MIR-9 TARGETS OC2 IN PROLIFERATING AND DIFFERENTIATING

NEURAL STEM CELLS

by

JORGE DAVILA

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ABSTRACT OF THE DISSERTATION

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MicroRNAs are key regulators of biological processes. In this thesis we identify mir-9 as a critical regulator during NSC proliferation and neuronal differentiation. Interestingly the role of mir-9 in NSCs differs depending on our experimental model.   In a rat multipotential NSC, exogenous expression of mir-9 alone can enhance their neurogenic capacity. Meanwhile, in human NSCs mir-9 plays a role in NSC maintenance. These results either suggest that the role of mir-9 is not conserved across species or most likely that mir-9 can exert different cellular functions depending on the cellular context. In H1 NSCs subpopulations of cells expressing different combinations of proliferation and/or differentiation markers in cultures of neuronal differentiating cells were identified. The combined expression of differentiating and proliferating markers is a clear indication that differentiation is a "fluid" process that requires multiple overlapping steps to reach a specific phenotype. In addition, we identified OC2 as an anti-neuronal gene. Its mRNA is associated with the RISC complex during NSC neuronal differentiation where mir-9 serves to reduce OC2 protein output. These data together show that mir-9 serves to

regulate NSCs in a context dependent manner and that it can canalyze neuronal differentiation by inhibiting genes that would prevent or retard neuronal differentiation.

PREFACE

I would like to state that this thesis represents the completion of a series of scientific endeavors that were begun when I started graduate school, but that would not be correct. Scientific studies are never truly finished, the acquisition of data most of the time only leads to new and more interesting questions. One is taught to do science by following the scientific method: create a hypothesis and then develop and perform a series of experiments to prove or disprove the hypothesis. But the reality is that biological research is not a linear progression of events. Most experiments will lead to several bigger questions that divert from the original goal of the project. The work presented in this thesis is just a portion of the labor I have accomplished over the last few years. It represents experiments that were independently designed and implemented by myself. In addition, I have worked on several other collaborative projects while in graduate school.

The general theme throughout my graduate career has been to decipher the roles of microRNAs in different cellular contexts. During my tenure in the Hart lab I gained expertise which has allowed me to collaborate closely with several groups. These collaborations have lead to co-authorships on several publications. In this preface I will briefly describe a few projects that I have participated in.

One of the first projects I was involved with when I started in the Hart lab entailed mRNA expression profiling studies of a series of rat NSC clones via microarray analysis. Dr. Hedong Li was responsible for the isolation several rat NSC clones including the

neuronal restricted precursor, L2.2, and, the multipotential NSC, L2.3. Both of these clones were utilized during the course of this thesis. In the interest of defining both L2.2 and L2.3 as valid models 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 gene expression assays that ultimately were important for the identification of L2.2 as a specific interneuron precursor (Li et al., 2008a). These data are referenced within my thesis.

The microarray data sets that I performed in the above project were also used in an additional study in which we performed a bioinformatic analysis of NSC differentiation (Goff et al., 2007). In this study, AB1700 Rat Genome Survey arrays (Applied Biosystems, Foster City, CA) were employed. 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, Dr. Loyal Goff re-mapped all of the probes on this array 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.

In conjunction with members from the Rutgers Stem Cell Research Center, we used Solid deep sequencing of small RNAs from Ago2 RNA immunoprecipitations to identify novel miRNAs expressed in human ESCs, IPSCs and NSCs (Goff et al., 2009). This study mixed a combination of bioinformatic and biochemical assays to identify 146

novel miRNAs.   For this publication I optimized and performed the Ago RNA immunoprecipitations as optimized the miRNA qPCR validation assays.   I also contributed intellectually by helping with the experimental design and data interpretation. In this study we identified specific groupings of miRNAs which expression differed between iPSCs and hESCs. These observations were further described in a review that I co-authored (Lakshmipathy et al., 2010).

In a more recent collaboration with the Schachner lab, I worked closely with Young Mi Yu in dissecting the role of mir-133b during the spinal cord regeneration in *D. renio*. Zebrafish have the endogenous capacity to regenerate their spinal cords after injury. In this study we identified mir-133b as one of the key regulators of this process. In these studies I helped Young Mi with experimental designed and wrote a significant portion of the final manuscript.

Besides these projects there are several other collaborations which are ongoing investigations regarding the roles of miRNAs in different cellular context that have not been published yet. In my opinion collaborations such as the ones stated are what make a scientific career entertaining and satisfying. The knowledge that every day I possibly will be working on something completely novel is the driving force that makes me continue this line of work.

ACKNOWLEDGEMENTS AND DEDICATION

This thesis would not have been possible without the support, encouragement and cooperation of multiple people throughout my graduate career. During my years with the Hart group, lab members have become more than just colleagues. They have become friends and an extended family. They have been there to help and encourage me during stressful situations and moments of self doubt. I would first like to thank my advisor and mentor Dr. Ronald Hart. I will be eternally grateful for the opportunity that Dr. Hart gave me to take this scientific endeavor under his mentorship. I can guaranty that this has not been an easy journey for either of us. I would probably not have succeeded in completing my graduate studies if we're not for Dr. Hart's great patience and support.

I want to thank Chris Ricupero for the long scientific and non-scientific discussions that sometimes seemed to never end. He was a major source of entertainment and stress relief during the course of my graduate studies. I will definitely miss his eclectic musical taste. I must say that Chris has been of great help with the rat NSC studies; always helping me with culturing of the cells and optimizing protocols such as the FACS analysis. Chris has always been there when I needed anything in or out of the lab. He has become a good friend that I will miss having across the bench from me.

I also want to thank Dr. Jennifer Moore "The Stem Cell Master" who essentially taught me everything I have learned regarding handling and maintenance of the hESCs. Without her help and expertise I would probably still be struggling to differentiate hESCs into NSCs. Jennifer has been very generous with her time and knowledge. She has

always gone out of her way to help me overcome any experimental obstacle that I have confronted regarding the culturing of hESCs and NSCs. I consider Jennifer a good friend and I will miss our debates that most of time made no sense but were fun to have anyway.

I want to thank Mavis Swerdel for several of the gene expression assays performed in this thesis. The reality is that Mavis is much more than a lab tech for the Hart group. She is the one that keeps the lab running smoothly in every imaginable aspect. I could not imagine the Hart lab without her. In my case, she was the person that kept me grounded during the last few years. Anytime, I needed to vent or just get away and talk she was the person I would go to. I must thank her for just being there to listen to my rants and diffusing small disagreements between lab members that could have escalated into much bigger confrontations. I will miss the long conversations with her.

Alana Toro-Ramos also helped with multiple of the assays done in this thesis specifically experiments that were performed in the human NSCs. I will forever be thankful to her for always going out of her way to lend a helping hand, be it culturing cells, doing FACS or a number of things that were not related to the lab. She has become a very good friend over the years and I wish her the best.

Finally, I want to dedicate this thesis to my parents, Jorge and Judy, who have unconditionally supported me over the years and have had to sacrifice so much for me. Love you both. I also want to dedicate this thesis to Tanya my partner, best friend and wife to be. Thanks for putting up with all my crap over the years. You are the reason that I have been able to complete this journey. Thanks. I love you.

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ABBREVIATIONS USED

Ago1   Argonaute1

Ago2   Argonaute2

bHLH   Basic Helix-Loop-Helix

BMEL   Bipotential mouse embryonic liver cells

Bps   Base pairs

cDNA   Complementary DNA

CNS   Central nervous system

Cre   Cre-recombinase

Dnmts   DNA methylation and methyltransferases

EST   Expressed sequence tag

FACS   Fluorescence-activated cell sorting

FL   Firefly luciferase

FoxA2   Forkhead box A2

GAPDH   Glyceraldehyde 3-phosphate dehydrogenase

GFAP   Glial fibrillary acidic protein

GO   Gene ontology

GRP	Glial restricted precursor
hESC	Human embryonic stem cells
HMW	High molecular weight
Hrs	Hours
IP	Immunoprecipitation
LMW	Low molecular weight
MBP	Myelin basic protein
MG	Matrigel
miRNA	MicroRNA
miRNP	miRNA associated ribonucleoprotein complexes
MRE	MiRNA response elements
mRNA	Messenger RNA
MT	Mutant
Ngn3	Neurogenin3
NINDS	National Institute of Neurological Disorders and Stroke
NRP	Neuronal restricted precursor
NSC	Neural stem cell

NSE	Neuron specific enolase
NTC	No template control
OC1	Onecut1
OC2	Onecut2
OC3	Onecut3
OPC	Oligodendrocyte precursor cells
PLP	Proteolipid protein
pri-miRNA	Primary transcripts of miRNA
REST	RE1 silencing transcription factor
RIP	RNA immunoprecipitation
RISC	RNA-induced silencing complex
RL	Renilla luciferase
RRQ	Relative relative quantity
UTR	Untranslated
VPA	Valproic acid
WT	Wild type

I.   INTRODUCTION

The number of individuals afflicted with neurological disorders in the United States continues to increase, creating a tremendous burden on the health care segment of the economy as well as great human suffering. With an increase in the aging population, neurological disorders have become a major cause of morbidity and mortality. One of the main goals of our lab is to develop potential therapeutic treatments for individuals with spinal cord injuries.   According to the National Institute of Neurological Disorders and Stroke (NINDS), there are an estimated 10,000 to 12,000 spinal cord injuries every year in the United States.   A quarter of a million Americans are currently living with a spinal cord injury. The cost of managing the care of these patients approaches $4 billion each year (http://www.ninds.nih.gov/disorders/sci/detail_sci.htm). These staggering numbers demonstrate the need for a cure. Stem cell transplantation therapy is becoming an enticing potential therapeutic strategy for spinal cord injured patients. There have been several advances in utilizing neural stem cells in spinal cord injuries. Members of a collaborating lab have shown that transplantation of radial glial cells following spinal cord contusion promotes functional recovery in rats (Hasegawa et al., 2005). The transplanted cells have the ability to migrate and form bridges across the spinal cord lesion. Furthermore, in 2010 Geron began the first clinical trials utilizing oligodendrocyte precursor cells (OPC) derived from human embryonic stem cells (hESC) for spinal cord injuries. They have shown in pre-clinical studies that these cells, when injected into the spinal cords of injured adult rats, can migrate throughout the lesion site, mature into functional oligodendrocytes that remyelinate axons and produce

neurotrophic factors resulting in improved locomotion in the treated animals (Zhang et al., 2006). These studies indicate the potential of stem cell derived therapies.

A major limiting factor to utilizing stem cells as a treatment for spinal cord injury is the uncertainty of the fate of the cells once transplanted. Stem cells have the ability to differentiate into a wide range of cell types or to continue to proliferate without differentiating into a mature phenotype (Lin and Schagat, 1997). Therefore, if we intend to harness these cells for therapeutic treatments we will need to overcome the uncertain fate these cells exhibit in their undifferentiated state. This can be done by utilizing cells that have been differentiated to a lineage specific precursor stage. To reliably obtain large quantities of pure populations of lineage restricted precursors for transplantation purposes, we must understand their internal cell mechanisms and programming, this will lead to methods for stabilizing restricted differentiation and/or promoting differentiation towards desired phenotypes.

The two predominant cell types of the brain, neurons and glia, both have potential therapeutic value in injured spinal cord. For example, directed differentiation of oligodendrocytes would be beneficial to remyelinate axons after injury. Sharp *et al.* have shown that transplantation of hESC-derived oligodendrocyte precursor cells (OPC) attenuated lesion pathogenesis and improved recovery of forelimb function in cervical injured rats (Sharp et al., 2010). This study and others similar to it have led to the current human clinical trials managed by Geron. Meanwhile, transplantation of motor neurons could serve to replace neurons that died due to the injury. Transplantation of hESC derived motor neuron progenitor cells have also been shown to enhance functional recovery of cervical injured rats (Rossi et al., 2010). Therefore understanding

the mechanisms that control lineage specific neural differentiation is critical for optimizing protocols to obtain these cells. In this thesis we identify key regulators of neural stem cell (NSC) differentiation to advance protocols for NSC transplantation.

Recent studies have begun to clarify molecular interactions involving genes such as the basic helix-loop-helix-family of transcription factor that regulates differentiation of neuronal or oligodendrocytic cell lineages and creates unique transcriptional signatures for each of these differentiation pathways. Other studies have also identified microRNAs (miRNA) as regulators of developmental processes in plants and animals. The explosion of small non-coding RNA discoveries in recent years has 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.

Temporal regulation of ESCs and NSCs.

Stem cells are defined by their ability to produce many (pluripotent) or all (totipotent) cell types upon differentiation, and to produce new stem cells by cell division. The first proof of the existence of stem cells arose from the work of Till and Becker, who were studying hematopoietic stem cells derived from bone marrow (Till and McCullock, 1961; Becker et al., 1963), as well as Altman (Altman, 1962; Altman and Das, 1965). This pioneering research led directly to the use of bone marrow transplantation as therapy for human leukemias. By the 1980's, Evans and colleagues had created stem cells from teratocarcinomas or early embryos (Martin and Evans, 1974; Evans and Kaufman, 1981). Other studies have demonstrated a broad variety of potential sources

and culture methods for preparing stem cells as potential CNS therapies, including cultured stem cells from early embryos (Kim et al., 2002a; Ying et al., 2003), bone marrow (Woodbury et al., 2000; Woodbury et al., 2002), umbilical cord blood (Saporta et al., 2003) and adult tissues (Shihabuddin et al., 2000).

A major limitation to utilizing stem cells for therapeutic purposes arises from the same characteristic which makes them so attractive for therapeutics, their pluripotent capacity. Controlled and directed generation of neurons or glia to replace lost or damaged tissue will be a critical component to stem cell based therapies designed to treat neurodegenerative diseases and neurological disorders. The self-renewing capacity of neural stem cells (NSC) and their potential to generate the main cellular phenotypes of the nervous system, neurons and glia, make them the ideal cell type to produce functional mature neural phenotype cells.

When working with differentiating NSCs or any type of stem cell, it is important to be certain of the cell population one is culturing. Multiple cellular markers have been identified over the years that serve to confirm the cellular identity and maturity of the cultured cells. In pluripotent ESCs, the expression of transcription factors such as Oct4 and Nanog or cell surface markers such as SSEA-1 and Tra-1-60 are a clear indication that the cells are ESCs and are in a pluripotent state (Reviewed in (Nagano et al., 2008)).

One of the most widely used markers for NSC identification is the cytoplasmic protein Nestin (Hockfield and McKay, 1985). In the mammalian nervous system, Musashi, a RNA binding protein, is also expressed in neural precursor cells and NSCs

(Sakakibara et al., 1996). Both of these markers serve to identify cells that have begun to differentiate towards a neural lineage but have not committed to a specific mature cellular phenotype. In the developing and adult CNS, a portion of the multipotential NSC will become more restricted in terms of their differentiation potential. Spatial and temporal cues regulate the transition in the cells differentiation capacity (Li et al., 2004; Li and Grumet, 2007). The restricted differentiation is apparent in neuronal restricted precursors (NRPs) and glial restricted precursors (GRPs). Although they can still self-renew, they are limited to a specific phenotypic outcome upon differentiation. These cells can be identified by a series of specific cell surface markers. NRPs express polysialylated NCAM and can be identified using the monoclonal antibody 5A5 (Mayer-Proschel et al., 1997). GRPs can be identified with the A2B5 antibody, which recognizes a different carbohydrate antigen than 5A5 (Mayer-Proschel et al., 1997). As cells mature and terminally differentiate they lose these markers and acquire other ones depending on their differentiation stage and phenotype.

The antibody TuJ1, which identifies the Neuronal Class III β-Tubulin, has been used widely as a marker of neuronal differentiation because it is one of the earliest known neuronal proteins to appear upon differentiation (Li et al., 2008a; Chambers et al., 2009; Vierbuchen et al., 2010). Our experience with human ESC and NSC cultures has shown, that because TuJ1^{+} cells can be very immature neurons this marker can readily identify cells that have spontaneously differentiated in cultures and sometimes gives a high degree of background. Due to this issue, mature neuronal markers such as neuron specific enolase (NSE) (Schmechel et al., 1980) and microtubule-associated protein 2 (MAP2) (Izant and McIntosh, 1980) are better to assess levels of neuronal differentiation in culture. Even though, we describe these markers as clear temporal identifiers of

differentiation stages, the reality is that differentiation is a very fluid phenomenon in which cells can co-express different stage markers during transition periods. This just illustrates the complexity of the regulatory mechanisms that are occurring during differentiation.

MiRNAs are post-transcriptional regulators of gene expression.

MiRNA are single-stranded RNAs approximately 21 nucleotides long that are found in a wide variety of organisms, from plants to insects to humans (Ambros, 2001; Bartel, 2004). The primary transcripts of miRNA (pri-miRNA) are processed by the Microprocessor (Drosha-DGCR8) complex to yield a stem-loop precursor miRNA (pre-microRNA) (Han et al., 2004; Zeng et al., 2005). In animals, pre-miRNAs are exported from the nucleus to the cytoplasm by Exportin-5, where they are processed by Dicer to yield a double-stranded 19-25 nucleotide mature miRNA (Bartel, 2004; Zeng and Cullen, 2004). One of the strands of the mature miRNA is finally incorporated into the RNA-induced silencing complex (RISC) and directed to 3' untranslated region of mRNAs, targeting them for degradation, or suppressing or activating translation (Carrington and Ambros, 2003; Dykxhoorn et al., 2003; Pickford and Cogoni, 2003; Vasudevan et al., 2007). Complexes containing miRNAs and the RISC 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. MiRNA regulation in animals is mediated by imperfect binding to miRNA response elements (MRE) in mRNA targets (Bartel, 2004). Although miRNA-mRNA interactions are imperfect, there is evidence that the specificity of the targeting is primarily due to the miRNA "seed" sequence, which is

nucleotides 2-7 of the mature sequence (Grimson et al., 2007). The seed sequence is commonly identified as nucleotides two through seven on the 5'end of the miRNA. These tend to have a perfect match with the target mRNA, even though there are specific instances in which the 3'end of the miRNA is more important for miRNA targeting specificity (Bartel, 2009). The miRNA/mRNA interaction 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., 2004b; 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), and/or mRNA sequestration to P-bodies (Liu et al., 2005b; Liu et al., 2005a; Rehwinkel et al., 2005; Behm-Ansmant et al., 2006; Lian et al., 2006; Pauley et al., 2006). Estimates suggest there are about 400 miRNA genes in each invertebrate species, and approximately 1000-1500 genes 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). 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 miRNAs (Lewis et al., 2005; Miranda et al., 2006).

MiRNAs in ESCs and their functional targets.

The importance of miRNA function in stem cells has been established in C. elegans, Drosophila, Danio (zebrafish) and mice, where disruption of miRNA processing enzymes such as Dicer or DGCR8 leads to defects in cell proliferation and embryo development (Bernstein et al., 2003; Wienholds et al., 2003; Hatfield et al., 2005; Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007; Nimmo and Slack,

2009). In mouse, Dicer-null animals are embryonic lethal demonstrating its critical role during early embryonic development (Bernstein et al., 2003). However, dicer-null mice ESCs are viable albeit with marked defects in proliferation and differentiation. Notably, dicer knockdown in hESCs cause a prolonged G0 and G1 phases of the cell cycle (Qi et al., 2009) and this defective cell cycle progression could have an effect on other processes such as differentiation. Dicer-null ESCs are defective in undergoing differentiation upon induction and fail to express differentiation markers such as HNF4a, BMP4 and GATA1 (Kanellopoulou et al., 2005). Further Dicer deficient cells show decreased levels of DNA methylation and methyltransferases (Dnmts) (Benetti et al., 2008; Sinkkonen et al., 2008) and increases telomerase recombination and elongation (Benetti et al., 2008). This defect in DNA methylation leads to incomplete and reversible silencing of the Oct4 pluripotent gene, thereby resulting in lack of differentiation (Benetti et al., 2008; Sinkkonen et al., 2008). Since Dicer is needed for miRNA and endo-siRNA biogenesis, it can be argued that the phenotype may not be solely caused by lack of miRNA. One study reports that Dicer mutants had altered profiles of miRNA and not of other small RNAs, and that half of the miRNAs detected were known regulators of cell cycle and oncogenesis (Calabrese et al., 2007). Even though Dicer potentially has other roles in addition to miRNA processing, studies using Dicer-null ESC models suggest that miRNAs would serve a dual role. Firstly, miRNAs serve to regulate negative cell cycle modulators to ensure proper replenishment of the stem cell population. Secondly, miRNAs are required for adequate differentiation, potentially serving to remove genes that would required for ESC maintanace.

More stringent evidence for miRNA requirement in ESC self-renewal and differentiation comes from the DGCR8-deficient ESC. DGCR8 knock out mouse ESC

show phenotypes similar to Dicer-deficient mouse ESC with reduced cell proliferation, abnormal cell cycle control and deficiencies in differentiation (Wang et al., 2007). DGCR8-null ESC arrest in the G1 phase, implicating a role for miRNA in ES cell cycle in promoting transition from G1 to S phase (Wang et al., 2007). These cells cannot fully silence the expression of self-renewal genes such as Oct4, Rex1, Nanog and Sox2 and subsequently show reduced expression of differentiation markers (Wang et al., 2007). DGCR8-mutant ESC when injected into host mice do not differentiate into the three germ layers to form teratomas, a features characteristic of normal embryonic stem cells (Bodnar et al., 2004; Keller, 2005; Menendez et al., 2006). Taking advantage of the simplified microRNA background in DGCR8-null mouse ESCs, Blelloch and colleagues have discovered specific functions for families of microRNAs controlling cell cycle and self-renewal (Wang and Blelloch, 2009; Melton et al., 2010), to be described in detail below. These studies illuminate the critical role of miRNAs during ESC differentiation and how these molecules are required for cells to be able to change their phenotypic states during differentiation.

However several differences were observed between the Dicer and DGCR8 mutant cells. While the Dicer-deficient ES cells did not express any differentiation markers (Kanellopoulou et al., 2005), DGCR8 deficient ESC do express some differentiation markers and the defects in cell proliferation and cell cycle progression were less pronounced than Dicer-mutant cells (Wang et al., 2007). These studies confirm the essential regulatory role of miRNAs in ESC proliferation, cell cycle and differentiation but also suggest that other small RNAs may play a role in this process as well.

Since miRNA synthesis has been shown to be critical for progression of differentiation, ESCs ought to express unique patterns of miRNAs to support pluripotency.   Embryonic stem cells have been reported to express a small subset of unique miRNAs (Houbaviy et al., 2003; Suh et al., 2004; Calabrese et al., 2007; Lakshmipathy et al., 2007; Morin et al., 2008). Most of these ES-specific miRNAs occur as two clusters. The human miR-371 cluster is located on chromosome 19 and is analogous to the mouse miR-290 cluster and the miR-302 cluster located on chromosome 4 is associated with both murine and human ESC (Houbaviy et al., 2003; Suh et al., 2004; Strauss et al., 2006). Two additional clusters, miR-17 on chromosome 13 and the miR-106a cluster on chromosome X, have also been shown to be upregulated in ESCs (Laurent et al., 2008). These unique expression patterns of miRNAs suggest that miRNAs are not only essential for cells to differentiate but also are critical to maintain a cell in a desired phenotypic state.

Neural associated miRNAs and their roles in CNS development.

Differentiation into specific cell types has been found to correlate with regulated changes in miRNA expression patterns, presumably to promote differentiation or to stabilize new cell types. This role of miRNAs is retained in neural differentiation during development. Conditional Dicer knockout mice with the Cre-loxP system have allowed several groups to shed light on the global roles of miRNAs during mouse neural development. Conditionally knocking out Dicer in neural progenitor cells using specific promoter driven Cre-recombinase (Cre) mice lines (Emx-1-Cre and Nestin-Cre), shows that neural progenitors undergo cell death and abnormal differentiation in the cortex and striatum (Kawase-Koga et al., 2009). A marked reduction in radial thickness starting at

E13.5 due to neuronal apoptosis was observed in Dicer conditional knockouts using a Emx1-Cre mouse line (De Pietri Tonelli et al., 2008). They also observed postnatal defective cortical layering which is attributed to an impairment of neuronal differentiation. Interestingly, Dicer ablated neuroepithelial cells, and the neurogenic progenitors derived from them, were unaffected by miRNA depletion with regard to cell cycle progression, cell division, differentiation and viability during the early stage of neurogenesis, and only underwent apoptosis starting at E14.5 (De Pietri Tonelli et al., 2008). Two different groups have established Dicer deficient mouse NSCs, void of miRNAs, and capable of self-renewal and expansion in cell culture (Andersson et al., 2010; Kawase-Koga et al., 2010). Both of these lines undergo cell death in the absence of mitogens and lack the ability to differentiate. This phenotype can be rescued by replacing Dicer (Andersson et al., 2010). These studies suggest progenitor cells are less dependent on miRNAs than their differentiating progeny or that changes in the global miRNA population are critical to transition from progenitor state to a differentiated one.

Unique signatures of miRNA expression would be required for efficient neural differentiation. Previous work identifying new miRNAs and their expression profiles has established distinct subset of miRNAs with enriched or specific expression in neural tissues (Wienholds et al., 2005; Kapsimali et al., 2007). Embryonic stem cell specific miRNAs are down-regulated during RA-induced differentiation (Houbaviy et al., 2003; Suh et al., 2004; Song and Tuan, 2006) of neuronal precursor cells. Several miRNAs have been identified as brain-enriched or specific (Krichevsky et al., 2003; Miska et al., 2004), miR-9 and miR-153 are expressed in proliferating and differentiating neural cells (Mortazavi et al., 2006; Kapsimali et al., 2007) while miR-124 expression is restricted to differentiating neurons (Kapsimali et al., 2007; Yu et al., 2008). The contrasting

expression profiles of these two groups of miRNAs suggest that they may in fact have opposing functions in development. For example, mir-302, an ESC specific microRNA, regulates the cell cycle in pluripotent ESCs, by inhibiting cell cycle regulators, such as cyclin D1 (Card et al., 2008). This regulation serves to maintain stemness in pluripotent ESCs. On the other hand, upon neural differentiation, mir-124 and mir-9 are induced and have been shown to inhibit the anti-neural REST/SCP1 pathway, allowing for neurogenesis to occur (Visvanathan et al., 2007).

The RE1 silencing transcription factor (REST) serves as a transcriptional repressor which plays a critical role in regulating neuronal gene expression and promoting neuronal fate (Chong et al., 1995; Schoenherr and Anderson, 1995) . REST normally interacts with two corepressors, CoREST and mSin3a, to recruit DNA-binding protein MeCP2, histone deacetylases (HDAC), and other silencing proteins, to alter chromatin conformation to a heterochroamatin or inactive state (Andres et al., 1999; Grimes et al., 2000; Lunyak et al., 2002; Ballas and Mandel, 2005). Recruitment of REST is known to target promoters of pro-neuronal genes, and limits their expression exclusively to neuronal tissues by repressing their transcription in non-neuronal cells (Lunyak et al., 2002). Ballas *et al.* (2005) showed that REST is a key regulator in the transition from embryonic stem cells to neural progenitors and from neural progenitors to neurons. Its expression level is progressively reduced as the cell transitions from a pluripotent stem cell to a NSC and finally to a post-mitotic neuron (Ballas et al., 2005). MiRNA regulation of REST would be an example of how up-regulated miRNAs are responsible for inducing a translational shift that would promote a mature neural or neuronal phenotype.

Mir-9 and its neural role during proliferation and differentiation.

One intriguing neuronal miRNA that potentially can regulate the transition from a NSC to a neuron during differentiation is miR-9. Mir-9 is expressed in proliferating and differentiating neural cells (Mortazavi et al., 2006; Kapsimali et al., 2007). Several groups have begun to decipher the role of this gene in NSC proliferation and neuronal differentiation. Mir-9 is highly conserved across species and shows CNS regional specificity in its expression (Wienholds et al., 2005; Kapsimali et al., 2007). Interestingly, there are discrepancies about the role of mir-9 in NSC proliferation and differentiation. Mir-9 expression in late embryonic zebrafish brains shows spatial specificity, avoiding expression in the midbrain-hindbrain boundary (MHB) region, a non-neurogenic boundary zone containing a pool of progenitor cells that contributes neurons to the midbrain-hindbrain domains. This spatial specificity has been proposed to be critical for regulation of FGF signaling and the maintenance of a neural progenitor state *in vivo* (Leucht et al., 2008). Mir-9 over-expression was shown to promote premature neuronal differentiation in the MHB; meanwhile, knockdown of mir-9 with modified antisense oligonucleotides (morpholino) had the opposing effect by increasing the MHB area size and region specific markers (Leucht et al., 2008). Similarly, in the mammalian embryonic brain, Shibata *et al.* showed by gain and loss of function experiments that mir-9 regulates differentiation of Cajal-Retzius cells in the medial pallium by targeting Foxg1 (Shibata et al., 2008). Mir-9 knockdown caused a reduction of Cajal-Retzius neurons but did not have an effect on progenitor cells (Shibata et al., 2008). In another study, Zhao *et al.* have shown that knock down of mir-9 in adult mouse NSCs caused a small increase in proliferating cells (1.37-fold) and that over-expression of mir-9 leads to a decrease in proliferation of precursor cells and an increase in both glial and neuronal differentiation (Zhao et al., 2009). Meanwhile, miR-9 knockdown caused a reduction in

differentiating neurons from neural progenitor cells derived from mouse ES cells, accompanied by a slight increase in GFAP$^+$ astrocytes, although the effects on proliferation were not directly tested in this study (Krichevsky et al., 2006). In neural progenitors derived from human ES cells, loss of miR-9 has been shown to suppress proliferation. In this model, loss of miR-9 promotes migration of neural progenitors but has no effect on differentiation (Delaloy et al., 2010). From these studies we can conclude that in most systems, miR-9 is critical for neuronal differentiation, but the effect on proliferation is variable.

The differences among studies can partially be attributed to differences in the model systems or growth conditions, but, these discrepancies also raise the possibility that the function of miR-9 in neurogenesis and proliferation is context dependent. Bonev and collegueas have shown that mir-9 is expressed in neural progenitor cells of *X. tropicalis*, and its knockdown results in an inhibition of neurogenesis along the anterior-posterior axis. However, the underlying mechanism differs--in the hindbrain, progenitors fail to exit the cell cycle, whereas in the forebrain they undergo apoptosis, counteracting the proliferative effect (Bonev et al., 2011). This study illustrates for the first time that the spatial context in which mir-9 is expressed will determine its biological role within an organism. Furthermore, these data hint at the possibility that mir-9s variable context dependent bioactivity might also vary across species. The bigger question would be; are mir-9 targets conserved across species?

Onecut family of transcription factors

Among the predicted targets of mir-9 is Onecut 2 (OC2). It has been shown that mir-9 targets OC2 in rat INS-1E insulinoma cells (a pancreatic beta-cell model)

(Plaisance et al., 2006). Mir-9 acts by diminishing the expression of the transcription factor OC2 and, in turn, increases the level of Granuphilin/Slp4, a Rab GTPase effector associated with beta-cell secretory granules that exerts a negative control on insulin release. This study confirmed OC2 regulation by mir-9, a neuronal associated microRNA (Krichevsky et al., 2006). This leads us to wonder if this regulation is conserved in other systems, particularly in NSCs of the developing CNS.

Onecut family members are a series of transcription factors that contain a cut domain and a homeobox domain, which are both involved in DNA binding (Hong et al., 2002). These genes are conserved across multiple species, including humans, rodents, C. elegans, D. melanogaster, D. rerio and S. purpuratus (sea urchin). Mammalian genomes contain three members of this family, Onecut 1 (OC1), also known as HNF-6, OC2 (Jacquemin et al., 1999) and Onecut 3 (OC3) (Vanhorenbeeck et al., 2002). The sequence conservation in their cut and homeodomains suggests that these paralogs may regulate the same genes, and indeed many OC1 binding sites on DNA are recognized by OC2 and OC3 (Jacquemin et al., 1999; Vanhorenbeeck et al., 2007). Neither OC2 nor OC3 is required for pancreas specification (Vanhorenbeeck et al., 2007). However, Onecut 2 plays partially redundant roles with OC1 in pancreas morphogenesis and in the differentiation of endocrine precursors. Interestingly, it has been shown that OC3 expression is dependent on OC1 in OC1 KO mice (Pierreux et al., 2004; Vanhorenbeeck et al., 2007). Therefore, by knocking out OC1, one would be knocking out OC3 as well. The developmental roles of the OC family of transcription factors have been associated primarily with endodermal development, specifically cell differentiation in liver and pancreas (Jacquemin et al., 2003a; Briancon et al., 2004; Hara et al., 2007; Matthews et al., 2008). OC1 and OC2 have been shown to regulate the

transcriptional activity of neurogenin 3 (Ngn3) and of the forkhead box A2 (FoxA2), also known as HNF-3β, during endodermal cell differentiation (Landry et al., 1997; Jacquemin et al., 2000). These genes are required for endoderm maturation and differentiation (Wang et al., 2009). Given the broad functional redundancy between OC factors (Jacquemin et al., 1999; Vanhorenbeeck et al., 2002; Clotman et al., 2005), these observations underline that these proteins may exert overlapping functions.

Onecut family of transcription factors are expressed in the CNS during neural development.

Currently little is known about the role of OC genes in neural development, except for a few studies which indicate temporal and spatial expression specificity during neural development in the CNS. OC1 is expressed in many parts of the central nervous system (CNS), including the ventral half of the rhombencephalon, the whole mantle layer of mesencephalon, the telencephalon and the diencephalon during development (Landry et al., 1997; Rausa et al., 1997). In situ hybridizations identified OC1 as present in the mantle layer but absent from the ventricular zone. This would suggest a role in cell specification or differentiation and not a proliferative or stemness maintenance role. OC2 expression is present across most of the mouse CNS. E9.5 embryos show OC2 expression along the cephalocaudal axis of the nervous system and along the neural tube. At E10.5 embryos show expression in the neural tube and the dorsal root ganglia. Later, at E12.5 OC2 is expressed in the ventral horn of the spinal cord, dorsal root ganglia, thalamus and the hind brain; and at E15.5 expression is present in the cerebellum, mammillary body, the optic chiasma and suprachiasmatic nucleus

(Jacquemin et al., 2003b). The developmental timing of the expression of these genes coincides with both neurogenesis and gliogenesis in mouse embryos.

In contrast to mammalian OC genes, the product of Drosophila D-OC is expressed exclusively in the nervous system throughout the life span of the flies and might play a role in neural differentiation and maintenance (Nguyen et al., 2000). The Drosophila D-OC was also shown to regulate photoreceptor cell differentiation but have an effect on early cell specification during eye development.   Another homolog of OC1 has been identified in H. roretzi (sea squirts) which is exclusively expressed solely in the CNS (Sasakura and Makabe, 2001). This homolog was shown to have a role in the speciation of the neural tube. Hong *et al.* searched for the zebrafish homolog of OC and found that it displays a highly dynamic expression pattern in the primary neurons of the brain and spinal cord during zebrafish embryogenesis (Hong et al., 2002). A more recent report by Francius and Clotman shows a correlation between the expression of OC family members and a subpopulation of newly-born and differentiating spinal motor neurons within the four motor columns of the mouse spinal cord (Francius and Clotman, 2010) . These studies suggest that OC family members potentially regulate neuronal differentiation in the spinal cord in a positive manner. This is critical to mention because, these observations will contrast with some of our data.

Downstream effectors of Onecut genes have roles in glial differentiation.

OC1 and OC2 have been shown to regulate the transcriptional activity of Ngn3 and of FoxA2 during endodermal cell differentiation (Landry et al., 1997; Jacquemin et al., 2000). Loss of function of FoxA2 in zebrafish leads to defective development of

oligodendrocytes, serotonergic raphe nucleus and several cranial motor nuclei in the floorplate (Norton et al., 2005) . It is important to mention that the floorplate is induced but fails to differentiate. The loss of FoxA2 also causes a severe reduction of prospective oligodendrocytes in the midbrain and hindbrain. The reduction of oligodendrocytes in different regions of the CNS would suggest that FoxA2 has a role in glial differentiation, specifically oligodendrocyte specification.

Ngn3-null mice showed a loss of expression of Nkx2.2, a transcription factor required for proper oligodendrocyte differentiation (Lee et al., 2003). There is also a reduction in the expression of myelin basic protein (MBP), proteolipid protein (PLP), and glial fibrillary acidic protein (GFAP), markers for mature oligodendrocytes and astrocytes in these animals. Ngn3 was identified as a transcriptional regulator of PLP. Oligodendrocytes can arise from two regions or cell populations in the ventral neural tube, a Ngn3 / Nkx2.2 population or a Olig2 / Sox10 / PDGFRα population (Liu et al., 2002). Neither of these populations is positive for mature glial markers. These observations suggest that Ngn3 may regulate glial differentiation at a developmental stage prior to the segregation of the oligodendrocyte and astrocyte lineage. If OC genes regulate Ngn3 and FoxA2 in the CNS, that would signify that Onecut genes have a role in glial differentiation also.

The Onecut transcriptional network is targeted by neuronal associated miRNAs.

As stated previously, mir-9 targets OC2 in rat INS-1E insulinoma cells (a pancreatic beta-cell model) (Plaisance et al., 2006). Mir-9 acts by diminishing the expression of the transcription factor OC2 and, in turn, increases the level of

Granuphilin/Slp4, a Rab GTPase effector associated with beta-cell secretory granules that exerts a negative control on insulin release. This study confirmed OC2 regulation by mir-9, a neuronal associated microRNA (Krichevsky et al., 2006).   FoxA2, a downstream effector of Onecut genes, has been validated biochemically as a target of mir-124 in MIN6 beta-cells (Baroukh et al., 2007).   MiR-124 regulated Foxa2 gene expression, and that of its downstream target, pancreatic duodenum homeobox-1 (Pdx-1). Mir-124 expression is associated with differentiating neurons (Kapsimali et al., 2007; Yu et al., 2008).   These two studies suggest that OC2 and FoxA2, members of a potentially pro-glial network, are regulated by miRNAs that have roles in neuronal differentiation. This presents the possibility that specific neuronal microRNAs inhibit glial related genes during neuronal differentiation.

miRNAs are predicted to canalize differentiation processes.

Eran Hornstein and Noam Shomron propose that miRNA interactions with the network of protein-coding genes evolved to buffer stochastic perturbations and thereby confer robustness to developmental genetic programs (Hornstein and Shomron, 2006). At the root of their hypothesis, they suggest that miRNA regulatory networks evolved under natural selection in order to stabilize the phenotype and decrease the variability of specific traits.   This view of the proposed role for miRNAs arose from Waddington's original canalization hypothesis (Waddington, 1959).   This hypothesis can be extrapolated beyond populational evolution and can be applied to cellular processes such as ESC differentiation. MiRNAs would serve to channel differentiation to a specific phenotypic outcome. In the case of neurogenesis, it is possible that specific groupings of neurogenic miRNAs would serve to inhibit genes involved with alternate phenotypes,

such as gliogenic genes. In this thesis we will show that the role of mir-9 during NSC differentiation varies according to species and cellular context. In a rat NSC model it serves to modulate or canalyze neurogenesis by inhibiting genes, such as OC2, which serves an anti-neuronal role. Meanwhile in human NSC, mir-9 contributes to the maintenance of a NSC state and does not promote neuronal differentiation.

II. RESULTS

Section A.

Mir-9 canalyzation of rat NSC neuronal differentiation via regulation of OC2

In an effort to determine various regulatory mechanisms driving the formation of each neural phenotype, we have established two *in vitro* models that recapitulate neural stem cell differentiation; one producing primarily neurons, the other, cells with a more mixed phenotype. We have used 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. Results obtained from the following studies should be directly applicable to human stem cell biology, leading to therapeutic NSC transplantation protocols for spinal cord injury patients.

An *in vitro* model of rat neural stem cell specification.

In order to generate a reproducible model for neural stem cell differentiation, Dr. Hedong Li isolated two immortalized 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. Cells were cultured in the presence of FGF2 and LIF for two days and then immortalized with the PK-VM-2 retrovirus expressing v-myc. (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 transduced NSC clones. One of these clones, named 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 grown on laminin and send out multiple processes upon differentiation (Li et al., 2008a).

Upon withdrawal of FGF2, L2.2 readily differentiates into β-III tub+ (Beta-III Tubulin) neuronal precursor cells as measured by immunostaining (Fig.1), qRT-PCR and western blots (Li et al., 2008a) 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, $15.7\pm7.5\%$ of the recorded L2.2 cells exhibit action potential, and $43.7\pm10.9\%$ 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., 2008a). This cell clone serves as a model to study neuronal differentiation and allows us to observe neuronal specific differentiation events.

Alternatively the clone labeled L2.3 presents a more mixed phenotype, including astrocytes, oligodendrocytes, as well as potentially glutamatergic neurons upon differentiation (Fig.1). This clone was characterized in detail by 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). By contrasting the two clones we will be able to identify critical molecular pathways that regulate neuronal differentiation.

mRNA and miRNA expression profiling of rat NSCs and NRPs.

In an attempt to identify temporally regulated transcripts associated with NSC differentiation we profiled mRNA and miRNA expression patterns during FGF removal of the neuronal restricted precursor, L2.2, and, the multipotential NSC, L2.3 by microarray analysis (Goff et al., 2007; Li et al., 2008a). Triplicate cultures were prepared from the neurogenic L2.2 NRP clone and the multipotential L2.3 NSC clone prior to (0 days) or 1 or 3 days following bFGF withdrawal. Low molecular weight fractions of RNA were prepared and assayed on the NCode miRNA microarray (Goff et al., 2005) (Invitrogen, Carlsbad, CA) by Dr. L. Goff. We also prepared high molecular weight fractions from the same samples, labeled them by incorporation of biotinylated nucleotides into a cDNA reaction, and hybridized them to the Applied Biosystems 1700 rat genome survey microarrays (Goff et al., 2007). In collaboration with Rebecka Jörnsten, from the Statistics Department at Rutgers, data from both sets of arrays were quantile normalized and filtered by ANOVA at 5% FDR (mRNA) or 10% FDR (miRNA), yielding 3,181 regulated mRNAs and 39 regulated miRNAs.

A select set of differentially expressed transcription factors, primarily members of the basic Helix-Loop-Helix (bHLH) family, and several of the regulated miRNAs were interrogated by qPCR in order to confirm the microarray results. bHLH transcription factors have been shown to have critical roles in neural development and specification (Lee, 1997; Kageyama et al., 2005; Sugimori et al., 2007). Among the genes

interrogated were Pax6, Olig2, Ngn2 and Ngn3. Their expression patterns correlated well with the microarray results (Data not shown).

Among regulated miRNAs, we see that mir-9 and mir-124a are both induced upon the differentiation of the neurogenic clone, L2.2. This observation was confirmed by qPCR (Fig. 2). Previous studies support these data; mir-9 and mir-124a have been shown to have roles in the differentiation of neural progenitor cells both in vitro and in vivo (Krichevsky et al., 2006; Cheng et al., 2009; Zhao et al., 2009). These results correlate well with the expression patterns seen on our microarray analysis. We are confident that the exploratory lists of gene expression changes detected on our microarrays are accurate.

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

It is well documented that miRNAs exhibit temporal and tissue specific expression patterns, and have been implicated in developmental roles, including adipocyte, hematopoietic and neuronal differentiation (Brennecke et al., 2003; Krichevsky et al., 2003; Kuwabara et al., 2004; Sempere et al., 2004; Krichevsky et al., 2006). We hypothesized that the expression of specific combinations of miRNAs determines the final phenotypic state upon differentiation. We were particularly interested in identifying specific groups of miRNAs involved in neuronal differentiation. We believed that if we identified common regulation patterns between miRNAs and transcription factors, we would be able to identify these miRNA groupings. The roles of transcription factors, particularly those of the bHLH family, are much better characterized

in NSC differentiation than the roles of miRNAs (Lee, 1997; Kageyama et al., 2005; Sugimori et al., 2007). Therefore, we believed that by correlating miRNA expression to the mRNA expression of transcription factors would shed light on the potential functional roles for particular groups of mRNAs and miRNAs.

In order to predict miRNA-mRNA mechanisms in the context of NSC differentiation, we have cross-correlated the expression patterns of miRNAs and a subset of transcription factor mRNAs from our list of significantly expressed genes (Fig. 3) (Goff et al., 2008). Pearson correlation coefficients were calculated using the expression values for each mRNA-miRNA pair across the 12 samples (3 replicates each of 0 and 3 days in both L2.2 and L2.3). Two-dimensional hierarchical clustering of the resulting matrix of correlation coefficients, on both mRNA and miRNA axes, produced clusters of mRNAs and miRNAs with strong relationships across the 12 samples that may describe networks of interactions between the two molecule types, as well as help ascribe miRNAs to key biological functions. By only using the expression data of mRNA transcripts of transcription factor in the cross-correlation we can focus on genes that have a direct influence over transcription rates. For example, when looking at positively correlated transcription factor mRNA and miRNA groups, green on the heatmap, it would be possible to identify potential transcriptional regulatory networks working upon specific miRNA subgroups. On the other hand, we hypothesize that several of the negatively correlated miRNA-mRNA combinations, seen as red on the heatmap, would be indicative of mRNA degradation by a specific miRNA via the RISC complex.

We were interested if any meaningful relationships among mRNAs could be inferred from their correlation to miRNAs. Interestingly, there is a clear clustering of

mRNAs based on functionality, based on gene ontology (GO) and a literature search. The highest-level cluster separation adequately distinguishes between neurogenic and gliogenic mRNA. The side colorbar in (Fig.3) indicates mRNAs that are associated with neurogenesis (blue), gliogenesis (red), or stem cell maintenance (yellow).

When we focus on the miRNA axis, we can see a close proximity of miR-9 and miR-124a, both well known neuronal miRNAs (Krichevsky et al., 2006; Cheng et al., 2009; Zhao et al., 2009). mir-9 and mir-124 also cluster with mir-153 and 182. We believed these four miRNAs to be a pro-neuronal group. This group showed a strong positive correlation to known neurogenic transcription factors, such as Ngn2 and members of the NeuroD family (Lee, 1997). This provides additional evidence that these miRNAs are expressed during neuronal specification and are required for acquisition of a neuronal phenotype. It is important to keep in mind that these groupings were based not on expression profiles but rather correlation with significant miRNAs.

A neurogenic group of miRNAs can promote neuronal differentiation in a multipotential NSC.

We identified mir-9, mir-124a, mir-182, and mir-153 as primary members of a potential group of neurogenic miRNAs in our cross-correlation analysis. Among those identified as brain-enriched miRNAs by previous reports, mir-9 and mir-153 are expressed in proliferating and differentiating neural cells (Mortazavi et al., 2006; Kapsimali et al., 2007) while mir-124 expression is restricted to differentiating neurons (Kapsimali et al., 2007; Yu et al., 2008). To confirm the expression profile of this particular group of neurogenic miRNAs in our neural precursor clones, we performed

qPCR on the same LMW RNA used for the miRNA microarray analysis. With the exception of mir-182, the trend was increased expression of mir-9, mir-124, and mir-153 upon differentiation of L2.2 and L2.3 (Fig.2). Specifically, miR-9 showed a significant increase in expression upon differentiation of both clones, especially in the L2.2 clone, and mir-124a expression was significantly up-regulated after 3 days of differentiation in the L2.3 clone (p<0.05). Statistical significance for expression of mir-153 and mir-182 could not be determined due to a single outlier. Nevertheless, qPCR analysis showed mir-182 expression remained relatively expressed in the L2.2 clone (Fig. 2).

Gain- and loss-of-function analyses of mir-9 and mir-124 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 the decrease of the glial marker GFAP in differentiating ES cells, suggesting mir-9 plays a role in promoting a neuronal phenotype. To test whether these miRNAs are capable of directly affecting the phenotype of differentiating neural precursor cells, we transfected strand-specific PremiRs (Ambion, Austin, TX) 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 miRNA molecules, can be appropriately loaded into miRNP complexes, and allow for the over-expression of a strand-specific miRNA sequence. We hypothesized that if these miRNAs were capable of inducing a neuronal phenotype (TuJ1^{+}), then they should be able to increase the percentage of neuronal cells produced during differentiation of this multipotential clone.

We electroporated PremiRs (Ambion, Austin, TX) of mir-9, 124, 153 and 182 individually or as a mixture into replicate (n=3) cultures of L2.3 and allowed the cells to

recover for approximately 4 hours prior to differentiation (-bFGF). At 72 hours post FGF removal, cells were fixed and analyzed for TuJ1 expression by flow cytometry. The results from this FACS analysis demonstrate a significant increase in the percentage of TuJ1+ cells at 3 days post-differentiation after exogenous expressing mir-9, mir-153, or overexpressing all four neurogenic miRNAs simultaneously (Fig.4; p<0.05, Student's t-test). However, over-expressing the mixture of neurogenic miRNAs did not yield a synergistic increase in the percentage of TuJ1+ cells suggesting redundancy exists among these particular miRNAs (Plasterk, 2006; Miska et al., 2007; Wang et al., 2008) (Fig.4). To further test this possibility of redundancy, we also tested the requirement of these neurogenic miRNAs for induction of the neuronal phenotype by transfecting Anti-miR (Ambion, Austin, TX) miRNA inhibitors with the aim of blocking the specific activity of each of these miRNAs during L2.3 differentiation. We observed a slight reduction in TuJ1+ cells by inhibition of these endogenous neurogenic miRNAs but this difference has not been confirmed as significant (Fig.4). These results suggest that exogenous expression of these miRNAs is sufficient to enhance a pro-neuronal effect in uncommitted neural precursor cells. The inability to reverse this effect also suggests that there are potentially other miRNAs that have pro-neuronal roles and function in parallel pathways.

Cross-correlation of mRNA and miRNA expression profiles identifies the OC family of transcription factors as potential regulators of neural differentiation.

We were interested in identifying which mRNAs are regulated by these pro-neuronal miRNAs during neurogenesis. Since miRNAs have been shown to mediate mRNA degradation, it is reasonable to suggest that this activity could be identified in a

subset of mRNA:miRNA pairs demonstrating strong negative correlations across multiple conditions. We began to dissect the potential mRNA:miRNA interactions that were predicted from the cross-correlation matrix (Fig.3). Focusing only on the interactions between the neurogenic cluster of miRNAs and the regulated mRNAs, we identified OC2 as a potential target of the neurogenic cluster (Fig.5). OC2 shows a negative correlation with all members of the pro-neuronal miRNA cluster, which could be indicative of targeting (Fig.5). In addition, OC2 also clusters with genes that have well documented pro-gliogenic roles, as identified in the red colored side bar (Fig.5). We speculated that OC2 would have an opposing role to neuronal differentiation and would be a logical target for pro-neuronal miRNAs. Furthermore, a literature search indicated that OC2 was a direct target of mir-9 in rat INS-1E cells (Plaisance et al., 2006), supporting our hypothesis that OC2 is targeted by mir-9 and other pro-neuronal miRNAs during neurogenesis. This led us to investigate this interaction in the context of NSC differentiation and question if the other regulated OC members have a role in neural development.

In the microarray data set used to create the cross-correlation matrix mentioned previously, we observed an increase in mRNA expression of all three OC family members in the multipotential clone L2.3, which produces a mixture of phenotypes upon bFGF withdrawal; but no difference in the neuronal-restricted-precursor clone L2.2. To confirm these data we assayed mRNA expression for all three OC members during differentiation of our model cells by qPCR. Total RNA was extracted from differentiating L2.2 and L2.3 cells (0, 1 and 3 days post FGF withdrawal, n=3). The main purpose of this assay was to confirm the microarray results discussed previously. In general, expression patterns concur with the previous results. qPCR results demonstrate that

OC1 and OC2 are both induced after FGF removal from L2.3, multipotential NSCs (p<0.05, T-test) (Fig.6). Meanwhile, there was no change in expression in the NRP clone, L2.2 (Fig.6). One cannot speculate on the role of these genes based on the expression patterns in each cell clone individually. But when one compares the mRNA expression levels in the two cell clones, one might predict that OC1 and OC2 have no role or a very limited one during neuronal maturation, which would be reflected by the lack of regulation in the NRP clone, L2.2. Alternatively, because OC1 and OC2 are induced in the multipotential NSC, L2.3, they might serve an anti-neuronal or pro-gliogenic role. It is important to remember that the multipotential NSC L2.3 gives rise to a mixed population of glia and neurons upon differentiation.

Enhanced glial differentiation protocol increases mRNA expression of OC family members.

The cross-correlation data predicted that OC family members may serve an anti-neuronal or pro-glial role. If this is true we would expect to see an increase in expression of the OC family during differentiation protocols that would promote non-neuronal phenotypes, such as glial cells. To test this hypothesis, we differentiated the L2.3 multipotential rat NSCs in the presence of 1% FBS. Serum enhances glial differentiation, as can be seen in the drastic increase in GFAP positive cells 3 days after FGF removal (Fig.7A). We then assayed these cells for mRNA expression of OC1 and OC2 genes by qPCR (n=3), as described previously. mRNA levels of OC1 and OC2 genes increase significantly when the multipotential NSC clone L2.3 is differentiated in the presence of serum (Fig.7B ; p<0.05, Student's t-test). This observation correlates well with the hypothesis that OC family members serve an anti-neuronal role upon NSC

differentiation. Protein levels could not be ascertained in the rat model due to the lack of rat specific antibody, so these experiments were performed in the human NSC model described in the results section B.

Inhibition of Onecut1 and 2 by siRNA knock-down shows an anti-neuronal role.

If OC family members serve an anti-neuronal or pro-glial role during NSC differentiation we would expect that knock down of these genes would lead to enhanced neuronal differentiation and a reduction in glial differentiation. In order to identify the role of the OC family upon NSC differentiation, we performed shRNA knock down experiments in the L2.3 multipotential NSCs during FGF removal and interrogated the phenotypic outcomes by FACS analysis after staining for the NSC marker Nestin, the neuronal marker TuJ1 and the glial marker GFAP. We used gene specific shRNAs to knock down OC1, OC2, or a combination of both (n=3). Results show that at three days post FGF removal, knock down of OC2 causes a significant increase in the number of TuJ1$^+$ cells compared to cells treated with a scrambled shRNA (p<0.05, n=3, T-test) (Fig.8A). In addition, knock down of OC2 alone or in combination with OC1 causes a significant decrease in Nestin$^+$ cells (p<0.05, n=3, T-test). After OC2 knock-down, there is an increase in the neurogenic capacity of these cells, suggesting, a pro-gliogenic or anti-neurogenic role for this gene. This was most noticeable when we plotted the ratio of the percent of TuJ1$^+$ cells to the percent of Nestin$^+$ cells (Fig.8B). This indicates that the increase in TuJ1$^+$ cells is at the expense of Nestin$^+$ cells, suggesting that these cells are potentially differentiating and leaving the cell cycle faster. We could not assay for GFAP$^+$ cells at three days because under these growth conditions GFAP is not expressed until five days after FGF removal. We then decided to look at the number of

TuJ1$^+$ and GFAP$^+$ cells after five days of differentiation to interrogate if OC2 had a pro-glial role. The FACS results indicated that knock down of OC1 and OC2 together causes a small yet significant decrease of 7% in GFAP$^+$ cells (p<0.05, T-test) (Fig.8C). Meanwhile, knock down of OC2 alone or in combination with OC1 still caused a significant increase in TuJ1$^+$ cells. Plotting the ratio of the percent of TuJ1$^+$ cells to the percent of GFAP$^+$ cells shows that OC2 knockdown causes a shift in these cells towards a neuronal lineage (Fig.8D). These data indicate that OC2 seems to have a more predominant role in regulating neural specification upon differentiation and serves an anti-neuronal role. We can speculate that by inhibiting OC2, we remove a barrier of neuronal differentiation. This could be the reason these cells leave the NSC state, as seen by a reduction in Nestin$^+$ cells, and increase their neuronal capacity. Interestingly, these results mimic the results obtained when mir-9 was exogenously over-expressed in the L2.3 multi-potential NSC.

The transcription factor OC2 is a target of the pro-neuronal miRNA mir-9 in rat NSCs.

As shown previously, exogenous expression of mir-9 can increase the neurogenic differentiation capacity of the multipotential NSC L2.3. We have also shown that mir-9 is endogenously induced upon neuronal differentiation in the L2.2 NRP clone. Mir-9 is an active modulator of neuronal differentiation. We propose that mir-9 serves to canalize the differentiation process. This miRNA exerts its function by repressing either inhibitors of a specific phenotypic outcome, in this case, inhibitors of neurogenesis, or by inhibiting genes that would lead to undesirable phenotypic outcomes, such as pro-glial genes.

We began to question the relationship between mir-9 and the OC2 transcription factor that was described in the cross-correlation matrix (Fig.5). OC2 clustered well with pro-glial transcription factors, supporting a role of an anti-neuronal gene. It also showed a negative correlation with mir-9, a pro-neuronal miRNA. As we proposed earlier, genes expressing a negative correlation with a particular miRNA could be predictive of targeting. Studies have shown that mir-9 targets OC2 in rat INS-1E cells (Plaisance et al., 2006). We were curious if this is also the case during neural differentiation. We contrasted the mir-9 and OC2 expression patterns in both L2.2 NRP and L2.3 NSC cells in order to compare between a neurogenic and a more multipotent differentiation event. Briefly, in the L2.2 NRP, the clone with greater mir-9 abundance upon differentiation, there is no change in the expression levels of OC2. On the other hand, OC2 is induced upon differentiation of the multipotential L2.3 NSC clone, but mir-9 does not show a robust induction (Fig.9). We can speculate that an increase in mir-9 during neuronal differentiation would lead to inhibition of OC2.

We examined other predicted mir-9 miRNA response elements (MRE) in the 3' UTR of the OC2 gene using a target prediction algorithm, TargetScan (Fig.10). TargetScan computes predicted biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA (Friedman et al., 2009). We noticed that the predicted rat OC2 mRNA annotation in the NCBI database did not include a predicted 3' UTR. TargetScan identified mir-9 MREs in the rat genomic sequence that flanked the 3' end of the predicted OC2 mRNA and aligned well with the human OC2 3' UTR. When we looked at the annotated genomic context of the predicted OC2 gene (EMBL accession number ENSRNOT00000024631) using the UCSC genome browser online tool (Fujita et al., 2011) we noticed that the

human and mouse OC2 mRNAs have surprisingly long 3' UTRs which are highly conserved across species other than rat (Fig.11). The human 3' UTR extends 14,575 base pairs (bps) and the mouse one extends 11,857 bps. It is also clear that there are multiple rat ESTs that align with the 3' ends of the mouse and human OC2 gene (Fig.11). This would suggest that the rat OC2 3' UTR is potentially much larger than what has been annotated.

We mapped a portion of the rat OC2 mRNA 3' UTR by a rapid amplification of cDNA 3' end (3' RACE) assay (Li et al., 2005). We were not able to amplify products that are comparable in size to the mouse or human 3' UTR counterparts. The longest amplicon we obtained was approximately 2.8 Kb long. Within this 2.8 Kb fragment of the Onecut 2 3' UTR there are multiple predicted miRNA targeting sites for mir-9 and mir-153 (Fig. 10 and 11). Interestingly, in addition to this 2.8 Kb amplicon, we obtain multiple smaller size amplicons after the PCR (Fig.12). At first we believed that these might be splice variants or non-specific products, but when we mapped the size of PCR products to the genome starting from the site for the forward PCR primer, we realized that they all ended in genomic regions containing stretches of adenines. When we take a closer look at the rat genomic sequence that aligns with the mouse and human OC2 3' UTRs, we identify multiple internal stretches of 10 or more adenines (Fig.12). We believe that internal stretches of adenines are serving as complimentary sequences for the oligo dT primer we use for the reverse transcription reaction or the anchored poly A primer we use for the 3' RACE PCR. These interactions are competing with the poly(A) tail at the 3' end and hindering us from obtaining longer PCR products. This problem probably explains why this gene's 3' UTR is so poorly annotated in the rat genome. Based on homology to the mouse and human OC2 genes, it is likely that the 3' UTR of

the rat OC2 gene is much longer than the 2.8Kb fragment that was obtained by 3' RACE PCR. By performing these PCRs we have expanded the region of known 3' UTR and have confirmed the presence of several of the computationally-predicted mir-9 MREs within the 3' UTR of OC2.

To prove the hypothesis that OC2 transcripts are negatively regulated by mir-9 in rat NSCs, we cloned two of the predicted OC2 mir-9 MRE into the 3' UTR of a firefly luciferase gene (FL) in the pMir-Glo luciferase reporter plasmid to test bioactivity (Promega, Madison, WI) (Fig.13A). In addition to plasmids containing the wild type (WT) MRE sequences, we also constructed mutated versions of the MRE sequences by altering the seed binding region (MT) (Fig.13A). The pMir-Glo backbone has the advantage that it also expresses a renilla luciferase gene (RL) that will serve to normalize for transfection efficiency across samples. The luciferase reporter constructs were nucleofected into L2.3 multipotential NSC with PremiR-9, AntimiR-9 or a scrambled sequence, which would not target the reporter, as a negative control and assayed for luciferase activity 24 hrs after transfection (n=6) (Fig.13B). The WT construct assayed in the presence of PremiR-9 showed a reduction in the normalized FL/RL ratio (0.30 ±0.011; mean ± SEM) when compared to the negative control (1.00 ±0.033; mean ± SEM) (Fig.13B). This reduction was reversed when the WT construct was assayed along with AntimiR-9. The normalized FL/RL ratio (1.65 ±0.071; mean ± SEM) increased above the negative control (1.00 ± 0.033; mean ± SEM). Since the inhibition was reversed by addition of an antagonist, this increase above the negative control is due to the inhibition of endogenous mir-9 in the L2.3 NSCs. Furthermore, we observe no reduction in luciferase activity from the mutated form of the OC2 mir-9 MRE when we assay in the presence of PremiR-9. These results show that mir-9 post-transcriptionally

acts in trans to negatively regulate a cis OC2 mir-9 MRE. By regulating luciferase translation, we have shown that OC2 mRNA is capable of being inhibited by exogenous mir-9. Furthermore, by repressing endogenous mir-9, we have demonstrated bioactivity of the inhibitor. Taken together, this indicates that OC2, at least at one of the predicted target sites, is likely to be inhibited by mir-9 during neurogenesis.

Summary of Section A: Mir-9 canalyzation of rat NSC neuronal differentiation via regulation of OC2.

In this section we have identified specific miRNAs that can modulate neurogenesis in rat NSCs. Amongst these miRNAs is mir-9, which alone can enhance the neurogenic capacity of a multipotential rat NSC. We also identified and biochemically validated OC2 as a putative target of mir-9 during rat NSC differentiation. OC2 shRNA knock-down can enhance the neurogenic capacity of the rat NSCs, suggesting an anti-neuronal role for this gene. Interestingly, these results mimic the observations obtained when exogenously over-expressing mir-9. These data support the hypothesis that mir-9 serves to canalyze neuronal differentiation by inhibiting genes that would be deleterious for neuronal differentiation.

Section B.

Mir-9 regulation of OC2 during human NSC neuronal differentiation.

The studies performed in the rat NSC model system provided us with a good understanding of what we believed to be the role of mir-9 during neuronal differentiation.

Even though, the rat NSC clones were a simpler system to work with, we decided to pursue the rest of this project utilizing neuronal differentiation protocols in hESCs. One of the reasons we decided to change systems was because several experiments where technically impossible to perform in rat cells due to the lack of rat specific antibodies. This was not a problem in the human system. We also believed that results obtained from studies in human cells would be more relevant due to their applicability to potential therapeutic uses in individuals with spinal cord injuries or with neurodegenerative conditions.

Human H1 hESC NSC differentiation optimization.

To study the role of mir-9 and its relationship with OC2 during human NSC neuronal differentiation we first needed to optimize our protocols to reproducibly make high quality human NSCs. The optimization of this process was done in close collaboration with Dr. Jennifer Moore. We preferentially induced human NSC formation from H1 hESCs by inhibiting TGF-β signaling in the presence of noggin (Chambers et al., 2009; Zhou et al., 2010). To ascertain that the cells resulting from these cultures were true NSCs, expression of specific NSC markers were ascertained by FACS analysis. As a first pass we wanted to determine how homogeneous were these cultures by measuring the percentage of cells expressing the NSC markers Nestin and Musashi (Fig.14 A-B). Approximately 75% of the NSCs were Musashi positive, meanwhile H1 ESCs had only 2.5% of the cells positive for Musashi. We assayed for the NSC marker Nestin, which was expressed in approximately 50% of the NSCs (Fig.14A). Unfortunately, confirmation of the specificity of this antibody could not be confirmed because we would consistently see high levels of expression in cultures of

pure populations of undifferentiated H1 hESCs and cultures of differentiated NSCs (data not shown). Therefore, we continued to use Musashi as our primary NSC marker. We also measured the expression of Sox2, another marker that has been shown to be expressed in both ESCs and NSCs (Zappone et al., 2000; Avilion et al., 2003). We see that nearly all H1 hESCs express Sox2 and that this expression goes down to approximately 50% in NSCs (Fig.14A). This reduction in Sox2 indicates that even though most cells are positive for Musashi there is a heterogenous population of NSCs. Potentially these cells are at different stages of differentiation or it is possible that there are different populations of restricted precursor cells in these cultures. This issue will be addressed later in this thesis.

As another validation that these cells have differentiated to NSCs we assessed the levels of mir-9. As stated previously mir-9 has been shown to be expressed in proliferating neural progenitor cells. We ascertained the levels of this miRNA by qPCR. Mir-9 levels are tenfold higher in the NSCs than in the undifferentiated H1 hESCs (Fig.14C). This is a clear indication that these cultures for the most part have differentiated towards a neural lineage.

It is essential to confirm that these cells not only express NSC markers, but have also lost their pluripotency capacity. We wanted to ascertain if there was a population of cells expressing the pluripotency marker Oct4, which would be indicative of incomplete differentiation towards the ectodermal neural lineage. The absence of pluripotent cells was verified by FACS and qPCR (Zhang et al., 2001; Chambers et al., 2009; Moore et al., 2010). FACS analysis on H1 hESCs and NSCs showed that nearly 100% of H1 hESCs are positive for Oct4, meanwhile practically all NSC are devoid of the marker

(Fig.14A). As another test for the loss of pluripotency, we assessed by qPCR the levels of mir-302, an ESC specific miRNA. We see a major decrease in the levels of mir-302 upon NSC formation (Fig.14C). These results confirm that these cells have differentiated beyond the pluripotent hESC state.

After ascertaining that these cells have differentiated from a pluripotent hESC state to a neural lineage, we wanted to confirm that these cells have not completely differentiated to a mature neuronal phenotype. To test the levels of spontaneous differentiation we tested the percentage of cells that are positive for neuron specific enolase (NSE) and TuJ1 by FACS analysis. We see that H1 NSC cultures are devoid of NSE positive cells (Fig.16) and contain less than 20% of TuJ1 positive cells (Fig.14D). The absence of NSE indicates that there are no mature neurons in these cultures. Furthermore, the relatively low level of TuJ1 positive cells is not surprising because some hESC and hNSC tend to spontaneously differentiate and express the TuJ1 marker. This has been seen by immunostaining of the different culture conditions (data not shown). These TuJ1 positive cells are typically removed upon passaging of the cultures because they tend not to stick to the plate after the cell passage. We can conclude that these cell cultures have differentiated towards a neural ectodermal lineage and are probably NSCs. To confirm that they are truly NSC we need to show that these cells can give rise to the two major neural lineages, neurons and glia.

Human H1 NSC neuronal differentiation.

The overall goal of section B of the thesis was to identify the role mir-9 during neuronal differentiation and test if the negative regulation of OC2 by mir-9 was conserved across species. NSCs can give rise to three major neural cell types, neurons, astrocytes and oligodendrocytes. To enhance neuronal specific differentiation we first needed to optimize neuronal differentiation protocols. We used more than one neuronal differentiation method, with the idea that if the regulatory mechanisms are critical for neuronal differentiation then they would be observed in different differentiation protocols. Even though we will not address this topic in this project it is possible that the two differentiation protocols could lead to different subtypes of mature neurons, but we believe that the mir-9 regulation of OC2 is not limited to a specific subclass of neuron and is a general neuronal differentiation phenomena. Thus, it is important to compare more than one neuronal differentiation protocol to confirm that the observations that we make are general occurrences during neuronal differentiation and not mere artifacts caused by a specific protocol or neuronal subtype. To be able to test this hypothesis, we first needed to confirm the effectiveness of our neuronal differentiation protocols.

H1 NSCs were expanded in NPM media prior to differentiation. Approximately every three to four days when the H1 NSCs would reach ~90-95% confluence, the cells were passaged 1:3 or 1:4 depending on the density and plated in NPM. The following day the media was changed to one of two neuronal differentiation media The two neuronal differentiation media used are NDM (NDM-neurobasal media, 1x B27 and 10 ng/mL BDNF) or NBM+VPA (NBM+VPA -neural basal, 2% B-27, 1% N2, 1% ITS, 2 mM L-glutamine, 1 mM valproic acid (VPA)). Media was replaced every other day.

To confirm that these protocols were enhancing neuronal differentiation of the H1 NSCs, we tested differentiating cell populations for the appearance of neuronal markers and the reduction of neural stem cell markers. Yu and colleagues have shown that VPA treatment results in a strong inhibition of cell proliferation and induction of neuronal differentiation in the mouse embryo hippocampus (Yu et al., 2009). Thus, we first wanted to confirm that VPA serves as an enhancer of neuronal differentiation or at least enriches NSC differentiating cultures for neuronal phenotypes in our model system. To test this, H1 NSCs were passaged as described and plated in NPM media. A day after passaging media was changed to NBM or NBM+VPA. FACS analysis of TuJ1 stained cultures show that there is approximately a two fold increase in TuJ1$^+$ cells seven days after differentiation in NBM+VPA (36.00% TuJ1$^+$) media when compared to NBM (17.76% TuJ1$^+$) media (Fig.15A-B). Cultures treated with NBM+VPA showed an increase in the number of cells positive for Map2 (Izant and McIntosh, 1980), a mature neuronal maker (Fig.15A-B). The presence of Map2$^+$ cells in the NBM+VPA treated cultures signifies that VPA does not only enhance neuronal differentiation but also accelerates the differentiation process. As a side note, images of TuJ1-stained differentiated H1 NSCs show clear neuronal morphology (Fig.15C). These results mimic observations by Yu *et al.* (2009), in which VPA served to enhance neuronal differentiation.

To ascertain the efficiency of the neuronal differentiation protocols, we further characterized the cells produced upon differentiating. H1 NSCs were differentiated in NBM+VPA or NDM media and assayed by FACS analyses at seven days after the beginning of neuronal differentiation. Cultures were stained for Musashi and NSE. We expected these markers to be mutually exclusive because Musashi should identify

proliferating NSCs and NSE is a marker for differentiated neurons. Interestingly, we observed a very heterogeneous population of cells in the differentiating cultures. For example, cultures of H1 NSCs grown in NPM media showed that approximately 94% of the cells expressed Musashi and these cultures were essentially devoid of NSE positive cells in the gated fractions (Fig.16). As to be expected, seven days after differentiation Musashi levels drop in both differentiation conditions. We did not expect to see Musashi and NSE to be expressed in the same cells. In the NBM+VPA differentiation condition, we observed 38% of the cells stain solely for Musashi, 19% co-stain for both Musashi and NSE, and 3% stain uniquely for NSE (Fig.16). During NDM mediated differentiation we observe a similar trend, 54% of the cells stain solely for Musashi, 12% co-stain for both Musashi and NSE, and 1.5% stain uniquely for NSE (Fig.16). These results indicate that there is a transitional stage during neuronal differentiation where differentiating neurons co-express both of these markers.

To further define these populations of cells, we also measured levels of the proliferative marker Ki67 (Gerdes et al., 1983). We expected that cells that had begun to differentiate and were committed to a neuronal fate would exit the cell cycle and be devoid of Ki67. To test this hypothesis, we analyzed undifferentiated H1 NSCs expanded in NPM and seven day old differentiated H1 NSCs that were stained with NSE and Ki67 by FACS. To our surprise, we saw that our cultures of NSCs expanded in NPM only showed a 45% of the population to be positive for Ki67 (Fig.16). This indicates that more than 50% of the cells in these cultures have left the cell cycle and have begun to spontaneously differentiate, even though these cells are grown in the presence of FGF. These cells also show no positive staining for NSE (Fig.16) and very low levels of TuJ1 (Fig. 14D). When we analyze the seven day differentiated cultures

we see that there are fewer Ki67 positive cells in both the NBM+VPA and NDM differentiated cultures (Fig.16). We observe in NBM+VPA differentiated cultures that 10% of the cells stain solely for Ki67 and that there are 3% that costain for both Ki67 and NSE (Fig.16). During NDM mediated differentiation we observe a similar trend, 13% of the cells stain solely for Ki67 and .5% costain for both Ki67 and NSE (Fig.16). We had expected to observe many more Ki67$^+$ cells in the undifferentiated cultures grown in NPM. These numbers reflect that approximately 50% of the cells have exited the cell cycle but have not fully differentiated towards a neuronal phenotype. It is possible that if these cells were left in culture for an extended period of time they might reach a mature phenotype. Interestingly, the proliferating Ki67$^+$ cells do not take over the culture. Therefore, after each passage there is a percentage of NSCs that will leave the cell cycle, possibly due to spontaneous differentiation or an asymmetric cell division (Walczak et al., 2007). The co-expression of NSC and neuronal markers signify that during differentiation there is a transitional period even while the cell is still within the cell cycle.

To further validate some of these results we performed western blot analysis on the NBM+VPA differentiated H1 NSCs. Musashi protein levels decrease by day one after differentiation and remains down at three and seven days post differentiation (Fig.17). Furthermore, we see the inverse trend with TuJ1. TuJ1 protein continually increases until day seven post differentiation (Fig.17). The reduction of NSC specific markers, the evidence of cells leaving the cell cycle and an increase in neuronal markers makes us confident that the neuronal differentiation protocols are enhancing neuronal differentiation in the H1 NSCs. Therefore, these protocols serve as an efficient in vitro model of neurogenesis.

Mir-9 levels increase during NSC neuronal differentiation.

We have shown that mir-9 has a pro-neuronal role during neuronal differentiation of rat NSCs. This miRNA has been shown to have different roles depending on its temporal and spatial expression (Bonev et al., 2011; Shibata et al., 2011). In some mouse CNS cellular contexts, mir-9 exerts a pro-neuronal role and in others it serves to regulate proliferation (Shibata et al., 2011). Therefore we wanted to ask what the role of mir-9 is during human NSC neuronal differentiation. If mir-9 serves to modulate and canalyze neuronal differentiation in human NSCs, then we would expect to see an increase in mir-9 expression upon neuronal differentiation of H1 NSCs. To begin to test this hypothesis we assayed the levels of mir-9 during the differentiation of H1 NSCs. Mir-9 specific probes were used to determine changes in the relative abundance of mir-9 during H1 NSC neuronal differentiation by qPCR. We assayed H1 NSC samples grown in NDM, NBM, NBM+VPA or NBM+10%FBS for 1, 3 and 7 days after removal from NPM (Fig.18). Mir-9 levels increase by day 7 in all of these culture conditions, except for when the cells are grown in the presence of serum. This observation is consistent with the hypothesis that mir-9 acts as a pro-neuronal role because serum has been shown to promote non-neuronal phenotypes (Fig.7). Therefore, we conclude that when human NSCs are exposed to a neuronal differentiation environment there will be a population of cells in which mir-9 will be induced. Interestingly, mir-9 levels were higher in cells grown in NBM only, when compared to cells grown in the presence of VPA or NDM. The cells in NBM cultures continue to proliferate and over time start to spontaneously differentiate (data not shown). This observation could mean two things. First, the primary role of mir-9 in human NSC is to regulate their proliferative state. These results tend to correlate

with observations made by Delaloy *et al.* (2010). Delaloy and colleagues elegantly showed in neural progenitors derived from human ESCs, loss of miR-9 suppresses proliferation (Delaloy et al., 2010). In their model, loss of miR-9 promotes migration of neural progenitors but has no effect on differentiation. Secondly, it is possible that these cells begin to differentiate or become lineage restricted precursors, but due to the lack of the correct environmental cues in the media cannot complete the differentiation process. Therefore, it is possible that mir-9's biological role in human NSCs neuronal differentiation is different than the one observed in rat NSCs.

Exogenous expression of mir-9 in differentiating H1 NSCs reduces neuronal differentiation and retains cells in a NSC state.

Results show that mir-9 levels increase in H1 NSC cultures when they are removed from the NPM media containing FGF. Interestingly, mir-9 levels were higher in cells grown in NBM only, a condition in which the cells continue to proliferate but show low levels of spontaneous neuronal differentiation, even in the absence of neurogenic stimulators. These data contrast with our previous observations in rat NSCs, where mir-9 expression increases in cells that are differentiating and have stopped dividing. This led us to question the role of mir-9 in H1 NSCs. If mir-9 serves to modulate or enhance neuronal differentiation as was seen in rat NSC, then we would expect to see an increase in TuJ1$^+$ cells and a decrease in Musashi$^+$ cells when we exogenously express mir-9. On the other hand, if the primary role of mir-9 is to regulate the proliferation, as was shown by Delaloy *et al.* (2010), then we would see the opposite effect when exogenously expressing mir-9. To shed light on the role of mir-9 in H1 NSCs, we

decided to take a gain of function approach and over-express mir-9 in the H1 NSCs. We nucleofected H1 NSCs with PremiRs (Ambion, Austin, TX) for mir-9 or negative control scrambled sequence for comparison. The cells were plated in NPM and left to recover overnight. The next day medium was changed to NBM or NBM+VPA to induce differentiation. Seven days after removal from NPM medium, cells were stained for TuJ1 and Musashi and assayed by FACS analysis. As expected, cultures grown in NBM+VPA had more TuJ1$^+$ cells and less Musashi$^+$ cells than cultures grown in NBM only (Fig.19). This showed that differentiation had occurred as predicted. Comparing NBM cultures of the mir-9 nucleofected cells to NSCs nucleofected with a scrambled negative control, the exogenous mir-9 leads to a slight but significant increase in the number of Musashi$^+$ cells and a decrease in the number of TuJ1$^+$ cells (Fig.19). This indicates that mir-9 in H1 NSCs serves to retain the cells in a progenitor state. Interestingly, this phenomenon was not observed in the cultures that were grown in NBM+VPA. We could speculate that in this experiment the effects of VPA overcome the pro-proliferative role that exogenous over-expression of mir-9 has during differentiation. This effect shows that miRNAs serve to modulate and fine tune biological states and not drive a particular outcome.

OC2 expression levels decrease upon neuronal differentiation.

Previously, we showed that OC2 serves an anti-neuronal role in rat NSCs. If OC2's role is conserved across species we would expect that the OC2 genes would exhibit a high degree of homology. Alignment of the protein sequences from the mouse, rat and human OC2 genes show conservation and suggest that they are orthologs

(Fig.20). The functional domains consisting of the TP Box, the cut domain and the homeo box are 100% conserved. The TP box has been shown to contribute to transcriptional activity, while the cut domain and the homeo box are DNA binding domains (Lannoy et al., 2000). Therefore, we can predict that the biological roles of OC2 are potentially preserved in human cells.

If OC2's anti-neuronal role is conserved during human neuronal differentiation, then we would expect to see a decrease in the levels of OC2 upon neuronal differentiation of human NSCs. A drop in OC2 protein levels at day one of the neuronal differentiation protocol was determined by western blot analysis (Fig.17). OC2 protein levels remain low up to seven days into neuronal differentiation. This result is consistent with the hypothesis that OC2 has an anti-neuronal role during NSC differentiation. Interestingly, when we assayed for mRNA levels of OC2 we see a contradictory trend. MRNA levels of OC2 rise upon differentiation of the NSCs (Fig.21A). This opposing trend would suggest that there is a post-transcriptional regulatory mechanism that is disrupting protein output. It is possible that this reduction in the protein level of OC2 is mediated by a post-transcriptional mir-9 regulation similar to the one presented previously in the rat NSC's.

To test the relationship between the mRNA levels of OC2 and levels of mir-9 after 7 days in different neuronal and non-neuronal differentiation protocols we plotted the RRQ values from qPCR results (Fig. 18 and 21A) on a scatter plot and performed a linear regression analysis to determine the correlation coefficient (Fig. 21B). The R^2 equaled 0.2615 indicating that there was a negative correlation between the levels of mir-9 and OC2 RNA across the different protocols. Even though this analysis did not reach statistically

significance; there was a clear trend which indicated that mir-9 expression does not correlate well with OC2 mRNA expression. This analysis fortifies the hypothesis that human OC2 mRNA is potentially regulated by mir-9 in a similar manner as we showed in rat NSCs.

OC2 mRNAs associate specifically with the RISC complex via Ago1 and Ago2.

Our previous results in rat NSCs showed that OC2 mRNAs are post-transcriptionally regulated by mir-9. If OC2 mRNAs are regulated by miRNAs in human NSC, then we would expect to see OC2 mRNA associated with the RISC complex. This association would confirm that OC2 transcripts are found in a complex known to suppress translation, consistent with a model of being post-transcriptionally regulated, by mir-9 and/or other miRNAs. RNA immunoprecipitations (RIP) of Ago1 and Ago2, components of the RISC complex, were done to show this association. There are four human Ago proteins (Ago1-4). Interestingly, studies regarding the miRNA binding specificity of these proteins have presented contradicting results. Some studies have shown that each Ago binds miRNAs without selectivity (Meister et al., 2004a; Landthaler et al., 2008), but a more recent study has shed evidence suggesting some preferences (Burroughs et al., 2011). We decided to test more than one Ago proteins to avoid the possibility of any selectivity.

To identify components of RISC complexes it was important to demonstrate that Ago antibodies would immunoprecipitate their epitope-containing proteins. RIP protocol optimization was critical to obtain the desired results. When these experiments were started there were no reliable commercially available Ago1 and Ago2 specific antibodies that had been shown to work for RIPs. After testing several antibodies, we found that

the anti-Ago1 (Abcam, #ab5070, Cambridge, MA) and anti-human Ago2 (11A9; Ascenion GmbH, Helmholtz Zentrum, München) worked best with our protocols. The Ago1 and Ago2 antibodies were tested for specificity by immunoprecipitations followed by western blotting, which produced bands of approximately 100 kDa corresponding to either Ago1 and Ago2 (Fig.22A-B). These assays show the specificity of the antibodies and their capacity to IP Ago proteins.

In order to detect specific mRNA and miRNA components of the immunoprecipitated RISC complexes, we tested whether Ago IPs would retain these molecules after elution. We needed to optimize various parameters, including the wash, elution and cross-linking conditions until we found a protocol that suited our experimental needs. Several aspects of the finalized protocol are based on the PAR-CLIP protocol developed by Dr. Markus Hafner from Dr. Tom Tuschl's lab (Hafner et al., 2010). The final optimized protocol (Fig.23) is described in detail in the method section.

As stated previously when we began this project there were no reliable Ago antibodies for RNA immunoprecipitations, therefore a good portion of the initial optimization of the RIP protocols were done using a myc-tagged Ago2 construct which were transfected into 293T cells. By using the tagged protein we could use an anti-myc-tag specific antibody instead of the Ago specific antibody to pull down RNAs that were associated with the RISC complex. As a confirmation that our RIP protocol was pulling down RNAs associated with the RISC complex we used a control mRNA that was known to be regulated by a specific miRNA. The control mRNA was processed from a pRL plasmid containing a renilla gene fused to the 3' UTR of c-Myc with wild type or mutated

forms of a let-7 MRE (Kumar et al., 2007). We confirmed that these are regulated in 293T cells as seen in a luciferase assay (Fig.24A).

293T cells were cotransfected with the WT or MT pRL-c-Myc-3' UTR plasmids and with the myc-Ago2 plasmid and. These cells were then used for RIP assays using an anti-myc tag antibody. As a negative control 293T cells were only transfected with the pRL-c-Myc-3' UTR plasmids. Without a myc-tagged Ago2 protein there should be no pull down of miRNAs or mRNAs. This would measure background levels of non-specific binding to the beads and antibody. Eluted fractions were assayed for the presence of miRNAs and mRNAs. We first assayed for the presence of let-7, a miRNA regulator of the c-Myc 3' UTR, by qPCR and determined that it selectively was retained in the eluted fractions from cells that were transfected with the myc-Ago2 plasmid but was absent from the cells that were missing the tagged Ago2 proteins (Fig.24B). To confirm that the RIPs were also capable of selectively pulling down mRNAs we assayed the eluted fractions for the presence of mRNAs for the renilla gene that were fused to the 3' UTR of c-myc which contains a let-7 MRE. QPCR results show that we were able to retain mRNAs in the eluted fractions (Fig.24B). This confirmed that our protocol worked for RIPs. We later started to use an Ago2 specific antibody (11A9; Ascenion GmbH, Helmholtz Zentrum, München) and comfirmed that they were able to retain miRNAs (Goff et al., 2009).

To show that OC2 mRNAs are associated to the RISC complex during H1 NSC neuronal differentiation we RNA immunoprecipitated with anti-Ago1 and anti-Ago2 antibodies. Because the reduction in OC2 protein begins within 24hrs of neuronal differentiation we speculated that the post-transcriptional regulation of OC2 mRNAs

would occur early during the differentiation protocol. To test this hypothesis, we RNA immunoprecipitated within ~14 hours after the beginning of neuronal differentiation. Therefore, we added the 4-thio-uridine to the neuronal differentiation media when we began the neuronal differentiation. After the elution step of the RIPs, RNA was extracted and assayed for the presence of OC2 mRNAs by qPCR. The mRNA abundance was calculated by comparing to a standard curve prepared from dilutions of the input sample that was retained prior to the RIP. This method allowed us to determine enrichment of a particular mRNA in the RIP samples with either Ago1 or Ago2 antibodies relative to their respective isotype control.

QPCR for OC2 mRNAs from a single RIP shows that this transcript was enriched in eluted fractions from samples immunoprecipitated with Ago1 or Ago2 antibodies relative to their respective isotype negative control (Fig.25 and 26). As a positive control, we tested for enrichment of the anti-neural REST mRNA, which has been previously shown to be regulated by mir-9 and mir-124 during neuronal differentiation (Conaco et al., 2006; Visvanathan et al., 2007; Packer et al., 2008; Laneve et al., 2010). The anti-neural REST mRNA was found to be enriched in the RIP samples when compared to the isotype control (Fig.25 and 26). As a negative control, we assayed for the presence of GAPDH mRNA in the Ago RIP samples. GAPDH mRNAs are not predicted to be regulated by any of the well characterized neural miRNAs and should not be associated with the RISC complex or any of the Ago proteins. We did not see an enrichment of GAPDH mRNA in the Ago RIP samples relative to their isotype control (Fig.25 and 26).

These data just presented were based on one single RIP. To measure biological reproducibility and significance of this phenomena we repeated the Ago2 RIPs with new differentiating cultures (n=3). The Ago2 RIPs showed a statistically significant enrichment for OC2 mRNAs when compared to an IgG control (Fig.27). REST mRNA appeared to be enriched in the Ago2 RIPS but did not reach our threshhold to consider it statistically significant. Meanwhile, there was no enrichment of GAPDH mRNAs in the Ago2 RIPs when compared to IgG. When we repeated the Ago1 RIPs we were not able to determine statistical significance (data not shown). Therefore, we can conclude that OC2 mRNAs are consistently associated with Ago2 proteins during neuronal differentiation of H1 NSCs. Regarding Ago1, we cannot confirm that there is enrichment of OC2 mRNAs coupled with protein. The selective loading of specific RNAs into the different human Ago proteins is a hotly debated topic. Evidence for selective or non-selective loading of miRNAs into the Ago proteins has been reported. Some studies have shown that each Ago binds miRNAs without selectivity (Meister et al., 2004a; Landthaler et al., 2008), but a more recent study has shed evidence suggesting some preferences (Burroughs et al., 2011). It is possible that this occurs with the mRNAs as well. If there is selective loading, this would explain the statistically non-significant results obtained with the Ago1 RIPs.

The association between Ago2 and OC2 mRNA most likely leads to a post transcriptional regulation that would explain the opposing levels of OC2 mRNA and protein. This regulation would serve to reduce OC2 protein levels in NSCs during neuronal differentiation even though there is mRNA present. This is a clear example of how miRNAs serve to canalyze biological processes such as neuronal differentiation by removing genes that would be deleterious to a particular biological outcome.

OC2 mRNAs are post-transcriptionally targeted and negatively regulated by mir-9 in human H1 NSCs.

We have shown that OC2 mRNA is associated with Ago1 and Ago2, and is post-transcriptionally regulated during H1 NSC neuronal differentiation, but to test the hypothesis that this regulation is specifically mediated via mir-9 several approaches were taken. First, we tested if exogenous expression of mir-9 would alter the endogenous levels of OC2. If OC2 is targeted by mir-9 in these cells we would expect that exogenous mir-9 would cause a decrease in the expression of OC2, particularly at the protein level. To exogenously express mir-9 in the H1 NSCs, cells were transfected with mir-9 PremiRs. OC2 RNA levels were assayed by qPCR. We observed a reduction in the abundance of the human OC2 mRNA in samples treated with PremiR-9 (0.483 ±0.035; mean ± SEM) when compared to cells treated with a scrambled negative control (1.000 ±0.207; mean ± SEM) (Fig.28A). Furthermore, when protein abundance was assayed by western blot analyses, a large reduction in OC2 protein is observed in samples treated with the mir-9 mimic (Fig.28B). These observations show that when H1 NSCs are treated with exogenous mir-9 there is a decrease in the level of the OC2 protein. The previous results showing that OC2 mRNA is associated with Ago2 supports the observation of reduced levels of OC2 mRNA upon exogenous expression of mir-9 because Ago2 is the only Argonaute with RNA slicing capacity (Meister et al., 2004b).

As a secondary experimental method to confirm the hypothesis that OC2 transcripts are directly targeted by mir-9 in H1 NSCs, we cloned two of the predicted

OC2 mir-9 MREs into the 3' UTR of a FL gene in the pMir-Glo luciferase reporter plasmid (Promega, Madison, WI) to test their capacity to be regulated in human NSCs (Fig.28C). In addition to the constructs containing the WT MRE sequences, we also constructed mutated versions of the MRE sequences by mutating seed binding region (MT) (Fig.28C). The pMir-Glo backbone has the advantage that it also expresses a RL gene that will serve to normalize for transfection efficiency across samples. The luciferase reporter constructs were nucleofected into H1 NSCs with PremiR-9, AntimiR-9 or a scrambled sequence as a negative control and assayed for luciferase activity 24hrs after transfection (n=6) (Fig.28D). To test if the predicted OC2 mir-9 MRE is regulated by the endogenous mir-9 in the H1 NSCs, relative luciferase activites from samples transfected with either the WT or the MT reporter were compared (Fig.28D). There is a 27% reduction in the activity of the WT version of the OC2 mir-9 MRE when compared to the MT (p-value<0.001). This signifies the MRE that was cloned into the reporter has a negative effect on the reporter activity. To test if this negative effect is directly responsible by mir-9 we assayed the WT MRE reporter activity when regulating mir-9. When the WT OC2 mir-9 MRE construct was assayed in the presence of exogenous PremiR-9 there was a 42% reduction in luciferase activity when compared to the negative control (p-value<0.001) (Fig.28D). This reduction was reversed when the WT construct was assayed with the antagonist AntimiR-9. These results show that mir-9 post-transcriptionally acts in trans to negatively regulate a cis OC2 mir-9 MRE in human H1 NSCs. The use of these two approaches in combination confirms that the regulation of OC2 by mir-9 is not mediated by indirect regulatory mechanisms. Along with the previous data that shows that OC2 mRNAs associate selectively to the RISC complex via Ago 1 and Ago2, we can conclude that OC2 mRNAs are negatively regulated by mir-

9 during human neural differentiation. This inhibition is an example of how miRNAs serve to modulate and canalyze neuronal differentiation.

Summary of Section B: The role of mir-9 in human H1 NSC neuronal differentiation.

In this section we presented an optimized culture method to derive populations of NSCs from H1 hESCs using Noggin to inhibit TGF-β signaling (Chambers et al., 2009). We show that VPA, a HDAC inhibitor, serves to enhance neuronal differentiation of NSCs, suggesting that changes in histone acetylation patterns are critical for neuronal differentiation. Subpopulations of cells expressing different combinations of proliferation and/or differentiation markers in cultures of neuronal differentiating cells were identified. The combined expression of differentiating and proliferating markers is a clear indication that differentiation is a "fluid" process and that the epigenetic changes regulated by VPA occur in a stepwise manner. Using this neuronal differentiation protocol we were able to test the role of mir-9 during neuronal differentiation. Mir-9 regulates H1 NSC proliferation and differentiation. Exogenous expression of mir-9 is capable of enhancing the pluripotent state of H1 NSCs, observed by an increase in Musashi$^+$ cells and a decrease in TuJ1$^+$ cells. These results suggest that there is possibly a cross talk between epigenetic regulation and miRNA regulation. In addition, OC2 was confirmed among the genes that are post-transcriptionally regulated and that OC2 mRNA is associated with the RISC complex during the differentiation process. OC2 mRNA was also biochemically validated as a target of mir-9 during H1 NSC differentiation, confirming that this regulation is conserved across rat and human. In this section, we have shown that even though mir-9's sequence is perfectly conserved across species

there are context specific differences that lead to it having different roles in rat and human NSCs.

III. DISCUSSION

Pluripotent stem cells hold immense promise for regenerative medicine due to their self renewal and potential for differentiation. A major limiting factor to utilizing stem cells as a treatment for neurological conditions, such as spinal cord injury, is the uncertainty of the fate of the cells once transplanted. Stem cells have the ability to differentiate into a wide range of cell types or to continue to proliferate without differentiating into a mature phenotype (Lin and Schagat, 1997). Unchecked proliferation and/or differentiation could lead to several adverse outcomes such as tumor and teratoma formations (Reviewed in (Li et al., 2008b)). Therefore, if we intend to harness these cells for therapeutic treatments we will need to overcome the uncertain fate these cells exhibit in their undifferentiated state. This can be done partially by utilizing more restricted cells that have been differentiated to a lineage specific precursor stage. To reliably obtain large quantities of pure populations of lineage-restricted precursors for transplantation purposes, we must understand their internal cell mechanisms and programming. This will lead to methods for stabilizing restricted differentiation and/or promoting differentiation towards desired phenotypes. Understanding the molecular mechanisms that control lineage specific neural differentiation is critical for optimizing protocols to obtain cells suitable for transplantation. As this research progresses, the roles of small non-coding RNAs must not be ignored. In this thesis we have added to the collective knowledge about the role of miRNAs during NSC proliferation and neuronal differentiation. This information will be critical for the advancement of clinical applications for cell transplantation therapies for neurological conditions, such as spinal cord injury.

The role of miRNAs in NSC maintenance and neuronal differentiation.

Several miRNAs identified in rat NSC serve to enhance the neuronal capacity of these cells upon neuronal differentiation. Exogenous over-expression of all four miRNAs (mir-9, 124, 153 and 182) together caused an increase in the neurogenic capacity of rat NSC upon differentiation. We speculate that these miRNAs serve to canalyze neuronal differentiation by targeting and inhibiting genes that would be deleterious to a neuronal phenotype upon neuronal differentiation or genes that would be required for NSC maintenance.

The idea that miRNAs serve to canalyze biological processes was originally proposed by Eran Hornstein and Noam Shomron (Hornstein and Shomron, 2006). They hypothesize that miRNAs interacting with the network of protein-coding genes evolved to buffer stochastic perturbations and thereby confer robustness to developmental genetic programs. At the root of their hypothesis, they suggest that miRNA regulatory networks evolved under natural selection in order to stabilize phenotype and decrease the variability of specific traits. This view of the proposed role for miRNAs arose from Waddington's original canalization hypothesis (Waddington, 1959). This hypothesis can be extrapolated beyond populational evolution and can be attributed to cellular processes such as NSC differentiation. MiRNAs serve to channel differentiation to a specific phenotypic outcome. In the case of neurogenesis, it is possible that specific groupings of neurogenic miRNAs would serve to inhibit genes involved with alternate phenotypes. Mir-9 and mir-124 have been shown to target anti-neural and anti-neuronal genes. For example, these miRNAs inhibit the anti-neural REST/SCP1 pathway, allowing for neurogenesis to occur (Visvanathan et al., 2007; Packer et al., 2008). Mir-9 also inhibits the orphan nuclear receptor TLX (Zhao et al., 2009), which is essential for

NSC proliferation (Shi et al., 2004). These are two examples of how miRNAs serve to canalyze a particular outcome upon differentiation of NSCs.

The neurogenic miRNAs were also individually over-expressed in rat NSCs. A significant increase in the percentage of TuJ1$^+$ cells after exogenous expression of mir-9 and mir-153, but not mir-124 and mir-182 was observed. Interestingly, over-expressing the mixture of neurogenic miRNAs did not yield a synergistic increase in the percentage of TuJ1$^+$ cells suggesting that redundancy exists among these particular miRNAs. Studies have shown that the role of individual miRNAs is limited during development due to compensatory or redundant roles of other expressed miRNAs   (Plasterk, 2006; Miska et al., 2007; Wang et al., 2008). We speculate that these miRNAs regulate neurogenesis by targeting the same genes or genes that are in common pathways. This suggests that there are multiple miRNAs that would serve redundant roles that would promote robust regulatory mechanisms to canalyze a particular outcome. This point will be addressed in more detail later in the discussion.

We were surprised that mir-124 was not able to enhance the neurogenic capacity of the differentiating rat NSCs because previous studies have shown mir-124 to stimulate neurogenesis in the sub-ventricular zone (Cheng et al., 2009). Another study has shown that mir-124 along with mir-9* serves to repress BAF53a during neuronal differentiation (Yoo et al., 2009). BAF53a (also known as ACTL6a) is a subunit within Swi/Snf-like neural-progenitor-specific BAF (npBAF) complexes. For complete development of post-mitotic neurons, BAF53a needs to be replaced by the homologous BAF53b (ACTL6b) subunit within the neuron-specific BAF (nBAF) complexes (Yoo et al., 2009). This switch regulates an ATP-dependent chromatin-remodelling mechanism that

coincides with the final mitotic division of neurons. We speculate that the opposing result with mir-124 can be attributed to different cellular contexts. The cellular environment will determine the functionality of a specific miRNA to modulate a particular phenotypic outcome. MiRNA counteracting RNA-binding protein (RBP) are an example of how the cellular environment or context could influence the regulatory role of miRNAs. Dead end 1 (Dnd1), an evolutionary conserved RBP, negates the function of several miRNAs in human cells by binding to target mRNAs and blocking the access of miRNAs (Kedde et al., 2007; Kedde and Agami, 2008). The presence or absence of MREs in the mRNAs can be regulated by alternative splicing, and lead to changes in the miRNA regulation (Pietrzykowski et al., 2008). Therefore, changes in the environment which could block or remove a MRE from being targeted would control the relevance that a miRNA would have in a particular cell context. We have seen that serum cancels any affect exogenous expression of miRNAs would have on rat NSCs (data not shown). Morphogens such as growth factors are probably the main driving force behind the different phenotypic outcomes upon NSC differentiation. These will lead to the activation of signal transduction pathways and enhanced expression of specific transcription factors that would drive a particular differentiation pathway. In other words, if critical pro-neuronal transcription factors are not expressed during NSC differentiation, it would not matter if we increase the levels pro-neuronal miRNAs since the cell will not be able to differentiate towards a neuron without the transcription factors. This exemplifies how miRNAs probably do not drive neurogenesis or any other biological process but serve to modulate the cellular environment to make it favorable for a particular outcome.

The context-specific roles of miRNAs are clearly seen with mir-9. We have shown that mir-9's function in rat NSCs is to modulate and canalyze neuronal

differentiation. On the other hand, we have also shown that mir-9 promotes a proliferative and NSC state in human H1 NSCs. The sequence of mir-9 is perfectly conserved across species. What would explain these apparently opposing roles? Again, we must take into consideration the context in which the miRNA is acting. For a miRNA to have the same regulatory role across species its targets need to be conserved and have the same functions. It is possible that MREs of specific targets are not conserved and that would explain why a miRNA would have different roles in different species. Bioinformatic methods have identified a significant number of experimentally determined non-canonical and non-conserved MRE sites (Betel et al., 2010). This illustrates how one cannot assume that because the sequence of a miRNA is conserved its functions will be conserved across species.

Mir-9 has been shown to have different roles within the same organism. Bonev *et al.* (2011) have shown that mir-9 is expressed in neural progenitor cells of *X. tropicalis*, and its knockdown results in an inhibition of neurogenesis along the anterior-posterior axis. However, the underlying mechanism differs--in the hindbrain, progenitors fail to exit the cell cycle, whereas in the forebrain they undergo apoptosis, counteracting the proliferative effect (Bonev et al., 2011). In a more recent study, null-mir-9-2/3 mice show that mir-9 has multiple roles depending on the temporal-spatial context in the developing brain (Shibata et al., 2011). These animals show an increase in progenitor cells in the subpallium, meanwhile, there is a decrease in proliferation of progenitor cells in the sub-ventricular zone at a later developmental stage. Shibata *et al. (2011)* propose that mir-9 functions are modulated by RBPs such as Elavl2 and Msi1 in the WT animal. Elav2 associated with Foxg1 3′ UTR, and it countered the Foxg1 suppression by miR-9. These study illustrated that the spatial context in which mir-9 is expressed will determine

its biological role within an organism. These data illustrate how important the cellular context is to the biological activity of a particular miRNA.

Our results show that addition of mir-9 to differentiating H1 NSC cultures causes a slight but significant increase in the number of Musashi$^+$ cells and a decrease in the number of TuJ1$^+$ cells (Fig.19). These observations indicate that mir-9 serves a role to maintain cells in a proliferative and NSC state in human H1 NSCs. This is consistent with reports from other groups. Delaloy *et al.* (2010) showed that in neural progenitors derived from human ES cells, loss of miR-9 suppresses proliferation and promotes migration of neural progenitors but has no effect on differentiation. They show that the role of mir-9 in hNSC is largely attributed to its negative regulation of stathmin, which promotes microtubule instability (Delaloy et al., 2010). The notion that mir-9 serves to enhance a NSC state seem to be counterintuitive because upon placing the H1 NSCs into neuronal differentiation media we observe an increase in the levels of mir-9 (Fig.18). We speculate that this increase in mir-9 is not attributed to the differentiating NSCs but the cells that have failed to differentiate and remain in a NSC state. We show that the cultures of NSCs and differentiating neurons consist of heterogeneous populations of cells. Even though, these cells are in differentiation media there still is a population of cells that are proliferating and Musashi positive (Fig.16). It is technically difficult to conclude which cells are specifically expressing mir-9 and at what levels they are expressing mir-9. Delaloy *et al.* showed that mir-9 was expressed not only in human NSCs but in differentiated Map2$^+$ neurons and S100β$^+$ astrocytes by in situ hybridization (Delaloy et al., 2010). Unfortunately, they did not assess if there were differences in the levels of mir-9 in these different cell types. We have tried a similar approach to identify mir-9 expressing cells in differentiating H9 NSCs but did not have success (data not

shown). A way of circumventing the obstacles we have encountered would be to develop a stable hESC line with a reporter for mir-9 activity. This reporter system would be beneficial to measure mir-9 activity during NSC and neuronal differentiation. It is possible that mir-9 has multiple roles and these roles are dependent not only the cellular context but on the fine tuning of the levels of mir-9. With a quantitative output from mir-9 reporter system (GFP or Luc), we would be able to match mir-9 levels to a biological role.

A possibility that was not addressed in this study is that mir-9 could serve to partially differentiate and maintain the NSCs as neuronal restricted precursors (NRP), a cell that will only differentiate towards neurons but still has the capacity to proliferate. In rat cells the transition through this stage might not be evident because of the efficiency of the differentiation conditions. Meanwhile, in our model of human NSCs, the growth conditions cause the cells to differentiate slower than and not as efficiently as the rat progenitor cells. Therefore, when we treat the human NSCs with mir-9, we might be increasing the number NRP cells in the culture which probably would be Musashi[+]. We only looked at NSC and neuron markers but did not focus on any of the NRP markers such as PSA-NCAM. It would be interesting to focus on more specific cell populations within the heterogeneous cultures to determine if this is the case with the human cells. Mir-9 might have a conserved role across species to regulate the formation of restricted precursor cells and by default increase the number of neurons that are formed. This possibility needs to be further addressed in future experiments.

MiRNAs have been shown to exert a fairly modest effect on the overall gene expression of miRNA genes. Most miRNAs induce less than twofold changes in target

gene expression (Baek et al., 2008; Selbach et al., 2008). Changes in the levels of mir-9 most likely serve as an important buffering system to ensure the precision of gene regulation and fine-scale adjustments to protein output. We speculate that fluctuations in the levels of mir-9 cause the cellular environment to shift from a NSC state to a more neuronal state; in which mir-9 is important for buffering both phenotypic states. Future work is needed to identify molecules and conditions that regulate the activity of mir-9. By illustrating that the role of mir-9 is context dependent, we have added important input to the collective knowledge of the role of mir-9 during NSC differentiation, which will help to develop protocols to regulate human NSC expansion and differentiation.

Epigenetic regulation of neuronal differentiation

We show that VPA, a HDAC inhibitor, serves to enhance neuronal differentiation of NSCs, suggesting that changes in histone acetylation patterns are critical for neuronal differentiation. These results confirm a study from Yu and colleagues, in which they shown that VPA treatment results in a strong inhibition of cell proliferation and induction of neuronal differentiation in the mouse embryo hippocampus (Yu et al., 2009). Because VPA is thought to globally inhibit HDACs and not specifically regulate the acetylation pattern of any particular gene, we can speculate that in these cells pro-neuronal genes are "set" or "primed" to readily be activated by the inhibition of the HDAC inhibitor. This regulation probably works in a context specific manner. For example, if we were to treat glial restricted precursors with VPA it is possible that the chromatin state of the cell would prefer glial differentiation instead of neuronal differentiation. Therefore, the epigenetic changes that are caused by VPA do not act alone but in unison with other epigenetic marks such as methylations, glycosylations and ubiquitination. The

combination of all the marks would create a signature that would determine phenotypic fate upon differentiation. VPA treatment could possibly just accelerate the activation of genes that are already poised to push the cell towards a specific differentiation outcome by relaxing the chromatin structure of specific genes and allowing transcription activation.

Recently, relationships have been proposed between microRNA regulation and epigenetic marks (see reviews: Chuang and Jones, 2007; Saetrom et al., 2007; Iorio et al., 2010). HDAC inhibition leads to rapid changes in microRNA expression (Scott et al., 2006). For example, misregulation of the transcription of mir-9 in colorectal cancer has been associated to epigenetic alterations (Bandres et al., 2009). HDAC inhibitors can reestablish transcriptional activity of different mir-9 loci in colorectal cancer cell lines (Bandres et al., 2009). Interestingly, we see an increase of mir-9 one day after VPA treatment but then this effect is lost by three days after the initiation of differentiation (Fig.18). In fact we observe a decrease in the levels of mir-9 in cultures that are treated with NBM+VPA at three and seven days into the neuronal differentiation process. This would suggest that there are other factors besides the acetylation status of the mir-9 genomic location that also regulate the expression of mir-9.

Alternatively, it is possible that VPA does not truly enhance neuronal differentiation but only enriches the cultures for neuronal cells. We did not fully address this in this thesis because for our experimental needs all we needed was a way of assaying a more homogeneous cell culture population of neurons after NSC differentiation. Even with VPA we still did not obtain a completely homogeneous population in our differentiating cultures. The combined expression of differentiating and

proliferating markers (Fig. 16) is a clear indication that differentiation is a "fluid" process and that the epigenetic changes during neurogenesis occur in a stepwise manner.

OC2 is a target of mir-9 during NSC differentiation.

   To the best of our knowledge, our results are the first to illustrate a functional role for OC2 in differentiating NSCs. We have confirmed that OC2 serves an anti-neuronal role during the differentiation of rat NSCs derived from embryo fore-brains and that this gene is post-transcriptionally regulated by mir-9 in both human and rat NSC neuronal differentiating cells. The developmental roles of the OC family of transcription factors have been primarily associated with endodermal development, specifically cell differentiation in liver and pancreas (Jacquemin et al., 2003a; Briancon et al., 2004; Hara et al., 2007; Matthews et al., 2008). Very few studies have looked at the role OC2 and the other family members in the CNS. OC2 expression is present across most of the developing mouse CNS (Jacquemin et al., 2003b). The developmental timing of the expression of OC2 coincides with both neurogenesis and gliogenesis in mouse embryos (Jacquemin et al., 2003b). Interestingly, the fly and sea squirt orthologs of OC2 are expressed exclusively in the nervous system (Nguyen et al., 2000; Sasakura and Makabe, 2001). A more recent report by Francius and Clotman show a correlation between the expression of OC family members and a subpopulation of newly-born and differentiating spinal motor neurons within the four motor columns of the mouse spinal cord (Francius and Clotman, 2010). There has been no conclusive study that has looked beyond gene expression patterns of OC2 or any other member of the OC family in the CNS. Studies have primarily focused on associations of gene expression by in

situ hybridizations or immunohistochemistry, and have neglected to identify the functional roles of OC2 in neural tissue.

Our studies show that OC2 has an anti-neuronal role during rat NSC differentiation. ShRNA knockdown of OC2 increases the neurogenic capacity of the multipotential rat L2.3 NSC. The increase in the number of TuJ1^{+} cells demonstrates that OC2 serves a role during the early differentiation process to regulate the phenotypic fate of the cell. At three days post differentiation we observe that there is an increase in the number of TuJ1^{+} cells at the expense of Nestin^{+} cells. At a later time point (5days) we see that the increase in the TuJ1^{+} cells is at the expense of GFAP^{+} cells. These results mean that the anti-neuronal role of OC2 could be due to a potential pro-glial role during differentiation. The decrease in the number of GFAP^{+} cells upon knock down of OC2, and the increase in expression of OC2 in the rat multipotential NSC clone, L2.3, during differentiation which is not evident in the NRP clone, L2.2, would support this hypothesis. OC2 most likely is working as a molecular switch that would help determine the final phenotypic fate of a cell during early differentiation. In the same manner that the anti-neural REST/SCP1 pathway serves to regulate neural fate, OC2 might serve to regulate NSC differentiation.

The OC family has been shown to regulate the transcriptional activity of Ngn3 and FoxA2 during endodermal cell differentiation (Landry et al., 1997; Jacquemin et al., 2000). Both of these genes have roles in regulating glial differentiation (Liu et al., 2002; Lee et al., 2003; Norton et al., 2005), specifically oligodendrocyte differentiation. Interestingly, these potential downstream targets of OC2 are regulated during NSC differentiation. We interrogated the mRNA expression of Ngn3 and FoxA2 during

differentiation of the multipotential rat NSC clone L2.3 and the NRP clone L2.2 (data not shown). Neither of the genes increases their expression in the neurogenic L2.2 clone during differentiation. Meanwhile, both Ngn3 and FoxA2 show incremental expression during the differentiation of the multipotential NSC clone, L2.3. As mentioned previously, OC2 is induced during the differentiation of the L2.3 NSCs but not in the L2.2 NRPs. We speculate that OC2 is regulating Ngn3 and FoxA2 during NSC differentiation. This regulation would support a role for OC2 during glial differentiation which would reinforce the anti-neuronal role we observed in our assays.

OC2 mRNAs post trancriptional regulation during NSC neuronal differentiation was confirmed by demonstrating that OC2 mRNAs are associated with the RISC complex. We also biochemically validated OC2 as a target of mir-9 during NSC differentiation in both rat and human cells. In differentiating multipotential rat NSCs we observe similar effects when we exogenously express mir-9 or shRNA knock-down OC2. We speculate that by knocking down OC2 in differentiating rat NSCs we are actually mimicking or enhancing the role of mir-9 during the differentiation process. If this is the case then OC2 is one of the critical targets of mir-9 during the differentiation process. As stated previously, one miRNA can potentially target hundreds of genes. This would mean that out of all the targets of mir-9, OC2 is one of the more critical ones during the differentiation process. This illustrates how miRNAs, particularly mir-9, serve to canalyze the differentiation process.

OC2 has also been shown to be targeted by mir-495 and mir-218 in bipotential mouse embryonic liver (BMEL) cells (Simion et al., 2010). It is possible that there are other miRNAs that regulate this gene during neural development. Thus, in order to

predict if OC2 is targeted by other miRNAs we used the prediction algorithm TargetScan (Friedman et al., 2009). TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA. Not to our surprise, the algorithm predicts that OC2 is a target of mir-9 in human, mouse and rat. More interestingly, OC2 is predicted to be also targeted by mir-124, mir-153 and mir-182, the other three members of the pro-neuronal miRNA group assayed in rat NSCs (Fig. 10), in addition to dozens of other miRNAs. This supports the idea that there are specific groupings of co-expressed miRNAs with redundant roles that serve to regulate the same genes or genes that are part of the same pathway.

We could speculate that if groupings of miRNAs regulate specific pathways then downstream effectors of OC2 are possibly targeted by these miRNAs as well. So, are any of the downstream effectors of OC2 regulated by mir-9 or any of the other members of the group of pro-neuronal miRNAs? FoxA2, a potential downstream effector of OC2, has been validated as a target of mir-124 in pancreatic beta cells (Baroukh et al., 2007). We continued our literature search to identify reports of other downstream effectors targeted by the pro-neuronal grouping of miRNAs. No other studies have validated targeting interactions between known downstream effectors of OC2 and the pro-neuronal miRNAs, mir-9, mir-124, mir-153 and mir-182. Ngn3 is not predicted to be targeted by any of the pro-neuronal miRNAs, but two of its transcriptional targets are predicted to be regulated by the pro-neuronal miRNAs. PLP, the predominant myelin protein present in the central nervous system (CNS), is predicted to be targeted by mir-124 and nkx2.2, a Homeobox protein, is predicted to be targeted by mir-182. If these interactions are confirmed, that would signify that the pro-neuronal grouping of miRNAs negatively regulate several steps in a transcriptional network that begins with OC2. This

supports our hypothesis that there are groupings of miRNAs that serve to canalize neurogenesis by inhibiting pathways which would promote alternative phenotypes.
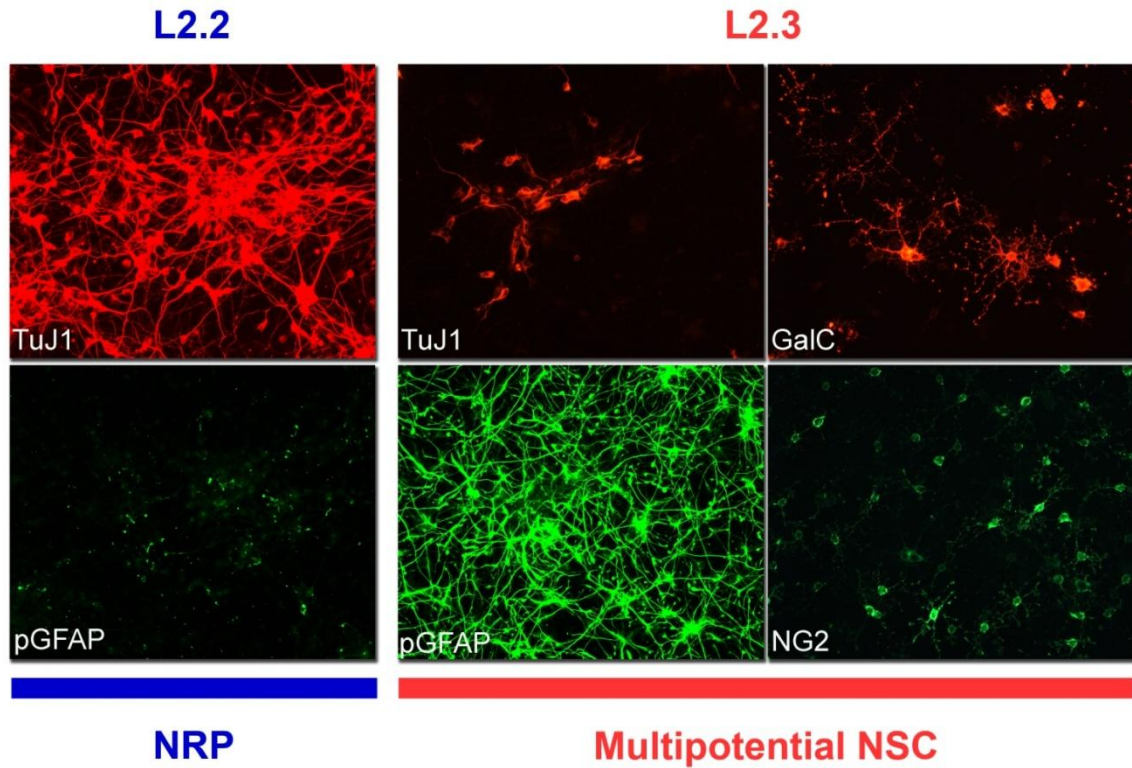
IV.   FIGURES

Fig. 1. *L2.2 and L2.3 NSC clones.* Two v-myc transduced 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^{+} GABAergic interneurons. The other, L2.3, exhibits a mixed phenotype. Most cells are GFAP[+], glial lineage, 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)
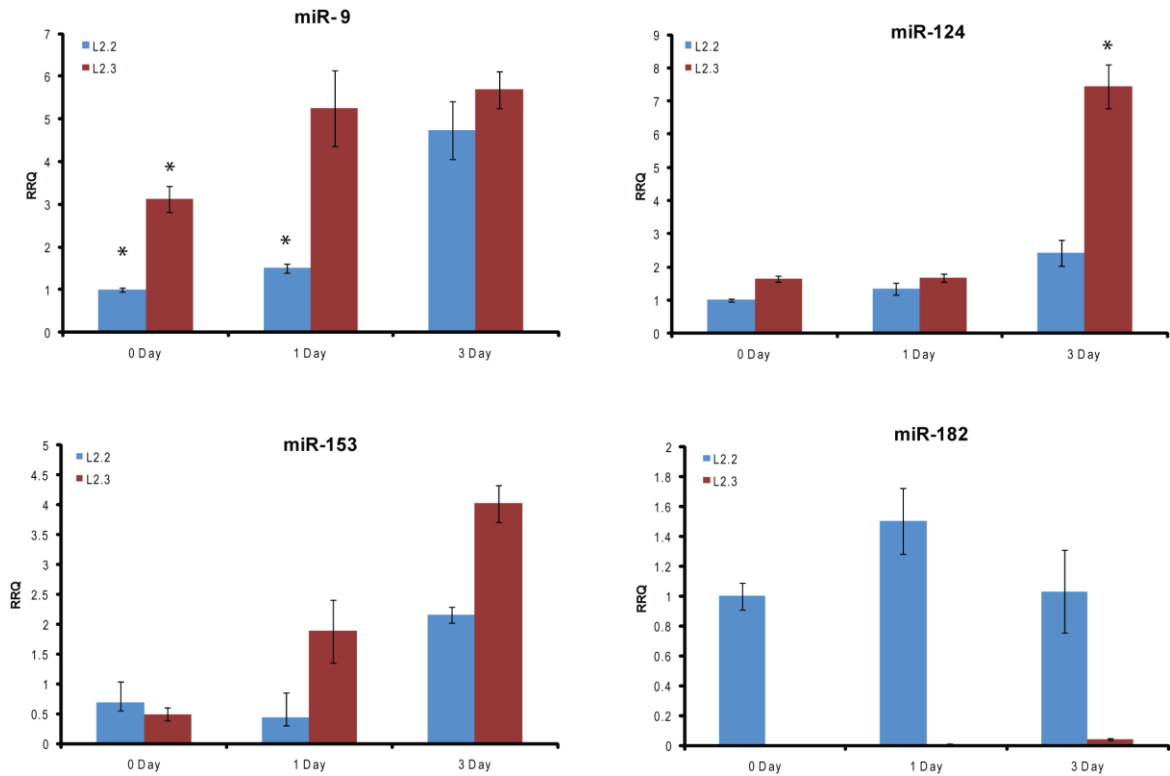
Fig. 2. Differentially expressed microRNAs during NSC differentiation. A select group of differentially expressed microRNAs were identified in a microarray analysis during the differentiation of the multipotential L2.3 NSC clone and the L2.2 NRP clone. Expression patterns were confirmed for 0, 1 and 3 days post differentiation by qPCR (n=3; *p<0.05). Statistical significance for expression of miR-153 and miR-182 could not be determined due to a single outlier. Nevertheless, qPCR analysis showed miR-182 expression remained relatively expressed in the L2.2 clone.
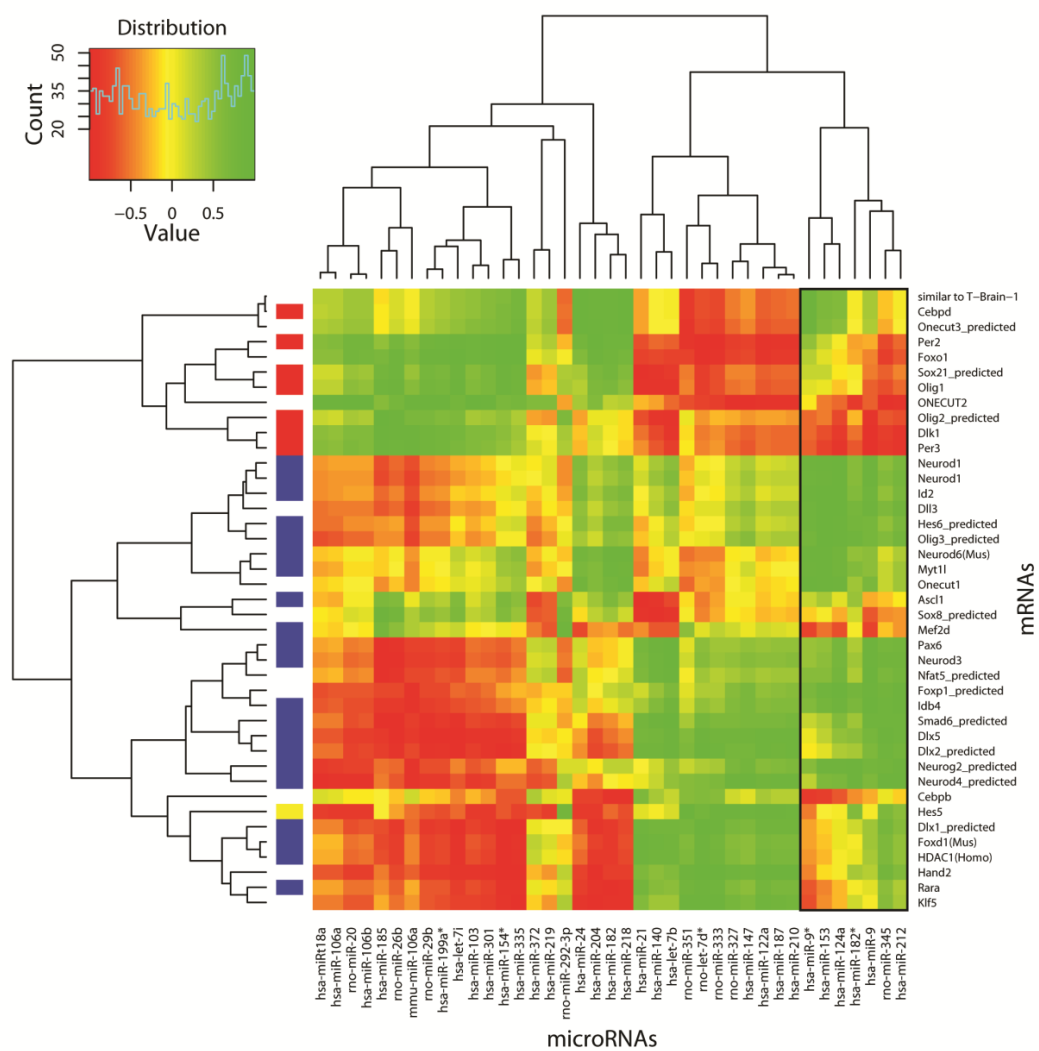
Fig. 3. *Cross Correlation of significant transcription factors and significant microRNA expression patterns identifies potential networks of mRNA:microRNA interactions.* Pearson correlation coefficient values were calculated between expression values for significant transcription factors and microRNA 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. 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. A side colorbar is provided to indicate mRNAs 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. A box marks a grouping of clustered microRNAs with known neurogenic roles. *(Produced in collaboration with Dr. Rebecka Jörnsten and Dr. Loyal Goff).*
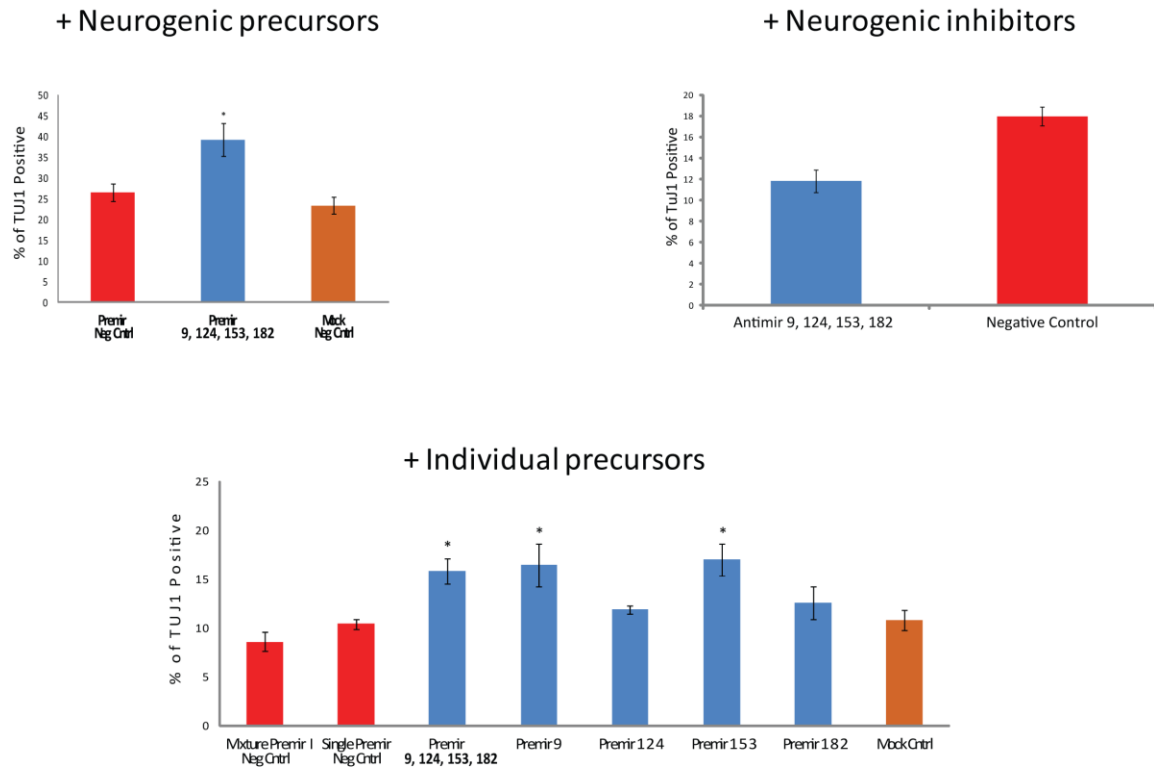
Fig 4. **Exogenous expression of a select group of microRNAs increases the neurogenic capacity of a multipotential NSC clone.** Gain or loss of function of mir-9, mir-124, mir-153 and mir-182 was assayed in the multipotential L2.3 NSCs. Individual or a mixture of Ambion Premirs[TM] were nucleofected into L2.3 clones prior to FGF withdrawal (n=4). After 72 hours of differentiation (-bFGF) cells were stained for TuJ1 and assayed via flow cytometry. (*p<0.05). Addition of the four predicted neurogenic microRNAs yielded a larger percentage of TuJ1^{+} (neurogenic) cells compared to the negative control. A mix of Antimirs for all four microRNAs was nucleofected and assayed similarly to the previous assay with the Premirs.
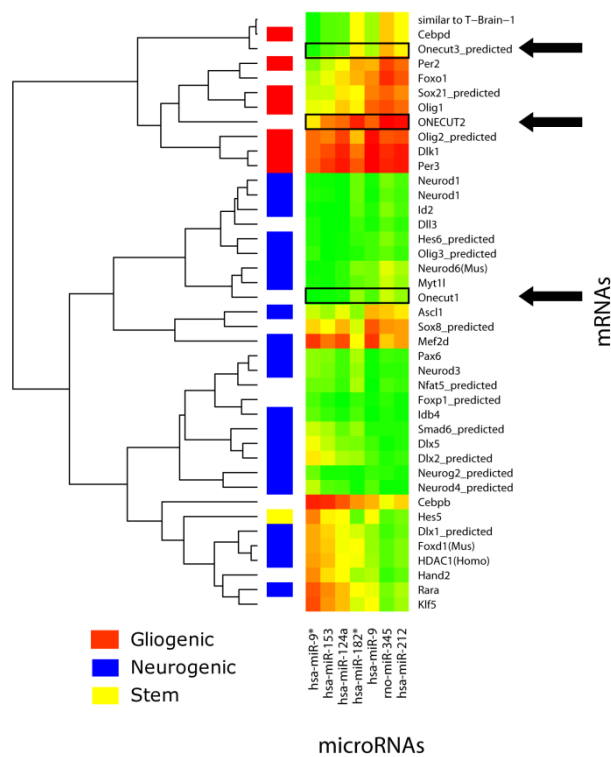
Fig. 5. Cross-Correlation matrix focusing on the pro-neuronal microRNAs. This figure is a portion of the Cross-correlation presented in fig. 3, focusing on the pro-neuronal microRNAs and the transcription factors. Arrows are identifying members of the Onecut family. Notice how OC2 shows a negative correlation (red) with all members of the pro-neurogenic grouping of microRNAs. A negative correlation would be predictive of miRNA targeting. It is also interesting to observe that OC2 also falls into the gliogenic grouping of transcription factors.
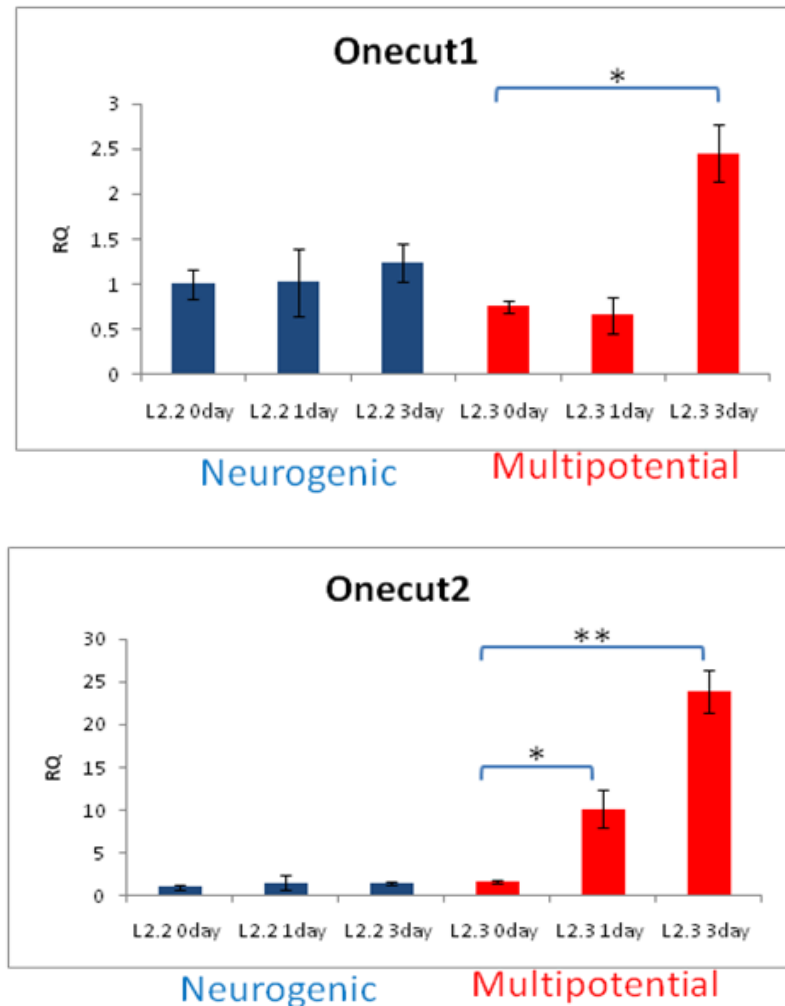
Fig. 6. Onecut 1 and Onecut 2 expression during rat NSC differentiation. OC1 and OC2 were identified as differentially expressed in a microarray analysis during the differentiation of the multipotential L2.3 NSC clone and the L2.2 NRP clone. Expression patterns were confirmed for 0, 1 and 3 days post differentiation by qPCR (n=3) (*p<0.05, **p<0.01 Student's t-test). The Onecut 1 and 2 genes showed significant regulation during NSC differentiation in the multipotential L2.3 NSC clone but not in the NRP L2.2 clone.
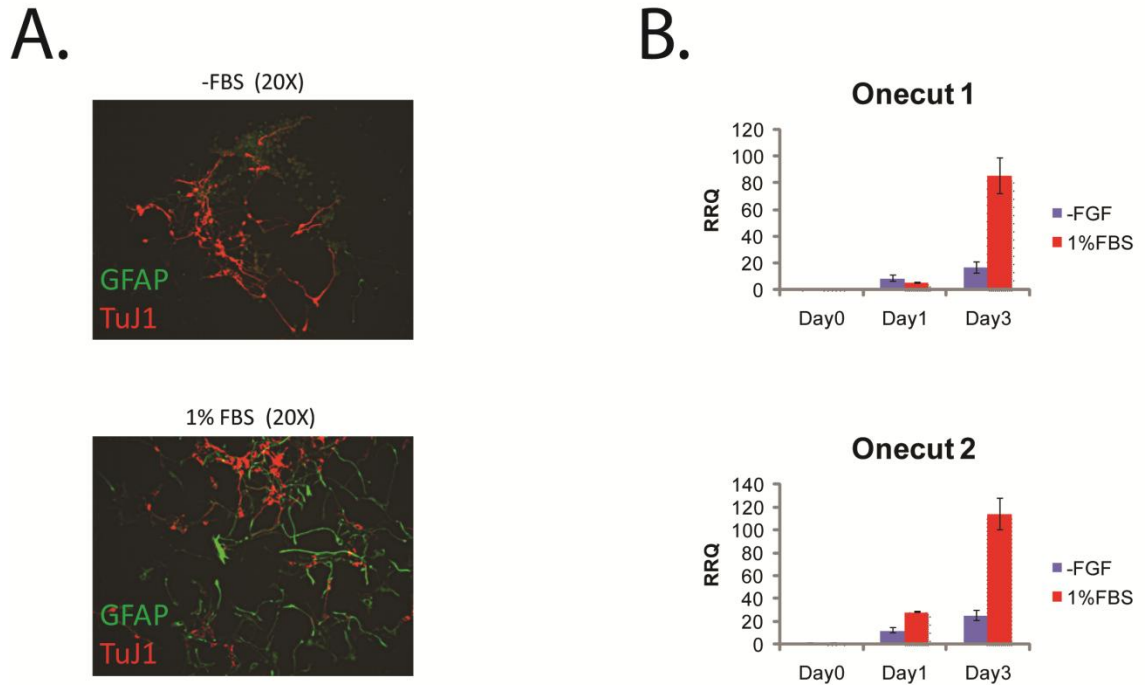
Fig. 7. OC1 and OC2 expression serum treatment. A) Immunostaining of glial marker GFAP (green) and neuronal marker TuJ1 (red) 3 days post differentiation. Differentiation media supplemented with 1%FBS enhances glial differentiation in multipotential rat NSCs, L2.3. B) Expression patterns of OC1 and OC2 mRNAs were tested at 0, 1 and 3 days post differentiation by qPCR (n=3) (*p<0.05, **p<0.01 Student's t-test). Levels of OC1 and OC2 genes showed a significant increase in the cultures treated with 1%FBS. The pro-glial environment increases OC gene levels.
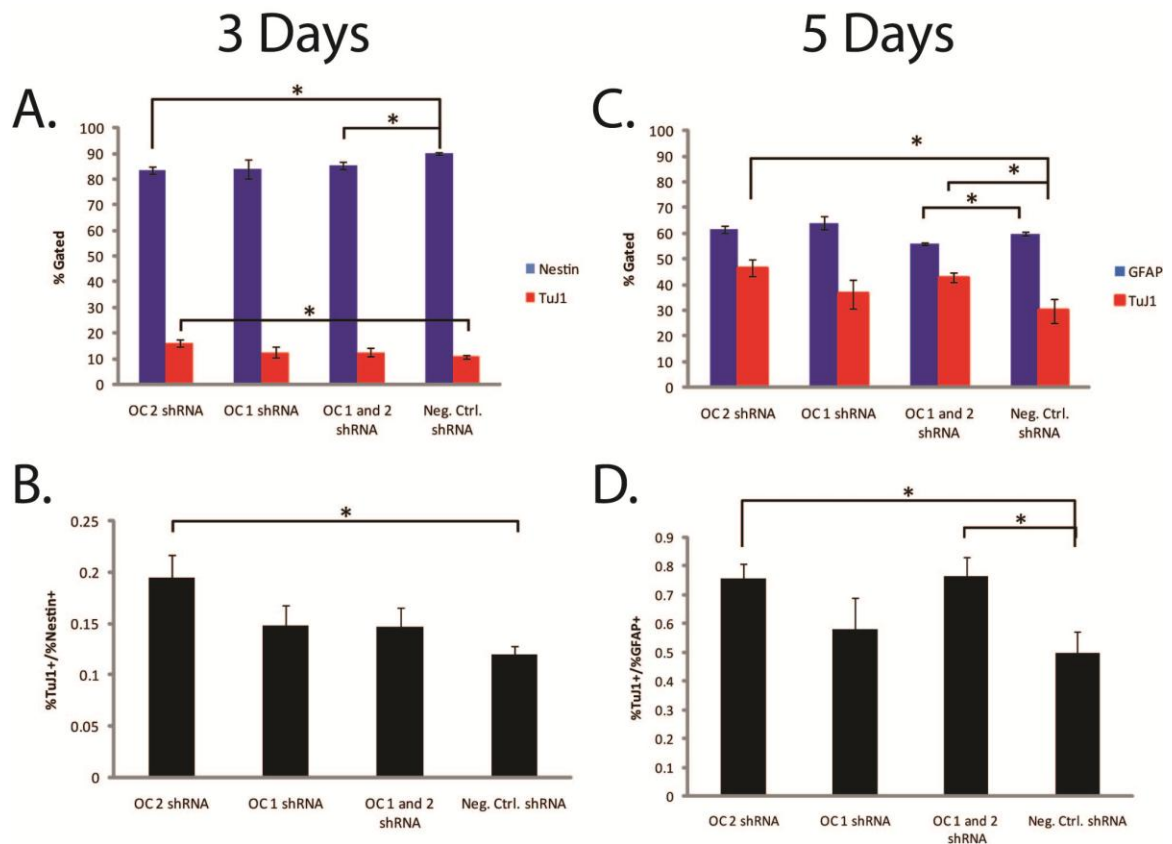
Fig. 8. shRNA knockdown of transcription factors, Onecut 1 and 2, increases the neurogenic differentiation capacity of a NSC clone at 3 days and reduces the glial outcome after 5 days of differentiation. shRNAs against Onecut 1 and 2 were nucleofected into the multipotential L2.3 NSC clones. Cells were harvested at 3 or 5 days post differentiation, fixed and stained for phenotypic markers Nestin (NSC), TuJ1 (neuron), GFAP (glia). Staining differences were then determined by FACS analysis. (n=3) (*p<0.05, Student's t-test)
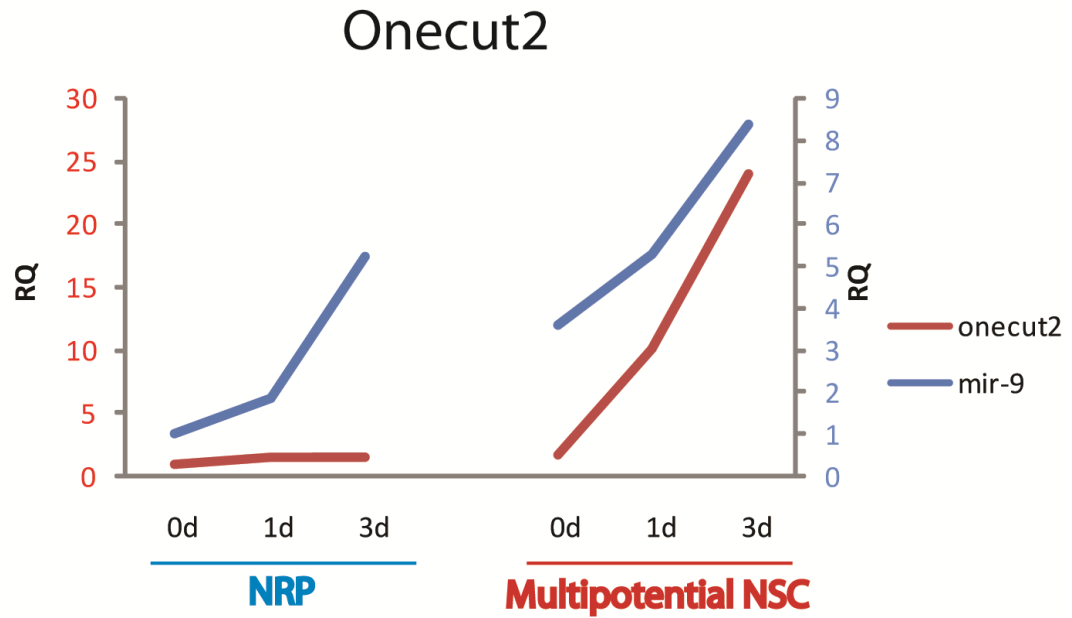
Fig. 9. OC2 expression relative to mir-9, show inverse or no correlation in the NRP clone upon neuronal differentiation. Relative expression patterns of Onecut family members in relation to mir-9 expression were determined by qPCR (n=3). RQ values of the mRNAS and the microRNA are plotted side by side. Note the lack of correlation between the OC2 gene and mir-9 expression patterns in the neuronally-restricted precursors. This contrasts with the correlated expression in the multipotential NSC clones. It is possible that mir-9 targets OC2 during neurogenesis and causes OC2 transcripts to be degraded.
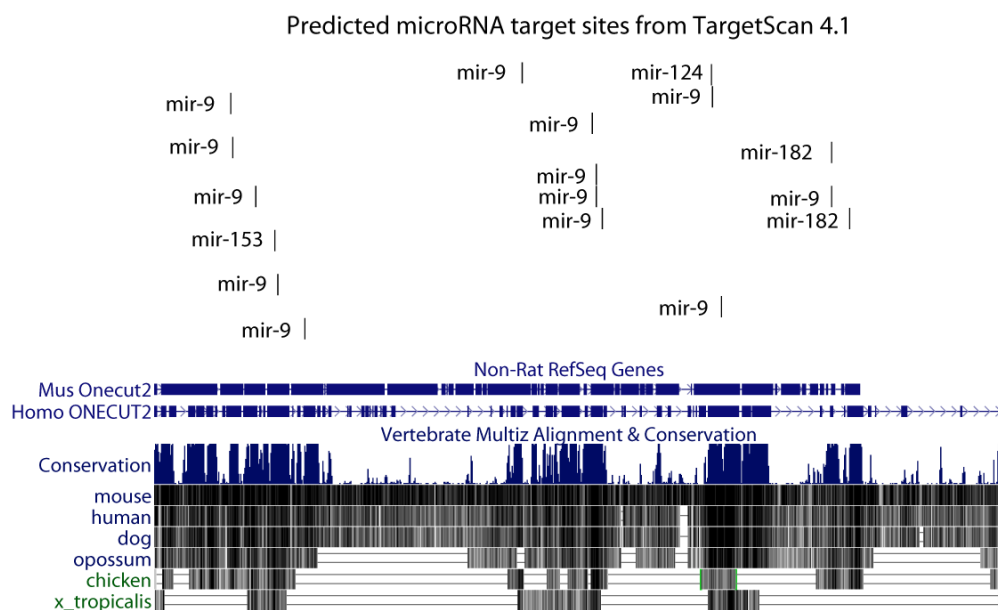
Rat Onecut2 3′UTR

Fig. 10. The microRNA target prediction algorithm Targetscan identifies OC1 and OC2 as potential targets of the pro-neuronal group of microRNAs. OC1 is predicted to be targeted by mir-9. OC2 is predicted to be targeted by all four members of the pro-neuronal microRNA group, mir-9, mir-124, mir-153 and mir-182. Notice the conservation amongst the human, mouse and rat potential MREs.
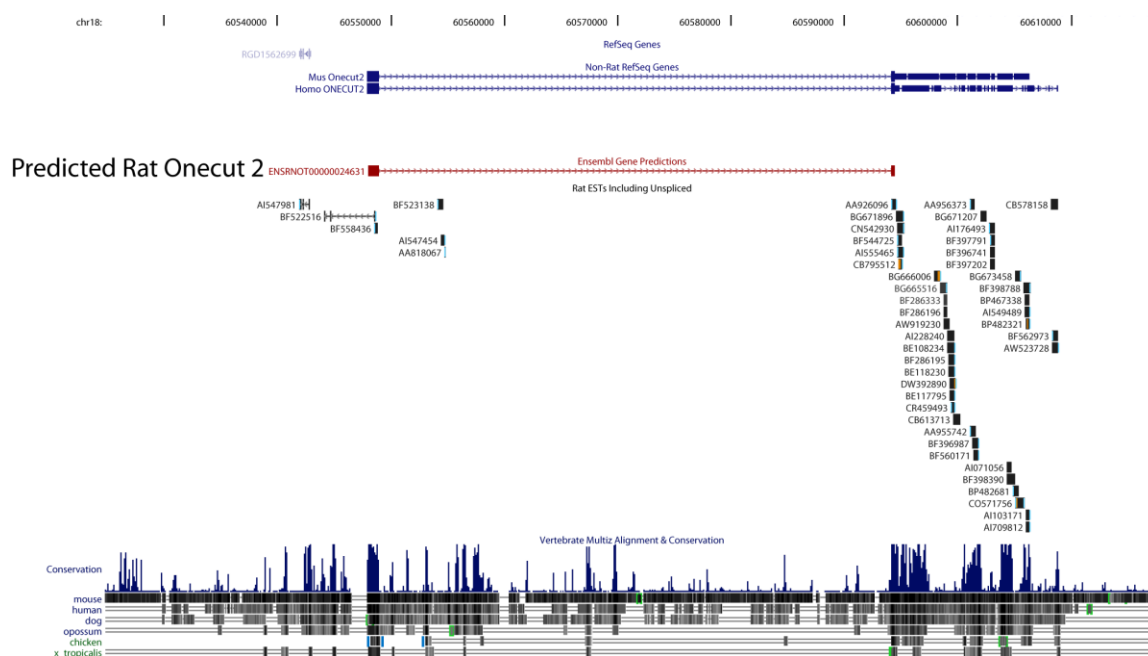
Fig. 11. OC2 3' UTR is poorly annotated in the rat genome. The human 3' UTR extends 14,575 base pairs (bps) and the mouse one extends 11,857 bps. The genomic regions of these 3' UTRs show high levels of conservation with the rat genome. Multiple rat ESTs align with the 3' ends of the mouse and human OC2 gene. Due to conservation we speculate that the rat OC2 3' UTR should be comparable in length to the mouse and human counterparts.
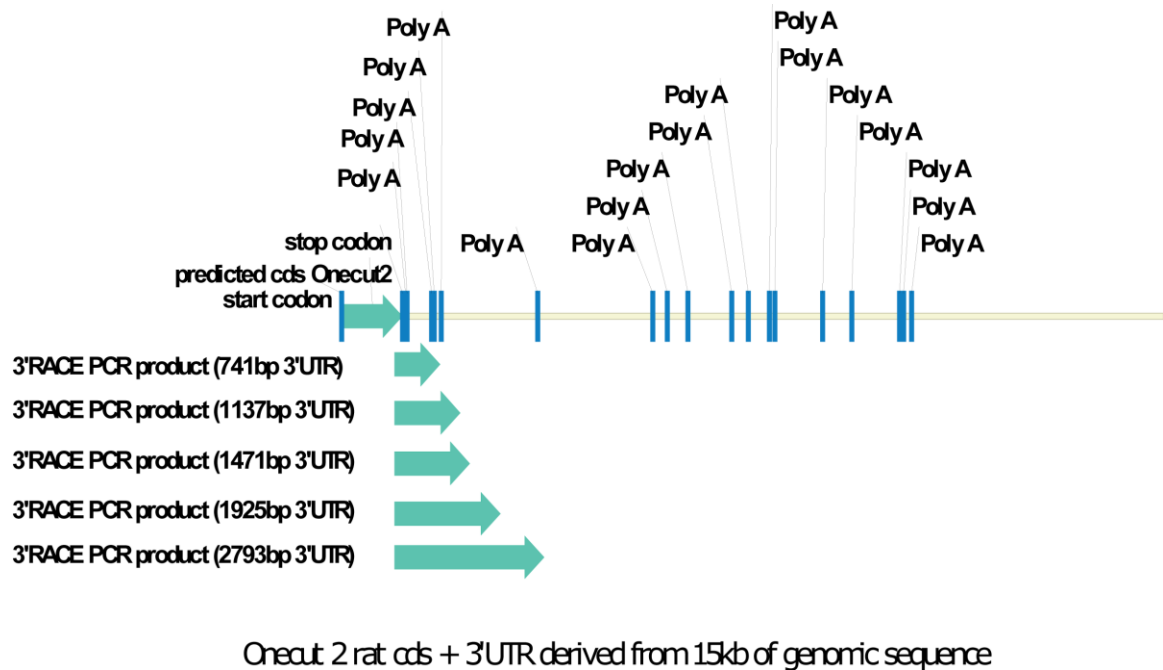
Onecut 2 rat cds + 3'UTR derived from 15kb of genomic sequence

Fig. 12. 3' RACE results of OC2 3' UTR. Using an anchored oligo dT prepared cDNA library from the multipotential L2.3 NSC; we performed a 3' RACE assay on the OC2 gene to identify its full length 3' UTR. Multiple size products were obtained, the longest corresponding to a ~2.8 Kb fragment. Interestingly most of these fragments aligned with internal stretches of ten or more A residues (polyA). It is possible that the oligo dT primers used to build the library non-specifically bound to these internal stretches and has not allowed us to identify the true end of the transcript.
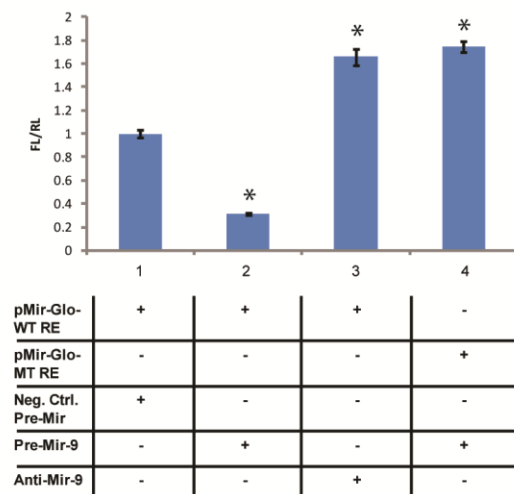
A.

B.

Fig. 13. **OC2 is targeted by mir-9 in rat NSCs.** A) WT and MT Cartoon representation of two OC2 mir-9 MREs which where fused to the 3'UTR of a firefly luciferase gene in the pMir-Glo vector (Ambion). B) Luciferase assays indicate that the tested OC2 mir-9 MRE is negatively regulated by mir-9 in rat NSCs.
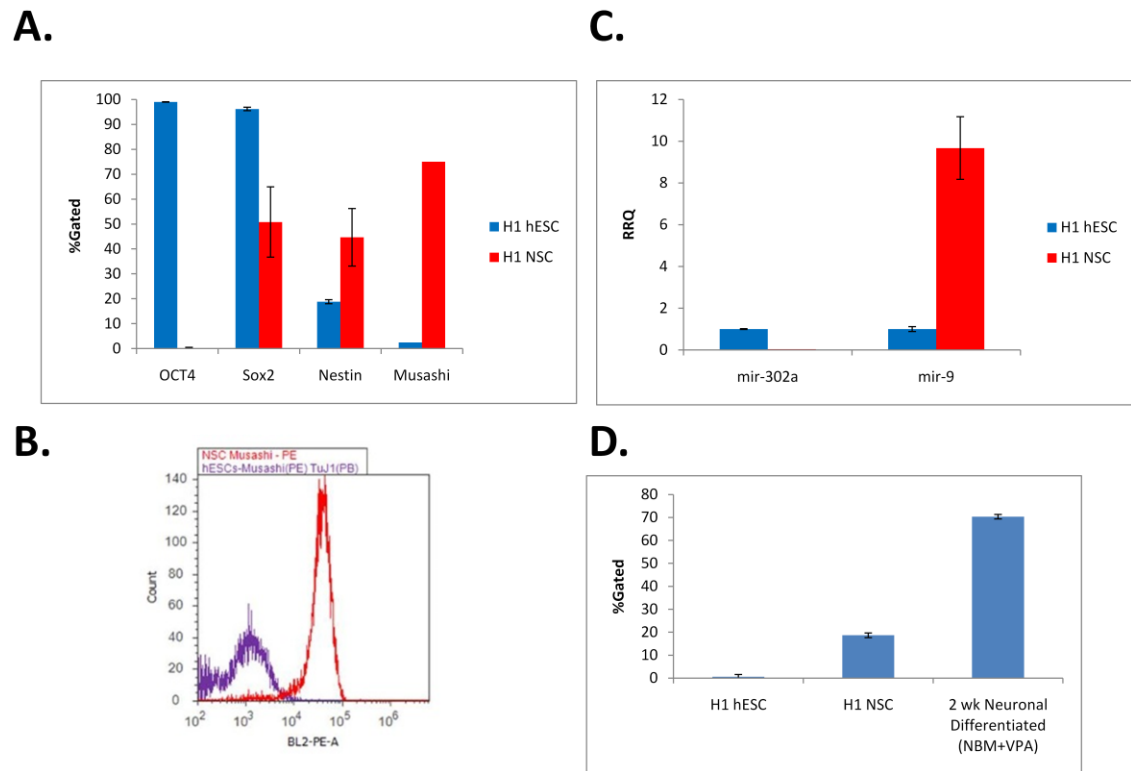
A.

B.

C.

D.

Fig. 14. Characterization of H1 NSCs. A) Graphical representation of FACS data. H1 hESCS and NSC were immunostained and assayed by FACS analysis for stage specific markers. H1 NSCs lose pluripotency marker OCT4 and gain NSC markers Nestin and Musashi. B) Histogram plot showing the increase in the number of Musashi^{+} cells in the NSC cultures when compared to H9 hESC. C) MiRNA levels of ESC specific mir-302 and neural mir-9 were tested by qPCR (n=3). D) Graphical representation of FACS data. H1 hESCs, H1 NSC and H1 derived neurons were immunostained and assayed by FACS analysis for the neuron specific marker, TuJ1.
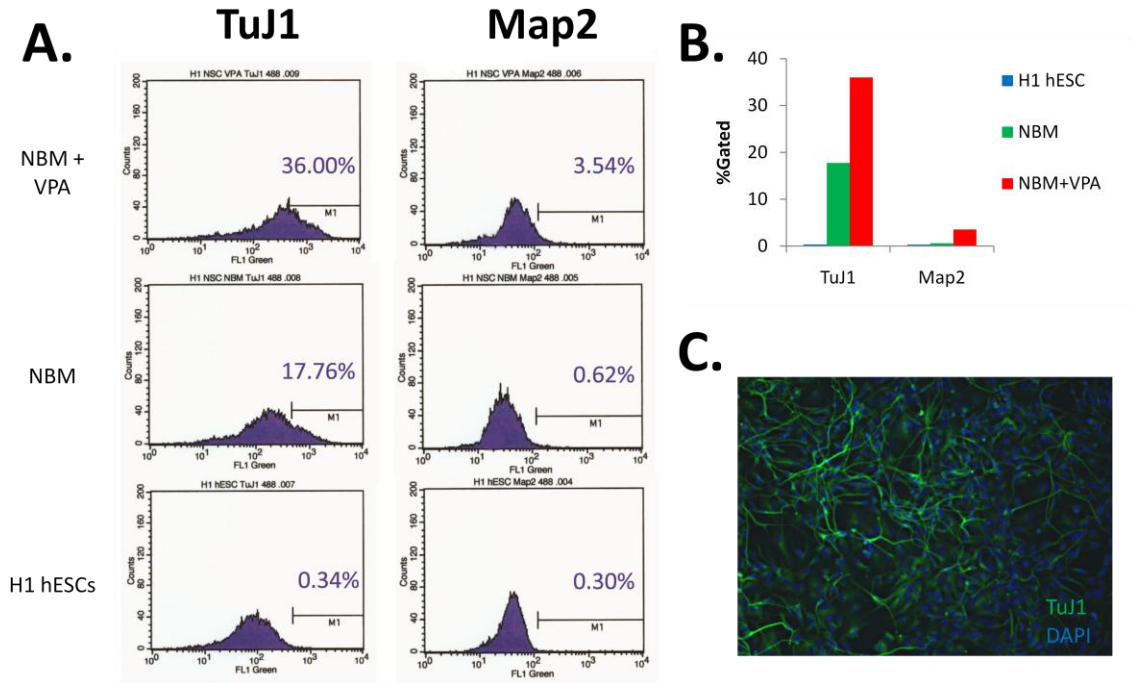
Fig. 15. VPA treatment serves to enhance neuronal differentiation of H1 NSCs.

A) FACS analysis for immunostained H1 hESCs and differentiated H1 NSCs in different media with neuronal markers TuJ1 and Map2. VPA treatment increases the percentage of TuJ1$^+$ and Map2$^+$ cells. H1 hESCs were assayed to show specificity of antibodies. Numbers represent means from three cultures. B) Graphical representation of the FACS data presented in A. C) Immunostaining of differentiated H1 NSCs treated with VPA, TuJ1 (green), DAPI (blue). One can notice a clear neuronal morphology in the TuJ1$^+$ cells.
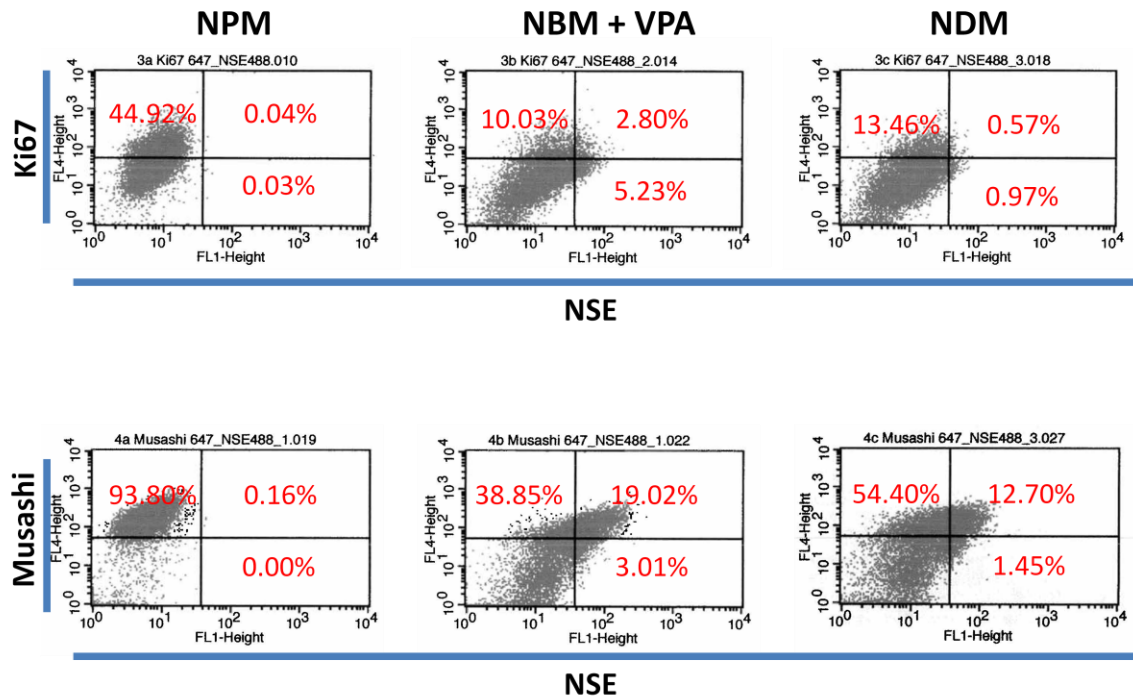
Fig. 16. Neuronal differentiation of H1 NSCs. FACS analysis of immunostained H1 NSCs cultured in NPM and seven day H1 NSC neuronal differentiated cells cultured in NDM or NBM+VPA with neuronal marker NSE, NSC marker Musashi and proliferation marker Ki67. Numbers represent means from three cultures. It is clear that differentiated cultures contain heterogeneous population of cells.
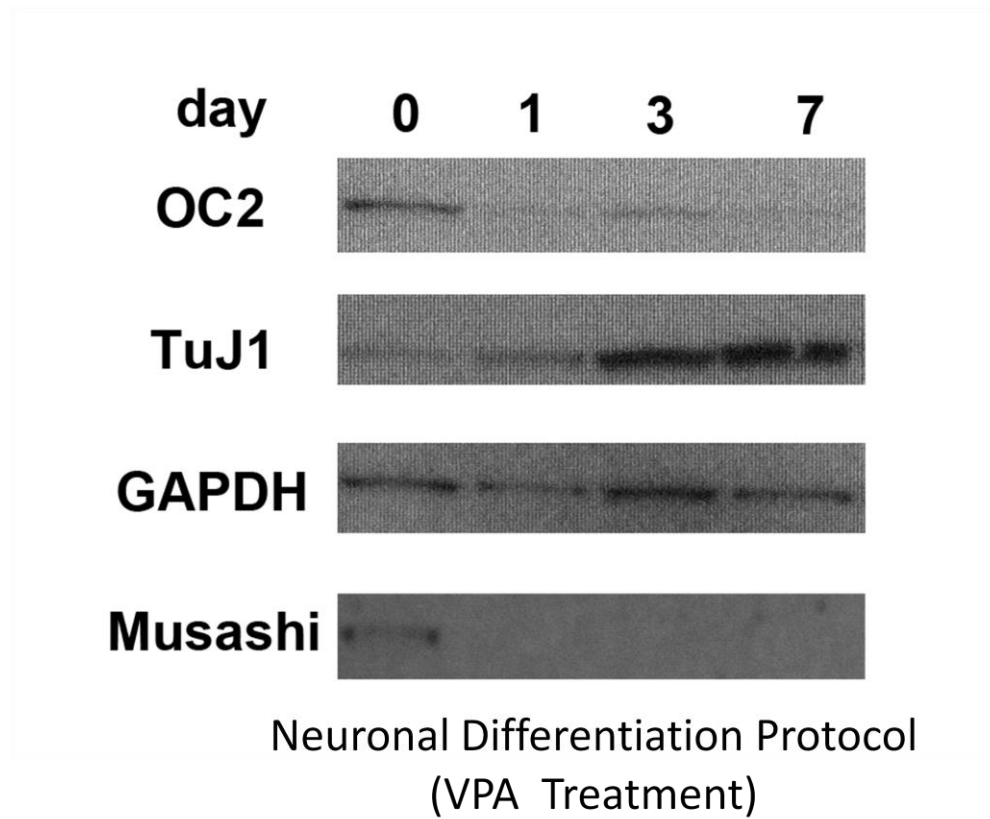
Fig. 17. Protein levels of NSC and neuronal markers during H1 NSC neuronal differentiation. Protein levels for select markers from H1 NSCs during NBM+VPA neuronal differentiation were assessed by western blot analyses. Blot was probed for OC2, TuJ1 and Musashi. GAPDH was used as a loading control. The loss of Musashi expression and the gain of TuJ1 is a clear indication that these cells are differentiating towards a neuronal phenotype.
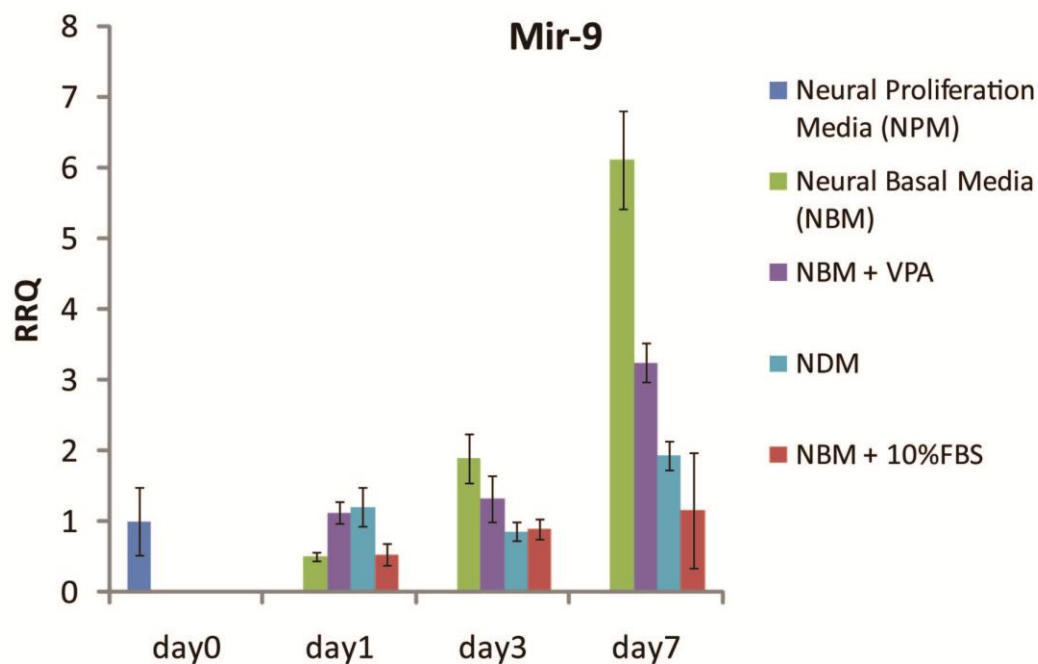
Fig. 18. Mir-9 levels upon differentiation of H1 NSCs. Levels of mir-9 were tested by

qPCR (n=3) for H1 NSCs grown in multiple growth conditions. NSCs were differentiated

in NDM, NBM, NBM+VPA and NBM+10%FBS.   Mir-9 levels increase in culture

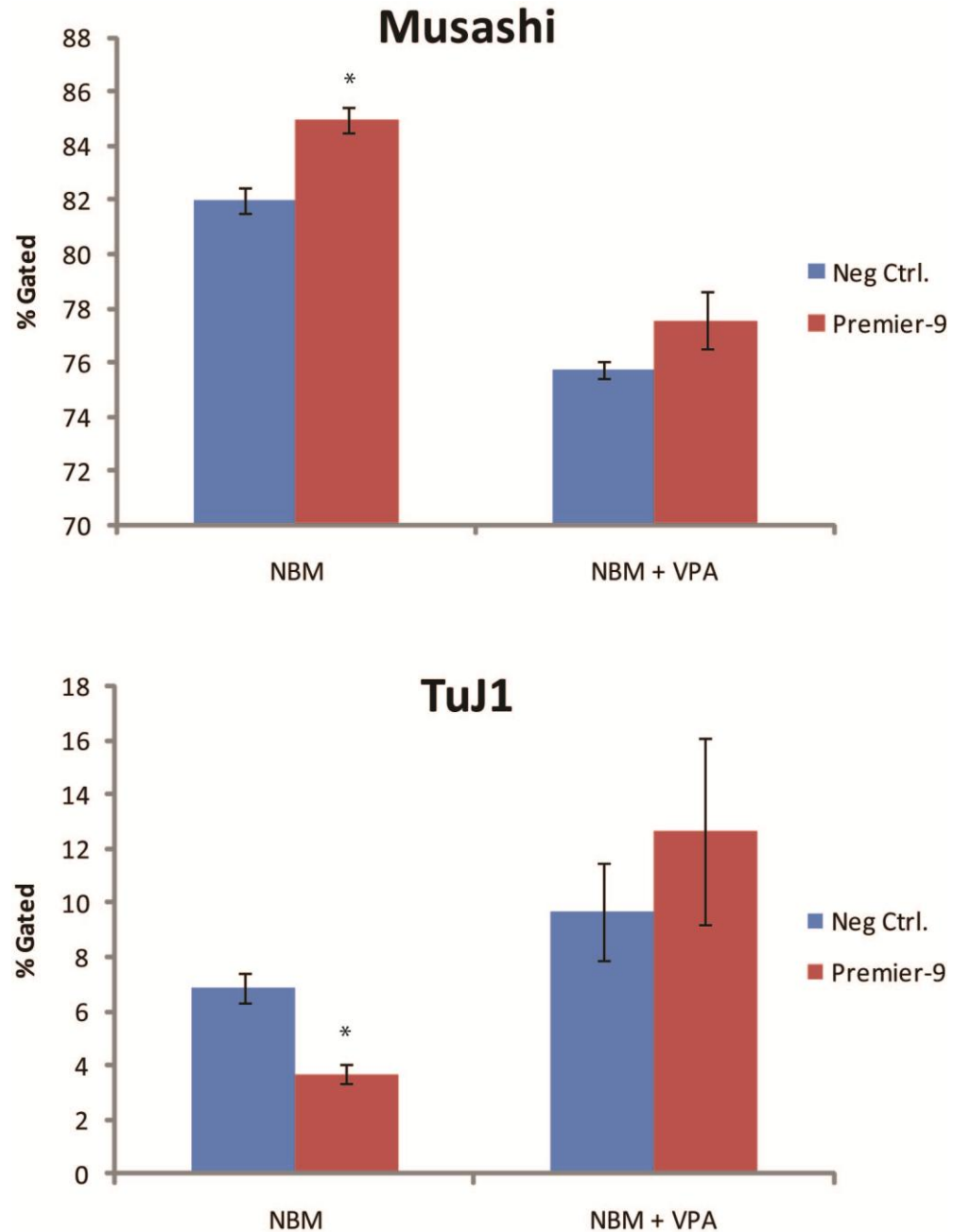conditions that enhance neuronal differentiation.

Fig. 19. Mir-9 exogenous expression in H1 NSCs. H1 NSCs were nucleofected with Premir-9 or a scrambled negative control miRNA mimics. Cells were then differentiated in NBM or NBM+VPA media. Seven days post differentiation, Musashi and TuJ1 immunostained cells were assayed by FACS analyses (n=3) (*p<0.05, Student's t-test).
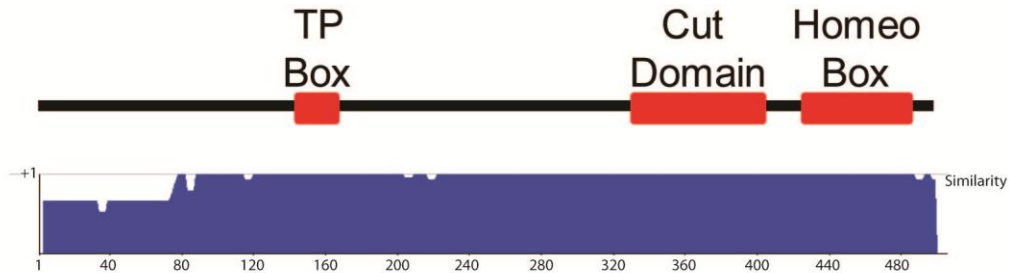
Fig. 20. Cartoon representation of the OC2 protein. Representation of OC2 with its functional domains. The TP box has been shown to contribute to transcriptional activity, while the cut domain and the homeo box are DNA binding domains (Lannoy et al., 2000). Bottom scale represents the amino acid sequence conservation of the protein across mouse, rat and human species. The protein is highly conserved across species, especially the cut domain and the homeo box regions that code for the DNA binding domains.
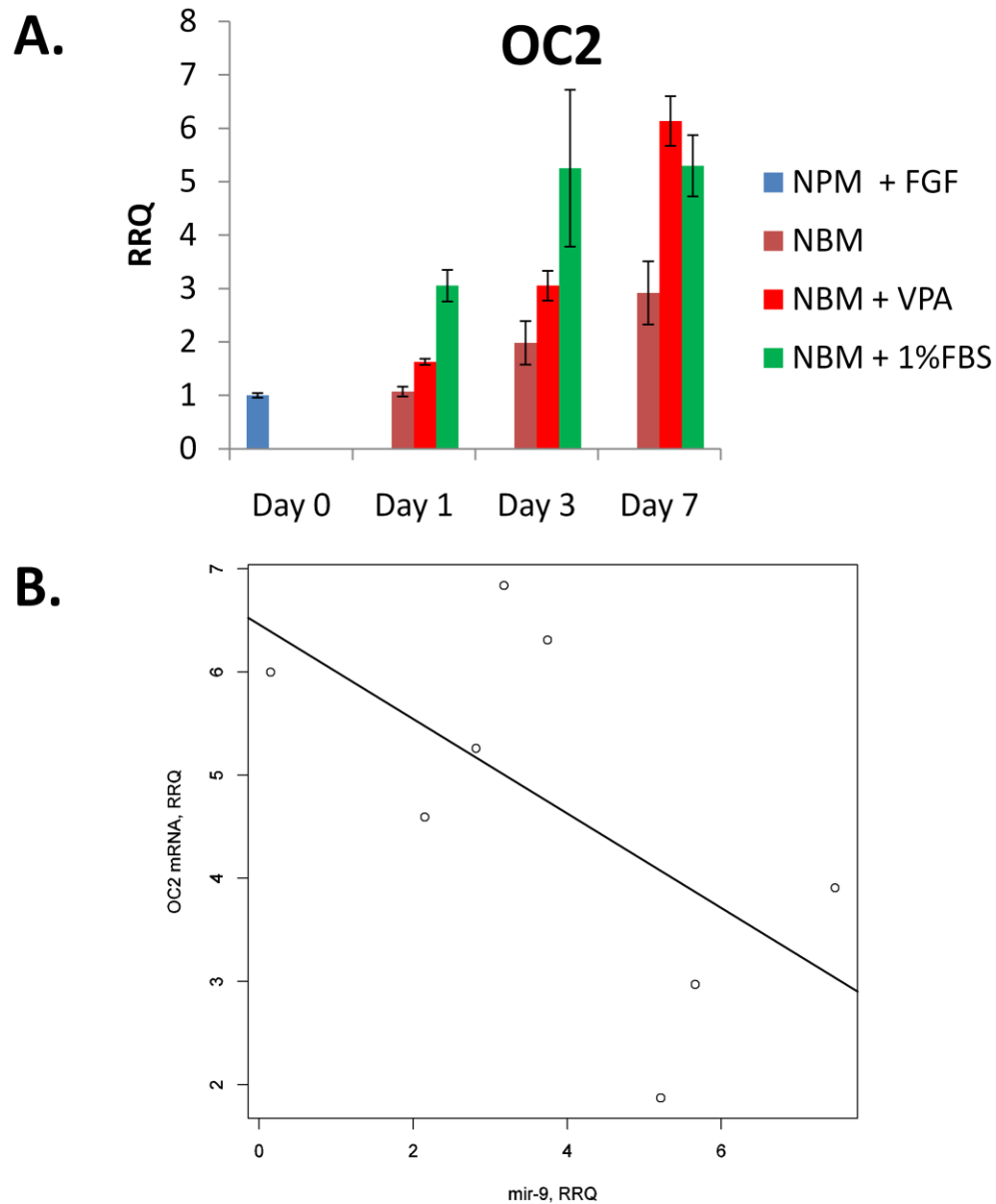
Fig. 21. OC2 mRNA expression and Correlation to mir-9 expression in differentiating NSCs. A) mRNA levels were tested in differentiating H1 NSCs in different medias at 0, 1, 3 and 7 days post differentiation by qPCR. B) Scatter plot representing OC2 and mir-9 relative expression values at day seven of differentiation.
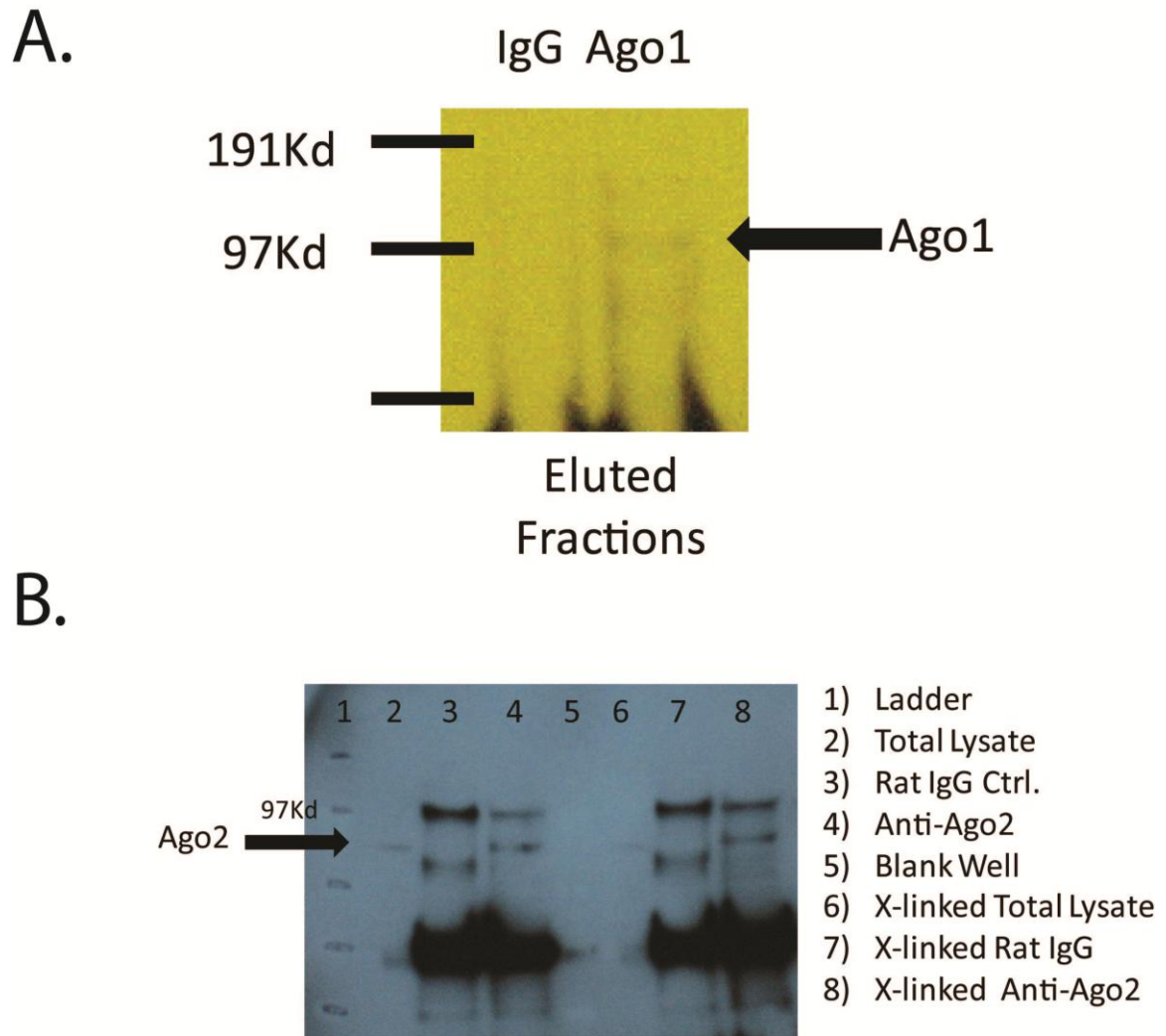
Fig. 22. Immunoprecipitations of Ago1 and Ago2 proteins. A) Ago1 IP using anti-Ago1 antibody. Eluted fractions were then blotted and probed with anti-Ago1 antibody. A band of approximately 100Kd representing Ago1 is apparent in the sample immunoprecipitated with the anti-Ago1 antibody and void in the isotype control. B) H1 NSCs treated with 4-thio-uridine for 16 hrs with or without UV cross-linking prior to Ago2 IP. Eluted fractions were then blotted and probed with anti-Ago2 antibody.

R.I.P. Protocol Overview
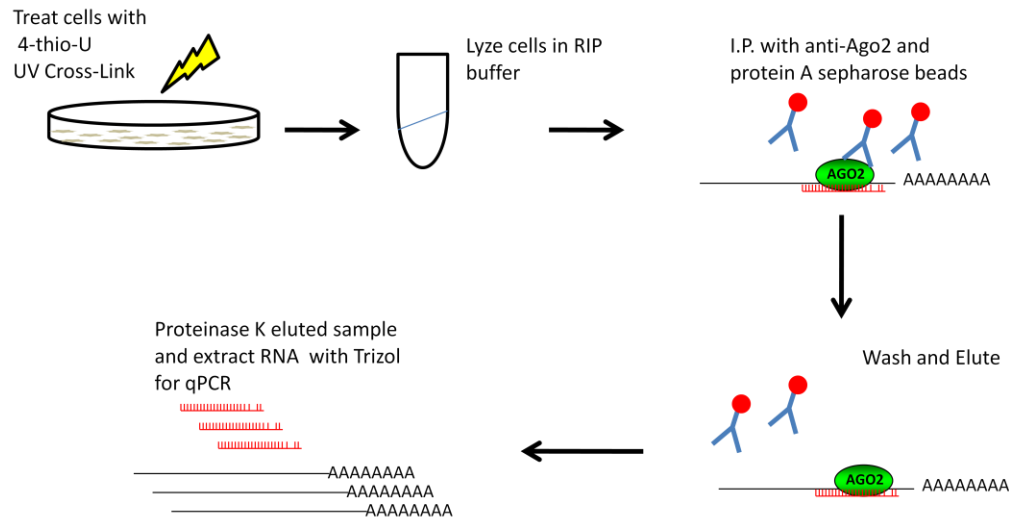
Fig. 23. RNA immunoprecipitation (RIP) assay overview. Cartoon representation of Ago1 and Ago2 RIPs. Cells were grown in the presence of 4-thio-uridine before UV cross-linking and lysing. RNA containing RISC complexes were then immunoprecipitated with anti-Ago1 or anti-Ago2 antibodies. After elution RNA was extracted and assayed for the presence of select mRNAs.
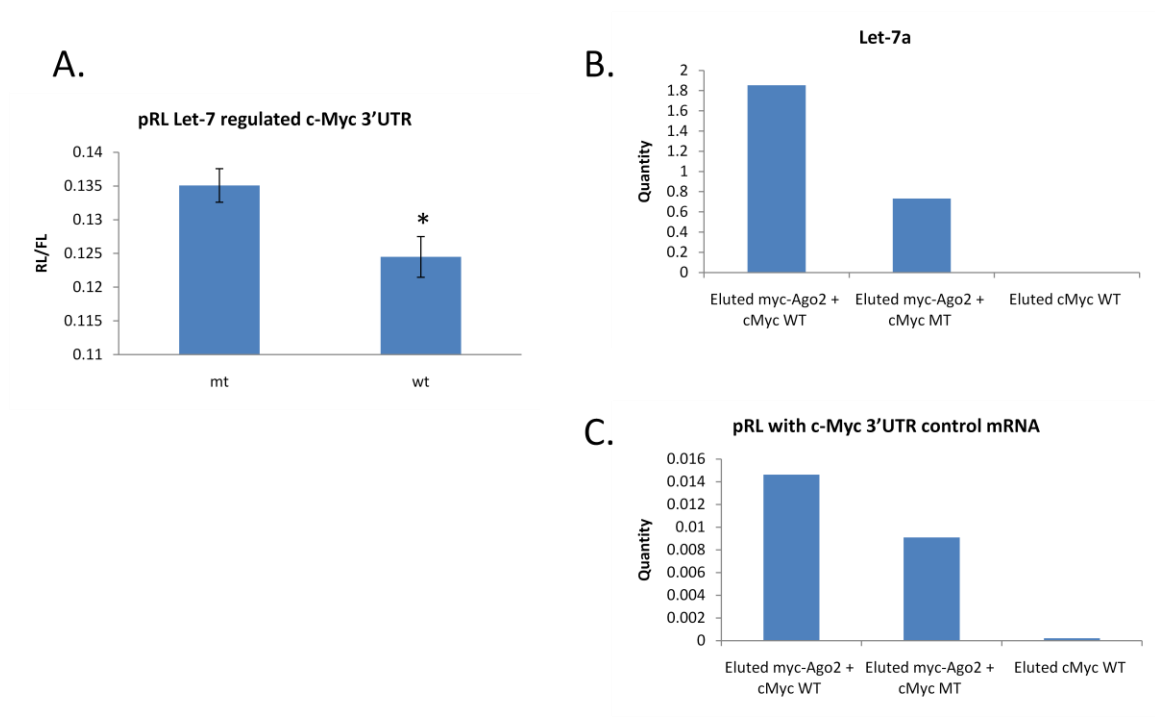
Fig. 24. Validation of RIP protocol. A) Luciferase assay using a pRL plasmid with the 3'UTR of c-Myc cloned into 3'UTR of renilla luciferase gene. The wild type (wt) let-7 MRE is negatively regulated compared to a mutant (mt) form of the MRE in 293T cells (n=6) (*p<0.05, Student's t-test). B) myc-Ago2 transfected 293T cells were used to optimize the RIP protocol. RIPs using an anti- myc-tag antibody were able to selectively retain miRNAs in the eluted fractions. C) myc-Ago2 transfected 293T cells were cotransfected with the pRL c-Myc 3'UTR, as a positive control. Same eluted fractions that were assayed for miRNAs contained mRNAs.
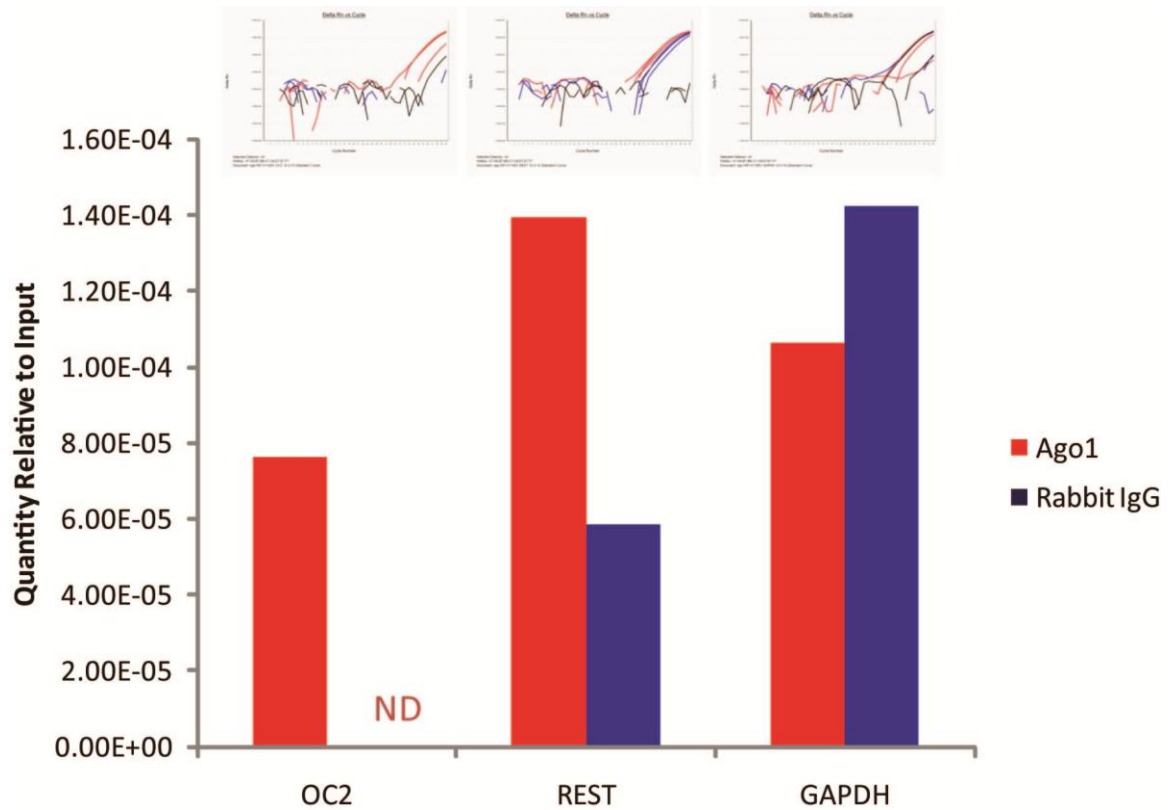
Fig. 25. Ago1 RIP in differentiating H1 NSCs. H1 NSCs were differentiated and treated for RIP assays. Samples were RNA immunoprecipitated using an anti-Ago1 antibody following protocols described in the text. Eluted fractions were then tested for the presence of OC2, REST and GAPDH mRNAs. One can observe enrichment of OC2 and REST of in the Ago1 eluted fraction relative to the isotype control, but not GAPDH (n=1).
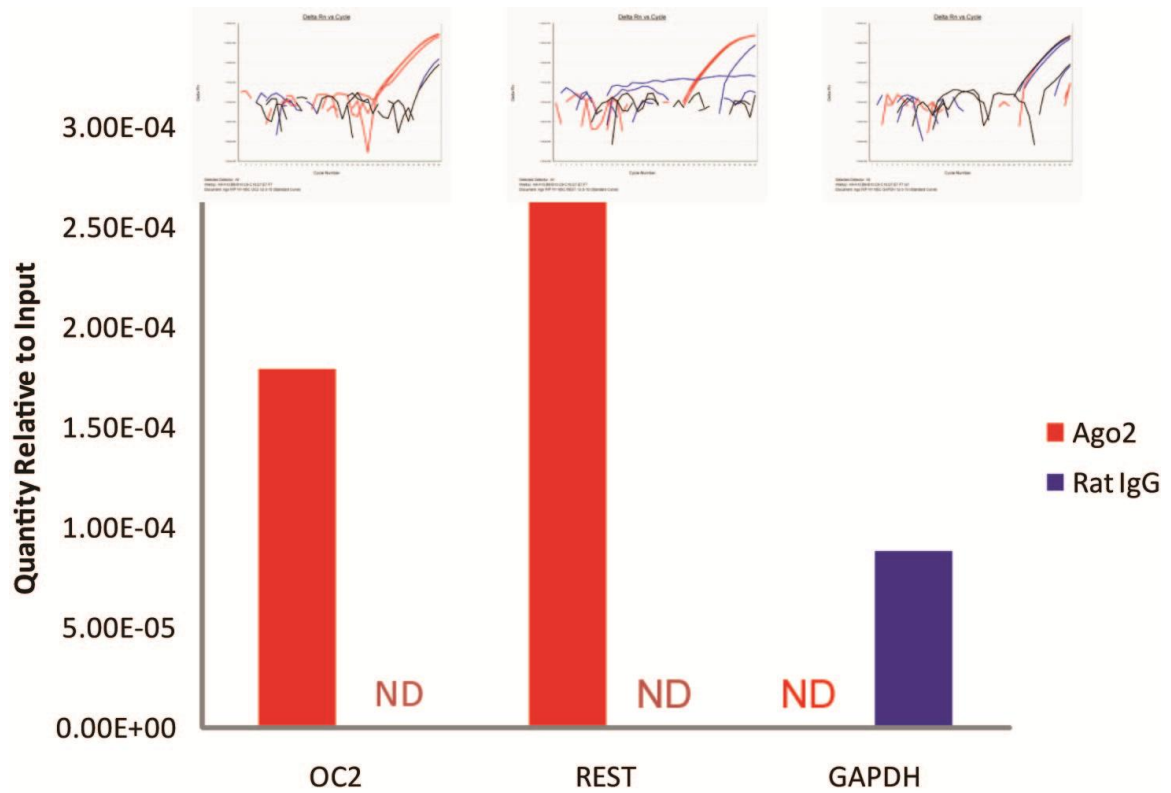
Fig. 26. Ago2 RIP in differentiating H1 NSCs. H1 NSCs were differentiated and treated for RIP assays. Samples were RNA immunoprecipitated using an anti-Ago2 antibody following protocols described in the text. Eluted fractions were then tested for the presence of OC2, REST and GAPDH mRNAs. One can observe enrichment of OC2 and REST of in the Ago2 eluted fraction relative to the isotype control, but not GAPDH (n=1).
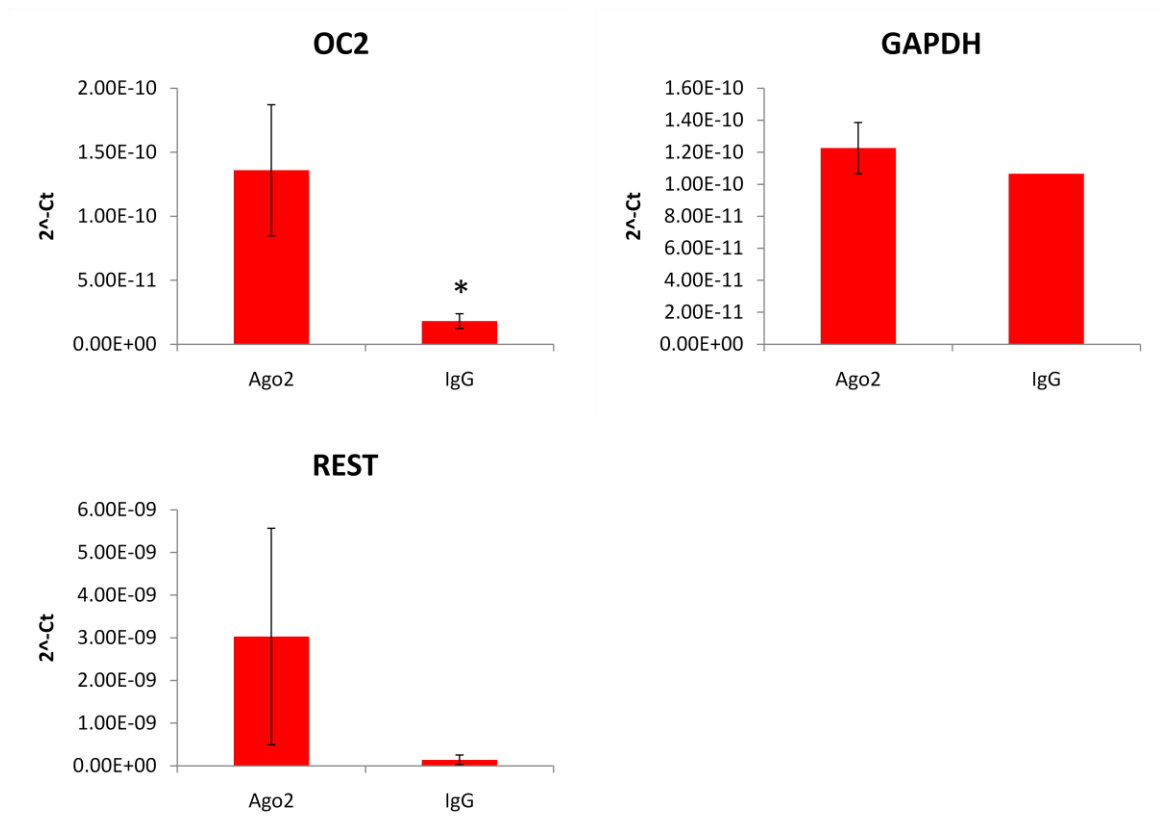
Fig. 27. OC2 is consistently enriched in Ago2 RIPS from H1 NSCs. Anti-Ago2 antibody was used in RIPs from differentiating H1 NSCs. Eluted RNA was assayed for enrichment of OC2, REST and GAPDH mRNAS in the Ago2 eluted fractions vs. an IgG control (n=6) (*p<0.05, Student's t-test)
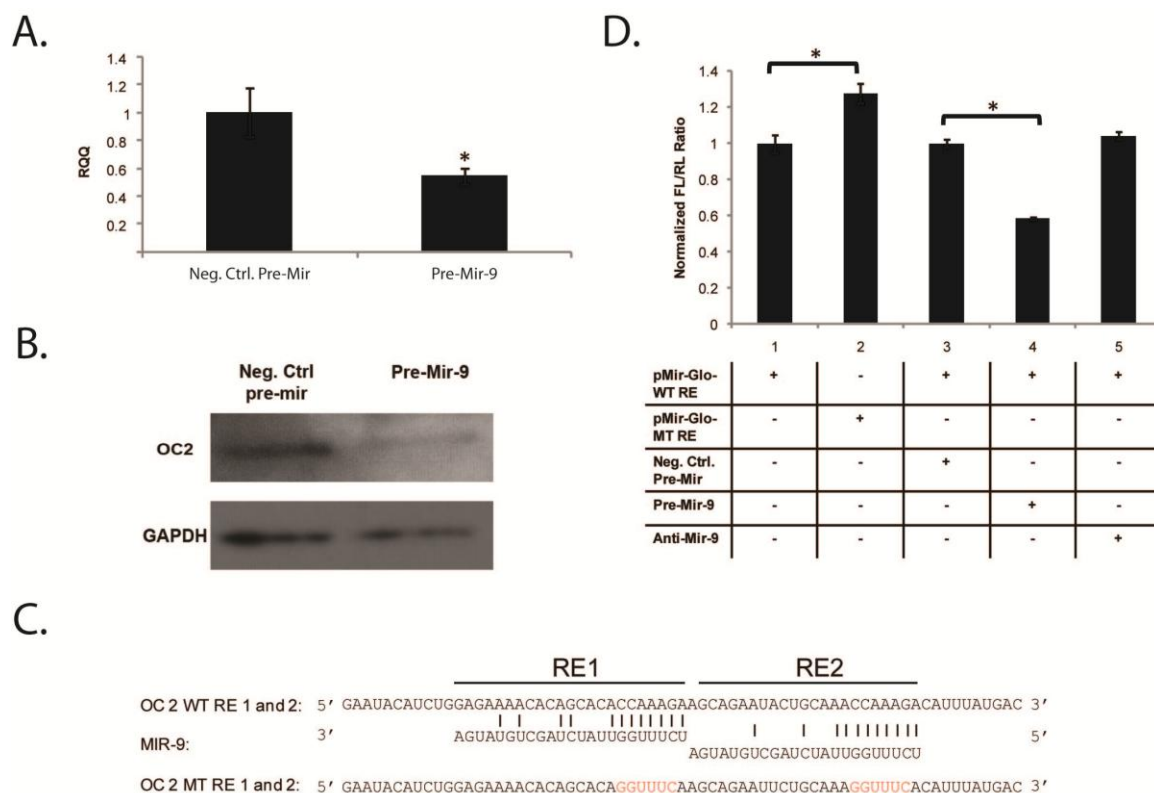
Fig. 28. OC2 mir-9 regulation in H1 NSCs. A) Relative OC2 mRNA levels in H1 NSCs treated with Pre-Mir-9 or a scrambled negative control, tested by qPCR 24hrs post transfection (n=3) (*$p<0.05$, Student's t-test). B) Western blot of H1 NSCs treated with Pre-Mir-9 or a scrambled negative control. OC2 protein levels were assayed with anti-OC2 antibody. GAPDH levels were assayed as a loading control. C) WT and MT Cartoon representation of two OC2 mir-9 MREs which where fused to the 3'UTR of a firefly luciferase gene in the pMir-Glo vector (Ambion). D) Luciferase assay with the human OC2 mir-9 MRE cloned into 3'UTR of firefly luciferase gene. The MRE is negatively regulated by mir-9 (n=6) (*$p<0.05$, Student's t-test)

V. TABLES

Table1. Functional roles of mir-9 in neural tissues

Function	Species	Target	Ref
Promotes proliferation of hESC derived hNSCs and limits migration of cells.	Human	Stathmin	(Delaloy et al., 2010)
Potential role in neuronal differentiation	Human	REST	(Laneve et al., 2010)
Reduces glial differentiation in mESC derived NSCs	Rodent	?	(Krichevsky et al., 2006)
Modulates proper differentiation of Cajal–Retzius cells in the medial pallium	Rodent	FoxG1	(Shibata et al., 2008)
Negative regulation of NSC proliferation and enhanced neural differentiation	Rodent	TLX	(Zhao et al., 2009)
Mir-9-1 and 2 KO mice show roles in both proliferation and differentiation depending on temporal spatial context.	Rodent	FoxG1	(Shibata et al., 2011)
miR-9 promotes neurogenesis in the midbrain-hindbrain	Zebrafish	Her5, Her9	(Leucht et al., 2008)

Table 1. Functional roles of mir-9 in neural tissues. Mir-9 serves different roles in proliferating and differentiating NSC depending on the cellular context.

VI.   METHODS

Rat 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.

H1hESC cell culture and NSC differentiation

H1 hESCs were grown on feeder free conditions in mTeSR (STEMCELL Technologies, Vancouver, BC, Canada). Once every week or when wells were 70-80% confluent, colonies were dissociated into clumps using 1 U/mL Dispase (BD Biosciences, Franklin Lakes, NJ)and platted on Matrigel (BD Biosciences, Franklin Lakes, NJ) coated plates in 1:12 dilution. Plating ratio was dependent on the well density prior to passaging. Before passaging, culture wells were manually cleaned of necrotic and differentiating cells using a flame polished glass pipette. Medium was changed every day.

To preferentially induce human NSC formation from H1 hESCs, we inhibited TGF-β signaling in the presence of Noggin (Chambers et al., 2009; Zhou et al., 2010). To start this process, 70-80% confluent cells were passaged by disaggregation into clumps of cells (approximately 50-100 cells per clump) with 1 U/mL dispase and plated at a ratio of 1:5 on matrigel coated dishes in a mixture of 50% mTesR/50% NBM (NBM -

neural basal, 2% B-27 (Gibco Life Science Technologies, Carlsbad, CA), 1% N-2 Supplement (Gibco Life Science Technologies, Carlsbad, CA), 1% ITS (Insulin, Transferrin, Selenium) (Gibco Life Science Technologies, Carlsbad, CA) , 2 mM L-glutamine) with 500 ng /mL Noggin (Peprotech, Rocky Hill, NJ). The medium was refreshed with 50% mTeSR/50% NBM containing 500 ng/mL Noggin every other day until day 6. From days 6 to 12, the medium was refreshed every other day with NBM containing 500 ng/mL Noggin. On day 12 the cells were manually passaged by dissociation into small clumps. To enrich for clumps of differentiating neural stem cells, the suspension of cell clumps was filtered with a 40 µM cell strainer. The clumps were platted on laminin coated dishes in NBM (without Noggin) at a ratio of 1:2 and the media is refreshed every other day with NBM. When these cells became ~70% confluent (around day 20) the media was changed to NPM (NPM- 50% DMEM/F12/50% Neural Basal, 0.5% N-2, 1% B-27, 20 ng/mL FGF). After the cells reached nearly 100% confluence, the cells were passaged with Accutase (STEMCELL Technologies, Vancouver, BC, Canada) and plated onto plates coated with ¼ the recommended concentration of Matrigel (BD Biosciences, Franklin Lakes, NJ) (¼ MG) in NPM. From this point onwards, when the cells reach 80-90% confluence, they were passaged at ratios of 1:3 to 1:4 onto ¼ MG and would remain in the neural stem cell state for at least 10-13 passages.

H1 NSCs cell culture and NSC neuronal differentiation

H1 NSCs were expanded in NPM media prior to differentiation. Approximately every three to four days when the H1 NSCs would reach ~90-95% confluence, the cells were passaged in a 1:3 or 1:4 manner depending on the density and plated in NPM.

The following day the media was changed to one of two neuronal differentiation media. The two neuronal differentiation media used were NDM (NDM-neurobasal medium, B-27 and 10 ng/mL BDNF) or NBM +VPA (NBM+VPA -neural basal, 2% B-27, 1% N-2, 1% ITS, 2 mM L-glutamine, 1mM valproic acid (VPA)). Medium was replaced every other day.

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).

NCode miRNA 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 900 miRNA 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 hybridized 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 miRNAs were selected as $p<0.05$ with an estimated 10% false discovery rate (FDR).

Cross correlation of mRNA and miRNA expression

The miRNA/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 time point. 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 qRT-PCR were designed using Primer Express 2.0 (Applied Biosystems Inc., Foster City, CA). Mature miRNA primers for NCode miRNA qRT-PCR (Invitrogen Inc., Carlsbad, CA) were designed according to manufacturer's recommendations. For each condition 2 µg 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 50 nM 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.

For mRNA assays, 1 µg of this RNA was reverse transcribed into cDNA and assayed by qPCR according to standard protocols. qPCR primers against the coding sequence were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Each assay consisted of 2 ng cDNA, 3 µl mixed primer pairs (50 nM final), and 5 µl SYBR Green PCR Master Mix (Applied Biosystems). Water was used as a "no template" negative control (NTC), and genomic DNA was used as a positive control. Plates were assayed on either the AB7900HT or the AB7500 Fast System. Data were analyzed by the ΔΔCt method for determining relative quantities (Ct=cycle threshold). All data were normalized to GAPDH and analyzed in the RQ manager software (Applied Biosystems, Foster City, CA). In previous studies we showed that GAPDH exhibited the least variability across samples during NSC differentiation compared to other housekeeping genes (data not shown). Primer and amplicon specificity were determined by amplicon dissociation curves.

For miRNA assays, 10 ng of RNA was reverse transcribed into cDNA using gene specific primers and assayed by qPCR following protocols for TaqMan MicroRNA Assays. Plates were assayed on either the AB7900HT or the AB7500 Fast System. Data were analyzed by the ΔΔCt method for determining relative quantities (Ct=cycle threshold). All data were normalized to RNU43 and analyzed in the RQ manager software (Applied Biosystems, Foster City, CA)..

Western Blot

Cell cultures were harvested and lysed using RIPA buffer (50 mM Tris, HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 0.1% SDS). 20-30 µg of protein were ran on Invitrogen NuPAGE gels and transferred using the IBlot system (Invitrogen, Carlsbad, CA) following manufactures protocols. Blots were then assayed using desired antibodies.

Transfections

Rat NSCs

Transfections into rat NSC clones were done via electroporation in the Amaxa 96-well shuttle system (Lonza) using the Rat Neuron Nucleofector Kit (VHPG-1003). Observed transfection efficiencies using the 96-well shuttle system were consistently >80% for all NSCs. Transfections were done following amaxa standard protocols. 5×10^5 rat NSCs were nucleofected per well using 500 ng of plasmid DNA or miRNA mimics. Cells were then plated in corresponding conditions.

H1 NSCs

H1 NSCs were washed twice with PBS and detached from the plates by 2-3 minutes of incubation with Accutase at 37°C. The detached cells were then dissociated by addition of 2 ml/well of PBS followed by gentle pipetting. The cells were pelleted by

centrifugation at 1000 × g for 5 min and resuspended to a density of 5×10^6 cells/ml in transfection solution from the Amaxa® Rat Neuron 96-well Nucleofector® Kit (Lonza). For each electroporation, 500 ng of PremiR-9 (Ambion, Austin, TX) in 2 µl was placed in one well of a 96-well microcuvette plate (Lonza). A total of 20 µl of cell suspension (1 x 10^6 cells) was added to the well and pipetted to mix. Electroporation was done using the EM-110 rat neuron high efficiency program on the Shuttle System. After electroporation, the contents of each microcuvette well were dispersed as rapidly as possible with 80 µl of pre-equilibrated NPM media, then transferred to 96-well plate. Cells were left to rest in the incubator for 10-15 minutes before plating on ¼ MG coated plates. Cells were harvested for RNA and protein 24 hrs post nucleofection.

Luciferase assays

For these experiments 500 ng of plasmid and 500 ng of PremiR-9, Antimirs or a scrambled negative control, were transfected into H1 NSCs.Cells were harvested and assayed for FL and RL activity following the Dual Luciferase Reporter Assay Protocol (Promega, Madison, Wi) using the GlowMax 20/20 Luminometer (Promega, Madison, Wi). Transfection variability was then normalized by taking the ratio of FL/RL. These ratios were then compared across conditions to quantify the relative activity of the reporter. Luciferase activity was tested between 12 and 24 hrs post transfection. On average 6 to 12 wells were assayed.

RNA immunoprecipitations (RIP)

The day prior to the RIP, medium was changed to one containing 100 µM 4-thio-uridine and left to grow overnight for approximately 14 hrs. The 4-thio-uridine will incorporate into nascent RNAs during this growth period. This modified nucleotide will

serve to create stable covalent bonds with RNA associated proteins after UV cross-linking, and therefore increase the efficient pull down of RNAs associated with the immunoprecipitated protein (Hafner et al., 2010). The cross-linking also enhances the immunoprecipitation of Ago2 protein (Fig.22B).

The next day, the antibody-coated beads were prepared for the RIPs. 60 µl of beads were washed twice with PBS. Collection of beads was always done by centrifugation at 500 x g for 1 min. After washing beads, they were incubated with the antibodies. In the case of Ago2 RIPs, a protein-G Sepharose bead slurry (ZYMED) was incubated with either 60 µg of anti-human Ago2 or 60 µg of rat IgG (Millipore, PP68) as a negative control; and for Ago1 RIPs, protein A agarose bead slurry (Invitrogen) was incubated with 60 µg of anti-Ago1 or 60 µg rabbit IgG as a negative control, in 750 µl PBS for 2 hrs at 4°C.

To prepare cell lysates, 15-20x10^6 cells were lysed in 1ml RIP buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1X Halt protease inhibitor [Pierce], 10 U/ml RNAse Out [Invitrogen]) for 10 min on ice. Lysates were cleared by centrifugation (16,000 x g) for 10 min at 4°C. 100 µl of the total lysate was retained and kept for use as total input sample for later analysis. The rest of the cleared lysate (900 µl) was split in two. 450 µl was added to the specific Ago coated bead slurry and the other 450 µl was added to its corresponding isotype control coated beads. These were incubated overnight at 4°C on a rocker. The next day beads were washed twice with lysis buffer for 10 min at 4°C and once more with PBS. Bound material was eluted from beads with 50 µl of 0.1 M glycine (pH 2.3) for 15 min at room temperature. Eluted fractions were neutralized immediately with an equal volume of 1 M

Tris-HCl (pH 8) and then treated with 20 U of proteinase K for 10 min at 65°C. The total

lysate sample was also treated with proteinase K.

The eluted fractions (~100 µl) and the total lysate (input) sample were placed in 1

ml of Trizol for RNA extraction following the manufactures protocol. 10 µg of linear

acrylamide (Ambion) was used as a carrier during the isopropanol precipitation step.

After the extraction, re-suspended RNA was reversed transcribed using the High

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Ca).

Samples where then assayed by qPCR for the presence of specific mRNAs.

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VIII.   CURRICULUM VITA

Education

Rutgers University, Piscataway, NJ
Ph.D. Cell and Developmental Biology   **2011**

Universidad del Sagrado Corazon, San Juan, PR
B.S. Biology   **2001**

Teaching Experience

Rutgers University, Piscataway, NJ
Teaching Assistant – to Dr. Diana Martin in "General Biology"

Related Experience

Rutgers University, Piscataway, NJ
Graduate Fellow,
Rutgers Stem Cell Research Center and
W.M. Keck Center for Collaborative Neuroscience

Univ. of Puerto Rico, San Juan, PR
Laboratory Technician
Sandra Peña de Ortiz Lab

Publications

Jonathan Davila, Christopher Ricupero, Jennifer Moore, Alana Toro-Ramos, Ronald P. Hart. Mir-9 Regulation of OC2 During Neuronal Differentiation. (Manuscript in Preparation)
Contribution: Experimental design, bench work and writing

Young-Mi Yu[#], Kurt M. Gibbs[#], Jonathan Davila, Neil Campbell, Simon Sung, Tihomira I. Todorova, Seiji Otsuka, Hatem E. Sabaawy, Ronald P. Hart and Melitta Schachner, MicroRNA miR-133b is essential for functional recovery after spinal cord injury in adult zebrafish. Accepted Euro. J. Neurosci
Contribution: Collaborated with experimental design and writing

Uma Lakshmipathy, Jonathan Davila and Ronald P. Hart. miRNA in pluripotent stem cells. Regen Med. 2010 Jul; 5 (4):545-55. Review
Contribution: Ideas and writing

Loyal A. Goff[#], Jonathan Davila[#], Mavis R. Swerdel, Jennifer C. Moore, Rick I. Cohen, Hao Wu, Yi E. Sun, Ronald P. Hart. Ago2 Immunoprecipitation Identifies Predicted MicroRNAs in Human Embryonic Stem Cells and Neural Precursors. PLoS ONE.

(2009) 4(9): e7192. doi:10.1371/journal.pone.0007192 (# these authors contributed equally to this work)
Contribution: Ideas, biochemical assays (RIPs) and writing

Hedong Li, Yu Han, Caixia Bi, <u>Jonathan Davila</u>, Loyal A. Goff, Kevin Thompson, Mavis Swerdel, Cynthia Camarillo, Christopher L. Ricupero, Ronald P. Hart, Mark R. Plummer, Martin Grumet. Functional differentiation of a clone resembling embryonic cortical interneuron progenitors. Dev Neurobiol. (2008) Dec; 68(14): 1549-1564
Contribution: microarrays, gene expression assays

Kinzy TG, De Stefano LA, Esposito AM, Hurley JM, Roy R, Valentin-Acevedo AJ, Chang K, <u>Davila J</u>, Defren JM, Donovan J, Irizarry-Barreto P, Soto A, Ysla RM, Liesel Copeland H, and Copeland PR. A Birth-to-Death View of mRNA from the RNA Recognition Motif Perspective Biochemistry and Molecular Biology Education (2008) Jan; 36(1): 1-8.
Contribution: Ideas and writing

Goff LA, <u>Davila J</u>, Jörnsten R, Keles S, Hart RP. Bioinformatic Analysis of Neural Stem Cell Differentiation. J Biomol Tech. (2007) Sep;18(4):205-12.
Contribution: Ideas and microarrays

Colón-Cesario M, Wang J, Ramos X, García HG, <u>Dávila J</u>, Laguna J, Rosado C, Peña de Ortiz S. The Pyrimidine Analog, Cytosine Arabinoside, Blocks Memory Consolidation, but not Reconsolidation, in Contextual Fear Conditioning J Neurosci. (2006) May; 26(20): 5524-33
Contribution: Ideas and optimization of mouse intracranial injections and preliminary results of study