BRAIN-SPECIFIC MICRORNAS INDUCE NEUROGENESIS THROUGH INDIRECT REGULATION OF MEF2C ACTIVITY

by

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ABSTRACT OF THE DISSERTATION

Brain-specific MicroRNAs Induce Neurogenesis

Through Indirect Regulation of Mef2C Activity

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MicroRNAs represent a group of functional non-coding RNAs (ncRNA) with a role in both translational repression and/or RNAi-mediated degradation of specific target mRNAs. Reports indicate that microRNA regulation plays an important role in numerous cellular processes. We have identified microRNAs regulated during induction of neurogenesis in an *in vitro* model, and have correlated the expression of these specific microRNAs to regulated mRNAs with the goal of describing potential functional interactions between the two sets of molecules. We demonstrate the active role these regulated microRNAs play in inducing a neuronal phenotype in uncommitted neural precursor cells. As a result of this analysis, as well as additional studies of regulated mRNA transcripts during neurogenesis, we have identified a regulated transcription factor, Mef2C, which is chromosomally adjacent to one of the regulated microRNAs. The unexpected appearance of this transcription factor and its relatively unexplored role in neural differentiation suggest that Mef2C may play a role in this cellular process. This single genomic locus containing an induced microRNA involved in neuronal fate determination, as well as Mef2C, is transcriptionally regulated during neuronal cell specification. In addition, HDAC4, a known repressor of Mef2C activity is validated as a target for miR-9

mediated post-transcriptional repression. Described here is a putative network of interactions that arise from transcriptional activation of this locus, with the end result contributing to the induction of a neuronal phenotype.

PREFACE

This doctoral thesis represents my academic and intellectual contributions to a large, ongoing collaborative effort at the W.M. Keck Center for Collaborative Neuroscience at Rutgers University. One of the primary goals of this group is to uncover molecular mechanisms underlying the differentiation of neural stem cells. The focus of this effort is to affect the differentiation program of stem cells to direct a population of these cells towards a desired phenotype. Once capable of direction, these cells would then be specifically adaptable for use in therapeutic stem cell transplants. The pretext for these studies lies in the hope that stem cell transplants will eventually prove a successful therapy for treatment of traumatic and/or degenerative spinal cord injury.

The broad and ambitious nature of this research necessitates a collaborative environment with diverse contributions from a range of individuals. The contributions from specific members of this group are outlined in detail in the next section. It should be noted however that the specific biological questions asked and addressed in the research presented here denote my exclusive scholarly contributions to these studies.
The productive nature of my tenure as a graduate assistant at Rutgers University has resulted in several publications from which some of the work presented here is drawn. In the interest of full disclosure, I will present briefly a select list of my prior publications that may overlap with material presented within this thesis.

As our group began to study the relatively young field of microRNAs, we were positioned in such a way that rapid development of a novel microRNA microarray was possible. In collaboration with the lab of Dr. Richard Padgett and the Research and Development group at Genisphere, Inc. (Hatfield, PA) under the direction of Dr. Robert Getts, we were able to design, produce, and validate the miRMAX platform for microRNA expression profiling. This achievement was detailed in (Goff et al., 2005), and a patent for the probe design algorithm has been filed with my name listed. This platform

provided the basis for our entry into the field of microRNA studies and is the source for much of the microRNA expression profiling data presented herein. This system has since been licensed for use by Invitrogen, Inc (Carlsbad, CA) and is sold commercially as the NCode microRNA profiling system.

Through collaboration with Invitrogen, Inc, we were given the opportunity to work with members of their Stem Cells and Regenerative Medicine group. Several microRNA profiling projects, championed by Dr. Uma Lakshmipathy, were devised and conducted by our research group. The first was a characterization of human microRNAs that are regulated during the differentiation of human embryonic stem cells (Lakshmipathy et al., 2007). The second study was able to uncover several microRNAs that are regulated during cell-type-specific differentiation of human mesenchymal stem cells. Here, microRNAs were both computationally predicted and validated as being regulated by downstream effectors of a specific biological pathway. This work is currently submitted for peer review and waiting publication. Portions of these studies incorporated within this body of work primarily include references to the expanding role of microRNAs in stem cell maintenance and differentiation as well as some of the computational analyses used to explore the relationships between microRNAs and mRNAs (i.e. cross-correlation and transcription factor binding site analyses).

As a result of our experience with microRNA expression data, we were asked to write a chapter on the methods and techniques for analyzing such datasets, and the specific challenges that arise from profiling these small RNA sequences. These methods and considerations were compiled and are again awaiting publication (Goff et al., 2008). Portions of this thesis work have been analyzed using methods described within this chapter including but not limited to the quantile normalization of microRNA array data and biclustering analysis of microRNA and mRNA transcripts.

In the course of the mRNA expression profiling studies, we employed the AB1700 Rat Genome Survey arrays (Applied Biosystems, Foster City, CA). Despite the high-quality data obtained from this platform, we realized early on that the available levels of probe annotation to the public rat genomes for this array platform were inadequate for our needs. To address this, and to allow further analysis of the mRNA array results, all of the probes on this array were re-mapped to multiple public repositories. The updated annotation was aggregated and is available in (Goff et al., 2007). This study included an interpretation of the re-annotated array data using a classification and regression tree (CART) analysis of the upstream transcription factor binding sites of regulated mRNAs. This analysis was conducted in conjunction with Dr. Rebecka Jörnsten and is included in the thesis work presented here.

Lastly, the characterization of the L2.2 neural stem cell clones described within this text is also represented in a recent paper that has been submitted for review. Dr. Hedong Li is responsible for the isolation of this and several other neural stem cell clones utilized during the course of these research studies. In the interest of defining both L2.2 and L2.3 as a valid model for the dissection of neural stem cell differentiation, it was essential that a transcriptome-level analysis be conducted. In this study, I contributed to the data analysis of the mRNA array data as well as the interpretations of the qRT-PCR data describing L2.2 as a specific interneuron precursor (Li et al., 2008). These data are referenced within my thesis as well.

ACKNOWLEDGEMENTS AND DEDICATION

This thesis would not be possible without the support, help, and contributions of a large number of individuals. Principally, I am forever grateful for the guidance and support given to me by my advisor Dr. Ron Hart. I am immensely grateful for the opportunities presented to me while at Rutgers University and the W.M. Keck Center. Specifically within the laboratory group of Dr. Hart, I have been given the opportunity and the freedom to explore numerous research interests including microarray development, neuroscience, stem cell biology, computational biology, high-throughput genomics, statistics, data analysis techniques, and most recently next-generation sequencing. I would like to thank Dr. Hart for providing direction, autonomy, and opportunity; without any of which I would not consider my graduate experience nearly as successful. I could not have imagined a better graduate experience encompassing so many fields, academic and institutional collaborations, and research topics. The added benefit of a mutual admiration for all things technological has made this a truly enjoyable few years.

Mavis Swerdel has not only embraced a well-needed maternal niche within our lab group, but has proven time and again to be an exceptional technician. Thank you Mavis for agreeing to offload much of the microarray printing and hybridization services, and for the immeasurable amount of help you have provided to both this research project and the countless others that have sprung up since you're arrival. As I'm sure you are aware, this lab would not operate nearly as well without you.

I would like to thank Jonathan Davila and Chris Ricupero for being a constant source of both entertainment and academic debate. More specifically, Jonathan is also responsible for the labeling and hybridization of the mRNA samples to the AB1700 arrays described herein. He has also been gracious enough to conduct several of the 3'UTR targeting assays and transfections. Chris has maintained and provided cells as needed for many of these experiments. In addition, it is Chris' experience on the

fluorescence activated cell sorting system that has allowed for the enumeration of cells in the differentiation studies. Chris has also contributed immunostaining results and transfections of both the pre- and anti-miRs.

Dr. Cynthia Camarillo has graciously contributed cells as needed for this research effort and continues to focus on the protein and antibody characterization for rat Mef2c and HDAC4. Cindy has also spent much of her time vetting a chromatin immunoprecipitation assays for Mef2c as well. As part of this research, I thank Cindy for developing the Mef2c-FLAG plasmid mentioned within this thesis.

The L2.2 and L2.3 model for neural stem cell differentiation would not be possible without the prior and continued efforts of Dr. Hedong Li. Hedong is responsible for the initial isolation and characterization of these two clones, as well as the initial cultures used for the differentiation time-course assay. I would like to thank him for the use of these cultures in this research. In addition, Hedong has been a wealth of information regarding the biology of embryonic neural development.

Dr. Rebecka Jörnsten from the Department of Statistics at Rutgers University is the person most directly responsible for my introduction to R. I would like to thank Rebecka for all of her help and patience in teaching me both the R-language and statistics in general. Her direct contributions to this work are evident in the CART analysis of transcription factor binding sites, as well as the model-based clustering of array results during the differentiation studies. In addition, the cross-correlation approach to miRNA:mRNA interaction elucidation was conceived in part by Dr. Jörnsten.

A strong collaboration has existed for several years now with the Research and Development group at Genisphere, Inc. under the direction of Dr. Robert Getts. I would like to thank Jessica Bowers, Kelly Sensinger, and Kevin Howerton for both the contributions made in the development of the NCode platform and for being a cheerful source of technical support as needed. I would also like to thank the Research and

Development, and the Stem Cells and Regenerative Medicine groups at Invitrogen Inc., specifically Christopher Adams, Kristin Wiederholt, Mark Landers, and Uma Lakshmipathy, for the opportunities to collaborate in numerous projects.

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Special thanks are in order for the five members of my thesis committee; Dr. Ron Hart, Dr. Martin Grumet, Dr. Richard Padgett, Dr. Robert Getts, and Dr. Rebecka Jörnsten.

Most of all I am deeply thankful for the love and support of my wife, Lauren. Your patience, understanding, and love helped to make this possible. I would not be here without you. It is to you Lauren, as well as my parents, brothers, and the rest of my wonderful family, that I dedicate this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED

miRNA	MicroRNA
mRNA	Messenger RNA
cDNA	Complementary DNA
LMW	Low molecular weight
HMW	High molecular weight
NSC	Neural stem cell
NSPC	Neural stem precursor cell
ES(C)	Embryonic stem cell
MEF2	Myocyte enhancer factor 2 family of transcriptional regulators
HDAC	Histone deacetylase family of transcriptional regulators
FACS	Fluorescence activated cell sorting
RNAi	RNA interference pathway/mechanism
TFBS	Transcription factor binding site(s)
PolII/III	RNA polymerase II or III
qRT-PCR	Quantitative real-time polymerase chain reaction
LIF	Leukemia Inhibitory factor
FGF(2)	Fibroblast growth factor

RISC	RNA-induced silencing complex
RA	Retinoic acid
GABA	Gamma aminobutyric acid
NMDA	N-methyl-D-aspartic acid
MAP2	Microtubule associated protein 2
NeuN	Neuronal nuclei protein
MASH1	Mammalian achaete-schute homolog 1
cAMP	Cyclic AMP
CBP	CREB binding protein
CARM1	Coactivator-associated arginine methyltransferase
HAT	Histone Acetyltransferase
MAPK	Mitogen-activated protein kinase
CDK	Cyclin-dependent kinase
βIII-Tub	Beta-III Tubulin
GalC	Glactosylceramidase
GFAP	Glial fibrillary acidic protein
TuJ1	Neuron-specific beta-III Tubulin antibody
BMP2	Bone-morphogenetic protein 2

SHH	Sonic Hedgehog protein
GAD	Glutamic acid decarboxylase
ANOVA	Analysis of variance
CART	Classification and regression tree
BLBP	Brain-lipid binding protein
PAX	Paired-box transcription factor
DLX	Distal-less homeobox transcription factor
NeuroD	Neurogenin
NPY	Neuropeptide Y
DARPP32	Dopamine and cAMP regulated phosphoprotein 32
TH	Tyrosine hydroxylase
Rhb	Rhesus blood group associated B glycoprotein
BAC	Bacterial artificial chromosome
FLAG	A specific polypeptide added to a recombinant expressed protein (N-DYKDDDDK-C)
VPA	Valproic acid

I. INTRODUCTION

The therapeutic potential of stem cell transplants is, at present, principally mitigated by our limited understanding of the cellular mechanisms governing the maintenance and differentiation potential of these cells. A greater perception of the molecular networks responsible for the pluripotency of stem cells, as well as their differentiation patterns, will prove invaluable to future research and foster new and innovative methods for treatments of disease and traumatic injuries. The potential benefits of stem cell therapies are immediately apparent in their applications to both neurodegenerative disorders and traumatic brain/spinal cord injuries. These conditions are characterized by a resulting decrease in efficacy of the adult nervous system, whether a result of progressive failure to properly function, or direct compromise of the integrity of this fragile organ system. In either case, an ideal therapy outcome would involve a direct engineering of a replacement or repair within the individual. When we understand how a dopaminergic neuron is formed from an uncommitted stem cell, we will be better positioned to direct the differentiation of a stem cell transplant in a Parkinson's patient to assume the role of dopamine-producing neurons. Likewise, a study in oligodendrocyte formation and myelination activity brings us closer to cell-based therapies targeting the demyelinated lesions of a multiple sclerosis patient.

Traumatic spinal cord injuries represent a break in the integrity of the peripheral nervous system. One proposed application of stem cell therapy would be to repair damage to a disrupted spinal cord across the injury site. Among the numerous processes involved in this repair would be the requirement for regeneration of neurons, their re-myelination, and a potential suppression of inflammation/necrosis associated with secondary injury characteristics. A comprehensive study into the mechanisms governing the differentiation of neural stem cells would allow for their directed differentiation towards a phenotype required for such repair.

It is our belief that no differentiation study is complete without incorporation of the rapidly-expanding field of non-coding RNA. For many years, molecular biology research has been focused on the 'central dogma' that the role of RNA was to act as an intermediate between the instructional DNA and the functional protein molecules. The explosion of small non-coding RNA discoveries in recent years has re-emphasized the importance of these molecules in the proper functioning, regulation, and operation of the cell. We present here, a novel role for specific small RNAs in neural stem cells, and outline a pathway by which they directly influence the differentiation potential of these cells.

MicroRNAs are small regulators of post-transcriptional gene expression.

The recent discovery of microRNAs ("miRNAs") has led to an explosion in the study of non-coding RNA regulation. Various types of small, non-coding RNAs exist as modulators of gene expression, affecting transcription rate (Kuwabara et al., 2004, Goodrich and Kugel, 2006, Kim et al., 2006a, Place et al., 2008), heterochromatin formation (Saito et al., 2006a, Matzke et al., 2004, Kim et al., 2006a, Kanellopoulou et al., 2005), transposon silencing (Aravin et al., 2007, Brennecke et al., 2007, Cao et al., 2006, Saito et al., 2006a, Schramke and Allshire, 2003), mRNA stability (Wu et al., 2006, Giraldez et al., 2006, Behm-Ansmant et al., 2006, Shyu et al., 2008), and mRNA translation into functional proteins (Felli et al., 2005, Liu et al., 2005, Pauley et al., 2006). Mature microRNAs are single-stranded RNA species of ~21 nucleotides that derive from a ~70-100 nucleotide precursor and are found in a wide variety of organisms, from plants to insects to humans (Ambros, 2001, Bartel, 2004). To date, miRNAs have not been formally identified in bacterial, archaea, or older eukaryotes (e.g. S. *cerevisiae*) but there is evidence to suggest that similar small RNA species are present with similar roles in these more ancient clades (Hannon et al., 2006). In higher eukaryotes, complexes

containing microRNAs and the RISC complex (RNA induced silencing complex) involved in RNAi are similar (Bartel, 2004), since endogenous microRNAs can cleave mRNAs with perfect complementarity (Yekta et al., 2004) and exogenously introduced siRNAs can attenuate translation of mRNAs having imperfect complementarity (Nielsen et al., 2007, Svoboda, 2007). In metazoans, the predominant form of microRNA regulation is mediated by imperfect pairing to a 3' UTR element in an mRNA target (Bartel, 2004). This action results in one of several identified mechanisms resulting in attenuation of protein production including, but not limited to, mRNA cleavage (Bartel, 2004, Liu et al., 2004, Meister et al., 2004, Yekta et al., 2004), rapid mRNA de-adenylation (Giraldez et al., 2006, Wu et al., 2006), inhibition of translation initiation (Humphreys et al., 2005, Pillai et al., 2005), post-initiation ribosome drop-off (Petersen et al., 2006), and/or mRNA sequestration to P-bodies (Lian et al., 2006, Pauley et al., 2006, Behm-Ansmant et al., 2006, Felli et al., 2005, Liu et al., 2005, Rehwinkel et al., 2005). Recent evidence suggests that microRNAs may play an equally important role in activating the translational activity of mRNAs as well. One example of this type of translational activation is the targeting of AU-rich elements (AREs) by miR-369-3 resulting in the recruitment of Ago2 and Fragile X mental retardation-related protein (FMRP/FXR1) (Vasudevan et al., 2007). There is additional evidence to suggest that microRNA activity as either a repressor or an activator is mutable depending on the cellular context (Vasudevan et al., 2007).

Conservative estimates put the number of miRNAs at ~400 for each invertebrate species, and ~1000-1500 miRNAs in mammals (Lewis et al., 2005, Lim et al., 2005), with some groups predicting as many as 10,000-20,000 per genome (Miranda et al., 2006). 541 microRNA hairpins have been validated and annotated in the human genome alone (Griffiths-Jones, 2006, Griffiths-Jones et al., 2006). Many of these hairpins produce functional mature miRNAs from either arm (5' and/or 3' ends), effectively

doubling the number of functional sequences. It is reasonable to assume that there are miRNAs that remain to be discovered within our genome, suggesting that the less conservative estimates of several thousand small RNA sequences per genome are not outrageous. Next-generation sequencing technologies are already beginning to provide insights into the number of small RNAs, and the extent of regulation by these molecules. Indeed, several novel classes of small RNAs have already been resolved from early datasets (Olson et al., 2008, Girard et al., 2006, Smalheiser and Torvik, 2005), indicating that as our limits of detection progress, we will uncover even more functional microRNA sequences. The widespread impact of this new layer of gene regulation is also becoming more apparent in that several groups estimate anywhere from ~30% to 95% of the genome may be targets for microRNAs (Miranda et al., 2006, Lewis et al., 2005). This type of gene control represents a novel regulatory mechanism, and is predicted to affect many crucial cellular processes and developmental programs, including neurogenesis.

MicroRNAs are implicated in a wide variety of cellular processes.

While only a few validated target mRNAs have been identified in animals, this information, combined with correlated tissue expression data and functional analyses, highlights some of the important roles for microRNAs. These small regulators have been shown to play pivotal roles in numerous cancers as both oncogenic components as well as tumor suppressors (Ovcharenko et al., 2007, Chang et al., 2007, Sun et al., 2007, Akao et al., 2006, Calin et al., 2002, Calin et al., 2005, Calin et al., 2004, Eder and Scherr, 2005, Felli et al., 2005, Gregory and Shiekhattar, 2005, Hayashita et al., 2005, He et al., 2005a, He et al., 2005b, Hossain et al., 2006, Johnson et al., 2005, Kluiver et al., 2006, Kutay et al., 2006, Roldo et al., 2006, Saito et al., 2006b, Tam and Dahlberg, 2006, Voorhoeve et al., 2006, Tavazoie et al., 2008), cardiac hypertrophy/failure (van Rooij et al., 2006, Chen et al., 2008), Alzheimer's disease (Niwa et al., 2008, Wang et

al., 2008), Huntington's disease (Johnson et al., 2007), schizophrenia (Hansen et al., 2007, Burmistrova et al., 2007, Perkins et al., 2007) and several other disorders. Additionally, cellular processes such as fat metabolism (Esau et al., 2006, Xu et al., 2003), insulin regulation (Plaisance et al., 2006, Poy et al., 2004), apoptosis (Baehrecke, 2003, Chan et al., 2005, Cimmino et al., 2005, Xu et al., 2004a), cell cycle regulation (Hatfield et al., 2005, Lian et al., 2006, Shcherbata et al., 2006), maternal-zygotic transition (Giraldez et al., 2006, Mishima et al., 2006, Wienholds et al., 2003), viral defense (Lecellier et al., 2005, Cullen, 2004), axis specification/patterning (Giraldez et al., 2005, Harfe et al., 2005, Johnston and Hobert, 2003, Mansfield et al., 2004, Hornstein et al., 2005), tissue formation (Chen et al., 2006, Frederikse et al., 2006, Naguibneva et al., 2006, Yi et al., 2006), as well as stem cell specification and differentiation (Hatfield et al., 2005, Houbaviy et al., 2005, Houbaviy et al., 2003, Lee et al., 2005, Shcherbata et al., 2006, Song and Tuan, 2006, Suh et al., 2004, Zhao et al., 2006, Anderson et al., 2006, Bentwich, 2005, Brennecke et al., 2005, Chen et al., 2006, Esau et al., 2004, Kim et al., 2006b, Naguibneva et al., 2006, Rao, 2004, Sweetman et al., 2006, Wu and Belasco, 2005) have all been associated with microRNA activity. It is these last few roles in development and differentiation that have been the impetus for the majority of the research into microRNA regulation of neural stem cell specification outlined here.

Shortly after the identification of microRNAs, tissue surveys were conducted to assess the potential impact of these small inhibitors (Krichevsky et al., 2003, Nelson et al., 2004, Thomson et al., 2004, Barad et al., 2004, Babak et al., 2004). It became immediately apparent that most microRNAs are highly tissue-restricted, with only a slight overlap between tissues for any given microRNA. A few microRNAs, such as the let-7 family, are ubiquitously expressed in all tissue types (Thomson et al., 2004, Nelson et al., 2004, Barad et al., 2004), suggesting a role in regulating the more basal, and

therefore common processes within the cell, such as cell-cycle regulation (Johnson et al., 2005). More commonly, a set of tissue-specific microRNAs are associated with tissue-specific cellular functions. This correlation, while only predictive of a microRNA's role, is strengthened by associated functional analysis of microRNA activity via over-expression (Poy et al., 2004, Johnson et al., 2005, van Rooij et al., 2006), and inhibition studies (Esau et al., 2004). For example, antisense targeting of miR-122, a liver specific microRNA, leads to dysregulation of lipid metabolism in the liver (Esau et al., 2006). Using similar approaches of combining tissue-specific expression data with functional assays has led to a greater understanding of the impact of microRNAs in the cell, without the need for specifically identifying valid mRNA targets. Using this approach, groups have ascribed general functions for given microRNAs in context, such as the requirement for miR-125b for the proliferation of differentiated cells (Lee et al., 2005), miR-143 for the regulation of adipocyte differentiation (Esau et al., 2004), the role of miR-134 in regulation of dendridic spine formation in hippocampal neurons (Schratt et al., 2006), and numerous others (Tsuchiya et al., 2006, Kim et al., 2006b, Tuddenham et al., 2006, Taganov et al., 2006, Naguibneva et al., 2006). It would be shortsighted at this point to restrict the potential roles that microRNAs may be performing in the cell as new roles are being unraveled at an accelerated rate. The diversity in the already established roles for microRNAs, demonstrate that this class of small regulatory RNA molecules plays an integral role in numerous biological pathways and suggests that they will play an important role in other cellular processes including differentiation.

Regulation of microRNA expression.

While little is known about the functions of expressed microRNAs, even less is known about the mechanisms governing the regulation of microRNAs themselves. microRNAs are derived from both spliced intronic regions of mRNAs (Ying and Lin,

2004, Ying and Lin, 2005, Lin et al., 2006), as well as from unique transcripts located in intergenic regions (Lagos-Quintana et al., 2003, Ying and Lin, 2006, Gu et al., 2006). Evidence now also suggests that microRNAs can arise from processed pseudogenes as well (Devor, 2006). Transcription of intronic microRNAs is believed to be regulated by the same mechanisms that regulate the abundance of the host transcript. The identification of additional microRNA processing steps (Diederichs and Haber, 2007, Wulczyn et al., 2007), and the requirement for spliceosome components for liberation of microRNA hairpins from intronic regions (Okamura et al., 2007) suggests that intronic microRNAs may be processed at different rates than their intergenic counterparts. Intergenic microRNAs have been shown to be transcribed by either RNA Polymerase II (Pol II) (Lee et al., 2004) or more recently in some cases by Pol III (Borchert et al., 2006). The Pol II transcripts are 5'-capped and poly-A-tailed (Lee et al., 2004, Cai et al., 2004) in a similar fashion to known mRNAs. A large number of intergenic microRNAs exist as poly-cistronic clusters. These clusters are often transcribed as a single unit and summarily processed into individual microRNA precursors after nuclear export (Krichevsky et al., 2003, Houbaviy et al., 2003, Suh et al., 2004, Tanzer and Stadler, 2004, He et al., 2005b, Altuvia et al., 2005, Hayashita et al., 2005). Interestingly, early sequence analysis of upstream regions of intergenic microRNAs failed to identify common Pol II minimal promoter elements or similarities to known mRNA promoter elements (Cai et al., 2004, Houbaviy et al., 2005, Lee et al., 2004). A few groups, including our lab, have since cloned and validated promoter regions for a small number of microRNAs, and demonstrated that similar regulatory mechanisms do in fact exist to control the transcription rates of intergenic microRNA. These mechanisms include conserved and occupied transcription factor binding sites as well as chromatin modifications, both of which have been shown to exert a regulatory pressure on the cloned promoters (Fazi et al., 2005, Scott et al., 2006, Taganov et al., 2006, Plaisance et

al., 2006, Saito et al., 2006b). Since microRNA regulatory mechanisms are similar to currently understood transcription mechanisms, we were able to study their function using standard techniques.

microRNAs in stem cells and development.

MicroRNAs have been ascribed numerous functions in the cell. One of the more interesting roles suggested for these small inhibitors of translation is the regulation and specification of stem cells. Several studies have attempted to determine the global role of microRNAs in development by selective knockdown of required components of the microRNA/RNAi pathway (Bernstein et al., 2003, Kanellopoulou et al., 2005). A few groups have determined that Dicer, the RNase III enzyme responsible for processing microRNAs and therefore required for microRNA activity, is required for murine cell differentiation and specification (Bernstein et al., 2003, Murchison et al., 2005, Kanellopoulou et al., 2005). Evidence shows that a Dicer-1 null mutant mouse was embryonic lethal due to a depletion of stem cells (Bernstein et al., 2003), as well as a failure of existing stem cells to adequately differentiate (Kanellopoulou et al., 2005). Interestingly, a similar study was conducted in zebrafish where it was shown that embryos with a maternal-zygotic Dicer mutant are capable of stem cell maintenance and differentiation but are defective in patterning, morphogenesis, and organogensis, suggesting that the role of microRNAs in stem cell regulation may have changed dramatically during the course of evolution (Giraldez et al., 2005). Furthermore, knockout mice lacking Argonaute2, the catalytic component of the RISC complex, exhibited severe defects in neural development, including a failure to close the neural tube (Liu et al., 2004). These experiments highlight the critical, if not yet well understood role that microRNAs play during development.

Early on, it was noted that stem cells express unique populations of microRNAs that were not present in any adult tissues (Houbaviy et al., 2003, Suh et al., 2004, Houbaviy et al., 2005, Giraldez et al., 2006, Mishima et al., 2006), some of which are additionally species specific. A conserved eutherian microRNA cluster is expressed exclusively in undifferentiated stem cells and is immediately downregulated upon induction of differentiation (Houbaviy et al., 2005, Houbaviy et al., 2003). Since microRNAs have been shown to exhibit a repressive role during proliferative cell phases (Vasudevan et al., 2007), it is reasonable to speculate that these microRNAs are responsible for maintaining a stem-like state through repression of pro-differentiation factors. A similar group of microRNAs, although with distinctly unique sequences and genomic locations, is evident in differentiating human embryonic stem cells (Suh et al., 2004). In one of our research efforts involving human embryonic stem cells we were able to describe significant differences in microRNA expression and regulation between undifferentiated human ES cell lines and their corresponding differentiated cells (Lakshmipathy et al., 2007). In addition, we were able to outline a network of interactions between regulated microRNAs and biologically relevant mRNAs that suggests a role for a subset of these microRNAs in maintenance of the undifferentiated pluripotent state. The presence of these embryonic stem cell-specific microRNAs, and their clearance during differentiation, immediately suggests a role in restricting cell differentiation.

In contrast, populations of tissue specific microRNAs are coordinately induced during differentiation and specification of stem cells (Kawasaki and Taira, 2003, Krichevsky et al., 2003, Miska et al., 2004, Wienholds et al., 2005, Giraldez et al., 2005, Chen and Lodish, 2005, Kwon et al., 2005, Chen et al., 2006, Kim et al., 2006b, Sweetman et al., 2006). Conserved microRNAs miR-1 and miR-206 are both induced during, and required for muscle cell differentiation and specification in mammals (Chen et al., 2006, Nakajima et al., 2006) as well as birds (Sweetman et al., 2006). Expression

of miR-181 in hematopoietic stem cells is associated with an increase in B-cell specification (Chen and Lodish, 2005), while other hematopoietic microRNAs (miR-142, and miR-155) are also induced during blood cell specification (Chen and Lodish, 2005, Ramkissoon et al., 2006, Song and Tuan, 2006). The requirement for microRNA activity during development and the influence that specific microRNAs have upon differentiation supports the hypothesis that microRNAs are required for establishing and perhaps maintaining a differentiated state.

Brain-enriched microRNAs.

One recurring theme in many of the early microRNA tissue studies was that neural tissues, primarily the brain and spinal cord, have a significantly higher number of unique microRNA sequences than most other tissues. In addition, it was immediately apparent that the majority of these sequences were restricted in their expression exclusively to neural tissues. These two observations suggest that microRNAs have a prominent role in the proper functioning of the nervous system. In fact microRNAs have been associated with proper neural development (Fiore et al., 2008, Fiore and Schratt, 2007, Goff et al., 2007, Krichevsky et al., 2006, Giraldez et al., 2005, Rogaev, 2005, Krichevsky et al., 2003), and miRNA dysregulation has been demonstrated in several neurodegenerative disorders (Wang et al., 2008, Nelson et al., 2008, Fiore et al., 2008, Rogaev, 2005). Previous work identifying new microRNAs and their expression profiles has established a distinct subset of microRNAs with enriched or specific expression in neural tissues. Several embryonic stem cell specific microRNAs are down-regulated during RA-induced differentiation of neuronal precursor cells (Houbaviy et al., 2003, Song and Tuan, 2006, Suh et al., 2004). Concurrently, brain-enriched microRNAs such as miR-9, miR-124a, miR-125, and numerous others, are induced in developing neural tissues (Krichevsky et al., 2003, Nelson et al., 2004, Rogelj and Giese, 2004, Miska et

al., 2004, Sempere et al., 2004, Schratt et al., 2006). This induction has been described in primary neural tissues, differentiating primary neurons (Krichevsky et al., 2003) ,as well as P19 cells (Sempere et al., 2004, Wu and Belasco, 2005), and our own neural stem cell clones. The contrasting expression profiles of these two groups of microRNA suggest that they may in fact have opposing functions in development; with some downregulated microRNAs responsible for maintenance of stemness, and other up-regulated microRNAs responsible for inducing a translational shift that would promote the neuronal condition and/or confer functional properties of a mature neural phenotype.

Myocyte enhancer factor 2 family of transcription factors.

Originally identified in differentiating myocytes (Yu et al., 1992), the Mef2 family of genes comprises a group of DNA-binding transcription factors belonging to the minichromosome maintenance 1-agamous-deficiens-serum response factor (MADS) family (Figure 1). Members of this family contain the highly conserved N-terminal MADS domain which mediates dimerization and binding activity to the A/T rich consensus sequence CTA$(A/T)_4$TAG/A (McKinsey et al., 2002). This domain is minimally required for DNA binding, although a neighboring, and similarly conserved, Mef2 domain additionally conveys high DNA binding affinity as well as mediates cofactor binding (McKinsey et al., 2002). This Mef2 domain is the defining characteristic of the Mef2 family. A strongly conserved nuclear localization signal is present at the C-terminus of the majority of Mef2 isoforms as well, suggesting the proteins are localized primarily in the nucleus. A greater divergence is seen among different isoforms in the transcription-activating C-terminal domains of Mef2 family members (McKinsey et al., 2002). Gene duplications have resulted in four Mef2 isoforms (Mef2A-D) in higher vertebrates, diverged from a single representative member in lower organisms such as *C. elegans* and *D. melanogaster*. Extensive post-translational modification sites are strongly

conserved both across lineages as well as between various family members. Adding to the complexity of the Mef2 family tree, multiple splice variants have been identified for many of the isoforms (Yu et al., 1992, Zhu et al., 2005). Despite being initially described as exclusive to differentiating muscle, members of the Mef2 family of transcription factors have more recently been shown to be expressed in the developing brain, suggesting an additional and/or complementary function in this tissue.

Mef2 isoforms are expressed in the brain during neural development.

Shortly after the identification of Mef2 genes in developing muscle (Yu et al., 1992), related studies indicated the presence of various Mef2 isoforms in the nervous system as well (Yu et al., 1992, Leifer et al., 1993, McDermott et al., 1993, Leifer et al., 1994, Lyons et al., 1995, Schulz et al., 1996, Lin et al., 1996). Mef2 is expressed in the neurons of *C. elegans* and in Kenyon cells of *D. melanogaster* (Dichoso et al., 2000, Schulz et al., 1996), however, it is not known what effect (if any) this expression has on neural differentiation in these model organisms. In mammals, Mef2 family members are expressed in neural crest cells as early as E8.5 and in the brain by E12.5 (Edmondson et al., 1994). All four isoforms (A-D) are present in developing cortex and olfactory bulb although their expression patterns do not necessarily overlap. Mef2A has additionally been identified in hippocampus, thalamus, and the internal granular layer of the cerebellum, where it is associated with granule neuron differentiation markers such as gamma aminobutyric acid (GABA) (Lin et al., 1996). Mef2B follows a similar expression pattern but is only found in the cortex, olfactory bulb, and dentate gyrus after development (Lyons et al., 1995). Mef2D can be located throughout the developing nervous system and through adulthood (Lyons et al., 1995). Expression of Mef2C in the developing brain is slightly different. This isoform is expressed in select cortical neurons present in the external granular layer(II), the internal granular layer(IV), and the fusiform

layer(VI) exclusively. This expression pattern is seen both during development as well as in the adult animals (Leifer et al., 1994, Leifer et al., 1993, McDermott et al., 1993), and strongly associates Mef2C with the presence of interneurons. The temporo-spatial expression of the Mef2 genes in the mammalian brain, along with a validated role in myogenesis, supports the hypothesis that this family plays a crucial role in the induction of neurogenesis in the developing brain.

Mef2 genes regulate the expression of pro-neuronal genes and exhibit a role in calcium-modulated and trophic factor-sensitive neuronal survival.

While only recently found to be expressed in the developing cortex, Mef2 gene activity has already been associated with key pathways in neural development. Members of this gene family have been shown to promote neuron survival in both Ca^{+}-dependent (Mao and Wiedmann, 1999, Linseman et al., 2003) and neurotrophin-mediated (Cavanaugh et al., 2001, Shalizi et al., 2003, Wang et al., 2007) mechanisms, act as a switch controlling post-synaptic dendrite differentiation (Shalizi et al., 2003), and suppress neuronal apoptosis in NMDA-induced excitotoxicity (Okamoto et al., 2002). Interestingly, additional roles for Mef2 genes in regulating the acquisition of the neuronal phenotype are beginning to emerge as well.

Recently, one group has demonstrated that a dominant-negative Mef2 is able to reduce expression of the neuronal marker MAP2 in retinoic acid-treated P19 cells (Skerjanc and Wilton, 2000, Okamoto et al., 2000). In addition, overexpression of Mef2C in P19 cells results in expression of NeuN, neurofilament, and MASH1 (a potent activator of neurogenesis) (Skerjanc and Wilton, 2000). These results implicate the Mef2 family in key neuronal pathways, and describe one mechanism by which a specific isoform is capable of inducing neurogenesis. With a demonstrated expression during *in vivo* neurogenesis, a previously established role in neuron-specific pathways, and a

known association with strong pro-neuronal genes (e.g. MASH1), the Mef2 family of transcription factors is appropriately positioned to contribute to neurogenesis in the developing brain. We present here compelling evidence that the role of this family during neurogenesis has been largely understated and that the mechanism by which Mef2-induced neurogenesis largely overlaps with the expression of brain-specific microRNAs.

Regulatory activity of Mef2 proteins is dependent on binding partners.

As with many other MADS-containing genes, Mef2 proteins interact with a wide range of transcription factors and various other modifying proteins. The wide array of binding partners creates a diverse population of genes that are affected by Mef2 activity downstream. Distinct subsets of target mRNAs are regulated depending on the Mef2 cellular context. MEF2 proteins bind DNA as both homo- or hetero-dimers and exhibit a bifunctional activity. In the absence of transacting modifications, Mef2 genes actively bind and recruit select class IIa histone deacetylases (HDACs) to promote heterochromatin formation as well as general repression of target transcription activity (McKinsey et al., 2002). The deacetylase activity of associated HDACs is not a requirement, however, for target gene repression, as the bound HDACs are capable of recruiting additional regulatory factors responsible for de-acetylation (Zhang et al., 2002, Zhang et al., 2001, Chan et al., 2003, Dressel et al., 2001). It has also been shown that Mef2-bound HDACs are capable of sumoylation of a specific conserved residue of Mef2d which then recruits additional class I HDACs to further repress target gene transcription (Riquelme et al., 2006, Kang et al., 2006). HDAC regulation of Mef2 activity is known to play a crucial role in muscle development as well (Leifer et al., 1994, Sparrow et al., 1999, Dressel et al., 2001, Zhu et al., 2005, Mejat et al., 2005). Indeed it has been suggested that specific class II HDACs regulate distinct aspects of muscle differentiation (Potthoff et al., 2007). If HDACs are capable of binding to and repressing

the transcriptional activation potential of Mef2 family members in muscle, than the expression of Mef2 genes in the brain implies that similar mechanisms may exist to regulate neural differentiation.

MEF2 proteins are also capable of transcriptional activation, but are again dependant on co-activator binding. CBP, CARM1, p300, and select other activating HATs can bind Mef2 directly via the Mef2 domain (Sartorelli et al., 1997, De Luca et al., 2003). Binding by any of these factors to this domain occludes the HDAC binding site. In addition to binding chromatin-modifying activators, MEF2 proteins can directly be activated via phosphorylation of key conserved residues. Phosphorylation of various residues has been shown to increase affinity for DNA, increase protein stability, and promote transcriptional activation (Gregoire et al., 2006). This activation is mediated primarily by select members of the MAPK and Cdk families of protein kinases. Under conditions of osmotic stress or inflammation, MEF2 is phosphorylated by p38 (Zetser et al., 1999, Marinissen et al., 1999, Han et al., 1997) and in situations of oxidative stress or as a result of serum treatment, ERK5 is responsible for phosphorylation of MEF2 (Kato et al., 1997, Marinissen et al., 1999, Fukuhara et al., 2000, Suzaki et al., 2002). Interestingly, both kinases have well defined roles in neuronal differentiation and survival. The multitude of mechanisms responsible for MEF2 activation hints at the importance of this family of proteins in enhancing the expression of numerous target genes.

In a biological system where microRNAs exert a post-transcriptional regulation on specific target mRNAs, each of these co-activators and co-repressors becomes a potential target for regulation. In fact, each of these genes contains several putative microRNA response elements suggesting that their expression may be attenuated in the presence of targeting microRNAs. microRNAs that directly modulate the expression of

specific activators and/or inhibitors of Mef2 could therefore provide an indirect mechanism to control the transcriptional activity of bound Mef2.

The availability of both microRNAs and Mef2 family members during neurogenesis, and the predicted interactions between regulated microRNAs and regulators of Mef2 activity support the hypothesis that microRNAs regulate the binding partners of Mef2 family members by attenuating the production of those target proteins capable of exerting a regulatory effect on Mef2 protein activity. We suggest here that Mef2 proteins and associated regulatory microRNAs interact to regulate neural differentiation in a previously undescribed mechanism. We identify a conserved genomic locus containing both a Mef2 gene and a microRNA implicated in neurogenesis and demonstrate that this locus is transcriptionally active during differentiation of neuronal precursors. Furthermore, we expound on a mechanism by which expression of this microRNA results in a switch in activity of the neighboring Mef2 gene, via targeted repression of a Mef2 binding partner, capable of further inducing transcription of the locus in a feed-forward mechanism. In addition, we examine the effect of expression of both transcripts on the induction of the neuronal phenotype in undifferentiated neural precursors.

II. RESULTS

We have been interested in identifying potential mechanisms of spinal cord injury repair. One focus of this research has been on therapeutic stem cell transplants. Several groups have attempted stem cell transplantation in spinal cord injuries with goals as diverse as neuronal replacement, remyelination of denuded axons, limitation of secondary injury, and generation of a migration scaffold (Hasegawa et al., 2005). Other members of our group have demonstrated success in transplantation of a NSC clone into damaged spinal cord. Transplantation of the radial glial cell clone RG3.6 into the spinal cord of contusion-injured rats shows a bridging of the injury site by the transplanted cells, as well as a modest functional recovery achieved through an associated neuroprotective effect (Hasegawa et al., 2005). While the therapeutic potential of NSCs is exciting, several significant hurdles still remain, including but not limited to prevention of tumorigenesis, regulating the in vivo environment, and directing and restricting the differentiation potential of the transplanted cells. In order to address the latter, a solid understanding of the native mechanisms of NSC specification is required. The two predominant cell types of the brain, neurons and glia, both have potential therapeutic value in injured spinal cord. In an effort to determine various regulatory mechanisms driving the formation of each, we have established and validated two reproducible *in vitro* models of neural stem cell differentiation; one neurogenic, the other, a more mixed phenotype. We continue to use these two cell clones to dissect the network of interactions during differentiation of neural stem cells, with the ultimate goal of directing this differentiation towards or away from a given phenotype.

An in vitro model of neural stem cell specification.

In order to generate a reproducible model for neural stem cell differentiation, Dr. Hedong Li isolated two neural stem cell clones (Li et al., 2004). Briefly, E14.5 rat

forebrains were dissociated into single-cell suspensions. Cells demonstrating growth as neurospheres were selected and trypsinized again. Cells were cultured in the presence of FGF2 and LIF for two days and then immortalized with the PK-VM-2 retrovirus (Villa et al., 2000). Cells were infected twice and then selected by resistance to G418. A single colony (L2) was subcloned further and yielded two v-myc transduced NSC clones. One of these clones, L2.2, was initially described as BLBP-, while the other, L2.3, was BLBP+. These two clones are both Nestin+ in their undifferentiated state, and propagate as neurospheres in culture. These cells express a polygonal morphology when coated on laminin and send out multiple processes upon differentiation (Li et al., 2008). Upon withdrawal of FGF2, L2.2 readily differentiates into ß-III tub+ (Beta-III Tubulin) neuronal precursor cells (Figure 2) as measured by immunostaining, qRT-PCR and western blots (Li et al., 2008), with little to no expression of GFAP or oligodendrocyte markers (GalC). Differentiation of L2.2 cells was inhibited by BMP2 and enhanced by SHH similar to cortical interneuron precursors.

At two days after FGF2 withdrawal, 15.7±7.5% of the recorded L2.2 cells exhibit action potential, and 43.7±10.9% demonstrated electrical activity when cultured in the presence of radial glial cells. In co-cultures, L2.2 cells expressed GAD and calbindin after 6 days of differentiation indicating their potential to differentiate into GABAergic interneurons. This conclusion was strengthened by the observation that differentiated L2.2 culture also expressed higher level of markers for interneuron subtypes including calbindin, calretinin, neuropeptide Y, tyrosine hydroxylase, somatostatin, neurotensin (Li et al., 2008).

Alternatively the clone labeled L2.3 presents a more mixed phenotype, including astrocytes, oligodendrocytes, as well as potentially glutamatergic neurons upon differentiation (Figure 2). This clone was characterized in detail by Li et al (Li et al., 2004). These two studies thoroughly detail the properties that suggest clone L2.2

resembles ventrally derived GABAergic interneuron precursors thus providing a stable and reproducible *in vitro* model for neurogenesis (L2.2) as well as a contrasting pooled phenotype model (L2.3).

mRNA expression profiling of L2.2 and L2.3 NSC clones.

With the goal of identifying regulated transcripts during differentiation of neuronal and mixed phenotype precursors, we performed mRNA microarray analysis on the L2.2 and L2.3 NSC clones. RNA from three replicates of L2.2 and L2.3 0, 1, and 3 days after FGF withdrawal was isolated using the miRvana kit (Ambion, Austin, TX). This method was chosen as it allows for the separate enrichment of high molecular weight (HMW) and low molecular weight (LMW) fractions, and retains microRNAs in the latter. RNA quality was measured on the 2100 Bioanalyzer (Agilent, Santa Clara, CA) and a minimal 260/280nm ratio of absorbance of 1.8 was observed. HMW RNA was labeled and hybridized to AB1700 Rat Genome Survey arrays according to manufacturer's guidelines. Data were collected and normalized using the ABArray package of R modules provided directly by Applied Biosystems (http://bioconductor.org/packages/1.9/bioc/html/ABarray.html). As part of this package, a quantile normalization is performed to scale the data and reduce variability across arrays. Due to poor and inconsistent data quality from the corresponding 1 day microRNA arrays in both cell lines, the 1 day mRNAs arrays were removed from the analysis. 3,181 probes (11.8%) from the mRNA array were found to be significantly regulated between cell clones and/or during the course of differentiation, as measured by two-way ANOVA (cell line and days after FGF withdrawal) and corrected for multiple testing (5% FDR Benjamini-Hochberg).

Original probe design and annotation for the AB1700 Rat Genome Survey Array was derived from the now obsolete Celera Discovery System and the Panther Protein

DB (derived from the Celera rat genome annotation). This protein-based annotation system was limited in scope and did not readily link to more reliable and curated public annotation. In fact, the original annotation provided by the array manufacturer provided direct mapping to public sequences for only 40% of the probes on the array. In an attempt to increase this number, we obtained the sequences directly from Applied Biosystems for all 26,857 probes. We conducted BLAST (Altschul et al., 1990) alignments of each probe sequence to all available public rat transcripts from the RefSeq (http://www.ncbi.nlm.nih.gov/projects/RefSeq), GenBank (http://www.ncbi.nlm.nih.gov/Genbank), dbEST (http://www.ncbi.nlm.nih.gov/projects/dbEST), and Ensembl (http://www.ensembl.org/Rattus_norvegicus). Additionally probe sequences were aligned to public rat genome sequences. Alignments were stringently selected for only those demonstrating 100% identity across the entire length of the probe. Despite this stringency, we were able to re-annotate 97.3% of the probe sequences to current public records (Goff et al., 2007). This detailed, updated annotation was then utilized to more adequately describe the mRNA sequences contributing to probe signals in the neural stem cell differentiation studies.

The 3,181 significant probes were K-means clustered (k=6; selected to maximize explained variability) to identify parallel biologically-meaningful expression patterns among regulated mRNA. A heatmap was constructed and juxtaposed next to the k-means clusters to visualize the contributions of probe groups to the cluster centers (Figure 3). As hypothesized, K-mean clusters represent biologically meaningful groups of mRNA. Genes in cluster 4 are expressed at relatively higher levels in undifferentiated L2.3 than in L2.2 cells and include the neural stem cell markers nestin and prominin (Li et al., 2008), supporting the idea that L2.3 are NSPC. Clusters 2 and 5 contain genes expressed at relatively higher levels in L2.2 compared to L2.3 cells including β-III

Tubulin, Pax6, DLX-5, NeuroD-1 and -3, supporting the notion that clone L2.2 is neurogenic. In contrast, genes in cluster 1 show relatively higher levels in undifferentiated L2.3 vs. L2.2 cells including Olig-1 and –2, and BLBP/Fabp7, which are associated with glial differentiation, and these genes are down-regulated in L2.2 cells during differentiation. Genes in cluster 3, including GFAP, S100ß, and tenascin-R, show much higher up-regulation in L2.3 than in L2.2 cells and represent markers of astroglial differentiation. These results provide additional support for the idea that clone L2.2 is neurogenic whereas clone L2.3 is both neurogenic and gliogenic.

Interestingly, members of the DLX family of transcription factors are specific markers for the cortex-invading interneurons. Surprisingly, we found that several DLX family members (DLX-1, -2, and -6) show much higher expression level in L2.2 than in L2.3 cells during their time course differentiation. (Cluster 5), suggesting that L2.2 may be an interneuron precursor clone. Interneurons are derived primarily from the ventral forebrain in developing rats (Anderson et al., 1997, Anderson et al., 2001, Pleasure et al., 2000, Xu et al., 2004b). These cells then migrate into dorsal cortex and synapse with the existing projection neurons. The DLX family of transcription factors has been used as specific markers for these cortex-invading interneurons (Anderson et al., 1997). The additional observation of DLX-5 and somatostatin expression provided further hints that L2.2 may represent a ventrally derived interneuron precursor. To test this idea further the expression of interneuron markers between the two clones and with primary cortical interneurons and their precursors was examined. 5A5+/A2B5- cells were isolated by Dr. Cynthia Camarillo from dissociated rat embryonic forebrain and enriched via magnetic bead sorting. These primary cells expressed higher levels of GAD-1, DLX-1, -2, -5 and -6 than unsorted forebrain indicating that 5A5+/A2B5- sorting enriched for genes associated with cortical interneuron precursors. These primary cells are believed to be the cells from which the L2.2 clone was initially derived. Importantly, L2.2 cells showed

higher expression of these genes than L2.3 cells, and the levels increased further with differentiation. Compared to L2.3 cells, L2.2 also expressed higher levels of interneuron subtype markers including calretinin, calbindin, NPY, somatostatin, DARPP32 and TH. Increased expression of DLX-2, GAD and calretinin proteins was confirmed in L2.2 cells by western blot analysis. Immunostaining of 6-day differentiated L2.2 cultures showed that nearly half (48 ± 6.8%) of these cells expressed calretinin and smaller percentages of cells expressed other interneuronal markers including calbindin (20.4 ± 3.2 %), neuropeptide Y (11.5 ± 2.8 %) and somatostatin (11.5 ± 1.6 %). Correlations between samples, across select markers were determined for each of L2.2, L2.3, whole embryonic forebrain, and 5A5+/A2b5- sorted cells. As hypothesized, the relationship between L2.2 and the sorted primary cells was the closest (Li et al., 2008).

One of the obstacles in our experimental design is the heterogeneous population of cells in the L2.3 clones upon differentiation. Since a neuronal sub-population is present, clear distinctions between mRNAs contributing to either neurogenesis or gliogenesis are not immediately perceptible, and neurogenic mRNA are not induced exclusively in the L2.2 clone. This distinction between gliogenic, neurogenic, and other significant mRNAs becomes much more apparent during analyses that will be discussed further below. Regardless of this complication, the L2.2 and L2.3 neural stem cell clones provide an adequate model for analysis of *in vitro* NSC differentiation. The explicit characterization of the L2.2 as an interneuron precursor provides additional information that can be incorporated into the analysis of this cell in a neurogenic context. The mixed phenotype L2.3 cell is used here to provide contrasts during differentiation (as observed and contributed by the glial components of this clone).

microRNA expression profiling of L2.2 and L2.3 NSC clones.

Low molecular weight (LMW) RNA from the same samples was prepared and hybridized to custom microRNA microarrays for expression analysis. RNA was labeled using the Array 900 microRNA Direct labeling kit from Genisphere, Inc. (Hatfield, PA). Cy5 labeled samples were hybridized to our custom miRMAX v2.0 arrays using an optimized protocol (Goff et al., 2005), and data were quantified using a GenePix Pro Scanner (Molecular Devices, Sunnyvale, CA). We used single-channel arrays to mimic the data obtained from the ABI 1700 chemiluminescent arrays. As an added benefit, we were able to avoid inclusion of additional noise from a control channel, since no direct comparison was being made between two samples. Data from 4 arrays from the 1 day time points were discarded due poor quality. As a result of the removal of these samples, there was not enough power to include the remaining 1 day time point samples in the statistical analysis either. All further microRNA studies were conducted using only the 0 and 3 day time point samples from each of the NSC clones.

Work in characterizing the microRNA expression profiles of these two rat neural stem cell clones (designated L2.2 and L2.3) during differentiation agrees with and extends previously published studies and identifies a subset of microRNAs induced during differentiation of the neurogenic clone L2.2. At least two of these microRNAs, miR-9 and miR-124a are consistently up-regulated in the neuronal phenotype and induced upon differentiation, further supporting a previously-suspected role in neurogenesis. High resolution *in situ* hybridizations using RNA oligonucleotides conjugated to a fluorescein hapten have demonstrated the expression of both miR-9 and miR-124a in mouse neurons (Deo et al., 2006). miR-9 was also observed in differentiating neural progenitor cells (Deo et al., 2006). Functional studies (gain- and loss-of-function analyses) of these two microRNAs in differentiating mouse ES cells

have demonstrated an effect on the expression of TuJ1 and GFAP markers, as compared to an untransfected control (Krichevsky et al., 2006). Specifically, the over-expression of miR-9 results in a decrease in the glial marker GFAP in differentiating ES cells, supporting the hypothesis that miR-9 plays a role in promoting a neuronal phenotype.

microRNA array data processing, normalization, and analysis.

Many novel algorithms have emerged to deal with multivariate microarray data. Combining data from multiple arrays requires some method of scaling and/or normalization. We have spent much time determining an optimal work flow for microRNA array analysis (Goff et al., 2008) . Most of the "traditional" normalization methods, such as loess normalization (Smyth and Speed, 2003), are based on assumptions that do not hold true for the data obtained from the miRMAX microarrays. For previous array experiments on mRNA platforms, this was the default method for normalization. The use of loess however, as with most model-fitting normalization methods, is only indicated when the data meet certain assumptions; including 1) high number of variables (genes), 2) minimal change between samples/treatments for the majority of genes, and 3) the number of up- and down-regulated genes on an array are roughly equal. In fact, the microRNA data do not meet any of these assumptions. To address this issue, a non-parametric, rank-based normalization method was employed. First applied to microarray data by Terry Speed's lab (Bolstad et al., 2003), quantile normalization forces the distributions of each sample to take the mean distribution across all arrays effectively scaling all samples. The main drawback of this approach to normalization is the strong assumption that the distributions of probe intensities are close to identical (even if individual probes differ in their positions in the distribution). This is true for low abundance genes, and to a fairly good approximation for genes of moderate abundance,

but does not necessarily hold true for the few high-abundance genes, whose typical levels vary noticeably from sample to sample. Not enough is known about the expression patterns of microRNAs to determine if this assumption is not met. However, we chose quantile normalization for the microRNA data as fewer assumptions were required for use, and the measured variability of the normalized data was decreased.

As an added benefit, the use of quantile normalization on the microRNA expression data helps to maintain consistency between the two array types, as the microRNA chip data were quantile normalized using R (http://www.r-project.org) and BioConductor (http://www.bioconductor.org/), employing an analogous method to that used by the ABarray package for the ABI arrays. Post-normalization data were tested for significance and 45 microRNA genes were determined to be significant between cell lines and/or time points (FDR 10%) by ANOVA of model parameterizations. microRNA genes were also clustered using the same model-based method applied to the mRNA data. A subset of significant microRNAs, including miR-9 and to a lesser extent, miR-124, belong to models demonstrating increased expression in the neurogenic clone L2.2. The regulation of these microRNAs in L2.2 and L2.3 differentiation, their previously described association with neurons (Deo et al., 2006, Krichevsky et al., 2006), and the understanding that microRNA activity has been demonstrated to strongly influence differentiation in various other cell types, suggest that these microRNA are directly involved in neurogenesis.

Cross-correlation of mRNA and microRNAs expression identifies putative regulatory networks in neurogenesis.

In order to assess the potential interactions between the significant mRNAs and significant microRNAs, we described the relationship between their expression profiles in a pairwise manner. Since microRNAs have been shown to mediate mRNA degradation,

it is reasonable to suggest that this activity could be identified in a subset of mRNA:microRNA pairs demonstrating strong negative correlations across multiple conditions. Inversely, microRNAs and mRNAs sharing similar transcriptional regulatory mechanisms may be identified as microRNA:mRNA pairs demonstrating a positive correlation across the same conditions. To this end, the Pearson correlation coefficient was calculated using the expression values for each mRNA-microRNA pair across the 12 samples (3 replicates each of 0 and 3 days in both L2.2 and L2.3). We had initially expected this analysis to produce a normal distribution of R-values across all mRNA:microRNA pairs with a few relationships either strongly correlated or strongly anti-correlated. 2D hierarchical clustering of the resulting matrix of values, on both mRNA and microRNA axes, surprisingly produced clusters of mRNA and microRNA with strong relationships across the 12 samples, and may describe networks of interactions between the two molecule types, as well as help ascribe microRNAs to key biological functions. We tested whether this clustering was a result of the inherent behavior of the two-dimensional hierarchical clustering, by randomly permuting the data in the cross-correlation matrix. The permuted data were re-clustered on both axes and the matrix yielded only noise with no discernable relationships either between or within either axis (not shown). We noticed that this cross-correlation analysis was able to adequately separate mRNAs into distinct clusters. Based solely on their relationship to the significant microRNAs, mRNAs with common roles were grouped together. By selecting a subset of the significant mRNAs that are known transcription factors (Figure 4), we, by definition, limit the mRNAs to those that have a direct influence over transcription rates. Clusters of microRNAs that are positively correlated with known transcriptional activators may in fact be direct transcriptional targets for the given mRNAs. The inverse would also apply to mRNAs with a known role in transcriptional repression. These correlations, combined with transcription factor binding site predictions, begin to describe a network of

microRNA transcriptional regulation as well as post-transcriptional regulation of mRNAs by significant microRNAs.

To begin exploration of the cross-correlation matrix, we asked whether any meaningful relationships among microRNAs could be inferred from their correlations to mRNAs. Prior to clustering, mRNAs were characterized as either neurogenic (blue colorbar), gliogenic (red colorbar), or otherwise involved in stem cell maintenance (yellow colorbar). Those that could not be directly characterized based on available literature were left blank. Interestingly, one subset of significant mRNAs, identified as the highest-level branch of the mRNA dendrogram, contained genes with a strong association to neurogenesis (blue colorbar), while another subset contained genes primarily associated with gliogenesis. It was interesting to note that these associations were so immediately apparent and yet the groupings were based not on expression profiles but rather correlation with significant microRNAs. If we extend this association to the microRNA axis, we can readily identify a high-level branch of the dendrogram grouping several microRNAs together (outlined in black); each of which demonstrates a strong positive correlation to the majority of the members of the neurogenic mRNA cluster. Based on this positive correlation, it can be concluded that these microRNAs (miR-9, miR-124a, miR-182, miR-153, and two others expressed at significantly lower levels) exhibit expression patterns that are parallel to those of known neurogenic mRNAs in the context of the neural differentiation model, suggesting involvement in neurogenesis. As described previously, both miR-9 and miR-124a have previously been shown to directly influence the differentiation of p19 cells (Krichevsky et al., 2006), so their presence in this cluster lends credence to this conclusion.

microRNAs associated with neurogenesis play an active role in induction of the neuronal phenotype

We have identified miR-9, miR-124a, miR-182, and miR-153 as primary members of a group of neurogenic microRNAs. Their expression levels in differentiating interneuron precursors correspond with the onset of a neurogenic phenotype, and they are strongly correlated with known neurogenic mRNAs, as estimated by the cross-correlation analysis. To test whether these microRNAs are capable of directly affecting the phenotype of a differentiating neural precursor cell, we transfected strand-specific pre-miRs (Ambion) for each of these small RNAs into replicate cultures of the mixed phenotype clone L2.3. These double stranded molecules are mimics of functionally mature microRNA molecules, can be appropriately loaded into miRNP complexes, and allow for the over-expression of a strand-specific microRNA sequence. We hypothesized that if these microRNAs were capable of inducing a neuronal phenotype, then they should be able to increase the percentage of neuronal cells produced during differentiation of this clone, or conversely decrease the number of cells expressing glial markers during the same time period.

We electroporated pooled pre-miRs into replicate (n=3) cultures of L2.3 and allowed the cells rest for 24 hours. Additional transfected cultures were differentiated for 3 or 5 days by FGF2 withdrawal. Coverslips were formalin fixed and immunostained for TuJ1 and GFAP. Percentage of $TuJ1^+$ and $GFAP^+$ cells were hand counted in 12 representative fields from each sample. While the aggregated results suggested both an increase in TuJ1+ cells at both 3 and 5 days post-FGF and a decrease in GFAP+ cells at 5 days post-FGF (not shown), this did not prove significant. This is most likely due to the high variability amongst those counting the cells. To gain power in the assay and remove this source of variability, we repeated this experiment and counted stained cells by

fluorescence activated cell sorting (FACS). Pooled pre-miRs were electroporated into L2.3 cells using the Amaxa 96-well shuttle nucleofector. Cultures were plated and FGF was again removed to induce differentiation.

The results from this FACS analysis demonstrate a significant increase at 3 days after FGF withdrawal in the number of TuJ1+ cells in the samples that were transfected with the mixture of pre-miRs (Figure 5). We have shown a reproducible increase from 23.4±1.96% to 39.2±4.01% of $TuJ1^+$ cells ($p<0.033$). A corresponding, although not significant, decrease in GFAP+ cells was observed at 5 days. Therefore, the observed association of these microRNAs with neurogenesis is, at least in part, a causative relationship wherein the microRNAs are actively promoting the neuronal phenotype. Individual pre-miRs are currently being examined in the same assay to identify microRNAs in the cluster sufficient to influence the differentiation phenotype alone. Preliminary evidence indicates that individual overexpression of three out of the four microRNAs will affect the differentiation potential of L2.3 in the absence of FGF. We are also testing the requirement of these neurogenic microRNAs for induction of the neuronal phenotype by transfecting anti-miR (Ambion) microRNA inhibitors with the aim of blocking the specific activity of each of these microRNAs during L2.3 differentiation. We have observed a slight reduction in $TuJ1^+$ cells by inhibition of these endogenous neurogenic microRNAs but this difference has not been confirmed as significant. These results support the hypothesis that expression of these microRNAs is sufficient to induce a pro-neuronal effect in uncommitted neural precursor cells.

Classification and regression tree (CART) analysis of mRNA expression patterns and putative upstream regulatory elements identifies Mef2 binding sites as sufficient to describe dynamically changing mRNA profiles.

We have attempted to describe patterns of mRNA regulation via clustering analysis and in addition, asked whether enriched putative transcription factor binding sites upstream of regulated mRNAs may be an important factor in driving these clusters. 1Kb upstream regions from each significant mRNA (using the full and robust 0,1, and 3 day dataset) were searched using the "Match" (Wingender et al., 1996) program to find high-scoring putative transcription factor binding sites (TFBS) corresponding to vertebrate position-weight matrices in version 10.2 of the Transfac database (http://www.biobase.de) (Wingender et al., 1996). mRNA were re-assigned to clusters based on expression profiles using all 0,1, and 3 day time points (Figure 6). Cluster labels were treated as class labels for the purpose of detecting discriminating combinations of predicted TFBS. We explored different scoring mechanisms for the presence of a motif, and the best results were obtained using a measure that incorporates the number of hits as well as the score of a top hit. That is, the top 10 scores of each motif were recorded and a total score was obtained as the max(score)*range(10 scores). This measure is large if the top score is large and/or there are many moderate (multiple) hits in the promoter for this motif. Finally, to find the discriminating TFBS we fit a classification tree to the data using a modified CART algorithm (Jornsten, 2007). The CART method selects a sequence of TFBS that optimally separate the gene classes. The first split in the tree is thus the single TFBS (TF1) that best separates the gene classes. The next two splits find the two TFBS that further improves the gene class separation: TF2(1+) best separates the gene classes with TF1 present, and TF2(1-) best separates the gene classes with TF1 absent. The

tree is grown until no further improvement can be made. To protect against overfitting we use 10-fold cross-validation. We repeatedly randomly split the data into a training set (90%) and a test set (10%). We prune back the tree to obtain the tree with the lowest gene class prediction error rate on the test set.

In essence, this CART analysis allows us to discern meaningful separations and/or similarities between clusters of regulated genes. These results indicate that several enriched TFBS emerge as key regulators of differentiation (Figure 7) (Goff et al., 2007). Specifically, the presence of a Mef2 binding site is sufficient to distinguish between static clusters (whose mRNA levels do not necessarily differ dramatically during differentiation), and clusters of mRNAs that are dynamically regulated over time (Goff et al., 2007). This supports the hypothesis that Mef2 genes, and their activity, play an important role in neurogenesis.

Cross correlation of mRNAs with putative Mef2 binding sites and microRNAs meaningfully groups microRNAs.

The unexpected identification of Mef2 binding sites as a descriptive classifier of mRNA expression profiles led us to determine which mRNA genes might in fact be regulated by Mef2 transcription factors. The TFBS prediction algorithm Match (Wingender et al., 1996) was again performed on each of the significant mRNA, this time searching for any potential Mef2 binding sites upstream. Those with putative Mef2 binding sites were selected and cross correlated with significant microRNA (Figure 8). Interestingly, using the relationships to potential Mef2 targets alone was adequate to segregate microRNA identified as upregulated in the neurogenic clone L2.2 versus those that were downregulated (as measured by qRT-PCR) in a secondary cross-correlation matrix. This suggests that activity at the putative Mef2 binding sites plays a role in neurogenesis. Since multiple Mef2 proteins and splice variants exist, all of which are

capable of binding to these sites, it must be determined which of these molecules are expressed and/or regulated during neural cell specification.

A single miR-9 locus is upregulated during neurogenesis.

The brain-enriched and regulated rat microRNA miR-9 is a functional mature molecule, whose transcriptional origin can be traced back to three possible paralogous genomic regions. In mammals, miR-9 can be transcribed from any of these three different genomic loci (miR-9-1, miR-9-2, and miR-9-3). In *R. norvegicus* distinct miR-9 transcripts are produced from regions 2q21, 2q11, and 1q31 respectively (Figure 9). Each of these genes produce a unique primary transcript that can be 5'-capped and poly-adenylated, processed by the RNase III enzyme Drosha, and exported from the nucleus. Each of these exported microRNA precursors contain the sequence of the functional mature miR-9, but contain distinct flanking sequences. When processed by Dicer and loaded into a miRNP complex, each results in the identical functional miR-9 molecule. It is this mature molecule which is detectable by the microarray system, and therefore this assay is unable to determine the genomic origin of the mature molecule whose expression is increasing during neurogenesis. To answer the question of which genomic locus is actively transcribing miR-9 molecules, we designed quantitative real-time PCR (qRT-PCR) primers for each of the three miR-9 precursors, as well as each of three predicted transcripts flanking the miR-9 precursor sequences. qRT-PCR analysis of differentiating L2.2 cells with these primer pairs allows for the sequence-specific discrimination of the activated transcripts during neurogenesis (Figure 10). These results indicate that while primary transcripts are detectable for all three miR-9 genes, only the transcripts from the 2q11 region, corresponding to the miR-9-2 variant, are significantly ($p<0.05$) increased during neurogenesis. Additionally, comparison of the average C_t values from the qRT-PCR data suggests that the miR-9-2 primary transcript is

expressed ~8-fold higher than either of the other transcripts measured. One complication of the mixed-phenotype clone L2.3 is evident in this result. The percentage of neurons obtained can vary from culture to culture. In the specific cellular preparation used to conduct the mature miRNA qPCR for Figure 10, there is a significant increase in the miR-9 expression during differentiation of L2.3 as well as L2.2. We believe that this elevation was principally due to a higher percentage of neurons produced in this specific culture. This isoform is one of the two miR-9 isoforms that is immediately adjacent to a Mef2 gene. These transcripts, corresponding to the miR-9-2 isoform of miR-9, are directly responsible for the detectable increase in the mature miR-9 molecule during neuronal specification.

An inversion artifact is identified in the rat genome at 2q11.

Inspection of the Rat reference genome (Rat Genome Sequencing Consortium v3.4) (Gibbs et al., 2004) genomic position of miR-9-2 indicates that the microRNA gene appears to be intergenic with no identified rat transcripts located nearby (Figure 11). Interestingly, genome regions flanking the miR-9-2 gene indicated strong homology to portions of the human, murine, and bovine Mef2c genes. Alignment of the rat genome to the mouse genome in this region identified what appeared to be an inversion artifact located within the first of these regions of homology to Mef2c genes. This inversion divides the regions of homology to Mef2c in half, as well as change the orientation of a portion of the putative gene. Comparing this inversion feature to the bacterial artificial chromosomes that were used to sequence this portion of the rat genome, we noticed that the inversion artifact begins at the precise point where BAC CH230-394N10 begins. In addition, the 5’ and 3’ sequences of this BAC contain regions of poor resolution as well as regions of low complexity. It was postulated that this BAC was inserted into the rat genome in an anti-sense orientation and that the two adjacent regions of homology to

Mef2C that flank the miR-9-2 sequence, are in fact a single transcription unit containing the rat homolog of Mef2C. By inverting the sequence at the appropriate sites, we were able to adjust the genomic sequence and reconstruct the mRNA sequence of a putative rat Mef2C gene. PCR amplification of the full length rat Mef2C gene, as well as amplification across the “repaired” inversion site (data not shown) confirmed that the full length transcript is indeed present and in the correct orientation. As an added effect of this correction, the orientation of the rno-miR-9-2 gene, which was located within the inversion site, was corrected and now matches the orientation of the human and mouse sequences.

A hypothetical rat Mef2C gene was validated as a result of this correction. Mouse exons were mapped to the transcript, and alternative splice sites and untranslated regions were annotated. The hypothetical Mef2C mRNA presented an open reading frame that could be translated to produce a protein with several features including protein domains, regulatory sites, and select other attributes. These features were labeled based on homology to mouse and human as well (Figure 12). The reconstructed sequence of this resulting protein was aligned to the Mef2C genes from several other species as a measure of the accuracy of the reconstruction (Figure 13). With the exception of a single point mutation in *D. rerio*, the MADS-Mef2 domain is perfectly conserved. Additionally, alternative splice regions β and γ were identified as regions of poor conservation, due to their absence in several of the reference sequences. With the identification, annotation, and validation of the rat Mef2C gene, a panel of four Mef2 genes is now present in the rat genome, identified as Mef2A-D. This provides the superset of Mef2 proteins to test for expression, regulation, and involvement in neural differentiation.

In part to confirm the existence of this hypothetical rat Mef2c, primers were designed, in collaboration with Dr. Cynthia Camarillo, from the corrected sequence for

amplification of the full-length transcript. The primers incorporated a C-terminal FLAG epitope tag so that a cloned PCR product could be used to express a protein that could be traced. L2.2 cDNA was used as template DNA and upon amplification the product was cloned into the expression vector pSI. The resulting FLAG-tagged Mef2c mRNA plasmid was sequenced, and it was immediately apparent that the majority of the sequence was exactly as hypothesized from the gene reconstruction. The only exception was an alternate exon 3 that was later understood to be a brain-specific exon for Mef2c (Zhu et al., 2005). We have outlined the available splice variants, including the observed sequence (3F), in figure 14. Several clones were prepared in parallel to both ensure a successful outcome, but also to assess the diversity of splice variants for Mef2C in NSC. We were surprised to find the brain-specific exon 3 in each of ~10 clones observed. In contrast, the optional β-exon was present in only ~75% of sequenced clones. This corresponded to the observed proportions of PCR amplicons obtained from L2.2 cDNA across this alternative splice site (data not shown). These clones demonstrate for the first time the existence of a full-length functional Mef2c transcript in the rat and that this gene is capable of transcription in neural stem cells. The additional observation that mRNA splicing is actively producing alternative transcripts for Mef2c is noteworthy as well. Validation of the FLAG-tagged Mef2c protein was conducted via western blot using the ECS anti-FLAG antibody (Bethyl) and has successfully demonstrated the presence of the epitope tag in both HEK293 cells and L2.2 and L2.3 after transfection.

The rat miR-9 genes arose from gene duplication events and share common genomic neighbors, including Mef2 isoforms.

One of the more common origins of paralogous genes such as the miR-9 family of microRNAs is through genomic duplication. To ask whether or not the expansion of the miR-9 genes in rat was due to gene duplication events, we aligned the precursor

sequences for each miR-9 gene across all species in the Sanger registry v9.0. A phylogenetic tree of the evolutionary relationships between miR-9 genes shows that there have been several duplication events within the miR-9 family throughout the course of evolution (Figure 15). The mammalian miR-9-1 family is the most similar to the most primitive identified miR-9 gene, belonging to the flatworm *Schmidtea mediterranea*. This gene is most closely related to members of an early gene duplication event including several teleost and amphibian miR-9 genes. In early evolutionary history, there were many duplication events, as evidenced by the larger number of branches among more evolutionarily-ancient species. Around this time of prolific gene expansion, there was a duplication event that resulted in the mammalian miR-9-3 lineage.

Along with the miR-9 gene, a neighboring gene was also duplicated. Immediately downstream of rno-miR-9-1 and rno-miR-9-3 is a member of the Rhesus blood group-associated B glycoprotein (Rhb) family of genes (Figure 15C). Interestingly, Rhbg and Rhcg, the two paralogs adjacent to miR-9 genes, are distinct among the Rhb family members in that they are non-erythroid regulators of ammonium homeostasis, and are both moderately expressed in neural tissues. Significantly later in the course of evolution, another event occurred and the miR-9-1 gene was duplicated again to form the miR-9-2 gene branch. The preponderance for mammalian species within this new gene cluster along with a greater level of conservation amongst cluster members, leads to the conclusion that this duplication was a much more recent event. As before, a neighboring gene appears to also have been duplicated. Immediately upstream of rno-miR-9-1 and rno-miR-9-2, there are isoforms of the Mef2 family of transcription factors (Figure 15C). The Mef2d gene is located adjacent to the miR-9-1 gene at 2q34. Similarly, the Mef2c isoform is juxtaposed next to miR-9-2 at 2q11. The limited divergence of the most recent miR-9 branch, as compared to the miR-9-1 and miR-9-3 branches, suggests that this particular locus, miR-9-2, has been pressured to remain

unchanged, signifying a functional role within the cell. Others have suggested that the majority of miRNA genes arise not from duplication events but rather are 'born' via accelerated mutation rates across evolutionary time from non-miRNA sequences (Lu et al., 2008). While the origin of the first miR-9 gene cannot be determined from this analysis, it is clear that both the miR-9-2 and miR-9-3 genes arose from duplication events that also allowed for duplication of neighboring genes. This distinction is significant here due to the singular role played by the miR-9-2 locus in increasing the cellular levels of mature miR-9 during differentiation of neural precursor cells. The duplication of this microRNA implies a positive selection mechanism to retain this activity in the mammalian brain.

Mef2c and miR-9-2 are derived from adjacent transcriptionally-active genomic regions in neuronal differentiation.

To determine the expression and regulation of Mef2 family members during neurogenesis, we analyzed the mRNA levels of each isoform via standard qRT-PCR. RNA previously isolated from L2.2 and L2.3 for use with the array analysis was used. 2μg of HMW RNA from each of the three replicates of L2.2 and L2.3 at 0, 1, and 3 days post-FGF withdrawal was randomly-primed and reverse-transcribed into cDNA. 2ng of cDNA, oligo primers, and SYBR green master mix (Applied Biosystems) were combined for each qRT-PCR reaction. Primer sets were generated and assayed for all rat Mef2 isoforms. There was no amplification observed for Mef2B transcripts in either L2.2 or L2.3 although the primers were able to amplify genomic DNA (not shown). While there was expression of each of the remaining three isoforms of Mef2, the only isoform significantly increased during differentiation of L2.2 was Mef2C (Figure 16), supporting the hypothesis that a single Mef2 isoform is involved in neurogenesis. Copies of Mef2A

and Mef2D were expressed in both cell types, but demonstrated a significant decrease in the L2.3 clone during differentiation.

In addition to detailing the expression of the Mef2 genes, we have also assayed the expression of the neighboring miR-9 isoforms as mentioned previously. The microRNA qRT-PCR data indicates that the miR-9-2 isoform is the only significantly up-regulated miR-9 gene in the neurogenic L2.2 during differentiation (Figure 10). The close genomic proximity of miR-9-2 and Mef2C, and their similar expression patterns in differentiating neurons suggest the presence of these two transcripts on a region of chromatin that is transcriptionally active during neurogenesis.

Brain enriched microRNA upstream sequences contain functional Mef2 binding sites.

The brain-enriched microRNA miR-9 and miR-124 (Krichevsky et al., 2003, Kim et al., 2004, Krichevsky et al., 2006) each have three isoforms located throughout the rat genome. Since no protein coding sequence has been described surrounding any of these microRNAs, it is expected that they are in fact intergenic microRNAs with unique and identifiable promoter elements located upstream. We conducted a survey of the upstream region of each of these isoforms for putative transcription factor binding sites. High quality vertebrate position weight matrices (PWMs) from the Transfac 10.2 database (Wingender et al., 1996) were utilized to scan a 5Kb region upstream of the pre-microRNAs. As part of the Match algorithm, hits were selected to minimize the rate of both false positives and false negatives. As a further measure of stringency, results were selected as having a “core” nucleotide match of 100%, and tolerating a false negative rate of 10%. Interestingly, brain-specific microRNAs, including miR-9-2, are enriched for high-scoring Mef2 binding sites, as compared to a random subset of non-enriched microRNAs (Table 1). A conservation analysis of the region upstream of miR-9-2 indicates that at least two of these predicted Mef2 binding sites are in regions with a

high degree of conservation between human, mouse and rat (Figure 17) suggesting a conserved role and positive selective pressure to retain functional binding sites. Several other minimal promoter elements including a TATA box (Wasylyk, 1988), and an initiator sequence were also identified upstream of miR-9-2.

In order to determine whether the increase in detectable miR-9-2 transcripts during neuronal differentiation was directly related to transcriptional activation, we cloned a 5Kb region upstream of the rat miR-9-2 precursor into the promoter-less pGL4.10 luciferase expression vector (Promega). As a control, a 5Kb region of miR-9-1 was also cloned. These two plasmids along with pGL4.10 as a negative control, were electroporated (Amaxa) into L2.2 cells along the pRL Renilla luciferase transfection control vector. The ratio of Firefly luciferase to Renilla luciferase (FL/RL) showed little expression of luciferase in the cells transfected with the miR-9-1 promoter plasmid (Figure 18). In contrast, expression of luciferase in the miR-9-2-transfected L2.2 cells suggests that this promoter is present and indeed active, supporting the claim that miR-9-2 is the only regulated miR-9 family member during neuronal differentiation in L2.2.

As previously mentioned, a TFBS analysis of the 5Kb upstream region of miR-9-2 identified two high-scoring, strongly-conserved Mef2 binding sites, ‘Mef2.1’ and ‘Mef2.2’ respectively (Figure 17). These potential Mef2 binding sites may be responsible for activating the transcription of miR-9-2, either through epigenetic activation of the 2q11 region, or direct recruitment of enhancing TFs, during the specification of neurons. To determine the effect that either or both of these predicted Mef2 binding sites may have on the transcriptional activity of miR-9-2, we created deletion plasmids with either or both of the Mef2 binding sites removed. Using the QuikChange II site-directed mutagenesis kit (Stratagene), we deleted ~10-12mers from the 5kb upstream sequences of the miR-9-2 luciferase reporter plasmid corresponding to the putative Mef2 binding sites. An additional third plasmid was created with both sites removed. The deletion

plasmids, along with a promoter-less pGL4.10 positive control and the full-length 5kb upstream sequence as a negative control, were each transfected into L2.2 and FGF was subsequently withdrawn to induce differentiation of the interneuron precursor clone (Figure 19). The promoter-less luciferase reporter exhibited <10% of the activity of the full-length miR-9-2 upstream region, confirming the validity of the assay. Deletion of either the Mef2.1 or Mef2.2 binding sites resulting in a ~20% decrease in transcriptional activity from the 5kb upstream sequence, with only the Mef2.2 binding site demonstrating a significant decrease ($p<0.05$) from 100% activity. No doubt the effect observed from deletion of the Mef2.1 binding site would be determined significant in an assay with more statistical power. Interestingly, the plasmid with both conserved Mef2 binding sites removed demonstrated what could be considered a directly additive effect of a reduction in activity of 34%. This level was also determined to be significantly different ($p<0.05$) than the full-length miR-9-2 upstream sequence. While we do not want to insinuate that regulation by Mef2 is the only mechanism governing the transcription of miR-9-2, it is important to note that these two binding sites alone are capable of explaining up to one third of the transcription activity from the upstream region of miR-9-2 observed during differentiation of the interneuron precursor clone L2.2.

To confirm the hypothesized role that Mef2c, as opposed to other Mef2 proteins, plays in regulating the transcription of miR-9-2 in neuronal differentiation, we attempted to knock down the levels of Mef2c in differentiating L2.2 via short hairpin RNA (shRNA) targeting. shRNA inhibition exploits the endogenous RNAi mechanism to silence specific mRNAs through introduction of a plasmid capable of producing a specific short RNA transcript. This sequence is transcribed and folded into a hairpin RNA structure similar to endogenous miRNA. At this point the hairpin structure becomes a substrate for Drosha and Dicer processing into a siRNA. By controlling the sequence of the ~21mer region that will ultimately become the functional mature siRNA, one is able to specify a distinct

target for repression. The Mission shRNA library (Sigma), represents a large set of pre-defined shRNA sequences for a number of human, mouse, and rat mRNA sequences. In this case, the shRNAs are prepared inside a vector containing a U3 promoter. This allows for a ideally uniform expression via Pol III transcription. Unfortunately, since the rat isoform of Mef2C had yet to be described, pre-designed shRNAs for this precise sequence were unavailable. We instead analyzed shRNAs against the mouse Mef2c mRNA and compared them to the observed rat Mef2c mRNA to identify those targeting conserved sequences. Expression vectors containing shRNAs optimized for knockdown of rat Mef2c were co-transfected with the full length miR-9-2 luciferase reporter vector and a Renilla transfection control into L2.2 cells. FGF2 was withdrawn to stimulate neurogenesis and the resulting luciferase activity was measured using the Dual-luciferase reporter assay system (Promega) and recorded on the 20/20n luminometer (Turner BioSystems). To correct for differences in transfection efficiencies, values were expressed as a ratio of firefly luciferase to Renilla luciferase. Data indicate that the levels of luciferase reporter generated in the absence of Mef2c (as a result of knock-down by shRNAs) are significantly reduced from those observed in the presence of Mef2c (Figure 20). This was confirmed by the expression of a shRNA molecule containing a mismatch and thus unable to adequately silence Mef2c. The mismatched shRNA was unable to reproduce the reduction effect observed in the samples transfected with a functional shRNA expression vector.

The specific inhibition of Mef2C via shRNA in this study demonstrates not only that this protein plays a role in regulating the transcription of miR-9-2, but emphasizes the fact that this observed effect is mediated exclusively by this one specific member of the Mef2 family. The shRNAs are designed to be transcript-specific and therefore any observed effect is attributed principally to the reduction of that specific gene. In the case of the shRNA sequences used against the rat Mef2C, there is little to no homology with

either of the other expressed isoforms Mef2A or Mef2D. These results agree with the hypothesis that the induction of miR-9-2 during neurogenesis is in part due to the activity of Mef2C, the only regulated isoform of Mef2 during neurogenesis.

The requirement of the two Mef2 transcription factor binding sites to achieve full transcription activity, combined with the dramatic effect of Mef2C knockdown on the expression of luciferase in this assay supports the hypothesis that Mef2C binding to the upstream region of miR-9-2 is capable of affecting the expression of miR-9 *in vivo* and lends credence to the theory that Mef2C and miR-9 cooperatively interact to promote the neuronal phenotype.

Mef2c levels affect the differentiation potential of neural stem cells

We have shown that Mef2c is capable of directly affecting the transcription rate of miR-9-2 and that a subsequent shift in the levels of miR-9 can influence the differentiation potential of L2.3 cells. We then asked if the overall differentiation program could be altered through the direct modulation of Mef2c expression levels.

In order to test this it was necessary to inhibit Mef2c with great efficiency in the L2.3 clone. Using shRNA sequences as described previously, we were able to achieve modest transfection efficiencies in the NSC clones and the ability to inhibit Mef2C was further hindered by the basal levels of transcription from the U3 promoter on the Mission shRNA vector. To circumvent these obstacles, we decided to employ endogenous short interfering RNA (siRNA) sequences designed against Mef2C. Silencer siRNAs (Ambion) designed against the cloned 3F isoform of Mef2C were obtained. Unlike shRNAs, siRNAs are annealed, double-stranded ~21mers that are readily transfected directly into a cell. There is no additional transcription activity required, eliminating the problems with the U3 promoter in the shRNA vector. Due to their small size (relative to a full-sized plasmid), a significantly higher transfection efficiency is readily obtained. The specific

activity of these siRNAs was evaluated to determine their efficacy. Three siRNAs were introduced both individually and in a pooled sample to Mef2C-FLAG expressing HEK293 cells. Both siRNA sequences targeting coding regions of the rat Mef2C were able to virtually abolish the expression of Mef2C-FLAG protein as measured by western blot (data not shown). The siRNA sequence targeting the 3'UTR was unable to reduce the levels of Mef2C-FLAG protein as this portion of the Mef2C transcript was sacrificed to attach the FLAG sequence; serving as a very nice control for the specificity of the siRNAs.

A repressor of Mef2 transcription activity is a target for miR-9 and miR-124a.

With the goal of identifying potential feedback networks between Mef2C and miR-9, we analyzed the target predictions of several validated regulators of Mef2C activity. We selected TargetScanS (Lewis et al., 2003, Lewis et al., 2005) as a prediction algorithm due to the algorithm's success, although limited, at mirroring validated mRNA:microRNA interactions. Since we predict that Mef2C and miR-9 are coordinately involved in neurogenesis and since Mef2C is known to have inhibitory binding partners such as HDAC4 (Chan et al., 2003, Gregoire et al., 2006, Miska et al., 1999), we began by analyzing known repressor genes of Mef2 activity. Specifically, the class IIa HDACs, known repressors of Mef2C transcriptional activity, all contain multiple putative microRNA Response Elements (MREs) for both miR-9 and miR-124a in their 3'UTRs, suggesting regulation of these transcripts by the neuron-enriched microRNAs.

The rat HDAC4 gene contains a known 3'UTR of only 482 bp (Figure 21). However, known 3'UTRs for mouse and human HDAC4 genes extend much further. While it is not unprecedented that different species may have dramatically different length untranslated regions, the presence of a validated miR-1 response element in human HDAC4 (Chen et al., 2006) led us to believe that there may indeed be a longer

transcript available in these rat cells. As hypothesized, qRT-PCR analysis of regions past the 482 bp 3'UTR demonstrated that there was an available portion of the transcript which presented some difficulty in amplification. Inspection of the transcript at 482 bases past the stop codon showed an internal stretch of adenosine residues encoded in the genome, which may have acted as a priming site for oligo dT primed cDNA. Attempts to clone the 3'UTR of the rat HDAC4 using a random hexamer as a cDNA primer resulted in amplification of a ~1.2Kb fragment with significantly more information content, including multiple microRNA target sequences.

The 3'UTR of HDAC4 contained four predicted miR-9 response elements and seven miR-124a response elements (Figure 21). Alignments and secondary structure predictions between the MREs and their respective microRNAs indicated that several of these target predictions were strong candidates for microRNA:mRNA interactions (Figure 22), while others were less likely to be MREs due to weaker predicted interactions. The 1.2Kb fragment was cloned into the pmiR-Report (Ambion) luciferase expression vector. These plasmids were co-transfected with expression vectors for miR-9 and/or miR-124a (cloned into pSI and/or Block-it) in to HeLa cells. HeLa cells were chosen for their ease in transfection as well as inherently low background levels of both microRNAs. Data from these assays indicate that both miR-9 and miR-124a are capable of targeting the HDAC4 3'UTR (Figure 23). An additional cooperative effect was noted when miR-9 and miR-124a expression vectors were transfected together. This assay has been repeated on several occasions with similar results, indicating a reproducible interaction between these microRNAs and the HDAC4 3'UTR. This assay demonstrates that miR-9 and miR-124a can regulate expression levels of HDAC4 via 3'UTR binding. The implications of this regulation in neuronal development are such that an increase in miR-9 and miR-124a expression may be able to suppress the negative regulatory effect

of HDAC4 (and potentially other Mef2 inhibitors) on Mef2, thus promoting transcription at regions bound by Mef2C.

HDAC4 3'UTR mediates translational regulation during neural stem cell differentiation

After demonstrating that the 3'UTR can be directly targeted for repression by miR-9 and miR-124a in HeLa cells, we wanted to test whether this activity is present during the differentiation of neural stem cells. In collaboration with Jonathan Davila, a graduate student in the lab, the HDAC4 3'UTR luciferase reporter plasmid was co-electroporated with a Renilla control into replicate cultures (n=3) of the L2.2 and L2.3 clones. As a comparison, a luciferase plasmid containing the 3'UTR of a separate gene, Onecut2, was included. Negative control transfections of the pmiR luciferase vector with no added 3'UTR were included as well. Cultures were subject to differentiation by FGF withdrawal for 0, 1, or 3 days. At each time point, cells were harvested and luciferase levels were measured using the dual luciferase reporter assay (Promega). Firefly luciferase levels were corrected for transfection efficiency by normalization to Renilla expression levels. To correct for transcriptional variation as a result of FGF withdrawal, the FL/RL ratios were normalized to the FL/RL values observed for the control pmiR vector at each timepoint. As anticipated, the normalized firefly luciferase levels were significantly ($p < 0.01$) decreased in the HDAC4 3'UTR plasmid expressing L2.2 cells by 3 days of differentiation, confirming negative regulation via 3'UTR activity during neurogenesis (Figure 24). The opposite effect was observed at three days of differentiation of the corresponding L2.3 cultures, suggesting an activation of HDAC4 via its 3'UTR in the more mixed-phenotype clone. Neither effect was observed with the Onecut2 3'UTR indicating that this effect is specifically mediated by the HDAC4 3'UTR sequence.

To confirm that the observed luciferase assays predicted regulation of expected HDAC4 protein levels in differentiating neural stem cells, western blots for HDAC4 were conducted on replicate cultures of L2.2 and L2.3 in collaboration with Dr. Cynthia Camarillo. Cultures were either maintained in FGF (undifferentiated) or subject to 3 days of FGF withdrawal. As hypothesized, HDAC4 protein levels were reduced upon differentiation in the neurogenic L2.2 clone, predicting a reduced availability during neurogenesis (data not shown). As with the luciferase assays, it was observed that HDAC4 protein levels were elevated during differentiation of the mixed-phenotype L2.3. These results are consistent with a model of miR-9 and/or miR-124a mediated repression of HDAC4 during neurogenesis via targeting of the HDAC4 3'UTR.

HDAC4 inhibition of Mef2C transcriptional activity may be independent of histone deacetylase activity

In preliminary studies, we confirmed that HDAC inhibition leads to increased numbers of $TuJ1^+$ cells in a multipotential progenitor cell clone. A member of the research group, Christopher Ricupero, evaluated the role of several well-described HDAC inhibitor molecules in affecting the differentiation potential of the L2.3 neural stem cell clones. As reported by others using a variety of model systems (Balasubramaniyan et al., 2006, Siebzehnrubl et al., 2007), valproate (VPA, 0.1-0.4 mM), added to multipotent L2.3 cells, increased the percentage of $TuJ1^+$ cells within 3 days as determined by FACS analysis (data not shown). Based on the earlier work with mir-9 regulation of HDAC4 expression, we suspected that Type IIA HDACs might explain the increased neurogenesis after VPA inhibition. This was tested bioinformatically in collaboration with Dr. Ronald Hart by comparing upstream sequences of two sets of microRNAs based on their expression patterns in a neuronal progenitor clone. One population was observed to be upregulated during differentiation and the other was

expressed but not regulated. We hypothesized that the upregulated class of microRNAs would have an enriched number of predicted binding sites for transcription factors known to interact with HDAC4 or HDAC5 [tabulated in (Martin et al., 2007)]. 5 kb samples of genomic sequence upstream of the most likely transcription start site (predicted using enclosing RefSeq records, surrounding dbEST records or sequences adjacent to mature microRNA sequence) were retrieved. These sequences were searched for the appropriate position weight matrices specific for HDAC4/5 binding partners using MATCH software (BioBase, Wolfenbüttel, Germany). Comparing the summarized frequencies of finding sites, we used Student's t-test to determine whether the mean frequency of sites was different between regulated and non-regulated microRNAs. We found no difference, which is inconsistent with the hypothesis. However, the trial finding is supported by a recent publication demonstrating that Type IIA HDACs have little or no actual HDAC enzyme activity (Lahm et al., 2007) and so are not inhibited by compounds such as VPA. Substitution of a single amino acid in the active site (His->Tyr) caused a 1000-fold increase in deacetylase activity. Therefore, we reject the hypothesis that the increased neurogenesis observed with HDAC inhibitors is due to the deacetylase activity of type IIA HDACs and the future proposed studies will test the chromosomal effects of type I HDAC activity. This does not however exclude the possibility that type IIA HDACs are involved in neurogenesis as it has been shown that HDAC4 is capable of mediating Mef2 repression without a functional deacetylase domain (Chan et al., 2003) and that in general type IIA HDACs can recruit type I HDACs to bound complexes to affect deacetylation of adjacent chromosomal regions (Chan et al., 2003). We conclude, in agreement with others, that Class IIa HDACs are involved in neural cell specification, but that this involvement is independent of the deacetylase enzyme activity that is the defining characteristic of this class of genes. This is supported by other groups who have demonstrated the role of class IIa HDACs in active SUMOylation of proteins in key

differentiation pathways (Gregoire et al., 2006, Kang et al., 2006, Riquelme et al., 2006, Stankovic-Valentin et al., 2007) as one example of a secondary activity of this class of genes.

III. DISCUSSION

As research progresses into the means by which neurogenesis is induced in neural stem cells, the roles of small non-coding RNAs must not be ignored. The rapid pace at which new microRNA genes are being identified and characterized will allow better incorporation of these molecules into regulatory mechanisms. The importance of these genes should not be underestimated, as is evidenced by the central role of miR-9 described in this study.

We propose that Mef2C activation of miR-9-2, through upstream transcription factor binding sites, results in increased miR-9 expression, resulting in increased miR-9 targeting, and therefore attenuation of the Mef2 inhibitor HDAC4. This activity will stabilize the positive transcriptional activity of Mef2C and promote the neuronal phenotype through further miR-9 mediated neurogenesis as well as activation of previously established pro-neuronal genes. We have demonstrated that miR-9 expression, both as a member of a group of neurogenic microRNAs (Figure 5) or individually (not shown), is both associated with (Figure 4) and a cause of neurogenesis, that Mef2C is required for full transcription of miR-9 (Figures 19 & 20), and that expression of this microRNA will reduce the available levels of the HDAC4 protein (Figure 23).

miR-9 and Mef2C coordinately promote neurogenesis in differentiating NSCs

It is clearly evident in this research and in previous studies that the brain-specific microRNA miR-9 is involved in the neurogenic differentiation of stem cells. We have repeatedly confirmed the correlation of the mature miR-9 microRNA with the onset of the neuronal phenotype (Figures 4, 10, & 18). The over-expression of miR-9 both individually and in the context of other neurogenic microRNA is capable of increasing the number of $TuJ1^+$ cells at the expense of $GFAP^+$ cells in uncommitted neural stem cells

demonstrating a causative effect on neurogenesis (Figure 5). By using the strand-specific pre-miR for miR-9 over-expression we can determine that the induction of the neuronal phenotype is a result of the increase in miR-9 and not necessarily the miR-9* sequence produced from the opposite arm. In this study we delve further to describe the individual contributions of each distinct genomic locus to this enrichment. The duplication of miR-9 throughout the course of evolutionary history has provided the mammalian lineages with three functional miR-9 loci, each of which is capable of producing an identical mature microRNA (Figure 9). It is reasonable to theorize that not all of these loci are directly involved in mediating interneuron precursor differentiation, as we observed in L2.2. When we probe these loci to determine their unique roles in this context, we identify miR-9-2 as being the exclusive site for significant enrichment of miR-9 in differentiating interneuron precursor cells (Figure 10). A logical explanation for this exclusivity lies in the different transcriptional regulatory networks capable of activating each of these miR-9 loci, through divergent regulatory sequences.

A transcription factor binding site analysis of the upstream regulatory regions of the two rat miR-9 genes expressed in neural precursor cells, miR-9-1 and miR-9-2, reveals several common promoter elements in each (not shown). TATA-box, CCAAT-box, TFIIA, and Core binding factor sites are present in both 5Kb upstream sequences, suggesting that minimal Pol II promoter elements are involved in initiating transcription in both regions. Both miR-9-1 and miR-9-2 promoter regions have the potential to bind common activating transcription factors such as GATA, E2F, and FOX family proteins. A distinct difference is the presence of putative Mef2 binding sites in these transcriptional regulatory regions. The miR-9-2 promoter region contains three predicted binding sites for the Mef2 family of transcription factors, two of which represent a 100% match to the core promoter sequence associated with Mef2 binding (Table 1). These sites are all located in regions of the genome that demonstrate a high (>75%) conservation across

mammalian genomic sequences. It appeared oddly coincidental at first that a transcription factor with such conserved binding sites adjacent to miR-9-2 was computationally determined (Figures 7 & 8) to be important to the networks involved in L2.2 and L2.3 differentiation.

After validating both the existence (Figures 11 & 12), and expression of (Figure 16) the rat Mef2C gene in neural precursor cells, it was informative to note the differences between the cloned cDNA and the previously described Mef2C mRNAs in other organisms (Figure 14). The first observation of the high frequency (~75%) of the alternative β exon in the cDNAs cloned from L2.2 proved most revealing. This exon, encoding the acidic peptide N-SEDVDLLL-C, is present at higher levels during differentiation of muscle cells (Zhu et al., 2005). It is possible that this sequence is observed in a high frequency as a result of differentiation in the neural precursor clones as well. More importantly, inclusion of the β exon results in increase transcriptional activity as measured by Mef2 transcription reporters (Zhu et al., 2005). This suggests that this exon may enhance the transactivation activity of Mef2C during neural stem cell differentiation as well.

Despite the clear preference for the brain-specific exon 3 in the cDNA clones derived from L2.2, there is little known about the function of this peptide; specifically anything that may distinguish it from the activity of the muscle-specific isoform. Conserved domain scans using PantherDB, Prosite ExPASy, and CDD (NCBI) were only able to identify the conserved MADS-MEF2 domain and could not ascribe a function to any portion of this exon. Due to its location internally within the protein it is most likely not the product of an alternative 5' transcript end. Future analysis of the function of this exon may demonstrate its functional role in Mef2C induction of neurogenesis.

The identification of putative Mef2 binding sites in the promoter region of miR-9-2 led us to the hypothesis that Mef2C may be partially responsible for the increase in this

microRNA during neuronal differentiation. After demonstration of the requirement for the putative binding sites for full transcriptional activation of miR-9-2 during neurogenesis (Figure 19), and the resulting decrease in miR-9-2 promoter activity after RNAi-mediated reduction in Mef2C levels (Figure 20), this hypothesis was confirmed. By exploiting the specificity of the RNAi mechanism, we were able to further conclude that the reduction in miR-9-2 promoter activity was due exclusively to the activity of the Mef2C isoform (Figure 20). We conclude then that expression of Mef2C is not only capable of promoting neurogenesis through the previously described mechanism of MASH1 induction (Skerjanc and Wilton, 2000), but also by enhancing the expression of the neurogenic microRNA miR-9. This analysis serves as the first study into the transcriptional regulatory network governing the expression of miR-9 and demonstrates that this regulation is mediated by mechanisms similar to those employed by protein coding genes.

miR-9 reduces translational availability of the Mef2C inhibitor HDAC4

We have verified a pathway by which miR-9 is transcriptionally activated by Mef2C during neurogenesis. Due to the well-characterized roles of histone deacetylases in negatively regulating the activity of this class of transcription factors (Bertos et al., 2001, Chan et al., 2003, Lin et al., 1996, Linseman et al., 2003, Lu et al., 2000, McKinsey et al., 2000, Miska et al., 1999, Potthoff et al., 2007, Zhang et al., 2002), it seemed logical that we should test the interactions between HDACs and these two molecules in the context of neural stem cell differentiation. We initially asked whether any change was evident in the levels of HDAC4 mRNA in the L2.2 and L2.3 cells. Both microarray results and qRT-PCR indicated no change in the availability of HDAC4 mRNA in any condition. Despite this result, the expression of luciferase protein attached to the 3'UTR of HDAC4 showed a significant reduction during L2.2 neurogenic

differentiation (Figure 24). When we observed this HDAC4 3'UTR-mediated reduction in luciferase expression, we hypothesized that the HDAC4 protein may be differentially expressed both between L2.2 and L2.3 and during the course of FGF withdrawal-induced differentiation of these cells. As anticipated, the HDAC4 western results confirmed that despite a lack of change in mRNA levels, HDAC4 protein expression was reduced during neurogenesis, and conversely, increased during differentiation of the L2.3 clone (data not shown). A likely conclusion to be drawn from these results is that the levels of HDAC4 protein are repressed during differentiation of neuronal precursor cells and that this effect is mediated primarily via the 3'UTR.

We next tested the predictions that miR-9 and selected other neurogenic microRNAs are capable of attenuating the translation of HDACs. Building on the observation that the 3'UTR of HDAC4 is responsible for a portion of the reduction in HDAC4 protein levels during neurogenesis (Figure 24), we theorized that this reduction may be mediated through miR-9 and/or miR-124a binding to the 3'UTR of HDAC4. Using standard 3'UTR luciferase validation assays, we showed that this was indeed capable, and that luciferase-HDAC4 3'UTR levels were significantly reduced in HeLa cells after co-transfection with either or both miR-9 and miR-124a (Figure 23). This confirmed computational predictions that miR-9 and miR-124a can reduce the expression of HDAC4 protein (Figure 22). This result, in conjunction with the observation that HDAC4 protein levels are reduced through 3'UTR-mediated reduction during neurogenic differentiation, suggest that the inverse correlation that exists between miR-9/miR-124a and HDAC4 in our neural stem cell context is not coincidental. A logical interpretation of these results is that the observed reduction of HDAC4 protein during neurogenesis is at least partially mediated by specific microRNA regulation via the 3'UTR.

The combination of these observations is consistent with the hypothesis that the regulatory activity of Mef2C, which is known to be affected by HDAC4, is capable of being modulated by the expression of miR-9 and the resulting decrease in the inhibitor HDAC4. The de-repression effect of HDAC4 reduction on Mef2C activity in the context of neurogenesis is speculative at this point, however this has been demonstrated conclusively in other contexts (Lu et al., 2000, McKinsey et al., 2000), and is consistent with a previously described mechanism in muscle differentiation (Chen et al., 2006). In the Lu study, the researchers demonstrate a specific interaction between Mef2C, HDAC 4, and an A/T rich DNA element; and show that this complex was capable of inhibiting muscle differentiation through a repression of Mef2C activity (Lu et al., 2000). This inhibition is enhanced by overexpression of HDAC4. They co-immunoprecipitated a complex containing a GST-tagged HDAC4, ^{35}S-labeled Mef2C, and ^{32}P-labelled DNA demonstrating that HDAC4 repression of MEF2C activity is not a result of competition for the DNA binding domain of MEF2C. Chen and colleagues further extend this pathway by observing that a muscle-specific microRNA, miR-1, actively represses HDAC4 protein levels in myoblasts (Chen et al., 2006). This affects an increase in Mef2C transcriptional activity as measured by increased expression of MyoD, a known downstream effector of Mef2C-mediated muscle differentiation. To confirm that a parallel mechanism is at work in neural stem cell specification, we must first identify a similar MEF2C/HDAC4 protein complex via co-immunoprecipitation in the L2.2 and L2.3 NSC clones. The MEF2C-FLAG protein that we have cloned, in conjunction with both a FLAG antibody and more recently a custom generated polyclonal rat Mef2C antibody, will permit us to examine the actual binding partners of MEF2C protein during the course of neural differentiation.

An important first step will be to demonstrate that MEF2C co-immunoprecipitates with HDAC4 in the L2.2 and L2.3 cultures. We hypothesize that in the mixed-phenotype L2.3 cells, where HDAC4 protein levels increase during differentiation, a corresponding

increase would be observed in the amount of HDAC4 that is co-immunoprecipitated with MEF2C. Inversely, in the neurogenic L2.2, we expect that MEF2C:HDAC4 complexes would be significantly less abundant, and that the amount of HDAC4 bound to MEF2C would diminish during differentiation. These results would be consistent with a role for HDAC4 in suppression of Mef2C activity in neural cell differentiation.

A model for microRNA mediated regulation of Mef2 activity and induction of the neuronal phenotype.

The role of Mef2C during neurogenesis, its location alongside a microRNA identified as important in neuron specification, the co-regulation of these two transcripts, and the validation of a microRNA targeting a known Mef2 cofactor all support the model describing the potential roles each of these molecules may play in neural stem cell specification (Figure 25). We propose that activation of miR-9 results in the repression of factors inhibiting the transcriptional activity of available Mef2C, such as HDAC4. Once Mef2C is no longer inhibited by these repressors, it is free to bind activating co-factors, and induce the expression of pro-neuronal genes such as Mash1. Furthermore, Mash1 is capable of interacting with Mef2 and additionally enhancing its transcriptional activity. Activated Mef2 is capable of stimulating further expression of miR-9-2 through binding the upstream sequence, resulting in a stabilizing feedback loop. This self-stabilizing loop is a key aspect of the induction and maintenance of the neuronal phenotype in differentiating neural precursor cells.

Alternatively, if no miR-9 microRNA were present, Mef2 would remain a transcriptional repressor bound to chromatin-modifying and silencing HDACs. Mef2 would not be able to actively enhance transcription of Mash1 and/or other pro-neuronal genes, and would not be able to activate transcription of additional miR-9. Mef2 would in fact actively silence these genomic regions ensuring that they are not transcribed.

miR-9 and miR-124a are two miRNAs that have been the focus of many brain-microRNA studies since their identification. In one of the first papers to correlate the activity of a specific microRNA with a given phenotype, Krichevsky et al. describe the effects of introduction and inhibition of miR-9 and miR-124a in primary neural precursors (Krichevsky et al., 2006). Introduction of exogenous miR-9-like siRNAs into neural precursor cells was shown to affect the differentiation potential primarily through the reduction of $GFAP^+$ cells from the transfected population. In our study we see the parallel effect of an increase in neurogenesis, but in our model this is identified as a significant increase in the percentage of $TuJ1^+$ cells. While the result is the same (an increase in neuronal precursor cells) these are noticeably different metrics through which this change is observed. One explanation for this discrepancy is that the level of commitment to a particular phenotype may be inconsistent among the assays. In our model, the mixed-phenotype clone L2.3 still has the potential to differentiate into both neurons and glia, and retains the capacity to alter the proportion of cells with either phenotype upon differentiation. This is a reproducible model in which to evaluate the effect of miR-9 overexpression. In the Krichevsky study, the use of primary cells results in a more inconsistent model to test these effects. It cannot be clear what level of commitment to a particular phenotype each cell exhibits. Therefore, if a greater percentage of primary neural precursors are glial-restricted, then over-expression of a pro-neuronal microRNA such as miR-9 may not result in an increase in TuJ1 but may repress the expression of the glial marker GFAP. If the L2.3 model represents cells with a greater pluripotency, then expression of miR-9 will induce a neuronal phenotype in otherwise uncommitted cells, resulting in a detectable increase in $TuJ1^+$ cells.

One of the conclusions drawn by Krichevsky is that the pro-neuronal effect of miR-9 over-expression is due in part to a decrease in STAT3 phosphorylation (Krichevsky et al., 2006). The authors do not go into detail about the mechanism by

which this happens but merely suggest the correlation is important to neurogenesis. Indeed STAT3 repression has been shown to play a role in neuronal induction (Moon et al., 2002, Gu et al., 2005). It was surprising however, to find that treatment with trichostatin A (TSA), a potent inhibitor of HDAC activity, results in a decrease in transcriptional activity of STAT3 (Catania et al., 2006). This result, while attributed in this specific instance to abolition of deacetylase enzymatic activity, could also potentially be brought about through direct reduction in HDAC protein levels, effectively reducing HDAC enzymatic activity. It would not be unreasonable to hypothesize that the decreased phosphorylation of STAT3 observed in primary neural precursor cells after overexpression of miR-9 and miR-124a is mediated in part through direct targeting and repression of HDAC4 by these microRNA. While we do assert that the primary function of class IIa HDACs in inhibition of neurogenesis is deacetylase activity-independent, the relationship between STAT3 dephosphorylation and TSA-induced inhibition of HDACs suggests that this activity may be involved in a select few regulatory networks.

Undoubtedly, miR-9 and miR-124 are not the only microRNAs involved in neurogenesis. In this study we identified a group of four microRNAs capable of inducing the neuronal phenotype in an uncommitted neural precursor cell. Each of these microRNAs has numerous target mRNAs; the repression of which may help to promote the neuronal phenotype. What are the functions of miR-153 and miR-182 in the observed induction of neurogenesis? Do they function to promote neurogenesis or suppress aberrant phenotypes? Similar studies to those outlined here will help to elicit and expand the roles for these miRNAs.

In addition to the pathway described in this study, we have uncovered the modulation of several microRNAs (Figure 10) that have previously been associated with either cell proliferation (Cheng et al., 2005), stem cell maintenance (Houbaviy et al., 2003), or onset of a different phenotype. These changes represent a subset of the

microRNA contribution to neural stem cell differentiation. Clearly, the role of microRNAs in neural stem cell specification and differentiation is crucial to our understanding of the network of interactions contributing to the neuronal phenotype. Despite the depth and complexity of this system, it is clear from these results that the miR-9/Mef2C/HDAC4 pathway is involved in neurogenesis, and represents one mechanism by which neural stem cells can be directed towards a specific neuronal phenotype. Exploitation of this microRNA pathway to direct the differentiation of uncommitted neural stem cells to interneurons, as described here, provides important first steps for the development of a suitable cellular substrate for transplant into neurodegenerative diseases such as Parkinson's. Further exploration of both this pathway, and the broader topic of microRNA influence on stem cell specification, will help to facilitate the use of stem cells in therapies for numerous other neurodegenerative disorders and/or traumatic nervous system injuries.

IV. FIGURES

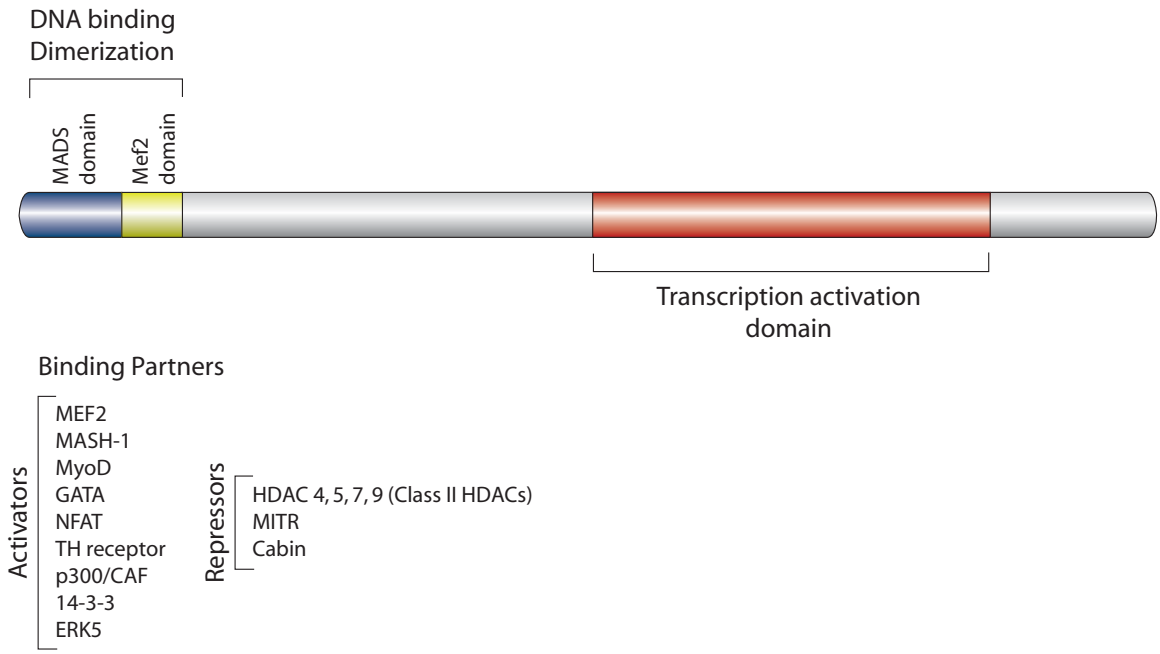

Figure 1: The *Mef2 family of transcription factors.* The mammalian Mef2 family of genes consists of three distinct and conserved domains. The N-terminal MADS domain confers binding activity to the consensus sequence CTA(A/T)$_4$TAG/A as well as dimerization. Immediately adjacent to the MADS domain is the highly conserved Mef2 domain responsible for additional DNA-binding affinity as well as cofactor binding. This domain serves primarily as a dynamic regulatory switch regulating the binding partners, and ultimately the specific activity of the Mef2 protein. The C-terminal end of the Mef2 genes is much less conserved and consists of a transcription activation domain. Additional C-terminal regulatory domains and alternative splice sites are available for a few of the Mef2 family members, and confer regulation activity by various MAP Kinases and or CDKs.

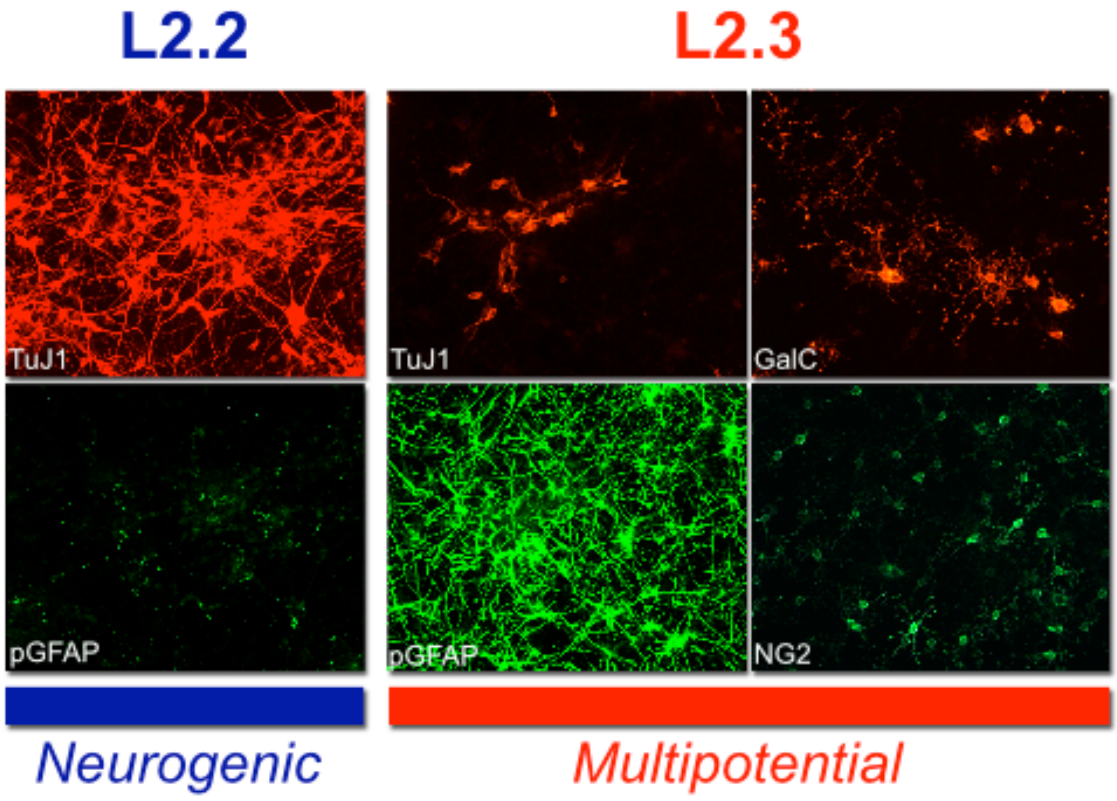

Figure 2: ***L2.2 and L2.3 NSC clones*****.** Two v-myctransduced NSC clones were derived from E14.5 dissociated rat cortex. Both cultures can be maintained as Nestin^{+} in the presence of FGF. When cultured in the absence of FGF, one clone, L2.2, differentiates predominantly into TuJ1^{+}GABAergicinterneurons. L2.3, exhibits a mixed phenotype. Most cells are GFAP^{+}astrocytes, while several show markers for other cell types including neurons (TuJ1), and oligodendrocytes (GalC, NG2). (Clonal selection, immunostaining, and figure courtesy of Dr. Hedong Li)

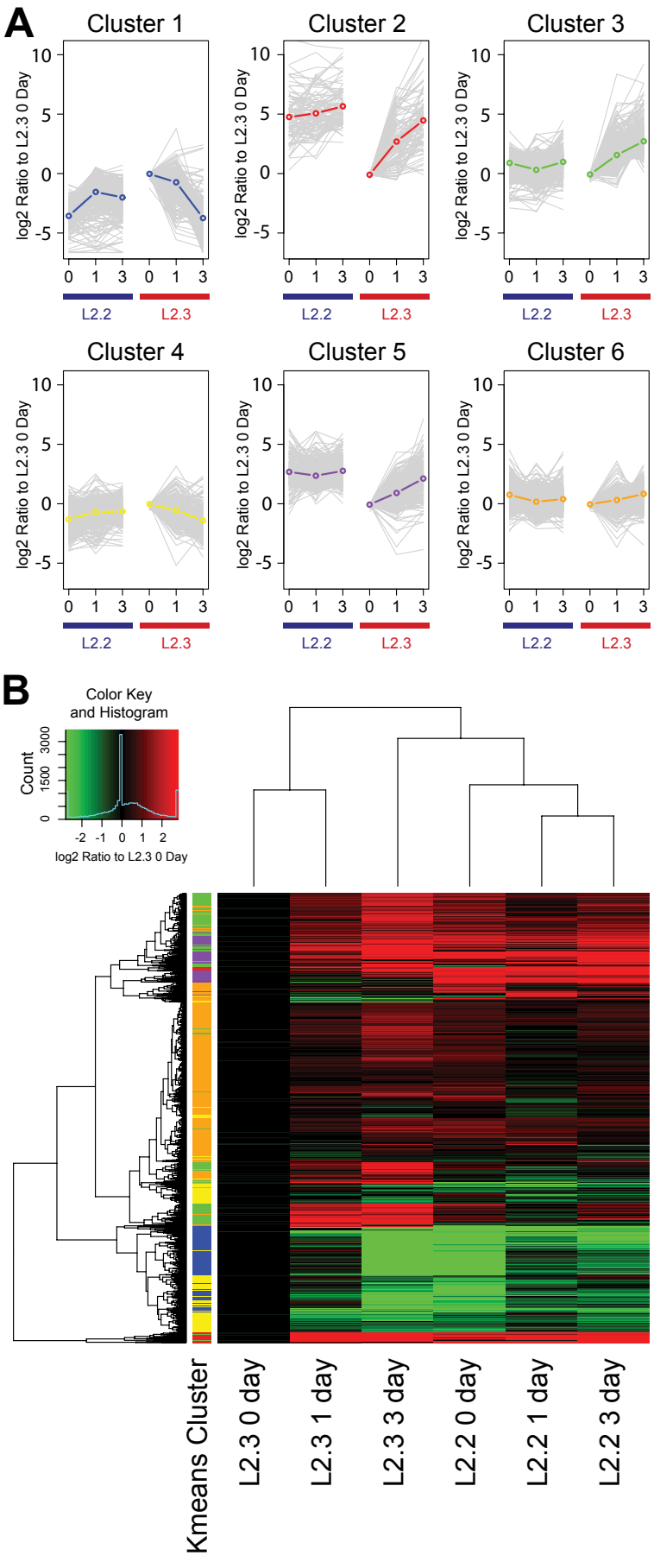

Figure 3:*Differences in gene expression patterns in L2.2 and L2.3 cells and during differentiation.* Upon FGF2 withdrawal, total RNA samples of L2.2 and L2.3 cultures were harvested after 0, 1 and 3 days and analyzed on rat genome survey chip (AB 1700). Probes were selected as significant ($p<0.05$ with 5% FDR) using two-way ANOVA for differences across cell line and time. Significant probes were k-means clustered (k=6) to identify similar expression patterns (A), and were also shown as the hierarchically clustered heatmap plotted using R (B).

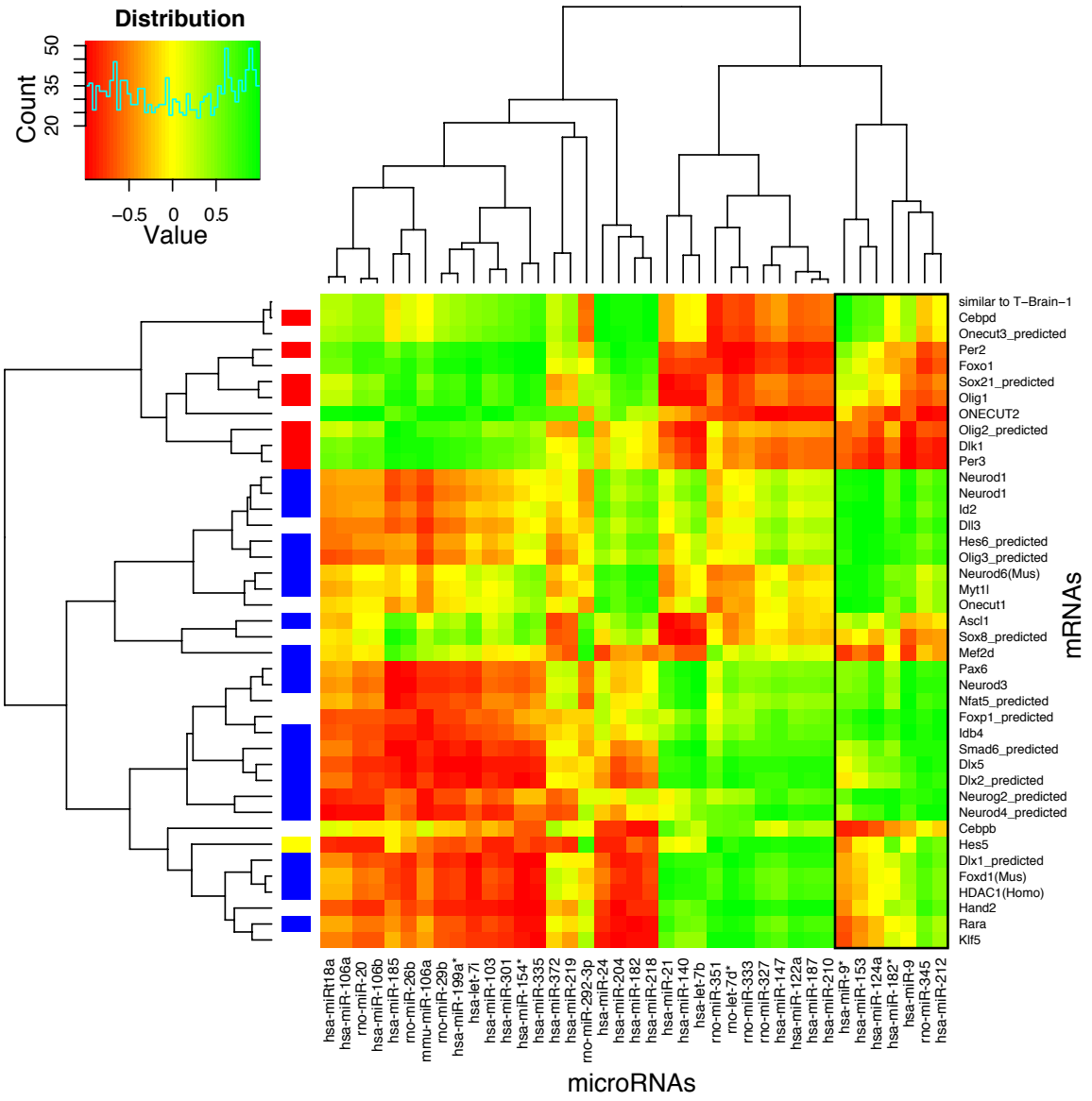

Figure 4: ***Cross Correlation of significant transcription factors and significant microRNA expression patterns identifies potential networks of mRNA:microRNA interactions.*** Pearson correlation coefficient values were calculated between expression values for significant transcription factors and microRNA and values were hierarchically clustered along both axes. Clusters of mRNA and microRNA with strong negative correlation (red) identify potential microRNA:mRNA interactions resulting in mRNA degradation and provide direction for future studies of microRNA targeting. Positively correlated mRNA and microRNA (green) represent transcripts that may be coordinately regulated during neurogenesis. A side colorbar is provided to indicate mRNA that are associated with neurogenesis (blue), gliogenesis (red), or stem cell maintenance (yellow). Interestingly, the highest-level cluster adequately distinguishes between neurogenic and gliogenic mRNA. The close proximity of miR-9 and miR-124a, along with a strong positive correlation to known neurogenic transcription factors, provides additional evidence that these microRNAs, along with other members of this cluster, are expressed during neuronal specification, and are required for acquisition of the neuronal phenotype.*(Produced in collaboration with Dr. RebeckaJörnsten)*

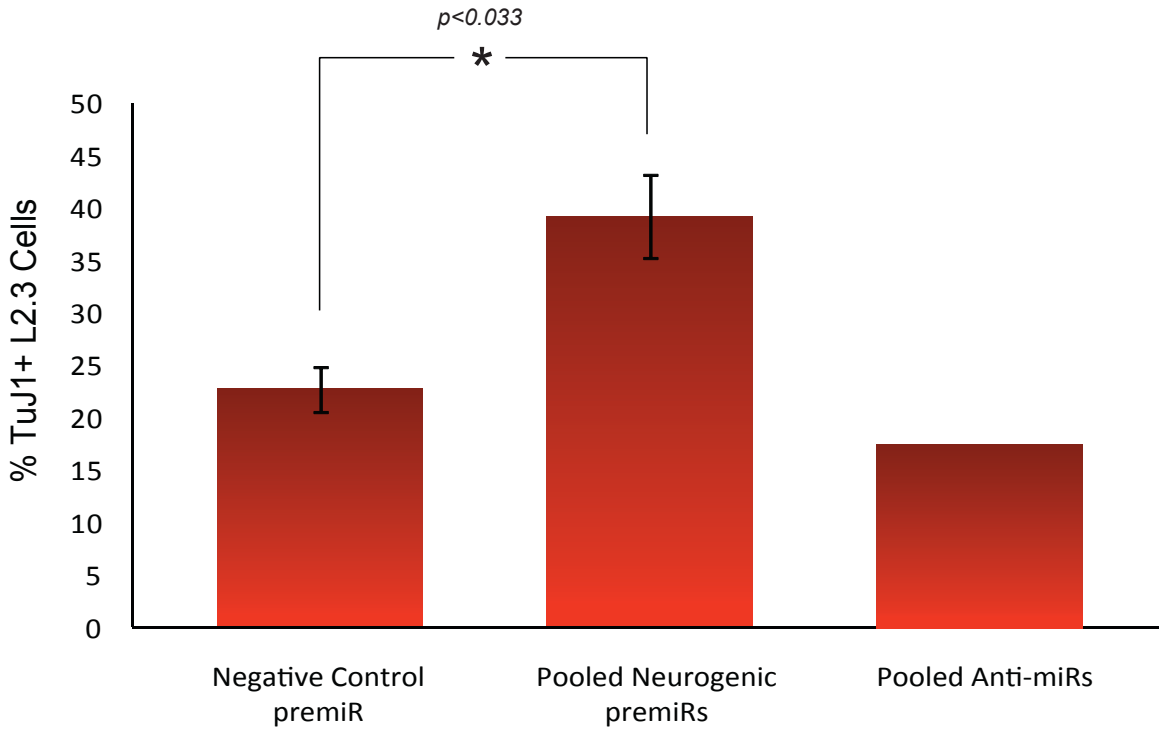

Figure 5:*Overexpression of neurogenesis-associated microRNAs induces the neuronal phenotype in uncommitted neural stem cells.* Pooled pre-miRs(Ambion) or anti-miRmicroRNA inhibitors (Ambion) for four neurogenesis-associated microRNAs (miR-9, miR-124, miR-182, and miR-153) were electroporated into three replicate cultures of the neural stem cell clone L2.3. Upon plating and withdrawal of FGF, cells were fixed and stained with TuJ1 antibody to determine a change in neurogenic potential. The pre-miR-transfected cultures demonstrated >25% increase in the percentage of cells staining positive for TuJ1. This was determined to be significant via Student's T-test with $p<0.05$ and no multiple testing correction. While there were insufficient replicates of the anti-miR-transfected samples to determine significance, we did detect an appropriately decreasing trend in the percentage of $TuJ1^+$ cells. These results confirm the role of these microRNAs in induction of the neuronal phenotype. (*Produced in collaboration with Chris Ricupero*)

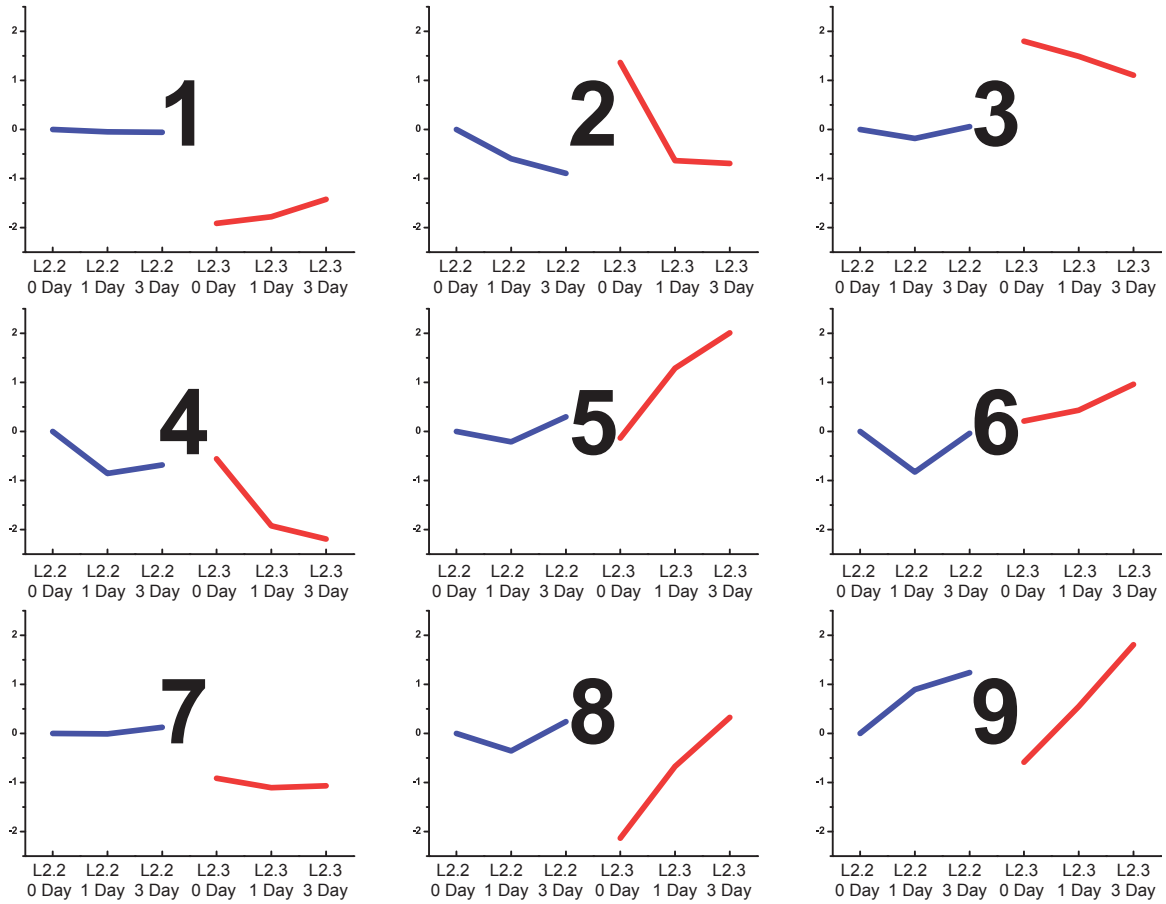

Figure 6: ***Clustering analysis of significant mRNAs identifies static differences between cell lines as well as dynamic differences during differentiation.*** A more robust analysis of the high quality mRNA array data (including 1 day timepoints) identified 2003 significant mRNA (FDR 5%) that were clustered using the same model-based clustering method. As a result, nine distinct shapes emerged representing mean expression values of each cluster member. Again, clusters are readily categorized as static and unchanging during differentiation (eg. 1,3,7), or dynamically regulated (eg. 4,5,8,9). This mRNA clustering analysis was used to drive the classification and regression analysis along with predicted transcription factor binding sites. (*Produced in collaboration with Dr. RebeckaJörnsten*).

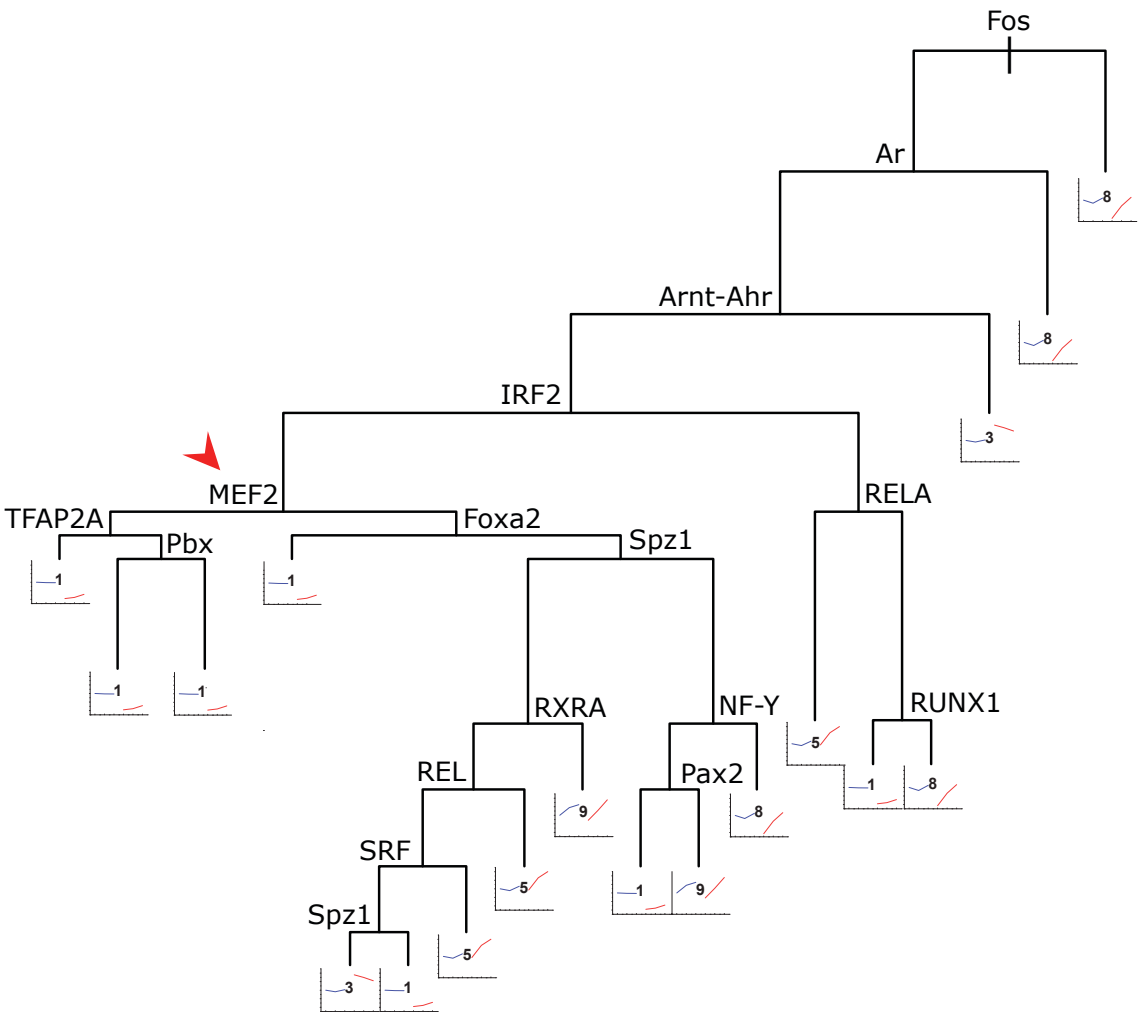

Figure 7: ***CART analysis identifies Mef2 binding sites as sufficient to distinguish between static and dynamic clusters of significant mRNA.*** We performed a classification and regression analysis on enriched TFBS upstream of significant mRNAs. Vertebrate position weight matrices from the Transfac 10.2 database were used to identify potential TFBS in a 1Kb upstream region of each significant mRNA. Cluster numbers were used as labels and cluster members were individually classified by the presence of significant TFBS to produce the above dendrogram. Early branches of the tree are not particularly informative in cluster discrimination, but we do notice that the statistical presence or absence of a Mef2 binding site is sufficient to discriminate between clusters of mRNAs that are unchanging during differentiation (static) and those whose levels are significantly modulated (dynamic). *(Produced in collaboration with Dr. RebeckaJörnsten).*

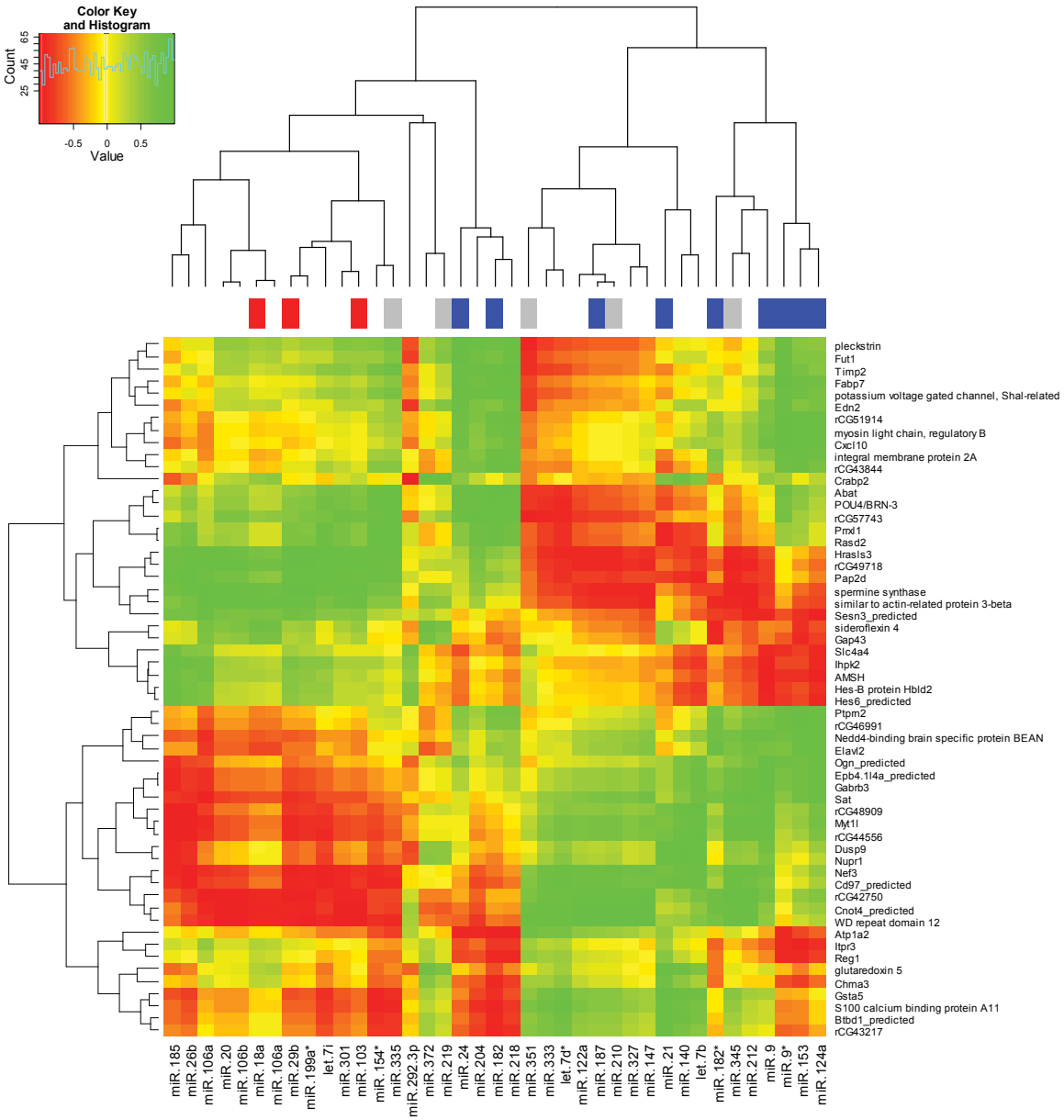

Figure 8: ***Cross-correlation matrix of significant mRNAs containing putative Mef2 binding sites, and all significant microRNAs.*** The 2003 significant mRNA were filtered for genes with putative Mef2 binding sites in a 5Kb upstream region. Cross-correlation with significant microRNAs again results in clusters of strong positive or negative correlation. The attached microRNA color bar indicates direction of change during differentiation of L2.2 cells as measured by qRT-PCR, with blue indicating an increase during neurogenesis, and red a decrease during differentiation. Grey microRNA had no directional change. Interestingly, these putative Mef2 targets alone are capable of segregating microRNA into reasonable clusters; those increasing during neurogenesis (blue) or decreasing during differentiation of L2.2 (red).

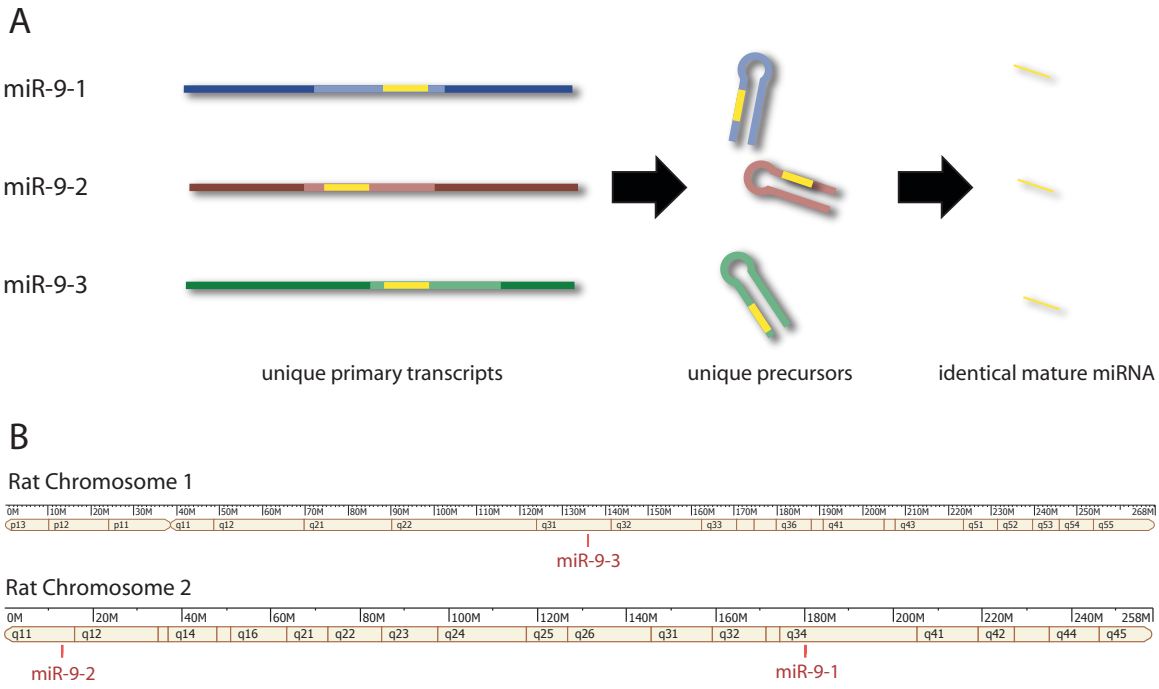

Figure 9: ***Identical mature miR-9 molecules can be derived from separate, unique primary transcripts and precursor molecules.*** (A) Distinct primary transcripts, through processing viz the RNAse III enzymes drosha and dicer, can produce identical mature molecules. While we cannot discern which loci are contributing to the expression of the mature molecules by direct quantitation, we can exploit the differences in the pri- and pre-microRNAs for qRT-PCR discrimination of the origin of mature microRNA molecules. (B)Mature rat miR-9 can be derived from multiple potential genomic loci. miR-9-1 and miR-9-2 are both located on chromosome 2 in the rat. miR-9-3 is relegated to chromosome 1 and notably, this region contains numerous ESTs derived from embryonic brain cDNA libraries (not shown).

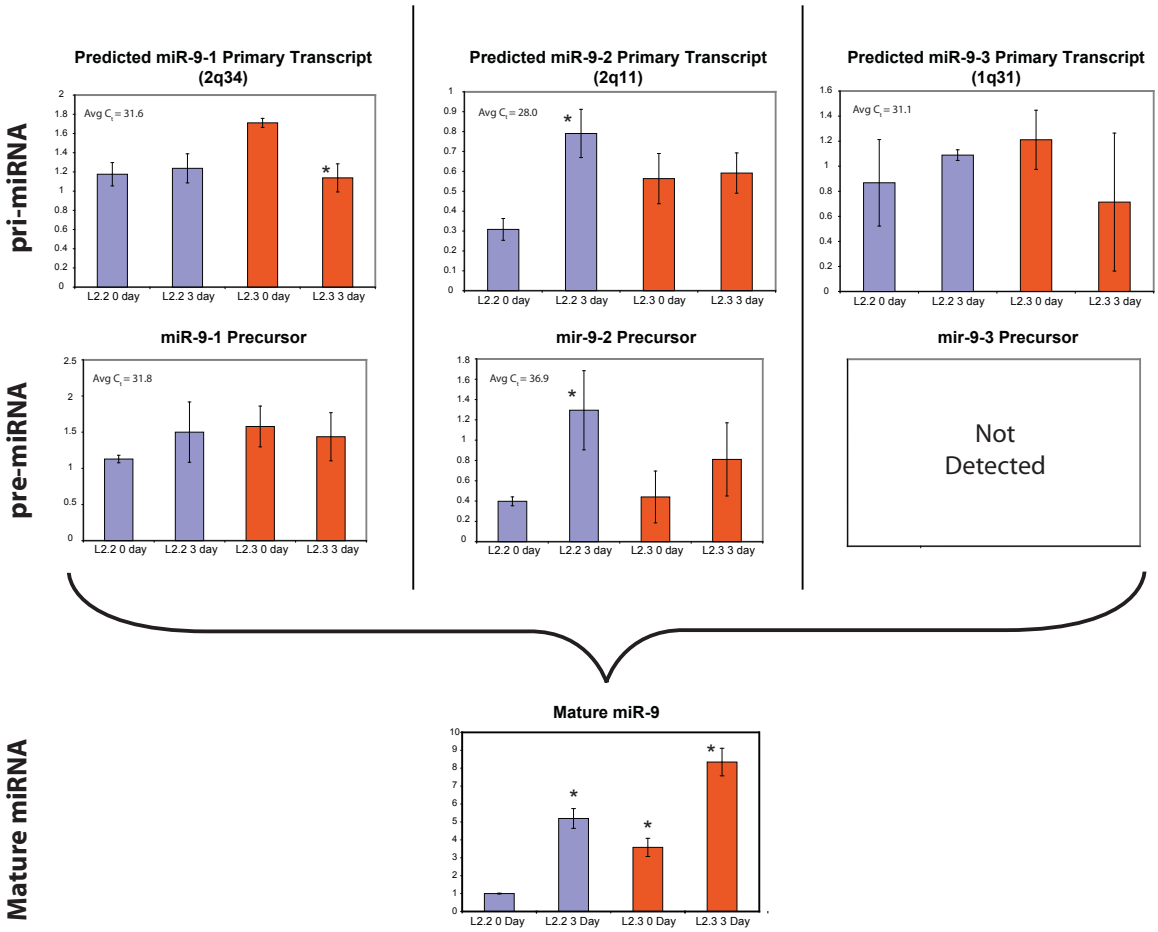

Figure 10: ***qRT-PCR analysis of separate miR-9 genomic loci identifies miR-9-2 (2q11) as the only significantly regulated genomic locus in differentiating L2.2 cells.*** Primers were designed for each of the primary and precursor miR-9 sequences. Additionally, qPCR was conducted using an assay designed for identification of the mature miR-9 product. While primary transcripts were detected for each of the three isoforms of miR-9, only pri-miR-9-2 is significantly increased during differentiation of the neurogenic clone. Despite the repeated failure of two separate pre-miR-9-3 assays (possibly due to lack of target sequence), the data from the remaining two precursor assays again mirror the expression pattern seen in the mature. This data confirms that the dynamic increase in mature miR-9 during neurogenesis is derived from the miR-9-2 transcript exclusively.

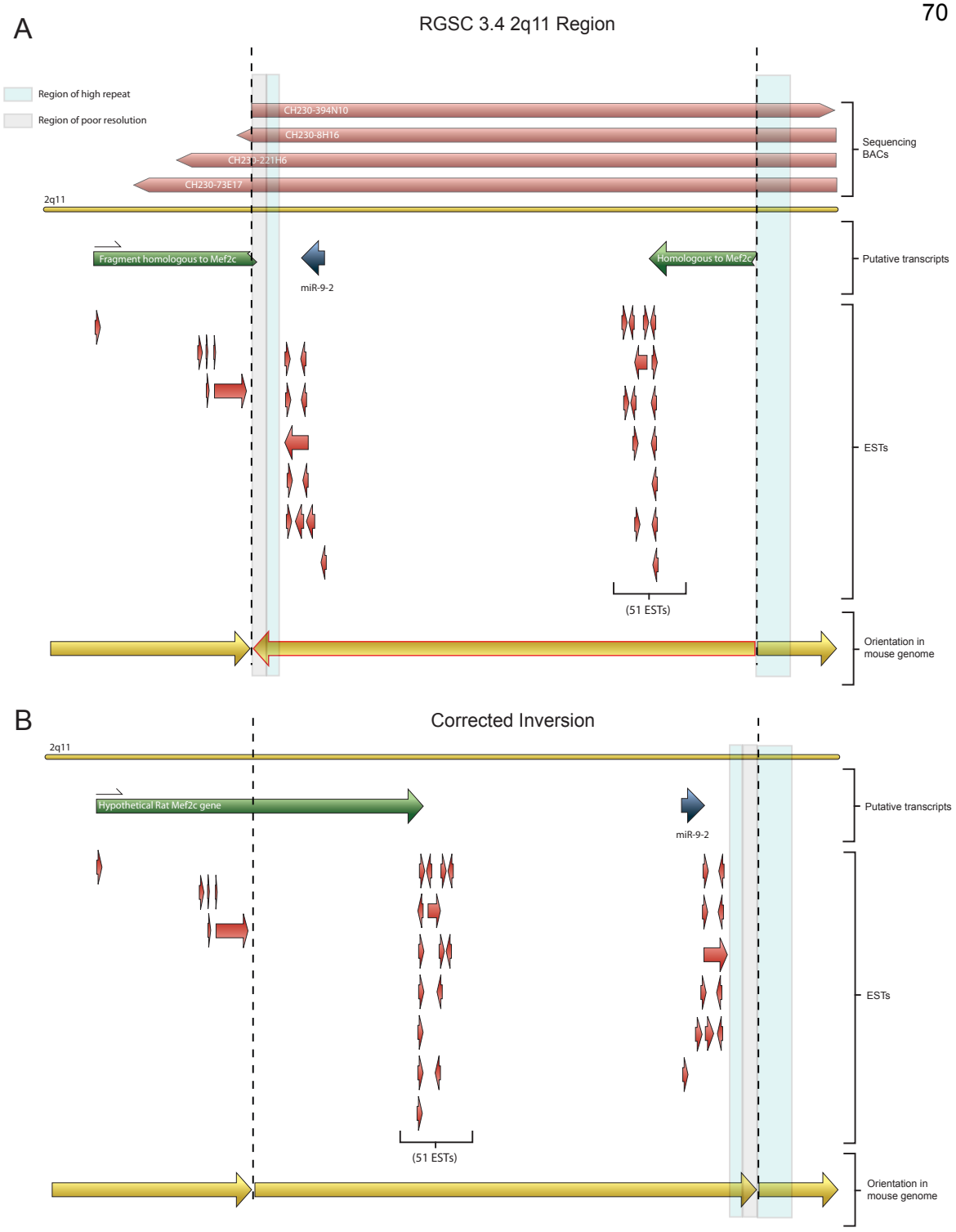

Figure 11: ***An inversion artifact at 2q11 in the RGSC v3.4 rat genome is identified and corrected***. Alignment of the 2q11 portion of the Baylor RGSC v3.4 rat genome to the corresponding portion of the mouse genome identified an inversion coincidentally located at the beginning of the sequencing BAC CH230-394N10. The poor sequence resolution at both ends of this BAC, combined with the supporting evidence from the homologous genomes, led us to suspect that this may be an artifact of the genomic sequencing. Additionally, ESTs located within this region (red) suggest that the true 3'

end of Mef2C was not included in the original transcript. This inversion was confirmed by PCR amplification across each of the “corrected” sites as well as amplification of a full length rat Mef2c gene, both of which would have failed if the actual genomic sequence were identical to the sequence listed in RGSC v3.4. As a result, the artifact was corrected, a full-length Mef2C gene was characterized, and the orientation of miR-9-2 was adjusted.

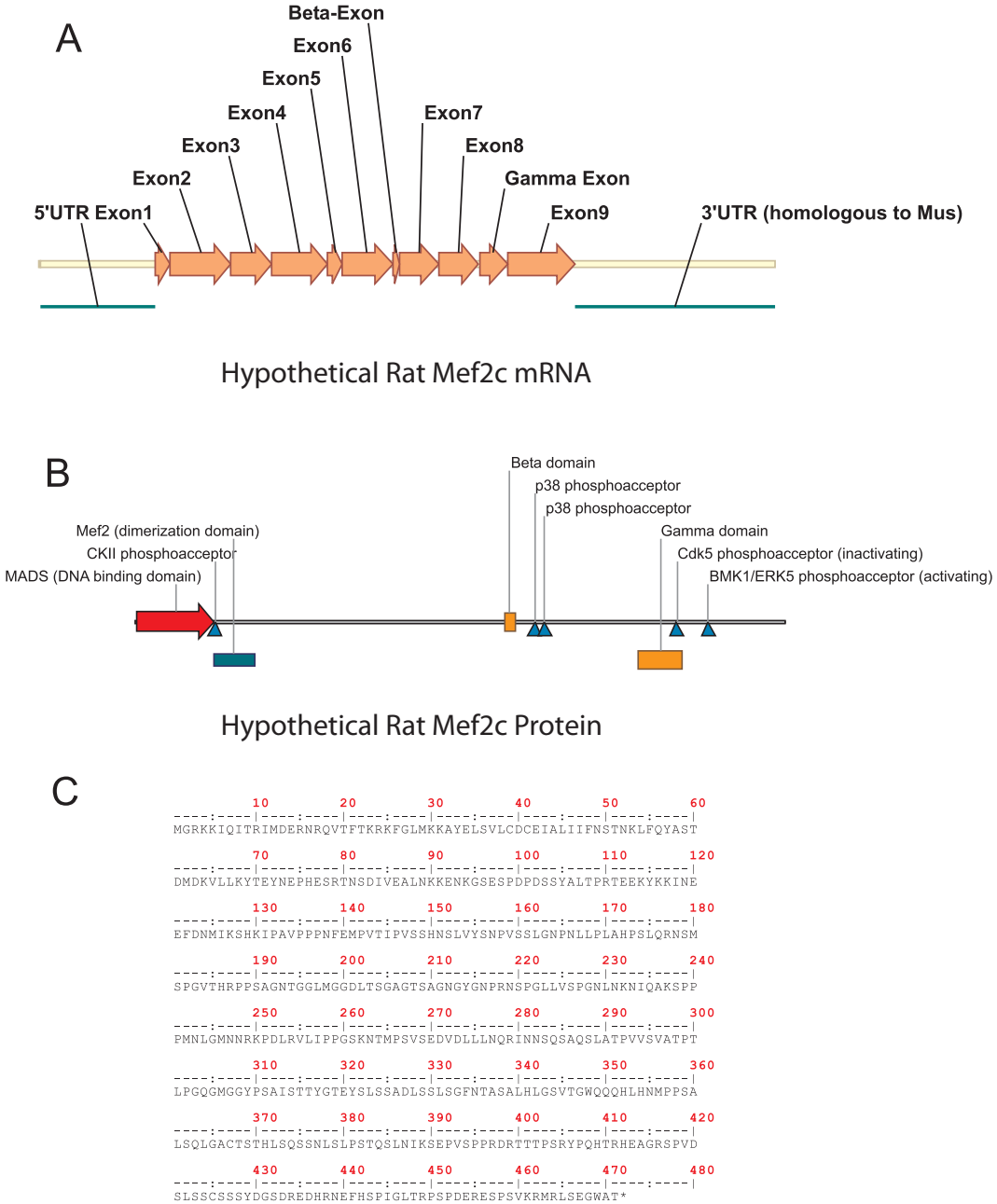

Figure 12: ***Reconstructed Hypothetical mRNA and Protein diagrams for Rat Mef2c***. As a result of the corrected inversion at 2q11, two regions of homology to mouse and human Mef2c needed to be combined to produce a new hypothetical transcript. Since no sequence overlap was available, the two fragments were joined in the appropriate orientation and key features of the new transcript were annotated based on homology to mouse and human. A) Known mouse exon-intron boundaries, as well as estimated 5' and 3' untranslated regions were mapped to the rat genomic region. B) The mRNA sequence was translated into a putative Mef2c protein sequence and key residues and domains were identified based on homology to the human Mef2c. C) The resulting full-length protein sequence is presented.

Figure 13: ***Mef2c conservation and estimation of reconstruction accuracy.*** As confirmation that the hypothetical rat Mef2C gene was constructed correctly, the protein sequence was aligned to 5 other Mef2C proteins from different species. While a few of these additional RefSeq proteins did not include known alternative splice sites (β and γ) resulting in regions of poor homology, the protein as a whole appears accurate. Interestingly, with the exception of a single point mutation in the zebrafish sequence, the MADS-Mef2 domain (black) is perfectly conserved.

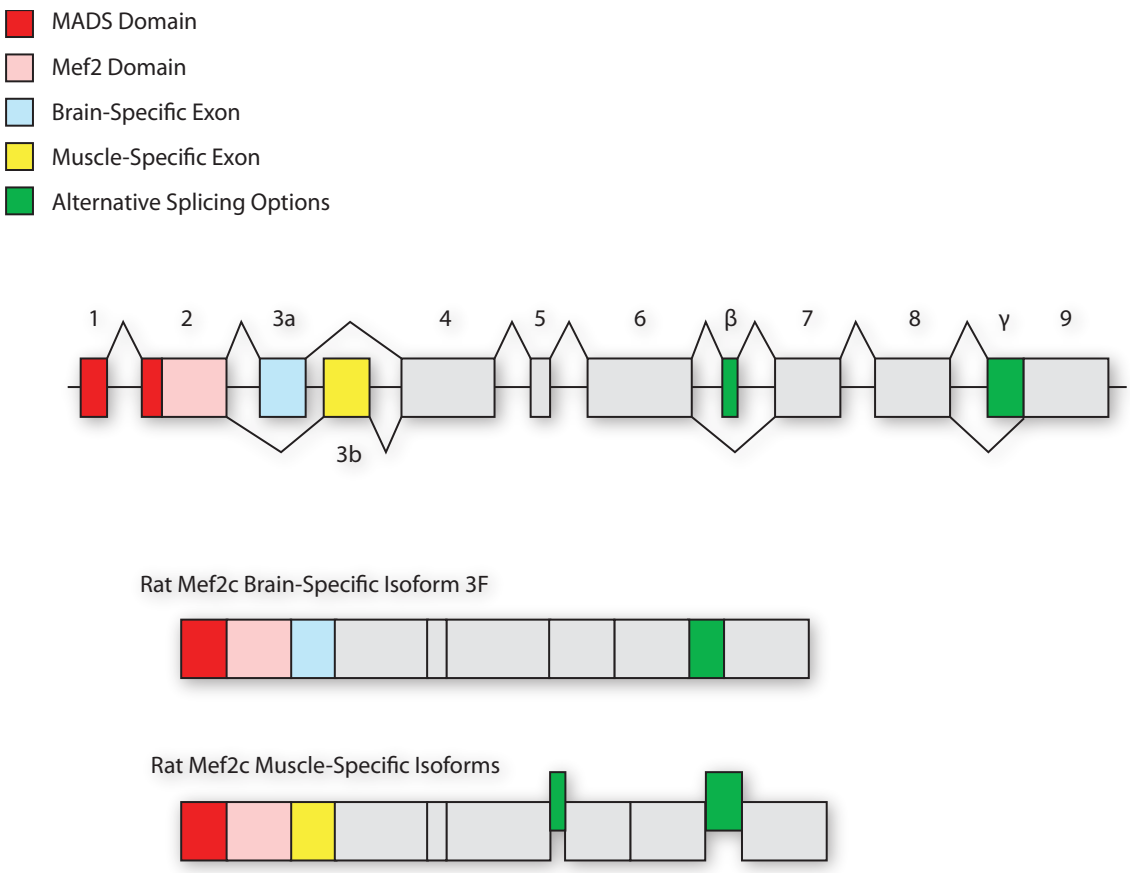

Figure 14: ***Splice variants of the mammalian Mef2c transcript.*** We have just described a previously uncharacterized rat homolog of the mammalian Mef2c gene. Through direct mapping to genome, and based on homology to other conserved mammalian Mef2c genes, we propose this model for available alternative splicing variants. Most interesting to note are the two alternative exons (β and γ) and the tissue-specific exon 3. Using undifferentiated L2.2 cDNA as template DNA, we successfully amplified and cloned the rat Mef2c brain-specific isoform 3F presented above. Interestingly, multiple clones were obtained for Mef2c from the L2.2 cDNA library and all were shown to contain the brain-specific isoform 3F, suggesting this is the exclusive form of Mef2C in neural stem cells. (*Mef2c cloned in conjunction with Dr. Cynthia Camarillo*).

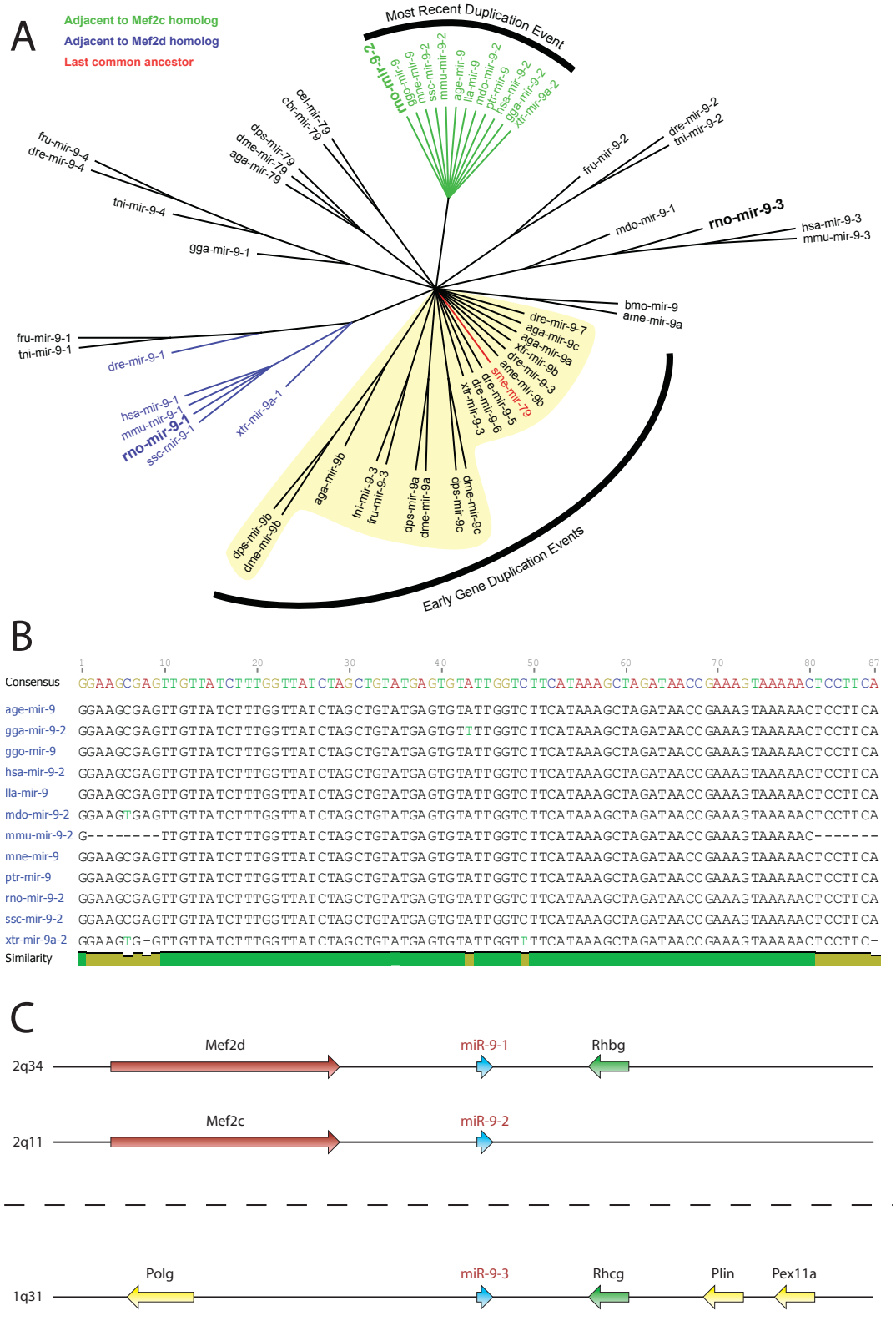

Figure 15: ***Evolutionary expansion of the miR-9 family of microRNAs.*** A) Cladogram describing the differences between all members of the miR-9 gene family (MIPF0000014) across all species present in the Sanger microRNA registry v9.0. The close proximity of rno-miR-9-1 to miR-9 genes in certain teleost fishes as well as in *X. laevis* indicates that this is most likely the ancestral miR-9 gene among mammals. Gene duplication events produce the branches containing miR-9-3 and miR-9-2, both of which carry

neighboring genes with them during duplication. The Mef2c isoform, located adjacent to rno-miR-9-2 is one of these genes carried over. The lack of divergence among the miR-9-2 isoforms indicates that this may have been the most recent duplication event. B) Alignment of the mammalian miR-9-2 precursors demonstrates how few changes have been introduced in this relatively new gene. Additionally, sequences corresponding to the mature microRNA sequence and the complementary sequence on the opposite arm are completely devoid of mutations indicating positive selection. C) Mature rat miR-9 can be derived from multiple potential genomic loci, two of which are immediately adjacent to known Mef2 genes. miR-9-1 and miR-9-2 are both located on chromosome 2 in the rat, and are immediately adjacent to two distinct Mef2 gene paralogs. miR-9-3 is relegated to chromosome 1 and does not have a neighboring Mef2 gene, but does however, retain a member of the Rhesus blood group associated family as seen next to miR-9-1. While no identified Mef2 isoform is near miR-9-3, the region contains numerous ESTs derived from embryonic brain cDNA libraries (not shown).

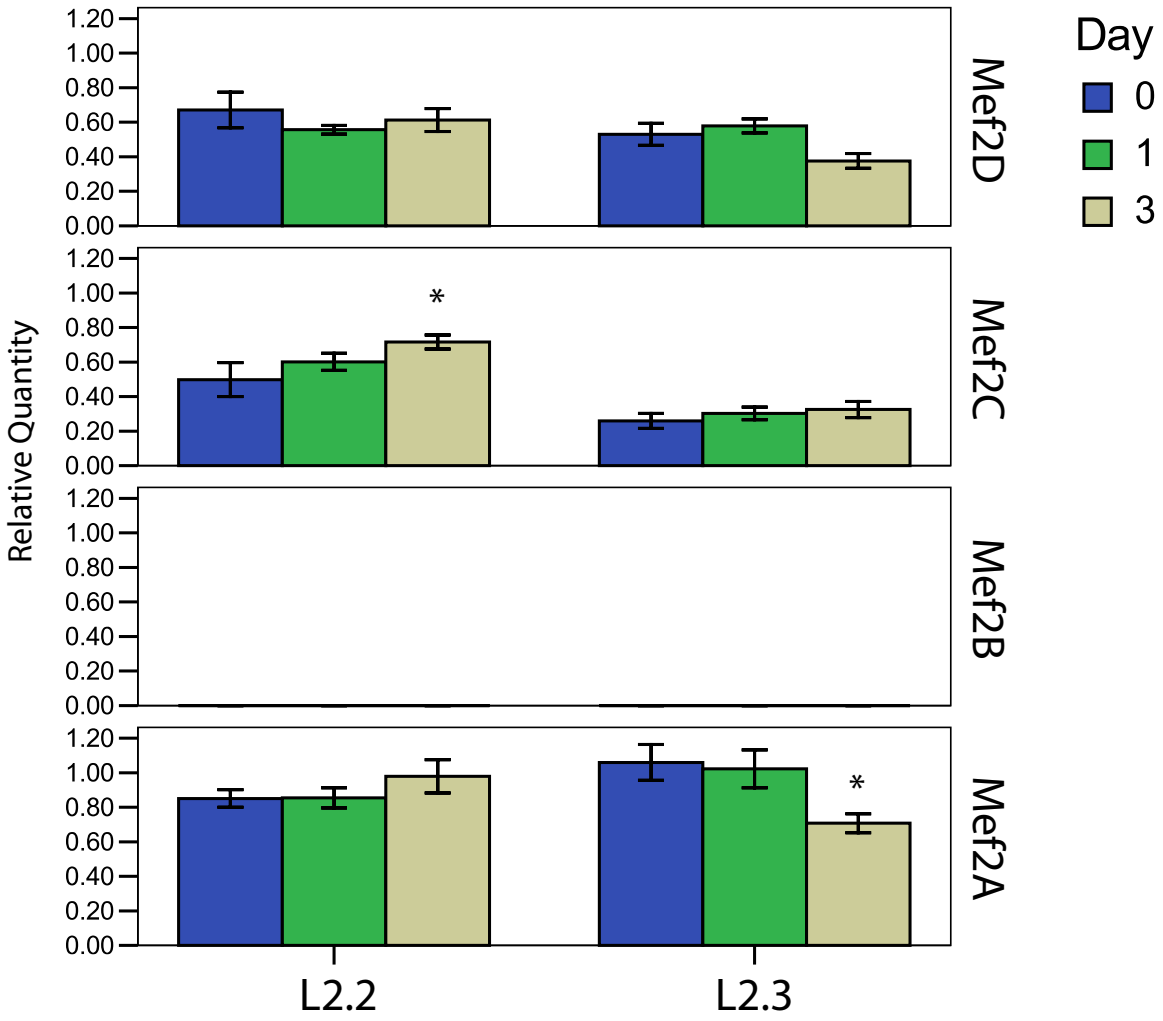

Figure 16: ***qRT-PCR analysis of rat Mef2 gene isoforms.***qRT-PCR analysis of each of the four Mef2 isoforms during differentiation of L2.2 and L2.3 cells. Comparisons were determined to be significantly differentvia Student's T-test with $p<0.05$ and no multiple testing correction. As anticipated, Mef2C is the only isoform that is significantly increased during neurogenesis in the L2.2 cells. Mef2A and Mef2D transcripts are both decreasing during differentiation of L2.3 cells. Mef2B transcripts were not detected, however primers were able to amplify a genomic DNA positive control.

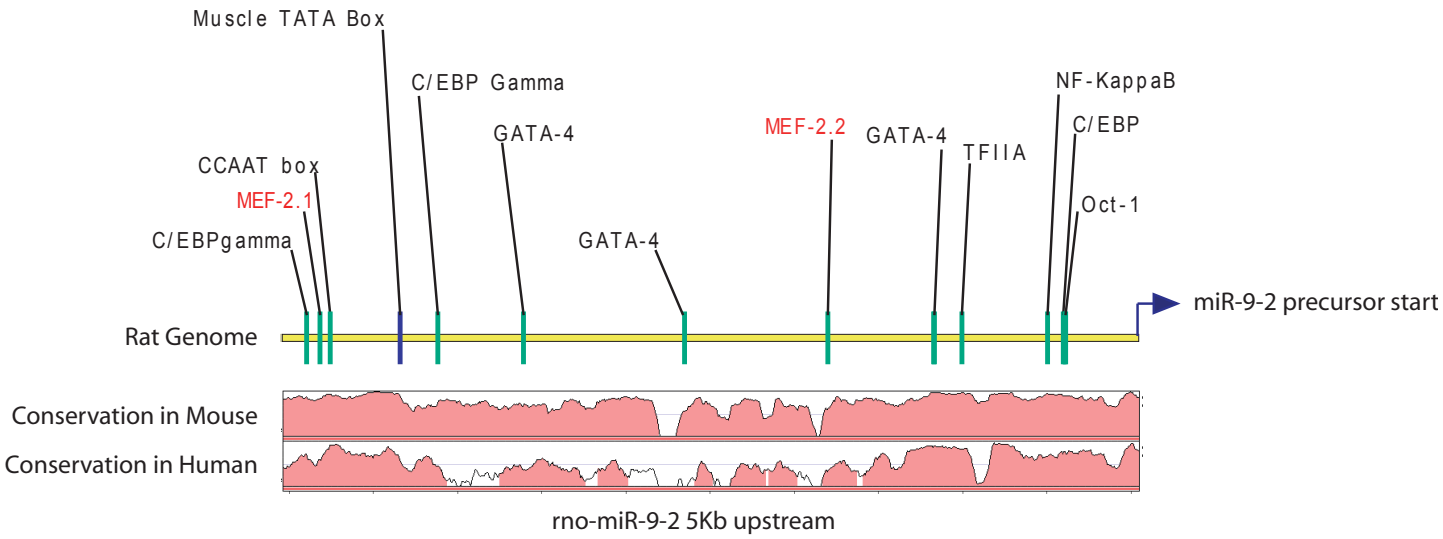

Figure 17: ***Transcription factor binding site predictions and sequence conservation of 5Kb upstream of miR-9-2.*** A 5Kb region upstream of the precursor sequence for miR-9-2 in rat was analyzed for potential transcription factor binding sites. Results are shown here including two putative Mef2 binding sites as well as a well defined TATA box and other minimal promoter elements. Conservation plots between mouse and human demonstrate that the majority of these key TFBS occur in regions of strong conservation (>90%).

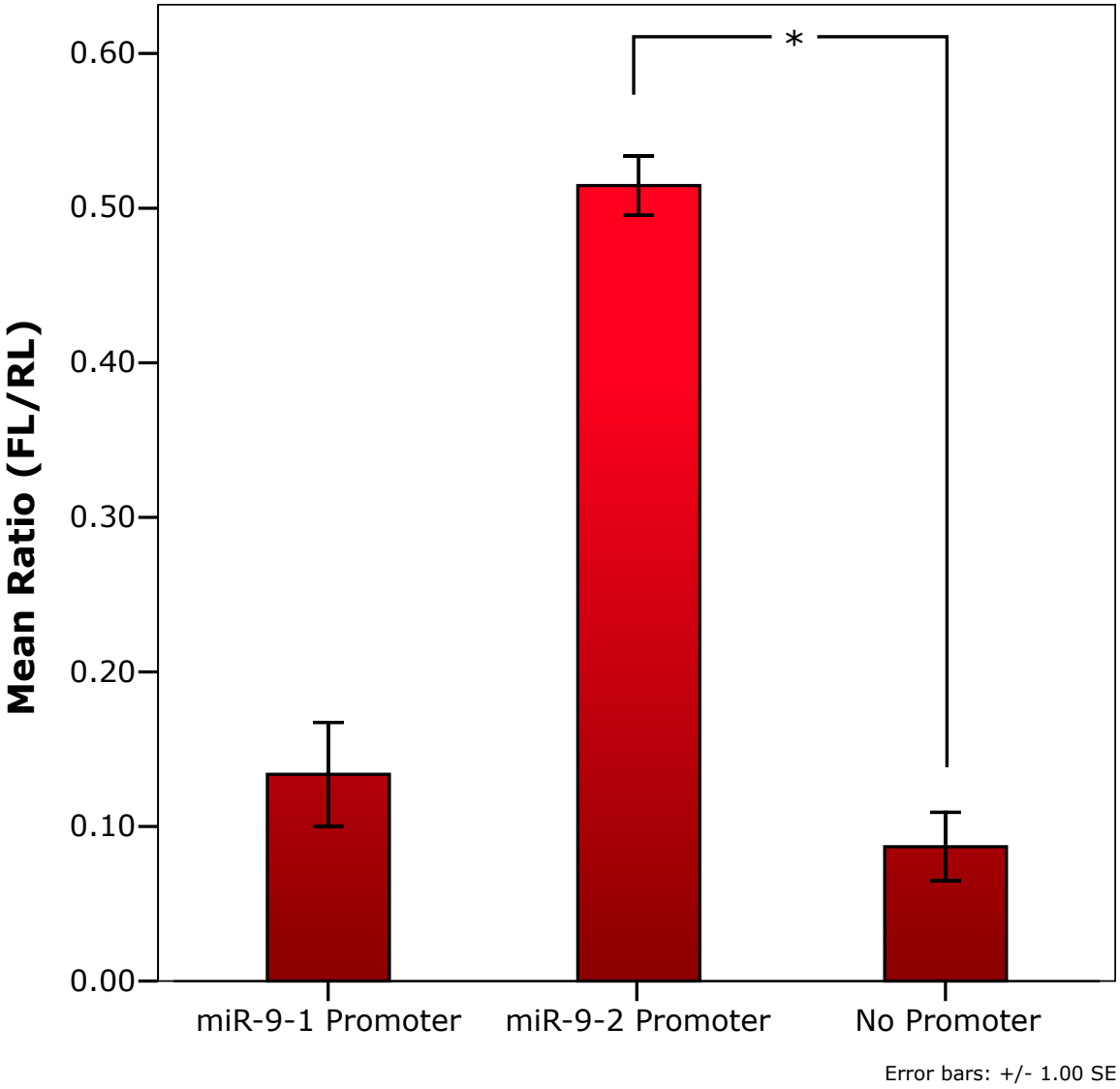

Figure 18: ***Luciferase reporter plasmids containing 5Kb upstream regions of two miR-9 genes demonstrate transcriptional activity of miR-9-2 only.*** Reporterplasmids were transfected, alongside Renilla control vectors, into L2.2 cells. Cells were grown in differentiation medium (FGF^-). After 48 hours, cells were harvested and luminescence was measured to determine transcriptional activation. Comparisons to the promoterless pGL4.10 were determined to be significant via Student's T-test with $p<0.05$ and no multiple testing corrections. While the 5Kb region from miR-9-1 expresses a moderate amount of luciferase, the levels were not significant as compared to the promoter-less pGL4.10. Alternatively, the 5Kb region from miR-9-2 was able to significantly increase cellular levels of luciferase, indicating active transcription in L2.2 cells.

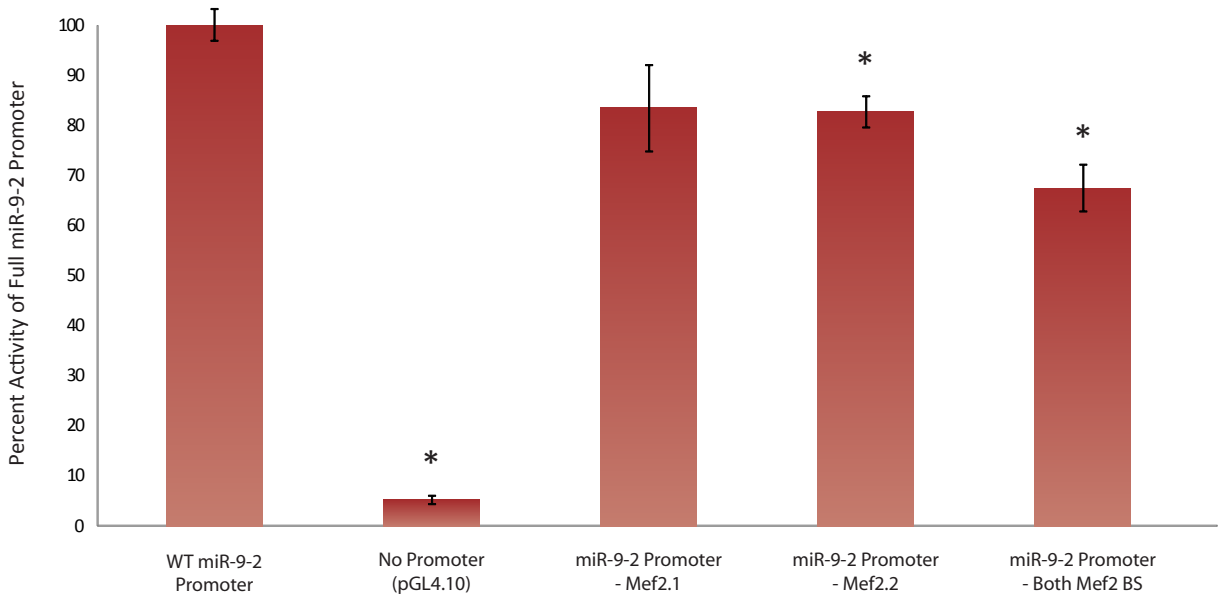

Figure 19:*Deletion of Mef2 binding sites in the upstream regulatory region diminishes transcription activity of the miR-9-2 gene locus.* A 5Kb upstream sequence of the rat miR-9-2 gene was cloned into the promoter-less pGL4.10 luciferase reporter vector. Two highly conserved Mef2 binding sites (Mef2.1 and Mef2.2 respectively) were deleted in separate plasmids and a double-deletion plasmid was constructed as well. Plasmids were co-transfected with a renillatransfection control reporter into undifferentiated L2.2 cultures. pGL4.10 and the full-length 5Kb upstream sequence were transfected as controls. Firefly luciferase/ renillaluciferase ratios (FL/RL) were determined using the dual-luciferase assay (Promega). Ratios are expressed here as percentage of full-length miR-9-2 upstream sequence activity and differences from wt promoter were determined to be significant via Student's T-test with $p<0.05$ and no multiple testing corrections. The promoter-less pGL4.10 exhibits <10% of the activity of the full-length promoter sequence. Deletion of both Mef2 binding sites individually results in an approximate 20% decrease in promoter activity. Interestingly, a combinatorial deletion demonstrated <70% of the activity of the full-length regulatory sequence, suggesting that these two binding sites are capable of affecting up to 30% of the transcriptional activity of the miR-9-2 gene.

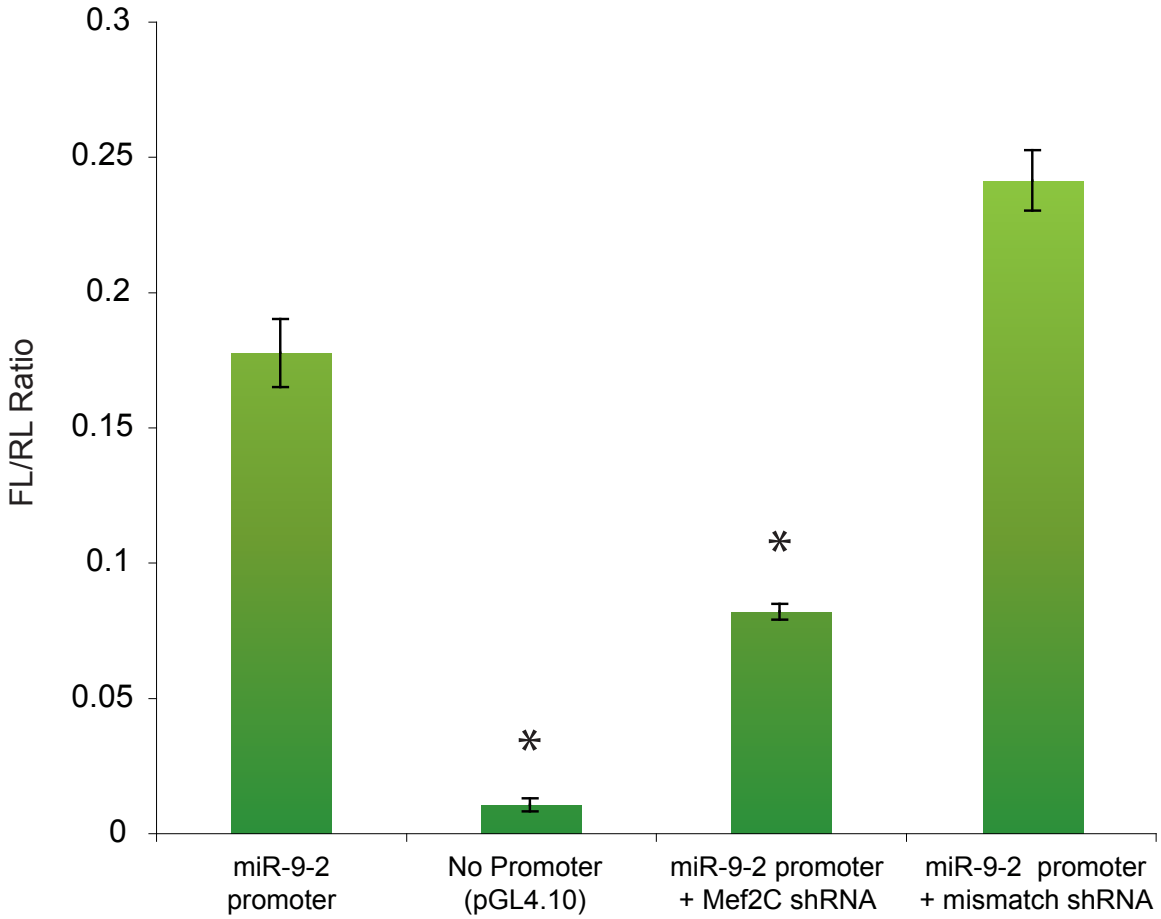

Figure 20:*Inhibition of Mef2C in neural precursors leads to a decrease in available miR-9-2.* FL/RL ratios were determined for replicate cultures of differentiated L2.2 cells with or without Mef2c shRNAs. For this assay, the firefly luciferase was produced by a 5Kb upstream regulatory region of rno-mir-9-2. We have previously demonstrated the requirement for the Mef2 binding sites for full transcriptional activation during neuronal differentiation. Significance in the observed difference from the wt miR-9-2 promoter was determined by Student's T-test with $p<0.05$ and no multiple testing corrections. In this assay,a significant decrease in the abundance of luciferase was detected in the cultures electroporated with inhibitors for Mef2c. This reduction was abolished with a control hairpin with a specific mismatch for the Mef2c protein. These results demonstrate the requirement of Mef2c for full activity of the miR-9-2 promoter.

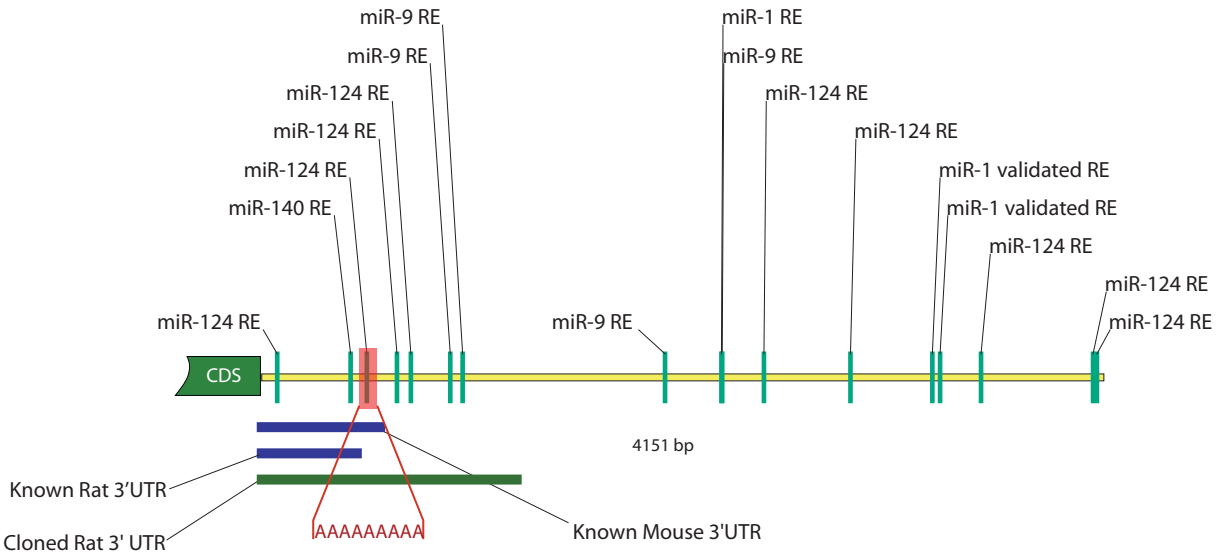

Figure 21: ***The 3'UTR of HDAC4, a known Mef2 co-repressor, is predicted to be targeted by miR-9 and miR-124a in addition to several other microRNAs.***A graphical representation of the 3'UTR of HDAC4 is presented. The full length sequence (yellow) corresponds to the validated human 3'UTR while the shorter blue sequences identify the length of 3'UTR sequences annotated in the public database for mouse and rat respectively. A genomically encoded stretch of adenosine residues sits immediately downstream of the abbreviated rodent 3'UTRs which may have acted as a priming site for dT primed assays. We were able to successfully amplify a 1250bp region of the rat 3'UTR (green) from random primed L2.2 cDNA and clone this region into the luciferase reporter vector pmiR-Report. Numerous brain-enriched microRNA are predicted to target this 3'UTR, several of which we have validated.

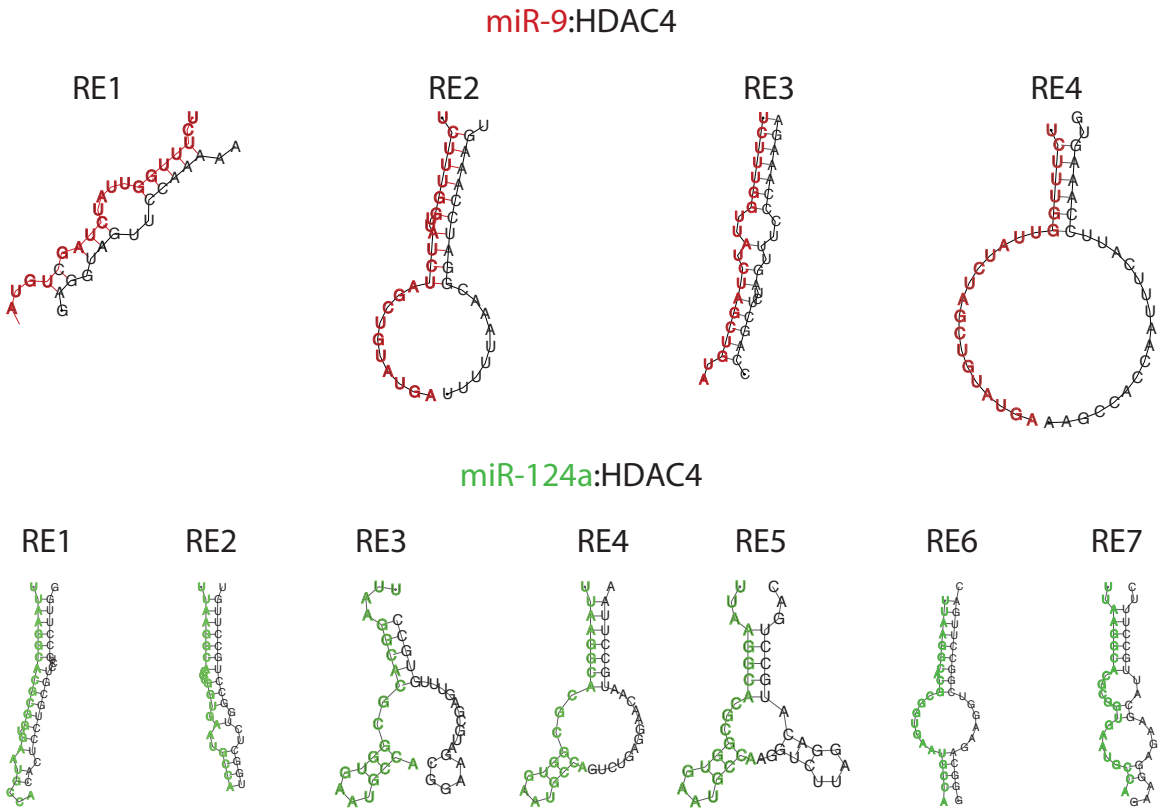

Figure 22: ***Alignments between microRNAs and predicted targeting sequences from the 3'UTR of HDAC4 show that some predicted MREsmay be more likely than others.*** Alignments of miR-9 and miR-124a to their respective response elements in the 3'UTR of the rat HDAC4 gene. While not explicitly predictive of an interaction, these alignments allow us to determine which MREs have a greater potential interaction with their corresponding microRNAs. For example, the tight interaction between miR-9 and RE1 and RE3 suggest that these two predicted MREs may in fact be actual targeting sites. Similar strength of binding is seen at RE1, RE2, RE6 and RE7 in the predicted miR-124a binding sites.

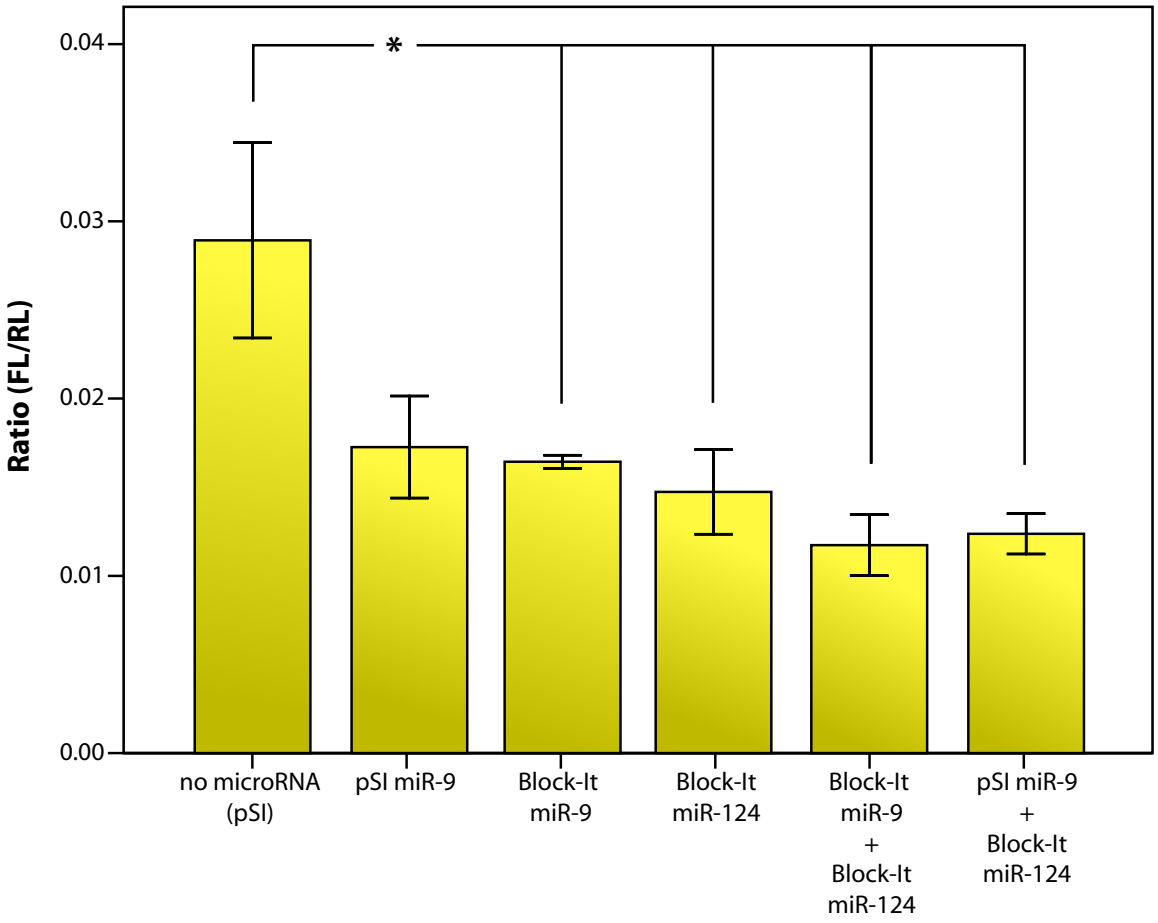

Figure 23: ***HDAC4 3'UTR is targeted for repression by the regulated microRNAs, miR-9 and miR-124a.*** Co-transfection of the HDAC4 3'UTR luciferase reporter plasmid with various combinations of miR-9 and miR-124a expression vectors demonstrates a significant regulatory effect on the expression of luciferase. Signficantly reduced expression levels were determined via on-tailed Student's T-test with $p<0.05$ and no multiple testing corrections. Individual expression of both miR-9 and miR-124a is sufficient to significantly alter luciferase expression as compared to an empty expression vector (pSI). Interestingly, a combinatorial effect is apparent when both miR-9 and miR-124a are expressed together, suggesting the two may act cooperatively to reduce the availability of HDAC4 transcripts in vivo.

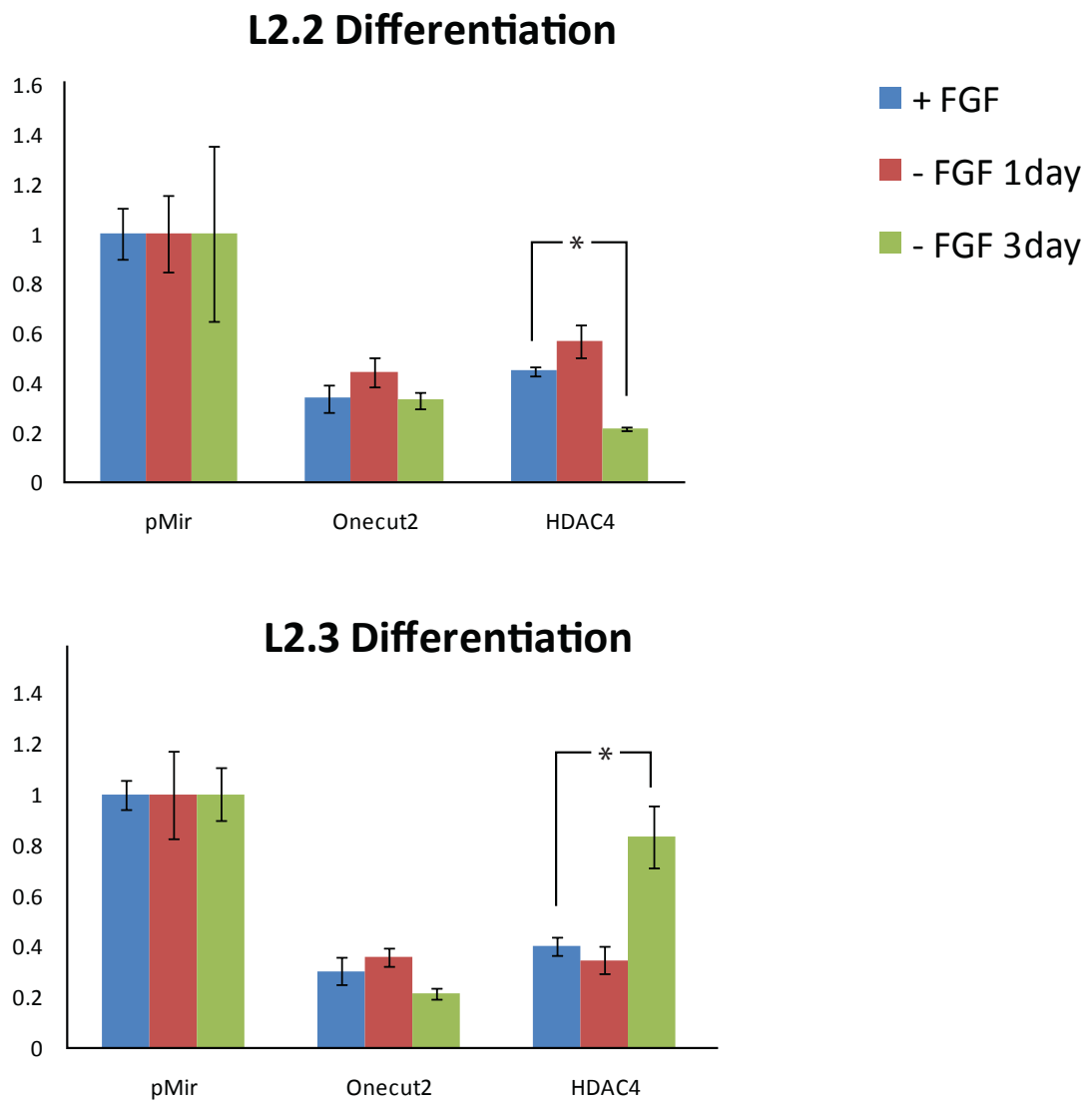

Figure 24:*HDAC4 is regulated via its 3'UTR during differentiation of neural precursor cells.* A portion of the 3'UTR of rat HDAC4 was cloned into the luciferase reporter vector pmiR. As a contrasting control, a similar portion of the Onecut2 gene was cloned into the same reporter. These plasmids were electroporated via 96-well shuttle (Amaxa), into replicate cultures of L2.2 and L2.3. After 0, 1, and 3 days of FGF withdrawal, cells were harvested and the levels of luciferase were measured using the 20/20n luminometer (Turner Biosystems). To correct for transfection efficiencies, the firefly luciferase levels were normalized to the renillaluciferase levels (transfected at equi-molar levels across all samples). To correct for changes in transcription rates as a result of FGF withdrawal, the FL/RL ratios were normalized to pMiR (no 3'UTR) control FL/RL levels.Signifcant differences to the time-point specific normalized values for the empty vector were determined via two-tailed Student's T-test with $p<0.05$ and no multiple testing corrections. In the neurogenic clone L2.2 we readily observe a significant reduction in firefly luciferase production by 3 days of differentiation, suggesting that the 3'UTR of HDAC4 is actively being repressed. We were excited to see that the inverse was true during the differentiation of L2.3 cells, in that luciferase levels increased by 3 days of differentiation, suggesting activation of the HDAC4 transcript via 3'UTR activity. These results confirm the presence of 3'UTR regulation of activity of HDAC4 during neural cell specification.

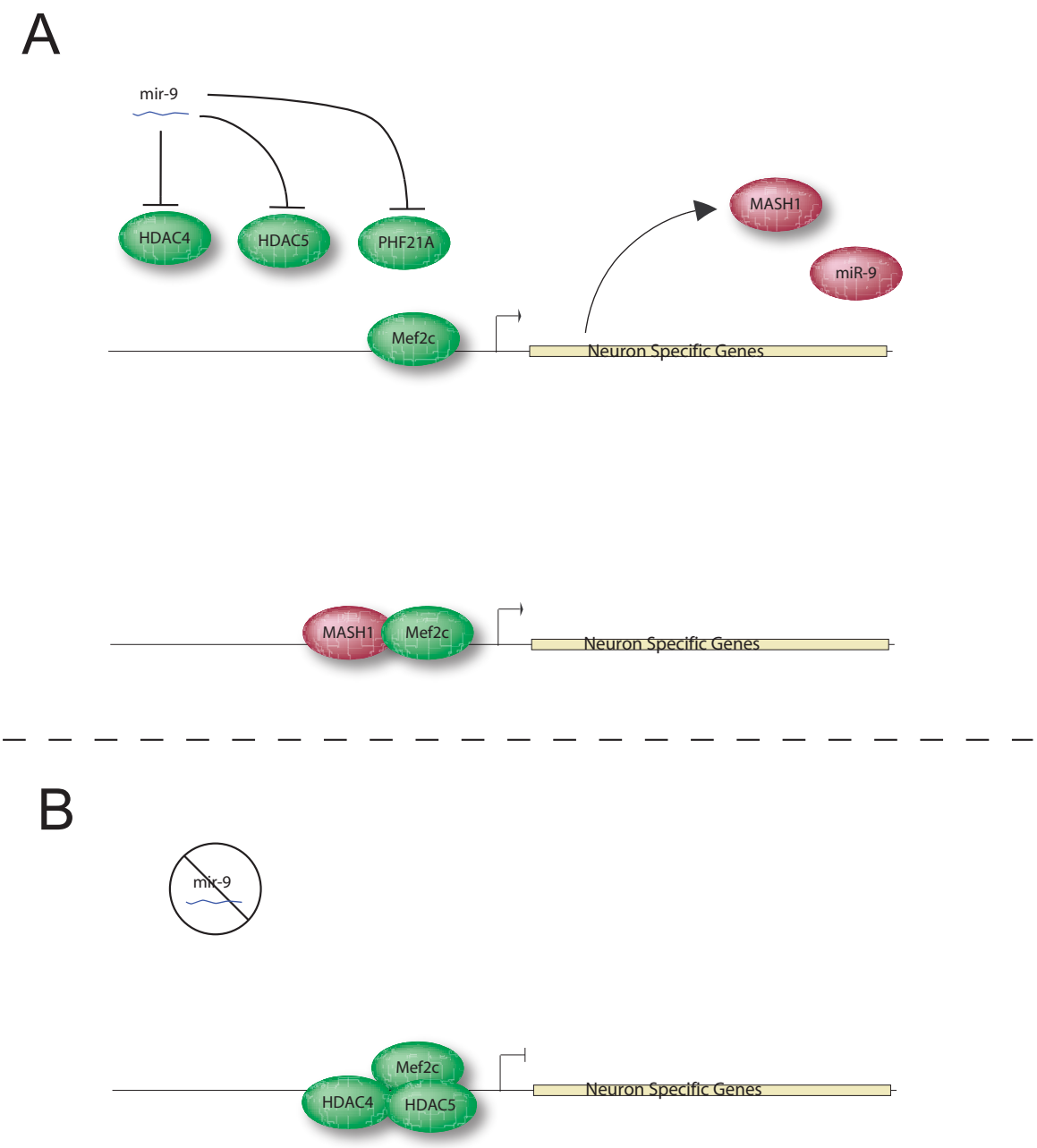

Figure 25: ***Model for miR-9/Mef2 mediated induction of neurogenesis*****.** A) In the presence of miR-9, Mef2 repressors such as HDAC4 and 5, are actively inhibited. This releases Mef2 to activate the expression of various pro-neuronal genes including Mash1. Mash1 and select other co-factors can dimerize with Mef2 to enhance is transcriptional activation activity and promoter further expression of neuron specific genes. Additionally, Mef2 would now be free to enhance the transcription of additional miR-9 thus creating a supportive feedback loop. B) In the absence of miR-9, transcriptional repressors bind to Mef2. Mef2 is still capable of binding DNA but now is actively recruiting silencing elements to its target genes. This results in limited or no expression of pro-neuronal transcripts

V. TABLES

Upstream region	*Predicted Mef2 binding sites*	*100% Core match to PWM*	*Matrix Match > 90%*
miR-9-1	1	1	1
miR-9-2	3	2	2
miR-9-3	1	0	0
miR-124a-1	1	1	0
miR-124a-2	2	2	1
miR-124a-3	0	0	0
miR-190	0	0	0
miR-34b	0	0	0
miR-142	0	0	0
miR-130b	0	0	0
miR-339	0	0	0
miR-25	0	0	0
miR-93	0	0	0
P-value	***0.017225***	***0.040859***	***0.101939***

Table 1: ***Predicted upstream Mef2 binding sites for a group of brain-enriched microRNAs and a random subset of microRNAs expressed but not regulated in differentiating L2.2 and L2.3 cells.*** Mef2 binding sites within a 5Kb upstream region of isoforms of the brain-enriched microRNAs miR-9 and miR-124 are enriched as compared to a random set of expressed microRNA upstream regions. The prevalence for Mef2 binding sites in these regions is highlighted by the sheer absence of predicted sites in the random subset.

VI. METHODS

Cell culture and differentiation

Generation of precursor clones (L2.2 and L2.3) from embryonic rat cortical cultures and their culturing conditions ws described previously (Li et al., 2004). Briefly, immortalized clones (e.g. L2.2, L2.3) were cultured overnight on laminin-coated glass coverslips in FGF2 containing serum-free medium, the medium was then removed and replaced with culture medium lacking FGF2. After maintenance for the number of days indicated, cultures were then fixed and stained with cell type specific markers.

mRNA expression analysis

L2.2 and L2.3 cells were cultured on laminin-coated 35 mm dishes in DMEM/F12 serum free medium containing FGF2 (10 ng/ml) at $3x10^5$ cells per dish. The next day, differentiation was initiated by changing to medium lacking FGF2 and including 0.5% fetal bovine serum (FBS). Triplicate cultures were harvested at day 0 (prior to FGF2 withdrawal), and 1 or 3 after differentiation. RNA was prepared from L2.2 and L2.3 cultures using the mirVana miRNA Isolation kit (Ambion/Applied Biosystems), which isolates and separates low molecular weight (LMW) from high molecular weight (HMW) RNA. 0.5 μg of HMW RNA was labeled using the NanoAmp™ RT-IVT Labeling Kit (Applied Biosystems) and hybridized to AB1700 Rat Genome Survey Microarrays following the manufacturer's protocols.

Array data were quality-assessed, aggregated, quantile-normalized, and analyzed using the ABarray Package for R (http://www.r-project.org/) and Bioconductor (http://www.bioconductor.org). Probes exhibiting a signal to noise ratio (S/N) < 3 were excluded from further analysis. A two-way ANOVA was performed on remaining probes using cell clone and time as factors. Significant probes were determined to have an

acceptable FDR of 5% using the Benjamini-Hochberg method. Significant probes were k-means clustered (k=6) to identify similar expression patterns. Cluster centers, along with the hierarchically clustered heatmap, were plotted using R. Gene-level interpretation of probe data was determined using annotation previously described (Goff et al., 2007).

AB1700 probe annotation

Sequences for 26,857 probes from the ABI 1700 Rat Genome Survey Array were provided by Applied Biosystems (Foster City, CA). All available curated rat transcripts were obtained from the following sources: NCBI RefSeq (http://www.ncnbi.nlm.nih.gov/projects/RefSeq/), NCBI Entrez Nucleotide DB (http://www.ncbi.nlm.nih.gov/entrez) (including dbEST, GenBank, and various other non-RefSeq rat transcripts), and Ensembl release 42 (http://www.ensembl.org/Rattus_norvegicus/). Rat genomic sequences from both the reference genome assembly (Gibbs et al., 2004) and the Celera assembly (Kerlavage et al., 2002) from the public repository at NCBI. All sequences and associated annotation were stored locally on our LAMP model bioinformatics server. Each ~60mer probe sequence was aligned to all available transcripts across all public sources using NCBI BLAST (Altschul et al., 1990) running on a dedicated Linux server. Probes were aligned using a minimum expect-value of 1.0×10^{-6}.

CART transcription factor binding site analysis

As described in (Goff et al., 2007), clusters were interpreted using potential regulatory sequences. Available 1Kb regions upstream of the 1,337 significant probes were searched for putative transcription factor binding sites (TFBS) using the Match Algorithm, and associated vertebrate position weight matrices (PWM) included in release 10.4 of the Transfac database (Biobase Corporation, Beverly, MA).

Cluster labels were treated as class labels for the purpose of detecting discriminating combinations of predicted TFBS. We explored different scoring mechanisms for the presence of a motif, and the best results were obtained using a measure that incorporates the number of hits as well as the score of a top hit. That is, the top 10 scores for each motif were recorded and a total score was obtained as the max(score)*range(10 scores). This measure is large if the top score is large and/or there are many moderate (multiple) hits in the promoter for this motif. Finally, to find the discriminating TFBS we fit a classification tree to the data using the CART software in R (Breiman, 1984). The CART method selects a sequence of TFBS that optimally separate the gene classes. The first split in the tree is thus the single TFBS (TF1) that best separates the gene classes. The next two splits find the two TFBS that further improves the gene class separation: TF2(1+) best separates the gene classes with TF1 present, and TF2(1-) best separates the gene classes with TF1 absent. The tree is grown until adding more splits until no further improvement can be made. To protect against over-fitting we use 10-fold cross-validation. We repeatedly randomly split the data into a training set (90%) and a test set (10%). We prune back the tree to obtain the tree with the lowest gene class prediction error rate on the test set.

NCode microRNA microarrays

LMW RNA was obtained from the same tissue samples for which HMW RNA was analyzed using the AB1700 array platform. Three replicates each of L2.2 and L2.3 NSCs at 0, 1, and 3 days post-FGF withdrawal were labeled using the Array 900miRNA direct labeling kit (Genisphere, Hatfield, PA) according to manufacturer's recommendations. 100 ng of LMW RNA was used as input for the labeling reaction. Labeled RNAs were hybridzed to NCode v2.0 arrays (Invitrogen, Carlsbad, CA) at 54˚C overnight.

Hybridized arrays were scanned, aligned, and median spot intensities were obtained using a GenePix 4000B scanner (Molecular Devices, Inc.)

The data were quantile-normalized and replicate spots were aggregated. Probes were selected as significant from a two-way ANOVA using both cell line and time as parameters. 39 significant microRNAs were selected as $p<0.05$ with an estimated 10% false discovery rate (FDR).

Cross correlation of mRNA and microRNA expression

The microRNA/mRNA data were jointly examined by computing pairwise Pearson correlations between the normalized fold-change levels, calculated as relative to the L2.3 0-day timepoint. Heatmap displays were constructed from these correlations using the gplots and marray packages in BioConductor (http://www.bioconductor.org/) and R (http://www.r-project.org).

qRT-PCR

Primers for all mRNA and pre-miRNA qRT-PCR were designed using Primer Express 2.0 (Applied Biosystems Inc., Foster City, CA). Mature miRNA primers for NCode microRNA qRT-PCR (Invitrogen Inc., Carlsbad, CA) were designed according to manufacturer's recommendations. For each condition 2μg of total RNA was used as input for first-strand cDNA synthesis. Template cDNA was amplified using Power SYBR Master Mix (Applied Biosystems) and designed primers at 50nM final concentration. qRT-PCR assays were performed on either the AB7900HT or the AB7500 Fast System. qRT-PCR data were analyzed in either R (http://www.r-project.org) or Excel.

Transfections

miR-9 and/or miR-124a expression plasmids (250ng each), along with the HDAC4 3'UTR reporter vector (500ng) and the transfection control vector pRL (250ng),

were transfected into HeLa cells. All transfections into HeLa cells used Lipofectamine 2000 according to the supplier's recommendations. Transfection into L2.2 and L2.3 NSC clones was effected via electroporation using either the Amaxa nucleofector, or the Amaxa 96-well shuttle system (Amaxa Biosystems, Gaithersburg, MD). Those transfections occurring in the nucleofector system were conducted using the Rat Neuron Nucleofector Kit (VPG-1003) using standard protocols. Observed efficiences differed between L2.2 and L2.3 clones but remained consistently between 40-60%. Transfections employing the 96-well shuttle system were conducted using the Rat Neuron 96-well Nucleofector Kit (VHPG-1003). Observed transfection efficiencies using the 96-well shuttle system were consistently >95% for both the L2.2 and L2.3 NSCs.

miR-9-2 promoter cloning and analysis

A 5Kb upstream region was arbitrarily selected from the start site of the known rat miR-9-2 precursor sequence. Primers were designed to allow for amplification and directional cloning into the pGL4.10 reporter vector (Promega). The upstream sequence was scanned for known core promoter elements and transcription factor binding sites using the Match algorithm provided with the Transfac database (Wingender et al., 1996). As a control, a 5Kb region directly upstream of the rat miR-9-1 precursor was also amplified and cloned into the pGL4.10 promoter. Upstream sequence and plasmid maps are available upon request.

Mef2C and HDAC4 3'UTR isolation and cloning

The predicted rat Mef2C mRNA was amplified using a forward primer consisting of an introduced NheI restriction site and the first 18 bases of the predicted mRNA. The reverse primer consisted of a MluI restriction site, a stop codon, the reverse complement of the FLAG antigen sequence (N-DYKDDDDK-C) and the reverse complement of the

last 24 bases of the predicted mRNA sequence. The resulting amplicon was 1.4Kb in length, as hypothesized, contained a C-terminal FLAG epitope tag, and allowed for directional cloning into the expression vector pSI. The SV40-driven Mef2C-FLAG tagged protein was observed in transfected HeLa, HEK293, L2.2, and L2.3 cells, and is detectable with both the M2-anti-FLAG antibody (Sigma) and our custom polyclonal anti-Mef2C antibody.

A fragment of the rat HDAC4 3'UTR was amplified using forward primers with a 5' end adjacent to the stop codon at the end of the HDAC4 CDS. Several reverse primers were evaluated to determine the maximal length of available 3'UTR, but the reverse primer producing the longest possible HDAC4 3'UTR resulted in an amplicons of ~1.2Kb. Random-primed L2.2 cDNA was used as an amplification template. The resulting amplicon was cloned into the 3'UTR-less luciferase reporter vector pMiR-Report (Ambion). Predicted microRNA response elements in the HDAC4 3'UTR were identified using PicTar microRNA target predictions (Krek et al., 2005), and free-energy of hybridization for predicted miRNA:HDAC4-3'UTR interactions was determined using RNAfold (Hofacker et al., 2002).

Luciferase assays

Target plasmids were cloned into either the promoterless pGL4.10 firefly luciferase reporter vector (Promega) or the 3'UTR-less pmiR-Report firefly luciferase reporter vector (Ambion). Vector sequences were provided from the suppliers. Construct sequences are available upon request. In most experimental conditions, plasmids were co-transfected with the Renilla luciferase control reporter vector pRL-SV40 (Promega) in a fixed concentration (0.5μg) to normalize for differences in transfection efficiencies. Cells were maintained in culture for at least 24 hours after transfection and then processed using the Dual-Luciferase Reporter Assay System (Promega) according to

manufacturer's recommendations. Luciferase levels were quantitated using a $20/20^{n}$ luminometer (Turner Biosystems). Data were expressed as the ratio of firefly luciferase (FL) to *Renilla* luciferase (RL) to normalize for differences in transfection efficiencies.

VII. REFERENCES

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VIII. CURRICULUM VITA

Education

Rutgers University, Piscataway, NJ
Ph.D. Cell and Developmental Biology **2008**

The College of New Jersey, Ewing, NJ
B.S. Biology **2001**

Teaching Experience

Rutgers University, Piscataway, NJ
Teaching Assistant – to Dr. Bruce Babiarz in "Histology"
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Publications

Li, H., Han, Y., Bi, C., Davila, J., Goff, L.A., Thompson, K., Swerdel, M., Camarillo, C., Ricupero, C.L., Hart, R.P., Plummer, M.R., Grumet, M., (Submitted) *Functional differentiation of an embryonic cortical interneuron precursor clone is promoted by co-culture with radial glial cells.*, Jthenal of Neuroscience

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