UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role Of Let-7 In Human Embryonic Stem Cell-Derived Neural Precursor Cells

A Thesis submitted in partial satisfaction of the requirements
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Biology

by

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2012

The Thesis of Connie Chen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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University of California, San Diego
2012

DEDICATION

I dedicate this thesis to the memory of my beloved parents, Kuo-Tong and Jan Bi Gi Chen, who taught me perseverance, sacrifice, and love.

EPIGRAPH

"And we know that in all things God works for the good of those who love him, who have been called according to his purpose."
Romans 8:28

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ABSTRACT OF THE THESIS

THE ROLE OF LET-7 IN HUMAN EMBRYONIC STEM CELL-DERIVED NEURAL PRECURSOR CELLS

by

Connie Chen

Master of Science in Biology

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Professor Alexey V. Terskikh, Chair

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SOX2 is a pan-neural transcription factor expressed in neural precursor cells (NPCs) independently of their regional identity. It plays a crucial role in both neural

development and in adult neurogenesis; however the molecular mechanisms underlying its function are poorly understood. Our previous data suggested that SOX2 controls LIN28, an inhibitor of let-7 miRNA biogenesis. We hypothesized that some of the pan-neural functions of SOX2 could be mediated by repression of let-7 miRNA activity. To identify miRNAs with pan-neural SOX2 dependency, I used NPCs with two different regional identities, dorsal and ventral. I modified a previously established human embryonic stem cell (hESC)-derivation protocol (which generated dorsal NPCs) by patterning NPCs with the Sonic hedgehog agonist purmorphamine, yieding ventral NPCs. Analysis of SOX2 targets in dorsal and ventral NPCs in addition to bioinformatics suggested that SOX2 represses the levels of let-7b and let-7i miRNA. Overexpression studies of let-7b and let-7i revealed that let-7b selectively suppresses NPC proliferation with no effect on neuronal differentiation, while let-7i abolishes neuronal differentiation without inhibiting NPC proliferation. These data suggest that the combined effect of let-7b and let-7i overexpression in NPCs photocopies the loss of SOX2 in these cells. Taken together, our results suggest that in our *in vitro* cultures, SOX2 suppresses the activity of let-7 miRNAs in NPCs. We propose that the function of let-7 miRNA family downstream of SOX2 may be a general mechanism for controlling both NPC proliferation and neurogenesis in developmental and adult contexts.

INTRODUCTION

Nervous System Development and Neural Precursor Cells

Mammalian embryonic neural development begins through a process called neurulation. At this stage, the embryo comprises three cell layers that will continue to migrate and differentiate: ectoderm (skin and neural tissues), mesoderm (muscle and bone), and endoderm (digestive and respiratory tract). During neurulation, the neural plate along the embryonic dorsal side folds to form a neural groove that will continue to deepen as the neural folds fuse to establish the neural tube, a structure that precedes the central nervous system (CNS). Neural precursor cells (NPCs) from the ectoderm are a multipotent neural stem cell population, capable of self-renewal and terminal differentiation into a variety of neuronal subtypes (Shakhova and Sommer 2010). As they migrate through the embryo to serve critical functions in the development of the nervous system and brain plasticity, different local environmental signals in different regions of the embryo direct NPC cell fate specification. In dorsal-ventral patterning, bone morphogenetic protein-4 (BMP4) is expressed in a dorsal to ventral gradient to induce dorsal fates while Sonic hedgehog (SHH) is expressed in a ventral to dorsal gradient to induce ventral fates (Wilson, P. A. and Hemmati-Brivanlou A., 1995; Chiang et al., 1996; Briscoe et al., 2001). Retinoic acid has also been found to pattern the CNS along the anterior-posterior axis (Schubert et al., 2006). These different signals are key to generating different types of neurons important for embryonic development while other molecules, such as the transcription factor SOX2, display a pan-neural expression and are important for neurogenesis in several systems (Kishi et al., 2000; Pevny et al., 2010; Rex et al., 1997).

Neural precursor cells can also be found in the adult nervous system where they serve as a multipotent stem cell population and continue to play crucial roles in cognitive function, nervous system homeostasis, and neurogenesis. Adult neurogenesis can be found in areas such as the lateral ventricles of the subventricular zone and the hippocampus where newborn neurons from precursors contribute to brain plasticity (Luskin 1993; Louis et al., 1996; Gage 2002; Ming and Song 2005). In addition, the decline of neural precursor cells has been associated with the decline of neurogenesis, learning and memory deficits, and neurodegenerative diseases (Kuhn et al., 1996; Ma et al, 2009; Winner et al., 2011; Jhaveri et al., 2012). Endogenous and transplanted NPCs have been found to contribute to functional recovery in neurodegenerative diseases such as multiple sclerosis (Snethen et al., 2008), spinocerebellar ataxia type 1 (Chintarwar et al., 2009), and Parkinson's disease (Pardal and LÓpez-Barneo 2012).

Neural precursor cells both in the developmental and adult contexts are characterized by their capacity for self-renewal and multipotency. These characteristics are tightly regulated by a dynamic network of factors such as epigenetic changes, extrinsic environmental signals, transcription factors, and microRNAs (miRNAs) (Ma 2006, Cremesi et al., 2003). Abnormal NPC gene expression patterns resulting from the deregulation of its complex neural molecular pathways have been implicated in a wide variety of pathologies such as fetal alcohol syndrome (Roitbak et al, 2011), anophthalmia (Taranova et al., 2006), autism (Li et al., 2008) and childhood leukodystrophies (Goldman 2001). Because SOX2 is expressed in all NPCs and plays important functions during neurogenesis, it is crucial to identify the molecular

mechanisms underlying its regulation of these types of cells, as this might unravel key pathways in neurodevelopment.

Transcription Factor: SOX2

Transcription factors, proteins that target specific DNA sequences for transcriptional regulation, play a critical role in mediating the pan-neural characteristics of neural progenitor cells. Induction of proneural genes such as the basic helix-loop-helix (bHLH) transcription factors MASH1 and NEUROGENIN 2, have been found to boost survival of transplanted NPCs in rat models (Yi et al., 2008). In knockout studies, neurons failed to develop as a consequence of the loss of NEUROG1 or NEUROD1 (Jahan et al., 2010). Among the transcription factors recognized in neural progenitor cells, SOX2 (SRY-related HMG-box gene 2) from the Sox family has been implicated in cell fate determination, embryonic development, and the maintenance of the proliferative and pluripotent potential of the neural progenitor pool (Schwartz et al., 2003; Graham et al., 2003; Favaro et al., 2009).

Neural precursor cells maintain a high endogenous level of SOX2 which is a crucial element for the maintenance of NPCs and normal neurogenesis in the CNS (Taranova, et al., 2006; Cavallaro et al., 2008; Favaro et al., 2009; Eminli et al., 2008; Puligilla et al., 2010; Wegner 2011). Its importance in neurogenesis is demonstrated by the defects observed in SOX2 loss of function studies in various neural contexts such as the adult hippocampus (Sisodiya et al., 2006), olfactory bulb (Cavallaro et al., 2008), cochlea (Puligilla et al., 2010), retina (Taranova et al., 2006), and embryonic dorsal root ganglia (Cimadamore et al., 2011). Human SOX2 haploinsufficiency has been observed in the pathologies such as anophthalmia which chiefly presents with

severe ocular underdevelopment and malformation (Fantes 2003), but also with abnormal hippocampal development (Hagstrom et al., 2005), oesophageal abnormalities (Bakrania 2007), and epilepsy (Sisodiya et al., 2006) among others. SOX2 deficiencies can also result in genital anomalies (Williamson et al., 2006), retarded growth, and sensorineural deafness (Hagstrom et al., 2005). It is to be noted that some of the pathological phenotypes associated with SOX2 mutations in humans (namely sensorineural deafness, retarded growth and oesophageal atresia) are very reminiscent of the neural-crest related pathology CHARGE, which suggests a role for SOX2 not only in central nervous system development but also in the human neural crest (i.e. the migratory population of cells that undergoes epithelial-mesenchymal transition (EMT) to give rise to the peripheral nervous system and contribute to the development of several organs such as heart and facial bones). Therefore, elucidating the mechanisms by which SOX2 supports self-renewal and neurogenic potential are key to understanding NPC biology.

MicroRNAs and the Let7 Family

In addition to transcription factors, microRNAs have been emerging as powerful regulators of gene expression. MicroRNAs (MiRNAs) are small noncoding RNAs about 20-25 nucleotides long that regulate gene expression through hybridization of the miRNA seed region (the 2-7 bases on the 5' end) to the 3' untranslated region (UTR) of target mRNAs to block translation or directly degrade mRNA targets (Valencia-Sanchez et al., 2006; Krol et al., 2010). Perfect complementarity is not necessary for target recognition, allowing miRNAs to regulate over hundreds of mRNAs as post-transcriptional regulators. First expressed in the

nucleus as primary microRNA (pri-microRNA), the immature double-stranded transcript is processed by enzymes Drosha-DiGeorge syndrome critical region gene 8 (DGCR8) and Pasha, then exported to the cytoplasm as precursor microRNA (pre-microRNA) (Lee et al., 2003; Gregory et al., 2006). Once exported, pre-miRNA is cleaved by RNase III Dicer into 20-25 nucleotide dsRNA duplex (miRNA/miRNA*) that unwinds with one strand becoming a guide strand that assembles to a microRNA ribonucleoprotein complex (miRNP) called RISC which targets mRNA (Figure 1A; Hutvagner et al., 2001; Ketting et al., 2001). Classically the other passenger strand has been understood to be degraded, but recent data suggest that it may also have other mediating functions elsewhere. For example mir-9, the guide strand of the miR-9-miR-9* duplex, inhibits NPCs self-renewal by targeting the nuclear receptor TLX while miR-9*, the passenger strand, regulates neuronal differentiation of fibroblasts by binding to the 3' UTR of BAF53a, a member of the SWI/SNF-like BAF chromatin remodeling complexes (Zhao et al., 2009; Yoo et al., 2011).

Expression array assays have allowed for identification of microRNA expression patterns in neurodevelopment to identify putative microRNAs involved in developmental and adult neurogenesis. For example, in embryogenesis the miR-290-miR-295 cluster is highly expressed in pluripotent mouse embryonic stem cells, but not differentiated cells (Houbaviy et al., 2003). In adult neural stem cells, mir-137, in coregulation with SOX2, and miR-184, through translational regulation of Numblike, have also been identified to mediate neural progenitor maintenance and proliferation while opposing differentiation (Szulwach et al., 2010; Liu et al., 2010).

The *lethal*-7 (let-7) family of miRNAs has also been identified as a key player in proliferation and differentiation. First identified in *Caenorhabditis Elegans* (*C. Elegans)* development, it was one of the first miRNAs to be discovered (Sulsten et al., 1977; Reinhardt et al., 2000). Let-7 is highly conserved across animal species with each isoform containing a slightly different sequence (Pasquinelli et al., 2000, Pasquinelli et al., 2003; Lagos-Quintana et al., 2003; Lee et al., 2007). Classically viewed as a tumor suppressor and prognosis indicator, let-7 family members have been found to be downregulated in many forms of cancer such as lung cancer (Takamizawa et al., 2004), ovarian cancer (Shell et al., 2007), and lymphomas (O'Hara et al., 2009), leading to uncontrolled proliferation and defects in differentiation. However, more recent studies have revealed that some members of the let-7 family may be upregulated in certain higher grade cancers (Lawrie et al., 2008; Lu et al., 2007; Brueckner et al. 2007). The varying of expression profiles of let-7 family member de-regulation in cancer suggest that they may hold slightly differing functions.

Let-7 family members also play a crucial role in development and stem cell populations. They have been shown to promote terminal differentiation in *C.* Elegans development and mature let-7a, let-7c, and let-7e have been found to be upregulated during mouse brain development (Reinhart et al., 2000; Wulczyn, et al., 2007). In addition, let-7b has also been found to inhibit neural stem cell proliferation by targeting the cell cycle regulator CYCLIN D1 (CCND1) and the stem cell regulator TLX (Zhao et al., 2010). The pluripotency factor LIN28 has been found to inhibit let-7

by blocking miRNA processing in both *C. elegans* development and mouse embryonic stem cells (Fig. 1A; van Wynsberghe et al., 2011; Hagan et al., 2009).

Human Embryonic Stem Cells as a Model for Human Development

Human embryonic stem cells (hESCs) serve as an important model for human development because of their ability to differentiate into diverse lineages from all three germ layers. Derived from the inner cell mass of a human blastocyst, they maintain pluripotency and can be differentiated to model human systems and diseases as opposed to "adult" stem cell models that can have a limited ability to differentiate into different cell types (Thomson et al., 1998; Odorico et al., 2001). Because of the difficulty in accessing primary human samples, hESCs serve as an excellent alternative model to studying neurodevelopment.

The mechanisms by which the let-7 family members mediate proliferation and differentiation in neural precursor populations are poorly understood. Because of the large diversity of NPCs affected by local signals in different regions, NPCs with different regional identities were investigated to identify let-7b as a miRNA generally controlled by SOX2 and important in neurogenesis. This study investigates the link between SOX2 defects and let-7 in hESC-derived NPCs to try to understand the complex neural network underlying self-renewal and neurogenic potential in neural precursor cells.

RESULTS

SOX2 downregulation results in proliferative and neurogenic deficits

Previous studies have revealed the crucial role for SOX2 in the acquisition of neuronal fates both *in vitro* in neural precursor cells and *in vivo* in the mouse and chick (Cimadamore et al., 2011). To further explore the mechanisms underlying SOX2 defects in NPCs, a previously published protocol for the derivation of NPCs from hESCs was used (Bajpai et al., 2009; Curchoe et al., 2010). SOX2 was downregulated for 3 days in NPCs carrying doxycycline-inducible SOX2 shRNA (shSOX2 cell line). In comparison to NPCs expressing a control shRNA (shCTRL), SOX2 knock-down resulted in an over 50% decrease in the cycling marker KI67 (Fig. 2A) as well as a decrease in transcript levels of self-renewal markers such as LIN28 and proliferation markers such as TLX, CCND1 and KRAS (Fig. 2C). Transcript levels for proneural genes such as NGN1 and MASH1 also decrease, corresponding with the inability of shSOX2 NPCs to make neurons under neurogenic conditions (Fig 2B).

Initial investigation of the molecular mechanisms underlying these SOX2 downregulation phenotypes led to a microarray screening performed in our laboratory in which SOX2 was identified as a positive regulator of the RNA binding protein LIN28, a well-known inhibitor of let-7 biogenesis and processing (data not shown). Together, these data suggested that LIN28 is under control of SOX2, thereby mediating some of the SOX2 functions in NPCs. Because LIN28 controls let-7 microRNA maturation, our preliminary data allowed us to speculate a connection between SOX2 and miRNA regulation in NPCs (Fig. 1B).

Establishing a ventral patterning protocol

The hESC-derived NPCs used in the previous experiment have been characterized to have a dorsal identity (Curchoe et al., 2010), as witnessed by the expression of markers such as PAX3 and SOX9 (Fig.3, Fig 5F-H). In order to study the pan-neural function of SOX2 in NPCs with different regional identities, I established ventral NPC cultures by exploiting the SHH pathway with its agonist purmorphamine. Neurospheres were generated from hESCs using previously published methods (Bajpai et al., 2009, Curchoe et al., 2010). At day 5 of differentiation, neurospheres were plated in self-renewal media containing the following concentrations of purmorphamine: 0µM, 0.3µM, 0.5µM, 1µM, and 3µM. Cells were fixed and stained at day 7 of purmorphamine treatment.

SOX2 and NESTIN stainings verified the NPC identity of cells at all concentrations (Fig. 4). PAX6 was also used as another general NPC marker. Its expression remained high up to 0.5 µM concentration of purmorphamine, then gradually decreased (Fig.4 and Graph 1B). Dorsal-ventral identity was assessed by evaluating the expression of PAX3 (dorsal marker), NKX2.2 and FOXA2 (both ventral markers). As previously reported (Curchoe et al. 2010), NPCs untreated with purmorphamine highly expressed PAX3 while lacking expression of ventral markers NKX2.2 and FOXA2 (Fig.4, Graph 1). Though increasing concentrations of purmorphamine resulted in a decrease in dorsal marker expression (PAX3 staining, Fig.4, Graph 1A), ventral marker expression was variable. NPCs treated with 0.3µM purmorphamine did not see a significant reduction in PAX3, nor did it show a significant increase in NKX2.2 or FOXA2 (Fig. 4B, Graph 1). A peak level of ventral

marker expression is observed at 0.5µM, but then steadily decreases with increasing concentration of purmorphamine (Figure. 4C-E, Graph 1C-D). In addition, the NPCs seemed to experience toxicity at purmorphamine levels above 0.5µM as evidenced by a decrease in cell density (Fig. 4D-E) and pyknotic nuclei (data not shown). Quantitative PCR analysis verified 0.5µM as the optimal concentration for ventralization, showing a decrease of transcript levels of dorsal genes GDF7 and PAX3 (Graph 2A) while showing an increase in ventral genes NKX6.1, OLIG2, and FOXA2 with a peak at 0.50µM (Fig. 3, Graph 2B). In conclusion, 0.5µM was chosen as the optimal purmorphamine concentration because of: 1) the lack of evident cytotoxic effects; 2) the strong reduction in dorsal marker expression (Graph 2A); 3) the pronounced increase in ventral marker expression (Fig.4C, Graph 2B); and 4) the high expression of general NPC markers (SOX2, NESTIN and PAX6, Fig. 4C). These experiments allowed us to establish human NPC cultures with very distinct regional identities (Fig. 5) that were subsequently utilized in high throughput screenings for the identification of SOX2-dependent miRNAs.

High-Throughput screening for SOX2 regulated miRNAs

Dorsal and ventral NPCs (dNPC and vNPC, respectively) were used in a microarray screening to identify SOX2 controlled miRNAs. Dorsal NPCs were propagated from neurospheres generated from shCTRL and shSOX2 lines plated onto Matrigel^{TM} at day 7, induced with 2µg/ml doxycycline at day 8 (to allow for shRNA expression), and harvested at day 11. Ventral NPCs were propagated from neurospheres generated from shCTRL and shSOX2 lines ventralized with 0.5µM purmorphamine at day 3, plated onto Matrigel^{TM} on day 7, induced with 2µg/ml

doxycycline without purmorphamine at day 12, and harvested at day 15. MiRNAs from control and SOX2 knock-down dNPCs and vNPCs were purified with the mirVana ™ kit.

Subsequent screening with Taqman® miRNA arrays revealed 282 miRNAs in vNPCs and 265 miRNAs in dNPCs with SOX2-induced global changes in expression of which 143 were in common (Fig. 6A) which led us to focus on let-7b as a commonly derepressed miRNA in both types of NPCs upon SOX2 downregulation (Fig.6B-C). Taqman qPCR for let-7b in dNPCs confirmed let-7b de-repression in SOX2 knock-down experiments (Fig. 6D). Bioinformatic analysis performed in our laboratory by Dr. Cimadamore found that the let-7i genomic locus is enriched for potential SOX2 binding sites, identifying let-7i as another potential target. This was confirmed by qPCR analysis of mature let-7i miRNA which was also derepressed upon SOX2 downregulation (Fig. 6D). Furthermore, subsequent ChIP-qPCR analysis identified an enriched SOX2 binding site on both let-7b and let-7i (data not shown). These results prompted investigation into the role of let-7b and let-7i in NPC proliferation and neuronal differentiation.

Generating hESC lines overexpressing let-7

Mature let-7b (Fig. 7A) and let-7i (data not shown) sequences were cloned between miR-30 regulatory regions of the pTRIPZ lentivector with the mammalian puromycin resistance for selection and turbo RFP for tracing inducible shRNA miRNA expression. The Tet-inducible promoter allows for doxycycline-induced exogenous let-7 expression independent of LIN28. Colony PCR verified the presence of the 114kb insert and let-7b clone #1 was chosen for lentiviral production after

sequencing confirmed the integrity of the insert (Fig. 7B). Lentiviruses containing selected clones were used for hESC transduction followed by puromycin selection to generate stable hESC lines expressing inducible let-7b (LET7B line) and let-7i (LET7I line).Verification of let-7b (Fig. 8A) and let-7i (Fig. 8B) overexpression was confirmed by qPCR.

Let-7b overexpression inhibits NPC proliferation

NPCs from shCTRL, LET7B, and LET7I lines were cultured under self-renewal conditions for four days in the presence of doxycycline to induce let-7 expression. In agreement with previous studies (Zhao et al., 2010), let-7b overexpression led to an over 30% decrease in cells positive for the proliferation marker KI67 (Fig. 9A, C) as well as an over 60% decrease in cells positive for M-phase marker Phospho-Histone H3 (PHH3) (Fig. 9B, D). These data are consistent with the loss of proliferation seen in SOX2 knock-down experiments (Fig.2A) and led us to speculate that the loss of mitogenic potential in the absence of SOX2 may be due to the increased activity of let-7b (Fig. 6B-D). In contrast, let-7i overexpression did not result in a decline in proliferation. I also observed that overexpression of let-7b or let-7i did not change neural precursor identity as documented by the expression of the progenitor markers SOX2 and NESTIN (Fig. 10). Therefore, we concluded that the let-7b mediated loss of proliferation in NPCs is not due to changes in cell identity (i.e. NPC differentiation) but it is very likely the result of direct repression of positive regulators of cell cycle [such as CCND1 and TLX, both of which have been shown to be let-7b targets (Zhao et al., 2010)]. Notably, these results are also in agreement with

the reduced expression of CCND1 and TLX observed in SOX2 knock-down experiments (Fig. 2C).

Let-7i overexpression inhibits neuronal differentiation

While let-7b overexpression recapitulated the cell cycle defects observed in SOX2 knock-down experiments, let-7i did not (Fig. 9). Since the loss of neurogenic potential is another well established phenotype associated with SOX2 downregulation, we investigated the effect of let-7 overexpression on NPC capability to differentiate into mature neurons. NPCs were cultured at high density under neurogenic conditions for 21 days. Let-7i overexpression resulted in a severe decrease in neuronal differentiation in contrast to let-7b (Fig. 11B), phenocopying the loss of neurogenic potential observed in SOX2 knockdown studies (Fig. 2B). In let-7i overexpressing cells, areas of higher cell density that classically differentiate into neurons remained negative for the mature neuronal marker MAP2 (Fig. 11A). The few cells that were still positive for MAP2 exhibited stunted morphology with shorter processes. Furthermore, let-7i overexpression under neurogenic conditions led to an over four-fold increase in apoptosis (Fig. 12) which likely contributes to the significant decrease in MAP2 positive neurons.

Because let-7i has not been well-characterized and its effect on neurogenic potential had not been previously studied, a qPCR analysis was performed to consider the molecular basis for the defects in neuronal differentiation. NPCs under self-renewal conditions at P2 were induced with 2µg/ml doxycycline for 3 days and then collected for mRNA extraction and qPCR analysis. Consistent with the observations

upon Sox2 downregulation (Fig. 2C), a significant decrease in proneural genes MASH1 and NGN1 transcript was observed (Graph 3). However, no effect on mitogenic genes CCND1 and TLX was observed. This is consistent with the absence of proliferation defects observed in let-7i overexpression studies (Fig. 9A-D).

Our finding in the hESC model suggests a mechanism for the dual roles of let-7 miRNAs as the mediators of SOX2 function in regulating NPC proliferation and neuronal differentiation through the SOX2–LIN28/let-7 axis (Fig. 13).

I would like to thank Dr. Alexey V. Terskikh for his support as my advisor and PI. I also appreciate the contributions of Dr. Flavio Cimadamore (Fig. 2: characterization of SOX2 knockdown phenotypes; Fig. 5: immunocytochemistry for NPCs; Fig. 6: Microarray preparation and analysis; Fig. 7: Let-7i cloning and quantitative PCR analysis). Figures and graphs from this thesis have been adapted for Cimadamore, Flavio; Chen, Connie; Amador-Arjona, Alejandro; Peran, Encarnacion M.; Huang, Chun-Teng; Terskikh, Alexey V. (2012). "Sox2-Lin28/Let7 Axies Regulates Human ES Cell-Derived Neural Precursor Proliferation and Neuronal Differentiation" currently in submission. The thesis author was the second author of this paper.

DISCUSSION

Preliminary results from our lab (such as the observed LIN28 dependency on SOX2 expression, Fig. 2C) let us speculate about a possible connection between SOX2 and microRNA regulation in NPCs. Because our model of human NPCs resembles dorsal neuroepithelial cells/neural crest cells (a particular type of NPC, responsible, among other functions, for the generation of the peripheral nervous system), the Sonic hedgehog pathway was exploited by using the Shh agonist purmorphamine to pattern NPCs toward ventral fates (Fig. 3, Fig. 5) in order to provide a comparative context for microarray screening. Though there were other published protocols for ventral neural cells from hESCs (Lee et al., 2007; Li et al., 2009; Fasano et al., 2010), we wanted to limit any confounding variables that may arise from differing protocols and also produce a more generalized population of ventral NPCs; therefore we did not choose a previously published protocol for ventral patterning and instead chose to optimize the use of purmorphamine as the only variable introduced in our published protocol. The optimal concentration of purmorphamine was 0.5µM, evidenced by an upregulation of ventral markers and downregulation of dorsal markers with immunocytochemistry (Fig. 4; Graph 1) and qPCR analysis (Graph 2). Also, the toxicity observed at the 1µM and 3µM concentrations were not observed at 0.5µM, a concentration sufficient to induce ventral fates.

In addition, I observed that subsequent enzymatic passaging resulted in a complete loss of ventral marker expression (NKX2.2 and FOXA2) while mechanical passaging maintained FOXA2 expression. However, FOXA2 was no longer active in

the nucleus, but became localized in the cytoplasm in its inactive form. Even with continual administration of purmorphamine, FOXA2 expression remained cytoplasmic, suggesting that FOXA2 has been phosphorylated and is no longer functional (Wolfrum et al., 2003). Furthermore, the loss of functional ventral marker expression did not result in an increase in dorsal marker expression, together pointing to a loss of ventral NPC patterning potential. Therefore ventrally patterned NPCs were used at passage 0 and compared with dorsal NPCs at passage 0 for microarray analysis.

In an initial microarray study I participated in with Dr. Cimadamore, LIN28 was identified as a protein regulated by SOX2 in NPCs. Additional data in our laboratory established the role of LIN28 in mediating SOX2 function. Because previous studies have shown that LIN28 inhibits let-7 microRNA maturation, we proposed that SOX2 may play a role in controlling microRNAs in NPCs (Hagan et al., 1999; van Wynsberge et al., 2011). Subsequent high through-put screening identified the highly conserved let-7 family member let-7b as a derepressed miRNA upon SOX2 downregulation in NPCs as suspected (Fig. 6A-C). The let-7 miRNAs are well known for their function in controlling proliferation in multiple model systems by opposing mitogenic pathways (Johnson et al., 2007; Kumar et al., 2008). More specifically, Let-7b had previously been found to block proliferation in melanoma cells by downregulating cyclins D1, D3, and A expression (Schultz et al., 2008) as well as regulate proliferation in neural stem cells through inhibition of CCND1 and nuclear receptor TLX (Zhao et al., 2010). In agreement with these findings, overexpression of let-7b inhibits proliferation in NPCs (Fig. 9). Notably, let-7b targets (CCND1 and

TLX) are downregulated in SOX2 knockdown experiments (Fig. 2C), which correlates well with the let-7b de-repression observed in the same context (Fig. 6D).

Let-7 has also been suggested to inhibit neuronal differentiation. For example, studies in mouse hippocampal neurons found that brain-derived neurotrophic factor (BDNF) induced LIN28, leading to inhibition of let-7 maturation in terminally differentiated neurons where LIN28-mediated degradation of let-7 was necessary for neuronal maturation (Huang et al., 2012). Furthermore, in retinal regeneration of *Danio renio*, Let-7 was reported to target and inhibit the MASH1 homologue Ascl1a (Ramachandran et al., 2010), a proneural gene necessary for neuronal differentiation (Guillemot et al., 1993). This was intriguing because in a previous study we identified that the proneural genes MASH1 and NGN1 were positively regulated by SOX2 (Fig. 2C; Cimadamore et al., 2011). Here I was able to elucidate the role of let-7i in abolishing neuronal differentiation of human NPCs (Fig. 11) by triggering apoptosis (Fig. 12) while not affecting NPC proliferation under self-renewal conditions (Fig. 9). Contrary to let-7b, let-7i is one of the less studied let-7 family members and, to our knowledge, its role in NPC biology and neurogenesis is completely unexplored. We therefore focused on understanding the molecular basis of let-7i-mediated inhibition of neurogenesis. We investigated whether the anti-neuronal effect of this miRNA could be explained by the loss of pro-neural gene expression, which is also observed in SOX2 knockdown experiments (Fig. 2C, see MASH1 and NGN1 expression). A strong downregulation of MASH1 and NGN1 transcripts was observed (Graph 3). Previous studies have described the requirement for these two pro-neural genes in the survival of young neurons during early phases of neurogenesis *in vivo* and *in vitro*

(Guillemot et al., 1993; Ma et al., 1999; Cimadamore et al., 2011). In fact, in line with these observations and in agreement with the reduced levels of MASH1 and NGN1, let-7i overexpressing NPCs undergo apoptosis when forced to differentiate into neurons (Fig. 12). It is worth mentioning that additional work performed in our laboratory focused on elucidating the mechanisms of SOX2-mediated repression of let-7. While accumulation of mature let-7b and let-7i are, as expected, inhibited by SOX2 *via* LIN28, preliminary data suggest that SOX2 might also repress let-7i by means of direct inhibition of pri-let-7i expression (i.e. the immature form of the miRNA). The meaning of this slightly different type of regulation is not clear; however, considering the differential effect exerted by let-7b and let-7i in NPCs, it is tempting to speculate that it might play some important function *in vivo.*

In the context of additional data from our laboratory, my data suggest that, as a family, let-7 antagonizes SOX2 function in neural precursor cells. The redundancy of the different let-7 members is not well understood (Boyerinas et al., 2010) and, in our model, let-7b and let-7i overexpression studies in human NPCs revealed their differential regulation of proliferation and neurogenic potential, respectively, which together phenocopies the deficiencies observed in SOX2 downregulation experiments. SOX2 maintains the proliferative capacity and neurogenic potential of NPCs by repressing, respectively, let-7b and let-7i through LIN28 (Fig. 13). The characterization of let-7 in NPC biology may lead to a better understanding of their function in regulating complex molecular pathways that govern NPC biology and mammalian neurogenesis.

I would like to thank Dr. Alexey V. Terskikh for his support as my advisor and PI. I also appreciate the contributions of Dr. Flavio Cimadamore (Fig. 2: characterization of SOX2 knockdown phenotypes; Fig. 5: immunocytochemistry for NPCs; Fig. 6: Microarray preparation and analysis; Fig. 7: Let-7i cloning and quantitative PCR analysis). Figures and graphs from this thesis have been adapted for Cimadamore, Flavio; Chen, Connie; Amador-Arjona, Alejandro; Peran, Encarnacion M.; Huang, Chun-Teng; Terskikh, Alexey V. (2012). "Sox2-Lin28/Let7 Axies Regulates Human ES Cell-Derived Neural Precursor Proliferation and Neuronal Differentiation" currently in submission. The thesis author was the second author of this paper.

MATERIALS AND METHODS

Human Embryonic Stem Cell Culture

The National Institutes of Health-approved hESC line, H9, was maintained on MEF feeders and Matrigel-coated (BD Biosciences #354230) tissue culture plates (Falcon #353046) in DMEM/F12 Glutamax (Gibco #10829), 20% Knockout Serum Replacement (Gibco #10828-028), 1X non-essential amino acids (Gibco #11140), 2mM L-glutamine (Gibco # 25030-081), 0.1 mM β-mercaptoethanol (Gibco #21985-023), and 1X Antibiotic/Antimycotic (Omega#AA-40) supplemented with 8 ng/ml bFGF (Sigma #F0291-25mg). HESCs were passaged every 5-7 days using 1mg/ml Collagenase type IV (Gibco# 17104-019) diluted in knockout DMEM and mechanical methods. Prior to passage, morphologically distinguishable differentiated colonies were manually removed. Medium was changed daily 48 hours after passage.

Derivation, maintenance and differentiation of human NPCs

Self-Renewal Conditions

Neurospheres were propagated from hESCs according to previously established protocols (Bajpai et al., 2009, Cimadamore et al., 2009, Curchoe et al., 2010). NPCs generated demonstrate a distinct dorsal identity (Fig. 5F-H). hES-derived NPCs were generated as monolayer cultures on Matrigel-coated (BD Biosciences #354230) tissue culture flasks (Corning #430639) in base medium (1:1 ratio of DMEM/F12 Glutamax (Gibco #10829) and Neurobasal medium [Gibco #21103-049], 1mM L-glutamine (Gibco # 25030-081), 1X Antibiotic/Antimycotic (Omega#AA-40), 2% B27 supplement without vitamin A [Gibco #12587-010], 10% BIT 9500 [StemCell Technologies], supplemented with 20ng/ml bFGF (Biopioneer #HRP-0011), 20ng/ml

EGF (Chemicon # GF001), and 5 mM nicotinamide (Sigma #N0636), 5 µg/ml insulin (Sigma11070-73-8 #). For NPCs P1 and later, 10ng/ml LIF (Millipore # LIF1005) is also added to the medium. hESC-derived NPCs were enzymatically passaged 1:2 or 1:3 every 5-7 days with Accutase (Chemicon #SCR005).

For ventralized cells, a seven day treatment of 0.5µM treatment of the SHH agonist purpmorphamine (Stemgent #04-0009) was given to NPCs at the rosette stage (5-7 days after neural induction).

Neurogenic Conditions

P2 hESC-derived NPCs were cultured at 500,000 cells per well on fibernectin-coated (Sigma #F2006) 48-well plates (Corning #CLS3548**)** under self-renewal conditions then switched to neurogenic media**,** base medium supplemented with 40ng/ml bFGF (Biopioneer #HRP-0011) and 40ng/ml BDNF (R&D Systems #248-BD/CF), the following day for 21 days.

Immunocytochemistry

Cells were given a PBS wash and fixed in 4% Paraformaldehyde (PFA/PBS) for 10 minutes at room temperature. Following a PBSAT (3% BSA and 0.5% Triton X-100 diluted in PBS) block for 1h at room temperature, cells were incubated overnight at 4°C with the primary antibodies (Table 1) diluted in PBSAT. Corresponding fluorochrome-conjugated secondary antibodies were used with each primary antibody at a 1:500 dilution. DAPI nuclear dye was used for nuclear co-staining at a 1:1000 dilution.

Quantitative PCR

Quantification of mRNA levels: Following total RNA extraction with RNeasy kit (Qiagen #74106), Quantitect kit (Qiagen #205311) was used to reverse transcribe 1 µg total RNA. Quantitative PCR was performed with SYBRGreen master mix (Invitrogen #4309155) with 2 µl of purified cDNA diluted 1:10 used as a template. HPRT was used for normalization. Data analysis was performed using the Δ(ΔCT) method. Primers used are listed in Table 2.

Quantification of mature microRNA levels - Taqman qPCR- :

The nucleospin miRNA kit (Macherey-Nagel # 740971.50) was used for purification of small RNA including the miRNA pool. The Taqman small RNA assay (Applied Biosystems # 4398987) was used to reverse transcribe 10ng of the purified small RNA pool for miRNA-specific cDNA preparation and qPCR quantification. Quantitative PCR was performed with Taqman universal PCR master mix and data were analyzed using the Δ(ΔCT) method and normalized to U6 snRNA.

Lentivirus-mediated shRNA and overexpression

Inducible SOX2 shRNA-expressing hES cells: hESC lines stably expressing doxycycline-inducible SOX2 shRNA and scrambled shRNA control were established as previously described {Cimadamore et al., 2011}.

Inducible Let-7 overexpression: Corresponding mature Let-7b or Let-7i sequences were cloned between the miR30' regulatory regions of the pTRIPZ lentivector (Open Biosystems #RHS4696), allowing for exogenous Let-7 expression independent of

LIN28 processing (Figure 8). Stable hESC lines were generated from vectors packaged into lentiviral particles the Viral Vector Core at the Sanford-Burnham Medical Research Institute (La Jolla, CA). Selection with puromycin (2.5µg/ml) for 10 days was used to eliminate hESC cells without the puromycin resistance gene in the pTRIPZ vector prior to use in experiments.

FIGURES

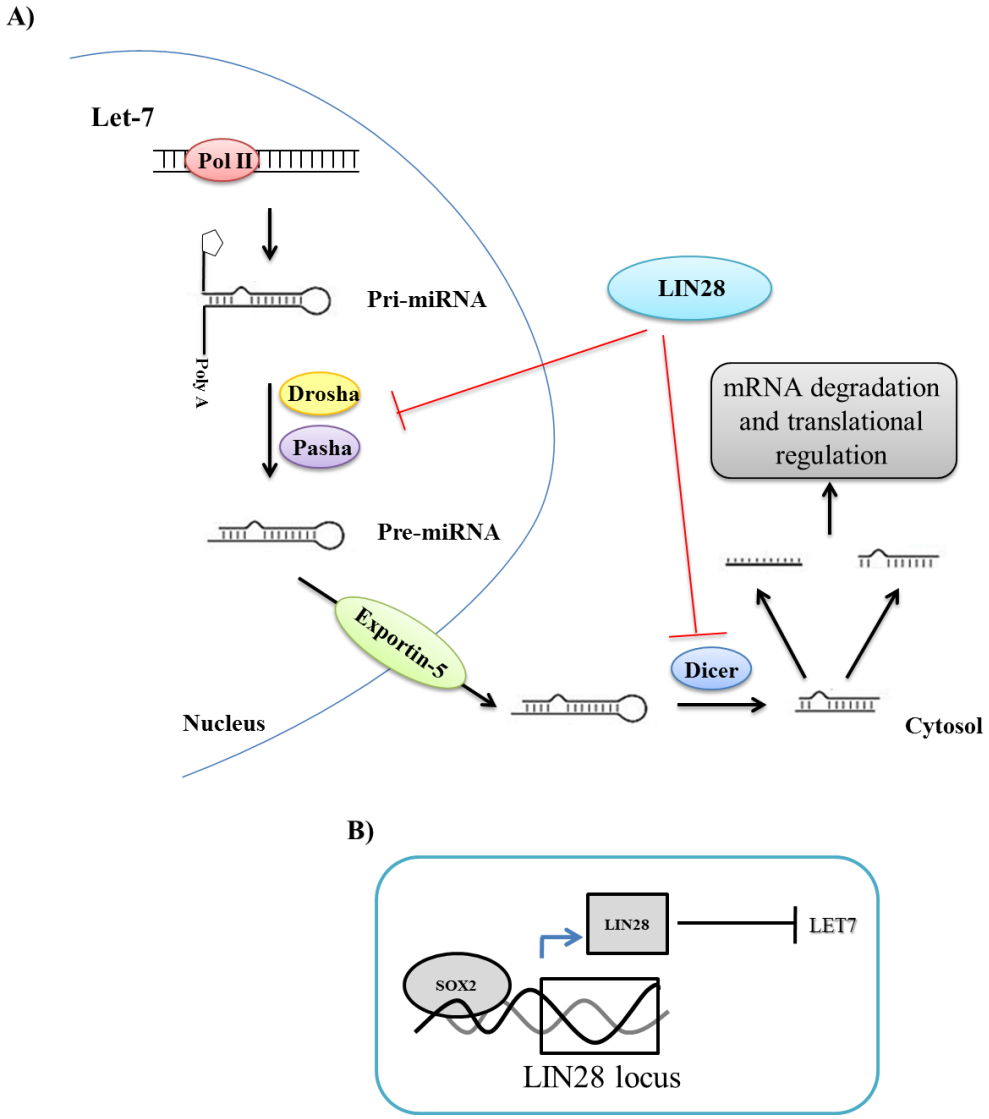

Figure 1: Let-7 family microRNA biogenesis. (A) Canonical microRNAs (miRNAs) are transcribed as primary transcripts (pri-miRNAs) by RNA polymerase II. Pri-miRNAs are further processed by Drosha and Pasha into precursor miRNAs (pre-miRNAs) which contain a short 2 nucleotide 3' overhang recognized by nuclear export factor Exportin-5, allowing for exportation to the cytoplasm. Further processing of pre-miRNAs by Dicer results in a miRNA duplex which comprise the mature miRNA strand and the passenger miRNA strand (miR*). These two strands serve different functions to mediate mRNA degradation and translational regulation. (B) Previous data showed that SOX2 is required for LIN28 expression. Because LIN28 is known to inhibit let-7 maturation, we speculated that SOX2 represses let-7 signaling through LIN28.

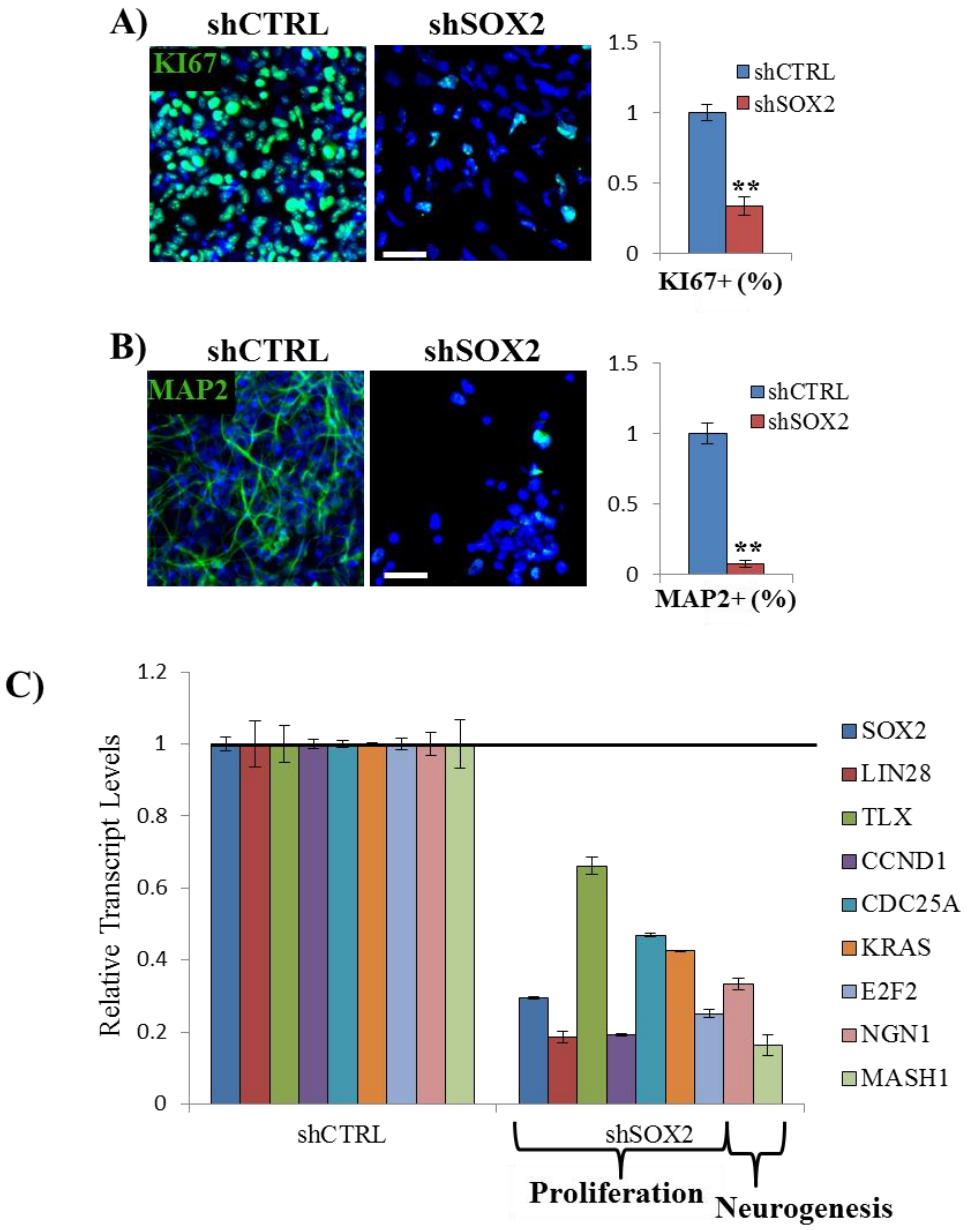

Figure 2: SOX2 downregulation results in proliferative and neurogenic defects. Immunocytochemical analysis (normalized to control) showing (A) reduced proliferation by KI67 staining and (B) reduced neuronal differentiation by the mature neuronal marker MAP2 in SOX2 knock-down NPCs (shSOX2) in comparison to shRNA control (shCTRL). Graphs to the right represent quantifications of KI67 and MAP2 expression. (C) Quantitative PCR analysis of SOX2 knockdown NPCs (shSOX2) normalized to control NPCs (shCTRL) and HPRT expression. Note the decreased expression of genes involved in proliferation and neuronal differentiation.

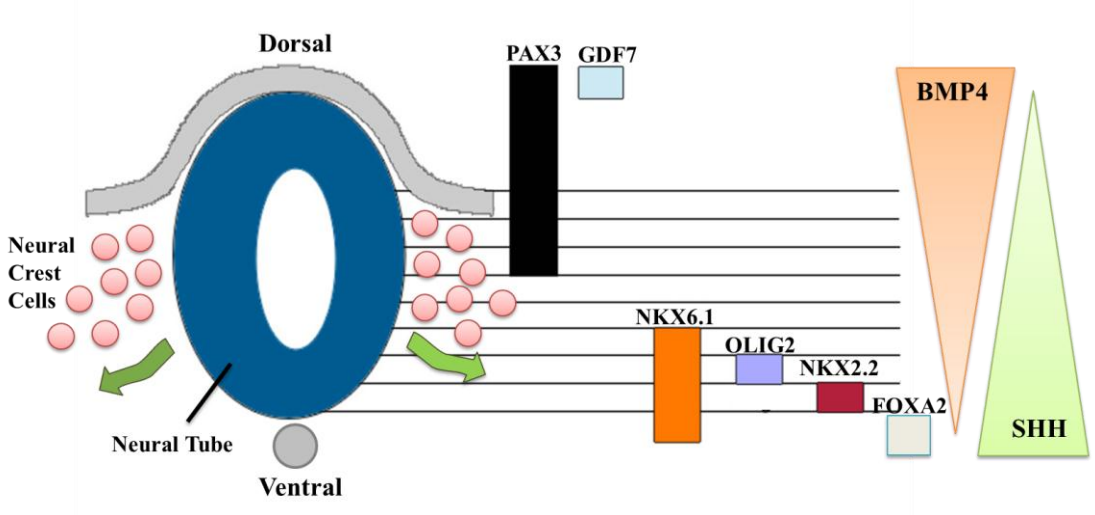

Figure 3: Dorsal-Ventral patterning of the neural tube. The newly developed neural tube is influenced by BMP4, expressed and secreted in a ventrally diffusing gradient from the roof plate cells, and Sonic hedgehog (SHH), expressed and secreted in a dorsally diffusing gradient from the floor plate cells and notochord. Different colored bars on the right represent areas of the neural tube expressing respective dorsal and ventral markers.

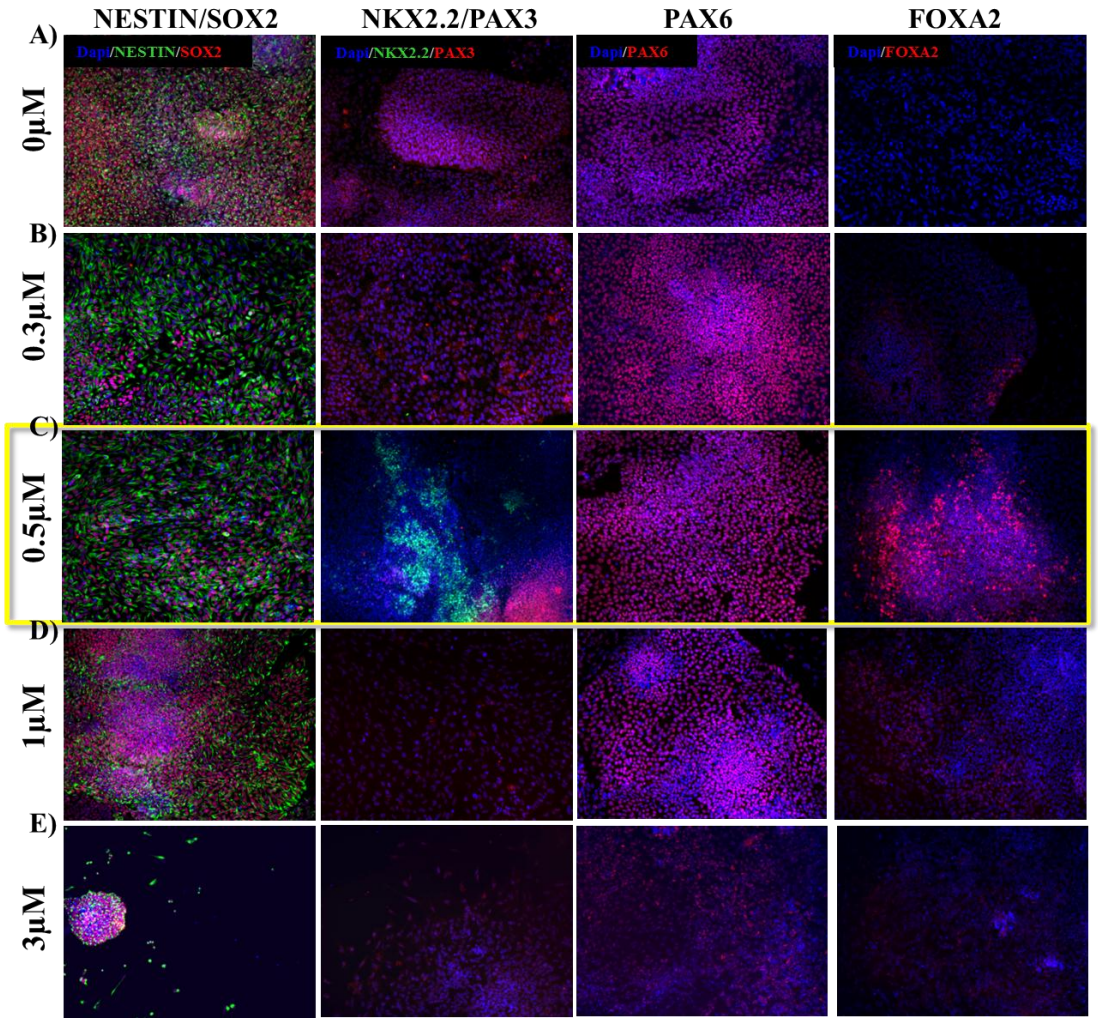

Figure 4: Dorsal and ventral expression in ventral patterning. HESC-derived NPCs were cultured under self-renewal conditions with the SHH agonist purmorphamine to induce ventral patterning at the following concentrations: 0µM, 0.3µM, 0.5µM, 1µM, and 3µM. (A-E) NPCs were fixed and stained for dorsal marker PAX3, as well as ventral markers NKX2.2 and FOXA2. NESTIN/SOX2 and PAX6 stainings confirmed that NPCs maintained their neurogenic and self-renewal capacity. (C) 0.5µM was the optimal concentration for maximal ventralization without inducing toxicity seen at 1µM and 3µM (D-E) to NPCs.

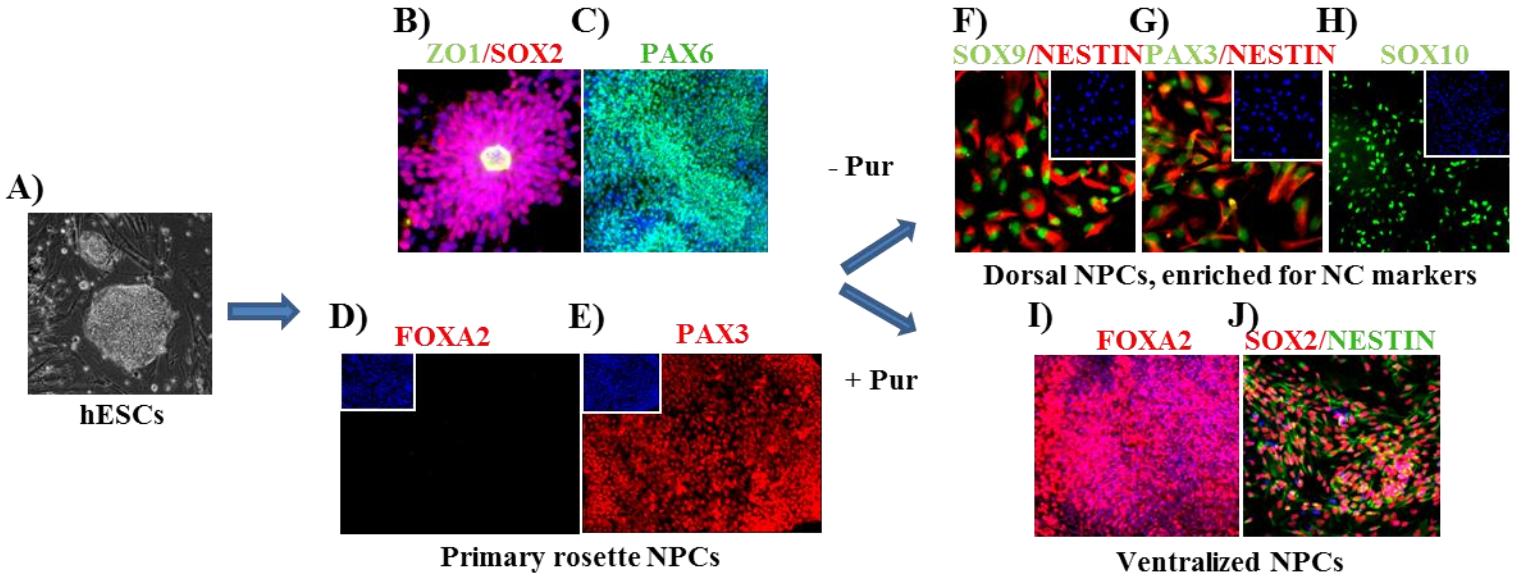

Figure 5: Derivation of dorsal and ventral NPCs from hESCs. (Adapted from S1 of pending paper Cimadamore et al.) (A) hESCs are cultured in suspension with neural induction media (NIM) for 5-7 days. (B) Resulting, rosette-forming NPCs (ZO1) are positive for neural markers SOX2 (B) and PAX6 (C). Rosette-forming NPCs exhibit a clear dorsal bias, staining negative for ventral marker FOXA2 (D) and strongly positive for dorsal marker PAX3 (E). (F-H) Continual culture of primary rosettes in NIM media results in dorsal NPCs positive for SOX9, PAX3, SOX10 (neural crest marker), and NESTIN (neuronal marker). (I) Patterning of primary rosettes with SHH agonist purmorphamine results in ventralized NPCs (vNPCs) positive for FOXA2. (J) vNPCs still retain their neural identity as confirmed by SOX2 and NESTIN stainings.

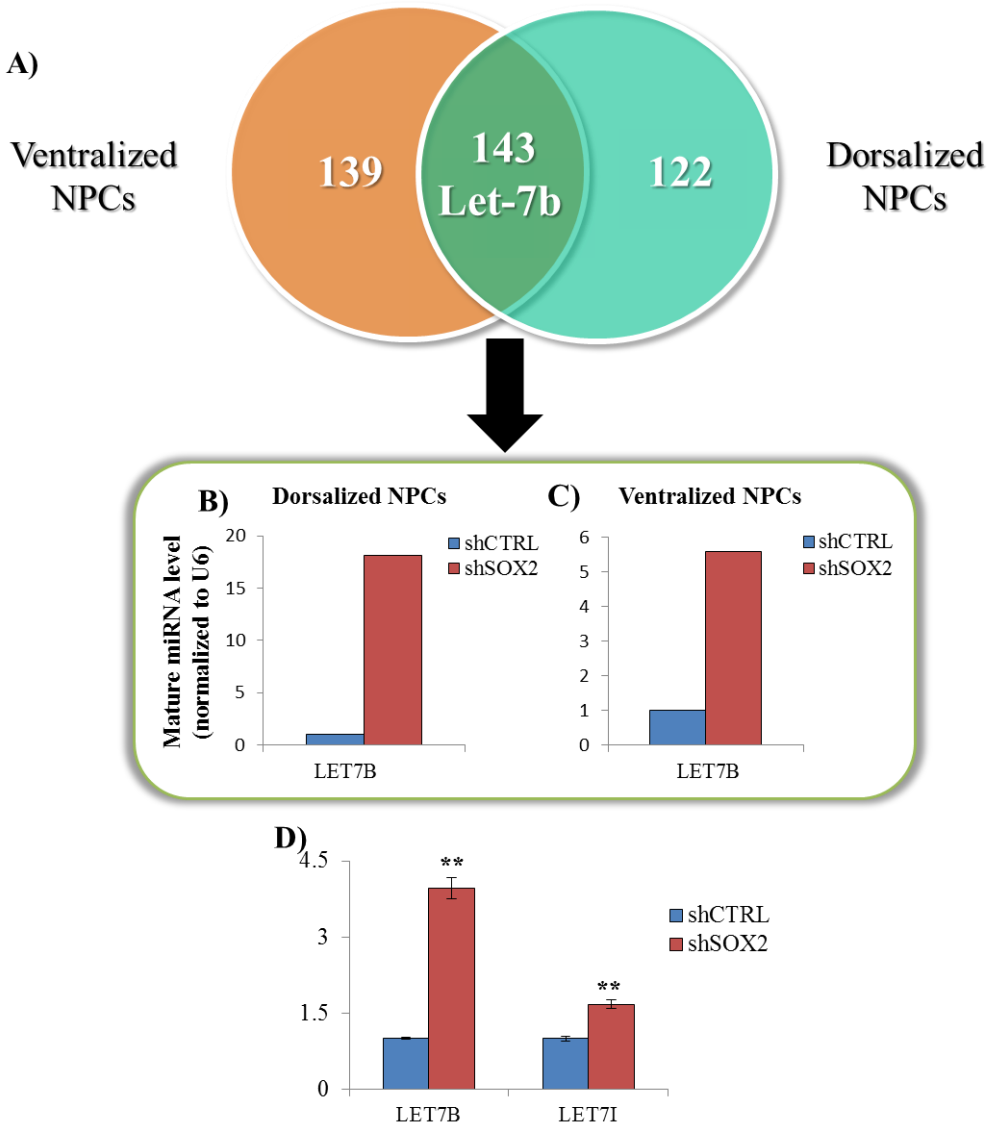

Figure 6: SOX2 represses let-7b in NPCs with different regional identities. Taqman miRNA arrays were used to quantify global changes in miRNA expression in both dorsal and ventral NPCs after 3 days of SOX2 downregulation. (A) Of the 282 miRNAs in ventral NPCs and 265 miRNAs in dorsal NPCs differentially expressed, 143 miRNAs were commonly regulated by SOX2 in both neural contexts. Let-7b was among those perturbed by SOX2 downregulation in both dorsal NPCs (B) and ventral NPCs (C). (D) Independent qPCR analysis in dorsal NPCs confirmed that let-7b and let-7i (another let-7 member identified by bioinformatics analysis) are derepressed in SOX2 knock-down experiments. (Student t-test, **Significant at $p<0.001$).

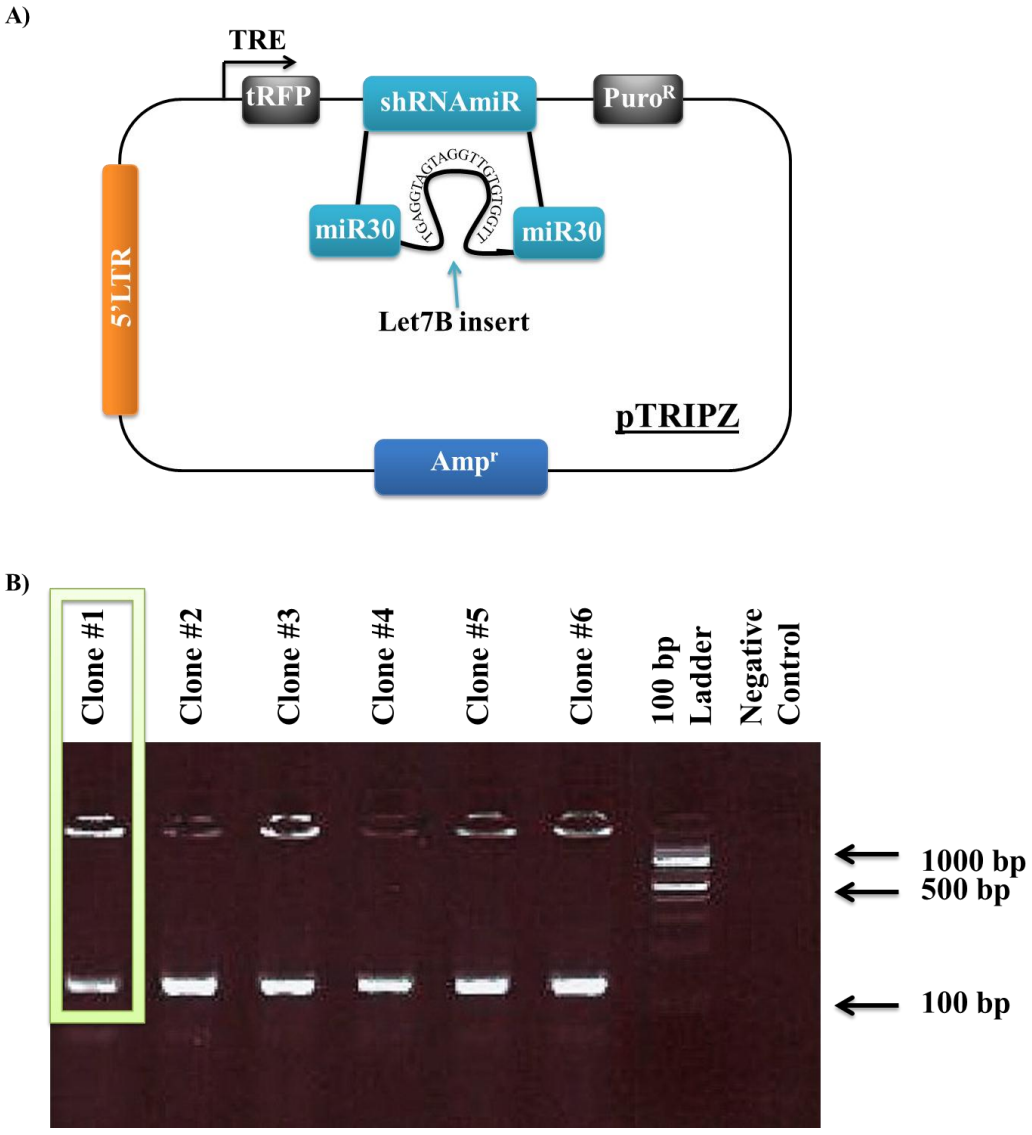

Figure 7: Let-7 overexpression in NPCs. The mature let-7b (A) and let-7i (data not shown) sequences were cloned between the miR-30 regulatory regions of the pTRIPZ lentivector to allow exogenous let-7 expression independent of endogenous Lin28. Expression in this vector is doxycylin-dependent. The puromycin resistance gene was exploited for selection of cells carrying the transgene. (TRE: Tet-inducible promoter; tRFP: turbo RFP marker to track inducible shRNA miRNA expression; $Puro^R$: mammalian puromycin selectable marker; Amp^R: bacterial ampicillin selectable marker). (B) PCR showing clones positive for the 114kb insert correspond to the mir30-let-7b-mir30 sequence shown in (A). Clone 1 was chosen after verification of the DNA sequence.

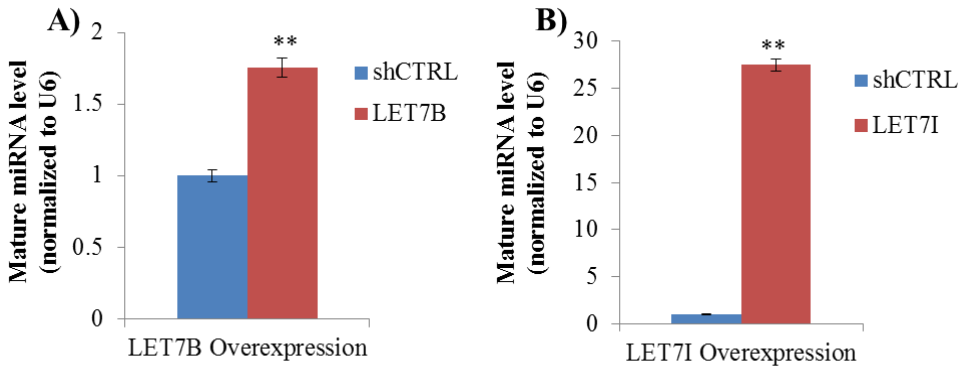

Figure 8: Validation of NPC lines overexpressing let-7b and let-7i. Quantitative PCR was used to verify let-7 overexpression in NPC lines carrying doxycycline-inducible let-7b (A) and let-7i (B). Cells were analyzed after 3 days of doxycycline treatment. Values are normalized to U6 small RNA and expressed as relative levels in comparison to cells expressing doxycycline-inducible control shRNA (shCTRL). (Student t-test, **Significant at p<0.001).

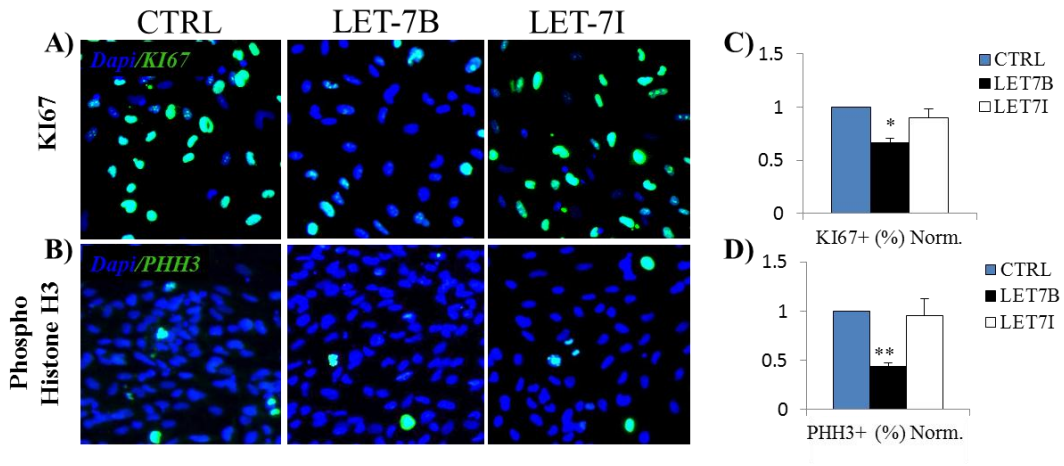

Figure 9: Let-7b NPCs exhibit defects in proliferation under self-renewal conditions. NPCs derived from the respective control and let-7 overexpression hESC lines were cultured under self-renewal conditions and induced with doxycycline for 4 days. Let-7b overexpression resulted in a significant decrease in the expression of the proliferative marker KI67 (A, C) and M-phase marker Phospho-Histone H3 (B, D), while let-7i did not in comparison to the shRNA control (shCTRL). (Normalized to shCTRL; Student t-test, *Significant at $p < 0.05$, **Significant at $p<0.001$).

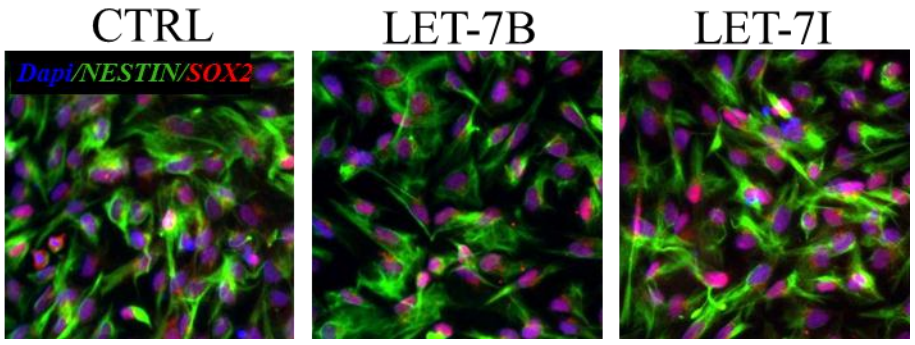

Figure 10: Let-7 overexpression under self-renewal conditions does not perturb NPC marker expression. In this experiment, let-7b and let-7i were induced with doxycycline for 4 days. Let-7b and let-7i overexpression in NPCs cultured under self-renewal conditions does not induce differentiation, as the cells retain expression of the neural precursor markers NESTIN and SOX2.

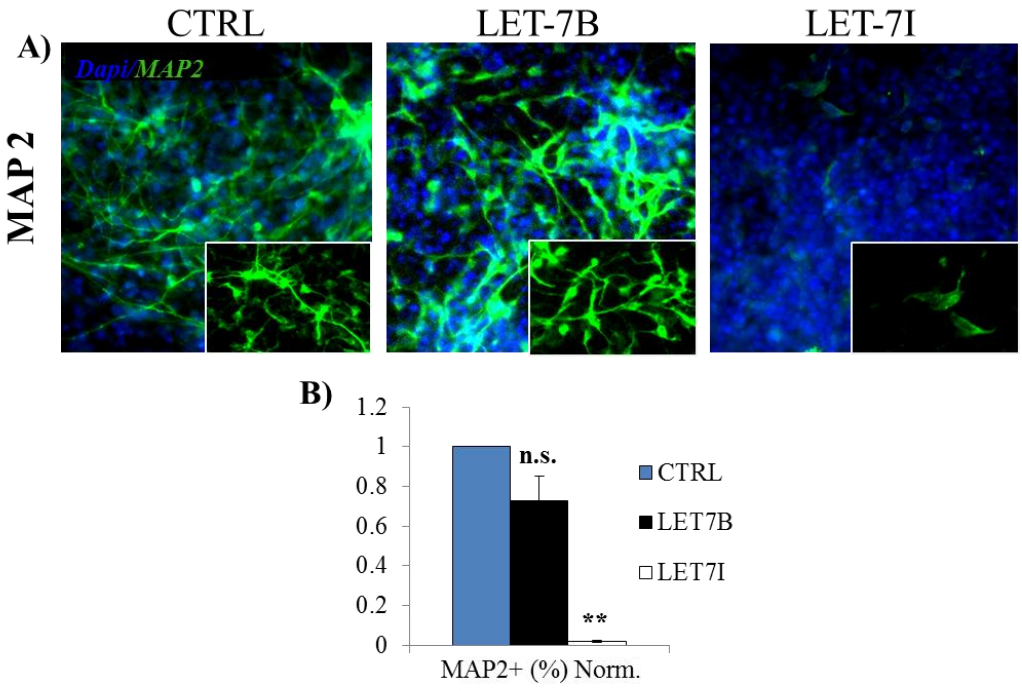

Figure 11: Effect of let-7b and let-7i overexpression in NPCs cultured under neuronal differentiation conditions. (A) Immunostaining for the mature neuronal marker MAP2 in let-7b and let-7i overexpressing NPCs cultured with doxycycline under neuronal differentiation conditions for 21 days. (B) Let-7i overexpression results in a significant decrease in MAP2 expression. (Normalized to shCTRL; Student t-test, **Significant at $p<0.001$).

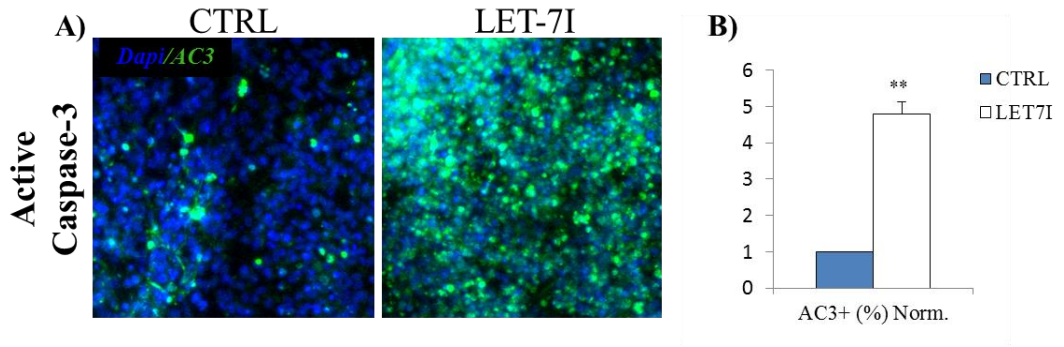

Figure 12: Let-7i induces apoptosis in NPCs cultured under neuronal differentiation conditions. (A) Immunostaining for the apoptotic marker Active Caspase 3 (AC3) in control and let-7i overexpressing NPCs cultured with docycycline for 21 days under neuronal differentiation conditions. AC3 immunoreactivity under the reported conditions is quantified in (B). (Normalized to shCTRL; Student t-test, **Significant at p<0.001).

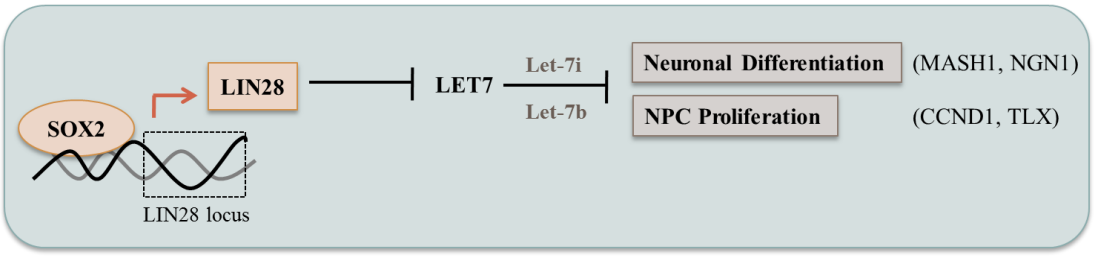

Figure 13: Schematics of the SOX2-LIN28/Let7 pathway regulating NPC proliferation and neuronal differentiation. Collectively, my data and additional data from our laboratory suggest that SOX2 sustains expression of genes required for NPC proliferation (CCND1, TLX) and neurogenic differentiation (MASH1, NGN1) by blocking let-7 maturation *via* LIN28.

I would like to thank Dr. Alexey V. Terskikh for his support as my advisor and PI. I also appreciate the contributions of Dr. Flavio Cimadamore (Fig. 2: characterization of SOX2 knockdown phenotypes; Fig. 5: immunocytochemistry for NPCs; Fig. 6: Microarray preparation and analysis; Fig. 7: Let-7i cloning and quantitative PCR analysis). Figures and graphs from this thesis have been adapted for Cimadamore, Flavio; Chen, Connie; Amador-Arjona, Alejandro; Peran, Encarnacion M.; Huang, Chun-Teng; Terskikh, Alexey V. (2012). "Sox2-Lin28/Let7 Axies Regulates Human ES Cell-Derived Neural Precursor Proliferation and Neuronal Differentiation" currently in submission. The thesis author was the second author of this paper.

TABLES

Table 1: Primary Antibodies

Name	Species	Company	Dilution	Application
FOXA2	ms	Developmental Hybridoma Bank	1:50	ICC
NKX2.2	ms	Developmental Hybridoma Bank	1:500	ICC
KI67	Rb	Vector labs	1:500	ICC
Active Caspase-3	Rb	Chemicon	1:500	IHC, ICC
MAP2	ms	Sigma	1:500	ICC
NESTIN	ms	Millipore	1:500	ICC
PAX3	Rb	Zymed	1:200	ICC
PAX6	Rb	Covance	1:500	ICC
PHH3	ms	Upstate	1:200	ICC
SOX10	ms	Wegner	1:100	ICC
SOX2	Rb	Millipore	1:500	ICC
SOX2	ms	R&D	1:100	IHC,ICC, ChIP
ZO-1	ms	BD transduction laboratories	1:100	ICC

Table 2: Quantitative PCR Primers

Gene	Sequence F	Sequence R
CCND1	TCATGGCTGAAGTCACCTCTTGGT	TCCACTGGATGGTTTGTCACTGGA
FOXA2 (HNF3ß)	AGACTCCTGCTTCTTCAAGCACCT	ACTTCCCTGCAACAACAGCAATGG
GDF7	CCACCATGTCCTCGTATTGCTTGT	TCATTCAGACGCTGCTCAACTCCA
HPRT	TGGAGTCCTATTGACATCGCCAGT	AACAACAATCCGCCCAAAGGGAAC
MASH1	AAGAGCAACTGGGACCTGAGTCAA	AGCAAGAACTTTCAGCTGTGCGTG
NGN1	CCAGCCACCACTTCAGTGTGATTT	TATTGTCAGCCGGCTCAAACCGAA
NKX6.1	GGACGACGACTACAATAAGCCTCT	GCTGCTGGACTTGTGCTTCTTCAA
OLIG2	TAAGCTGTTTGCTCACGTGACTGC	CTACAAAGCCCAGTTTGCAACGCA
PAX3	ACAACGCCTGACGTGGAGAAGAAA	ATCACAGACCGCGTCCTTGAGTAA
TLX	ACAGCCTGAGACTCTTCAATGCCT	AAGTGTAGGACGGTGTGTGTGTGT

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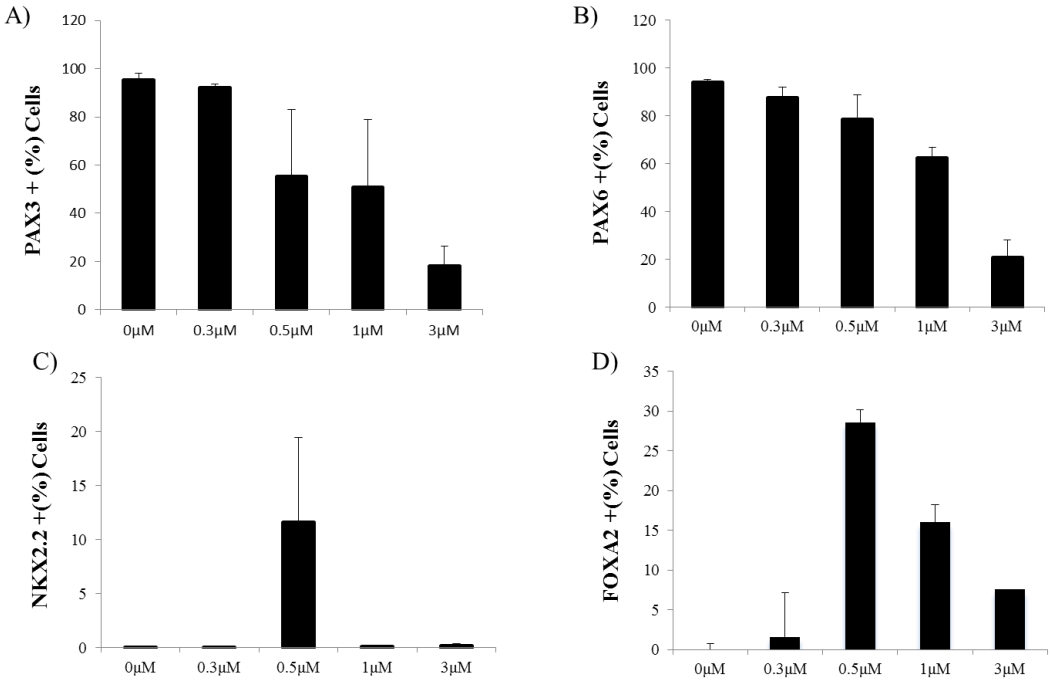
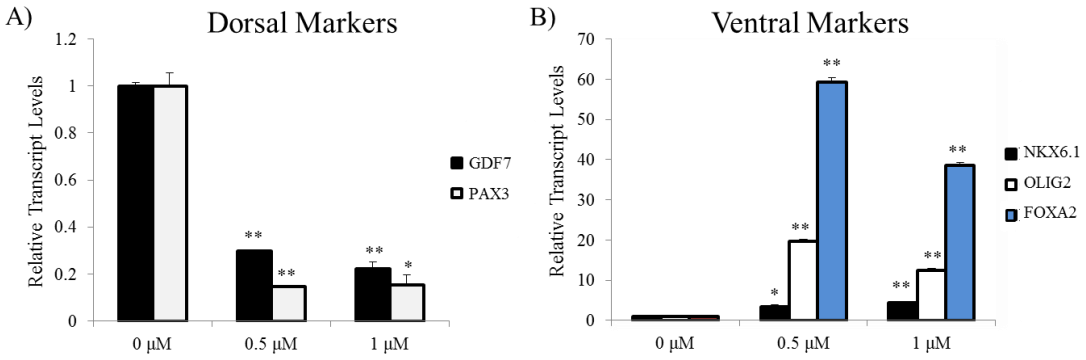
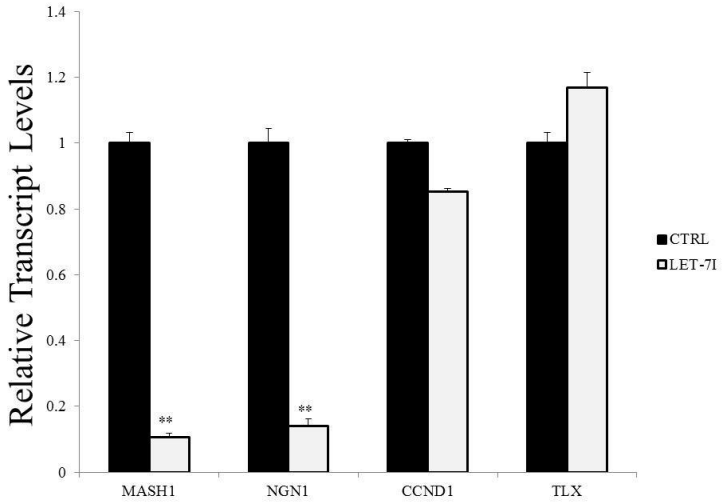
GRAPHS



Graph 1: 0.5μM purmorphamine favors ventral patterning without inducing NPC toxicity. Dorsal marker PAX3 (A) and more broadly expressed PAX6 (B) decrease with increasing concentrations of purmorphamine. 0.5μM is the most favorable concentration for ventral patterning as evidenced by ventral markers NKX2.2 (C) and FOXA2 (D).



Graph 2: 0.5μM is the optimal concentration of purmorphamine for ventral patterning. Quantitative PCR analysis of ventralized NPCs show a downregulation of dorsal genes GDF7 and PAX3 (A) and upregulation of ventral genes NKX6.1, OLIG2, and FOXA2 (B) in comparison to untreated NPCs (Student t-test, Significant at $p<0.001$).



Graph 3: Let-7i overexpression in NPCs abolishes neurogenic potential under self-renewal conditions. Quantitative PCR analysis of let-7i NPCs cultured under neurogenic conditions showed a strong downregulation of proneural genes MASH1 and NGN1 while genes involved in proliferation such as CCND1 and TLX were not significantly affected. (Normalized to CTRL, Student t-test, **Significant at $p<0.001$).

I would like to thank Dr. Alexey V. Terskikh for his support as my advisor and PI. I also appreciate the contributions of Dr. Flavio Cimadamore (Fig. 2: characterization of SOX2 knockdown phenotypes; Fig. 5: immunocytochemistry for NPCs; Fig. 6: Microarray preparation and analysis; Fig. 7: Let-7i cloning and quantitative PCR analysis). Figures and graphs from this thesis have been adapted for Cimadamore, Flavio; Chen, Connie; Amador-Arjona, Alejandro; Peran, Encarnacion M.; Huang, Chun-Teng; Terskikh, Alexey V. (2012). "Sox2-Lin28/Let7 Axies Regulates Human ES Cell-Derived Neural Precursor Proliferation and Neuronal Differentiation" currently in submission. The thesis author was the second author of this paper.

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