isl1-Lhx3 suppresses the generation of $Pax2^+$ or $Lhx1^+$ interneurons, while promoting the formation of ectopic $Hb9^+$ motor neurons in the dorsal spinal cord. The magnified images show that the $Hb9^+$ motor neurons are largely exclusive with $Pax2^+$ or $Lhx1^+$ interneurons, suggesting that Isl1-Lhx3 drives motor neuron formation at the expense of interneurons. +, electroporated side; -, unelectroporated control side.
(B,D) Quantification of the number of $Lhx1^+$ or $Pax2^+$ interneurons on the electroporated (elect) sides compared to unelectroporated (unelect) sides of the spinal cord. Error bars represent the standard deviation; ***$p < 0.0001$ in two-tailed Student's t-test; n = 3 embryos.
(E) In situ hybridization for miR-218 and immunohistochemical analyses with Pax2 and Hb9 antibodies on sequential sections revealed that ectopic $Hb9^+$ motor neurons are generated at the expense of $Pax2^+$ interneurons. The overlay of Pax2, Hb9 and miR-218 show that ectopic $Hb9^+$ motor neurons express miR-218 while lacking Pax2 expression.

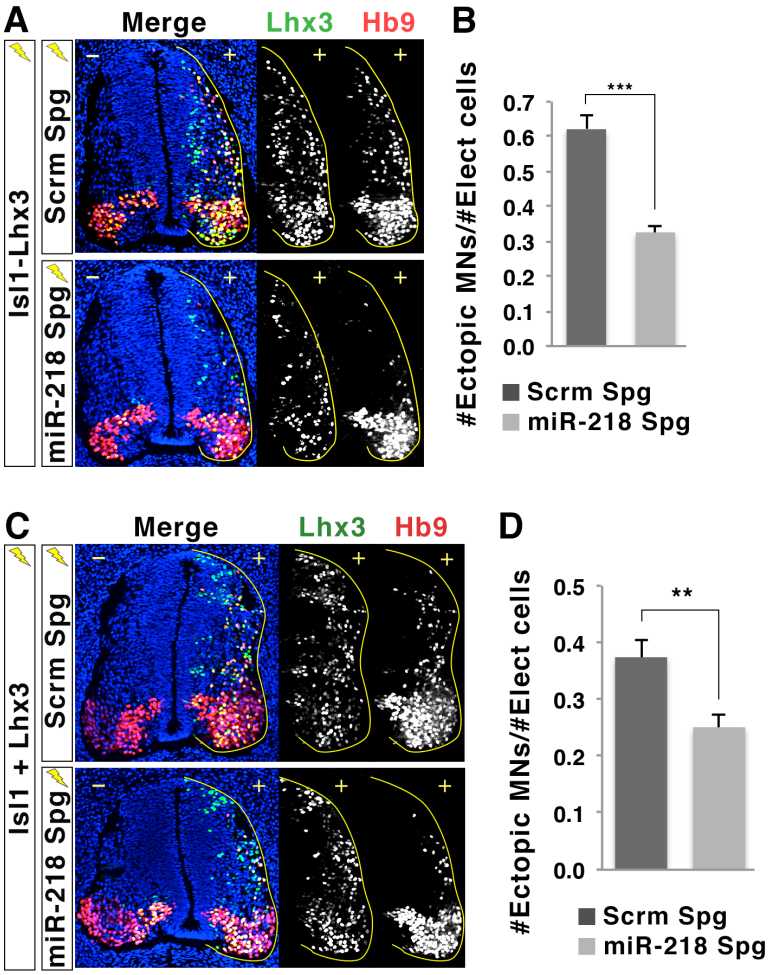


Figure 2.21. miR-218 is required for efficient generation of motor neurons by Isl1-Lhx3
(A,C) The analyses of ectopic motor neuron formation by Isl1-Lhx3 fusion construct (A) or Isl1 and Lhx3 separate constructs (C) in the presence of either miR-218 sponge inhibitor (miR-218 Spg) or scrambled sponge inhibitor (Scrm Spg) in the chick neural tube. +, electroporated side; -, unelectroporated control side. miR-218 inhibition reduces the efficiency of Isl1-Lhx3 in triggering ectopic motor neurons in dorsal neural tube. Immunohistochemistry using Lhx3 antibody shows electroporated cells in the dorsal spinal cord and Hb9 antibody is used to label ectopic motor neurons in the dorsal spinal cord and endogenous motor neurons.
(B,D) The effect of miR-218 inhibition on Isl1-Lhx3-induced motor neuron differentiation in (A,C) was quantified by the ratio of ectopic $Hb9^{+}$ motor neurons (MNs) over Lhx3-expressing transfected cells (Elect cells). Error bars represent the standard error of the mean; **p < 0.005, ***p < 0.0001 in two-tailed Student's t-test; n = 5-6 embryos.

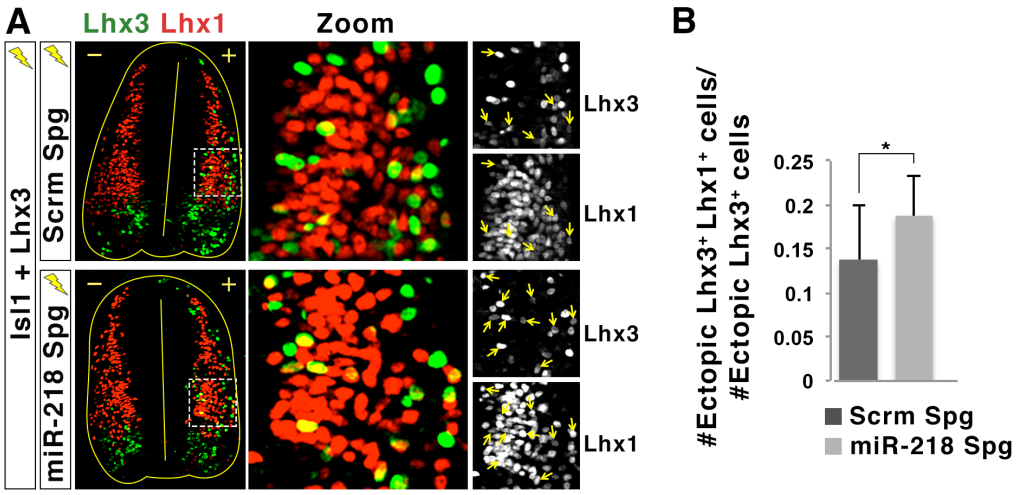


Figure 2.22. Inhibition of miR-218 downstream of Is1l and Lhx3 increases ectopic Lhx1 and Lhx3 double positive cells

(A) Immunohistochemical analyses using Lhx3 and Lhx1 antibodies in embryos electroporated with Isl1 and Lhx3 in the presence of either miR-218 sponge inhibitor (miR-218 Spg) or scrambled sponge inhibitor (Scrm Spg). Zoom panels show regions with ectopic $Lhx3^+$ cells and the arrows indicate $Lhx3^+$ and $Lhx1^+$ double positive cells. +, electroporated side; -, unelectroporated control side.

(B) The effect of miR-218 inhibitor on ectopic Lhx3 and Lhx1 double positive cells in (A) was quantified by the ratio of ectopic $Lhx3^+$ and $Lhx1^+$ double positive cells over the number of ectopic $Lhx3^+$ cells. Error bars represent the standard deviation; *$p < 0.05$ in two-tailed Student's t-test; n = 5-6 embryos.

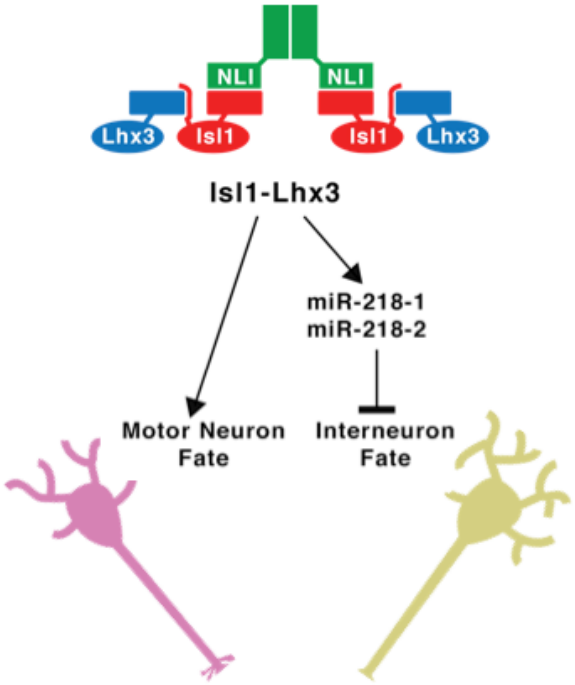


Figure 2.23. Model of Isl1-Lhx3 and miR-218 network in spinal cord motor neuron development
Model of Isl1-Lhx3 and miR-218 gene regulatory network in motor neuron development. While triggering the expression of many motor neuron-specific genes that drive motor neuron differentiation and maturation, Isl1-Lhx3 also directly induces the expression of miR-218-1 and miR-218-2, which are crucial to suppress unwanted interneuron genes in developing motor neurons.

DISCUSSION

During CNS development, neural progenitors undergo dramatic changes in gene expression to differentiate into diverse types of postmitotic neurons with distinct molecular and morphological phenotypes (Alaynick et al. 2011). One of the fundamental challenges in development is to understand the molecular mechanisms that drive this drastic and thorough transformation of the gene expression profile as neural progenitors acquire a specific neuronal identity. In the last decade, there has been an increase in our understanding of the role of miRNAs in neuronal development, where miRNAs have been shown to be important regulators of neuronal differentiation in numerous model systems (Kuwabara et al. 2004; Johnston et al. 2005; De Pietri Tonelli et al. 2008; Fineberg et al. 2009; Georgi and Reh 2010). However, the role of miRNAs in directing the differentiation of distinct neuronal cell types remains ambiguous. Here we report that miR-218 acts as an essential regulator of motor neuron fate specification that functions downstream of the Isl1-Lhx3 transcription factor complex. Our comprehensive analyses of miR-218 expression, function and direct targets provide strong evidence that miR-218 plays a crucial role motor neuron differentiation by repressing non-motor neuron fates (Figure 2.23).

miR-218, Isl1, and Lhx3 are crucial components of the gene regulatory network that controls motor neuron fate

The Isl1-Lhx3 hexamer complex functions as a fate-determining factor in motor neuron development (Thaler et al. 2002; Lee and Pfaff 2003; Lee et al. 2008; 2012; 2013). The motor neuron-inducing activity of Isl1-Lhx3 is substantially attenuated when miR-218 is inhibited in the spinal cord and embryonic stem cells (Figures 2.6 and 2.7), indicating that miR-218 is a key downstream effector for the Isl1-Lhx3 complex to induce motor neuron fate. Our miRNA array screen in Isl1-Lhx3 ESCs was used to identify miRNAs that function downstream of the Isl1-Lhx3 complex. The striking induction of miR-218 among hundreds of miRNAs, along with the presence of Isl1-Lhx3-bound peaks near both miR-218-1 and miR-218-2 genes show that miR-218 is a direct target of the Isl1-Lhx3 complex (Figures 2.1 and 2.3). The robust upregulation of miR-218 during the critical window of time when motor neurons are born may be required to establish the

gene expression profile and the cellular characteristics that are unique to motor neurons. In the future, it will be interesting to test whether co-expression of miR-218 could increase the efficacy of motor neuron generation by expression of Isl1, Lhx3, and a neurogenic basic helix-loop-helix transcription factor in stem cells or non-motor neuron cell types.

Our results demonstrate that miR-218 is an essential contributor to the gene regulatory network that controls motor neuron cell-fate specification. However, miR-218 does not appear to play an instructive role in motor neuron fate determination on its own, given that the misexpression of miR-218 alone is not sufficient to induce formation of ectopic motor neurons in chick neural tube (Figure 2.17 B). Instead, miR-218 promotes the timely transition of progenitor cells to postmitotic neurons by repressing target transcripts that promote neural progenitor characteristics and cell cycles. Moreover, miR-218 ensures the choice of motor neuron fate by suppressing interneuron genes. The generation of motor neuron progenitors (pMN), which give rise to motor neurons, has been attributed to the action of a sonic hedgehog morphogen gradient and the cross-repressive interactions between progenitor transcription factors, such as Olig2, Irx3 and Nkx2.2 (Briscoe et al. 1999; Jessell 2000; Lee and Pfaff 2001; Novitch et al. 2001). However, there is additional evidence that pMN cells maintain plasticity in choosing their cell fates. Inactivation of Isl1, Hb9 or LMO4 in motor neurons leads to the aberrant upregulation of interneuron genes and Olig2-lineage cells produce ventral interneurons in addition to motor neurons (Arber et al. 1999; Thaler et al. 1999; Dessaud et al. 2007; Lee et al. 2008; Chen et al. 2011). Thus, the mechanisms to suppress alternative cell fates need to operate continuously to block the erroneous gene expression during motor neuron differentiation. Together, our results support a model where miR-218 is directly upregulated by Isl1-Lhx3 to ensure proper motor neuron differentiation by repressing interneuron fates (Figure 2.23).

The role of miR-218 beyond motor neuron fate specification

miR-218 expression is detected at the onset of motor neuron differentiation and the expression is maintained exclusively in motor neurons throughout embryonic spinal cord development (Figures 2.4 C,D). Thus, miR-218 is likely to have roles in mature motor

neurons in addition to establishment of motor neuron fate. In this regard, it is interesting to note that many target transcripts identifed in the unbiased RISC-trap screen and GO analyses suggest a potential function for miR-218 in regulating neurite morphogenesis and synapse development. The miR-218 targets from our RISC-trap screen in this category include PTEN, NrCAM, CNTNAP2, EphA7, VCAN, MACF1, Clasp2, Robo1 and Robo2 (Supplementary Data 2 and Supplementary Data 3) (Song et al. 2010; Liu et al. 2012; Tu et al. 2013; Venkataraman et al. 2013). Future research on the function of miR-218 in motor neuron dendritogenesis and axogenesis may reveal crucial functions in developing and maintaining proper motor neuron circuitry.

miR-218 has previously been described as a tumor suppressor in the context of several types of cancers, such as medulloblastoma and glioma (Tu et al. 2013; Gao and Jin 2014). Consistent with this report, our miR-218 RISC-trap analysis revealed a highly significant cluster of miR-218 targets that are known to promote mitosis (Figure 2.9 D, Supplementary Data 3). Considering that miR-218 is expressed at a timepoint when motor neurons are transitioning from the mitotic progenitor to postmitotic status, miR-218 may play a role to promote cell cycle exit by downregulating these proliferation-related targets. This action of miR-218 would also be relevant to the role of miR-218 in cancers.

Potential combinatorial actions of miRNAs in motor neuron development

In addition to miR-218, our miRNA array study identified other miRNAs whose expression is significantly induced during Isl1-Lhx3-directed motor neuron differentiation (Figure 2.1 C). Those upregulated miRNAs include ten putative tumor suppressor miRNAs and three miRNAs that have been shown to play roles in reducing neural stem cell proliferation to stimulate neurogenesis: miR-26b, miR-200a, miR-224 (Dill et al. 2012; Peng et al. 2012; Bersten et al. 2014). The co-expression pattern of miRNAs in motor neurons raises the possibility that miR-218 functions in combination with other miRNAs, which are co-induced by Isl1-Lhx3, in controlling a subset of target genes. For example, miR-218 might cooperate with other tumor suppressor miRNAs to repress genes that facilitate mitosis and proliferation. The combinatorial actions of miRNAs may be important for the selection or robust downregulation of targets that

contain MREs for multiple miRNAs. This gene network consisting of Isl1-Lhx3 and multiple miRNAs during motor neuron development provides an interesting platform to test the paradigm that a transcription factor "code" and miRNA "code" cooperate to direct the precise differentiation of neuronal subtypes (Hobert 2008).

We also noted that several miRNAs implicated in neuronal development, such as miR-9 and miR-124, are highly expressed in Isl1-Lhx3 ESC-derived motor neurons (Supplementary Data 1) (Cao et al. 2007; Visvanathan et al. 2007; Yoo et al. 2009; Coolen et al. 2012; Dajas-Bailador et al. 2012). miR-9 is an interesting candidate to cooperate with miR-218. miR-9 exhibits fluctuating spatiotemporal expression in the embryonic spinal cord, with a brief period of expression in a subset of postmitotic motor neurons (Otaegi et al. 2011a; Luxenhofer et al. 2014). In our miRNA array screen, the expression levels of miR-9 were high in both Dox-untreated and treated conditions, suggesting that miR-9 is not a direct target of the Isl1-Lhx3 complex. miR-9 is known to play multiple roles in CNS development, such as controlling the timing of neurogenesis, axon extension and branching, and regulates motor columnar formation (Otaegi et al. 2011a; Coolen et al. 2012). While miR-218 expression was detected in all subtypes of motor neurons, it may control the differentiation of motor columns by collaborating with other miRNAs whose activity is specific to a motor neuron subtype. In light of this, it is notable that some of the miR-218 targets, such as Lhx1 and Onecut2, are known to have motor neuron subtype specific expression (Tsuchida et al. 1994; Francius and Clotman 2010). In the future, it will be interesting to further investigate the combinatorial actions of miRNA-218 with other miRNAs in motor neuron development.

Our study provides important insights into how miRNAs contribute to establishment of cell identity in CNS development and how they are interconnected with cell fate-determining transcription factors. The development of a unique cellular identity requires both activation and repression of genes, thereby building a gene expression profile unique to a specific cell fate. To achieve this, several transcription factors are known to function as both transcription activator and repressor in gene context- or cell context-dependent manner (Dasen et al. 2003). Our study suggests that employing miRNAs as downstream effectors of transcription activators to induce gene repression could be a prominent strategy in cell fate specification.

CHAPTER 3

Analysis of Multiple miRNAs in the Developing Spinal Cord

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*This chapter presents data that may be used in future projects and publications. It will not be submitted for publication as a single story and references are made to data presented in Chapter 2 of this thesis.

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ABSTRACT

In the last decade, our understanding of miRNA function in neuronal development has increased dramatically. miRNAs are essential regulators of neuronal differentiation and cell fate decisions in numerous model systems. However, the role of miRNAs in a gene regulatory network that determines cell fate is not well studied. Here we identify numerous miRNA candidates that might function in motor neurons and test whether they are expressed or active in developing spinal cord. Among many interesting miRNAs, we found that miR-153 has dynamic spatiotemporal pattern of expression in the spinal cord. Furthermore, we demonstrate that miR-153 and miR-218 cooperate to control the 3'UTR of axon guidance factor Robo2 in developing motor neurons. These results suggest that multiple miRNAs may work in combination to effectively repress target mRNAs and to promote proper motor neuron development and connectivity.

MATERIALS AND METHODS

DNA Constructs

The generation of the miRNA sensor plasmid was previously described (Cao et al. 2007). miRNA sensor MREs were cloned into the 3'UTR of sensor d4EGFP by multimerization of the following oligos: **miR-218 MRE** forward 5'- CGC GTA CAT GGT TAG ATC AAG CAC AAG, reverse 5'- CGC GCT TGT GCT TGA TCT AAC CAT GTA; **miR-145 MRE** forward 5'- CGC GTA GGG ATT CCT GGG AAA ACT GGA CG, reverse 5'- CGC GCG TCC AGT TTT CCC AGG AAT CCC TA. **miR-153 MRE** forward 5'- CGC GTG ATC ACT TTT GTG ACT ATG CAA G, reverse 5'- CGC GCT TGC ATA GTC ACA AAA GTG ATC A. **miR-181 MRE** forward 5'- CGC GTA CTCA CCG ACA GCG TTG AAT GTT G, reverse 5'- CGC GCA ACA TTCA ACG CTG TCG GTG AGT A. **miR-19 MRE** forward 5'- CGC GTT CAG TTT TGC ATG GAT TTG CAC AG, reverse 5'-CGC GCT GTG CAA ATC CAT GCA AAA CTGA A. **miR-135 MRE** forward 5'- CGC GTT CAC ATA GGA ATA AAA AGC CAT AG, reverse 5'- CGC GCT ATG GCT TTT TAT TCC TAT GTG AA. **miR-106 MRE** forward 5'- CGC GTC TAC CTG CAC TGT AAG CAC TTT TG, reverse 5'- CGC GCA AAA GTG CTT ACA GTG CAG GTA GA. **miR-367 MRE** forward 5'- CGC GTT CAC CAT TGC TAA AGT GCA ATT G, reverse 5'- CGC GCA ATT GCA CTT TAG CAA TGG TGA A. **miR-183 MRE** forward 5'- CGC GTA GTG AAT TCT ACC AGT GCC ATA G, reverse 5'- CGC GCT ATG GCA CTG GTA GAA TTC ACT A. **miR-let7g MRE** forward 5'-CGC GTA ACT GTA CAA ACT ACT ACC TCA G , reverse 5'-CGC GCT GAG GTA GTA GTT TGT ACA GTT A . **miR-15 MRE** forward 5'-CGC GTC ACA AAC CAT TAT GTG CTG CTA G , reverse 5'-CGC GCT AGC AGC ACA TAA TGG TTT GTG A . **miR-23 MRE** forward 5'- CGC GTG GTA ATC CCT GGC AAT GTG ATG, reverse 5'- CGC GCA TCA CAT TGC CAG GGA TTA CCA. **miR-33 MRE** forward 5'- CGC GTT GCA ATG CAA CTA CAA TGC ACG, reverse 5'- CGC GCG TGC ATT GTA GTT GCA TTG CAA. **miR-148 MRE** forward 5'- CGC GTA CAA AGT TCT GTG ATG CAC TGA G, reverse 5'- CGC GCT CAG TGC ATC ACA GAA CTT TGT A. **miR-182 MRE** forward 5'- CGC GTC GGT GTG AGT TCT ACC ATT GCC AAA G, reverse 5'- CGC GCT TTG GCA ATG GTA GAA CTC ACA CCG A. **miR-96 MRE** forward 5'- CGC GTA GCA AAA ATG TGC TAG TGC CAA AG, reverse 5'- CGC GCT TTG

GCA CTA GCA CAT TTT TGC TA. **miR-101 MRE** forward 5'- CGC GTT TCA GCT ATC ACA GTA CTG TAG, reverse 5'- CGC GTT TCA GCT ATC ACA GTA CTG TAG. **miR-103 MRE** forward 5'- CGC GTT CAT AGC CCT GTA CAA TGC TGC TG, reverse 5'- CGC GCA GCA GCA TTG TAC AGG GCT ATG AA. **miR-26 MRE** forward 5'- CGC GTA GCC TAT CCT GGA TTA CTT GAA G, reverse 5'- CGC GCT TCA AGT AAT CCA GGA TAG GCT A. **miR-382 MRE** forward 5'- CGC GTC GAA TCC ACC ACG AAC AAC TTC G, reverse 5'- CGC GCG AAG TTG TTC GTG GTG GAT TCG A.

The partial Robo2 wild-type and MRE mutant 3'UTR sensors were cloned using the following primers with previously constructed Robo2 3'UTR wild-type and mutant luciferase plasmid templates: forward 5' – GCA CGC GTT AAC TGA GAG GGG ACA TAC AAA GA, reverse 5' – GGC GCG CTT GCC AAC ACC ATC ATT CCT TCG A.

In Ovo Electroporation

Expression constructs were injected into the lumens of chick embryonic spinal cords at Hamburger Hamilton stages 12-14 (Hamburger and Hamilton, 1951). Electroporation was performed using a square wave electroporator (BTX) as previously described (Nakamura and Funahashi, 2001). Incubated chicks were harvested and analyzed at Hamburger Hamilton stages 17-32, fixed in 4% paraformaldehyde, and cryosectioned at 12 μm. Quantification of chick electroporation data where n = number of embryos included in the analyses, with 2-3 sections quantified per embryo. The methods used for statistical analyses are listed within each figure legend.

Quantification of Pixel Intensity

In ovo electroporation of miRNA sensor plasmids using concentrations of 1 μg/μl for individual MRE and vector sensors, 1.2 μg/μl for Robo2 3'UTR sensors. Unsaturated images were acquired on a Zeiss Axio Imager.Z2 microscope, maintaining the same exposure time ratio of GFP and RFP for each section. Pixel intensity was determined using ImageJ program using unadjusted images (Chapter 2). All sensor electroporation images used in figures were adjusted using the same settings.

In Situ Hybridization Assay

For in situ hybridization analysis, embryos were harvested at indicated stage, fixed in 4% formaldehyde, embedded in OCT, and cryosectioned at 18 μm. Locked nucleic acid (LNA)-modified miR-218, miR-153 and miR-145 oligonucleotide probes (Exiqon) were labeled with digoxigenin according to the suppliers protocol (Roche) and use for in situ hybridization as described (Kloosterman et al. 2006).

Isl1-Lhx3 ChIP-seq Peak microRNA Analysis

Isl1-Lhx3 chromatin immunoprecipitation and deep sequencing (ChIP-seq) using motor neuron differentiated Isl1-Lhx3 ESCs was previously described (Lee et al. 2013). Isl1-Lhx3 ChIP-Seq peaks near miRNA genomic loci were identified using QuEST software with a ChIP cutoff score of 30. The miRNAs candidates were identified by the presence of Isl1-Lhx3 ChIP-seq peaks within 20 kb upstream and 20 kb downstream of each miRNA intergenic gene and miRNA intragenic genes were included with the parameters of peaks identified with upstream 20kb + gene body + downstream 20kb.

Isl1-Lhx3 ESC miRNA Array and Small RNA Quantitative RT-PCR

The generation and differentiation of Isl1-Lhx3 ESCs was previously described (Lee et al. 2012). The miRNA microarray assays were performed with TaqMan® Array Rodent MicroRNA Card A (Life Technologies). The miRNA Array analyzes 380 miRNAs and contains five endogenous controls and one negative control assay. RNA extraction and cDNA amplification for TaqMan® miRNA array were performed according to manufacturer's instructions. (http://www.lifetechnologies.com/us/en/website-overview/ab-welcome.html).

INTRODUCTION

miRNAs are essential for neuronal differentiation (Andersson et al. 2010; Zehir et al. 2010; Georgi and Reh 2010) and are key regulators in the regulatory networks that determine neuronal cell fates (Chapter 2) (Chen et al. 2011; Luxenhofer et al. 2014; Pérez-Martínez 2014). While several miRNAs have been identified as important molecules that regulate neurogenesis individually, it remains unclear whether multiple miRNAs function in combination to influence the development of distinct neuronal cell types.

One well-studied model of neuronal differentiation and fate specification is spinal motor neurons (Stifani 2014). Motor neuron differentiation is primarily controlled by transcription factors that induce the expression of terminal differentiation genes in a precise spatiotemporal pattern (Alaynick et al. 2011). In particular, the expression of two LIM-homeodomain factors, LIM homeobox 3 (Lhx3) and Islet-1 (Isl1), is essential for the early stages motor neuron fate specification (Tsuchida et al. 1994; Pfaff et al. 1996; Thaler et al. 2002; Lee and Pfaff 2003). Isl1 and Lhx3 form a transcription complex with nuclear LIM interactor (NLI) (Thaler et al. 2002; Lee and Pfaff 2003), and co-expression of Isl1 and Lhx3 triggers motor neuron differentiation in ESCs (Lee et al. 2012; Mazzoni et al. 2013; Lee et al. 2013). Previous studies have characterized the genomic binding sites of the Isl1-Lhx3 complex and downstream target mRNAs of Isl1-Lhx3 (Lee et al. 2012; 2013; Mazzoni et al. 2013). We recently identified many miRNAs that are upregulated by Isl1-Lhx3 (Figure 2.1, Supplementary Data 2). In particular, we discovered that the highest upregulated miRNA, miR-218, plays an essential role in determining motor neuron fate downstream of Isl1-Lhx3 (Chapter 2). In the future, it will be interesting to investigate whether other miRNAs that are upregulated by Isl1-Lhx3 play a role in developing motor neurons in vivo.

To assess whether other miRNAs are involved in motor neuron development, we generated a list of miRNA candidates by combining information from multiple datasets. We then performed a miRNA response element (MRE) sensor screen to test whether selected miRNA candidates downregulate target genes in the developing spinal cord. These results suggest that 18 miRNAs are active in many regions of the developing chick neural tube, but it remains to be determined whether these miRNAs are exclusively active

or expressed in developing spinal motor neurons. We further examined the expression pattern of two candidates, miR-153 and miR-145, and found that miR-153 has dynamic spatiotemporal expression in the developing spinal cord of mouse and chick. The in situ hybridization revealed that miR-145 is not expressed in the developing spinal cord, but is strongly expressed in the developing heart.

Previous studies raise a possibility that multiple miRNAs function in combination to dynamically regulate a single target mRNA. This hypothesis is driven by data showing that the majority of mRNAs have multiple conserved MREs (Lewis et al. 2003; Krek et al. 2005; Brennecke et al. 2005), as well as direct evidence from in vitro assays. In addition, knocking out an individual miRNA often has a subtle or unobserved phenotype (Liu et al. 2005; Miska et al. 2007; Xiao et al. 2007; Fineberg et al. 2009). Our data indicate that at least two miRNAs, miR-218 and miR-153, cooperate to suppress the 3'UTR of roundabout 2 (Robo2), a gene involved in axon guidance (Reeber et al. 2008; Zhang et al. 2012). Altogether this study provides preliminary data that will support further investigation of combinatorial actions of multiple miRNAs in spinal cord development.

RESULTS

Identification of miRNA candidates

To generate a list of miRNA candidates with potential roles in motor neuron development, I compared information from three datasets. These datasets include Isl1-Lhx3 ChIP-seq data, Isl1-Lhx3 miRNA array data, and a list of miRNAs with previously described expression in the developing spinal cord (Figure 3.1 A, B). The Isl1-Lhx3 ChIP-seq analysis revealed 82 miRNA genes that are linked to at least one Isl1-Lhx3-bound ChIP-seq peak. The discovery of Isl1-Lhx3-bound ChIP-seq peaks near miRNA genes suggests that the expression of these miRNAs may be regulated by the Isl1-Lhx3 complex. Next, we used a dataset generated from the Isl1-Lhx3 ESC miRNA array analysis, which showed that 32 miRNAs were significantly upregulated at least 2-fold in Isl1-Lhx3 ESC-derived motor neurons (Figure 2.1 and Supplementary Data 1). Finally, we identified 4 miRNAs that were previously reported to be expressed in the developing spinal cord (Darnell et al. 2006; Otaegi et al. 2011a; Wei et al. 2013).

By comparing these groups of miRNA candidates, I identified 9 miRNAs that were found in at least two overlapping datasets (Figure 3.1 A). miR-145, miR-140, miR-350, miR-135a, miR-99b, and miR-148b are promising candidates due to their significant upregulation by Isl1-Lhx3 during the motor neuron differentiation of Isl1-Lhx3 ESCs and the identification of Isl1-Lhx3-bound ChIP-seq peaks near their genomic loci (Figure 3.1 B). miR-9 and miR-106a are promising candidates due to the presence of Isl1-Lhx3-bound peaks, their high level of expression in Isl1-Lhx3 ESC-derived motor neurons, and their known expression in embryonic spinal cord (Figure 3.1 A,B) (Darnell et al. 2006; Otaegi et al. 2011a). miR-218 was the strongest candidate that was identified in this screen, and our study uncovered a crucial role for miR-218 in motor neuron development (Chapter 2). Additionally, other studies have shown that miR-153 is expressed in developing zebrafish motor neurons (Wei et al. 2013), and in the developing chick spinal cord (Darnell et al. 2006). Altogether, combining these datasets provided a list of miRNAs that may function in developing spinal motor neurons.

Analysis of miRNA response element sensors

One method to assess whether a miRNA is active in the developing spinal cord is to perform in ovo electroporation of miRNA response element (MRE) sensors (Figure 2.5) (Cao et al. 2007; Luxenhofer et al. 2014). To determine whether selected miRNA candidates exhibit activity in motor neurons, I generated 22 miRNA sensor plasmids, in which a cytomegalovirus/chicken β actin (CAG) promoter drives the expression of a destabilized nuclear GFP with a half-life of 4 hours (d4EGFP) that has multimers of complete complementary miRNA response elements (MREs) cloned in the 3'UTR. The MRE sensor plasmids also have another CAG promoter directing the expression of monomeric nuclear RFP (mRFPn) (Figure 3.2 A) (Cao et al. 2007). In ovo electroporation of MRE sensors detect endogenous miRNA regulation of GFP expression, where the GFP gene contains miRNA-specific MREs within the 3'UTR. The RFP labels transfected cells and is not subject to miRNA-dependent repression.

Among 22 different MRE sensors screened using in ovo electroporation, 6 sensors, which contain MREs for miR-218, miR-145, miR-153, miR-106, miR-19, miR-135, exhibited unique GFP expression patterns that are distinct from the GFP pattern of the

sensor vector control (Figure 3.2 C-I). 12 MRE sensors, which respond to miR-181, miR-148, miR-15, miR-16, miR-26, miR-23, miR-183, miR-34, miR-33, miR-let7, miR-103, or miR-182, showed moderate repression of GFP compared to RFP everywhere in the spinal cord (data not shown). Finally, 4 MRE sensors for miR-140, miR-367, miR-96, or miR-101 showed weak repression of GFP compared to RFP everywhere (data not shown). Consistent with previous results, the miR-218 MRE sensor showed a drastic downregulation of GFP specifically in motor neurons, compared to interneurons (Figures 3.2 C and 2.5 C). The miR-145 MRE sensor also showed a strong downregulation of GFP in motor neurons compared to interneurons (Figure 3.2 D), similarly to the miR-218 sensor (Figure 3.2 C). This result suggests that endogenous miR-145 may actively repress target genes with miR-145 MREs in developing motor neurons. Interestingly, some of the motor neuron miRNA candidate sensors, such as miR-19 and 135, had regions with increased GFP expression relative to RFP (Figures 3.2 G-I). The stabilization of sensor GFP suggests that these miRNAs may bind the MREs to facilitate gene expression, rather than promote the degradation of target mRNAs. Other miRNAs of interest include miR-153, which showed moderate to strong GFP repression everywhere (Figure 3.2 E, I), and miR-106, which consistently showed the strongest GFP repression everywhere in the spinal cord (Figure 3.2 F, I).

Analysis of miR-145 expression in embryos

miR-145 is one of the most promising motor neuron miRNA candidates that we identified because it was significantly upregulated 3-fold in Isl1-Lhx3 ESC-derived motor neurons and two Isl1-Lhx3-bound ChIP-seq peaks were identified within 50 kb of the miR-145 gene (Figure 3.1 B). Additionally, the GFP expression pattern from the miR-145 sensor suggests that miR-145 may be active in developing spinal motor neurons (Figure 3.2 D). This led us to test whether miR-145 is expressed in the developing spinal cord. In situ hybridization analyses across multiple developmental stages reveal that miR-145 is not expressed in spinal motor neurons of embryonic mouse or chick (Figure 3.3 A,B). Interestingly, miR-145 is strongly expressed in the developing dorsal aorta and atrial chamber tissue (Figure 3.3 A,B). This expression pattern is consistent between chick and mouse embryonic tissues.

Analysis of miR-153 expression in embryos

Another promising miRNA candidate is miR-153 (Figure 3.1 A). miR-153 expression was detected in zebrafish motor neurons via qPCR (Wei et al. 2013), and preliminary in situ hybridization analyses performed in the Lee laboratory suggested that miR-153 is expressed in motor neurons during early stages of chick spinal cord development (data not shown). Interestingly, however, the miR-153 sensor analysis revealed that the GFP expression controlled by miR-153 MRE was down-regulated everywhere in the spinal cord (Figure 3.2 E). These MRE sensor results suggest that endogenous miR-153 may be widely expressed in multiple locations at different time points during spinal cord development. To test this hypothesis, I performed in situ hybridization experiments for miR-153 at numerous developmental stages in mouse and chick spinal cord tissue. These experiments revealed that miR-153 is initially expressed in the entire neural tube, but later becomes restricted to dorsal, postmitotic cells in the spinal cord (Figure 3.4). This expression pattern suggests that miR-153 may play a role in spinal cord development.

Potential co-regulation of the Robo2 gene by miR-218 and miR-153

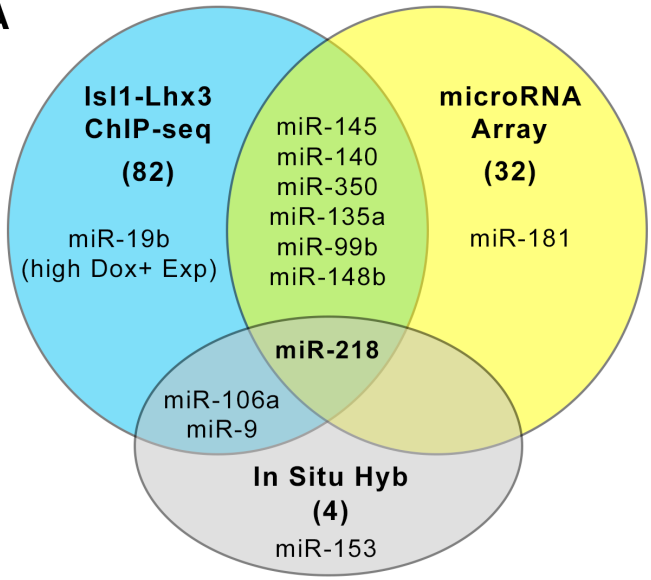
Previous studies have shown that Robo2 is a miR-218 target mRNA (Fish et al. 2011). Further work in the Lee laboratory confirmed that miR-218 can repress Robo2 expression, and the data also suggested that miR-153 may regulate Robo2 (data not shown). In the developing spinal cord, Robo2 mRNA is weakly expressed in motor neurons and strongly expressed in dorsal interneurons, where it plays a role in guiding the axons of commissural interneurons that cross the midline (Figure 3.5 A) (Sundaresan et al. 2003; Zhang et al. 2012). Robo2 mRNA expression is largely complementary to the expression of miR-218 in motor neurons (Figure 3.5 B,C), supporting the hypothesis that miR-218 represses Robo2 transcription. miR-153 expression is detected in the entire neural tube during the initial stages of neural tube development and at later stages, the expression is strongest in dorsal, postmitotic regions of both mouse and chick embryonic spinal cord (Figure 3.4 and 3.5 B,C). Interestingly, analyses of human and mouse Robo2 mRNA sequences revealed that they contain several miR-218 and miR-153 MREs (Figure 3.5 D). In particular, the Robo2 3'UTR contains strong and evolutionarily conserved MREs for

both miR-218 and miR-153 (Figure 3.5 D, 3.6 A). Our results show that miR-218 and miR-153 expression overlaps in motor neurons during the initial stages of neural tube development. The presence of conserved MREs, overlapping miR-218 and miR-153 expression in motor neurons, and the data generated by a former Lee lab member, suggest that miR-218 and miR-153 may co-regulate Robo2 in motor neurons.

miR-218 and miR-153 cooperate to repress the 3'UTR of Robo2 in vivo

Previous data generated in the Lee laboratory show that miR-218 and miR-153 may co-regulate Robo2 in vitro (data not shown). To test whether endogenous miR-218 and miR-153 could combinatorially regulate Robo2 in vivo, I cloned the partial 3'UTR of mouse Robo2 mRNA into the miRNA sensor vector for use in neural tube electroporations (Figure 3.6 B). In addition to generating a Robo2 wild-type (wt) 3'UTR sensor, I also cloned Robo2 3'UTR sensors that contain single mutations (mt) in the miR-218 MRE or miR-153 MRE and also a double mutant (DB), with mutations in both miR-218 and miR-153 MREs (Figure 3.6 A,B). In ovo electroporation of these constructs followed by analyses of GFP/RFP pixel intensity ratios uncovered in vivo regulation of the Robo2 3'UTR by endogenous miRNAs (Figure 3.6 C-G). I analyzed the chick embryos 2 days post electroporation at Hamburger Hamilton stage 20, which is a time point when both miR-218 and miR-153 are both expressed in motor neurons (Figure 3.5 B). Electroporation of the Robo2 wt sensor resulted in low GFP expression compared to RFP in motor neurons (Figure 3.6 C,G). In contrast, both the miR-218 mutant and miR-153 mutant sensors showed a significant de-repression of GFP in motor neurons, compared to the Robo2 wt sensor (Figure 3.6 E-G). Finally, electroporation of the Robo2 double mutant sensor showed a drastic de-repression of GFP in motor neurons compared to wild type or single MRE mutant sensors (Figure 3.6 D, G). These results suggest that endogenous miR-218 and miR-153 act in combination to repress the Robo2 3'UTR in developing motor neurons.

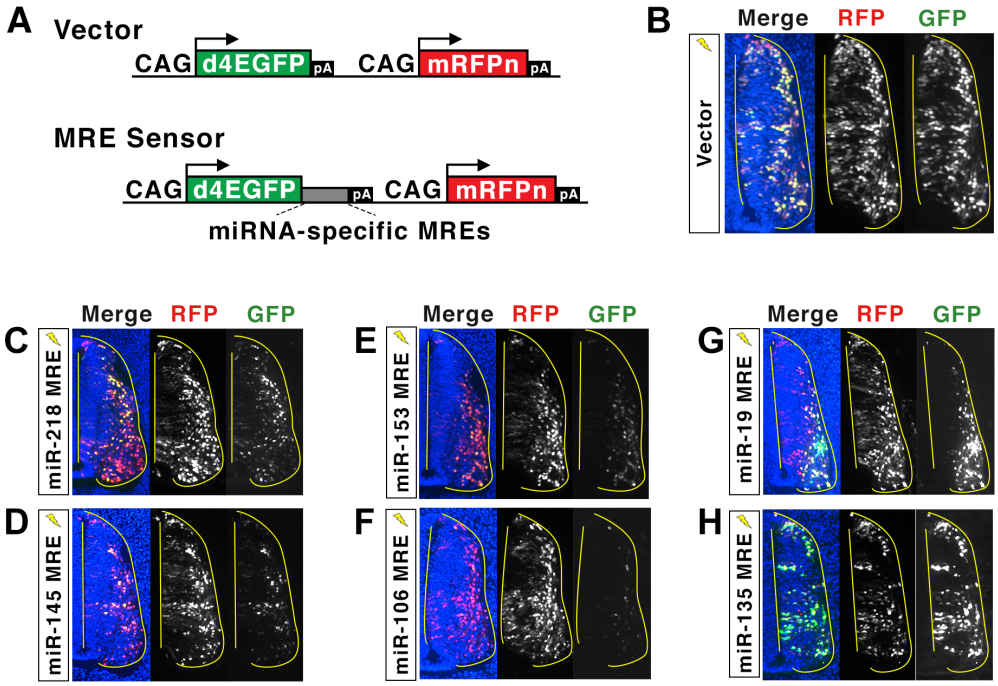
A



B

	Isl1-Lhx3 ChIP-seq Peaks		Isl1-Lhx3 miRNA Array		
microRNA	Location	Peak(s) [kb]	P Value	FC	Dox+ Exp
miR-218-2	Slit3 intron	46, 70	0.006	70.76	5
miR-181a-2	Nr6a1 intron	110	0.015	2.11	158
miR-145	Intergenic	9, 50	0.022	3.05	132
miR-140	Wwp2 intron	30, 43	0.024	2.24	45
miR-350	Cep17 exon	30	0.025	3.03	210
miR-135a-1	Glyctk exon	13.5	0.026	2.24	102
miR-99b	Intergenic	19, 100	0.050	3.35	55
miR-148b	Copz1 intron	8, 22, 50, 70	0.052	3.15	170
miR-19b-2	Intergenic	10	0.283	1.11	2
miR-106a	Kis2 exon	10	0.302	1.10	3
miR-9-2	RP23 exon	12, 23	0.423	0.85	31

Figure 3.1. Identification of miRNA candidates with potential roles in motor neuron development
(A) Venn diagram of three datasets that were used to identify candidate motor neuron miRNAs. (Blue) Analysis of chromatin immunoprecipitation and deep sequencing (ChIP-seq) data using motor neuron differentiated Isl1-Lhx3 ESCs revealed Isl1-Lhx3 genomic binding sites near 82 miRNA genes (Lee et al. 2013; data not shown). (Yellow) 32 miRNAs were significantly upregulated by Dox treatment (> 2-fold, $p < 0.05$), as determined by TaqMan miRNA arrays using Isl1-Lhx3 ESCs (Figure 2.1 and Supplementary Data 2). (Gray) Previously published miRNA in situ hybridization results reveal that 4 miRNAs are expressed in the developing chick or zebrafish spinal cord: miR-218, miR-153 and miR-106 (Darnell et al. 2006); miR-153 (Wei et al. 2013); miR-9 (Otaegi et al. 2011a).
(B) Table of top miRNA candidates sorted by Isl1-Lhx3 miRNA array P Value. Location indicates genomic location of the miRNA gene that was identified near an Isl1-Lhx3 binding site (peak) in the ChIP-seq analysis. The peak distance is the approximate distance between the miRNA gene and the Isl1-Lhx3-bound peak region. FC, fold change of miRNA expression in Dox-untreated versus Dox-treated Isl1-lhx3 ESCs; Dox+ Exp, describes the rank of relative expression levels of each miRNA in Dox-treated conditions.



I

microRNA	Sensor GFP Expression in Spinal Cord
miR-218	Strong repression in motor neurons
miR-145	Moderate to strong repression in motor neurons
mIR-153	Moderate to strong repression everywhere
miR-106	Strong repression everywhere
miR-19	Repression in progenitor zone, stabilization in postmitotic zone
miR-135	Strong stabilization everywhere

Figure 3.2. miRNA response element (MRE) sensor analysis
(A) Illustrations of vector and MRE sensor plasmids. The multimerized miRNA response elements (MREs) for miRNAs were inserted between the destabilized GFP (d4EGFP) gene and polyA (pA) sequences. The expression of both GFP and mononuclear RFP (mRFPn) is driven by two separate, ubiquitously active CAG promoters.
(B) The expression pattern of GFP and RFP in the chick spinal cord electroporated with miRNA sensor vector at Hamburger Hamilton stage 25. The relative expression levels of GFP and RFP are similar in all electroporated cells.
(C-H) The expression pattern of GFP and RFP at Hamburger Hamilton stage 25 in chick spinal cords electroporated with miR-218 (B), miR-145 (C), miR-153 (D), miR-106 (E), miR-19 (F), and miR-135 (G) MRE sensors. GFP expression is regulated by endogenous miRNA that binds to the miRNA-specific MREs present in 3'UTR of the GFP gene, while RFP expression depicts the electroporated cells.
(I) Summary of the observed GFP expression pattern of miRNA-specific GFP-MREs in the electroporated chick spinal cord that are shown in (C-H), compared to relative GFP/RFP expression observed with sensor vector alone (B). Other miRNA sensors that were tested show moderate GFP repression everywhere in the chick spinal cord: miR-181, miR-148, miR-15, miR-16, miR-26, miR-23, miR-183, miR-34, miR-33, miR-let7, miR-103, miR-182 (data not shown). Or weak GFP repression everywhere: miR-140, miR-367, miR-96, and miR-101 (data not shown).

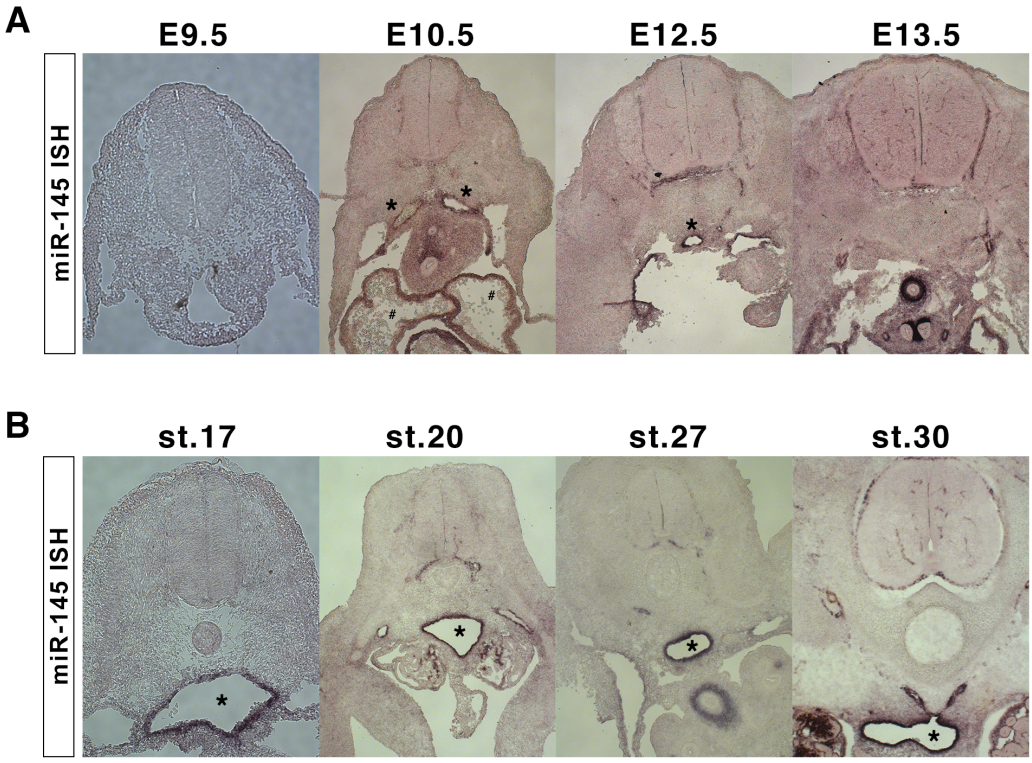


Figure 3.3. In situ hybridization analysis of miR-145 in embryonic spinal cord and trunk tissue
(A-B) miR-145 in situ hybridization in developing mouse (A) and chick (B) spinal cord and trunk tissue. Expression was detected in developing heart tissue. * indicates dorsal aorta, # indicates atrial chambers.

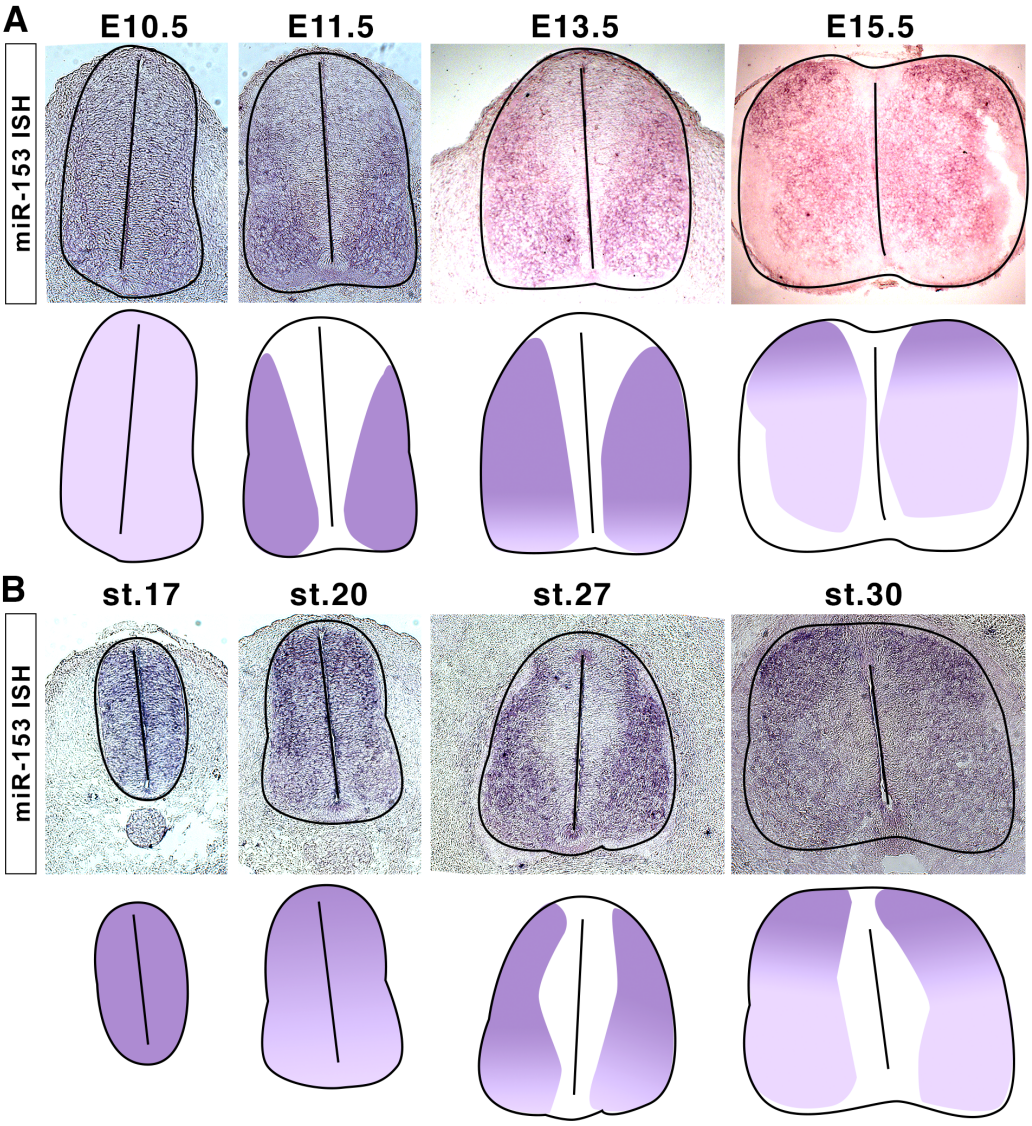


Figure 3.4. In situ hybridization analysis of miR-153 expression in embryonic spinal cord
(A) miR-153 in situ hybridization in the embryonic mouse spinal cord. miR-153 is initially expressed in the postmitotic, lateral regions of the spinal cord and expression is excluded from the progenitor zone in mouse embryonic day E10.5-E11.5. At later developmental time points, the strongest miR-153 expression is maintained in dorsal, postmitotic regions of the spinal cord.
(B) miR-153 in situ hybridization in embryonic chick spinal cord. miR-153 is initially expressed in the entire chick spinal cord at Hamburger Hamilton stages 17-20. At later developmental time points, the strongest miR-153 expression is maintained in dorsal, postmitotic regions of the spinal cord.

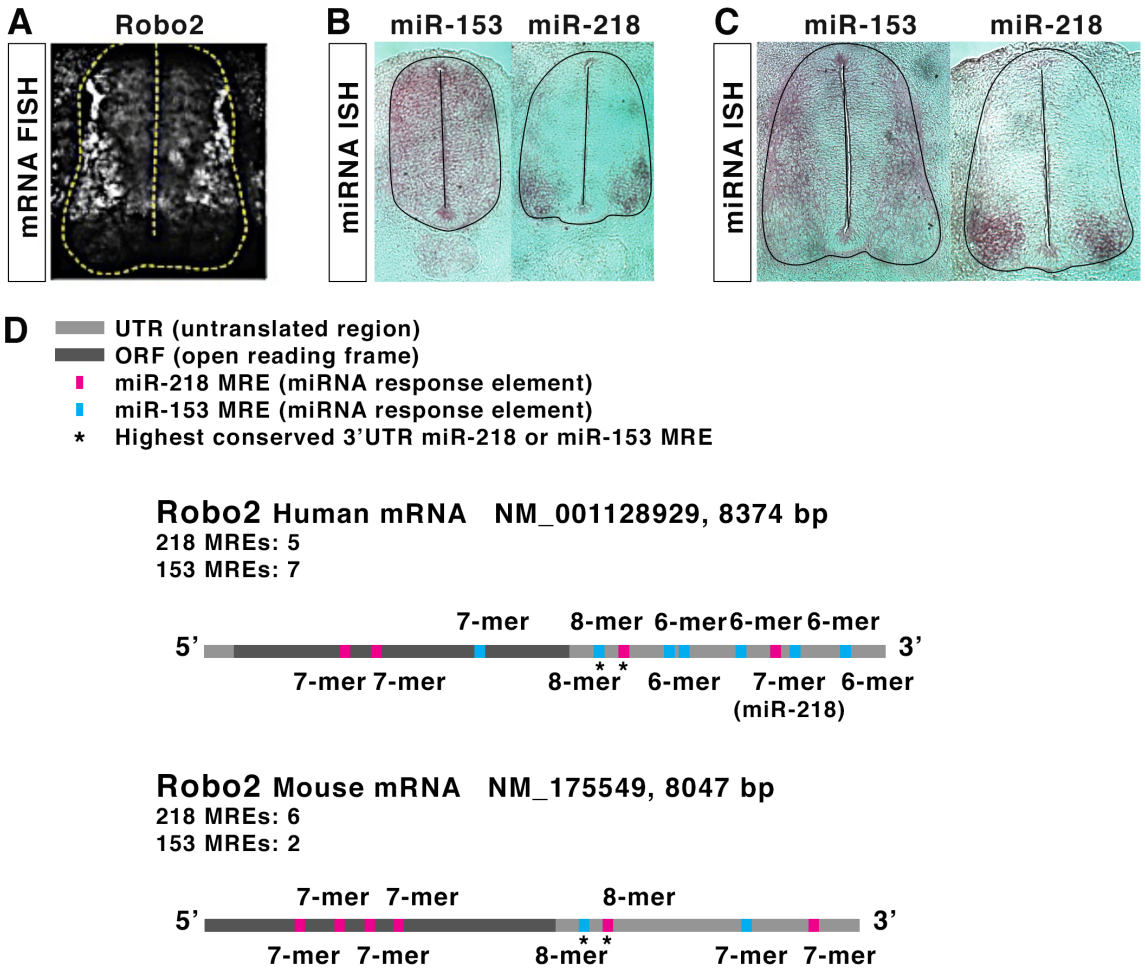


Figure 3.5. miR-218 and miR-153 may co-regulate Robo2
(A) Fluorescent in situ hybridization (FISH) for Robo2 mRNA. Robo2 mRNA expression is strongest in dorsal postmitotic interneurons in the developing spinal cord.
(B-C) In situ hybridization for miR-218 and miR-153 in chick embryos at Hamburger Hamilton stage 20 (B) and stage 25 (C). miR-153 is expressed in the entire neural tube during the initial stages of neural tube development and at later developmental time points, the expression is strongest in dorsal, postmitotic regions of the spinal cord. miR-218 expression is exclusive to motor neurons.
(D) Illustration showing the relative distribution of miR-218 and miR-153 MREs on human and mouse Robo2 mRNA sequences. The highest conserved miR-218 and miR-153 MREs in the 3'UTR were identified by TargetScan.

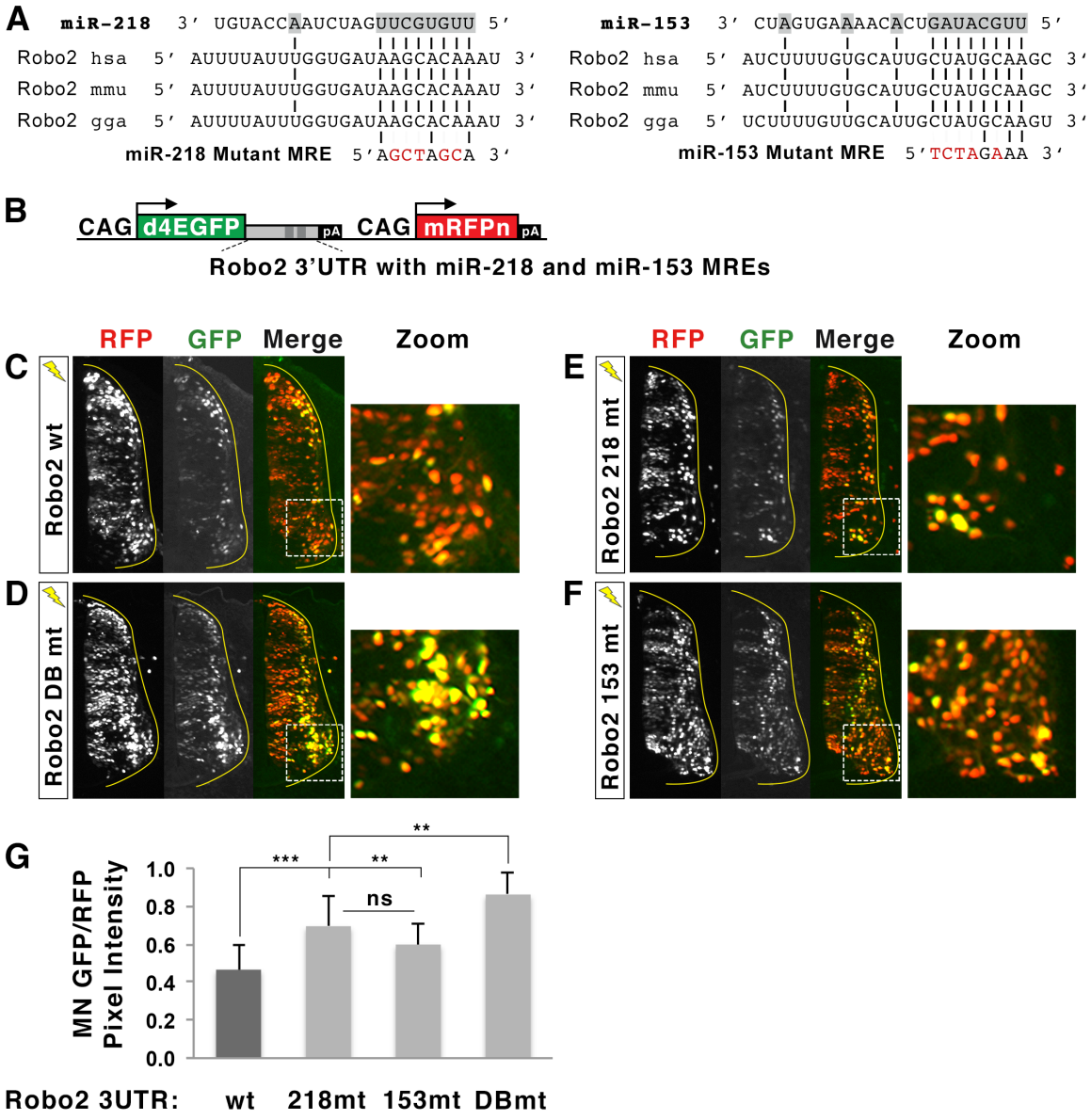


Figure 3.6. miR-218 and miR-153 cooperate to repress the 3'UTR of Robo2 in vivo

(A) The Robo2 3'UTR contains highly conserved miR-218 and miR-153 MREs, as identified by TargetScan. The miR-218 and miR-153 MRE mutations used for the Robo2 3'UTR sensor analysis are shown.

(B) Illustration of miRNA sensor plasmid, in which the partial 3'UTR of Robo2 that contains conserved miR-218 and miR-153 MREs, is cloned downstream of the d4EGFP gene. The expression of both GFP and mononuclear RFP (mRFPn) is driven by two separate, ubiquitously active CAG promoters.

(C-F) Robo2 3'UTR sensor analyses in Hamburger Hamilton stage 20 chick spinal cord electroporated with each miRNA sensor, as indicated. Only the electroporated side of the spinal cord is shown. GFP expression is regulated by the partial Robo2 3'UTR containing miR-218 and miR-153 wild type (wt) MREs, single MRE mutant (mt) or miR-218 and miR-153 double mutant (DB mt) MREs, while RFP is ubiquitously expressed in all electroporated cells. Motor neuron regions are magnified. The miRNA sensors show upregulation of GFP in motor neurons in wild type compared to mutated Robo2 3'UTR sensors.

(G) Quantification of relative pixel intensity of GFP/RFP in motor neurons (MN), as quantified using ImageJ program. Error bars represent the standard deviation; ***$p < 0.0001$, **$p < 0.005$ and ns, non-significant in two-tailed Student's t-test; n = 5-6 embryos.

DISCUSSION

Different neuron types serve as the building blocks of the central nervous system (CNS) and understanding the mechanisms that generate neuronal diversity and connectivity is an important goal in neurobiology research. Our analyses of multiple miRNA datasets and endogenous miRNA activity revealed candidate miRNAs that may have important roles in spinal cord development. Additionally, we show that miR-218 and miR-153 function in combination to repress Robo2. By identifying motor neuron-expressed miRNA candidates and assessing the combinatorial actions of miRNAs in vivo, we advance our understanding of miRNA function in CNS development.

Multiple motor neuron-expressed miRNA candidates

The ability to tease out the contribution of miRNAs to complex gene regulatory networks in the developing CNS using the multifaceted screens provides an exciting new paradigm to identify components in the regulatory pathways that determine neuronal cell fate. In our study, we reveal a group of miRNAs that may play a role in motor neuron development (Figure 3.1). We further tested whether selected miRNA candidates exhibit endogenous activity in the embryonic spinal cord using a miRNA sensor screen (Figure 3.2). The miRNA sensor screen revealed that the majority of the miRNA candidate MREs lead to a moderate downregulation of the target gene GFP in the entire spinal cord (Figure 3.2, data not shown). It is possible that this GFP downregulation reflects dynamic spatiotemporal expression of miRNAs in the spinal cord, including motor neurons. However, it remains to be determined whether the repression of the sensor GFP always reflects the activity of endogenous miRNAs and therefore the expression pattern of these miRNAs. For example, although the miR-145 MRE sensor showed promising downregulation of GFP in motor neurons, the in situ hybridization results show that miR-145 is expressed in developing heart tissue, but not in motor neurons (Figure 3.3). It is possible that other miRNAs may bind to the multimerized miR-145 MRE to direct downregulation of the miR-145 MRE sensor GFP in motor neurons. It is also possible that the multimers of miRNA-specific MRE sequences that are cloned into the 3'UTR of sensor GFP can contain other regulatory sequences that affect GFP expression. Further in

situ hybridization experiments will be needed to determine whether other miRNA candidates are expressed in developing spinal cord.

miRNA-mediated post-transcriptional upregulation

The miRNA sensor screen also revealed that two MRE sensors, miR-19 and miR-135, show a robust upregulation of GFP expression, compared to RFP and vector control, in developing chick spinal cord (Figure 3.2 B,G,H). Although miRNA-mediated downregulation of target genes is considered the primary function of miRNAs, increasing evidence suggests that miRNAs can also directly upregulate the expression of target mRNAs (Vasudevan and Steitz 2007). Some studies have shown that miRNAs can directly activate or stabilize mRNA translation depending on the cellular context and type of MRE within a target mRNA (Li et al. 2006b; Vasudevan and Steitz 2007; Lin et al. 2011; Truesdell et al. 2012). Our results that miR-19 and miR-135 MRE sensors show robust stabilization of GFP is intriguing and suggests that these miRNAs may act as activators of gene expression in the developing spinal cord. The fact that miRNAs can act as both repressors and activators adds a new layer of complexity in understanding miRNA function. In the future, it will be interesting to test whether miR-19 and miR-135 are expressed in the developing spinal cord and whether they can directly stabilize and increase target mRNA expression.

The regulation of Robo2 by miR-218 and miR-153

Robo2 is a membrane receptor that acts as a repressive cue to prevent commissural axons from re-crossing the midline (Long et al. 2004; Reeber et al. 2008). Robo2 mRNA is largely excluded from motor neurons during early stages of spinal cord development (Figure 3.5 A) and the Robo2 3'UTR contains highly conserved, 8 complementary base-pair MREs for both miR-218 and 153 (Figures 3.5 D and 3.6 A). Interestingly, we found that both miRNAs are expressed in developing motor neurons (Figure 3.5 B). Further investigation of the endogenous miRNA-dependent regulation of the Robo2 3'UTR revealed that miR-218 and miR-153 cooperate to regulate the expression of Robo2 sensor GFP in motor neurons (Figure 3.6). These results suggest that both miR-218 and miR-153 are important for robust repression of Robo2 in motor neurons. Further analysis is

needed to determine the functional significance of miRNA-mediated repression of Robo2 in motor neurons and whether miR-218 and miR-153 can regulate Robo2 expression in vivo.

Additionally, it is interesting to note that while Robo2 mRNA is strongly expressed in the cell bodies of commissural interneurons of dorsal spinal cord, Robo2 protein is only detected in the distal ends of axons (Long et al. 2004; Reeber et al. 2008; Mambetisaeva et al. 2005), suggesting that Robo2 mRNA is either translated in cell body and transported to developing axons or it is locally translated in axons. One intriguing hypothesis is that the location of Robo2 mRNA translation is regulated by miRNAs. Our Robo2 3'UTR sensor data show that mutating miR-218 and miR-153 MREs results in significant de-repression of sensor GFP in motor neurons (Figure 3.6 C-G). It is interesting to note that the Robo2 sensor with miR-153 MRE mutant alone also showed strong GFP de-repression in the somas of dorsal interneurons (Figure 3.6 F), where Robo2 mRNA, but not Robo2 protein, is detected (Figures 3.5 A). A potential mechanism for the selective localization of Robo2 protein in axons but not in cell bodies is that miRNAs, such as miR-153, function to repress Robo2 translation in the soma in order to ensure that this repulsive membrane receptor is only present in axons. Future loss-of-function and gain-of-function experiments with miR-153 may reveal important regulatory roles for miR-153 in controlling Robo2 expression and interneuron development.

Conclusion

Determining how multiple miRNAs function in networks in neuronal development is a challenging goal in neurobiology. Our understanding of miRNA networks is rapidly evolving and the concept of combinatorial miRNA codes has become an increasingly interesting research topic (Krek et al. 2005; Hobert 2008; Yoo et al. 2009; Trompeter et al. 2011). By further characterizing miRNA activity and the combinatorial function of miRNAs in the developing spinal cord, our study provides an essential foundation for further analysis of endogenous miRNA function.

CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

Thesis Summary

The initial goal of my thesis was to identify miRNAs that play a role in motor neuron development. Based on previous work by the Lee laboratory and the robust upregulation of miR-218 by Isl1-Lhx3 in my miRNA array screen, I focused my study on miR-218 function during spinal cord motor neuron development. The transition from neuronal progenitors to mature neurons requires the changes in gene expression that determine neuron subtype-specific attributes. Our data show that miR-218 is essential for motor neuron differentiation downstream of Isl1-Lhx3, and that miR-218 represses target mRNAs that promote neural progenitor and interneuron phenotypes (Chapter 2). Additionally, we identified numerous miRNA candidates that may function in spinal cord development, and we show that miR-218 and miR-153 combinatorially repress Robo2 (Chapter 3).

Expression of miR-218 alone is not sufficient for motor neuron generation

Our data show that miR-218 is essential for motor neuron differentiation, but our results also show that expression of miR-218 alone does not induce motor neuron generation. Following in ovo electroporation of miR-218 under the direction of the ubiquitous polymerase III promoter, U6, expression of miR-218 resulted in a significant decrease in interneuron, but no significant difference in the number of motor neurons compared to the vector control (Figure 2.17). This result indicates that miR-218 is not sufficient to direct motor neuron generation. Additionally, electroporation experiments with high concentrations of miR-218 resulted in a dramatic decrease in all postmitotic neurons, including motor neurons, whereas electroporations with low concentrations of miR-218 had no observed effect (data not shown). One possible explanation for this result is that miR-218 represses the expression of genes that are important for neural progenitor maintenance and initial stages of neurogenesis, such as Tead1, Sox21, and FoxP2 (Figures 2.11 – 2.18). Therefore, premature expression of miR-218 in the neural progenitors may reduce cell proliferation and/or the ability of progenitor cells to produce

postmitotic neurons. Notably, electroporations using medium to high concentrations of miR-218, did not reduce the number of neural progenitor cells (Figure 2.17 C,D and data not shown), suggesting that miR-218 expression does not reduce cell proliferation in the progenitor zone, but instead may inhibit spinal progenitor cells from differentiating into postmitotic neurons.

Our data show that miR-218 activity is essential for Isl1 and Lhx3 to drive the efficient formation of ectopic $Hb9^{+}$ motor neurons in the dorsal chick spinal cord (Figure 2.21 C,D). To test whether co-expression of miR-218 along with the Isl1-Lhx3 complex enhanced motor neuron generation, we co-electroporated the miR-218 expression construct with Isl1 and Lhx3. Rather, miR-218 expression appeared to reduce the formation of $Hb9^{+}$ ectopic motor neurons by Isl1 and Lhx3 (Figure 4.1 A,B). It remains to be tested whether the ubiquitous expression of miR-218 affected the expression of Isl1 and Lhx3, thereby influencing the generation of ectopic $Hb9^{+}$ cells. It is also possible that premature expression of miR-218, which was not subject to the induction by Isl1-Lhx3, suppresses neuronal differentiation and therefore reduced motor neuron differentiation.

Similar to results in the spinal cord, expression of miR-218 in ESCs was not sufficient to trigger motor neuron generation, but effectively suppressed the formation of spinal interneurons (Figure 2.19 C, data not shown). In mouse embryoid bodies, retinoic acid (RA) treatment results in the robust expression of spinal interneuron markers and limited expression of some motor neuron markers, such as Hb9 (Renoncourt et al. 1998; Wichterle et al. 2002). In our RA-mediated differentiation of miR-218 and miR-Ctrl expressing-ESCs we did not observe an increase in Hb9 protein levels (data not shown).

So far, we have only analyzed the effect of expression of miR-218 in the developing spinal cord and ESCs. Our laboratory and others have identified numerous miR-218 target mRNAs that function as mitotic cell cycle regulators and have shown that miR-218 negatively affects cancer stem cell proliferation, migration and self-renewal (Figures 2.9 A,D and 2.10) (Tu et al. 2013; Gao and Jin 2014). In the future, it will be interesting to test whether controlling the timing and context of miR-218 expression could enhance motor neuron differentiation. For example, the generation of a Dox-inducible miR-218 ESC line and a postmitotic neural promoter-driven miR-218 construct for in ovo electroporation would provide the experimental paradigms to test whether we

could improve motor neuron generation by selectively expressing miR-218 during neuronal differentiation.

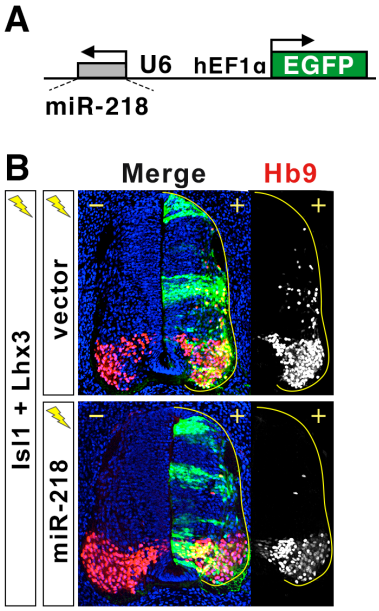


Figure 4.1 miR-218 expression reduces Isl1 and Lhx3-induced ectopic motor neuron generation
(A) Illustration of miR-218 expression construct, in which the miR-218 sequence is cloned into the hairpin structure of the EFU6 shRNA plasmid. The expression of miR-218 is regulated by the ubiquitously active U6 promoter and the EGFP gene is regulated by a separate, ubiquitously active hEF1α promoter.
(B) Analyses of ectopic motor neuron formation by electroporation of Isl1 and Lhx3 in the presence of either miR-218 expression or vector constructs in embryonic chick spinal cord at Hamburger Hamilton stage 25. GFP expression represents electroporated cells and Hb9 antibody labels motor neurons. +, electroporated side; -, unelectroporated control side.

Premature expression of miR-218 leads to stalled cell migration in the ventral spinal cord

In ovo electroporation of the miR-218 expression construct results in a significant decrease in interneurons and no significant changes in motor neurons (Figure 2.17). Interestingly, miR-218-electroporated cells failed to migrate to the lateral edge of the spinal cord compared to vector control. This migration phenotype is particularly strong in the motor neuron area, where transfected cells appear to be stalled in the progenitor zone (Figure 2.17 B,C). During CNS development, neural progenitor cells strike a balance between proliferation to produce new progenitors and neurogenesis, where differentiation is paired with cell detachment and migration away from the ventricular zone (VZ) (Cappello et al. 2006; Kadowaki et al. 2007; Zhang et al. 2010). In the spinal cord, this process of differentiation and detachment from the VZ has been shown to be regulated by transcription factor forkhead box proteins 2 and 4 (FoxP2, FoxP4) (Rousso et al. 2012). Rousso et al. showed that expression of FoxP4 promotes neurogenesis via downregulation of adhesion proteins, thereby stimulating the release of neural progenitors from the basement membrane of the VZ. Conversely, inhibition of FoxP2 or FoxP4 results in a decrease in neurogenesis and stalled neural progenitor migration. The migration phenotype that I observed with miR-218 expression studies is strikingly similar to the migration defect shown in FoxP2 and FoxP4 inhibition experiments. Interestingly, FoxP2 is a direct target of miR-218, both mouse and human FoxP4 transcripts contain 2-3 miR-218 MREs (Figures 2.9, 2.11-2.17, data not shown). These results suggest that the migration phenotype in the miR-218 expression experiments could be due to the repression of FoxP2 and FoxP4; in which premature downregulation of these transcription factors by miR-218 may decrease the ability of progenitor cells to detach and migrate away from the VZ. The ventral progenitor cells were more sensitive to the miR-218-induced migration phenotype than the electroporated dorsal progenitors, which often migrated all the way to the lateral edge of the spinal cord (Figure 2.17 B,C). Considering this result, I hypothesize that there may be additional miR-218 targets that are specifically expressed in the ventral spinal cord, whose premature downregulation leads to a selective decrease in lateral migration of ventral progenitor cells. Proper neurogenesis requires precise spatiotemporal regulation of gene expression. Our data

show that misexpression of miR-218 in ventral spinal cord progenitors results in stalled migration from the VZ, possibly due to the premature downregulation of neurogenesis promoting genes. These data also support the idea that miR-218 functions to repress transcripts that are initially upregulated to promote broad neurogenesis, such as FoxP2 and Foxp4, but ultimately need to be downregulated for proper motor neuron specification.

Biogenesis of miR-218

In Chapter 2, we provide evidence to support the hypothesis that miR-218 is directly upregulated by the Isl1-Lhx3 complex. miR-218 was robustly upregulated downstream of Isl1-Lhx3 in vitro (Figure 2.1) and in vivo (Figure 2.4 E,F). We also show that the Isl1-Lhx3 complex directly binds genomic regions near both miR-218-1 and miR-218-2 loci, within the introns of Slit2 and Slit3, respectively (Figure 2.3). However, these experiments do not determine whether miR-218 biogenesis is independent of the expression of Slit2 and Slit3. Correlative studies suggest that intronic miRNAs are co-regulated with their host genes (Rodriguez 2004; Baskerville 2005). However, there have also been reports of independent intronic miRNA regulation (Wang et al. 2009; Ramalingam et al. 2013).

Both Slit2 and Slit3 mRNAs are highly expressed in developing motor neurons in both chick and mouse spinal cord, and that miR-218 expression largely overlaps with their expression (data not shown). Additionally, mRNA deep sequencing (mRNA-seq) experiments using Isl1-Lhx3 ESCs show that Slit2 and Slit3 mRNAs are highly upregulated in motor neurons derived from Isl1-Lhx3 ESCs (Lee et al. 2012); the same conditions in which robust miR-218 upregulation was detected (Figure 2.1 C,D). Together, these data suggest that Isl1-Lhx3 directly upregulates the expression of Slit2 and Slit3 and that miR-218-1 and miR-218-2 gene upregulation may be dependent on host gene expression. However, the expression of Slit2 and Slit3 mRNAs in the developing spinal cord does not entirely overlap with miR-218 expression. For example, miR-218 expression was not detected in the developing floor plate, where Slit2 exhibits high expression and Slit3 shows weak expression (data not shown). Thus, I hypothesize that miR-218 is co-regulated with Slit2 and Slit3, but miR-218 may be degraded in

certain cellular contexts, such as the spinal cord floor plate. In future experiments, it would be interesting to test whether miR-218 expression is entirely coupled with the upregulation of Slit2 and Slit3 genes.

miR-218 target candidates identified in Isl1-Lhx3 mRNA-seq analysis

In addition to miR-218 targets that were uncovered in the RISC-trap screen, I also identified potential miR-218 target mRNAs by analyzing Isl1-Lhx3 ESC mRNA-seq data that was previously published by the Lee laboratory (Lee et al. 2012). The Isl1-Lhx3 mRNA-seq experiments were performed using the same protocol and ESC samples that I used for the miRNA array analysis, in which miR-218 expression was highly induced (Figure 2.1). Therefore, it is interesting to assess whether the mRNAs, which were significantly downregulated by Isl1-Lhx3 during differentiation of Isl1-Lhx3 ESCs to motor neurons, are miR-218 targets. For this analysis, I generated a list of 240 mRNAs that were significantly downregulated in motor neurons derived from Isl1-Lhx3 ESCs. Within this list of mRNAs, I identified 59 potential miR-218 target mRNAs that contained at least one miR-218 MRE within the transcript using TargetScan software or direct miR-218 MRE searches. Remarkably, this analysis revealed 12 mRNAs that were also identified as direct miR-218 targets in the RISC-trap screen (shown in blue) (Figures 4.2 and 2.9 A, and Supplementary Data 2). These target transcripts include Lhx1, Pou4f1, Bmpr1b, and Sox21, which I previously identified as relevant miR-218 targets based on their expression pattern in the developing spinal cord and well-established roles in spinal neural progenitors and interneurons (Figure 2.13).

Further analysis of these 59 miR-218 target candidates revealed 5 additional mRNAs with developmental expression patterns and functions that are also indicative of miR-218-dependent regulation: Pax2, Dbx1, Scrt2, Slain1 and Lrrn1 (shown in red) (Figure 4.2). I previously described Pax2 as a potential target in Chapter 2 of this thesis. Pax2 is essential for spinal dorsal interneuron differentiation and contains numerous miR-218 MREs (Figure 2.18). Additionally, miR-218 expression reduces Pax2 protein expression in both the developing spinal cord and ESCs (Figures 2.17 - 2.20). Dbx1, Scrt2, Slain1 and Lrrn1 are also interesting potential miR-218 target mRNAs in the context of spinal motor neuron specific regulation. For example, Dbx1 is a transcription

factor that is expressed in progenitor cells in the developing spinal cord and promotes interneuron differentiation (Jessell 2000; Okada et al. 2004). Scrt2 is a zinc finger transcription factor that is expressed in chick spinal progenitors (Vieceli et al. 2013), and has been shown to stimulate neurogenesis in the developing zebrafish spinal cord (Dam et al. 2011; Guez-Aznar and Nieto 2011; Rodriguez-Aznar et al. 2013). These potential miR-218 targets further support the role of miR-218 as a repressor of spinal interneuron and neural progenitor genes.

Two other candidate miR-218 targets, Slain1 and Lrrn1, are highly expressed in the developing nervous system, but less is known about their role in spinal cord development. Slain1 is a microtubule plus end tracking protein that promotes axon development in hippocampal neuron culture (Van der Vaart et al. 2012). Although high Slain1 expression has been detected in the spinal cord via whole mount in situ hybridization (Hirst et al. 2010), the detailed expression in spinal cord cross-sections has yet to be described. Lrrn1 is a leucine-rich repeat transmembrane protein that is highly expressed spinal cord progenitor cells and is important for the organization of the hindbrain and midbrain boundary (Andreae et al. 2007; Tossell et al. 2011). Further analyses are needed to determine whether these are authentic miR-218 targets and whether knockdown of these target mRNAs is important for motor neuron differentiation.

	Gene	FC	P Value		Gene	FC	P Value
1	Nmnat2	3.7	2.4E-11	31	Gadd45a	1.6	2.3E-03
2	Lhx1	2.9	3.3E-02	32	Ptchd2	1.6	4.3E-02
3	Pou4f1 (Brn3a)	2.8	6.8E-10	33	Slain1	1.6	1.6E-03
4	Fut9	2.6	1.2E-02	34	Sox21	1.6	2.9E-02
5	Pax2	2.6	1.5E-03	35	Hs6st2	1.5	2.4E-03
6	Ntrk3	2.6	2.1E-05	36	Lrrn1	1.5	1.3E-02
7	B3gat1	2.4	6.4E-05	37	Ptprn2	1.5	9.5E-03
8	6330527O06Rik (lamp5)	2.4	3.0E-03	38	Tbc1d30	1.5	3.4E-02
9	Robo3	2.3	8.6E-10	39	Scn8a (Nav1.6)	1.5	2.5E-02
10	Igfbpl1	2.2	2.8E-03	40	Alpl (TNAP)	1.5	2.9E-02
11	Bmpr1b	2.2	8.4E-04	41	Lin28b	1.5	2.2E-03
12	Nhlh2	2.2	1.5E-04	42	2610100L16Rik	1.5	1.8E-02
13	Dbx1	2.1	2.7E-07	43	Acpl2	1.5	4.9E-02
14	Scrt2	2.1	1.3E-03	44	Bcat1	1.4	1.3E-02
15	Pou3f3 (Brn1a)	2.1	5.3E-07	45	Nmral1	1.4	1.1E-02
16	Shisa6	2.0	4.1E-02	46	Stxbp6	1.4	3.5E-02
17	Rassf4	2.0	6.9E-04	47	Plxnc1	1.4	4.2E-02
18	Ebf3	2.0	6.1E-05	48	Onecut2	1.4	2.8E-02
19	Slc1a2 (EAAT2)	2.0	8.8E-03	49	Rassf2	1.4	4.0E-02
20	Ppp2r2c	1.9	1.0E-03	50	Prkar2b (Pkarb2)	1.4	1.3E-02
21	Boc	1.9	1.8E-02	51	Suv39h2 (KMT1B)	1.4	2.2E-02
22	Gria4	1.9	2.6E-02	52	Asf1b	1.4	4.0E-02
23	Celf4	1.8	4.1E-03	53	Cdca7	1.4	2.6E-02
24	Pcdh8	1.7	1.4E-02	54	Yeats4 (Gas41)	1.3	3.9E-02
25	Smpd3	1.7	2.5E-02	55	Nusap1 (LNP; ANKT)	1.3	3.9E-02
26	Ctnnd2	1.7	3.9E-03	56	Cdca7l (JPO2)	1.3	4.8E-02
27	Trim9	1.7	2.7E-02	57	Zfp238 (RP58)	1.3	3.4E-02
28	Nrarp	1.6	6.9E-04	58	Phf6	1.3	4.6E-02
29	Tub	1.6	3.6E-02	59	Lmnb1	1.3	3.5E-02
30	Gldc	1.6	2.9E-03				

Blue: RISC-trap miR-218 target mRNA
Red: Potential miR-218 target with previously published spinal cord expression

Figure 4.2 miR-218 target candidates identified in Isl1-Lhx3 mRNA-seq analysis
A list of mRNAs that are significantly reduced in motor neuron differentiated Isl1-Lhx3 ESCs ($p < 0.05$), as determined by analysis of a previously published mRNA-seq dataset (Lee et al., 2012). These targets also contain at least one miR-218 MRE within each transcript, as identified by TargetScan or direct miR-218 MRE searches. FC, fold change.

Thesis Conclusion

My dissertation shows that miR-218 is directly upregulated by the Isl1-Lhx3 complex and plays a fundamental role in ensuring motor neuron cell fate by repressing transcripts that promote neural progenitor and interneuron phenotypes (Figure 4.3). In the future, it will be interesting to know whether additional miRNAs, such as miR-153, are also essential for motor neurogenesis.

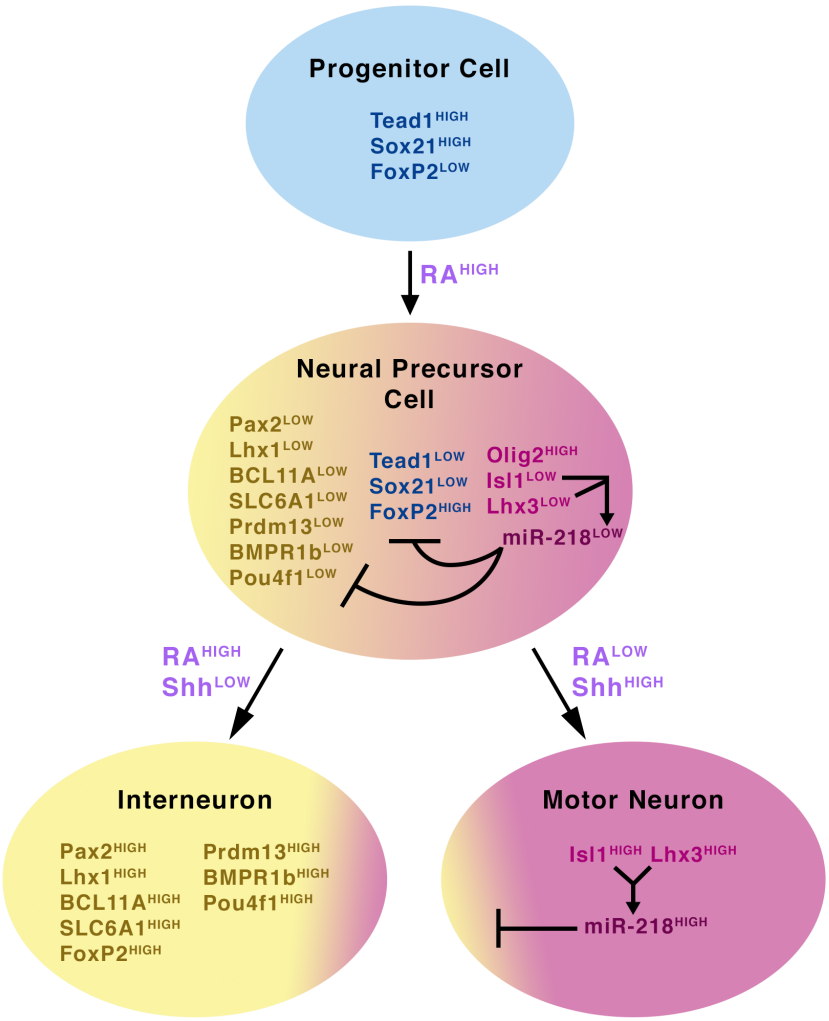


Figure 4.3 Model of miR-218 function in motor neuron development
Progenitor cells integrate morphogen signals, such as sonic hedgehog (Shh) and retinoic acid (RA), to induce the expression of genes that promote both motor neuron and interneuron development. During motor neurogenesis, miR-218 is directly upregulated by Isl1-Lhx3 and functions to support motor neuron differentiation by repressing target mRNAs that are essential for progenitor cell maintence, (Tead1, Sox21, FoxP2), and interneuron development, (Pax2, Lhx1, BCL11A, SLC6A1, FoxP2, Prdm13, BMPR1b, and Pou4f1).

APPENDIX: ABBREVIATIONS

3'UTR - 3' untranslated region

5'UTR - 5' untranslated region

Ago - Argonaute

ALS - Amyotrophic lateral sclerosis

BCL11A - B-Cell CLL/Lymphoma 11A

bHLH - Basic helix-loop-helix

BMP - Bone morphogenetic proteins

BMPR1b - Bone Morphogenetic Protein Receptor Type 1B

CAG - Cytomegalovirus/chicken β actin

CDS – Gene coding sequence

CNS - Central nervous system

d4EGFP - Destabilized nuclear GFP with a half-life of 4 hours

DB - Double mutant

Dbx1 - Developing brain homeobox 2

Dbx2 - Developing brain homeobox 2

dnGW182 - Dominant negative GW182

Dox - Doxycycline

EB - Embryoid bodies

ESC - Embryonic stem cell

Etv1 - Ets variant 1

FoxP1 - Forkhead box protein 1

FoxP2 - Forkhead box protein 2

FoxP4 - Forkhead box protein 4

GFP - Green fluorescent protein

GO - Gene ontology

Hb9 - Homeobox gene 9

hEF1α - Human enhancer of filamentation 1 alpha

HMC - Hypaxial motor column

HxRE - Hexamer response elements

IN - Interneuron

IP - Immunoprecipitation

Irx3 - Iroquois-class homeodomain protein 3

Isl1 - Islet 1

Isl2 - Islet 2

Lhx1 - LIM homeobox 1

Lhx1- LIM homeodomain 1

Lhx3 - LIM homeobox 3

LIF - Leukemia inhibitory factor

LMC - Lateral motor column

LMCl - Lateral motor column lateral

LNA - Locked nucleic acid

LOF - Loss of function

Lrrn1 - Leucine-rich repeat transmembrane protein 1

miRISC - miRNA-induced silencing complex

miRNA - microRNA

MMC - Medial motor column

MN - Motor neuron

MRE - microRNA response element

mRFPn - Monomeric nuclear red fluorescent protein

Ngn2 - Neurogenin 2

Nkx6.1 - NK6 homeobox 1

Nkx6.2 - NK6 homeobox 2

NLI - Nuclear LIM interacting protein

nNos - Neuronal nitric oxide synthase 1

Nxk2.2 - NK2 homeobox 2

Olig2 - Oligodendrocyte transcription factor 2

p0-p3 Progenitor ventral interneuron domains 0-3

Pax2 - Paired box protein 2

Pax6 - Paired box protein 6

pd1-6 - Progenitor dorsal interneuron domains 1-6

PGC - Preganglionic column

pMN - Progenitor motor neuron domain

Pou4f1 - POU Class 4 Homeobox

Prdm13 - PR Domain Containing 13

pSMAD - Phospho-mothers against decapentaplegic

RA - Retinoic acid

RFP - Red fluorescent protein

RFT1 - Requiring Fifty Three 1

Robo1 - Roundabout 1

Robo2 - Roundabout 2

Shh - Sonic hedgehog

Slain1 - SLAIN motif containing protein 1

SLC6A1 - Solute carrier family 6 member 1

SMA - Spinal muscular atrophy

Sox21 - Sex Determining Region Y Box 21

Tbx5 - T-box gene 5

Tead1 - TEA Domain Family Member 1

TRE - Tetracycline response element promoter

Tuj1 – Tubulin J 1

U6 - RNA polymerase III promoter U6

VZ - Ventricular zone

Wnt - Wingless-type MMTV integration site

Zeb2 - Zinc finger E-box binding homeobox

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