A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

# A role for microRNA-29b-mediated β-catenin signaling in the mouse brain development

뇌 발생과정중 microRNA-29b 에 의한 베타-카테닌 신호 전달의 기능연구

AUGUST 2014

서울대학교 대학원

협동과정 뇌과학전공

신 재 경

이학박사 학위논문

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# DEDICATION

I dedicate this dissertation to my parents and two sisters with love

# A role for microRNA-29b-mediated β-catenin signaling in the mouse brain development

**by**

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A dissertation submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Brain Sciences

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AUGUST 2014

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# ABSTRACT

**INTRODUCTION**: β-catenin has been widely implicated in the regulation of mammalian development and homeostasis. However, *in vivo* mechanisms by which Wnt/β-catenin signaling components regulate physiological events during brain development remain undetermined. Inhibitor of β-catenin and T cell factor (ICAT) interferes the association of β-catenin with T cell factor. Deficiency of ICAT in mice results in severe malformation of the forebrain and craniofacial bones, suggesting its critical role in regulation of the Wnt signaling in the CNS. The microRNA-29 family is abundantly expressed in the adult cortex, but not in the embryonic brain. However, its physiological role in neuronal development has not been well-studied. The purpose of this study is to determine if deregulated ICAT by miR-29 results in a defective neurogenesis toward neuronal cell lineages and whether these pathologies are due to impaired β-catenin-mediated signaling events.

**METHODS**: To mimic *in vivo* micro-environment, neural stem cells (NSCs) were cultured in 3D microfluidics device. Then qRT-PCR, BrdU-pulse and immunofluorescence were performed to characterize NSCs stemness in the niche, such as proliferation and self-renewal properties. In addition, luciferase activity was examined whether miR-29b directly targets the 3'-untranslated region (3'-UTR) of ICAT. Furthermore, *in utero electroporation* method was applied to investigate the roles of miR-29b *in vivo* during fetal corticogenesis under technical cooperation with a research lab at Hallym University.

**RESULTS**: miR-29b, but not miR-29a or c, is increased in differentiated NSCs which is reverse-correlated with the decreased levels of ICAT. I also found that miR-29b diminished NSCs proliferation and self-renewal capabilities, and controlled their fate, directing their differentiation along certain cell lineages. A luciferase reporter assay revealed that the 3'-UTR of the ICAT mRNA (also known as *CTNNBIP 1* gene) is the direct target of miR-29b. *In vivo* results show that applied anti-sense miR-29b by *in utero*

electroporation in corticogenesis led to the proliferative defect and premature outward migration.

**CONCLUSIONS**: I showed that miR-29b regulates neurogenesis by controlling Wnt/β-catenin signaling during brain development via inhibition of ICAT expression. Furthermore, these research findings may identify novel therapeutic approaches to modulate ICAT-mediated Wnt/β-catenin signaling to treat or prevent neurological disorders in humans.

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Keywords: neurogenesis, β-catenin, ICAT, microRNA-29b

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# CONTENTS

# LIST OF TABLES AND FIGURES

# LIST OF ABBREVIATION

**3'-UTR**, 3'-untranslated region

**EC**, endothelial cell

**ECM**, extracellular matrix

**EGF**, epidermal growth factor

**FGF**, Fibroblast growth factor

**FRS2α**, FGF receptor substrate 2

**GBM**, Glioblastoma multiforme

**GFAP**, glial fibrillary acidic protein

**GFP**, green fluorescent protein

**GSK-3β**, glycogen synthase kinase 3β

**ICAT**, inhibitor of β-catenin and T cell factor

**LEF**, lymphoid enhancer binding factor

**NEC**, neuroepithelial cell

**NSC**, neural stem cell

**OB**, olfactory bulb

**RISC**, RNA-induced silencing complex

**RMS**, rostral migratory stream

**SGV**, subgranular zone

**SVZ**, subventricular zone

**Shp2**, Src homology region 2 domain-containing phosphatase 2

**TCF**, T-cell transcription factor

**VEGF**, vascular endothelial growth factor

# INTRODUCTION

Neural stem cells (NSCs) that originate in the developing vertebrate brain are defined as their specialized capabilities to self-renew (Fuchs and Chen, 2013), proliferate and multipotent. NSCs can differentiate to multiple cell lineages to neurons, astrocytes, and oligodendrocytes (Alvarez-Buylla et al., 2002; Kriegstein and Alvarez-Buylla, 2009).

## 1. Cerebral cortical development

### 1.1 Corticogenesis in mammals

In the developing cortices, mitotic neural stem and progenitor cells located in neurogenic regions, such as ventricular or subventricular zone (SVZ) switch to post-mitotic neuroblasts. These neuroblasts migrated towards the cortical pial surface to form laminated cortical layers.

The vertebrate cerebral cortex is patterned into six layers of neurons late in development. Early-born neurons become postmitotic, migrate to the appropriate cortical layer, and differentiate into mature cortical neuron subtypes in corticogenesis. The well-known transcription factors (i.e. TBR-*1*, *Id-2*, and *Otx-1*) are preferentially expressed in specific cortical laminae. T-box brain-1 (TBR-1) is predominantly expressed in early-born cortical neurons of the preplate and cortical plate (layer 6) in the mammalian CNS (Bulfone et al., 1995; Hevner et al., 2001). Reelin (RELN) is expressed at high levels in the marginal zone, specifically in Cajal-Retzius cells. It is a secreted extracellular matrix (ECM) glycoprotein early in development.

**1.2 Characterization of the neurogenic niche**

In general, stem cells are defined as their specified abilities to self renew, proliferate and differentiate into multiple cell lineages. These stem cell

behaviors *in vivo* are strictly regulated for proper CNS development. It is believed that the distinctive nature of stem cells depends on extrinsic signals from the adjacent cells. These areas in the CNS are referred to as a 'neural stem cell niche (NSC niche)' or 'neurogenic niche'.

In the NSC niche, SVZ stem cells found in a vasculature-derived ECM-rich niche (i. e. laminin) (Shen et al., 2004). Endothelial cells (ECs), a crucial component of the NSC niche, secrete soluble factors to maintain SVZ stem cells "stem"-ness; self-renewal and neurogenic potential (Shen et al., 2008). EC-released pro-angiogenic factors can modulate the changes in miRNA profiles and responsive gene expression patterns in NSCs, leading to NSCs' cellular homeostasis.

To mimic the tissue-like microenvironment, three-dimensional (3-D) spheroid-cultured NSCs in an ECM hydrogel incorporated in a microfluidic platform are well established. The micro-engineered '3D NSC culture'

protocol has been proven to reconstitute an *in vivo*-like microenvironment that enables micro-scale cell to cell contact and/or cell to ECM interactions and quantitative investigation of NSC self-renewal and differentiation (Han et al., 2012; Shin et al., 2012).

### 1.3 Development of stem cells in the neurogenic niche

In the rodent embryo, an apical (ventricular)-basal polarized neuroepithelial cell (NEC) produces an actively proliferating SVZ stem cell that generates neuroblasts that migrate to the pial surface guided by elongated radial glia (Gotz and Huttner, 2005). NECs elongate early in development and convert to radial glial cells. Radial glia is thought to be progenitors toward astroglial cell lineages expressing glial fibrillary acidic protein (GFAP), $Ca^{2+}$-binding protein S100β (Raponi et al., 2007), vimentin, brain-lipid-binding protein (BLBP) and glutamate transporter (GLAST) (Doetsch, 2003; Mori et al.,

2006). Radial glial cell bodies with one ‘apical’ process attached to the ventricles and extend a longer ‘basal’ process to the pial surface of the neocortices (Doetsch, 2003). Radial glial cells form a scaffold for newborn neuroblasts to migrate late in development. Some radial glial cells change into SVZ astrocytes, and remain in a stem-like state within the adult brain.

In adults, the neurogenic regions were found to be highly restricted: 1) the SGZ of the dentate gyrus in the hippocampus that generates new granule cells (Gage et al., 1998); and 2) the SVZ in lateral ventricle where newly born neurons (neuroblasts) tangential migrate in the RMS to the olfactory bulb (OB) (Altman and Das, 1965; Alvarez-Buylla and Lim, 2004; Zhao et al., 2008).

The adult SVZ is a well-characterized neurogenic region in the CNS. It is located in the lateral ventricle, composed of an ependymal cell, types A, B, and C (Doetsch et al., 1997). SVZ astrocytes (type B cells), thought to be

stem-like cells in SVZ (Doetsch et al., 1999), are placed upon the ciliated ependymal layer. Epithelium-like ependymal cells line the ventricle wall. Apical type B cells give rise to transit-amplifying progenitors (TAPs, type C cells), which generate neuroblasts (type A cells). Type A cells migrate 3-8mm to the OB, where they form functional interneurons (Doetsch et al., 1997). These cells express polysialylated neural adhesion cell molecule (PSA-NCAM), TUJ-1 (β-tubulin) and doublecortin (DCX) (Yang et al., 2004). Inhibitory local-circuit interneurons in the OB divide into two main subtypes- granule cells and periglomerular cells (Lledo et al., 2006).

## 2. Signal transduction in the stem cell niche

### 2.1 Influence of growth factors and other extrinsic signals

Growth factors such as fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) are potent factors to maintain NSCs' stemness *in vitro*.

Both factors promote proliferation in germinal regions *in vivo*. FGF-2 (or basic FGF) is present at low levels in most organs, with higher concentrations observed in the brain and pituitary gland (Liekens et al., 2001). FGF receptors (FGFRs) transduce FGF signals to RAS-ERK and PI3K-AKT signaling cascades through FGF receptor substrate 2 (FRS2α) (Sato et al., 2010), the docking protein. FRS2α is ubiquitously expressed during development (Gotoh et al., 2004). FRS2α is a docking protein that facilitates FGFR signaling by recruiting the cytoplasmic protein tyrosine phosphatase, Shp2. Shp2 (Src homology region 2 domain-containing phosphatase 2, also known as *PTPN11*) is a tyrosine phosphatase with two Src-homology 2 (SH2) domains that acts to activate the RAS-ERK pathway by FGFs (Figure 1) (Neel et al., 2003).

FRS2α binds constitutively to the FGFR1 via its PTB domain and is heavily tyrosine phosphorylated upon ligand-induced activation. Tyrosine

phosphorylated FRS2 provides two docking sites for interaction with the tyrosine phosphatase, Shp2. FRS2 forms a complex with the N-terminal SH2 domain of the protein tyrosine phosphatase, Shp2 in response to FGF stimulation (Hadari et al., 1998).

The extrinsic signals are triggered by secreted molecules from adjacent ECs such as, both FGF and its polysaccharide cofactor, heparin. Heparin-binding growth factors such as basic FGF (bFGF) or FGF2, heparin-binding splice variants of vascular endothelial growth factor ($VEGF^{164}$ and $VEGF^{188}$) (Mentlein et al., 2004) or pleiotrophin (PTN, also known as HARP, heparin affinity regulatory peptide) (Mentlein and Held-Feindt, 2002) are associated to the ECM to attach to the cell-surface.

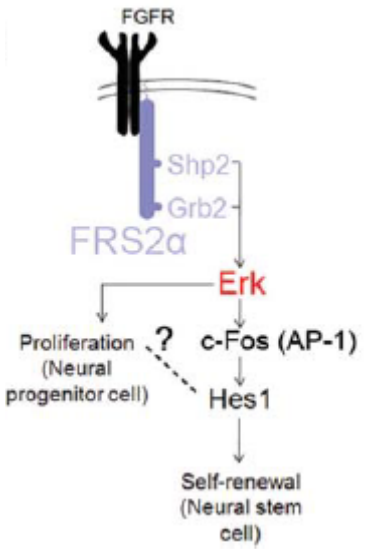


**Figure 1 FGF-induced ERK activation via FRS2α-Shp2 complex.** FGF-induced ERK activation via FRS2α-Shp2 complex contributes to both self-renew and proliferation of NSCs **(**adapted with permission from Gotoh N. et al.). FGFs regulate neurogenesis during early embryogenesis. The binding of FGFs to their cognate FGFRs results in FRS2α-Shp2 complex. FGFs-induced FRS2α-Shp2 complex formation leads to ERK activation.

## 2.2 An integrated shift: from FGF-receptor to GSK-3β

AKT inhibits the phosphorylation of β-catenin by glycogen synthase kinase (GSK)-3β. GSK-3β is a ubiquitously expressed serine/threonine protein kinase that phosphorylates at threonine 41, serine 33 and 37 (Kimelman and Xu, 2006) and inactivates β-catenin (Yost et al., 1996). β-catenin is a crucial downstream element of PI3 kinase/AKT cell survival pathway whose activity can be inhibited by AKT-mediated phosphorylation at serine 9 of GSK-3β (Cross et al., 1995). This inhibition prevents β-catenin degradation via the ubiquitin-proteasome (Aberle et al., 1997), resulting in its accumulation in the nucleus. β-catenin translocates to the nucleus, where it binds to the lymphoid enhancer binding factor (LEF)/T-cell transcription factor (TCF) family of transcription factors to induce expression of their responsive-target genes.

### 2.3 Wnt/β-catenin signaling related to intracellular mechanisms

Wnt signaling (www.stanford.edu/~rnusse/wntwindow.html) has been widely implicated in embryonic development and tumorigenesis (Logan and Nusse, 2004; Moon et al., 2004). β-catenin, a key element of the canonical Wnt signaling pathway, translocates to the nucleus and binds to the LEF/TCF family of transcription factors (Behrens et al., 1996) to initiate expression of Wnt-responsive genes such as cyclin D1 (Tetsu and McCormick, 1999) and *c-myc*. The inhibitor of β-catenin and T cell factor (ICAT, also known as *CTNNBIP1*) directly interacts with β-catenin via three helical domains at the N-terminus (Daniels and Weis, 2002). ICAT inhibits the interaction between β-catenin and TCF and interferes with Wnt signaling mediated by the β-catenin-TCF complex (Tago et al., 2000). *ICAT*$^{-/-}$ knockout mutant mice develop severe forebrain malformation beginning at embryonic day E11.5

(Satoh et al., 2004). *ICAT*-deficient mouse embryos exhibit a smaller overall brain size and abnormal forebrain morphologies.

## 3. Stem cells, Wnt/β-catenin and ICAT

### 3.1 β-catenin and associated mediators in the brain

The β-catenin mediated canonical Wnt pathway is a key regulator of progenitor cell proliferation in the developing cortical cortex. β-catenin is prominently expressed in neuroepithelial precursors across the period during brain development (Chenn and Walsh, 2002).

Together, β-catenin has been widely implicated in regulation of development and disease in humans, and is often mis-regulated in the various developmental abnormalities and pathological disorders (Clevers, 2006; Logan and Nusse, 2004).

### 3.2 Structural properties of β-catenin and its functions

Wnt/β-catenin signaling has been extensively implicated in embryonic development and tumorigenesis. β-catenin, a key component of the Wnt signaling pathway, translocates to the nucleus and binds to LEF/TCF family of transcription factors (Behrens et al., 1996) to initiate expression of Wnt-responsive genes such as cyclin D1 (Tetsu and McCormick, 1999) and *c-myc*. The ICAT directly interacts with β-catenin via three helical domains at the N-terminus (Daniels and Weis, 2002). This binding prevents the interaction of β-catenin with TCF and interferes with Wnt signaling mediated by the β-catenin-TCF complex. *ICAT*$^{-/-}$ knockout mutant mice develop severe forebrain malformation beginning at embryonic day E11.5 (Satoh et al., 2004). ICAT-deficient mouse embryos exhibit a smaller overall brain size and atypical forebrain morphologies (Figure 2).


**Figure 2 ICAT$^{-/-}$ results in multiple developmental defects**. *ICAT$^{-/-}$* embryo exhibits malformation of the forebrain and craniofacial bones (adapted with permission from Akiyama T. et al.). The depletion of ICAT results in multiple developmental abnormalities. (A-C) Wild-type (+/+) and ICAT$^{-/-}$ (-/-) embryos at E18.5 are shown in parallel. (F) ICAT$^{-/-}$ exhibited a cleft palate. (I-K) In ICAT$^{-/-}$ mice, the adrenal glands (arrowhead) also were defective.

Therefore, the negative regulation of Wnt signaling by ICAT via competition with TCF is critically integral to brain development, most likely through promotion of NSC proliferation and differentiation.

### 3.3 The negative regulatory effect of ICAT

*CTNNBIP1* maps to chromosome 1p36.22 in humans. ICAT include a N-terminal 3-helix bundle, bound to β-catenin at 'armadillo' (arm) repeats, and an extended C-terminal tail (Daniels and Weis, 2002). The C-terminal arm repeats of β-catenin bind to cadherins, TCFs, APC, and Axin (Daniels et al., 2001). ICAT interferes with the interaction of β-catenin with TCF-4 and inhibits to form a β-catenin-TCF-4 complex. ICAT binds to β-catenin and inhibits the canonical Wnt signaling cascades. ICAT induces G2 arrest followed by cell death in colorectal tumor cells (Sekiya et al., 2002). The ectopic induction of ICAT inhibits the expression of βIII-Tubulin (TUJ-1) and

thus neuronal differentiation in embryonal carcinoma P19 cells (Lyu et al., 2003).

## 4. Overview of microRNAs

### 4.1 microRNAs as an intrinsic regulator

MicroRNAs (miRNAs) are non-coding small RNAs of 22-24 nucleotides in length. Endogenous miRNAs can silence target mRNAs by triggering endonuclease cleavage or promoting translational repression. miRNAs regulate a variety of developmental and pathological processes. It is clear that miRNAs act as an intrinsic regulator of NSCs behavior (Cao et al., 2006). miRNAs that are expressed in NSCs can influence neurogenesis by fine-tuning gene expression. However, the most molecular targets of miRNAs remain uncertain.

**4.2 miRNA biogenesis, and its mechanism.**

The mammalian miRNA biogenesis proceeds into multiple steps (Figure 3) (Condorelli et al., 2010). First, miRNA gene is transcribed by RNA polymerase II into long hairpin-shaped primary miRNAs (pri-miRNAs) (Lee et al., 2004). pri-miRNAs are processed into ~70-80 nucleotide precursor miRNAs (pre-miRNAs) by the nuclear RNase III enzyme Drosha (Lee et al., 2003). pre-miRNA hairpins are exported from the nucleus by Exportin-5 in the presence of Ran-GTP as cofactor (Bohnsack et al., 2004; Yi et al., 2003). In the cytoplasm, the pre-miRNAs are processed into ~22-nucleotide duplex miRNAs by RNase III enzyme Dicer (Hutvagner et al., 2001). miRNA duplexes are unwound, the miRNA strand that has its 5' terminus at the end is called mature miRNA.

The mature miRNAs are incorporated into a ribonucleoprotein complex, the RNA-induced silencing complex (RISC). In RISC, miRNAs can mediate

repression of target gene by two modules: target mRNA cleavage or translational inhibition. Perfect matches results in cognate mRNA cleavage, followed by mRNA degradation, whereas partial miRNA mismatch with mRNA targets causes translational inhibition. miRNA-targeted mRNAs can be recruited to “processing” or “P”-bodies where they are depleted of the translation machinery and degraded.

miRNAs regulate approximately hundred and thousands target genes. A few DNA base-pairs in their 3’-UTRs corresponds to the seed sequences of miRNAs at the 5’-phosphate end. These 6-7 nt motifs are conserved at the 3’- UTR and are complementary to the seed sequences of miRNAs.

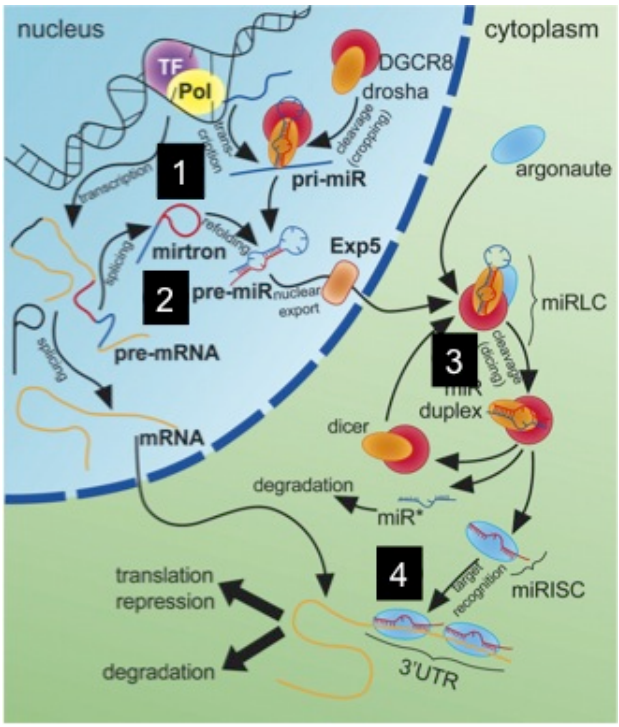


**Figure 3 A schematic diagram of miRNA biogenesis and action** (adapted with permission from the Oxford University Press). The mature miRNA sequence is given in red. TF, transcription factor; Pol, RNA polymerase II, Exp5, Exportin 5.

### 4.3 Identification of miRNAs in the developing mouse brain.

To address whether the miRNA expression pattern in the brain drastically changes during embryonic development, the miRNA profiles of embryonic and adult forebrain, including cortex and striatum, have been screened. Such studies showed that expression of the miR-29 family is absent in the embryonic brain, but is upregulated in the adult cortex and striatum (Figure 4) (Landgraf et al., 2007). The brain-enriched miR-29a, b, and c family is exclusively involved in neural development during early embryogenesis.

The brain-specific miRNAs fine-tunes corticogenesis at the early developmental stages. Brain-enriched miR-124, miR-128 has been exclusively studied. It could synergistically promote the developmental processes.

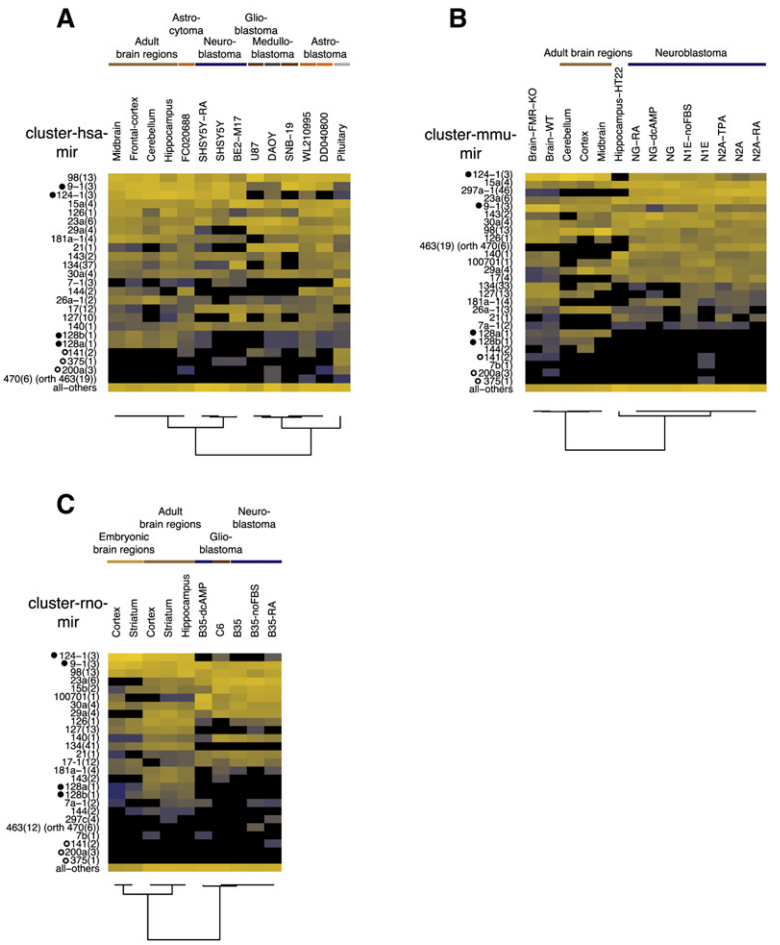


**Figure 4 miRNA expression profiles in CNS.** miRNA expression profiles in the CNS for human (A), mouse (B), and rat (C) (adapted with permission from the Elsevier). The neuronal-specific miRNA clusters are marked with black dot, the open circles indicate miRNAs enriched in varied tissues.

### 4.4 Classification of miR-29a, b, and c families and their targets

The mature miR-29a, b, and c sequences differ in 2-3 bases, but share an identical seed region (5'-AGCACCA-3'). miR-29a/b-1 is located in a genetic loci on chromosome-7, but miR-29b-2/c is on chromosome-1. The upstream regulatory promoter region of the miR-29a/b-1 gene contains several putative transcription factor (TF)-binding sites: Ap1RE (Ap1 consensus sequences), TIE (TGF-β inhibitory elements), and SBE (Smad binding elements). TF-binding sites and their interaction begins to PDGF-BB (i.p. of the Ap1RE sites) and TGFβ-linked Smad-2,3 signals (Noetel et al., 2012). Therefore, TGFβ families released by neighboring ECs at the niche can possibly regulate miR-29b expression pattern in NSCs.

The computational algorithms for miRNAs such as, TargetScan (http://www.targetscan.org) and microRNA.org (http://www.microrna.org) predict their targets.

# MATERIALS AND METHODS

## 1. Establishment of primary mouse embryonic neurosphere cultures

Timed-pregnant mice (e.g., CD1 albino, ICR) at E13.5 were purchased from animal care facilities, Harlan Laboratories, Inc. (Indianapolis, IN, USA). Experimental procedures were performed in accordance with protocols approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-130531-1).

To establish embryonic neurosphere cultures, mouse cerebral cortices were harvested at E13.5, dissociated, and resuspended (Rietze and Reynolds, 2006). Embryos at E13.5 were dissected, isolated the entire cortices and meninges were removed. And, dissociated, plated NSCs and, expended in response to basic FGF (early stem cell at E12), EGF (late “adult” stem cell at E17 to adult), or both (classic stem cell at E14 to E15) as

mitogens. Then test undifferentiated NSCs with widely employed molecular markers such as, Nestin, Bmi-1 (B-lymphoma Moloney murine leukaemia virus insertion region 1), Musashi-1, Sox-2 at mRNA level. bFGF, EGF-responsive NSCs were grown and then passaged.

**2. cDNA synthesis and qRT-PCR analysis**

The expression pattern of mature miR-29 a, b, and c was assayed by quantitative RT-PCR. Briefly, 100 ng of total RNAs, including small RNAs >18 nt in length, were reverse transcribed using miScript starter kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Specific RT primers for miR-29b sequence, guide strand (5'-UAGCACCAUUUGAAAUCAGUGUU-3', QIAGEN, Hilden, Germany), or U6 RNA were used. cDNA was amplified in an ABI StepOne system. Relative quantification (RQ) was carried out using the ΔΔCT method. Sample

variability was normalized to U6 RNA levels. To investigate miR-29b-regulated target mRNAs, 0.5 mg of total RNA was reverse transcribed, cDNA was synthesized, and PCR products were normalized to GAPDH levels.

### 3. Micro-device array fabrication and preparation

NSCs in the developing brain are dependent on signals from neighboring cells and ECM proteins. To mimic the surrounding microenvironment, I cultured NSCs in a three-dimensional (3-D) ECM hydrogel incorporated in a PDMS microfluidic assay (Chung et al., 2009; Huh et al., 2011).

The micro-device array consisted of eight units of micro-patterned polydimethylsiloxane (PDMS; Sylgard 184; Dow Chemical, MI) fabricated using a conventional soft lithography procedure. The sterilized micro-patterned PDMS and a glass coverslip substrate were treated with oxygen

plasma (Femto Science, Seoul, Korea) and bonded together, thus forming a closed micro-channel (140-160μm) system. A single unit is composed of one central channel with two side channels for 3D NSC culture and growth medium supplementation, respectively (Figure 5).

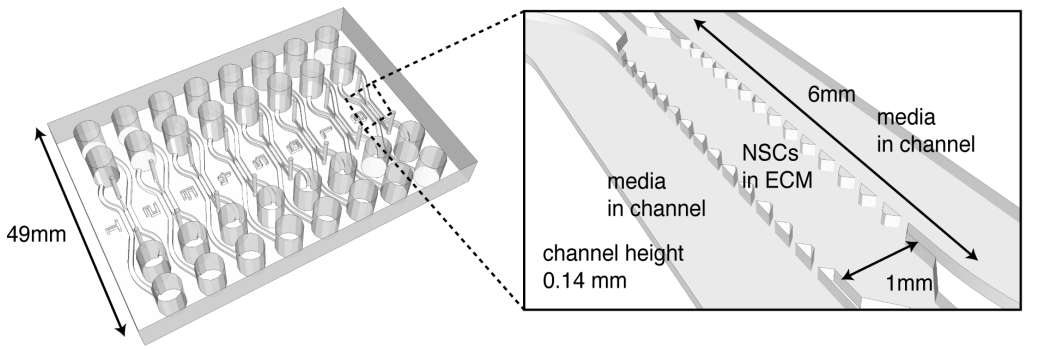


**Figure 5 Top view of the 3D microfluidic channels for qRT-PCR**. The microfluidic device has two side channels for supplying growth medium and one center channel for NSC culture in 3D micro-scale ECM hydrogels.

## 4. 3D NSC culture in the micro-device

For 3D NSC culture, collagen-based matrices (collagen type I, rat tail, BD Biosciences, Franklin Lakes, NJ, USA) were used as the ECM material. Collagen type I solution was diluted to 2 mg/ml in 10× phosphate-buffered saline (PBS; Thermo Scientific, MA, USA) and distilled deionized water, and the pH of the gel solution was adjusted to pH 7.4 with 0.5 N NaOH. E13.5 neurospheres were dissociated and suspended in ice-cold collagen solution at a density of $5 \times 10^6$ cells/ml. Cultivated NSCs in collagen solution were injected into the central channel and allowed to gel by incubating at 37°C for 30 min. For cell transfection, lipofectamine RNAiMAX (Invitrogen, CA, USA) was mixed with 100 nM siRNAs targeting ICAT (MISSION® siRNA, mouse, NM_023465, Sigma-Aldrich, MO, USA; siICAT#1, SASI_Mm01_00054131, sequence start at 949; siICAT#2, SASI_Mm01_00054132, at 825), or 50 nM miRNA (Qiagen, Hilden, Germany). After gelation, complete growth medium

and individual transfectants were added to the micro-device through both side channels and incubated overnight. The cells were washed and the medium refreshed daily. Specific gene expression of NSCs was quantified by qRT-PCR after 3 days in culture.

**5. Western blot analysis**

NSCs transfected with scrambled control siRNA and miR-29b (targets mature mmu-miR-29b-3p, 5'-UAGCACCAUUUGAAAUCAGUGUU-3' Cat# MSY0000127, QIAGEN, Hilden, Germany). 72 hours after transfection, NSCs were harvested and lysed in RIPA buffer. 30 - 50 μg proteins were loaded onto denaturing 4 - 12 % Bis-Tris gels (Invitrogen, CA, USA) and transferred to PVDF membranes (Millipore, MA, USA). Membranes were blocked with 3% milk in PBS with 0.05% Tween-20 for 1 h at room temperature, and then probed with adequate primary antibodies for

overnight at 4°C, followed by incubation with horse radish peroxidase (HRP) conjugated anti-rabbit or anti-mouse antibodies (1:700 - 2,000, Invitrogen, CA, USA). The HRP signals were visualized using an enhanced chemiluminescent (ECL) substrate (G-Biosciences, MO, USA) via LAS-3000 (GE ImageQuant, NJ, USA).

Western blot analysis was performed using anti-ICAT (1:500, Santa Cruz Biotechnology, Inc., CA, USA), anti-β-actin (1:5,000, Sigma-Aldrich, MO, USA) antibodies. *CTNNBIP1* gene product (ICAT) is a 9-kD protein. β-actin (Sigma-Aldrich, St. Louis, MO, USA) was used as loading control.

**6. Immunofluorescence**

A range of molecular markers has been used for IF such as, TUJ-1 (immature neuron), O4 (oligodendrocytes), GFAP, GLAST (astrocyte), Nestin (Rat 401, uncommitted neural stem cells). NSCs cultured in a micro-

device array were fixed in 4% (w/v) paraformaldehyde in PBS at room temperature for 15 min and washed with PBS. The fixed cells were incubated with 0.1% Triton X-100 for 5 min for permeabilization, blocked with 4% (w/v) bovine serum albumin (BSA) in PBS for 1 hour, and washed with PBS. After blocking, cells were incubated with antibodies against mouse monoclonal anti-TUJ1 (Millipore, MA, USA, 1:100), mouse monoclonal anti-O4 (Millipore, MA, USA, 1:100), rabbit polyclonal anti-GFAP (Abcam, Cambridge, England 1:100), rabbit polyclonal anti-ICAT (FL-81) (Santa Cruz, TX, USA, 1:100), and mouse monoclonal anti-active β-catenin (clone 8E7) (Millipore, MA, USA, 1:100) for 2 h at room temperature. Cell nuclei were stained with DAPI (Sigma-Aldrich, MO, USA).

## 7. Luciferase reporter assay

The full-length (2,173 bp) 3'-UTR of the mouse *CTNNBIP 1* gene (encodes ICAT) on a pEZX-MT01 backbone was purchased (GeneCopoeia™, MD, USA) (Figure 6). The mutated construct has TGCT to GTAG base mismatches within the miR-29-target binding site in the 3'UTR. HEK293T cells were seeded 24 h prior to co-transfection. Cells were co-transfected with 0.5 µg of reporter constructs (WT or MUT) and control siRNA or 50 nM mouse miR-29b-3p miRNA duplex (QIAGEN, Hilden, Germany). After 24 h, luciferase activity was measured using the dual-luciferase reporter assay system (Promega, USA) and normalized to *renilla* luciferase activity.

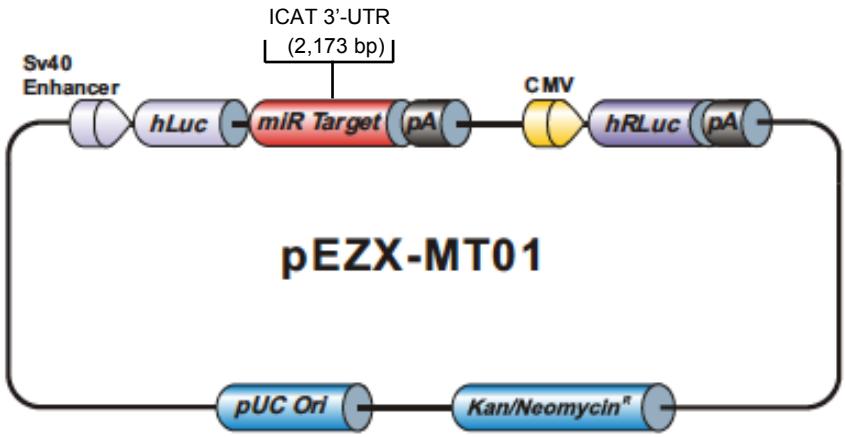


**Figure 6** Vector information for MmiT033050-MT01 (GeneCopoeia™, MD, USA).

## 8. BrdU incorporation assay

NSCs at E13.5 were seeded at $1\times10^5$ cells per well in laminin (Invitrogen, CA, USA)-coated 24-well plates. Cells were pulsed for 1 h with 10 μM 5-bromo-2'-deoxyuridine (BrdU) (BD Biosciences, Franklin Lakes, NJ, USA). BrdU is a thymidine (T) analog and it incorporates into the DNA while cells are at S-phase. The BrdU pulse-labeled cells were fixed and acid treated, followed by immunostaining with antibodies to anti-BrdU (Abcam, Cambridge, England 1:100).

## 9. Expansion and proliferation assays

Neurospheres were dissociated and plated with FGF-2, EGF in T25 flasks. At day 1, 3, 5, neurospheres were measured in diameter and counted selectively >50μm in diameter and also viable cells were identified by trypan blue.

## 10. *In utero* electroporation

Timed-pregnant C57BL/6N females at E13.5 were anesthetized with inhalational anesthetic, isoflurane (4% during induction, 2.5% during surgery), and embryos were exposed. The mature miR-29b RNA duplex anti-sense sequence is 5'-GCUGGUUUCAUAUGGUGGUUUA-3'. 1μl of small RNA (37.5 pmol control siRNA or anti-miRNA-29b) with plasmid DNA (0.625μg pCAGIG GFP-reporter plasmid, addgene, plasmid 11159) in PBS was prepared and microinjected into the lateral ventricle using mouse-controlled 90-mm glass capillaries (GD-1; Narishige, Tokyo, Japan) (Sun et al., 2011). Electroporation was performed into DNA-injected embryos with Tweezertrodes electrodes (diameter, 5 mm; BTX, Holliston, MA, USA). The electric pulses were delivered to the embryos with a duration of 50 ms per pulse (45V, 5 pulses, interval 950 ms) using a square-wave pulse generator

(ECM 830; BTX, MA, USA). The electroporated mice were sacrificed and analyzed at E16.5, 18.5 (Saito, 2006).

## 11. Histological approaches

To identify developmental defects at E16.5-18.5 based on molecular and morphological alterations, immunohistochemistry (IHC) on paraffin-embedded or frozen sections were performed. A various range of molecular markers have been used for IHC, including TBR-1 (for post-mitotic glutamatergic neurons), RELN. In addition, embryos dissected at E16.5, E18.5 to determine temporal differences of layers patterns in cerebral cortices.

## 12. Statistical analysis

Quantitative data were expressed as standard error mean (SEM), for n >3. Significant differences were tested by unpaired Student's *t*-test for comparison between two treatment groups. Asterisks indicate *p<0.05; **p<0.01; and ***p<0.001.

# RESULTS

## 1. Wnt signaling components are expressed in 3D cultured NSCs

NECs lie in the lateral ventricle, are responsible for generating most neurons and glias (Kriegstein and Alvarez-Buylla, 2009). To investigate the role of the miR-29 family in neuroepithelial cells (NECs), I performed quantitative real-time PCR analysis of miR-29a, b, and c subtypes in NSCs (Figure 7). miR-29b showed a prominent increase in abundance when differentiation was induced by commercial supplements (Stem Cell Technologies, BC, Canada) or 1% fetal bovine serum (FBS). To identify putative target molecules of the miR-29 family, I examined expression of ICAT and β-catenin. *ICAT* mRNA expression showed a reverse correlation with miR-29b expression (Figure 8).

## 2. miR-29b reduces NSC proliferation and self-renewal

To investigate the effect of miR-29 duplexes on NSCs, E13.5 cortical cells transduced with miR-29 or antisense against miR-29 were grown as neurospheres and plated in growth medium supplemented with EGF and FGF2. BrdU incorporation after 24 h revealed a 30% decrease in the growth rate of miR-29b treated NSCs, resulting in a decrease in the propagating cell population. In contrast, anti-sense against miR-29b promoted a 33% increase in the rate of cell proliferation (Figure 9).

EGF- and FGF2-responsive NSCs are present in the embryonic telecephalic germinal region as early as E13.5 (Reynolds and Rietze, 2005; Rietze and Reynolds, 2006). The primitive neural progeny cells mostly express the progenitor marker Nestin, a type VI intermediate filament protein, and a few early neurons express TUJ-1. To examine the differentiated cell subtypes generated from neurosphere-expanded stem cells, miR-29b transfected NSCs were analyzed by qRT-PCR to quantify expression of

markers specific to each cell type, such as Nestin (Figure 10), glial fibrillary acidic protein (GFAP), TUJ-1, and typical of oligodendrocytes O2 (data not shown). In the presence of miR-29b or siRNA against ICAT (siICAT#2), the expression of neural progenitor markers Nestin and GFAP, which is especially expressed in radial glial cells early in development (Doetsch, 2003; Frederiksen and McKay, 1988; Kriegstein and Alvarez-Buylla, 2009), was significantly reduced. Radial glia share features with neural stem and progenitor cells (NSPCs), which are earlier neuroepithelial cells present from E10.5 in embryonic development (Frederiksen and McKay, 1988).

**3. Nuclear β-catenin is necessary for NSC differentiation**

β-catenin translocates to the nucleus where it binds to the LEF/TCF family of transcription factors to control expression of their target genes (Behrens et al., 1996). β-catenin in the nucleus is essential for NSC differentiation

(Figure 11). ICAT colocalized with β-catenin in the cytoplasm but not at the plasma membrane while proliferate (Figure 12). Identical expression patterns in the colon epithelial cells were observed (Tago et al., 2000). Our results suggest that ICAT tightly cooperates with β-catenin in developing cortical layers.

I anticipated that miR-29 miRNAs would induce more neuronal-like progeny than glial cell lineages. At present, it is not clear how NSCs differentiate into each cell lineage, although it has been shown that ectopic induction of ICAT inhibits the expression of TUJ-1 and thus neuronal differentiation in embryonic carcinoma P19 cells (Lyu et al., 2003). Overall, little is known about the mechanisms controlling cortical development and the genetic pathways that regulate them.

**4. miR-29 directly targets the 3'-UTR of ICAT**

The TargetScan (http://www.targetscan.org) (Lewis et al., 2003) and microRNA.org (http://www.microrna.org) (Betel et al., 2008) algorithms confirmed that miR-29b targets the 3'-UTR sequence of *ICAT*. miR-29 family members (miR-29a, b, and c) were predicted to target a conserved seed sequence (5'-TGGTGCT-3') in the 3'-UTR of ICAT. To validate whether miR-29b targets ICAT through these putative target sites in the 3'UTR, I generated a luciferase reporter construct using the mouse ICAT 3'-UTR (2,173-bp) containing the predicted miR-29b target seed sequences. RNA duplexes of mature miR-29b were co-transfected into human embryonic kidney HEK293T cells with wild-type (WT) or mutant (MUT) miTarget miRNA 3'-UTR target clones (GeneCopoeia™, Rockville, MD, USA) as reporter constructs. Luciferase reporter gene activity decreased 20-30% when the WT reporter gene was co-transfected with miR-29b (Figure 13). To determine whether the predicted miR-29b target site in the *ICAT* 3'-UTR is

required for repression of ICAT expression, multiple base-pair mutations were introduced into the *ICAT* 3' UTR. These mutations of miR-29b target sites abolished miR-29b binding to the MUT construct. Activity of the reporter gene containing the mutant 3'-UTR was not affected by miR-29b. These results indicate that miR-29b represses ICAT protein expression by direct binding to the predicted target sites in the *ICAT* 3'-UTR.

**5. miR-29b antisense induces a profound proliferative defect during brain development**

To determine whether miR-29b plays a pivotal role in neuronal differentiation and migration *in vivo*, 2'-O-methyl modified anti-miR-29b inhibitor was microinjected into the left ventricle of mouse embryos at day E13.5. Inhibition of miR-29b by *in utero* electroporation of anti-miR-29b inhibitor into embryonic mouse brains led to a proliferative defect and premature outward

cortical migration (Figure 14). In contrast, the loss of function of ICAT induced by RNAi-mediated gene silencing *in utero* results in proper cortical migration. The miR-29b antisense inhibits TBR-1, a member of the brachyury (T-box) family of transcription factors. TBR-1 activates the RELN protein, which is secreted by Cajal-Retzius cells in cortical development (Hevner et al., 2001).

Cells transfected with control siRNA radially migrated at the cortical marginal zone and showed normal distribution of RELN expression. In contrast, a decrease in RELN-positive Cajal-Retzius neurons in the marginal zone was observed in brains with inhibition of miR-29b, presumably because TBR-1 expression in the cortical plate is suppressed in the presence of anti-miR29 (Figure 15). TBR-1 is an early pyramidal neuron marker that is expressed toward the pial surface and in the cortical plate (Hevner et al., 2001).

Malformations of cortical development can arise when preferentially labels projection neurons.

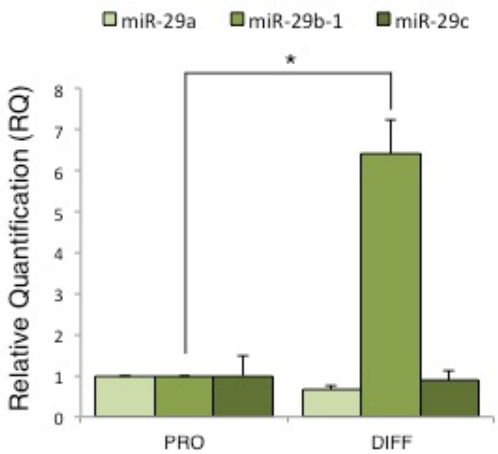


**Figure 7 Expression profiles of miR-29 subtypes in NSCs.** NSCs were harvested and total RNA was subjected to qRT-PCR to quantify expression of miR-29 subtypes a, b, and c. miR-29b, but not miR-29a or 29c, is significantly upregulated in 3D cultured NSCs, whereas ICAT is reduced as aged.

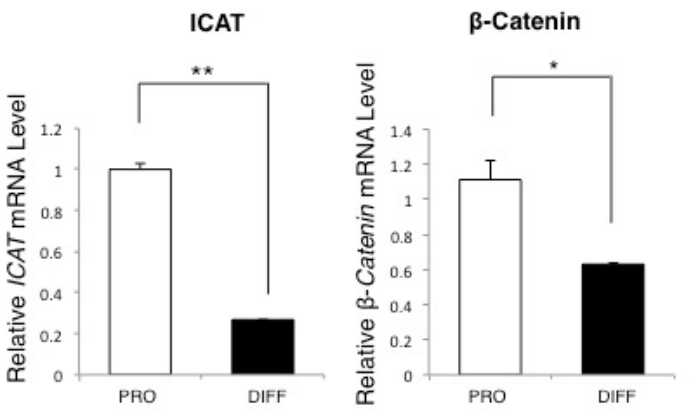


**Figure 8 Wnt signaling components expression pattern in NSCs** ICAT decrease results in an increase in the expression of miR-29b. ICAT and its binding partner β-catenin are highly expressed in fetal NSCs but expression declines with age (day 1, 3, 5). This decrease correlates with the increased expression of miR-29 family members, which are known to target and repress ICAT protein expression.

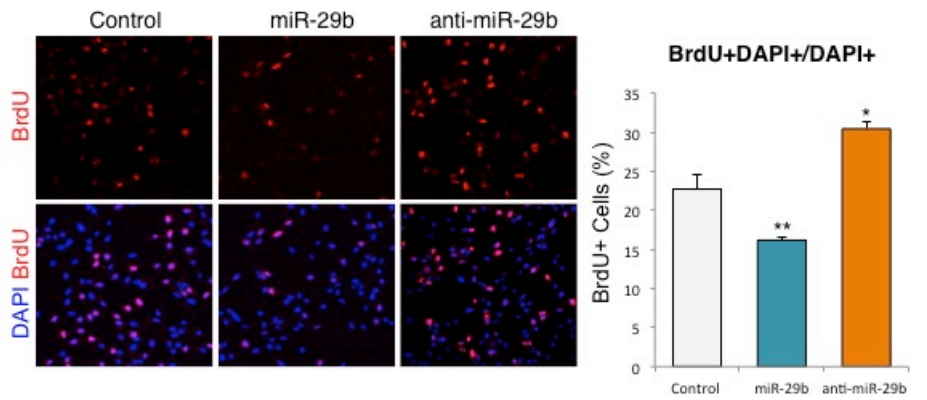


**Figure 9 miR-29b reduces NSCs proliferation**. NSCs from fetuses transduced with miR-29b or anti-miR-29b were plated on laminin-coated dishes, cultured in medium supplemented with EGF and basic FGF for 24 h, and labeled with a 1-h pulse of BrdU. Cells harboring a nuclear BrdU signal with DAPI (4,6-diamidino-2-phenylindole) were counted as positive. Quantification of BrdU incorporation was analyzed using Student's *t*-test. Approximately 1,000 cells were quantified for sections.

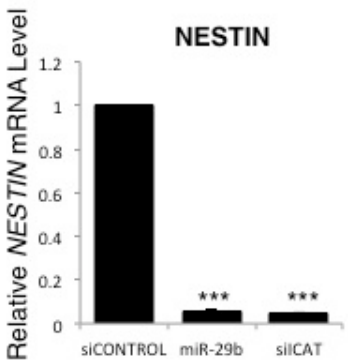


**Figure 10 miR-29b diminishes NSCs self-renewal**. ICAT-deficient NSCs showed a 90% reduction in expression of Nestin. Nestin is a type VI intermediate filament (IF) protein expressed in stem cells. Error bars show SEM of a triplicate experiment.

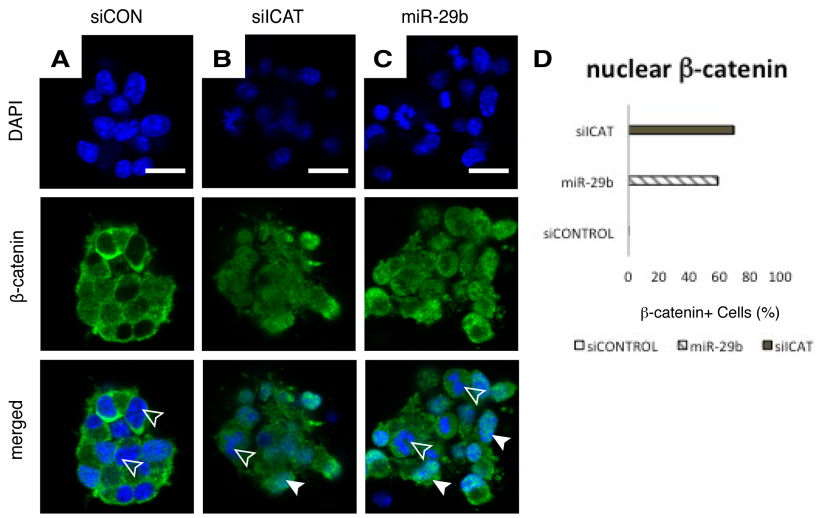


**Figure 11 Nuclear β–catenin is necessary for NSC differentiation** (A-C) β-catenin was enriched in the nuclei of differentiated NSCs. Scale bars indicate 15 μm Microscopy was performed on a Zeiss LSM 700 (Carl Zeiss, Germany) confocal laser scanning microscope or an Olympus FV1000-MPE confocal/multiphoton microscope. 3D reconstruction was performed using Imaris (custom software developed by Bitplane scientific software). (D) β-catenin is accumulated in the nuclei while miR-29b or siICAT was transfected in sphere-cultured NSCs. 60~70% of total cell population showed nuclear β-catenin.

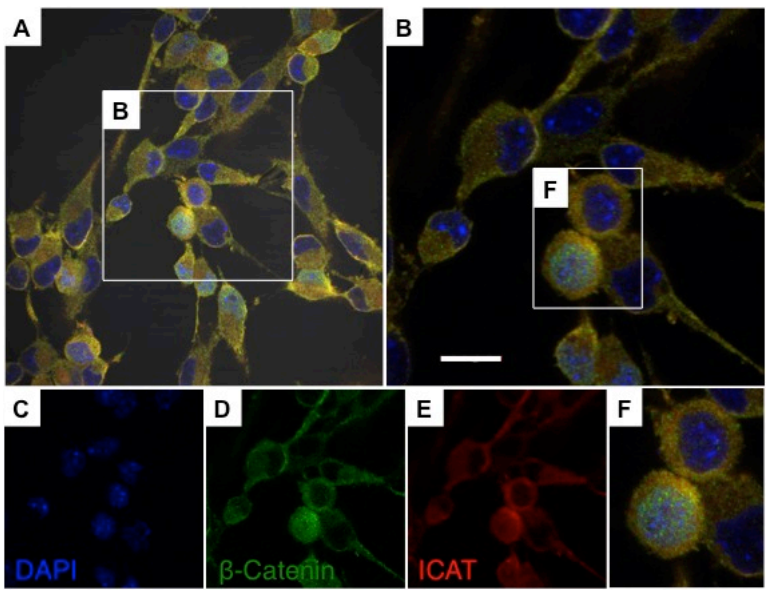


**Figure 12 ICAT co-localized with β–catenin in the nuclei in response to FGF-2, EGF** ICAT co-localized with β-catenin in the cytosol while basic FGF, EGF present. Scale bars indicate 10 μm (B). 100X magnification

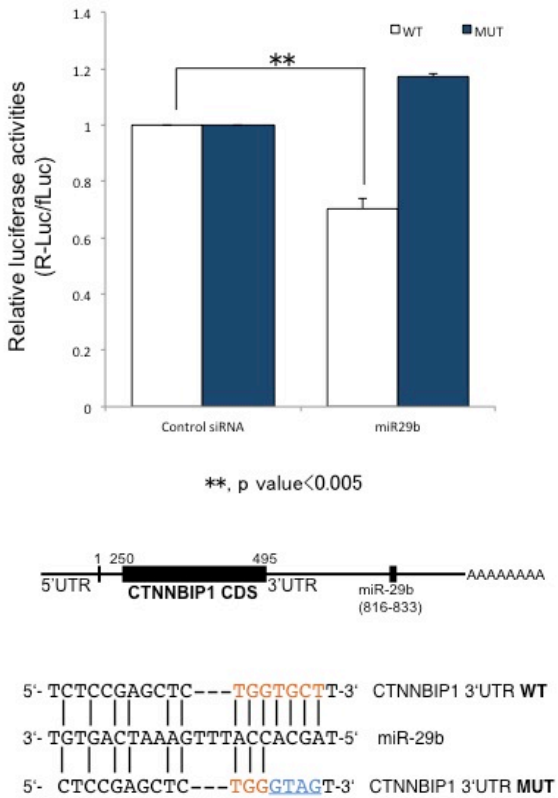


**Figure 13 miR-29b directly targets the 3'-UTR of ICAT.** HEK293T cells were transfected with luciferase reporters containing either wild-type 3'-UTR or the multiple-base mutant (TGCT to GTAG) 3'-UTR. Co-transfection of these reporters with miR-29b resulted in a decrease in the luciferase reporter activity of the wild-type 3'-UTR, but not the mutant 3'-UTR, indicating that miR-29b binds directly to ICAT 3' UTR. Firefly reporter luciferase activity was measured and normalized to *renilla* activity. complementary to seed sequences at 5'-end of miRNAs

miR-29b binding sites (5'-TGGTGCT-3') at 3'-UTR are indicated by red letters, and mutated bases are shown in blue.

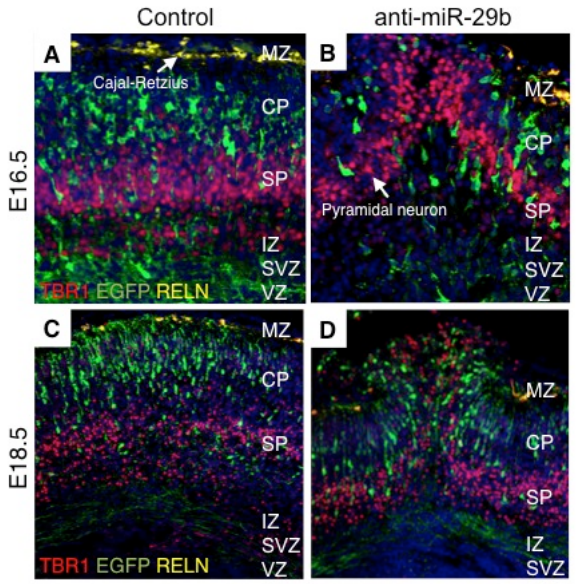


**Figure 14 Inhibition of miR-29b by *in utero* electroporation results in impaired corticogenesis.** TBR1 activates the RELN protein secreted by Cajal-Retzius cells during cortical development. The cortical plate at E16.5 shows TBR1-positive early pyramidal neuronal cells in red and RELN-expressing cells in the marginal zone in yellow. Inhibition of miR-29b by *in utero* electroporation of anti-miR-29b into embryonic mouse brains led to premature outward cortical migration. GFP-positive cells, transfected with control siRNA showed a normal distribution of RELN expression. In contrast, RELN-positive Cajal-Retzius neurons were decreased in brains microinjected with anti-miR-29b. MZ, marginal zone; CP, cortical plate; SC, subplate cells; IZ, intermediate zone, SVZ, subventricular zone; VZ, ventricular zone. Data are expressed as means ± SEM values (error bars) or assays performed in triplicate (*$p$<0.05; independent Student's $t$-test).

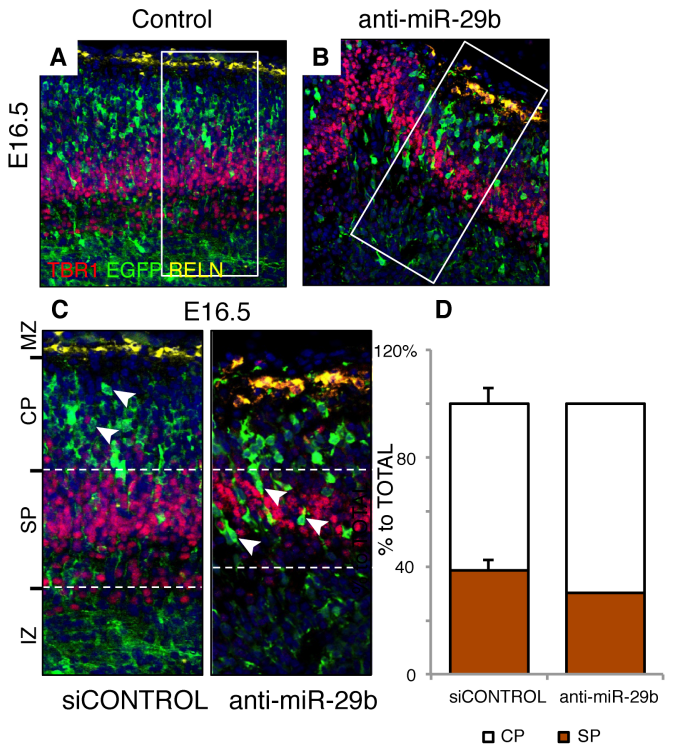


**Figure 15 Inhibition of miR-29b by *in utero* electroporation induced a profound defect in corticogenesis during mouse development.** During normal development, suppression of ICAT expression by miR-29 enhances neurogenesis *in vivo*. The cortical plate at E16.5 shows TBR1-positive early pyramidal neuronal cells (red) and RELN-expressing cells in the marginal zone (yellow). 2'-O-Me-modified anti-miR-29b oligonucleotides were microinjected into the left ventricle of mouse embryos at E13.5. TBR1 expression in the CP is suppressed when miR29 is inhibited. Genetic background: C57BL/6. Error bars show SEM; assays were performed in triplicate.

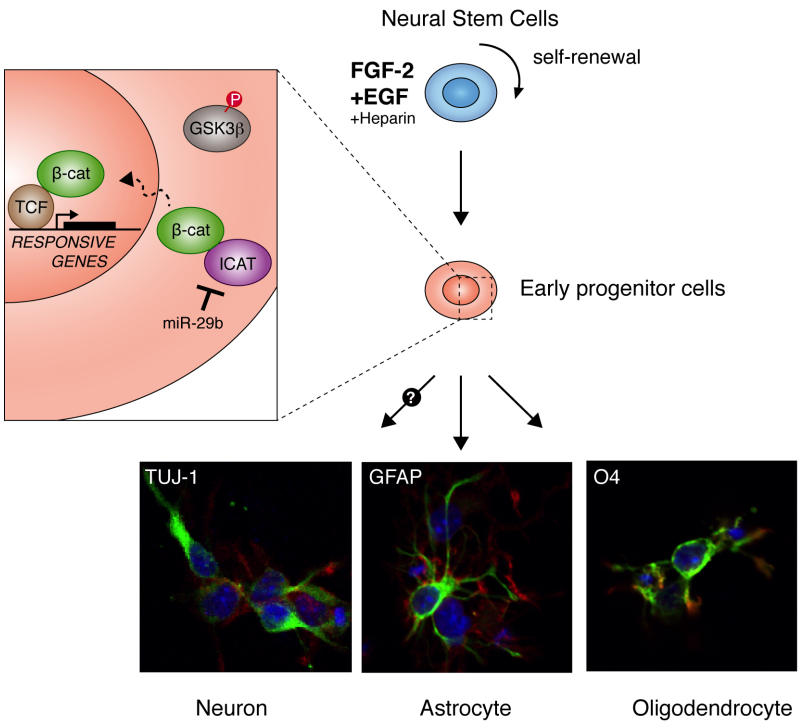


**Figure 16. Schematics of putative Wnt/β-catenin-mediated neurogenesis in mammals.** A potential schematic model of ICAT-dependent neurogenesis shows that miR-29b targets ICAT, thereby facilitating proliferation of neural stem cells in the developing brain.

# DISCUSSION

β-catenin is a well-known multifunctional protein involved in the regulation of mammalian CNS development. However, the mechanisms by which Wnt/β-catenin signaling components, such as ICAT regulate developmental disabilities in the CNS remain uncertain. Phosphorylated GSK-3β leads to β-catenin accumulation in the nucleus, where it couples with TCF. ICAT interferes with the association between β-catenin and TCF. *ICAT*$^{-/-}$ mouse embryos unveil severe malformation of the forebrain and craniofacial bones. This research aimed to investigate whether the miR-29 families regulate the expression of ICAT and, if so, whether dysregulation of ICAT by miR-29 results in defective neurogenesis and these pathologies are due to impaired β-catenin-mediated signaling events.

Here I demonstrate that miR-29b regulate ICAT-mediated Wnt/β-catenin signaling during fetal neurogenesis. The molecular nature of the downstream components of β-catenin is not well defined. Given the observations that: (1) miR-29 family members bind directly to ICAT; (2) β-catenin that is not bound to ICAT translocates to the nucleus; and (3) anti-sense against miR-29b alters the functional properties of ICAT, it seems likely that ICAT is a component of β-catenin-induced signal pathways (Figure 16). Although the genetic targets regulated by β-catenin and ICAT remain to be characterized, our data provide a link between miR-29b regulation and corticogenesis (Monuki and Walsh, 2001). Furthermore, these results may contribute to understanding the role of miR-29 miRNAs in the progression of many types of cancer (Park et al., 2009), especially brain cancer. miRNA molecules might also be considered as a putative therapeutic approach in human

patients with malignant glioblastoma multiforme (GBM), which represents the most common type of primary brain tumor.

NSCs and cancer stem-like cells share the morphological and functional similarities such as, self-renewal, extensive proliferation and invasiveness (Lee et al., 2006). These research findings may identify innovative therapeutic strategies to treat or prevent neurological diseases in humans, such as malignant GBM.

**Conclusions and perspectives**

There are diverse tumor types that arise from different cell types in the CNS, including neurons, glias, meninges and ependymal cells. The World Health Organization (WHO) has developed a scoring pattern in gliomas ranged from grade I to IV. GBM, categorized as grade IV tumors, is highly malignant and pro-mitotically active, and shows angiogenic pathologies. Gliomas

represent heterogeneity harboring multiple cell types including microglial cells originated from macrophages, ECs, pericytes, and smooth muscles cells (Charles et al., 2011).

The cancerous cells are thought to originate from multiple routes. First, they may arise from NSCs that undergo genetic mutations in tumor suppressor genes (p53, PTEN) (Duerr et al., 1998; Maintz et al., 1997) and oncogenes to alter “stem cell-like” properties through genomic instability, epigenetic dysregulation. Second, dedifferentiated non-neoplastic cells transform and produce astrocytic- and oligodendrocytic-based gliomas or neuroblastomas in adults.

**FGF- or EGF -related molecular pathologies in glioma**

It is observed overexpression of a variety of growth factors (i.e. PDGF (Guha et al., 1995), FGF2 (Takahashi et al., 1992), EGF) and their cognate

receptors in cancer. Epidermal growth factor receptor (EGFR) is amplified or hyperactivated via mutations in 30-50% of GBMs, it leads to signal transduction, such as the RAS-ERK, PI3K-AKT pathways, involved in cell proliferation and survival. To date, missense mutated EGFRs, known as EGFRvIII induced gliomas-like pathologies are well characterized, but FGFRs' are poorly defined.

**Pathological defects led by molecular alterations**

Many gene expression profiles and chromosomes are commonly altered in cancer. 81.8% of malignant gliomas (MGs) such as, anaplastic astrocytomas and GBMs (WHO grade III and IV) show mutation of the p53 gene (31.8%) or $p14^{ARF}$ deletion (54.5%). MDM2 overexpression (9.1%), EGFR amplification (50%), PTEN (phosphatase and tensin) mutations

(27.3%) and loss of heterozygosity (LOH) at the chromosomal regions 10q23.3 (86.4%) and 10q25-26 (100%) (Fulci et al., 2000).

Furthermore, $INK4A^{ARF}$ locus (encodes $p16^{INK4A}$ and $p14^{ARF}$) (Ichimura et al., 1996; Jen et al., 1994) deletion results in loss of inhibition of cyclin-dependent kinase 4 (CDK4) and MDM2. $p16^{INK4A}$ inhibits G1 transit to S-phase in the cell cycle. It causes an inhibition of retinoblastoma (Rb) and p53. p53 protein is often mutated in cancer, it altered cell-cycle arrest in G1.

In addition, PTEN, a tumor suppressor gene at 10q23 (*MMAC1*) (Myers et al., 1997) dephosphorylates phosphatidylinositol-3,4,5-trisphosphate (PIP3) to phosphatidylinositol-4,5-bisphosphate (PIP2), it results in the inhibition of AKT transactivation (Maehama and Dixon, 1998). To date, a majority of the changes in cell signal transduction become important in the progress to higher-grade gliomas.

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# 국문 초록

**서론:** 신경 줄기 세포는 중추신경계 발달 초기 미성숙, 미분화된 상태로 계속 증식할 수 있는 self-renewal 성격을 가지며, neuron 및 astrocyte 와 oligodendrocyte 로 분화가능한 다분화능을 지닌다. 본 학위논문에서는 FGF receptor 를 통한 신호전달 매개체 중 하나인 β-catenin 이 초기 뇌발생 과정 중 신경 줄기 세포 증식과 생존에 필수적인 역할을 한다는 가설 하에 실험을 진행하였다. FGF2 에 의해 활성화되어질 것으로 예상되는 intermediates 중 하나인 β-catenin 이 binding partner 인 ICAT (inhibitor of β-catenin and T-cell factor)에 의해 기능적으로 조절되어질 것이라고 예상하였으며 이 때 non-coding small RNA 인 microRNA 중 miR-29b 에 의해 조절되는 ICAT 의 발현에 미치는 영향을 연구, 그 생리적 의미를 파악하고자 하였다.

**방법:** 생체환경과 유사한 미세환경의 구현을 위해 최적화된 3D microfluidics platform 을 사용하여 qRT-PCR, immunofluorescence 및 luciferase 활성 측정

등의 방법으로 신경 줄기 세포의 분화와 증식에 관하여 평가하였다. 또한 한림대학교 약리학교실 연구팀과의 공동 연구로 13 일자 임신 쥐의 fetus 에 miR-29b 를 *in utero* electroporation 을 통해 주입한 후 뇌 형성에 미치는 영향을 조사하였다.

**결과:** 신경 줄기 세포의 3D 배양시 분화가 유도됨에 따라 miR-29b 의 발현은 통계적으로 유의하게 증가하였으나 miR-29a 또는 -29c 는 유의적인 변화가 없었으며 반면 ICAT 은 감소하였다. ICAT 3'-UTR construct 의 luciferase reporter 활성은 miR-29b 의 처리에 의하여 감소하였으나 scrambled control siRNA 는 reporter 의 활성에 영향을 미치지 않았다. 이 결과는 miR-29b 가 직접적으로 ICAT 의 3'-UTR 을 타겟한다는 것을 의미한다. 또한 miR-29b 는 신경 줄기 세포의 self-renewal 과 증식을 감소시키고 어떠한 세포 lineage 로 분화하는 지를 결정하였다. 또한, *in utero* electroporation 에 의한 생체 내 실험에서 miR-29b 의 저해는 마우스의 발생과정 중의 corticogenesis 에 심각한 결함을 초래한다는 사실을 발견하였다.

**결론:** 뇌발생 초기 miR-29b 는 ICAT 발현을 억제하여 Wnt/β-catenin 신호전달을 조절함으로써 발생과정 중의 설치류 마우스 신경세포 신생에 중요한 역할을 한다. 또한 이 결과는 miR-29b 가 ICAT 에 의한 Wnt/β-catenin 신호 전달을 조절함으로써 신경계질환을 치료 혹은 예방할 수 있는 새로운 치료전략으로서 활용될 수 있음을 시사한다.

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**주요어 :** 신경 줄기 세포, β-catenin, ICAT, **microRNA**-29b

**학 번 : 2012-30905**

A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

# A role for microRNA-29b-mediated β-catenin signaling in the mouse brain development

뇌 발생과정중 microRNA-29b 에 의한 베타-카테닌 신호 전달의 기능연구

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2014 년 08 월

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# DEDICATION

I dedicate this dissertation to my parents and two sisters with love

# A role for microRNA-29b-mediated β-catenin signaling in the mouse brain development

**by**

**JAEKYUNG SHIN, M.S.**

A dissertation submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Brain Sciences

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# ABSTRACT

**INTRODUCTION**: β-catenin has been widely implicated in the regulation of mammalian development and homeostasis. However, *in vivo* mechanisms by which Wnt/β-catenin signaling components regulate physiological events during brain development remain undetermined. Inhibitor of β-catenin and T cell factor (ICAT) interferes the association of β-catenin with T cell factor. Deficiency of ICAT in mice results in severe malformation of the forebrain and craniofacial bones, suggesting its critical role in regulation of the Wnt signaling in the CNS. The microRNA-29 family is abundantly expressed in the adult cortex, but not in the embryonic brain. However, its physiological role in neuronal development has not been well-studied. The purpose of this study is to determine if deregulated ICAT by miR-29 results in a defective neurogenesis toward neuronal cell lineages and whether these pathologies are due to impaired β-catenin-mediated signaling events.

**METHODS**: To mimic *in vivo* micro-environment, neural stem cells (NSCs) were cultured in 3D microfluidics device. Then qRT-PCR, BrdU-pulse and immunofluorescence were performed to characterize NSCs stemness in the niche, such as proliferation and self-renewal properties. In addition, luciferase activity was examined whether miR-29b directly targets the 3'-untranslated region (3'-UTR) of ICAT. Furthermore, *in utero electroporation* method was applied to investigate the roles of miR-29b *in vivo* during fetal corticogenesis under technical cooperation with a research lab at Hallym University.

**RESULTS**: miR-29b, but not miR-29a or c, is increased in differentiated NSCs which is reverse-correlated with the decreased levels of ICAT. I also found that miR-29b diminished NSCs proliferation and self-renewal capabilities, and controlled their fate, directing their differentiation along certain cell lineages. A luciferase reporter assay revealed that the 3'-UTR of the ICAT mRNA (also known as *CTNNBIP 1* gene) is the direct target of miR-29b. *In vivo* results show that applied anti-sense miR-29b by *in utero*

electroporation in corticogenesis led to the proliferative defect and premature outward migration.

**CONCLUSIONS**: I showed that miR-29b regulates neurogenesis by controlling Wnt/β-catenin signaling during brain development via inhibition of ICAT expression. Furthermore, these research findings may identify novel therapeutic approaches to modulate ICAT-mediated Wnt/β-catenin signaling to treat or prevent neurological disorders in humans.

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Keywords: neurogenesis, β-catenin, ICAT, microRNA-29b

Student number: 2012-30905

# CONTENTS

# LIST OF TABLES AND FIGURES

# LIST OF ABBREVIATION

**3'-UTR**, 3'-untranslated region

**EC**, endothelial cell

**ECM**, extracellular matrix

**EGF**, epidermal growth factor

**FGF**, Fibroblast growth factor

**FRS2α**, FGF receptor substrate 2

**GBM**, Glioblastoma multiforme

**GFAP**, glial fibrillary acidic protein

**GFP**, green fluorescent protein

**GSK-3β**, glycogen synthase kinase 3β

**ICAT**, inhibitor of β-catenin and T cell factor

**LEF**, lymphoid enhancer binding factor

**NEC**, neuroepithelial cell

**NSC**, neural stem cell

**OB**, olfactory bulb

**RISC**, RNA-induced silencing complex

**RMS**, rostral migratory stream

**SGV**, subgranular zone

**SVZ**, subventricular zone

**Shp2**, Src homology region 2 domain-containing phosphatase 2

**TCF**, T-cell transcription factor

**VEGF**, vascular endothelial growth factor

# INTRODUCTION

Neural stem cells (NSCs) that originate in the developing vertebrate brain are defined as their specialized capabilities to self-renew (Fuchs and Chen, 2013), proliferate and multipotent. NSCs can differentiate to multiple cell lineages to neurons, astrocytes, and oligodendrocytes (Alvarez-Buylla et al., 2002; Kriegstein and Alvarez-Buylla, 2009).

## 1. Cerebral cortical development

### 1.1 Corticogenesis in mammals

In the developing cortices, mitotic neural stem and progenitor cells located in neurogenic regions, such as ventricular or subventricular zone (SVZ) switch to post-mitotic neuroblasts. These neuroblasts migrated towards the cortical pial surface to form laminated cortical layers.

The vertebrate cerebral cortex is patterned into six layers of neurons late in development. Early-born neurons become postmitotic, migrate to the appropriate cortical layer, and differentiate into mature cortical neuron subtypes in corticogenesis. The well-known transcription factors (i.e. TBR-*1*, *Id-2*, and *Otx-1*) are preferentially expressed in specific cortical laminae. T-box brain-1 (TBR-1) is predominantly expressed in early-born cortical neurons of the preplate and cortical plate (layer 6) in the mammalian CNS (Bulfone et al., 1995; Hevner et al., 2001). Reelin (RELN) is expressed at high levels in the marginal zone, specifically in Cajal-Retzius cells. It is a secreted extracellular matrix (ECM) glycoprotein early in development.

**1.2 Characterization of the neurogenic niche**

In general, stem cells are defined as their specified abilities to self renew, proliferate and differentiate into multiple cell lineages. These stem cell

behaviors *in vivo* are strictly regulated for proper CNS development. It is believed that the distinctive nature of stem cells depends on extrinsic signals from the adjacent cells. These areas in the CNS are referred to as a 'neural stem cell niche (NSC niche)' or 'neurogenic niche'.

In the NSC niche, SVZ stem cells found in a vasculature-derived ECM-rich niche (i. e. laminin) (Shen et al., 2004). Endothelial cells (ECs), a crucial component of the NSC niche, secrete soluble factors to maintain SVZ stem cells "stem"-ness; self-renewal and neurogenic potential (Shen et al., 2008). EC-released pro-angiogenic factors can modulate the changes in miRNA profiles and responsive gene expression patterns in NSCs, leading to NSCs' cellular homeostasis.

To mimic the tissue-like microenvironment, three-dimensional (3-D) spheroid-cultured NSCs in an ECM hydrogel incorporated in a microfluidic platform are well established. The micro-engineered '3D NSC culture'

protocol has been proven to reconstitute an *in vivo*-like microenvironment that enables micro-scale cell to cell contact and/or cell to ECM interactions and quantitative investigation of NSC self-renewal and differentiation (Han et al., 2012; Shin et al., 2012).

### 1.3 Development of stem cells in the neurogenic niche

In the rodent embryo, an apical (ventricular)-basal polarized neuroepithelial cell (NEC) produces an actively proliferating SVZ stem cell that generates neuroblasts that migrate to the pial surface guided by elongated radial glia (Gotz and Huttner, 2005). NECs elongate early in development and convert to radial glial cells. Radial glia is thought to be progenitors toward astroglial cell lineages expressing glial fibrillary acidic protein (GFAP), $Ca^{2+}$-binding protein S100β (Raponi et al., 2007), vimentin, brain-lipid-binding protein (BLBP) and glutamate transporter (GLAST) (Doetsch, 2003; Mori et al.,

2006). Radial glial cell bodies with one ‘apical’ process attached to the ventricles and extend a longer ‘basal’ process to the pial surface of the neocortices (Doetsch, 2003). Radial glial cells form a scaffold for newborn neuroblasts to migrate late in development. Some radial glial cells change into SVZ astrocytes, and remain in a stem-like state within the adult brain.

In adults, the neurogenic regions were found to be highly restricted: 1) the SGZ of the dentate gyrus in the hippocampus that generates new granule cells (Gage et al., 1998); and 2) the SVZ in lateral ventricle where newly born neurons (neuroblasts) tangential migrate in the RMS to the olfactory bulb (OB) (Altman and Das, 1965; Alvarez-Buylla and Lim, 2004; Zhao et al., 2008).

The adult SVZ is a well-characterized neurogenic region in the CNS. It is located in the lateral ventricle, composed of an ependymal cell, types A, B, and C (Doetsch et al., 1997). SVZ astrocytes (type B cells), thought to be

stem-like cells in SVZ (Doetsch et al., 1999), are placed upon the ciliated ependymal layer. Epithelium-like ependymal cells line the ventricle wall. Apical type B cells give rise to transit-amplifying progenitors (TAPs, type C cells), which generate neuroblasts (type A cells). Type A cells migrate 3-8mm to the OB, where they form functional interneurons (Doetsch et al., 1997). These cells express polysialylated neural adhesion cell molecule (PSA-NCAM), TUJ-1 (β-tubulin) and doublecortin (DCX) (Yang et al., 2004). Inhibitory local-circuit interneurons in the OB divide into two main subtypes- granule cells and periglomerular cells (Lledo et al., 2006).

## 2. Signal transduction in the stem cell niche

### 2.1 Influence of growth factors and other extrinsic signals

Growth factors such as fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) are potent factors to maintain NSCs' stemness *in vitro*.

Both factors promote proliferation in germinal regions *in vivo*. FGF-2 (or basic FGF) is present at low levels in most organs, with higher concentrations observed in the brain and pituitary gland (Liekens et al., 2001). FGF receptors (FGFRs) transduce FGF signals to RAS-ERK and PI3K-AKT signaling cascades through FGF receptor substrate 2 (FRS2α) (Sato et al., 2010), the docking protein. FRS2α is ubiquitously expressed during development (Gotoh et al., 2004). FRS2α is a docking protein that facilitates FGFR signaling by recruiting the cytoplasmic protein tyrosine phosphatase, Shp2. Shp2 (Src homology region 2 domain-containing phosphatase 2, also known as *PTPN11*) is a tyrosine phosphatase with two Src-homology 2 (SH2) domains that acts to activate the RAS-ERK pathway by FGFs (Figure 1) (Neel et al., 2003).

FRS2α binds constitutively to the FGFR1 via its PTB domain and is heavily tyrosine phosphorylated upon ligand-induced activation. Tyrosine

phosphorylated FRS2 provides two docking sites for interaction with the tyrosine phosphatase, Shp2. FRS2 forms a complex with the N-terminal SH2 domain of the protein tyrosine phosphatase, Shp2 in response to FGF stimulation (Hadari et al., 1998).

The extrinsic signals are triggered by secreted molecules from adjacent ECs such as, both FGF and its polysaccharide cofactor, heparin. Heparin-binding growth factors such as basic FGF (bFGF) or FGF2, heparin-binding splice variants of vascular endothelial growth factor ($VEGF^{164}$ and $VEGF^{188}$) (Mentlein et al., 2004) or pleiotrophin (PTN, also known as HARP, heparin affinity regulatory peptide) (Mentlein and Held-Feindt, 2002) are associated to the ECM to attach to the cell-surface.

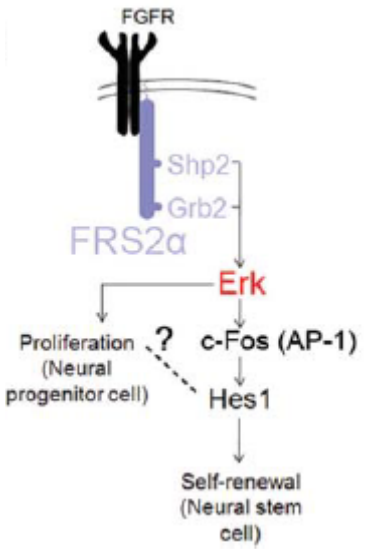


**Figure 1 FGF-induced ERK activation via FRS2α-Shp2 complex.** FGF-induced ERK activation via FRS2α-Shp2 complex contributes to both self-renew and proliferation of NSCs **(**adapted with permission from Gotoh N. et al.). FGFs regulate neurogenesis during early embryogenesis. The binding of FGFs to their cognate FGFRs results in FRS2α-Shp2 complex. FGFs-induced FRS2α-Shp2 complex formation leads to ERK activation.

## 2.2 An integrated shift: from FGF-receptor to GSK-3β

AKT inhibits the phosphorylation of β-catenin by glycogen synthase kinase (GSK)-3β. GSK-3β is a ubiquitously expressed serine/threonine protein kinase that phosphorylates at threonine 41, serine 33 and 37 (Kimelman and Xu, 2006) and inactivates β-catenin (Yost et al., 1996). β-catenin is a crucial downstream element of PI3 kinase/AKT cell survival pathway whose activity can be inhibited by AKT-mediated phosphorylation at serine 9 of GSK-3β (Cross et al., 1995). This inhibition prevents β-catenin degradation via the ubiquitin-proteasome (Aberle et al., 1997), resulting in its accumulation in the nucleus. β-catenin translocates to the nucleus, where it binds to the lymphoid enhancer binding factor (LEF)/T-cell transcription factor (TCF) family of transcription factors to induce expression of their responsive-target genes.

### 2.3 Wnt/β-catenin signaling related to intracellular mechanisms

Wnt signaling (www.stanford.edu/~rnusse/wntwindow.html) has been widely implicated in embryonic development and tumorigenesis (Logan and Nusse, 2004; Moon et al., 2004). β-catenin, a key element of the canonical Wnt signaling pathway, translocates to the nucleus and binds to the LEF/TCF family of transcription factors (Behrens et al., 1996) to initiate expression of Wnt-responsive genes such as cyclin D1 (Tetsu and McCormick, 1999) and *c-myc*. The inhibitor of β-catenin and T cell factor (ICAT, also known as *CTNNBIP1*) directly interacts with β-catenin via three helical domains at the N-terminus (Daniels and Weis, 2002). ICAT inhibits the interaction between β-catenin and TCF and interferes with Wnt signaling mediated by the β-catenin-TCF complex (Tago et al., 2000). *ICAT*$^{-/-}$ knockout mutant mice develop severe forebrain malformation beginning at embryonic day E11.5

(Satoh et al., 2004). *ICAT*-deficient mouse embryos exhibit a smaller overall brain size and abnormal forebrain morphologies.

## 3. Stem cells, Wnt/β-catenin and ICAT

### 3.1 β-catenin and associated mediators in the brain

The β-catenin mediated canonical Wnt pathway is a key regulator of progenitor cell proliferation in the developing cortical cortex. β-catenin is prominently expressed in neuroepithelial precursors across the period during brain development (Chenn and Walsh, 2002).

Together, β-catenin has been widely implicated in regulation of development and disease in humans, and is often mis-regulated in the various developmental abnormalities and pathological disorders (Clevers, 2006; Logan and Nusse, 2004).

### 3.2 Structural properties of β-catenin and its functions

Wnt/β-catenin signaling has been extensively implicated in embryonic development and tumorigenesis. β-catenin, a key component of the Wnt signaling pathway, translocates to the nucleus and binds to LEF/TCF family of transcription factors (Behrens et al., 1996) to initiate expression of Wnt-responsive genes such as cyclin D1 (Tetsu and McCormick, 1999) and *c-myc*. The ICAT directly interacts with β-catenin via three helical domains at the N-terminus (Daniels and Weis, 2002). This binding prevents the interaction of β-catenin with TCF and interferes with Wnt signaling mediated by the β-catenin-TCF complex. *ICAT*$^{-/-}$ knockout mutant mice develop severe forebrain malformation beginning at embryonic day E11.5 (Satoh et al., 2004). ICAT-deficient mouse embryos exhibit a smaller overall brain size and atypical forebrain morphologies (Figure 2).


**Figure 2 ICAT$^{-/-}$ results in multiple developmental defects**. *ICAT$^{-/-}$* embryo exhibits malformation of the forebrain and craniofacial bones (adapted with permission from Akiyama T. et al.). The depletion of ICAT results in multiple developmental abnormalities. (A-C) Wild-type (+/+) and ICAT$^{-/-}$ (-/-) embryos at E18.5 are shown in parallel. (F) ICAT$^{-/-}$ exhibited a cleft palate. (I-K) In ICAT$^{-/-}$ mice, the adrenal glands (arrowhead) also were defective.

Therefore, the negative regulation of Wnt signaling by ICAT via competition with TCF is critically integral to brain development, most likely through promotion of NSC proliferation and differentiation.

### 3.3 The negative regulatory effect of ICAT

*CTNNBIP1* maps to chromosome 1p36.22 in humans. ICAT include a N-terminal 3-helix bundle, bound to β-catenin at 'armadillo' (arm) repeats, and an extended C-terminal tail (Daniels and Weis, 2002). The C-terminal arm repeats of β-catenin bind to cadherins, TCFs, APC, and Axin (Daniels et al., 2001). ICAT interferes with the interaction of β-catenin with TCF-4 and inhibits to form a β-catenin-TCF-4 complex. ICAT binds to β-catenin and inhibits the canonical Wnt signaling cascades. ICAT induces G2 arrest followed by cell death in colorectal tumor cells (Sekiya et al., 2002). The ectopic induction of ICAT inhibits the expression of βIII-Tubulin (TUJ-1) and

thus neuronal differentiation in embryonal carcinoma P19 cells (Lyu et al., 2003).

## 4. Overview of microRNAs

### 4.1 microRNAs as an intrinsic regulator

MicroRNAs (miRNAs) are non-coding small RNAs of 22-24 nucleotides in length. Endogenous miRNAs can silence target mRNAs by triggering endonuclease cleavage or promoting translational repression. miRNAs regulate a variety of developmental and pathological processes. It is clear that miRNAs act as an intrinsic regulator of NSCs behavior (Cao et al., 2006). miRNAs that are expressed in NSCs can influence neurogenesis by fine-tuning gene expression. However, the most molecular targets of miRNAs remain uncertain.

**4.2 miRNA biogenesis, and its mechanism.**

The mammalian miRNA biogenesis proceeds into multiple steps (Figure 3) (Condorelli et al., 2010). First, miRNA gene is transcribed by RNA polymerase II into long hairpin-shaped primary miRNAs (pri-miRNAs) (Lee et al., 2004). pri-miRNAs are processed into ~70-80 nucleotide precursor miRNAs (pre-miRNAs) by the nuclear RNase III enzyme Drosha (Lee et al., 2003). pre-miRNA hairpins are exported from the nucleus by Exportin-5 in the presence of Ran-GTP as cofactor (Bohnsack et al., 2004; Yi et al., 2003). In the cytoplasm, the pre-miRNAs are processed into ~22-nucleotide duplex miRNAs by RNase III enzyme Dicer (Hutvagner et al., 2001). miRNA duplexes are unwound, the miRNA strand that has its 5' terminus at the end is called mature miRNA.

The mature miRNAs are incorporated into a ribonucleoprotein complex, the RNA-induced silencing complex (RISC). In RISC, miRNAs can mediate

repression of target gene by two modules: target mRNA cleavage or translational inhibition. Perfect matches results in cognate mRNA cleavage, followed by mRNA degradation, whereas partial miRNA mismatch with mRNA targets causes translational inhibition. miRNA-targeted mRNAs can be recruited to “processing” or “P”-bodies where they are depleted of the translation machinery and degraded.

miRNAs regulate approximately hundred and thousands target genes. A few DNA base-pairs in their 3’-UTRs corresponds to the seed sequences of miRNAs at the 5’-phosphate end. These 6-7 nt motifs are conserved at the 3’- UTR and are complementary to the seed sequences of miRNAs.

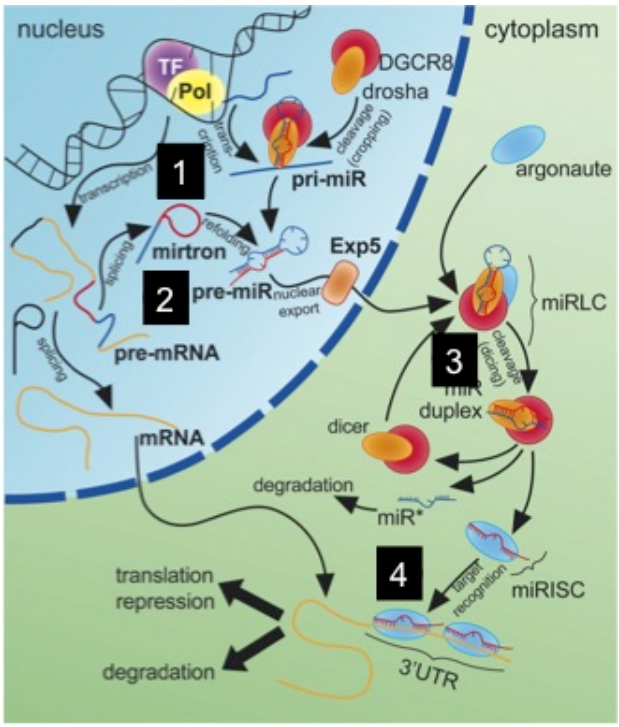


**Figure 3 A schematic diagram of miRNA biogenesis and action** (adapted with permission from the Oxford University Press). The mature miRNA sequence is given in red. TF, transcription factor; Pol, RNA polymerase II, Exp5, Exportin 5.

### 4.3 Identification of miRNAs in the developing mouse brain.

To address whether the miRNA expression pattern in the brain drastically changes during embryonic development, the miRNA profiles of embryonic and adult forebrain, including cortex and striatum, have been screened. Such studies showed that expression of the miR-29 family is absent in the embryonic brain, but is upregulated in the adult cortex and striatum (Figure 4) (Landgraf et al., 2007). The brain-enriched miR-29a, b, and c family is exclusively involved in neural development during early embryogenesis.

The brain-specific miRNAs fine-tunes corticogenesis at the early developmental stages. Brain-enriched miR-124, miR-128 has been exclusively studied. It could synergistically promote the developmental processes.

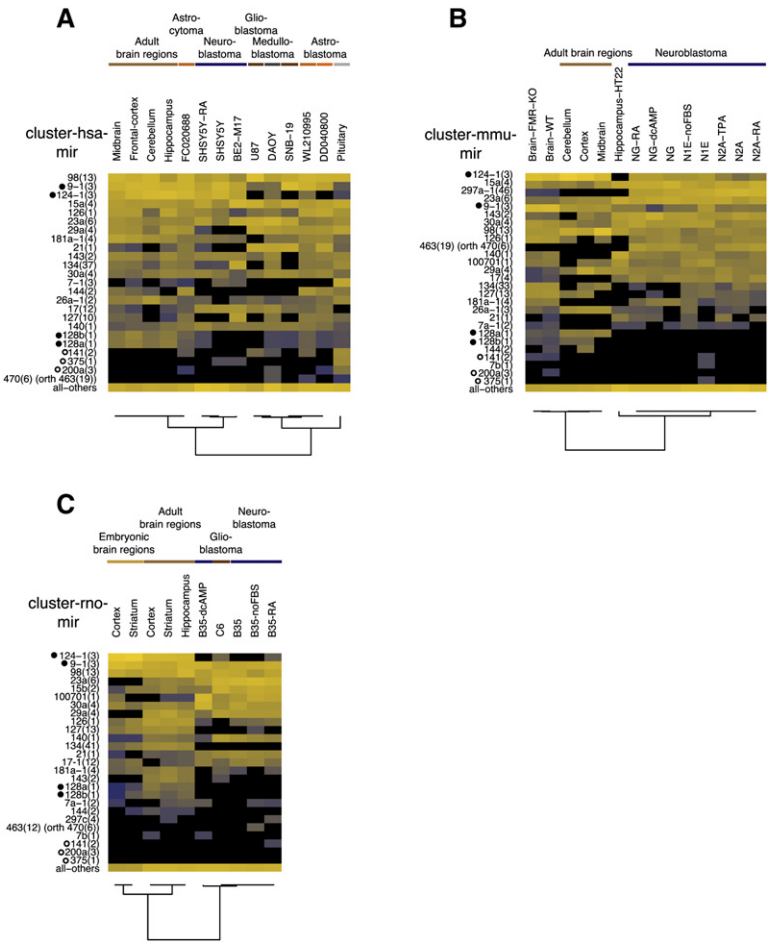


**Figure 4 miRNA expression profiles in CNS.** miRNA expression profiles in the CNS for human (A), mouse (B), and rat (C) (adapted with permission from the Elsevier). The neuronal-specific miRNA clusters are marked with black dot, the open circles indicate miRNAs enriched in varied tissues.

### 4.4 Classification of miR-29a, b, and c families and their targets

The mature miR-29a, b, and c sequences differ in 2-3 bases, but share an identical seed region (5'-AGCACCA-3'). miR-29a/b-1 is located in a genetic loci on chromosome-7, but miR-29b-2/c is on chromosome-1. The upstream regulatory promoter region of the miR-29a/b-1 gene contains several putative transcription factor (TF)-binding sites: Ap1RE (Ap1 consensus sequences), TIE (TGF-β inhibitory elements), and SBE (Smad binding elements). TF-binding sites and their interaction begins to PDGF-BB (i.p. of the Ap1RE sites) and TGFβ-linked Smad-2,3 signals (Noetel et al., 2012). Therefore, TGFβ families released by neighboring ECs at the niche can possibly regulate miR-29b expression pattern in NSCs.

The computational algorithms for miRNAs such as, TargetScan (http://www.targetscan.org) and microRNA.org (http://www.microrna.org) predict their targets.

# MATERIALS AND METHODS

## 1. Establishment of primary mouse embryonic neurosphere cultures

Timed-pregnant mice (e.g., CD1 albino, ICR) at E13.5 were purchased from animal care facilities, Harlan Laboratories, Inc. (Indianapolis, IN, USA). Experimental procedures were performed in accordance with protocols approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-130531-1).

To establish embryonic neurosphere cultures, mouse cerebral cortices were harvested at E13.5, dissociated, and resuspended (Rietze and Reynolds, 2006). Embryos at E13.5 were dissected, isolated the entire cortices and meninges were removed. And, dissociated, plated NSCs and, expended in response to basic FGF (early stem cell at E12), EGF (late “adult” stem cell at E17 to adult), or both (classic stem cell at E14 to E15) as

mitogens. Then test undifferentiated NSCs with widely employed molecular markers such as, Nestin, Bmi-1 (B-lymphoma Moloney murine leukaemia virus insertion region 1), Musashi-1, Sox-2 at mRNA level. bFGF, EGF-responsive NSCs were grown and then passaged.

**2. cDNA synthesis and qRT-PCR analysis**

The expression pattern of mature miR-29 a, b, and c was assayed by quantitative RT-PCR. Briefly, 100 ng of total RNAs, including small RNAs >18 nt in length, were reverse transcribed using miScript starter kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Specific RT primers for miR-29b sequence, guide strand (5'-UAGCACCAUUUGAAAUCAGUGUU-3', QIAGEN, Hilden, Germany), or U6 RNA were used. cDNA was amplified in an ABI StepOne system. Relative quantification (RQ) was carried out using the ΔΔCT method. Sample

variability was normalized to U6 RNA levels. To investigate miR-29b-regulated target mRNAs, 0.5 mg of total RNA was reverse transcribed, cDNA was synthesized, and PCR products were normalized to GAPDH levels.

### 3. Micro-device array fabrication and preparation

NSCs in the developing brain are dependent on signals from neighboring cells and ECM proteins. To mimic the surrounding microenvironment, I cultured NSCs in a three-dimensional (3-D) ECM hydrogel incorporated in a PDMS microfluidic assay (Chung et al., 2009; Huh et al., 2011).

The micro-device array consisted of eight units of micro-patterned polydimethylsiloxane (PDMS; Sylgard 184; Dow Chemical, MI) fabricated using a conventional soft lithography procedure. The sterilized micro-patterned PDMS and a glass coverslip substrate were treated with oxygen

plasma (Femto Science, Seoul, Korea) and bonded together, thus forming a closed micro-channel (140-160μm) system. A single unit is composed of one central channel with two side channels for 3D NSC culture and growth medium supplementation, respectively (Figure 5).

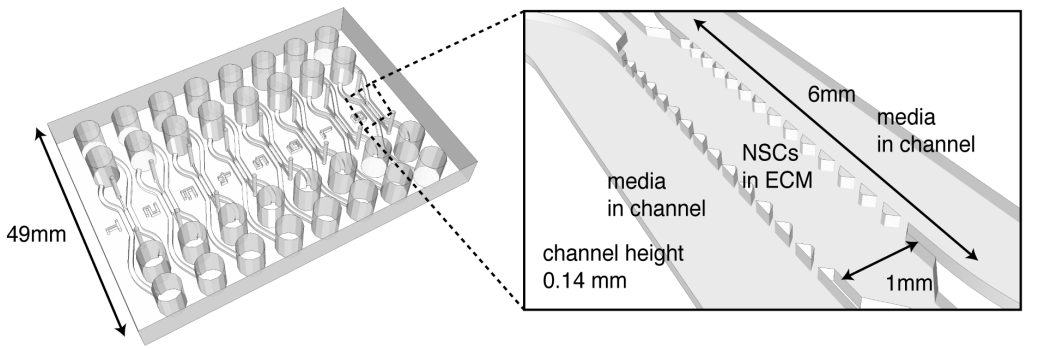


**Figure 5 Top view of the 3D microfluidic channels for qRT-PCR**. The microfluidic device has two side channels for supplying growth medium and one center channel for NSC culture in 3D micro-scale ECM hydrogels.

## 4. 3D NSC culture in the micro-device

For 3D NSC culture, collagen-based matrices (collagen type I, rat tail, BD Biosciences, Franklin Lakes, NJ, USA) were used as the ECM material. Collagen type I solution was diluted to 2 mg/ml in 10× phosphate-buffered saline (PBS; Thermo Scientific, MA, USA) and distilled deionized water, and the pH of the gel solution was adjusted to pH 7.4 with 0.5 N NaOH. E13.5 neurospheres were dissociated and suspended in ice-cold collagen solution at a density of $5 \times 10^6$ cells/ml. Cultivated NSCs in collagen solution were injected into the central channel and allowed to gel by incubating at 37°C for 30 min. For cell transfection, lipofectamine RNAiMAX (Invitrogen, CA, USA) was mixed with 100 nM siRNAs targeting ICAT (MISSION® siRNA, mouse, NM_023465, Sigma-Aldrich, MO, USA; siICAT#1, SASI_Mm01_00054131, sequence start at 949; siICAT#2, SASI_Mm01_00054132, at 825), or 50 nM miRNA (Qiagen, Hilden, Germany). After gelation, complete growth medium

and individual transfectants were added to the micro-device through both side channels and incubated overnight. The cells were washed and the medium refreshed daily. Specific gene expression of NSCs was quantified by qRT-PCR after 3 days in culture.

**5. Western blot analysis**

NSCs transfected with scrambled control siRNA and miR-29b (targets mature mmu-miR-29b-3p, 5'-UAGCACCAUUUGAAAUCAGUGUU-3' Cat# MSY0000127, QIAGEN, Hilden, Germany). 72 hours after transfection, NSCs were harvested and lysed in RIPA buffer. 30 - 50 μg proteins were loaded onto denaturing 4 - 12 % Bis-Tris gels (Invitrogen, CA, USA) and transferred to PVDF membranes (Millipore, MA, USA). Membranes were blocked with 3% milk in PBS with 0.05% Tween-20 for 1 h at room temperature, and then probed with adequate primary antibodies for

overnight at 4°C, followed by incubation with horse radish peroxidase (HRP) conjugated anti-rabbit or anti-mouse antibodies (1:700 - 2,000, Invitrogen, CA, USA). The HRP signals were visualized using an enhanced chemiluminescent (ECL) substrate (G-Biosciences, MO, USA) via LAS-3000 (GE ImageQuant, NJ, USA).

Western blot analysis was performed using anti-ICAT (1:500, Santa Cruz Biotechnology, Inc., CA, USA), anti-β-actin (1:5,000, Sigma-Aldrich, MO, USA) antibodies. *CTNNBIP1* gene product (ICAT) is a 9-kD protein. β-actin (Sigma-Aldrich, St. Louis, MO, USA) was used as loading control.

**6. Immunofluorescence**

A range of molecular markers has been used for IF such as, TUJ-1 (immature neuron), O4 (oligodendrocytes), GFAP, GLAST (astrocyte), Nestin (Rat 401, uncommitted neural stem cells). NSCs cultured in a micro-

device array were fixed in 4% (w/v) paraformaldehyde in PBS at room temperature for 15 min and washed with PBS. The fixed cells were incubated with 0.1% Triton X-100 for 5 min for permeabilization, blocked with 4% (w/v) bovine serum albumin (BSA) in PBS for 1 hour, and washed with PBS. After blocking, cells were incubated with antibodies against mouse monoclonal anti-TUJ1 (Millipore, MA, USA, 1:100), mouse monoclonal anti-O4 (Millipore, MA, USA, 1:100), rabbit polyclonal anti-GFAP (Abcam, Cambridge, England 1:100), rabbit polyclonal anti-ICAT (FL-81) (Santa Cruz, TX, USA, 1:100), and mouse monoclonal anti-active β-catenin (clone 8E7) (Millipore, MA, USA, 1:100) for 2 h at room temperature. Cell nuclei were stained with DAPI (Sigma-Aldrich, MO, USA).

## 7. Luciferase reporter assay

The full-length (2,173 bp) 3'-UTR of the mouse *CTNNBIP 1* gene (encodes ICAT) on a pEZX-MT01 backbone was purchased (GeneCopoeia™, MD, USA) (Figure 6). The mutated construct has TGCT to GTAG base mismatches within the miR-29-target binding site in the 3'UTR. HEK293T cells were seeded 24 h prior to co-transfection. Cells were co-transfected with 0.5 µg of reporter constructs (WT or MUT) and control siRNA or 50 nM mouse miR-29b-3p miRNA duplex (QIAGEN, Hilden, Germany). After 24 h, luciferase activity was measured using the dual-luciferase reporter assay system (Promega, USA) and normalized to *renilla* luciferase activity.

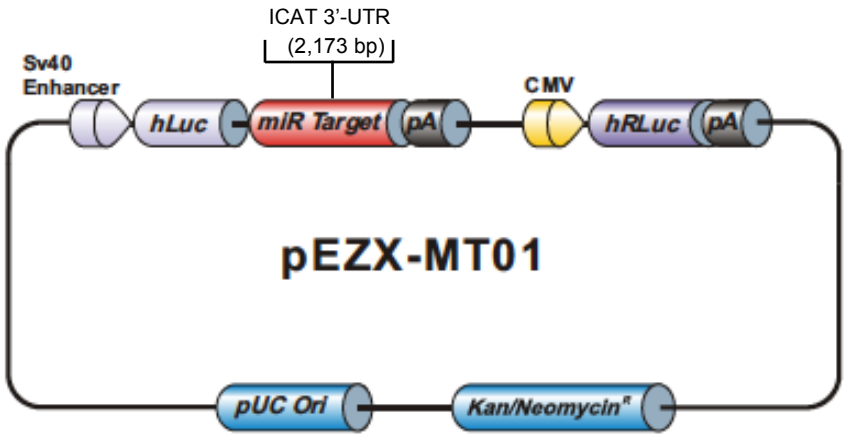


**Figure 6** Vector information for MmiT033050-MT01 (GeneCopoeia™, MD, USA).

## 8. BrdU incorporation assay

NSCs at E13.5 were seeded at $1\times10^5$ cells per well in laminin (Invitrogen, CA, USA)-coated 24-well plates. Cells were pulsed for 1 h with 10 μM 5-bromo-2'-deoxyuridine (BrdU) (BD Biosciences, Franklin Lakes, NJ, USA). BrdU is a thymidine (T) analog and it incorporates into the DNA while cells are at S-phase. The BrdU pulse-labeled cells were fixed and acid treated, followed by immunostaining with antibodies to anti-BrdU (Abcam, Cambridge, England 1:100).

## 9. Expansion and proliferation assays

Neurospheres were dissociated and plated with FGF-2, EGF in T25 flasks. At day 1, 3, 5, neurospheres were measured in diameter and counted selectively >50μm in diameter and also viable cells were identified by trypan blue.

## 10. *In utero* electroporation

Timed-pregnant C57BL/6N females at E13.5 were anesthetized with inhalational anesthetic, isoflurane (4% during induction, 2.5% during surgery), and embryos were exposed. The mature miR-29b RNA duplex anti-sense sequence is 5'-GCUGGUUUCAUAUGGUGGUUUA-3'. 1μl of small RNA (37.5 pmol control siRNA or anti-miRNA-29b) with plasmid DNA (0.625μg pCAGIG GFP-reporter plasmid, addgene, plasmid 11159) in PBS was prepared and microinjected into the lateral ventricle using mouse-controlled 90-mm glass capillaries (GD-1; Narishige, Tokyo, Japan) (Sun et al., 2011). Electroporation was performed into DNA-injected embryos with Tweezertrodes electrodes (diameter, 5 mm; BTX, Holliston, MA, USA). The electric pulses were delivered to the embryos with a duration of 50 ms per pulse (45V, 5 pulses, interval 950 ms) using a square-wave pulse generator

(ECM 830; BTX, MA, USA). The electroporated mice were sacrificed and analyzed at E16.5, 18.5 (Saito, 2006).

## 11. Histological approaches

To identify developmental defects at E16.5-18.5 based on molecular and morphological alterations, immunohistochemistry (IHC) on paraffin-embedded or frozen sections were performed. A various range of molecular markers have been used for IHC, including TBR-1 (for post-mitotic glutamatergic neurons), RELN. In addition, embryos dissected at E16.5, E18.5 to determine temporal differences of layers patterns in cerebral cortices.

## 12. Statistical analysis

Quantitative data were expressed as standard error mean (SEM), for n >3. Significant differences were tested by unpaired Student's *t*-test for comparison between two treatment groups. Asterisks indicate *p<0.05; **p<0.01; and ***p<0.001.

# RESULTS

## 1. Wnt signaling components are expressed in 3D cultured NSCs

NECs lie in the lateral ventricle, are responsible for generating most neurons and glias (Kriegstein and Alvarez-Buylla, 2009). To investigate the role of the miR-29 family in neuroepithelial cells (NECs), I performed quantitative real-time PCR analysis of miR-29a, b, and c subtypes in NSCs (Figure 7). miR-29b showed a prominent increase in abundance when differentiation was induced by commercial supplements (Stem Cell Technologies, BC, Canada) or 1% fetal bovine serum (FBS). To identify putative target molecules of the miR-29 family, I examined expression of ICAT and β-catenin. *ICAT* mRNA expression showed a reverse correlation with miR-29b expression (Figure 8).

## 2. miR-29b reduces NSC proliferation and self-renewal

To investigate the effect of miR-29 duplexes on NSCs, E13.5 cortical cells transduced with miR-29 or antisense against miR-29 were grown as neurospheres and plated in growth medium supplemented with EGF and FGF2. BrdU incorporation after 24 h revealed a 30% decrease in the growth rate of miR-29b treated NSCs, resulting in a decrease in the propagating cell population. In contrast, anti-sense against miR-29b promoted a 33% increase in the rate of cell proliferation (Figure 9).

EGF- and FGF2-responsive NSCs are present in the embryonic telecephalic germinal region as early as E13.5 (Reynolds and Rietze, 2005; Rietze and Reynolds, 2006). The primitive neural progeny cells mostly express the progenitor marker Nestin, a type VI intermediate filament protein, and a few early neurons express TUJ-1. To examine the differentiated cell subtypes generated from neurosphere-expanded stem cells, miR-29b transfected NSCs were analyzed by qRT-PCR to quantify expression of

markers specific to each cell type, such as Nestin (Figure 10), glial fibrillary acidic protein (GFAP), TUJ-1, and typical of oligodendrocytes O2 (data not shown). In the presence of miR-29b or siRNA against ICAT (siICAT#2), the expression of neural progenitor markers Nestin and GFAP, which is especially expressed in radial glial cells early in development (Doetsch, 2003; Frederiksen and McKay, 1988; Kriegstein and Alvarez-Buylla, 2009), was significantly reduced. Radial glia share features with neural stem and progenitor cells (NSPCs), which are earlier neuroepithelial cells present from E10.5 in embryonic development (Frederiksen and McKay, 1988).

**3. Nuclear β-catenin is necessary for NSC differentiation**

β-catenin translocates to the nucleus where it binds to the LEF/TCF family of transcription factors to control expression of their target genes (Behrens et al., 1996). β-catenin in the nucleus is essential for NSC differentiation

(Figure 11). ICAT colocalized with β-catenin in the cytoplasm but not at the plasma membrane while proliferate (Figure 12). Identical expression patterns in the colon epithelial cells were observed (Tago et al., 2000). Our results suggest that ICAT tightly cooperates with β-catenin in developing cortical layers.

I anticipated that miR-29 miRNAs would induce more neuronal-like progeny than glial cell lineages. At present, it is not clear how NSCs differentiate into each cell lineage, although it has been shown that ectopic induction of ICAT inhibits the expression of TUJ-1 and thus neuronal differentiation in embryonic carcinoma P19 cells (Lyu et al., 2003). Overall, little is known about the mechanisms controlling cortical development and the genetic pathways that regulate them.

**4. miR-29 directly targets the 3'-UTR of ICAT**

The TargetScan (http://www.targetscan.org) (Lewis et al., 2003) and microRNA.org (http://www.microrna.org) (Betel et al., 2008) algorithms confirmed that miR-29b targets the 3'-UTR sequence of *ICAT*. miR-29 family members (miR-29a, b, and c) were predicted to target a conserved seed sequence (5'-TGGTGCT-3') in the 3'-UTR of ICAT. To validate whether miR-29b targets ICAT through these putative target sites in the 3'UTR, I generated a luciferase reporter construct using the mouse ICAT 3'-UTR (2,173-bp) containing the predicted miR-29b target seed sequences. RNA duplexes of mature miR-29b were co-transfected into human embryonic kidney HEK293T cells with wild-type (WT) or mutant (MUT) miTarget miRNA 3'-UTR target clones (GeneCopoeia™, Rockville, MD, USA) as reporter constructs. Luciferase reporter gene activity decreased 20-30% when the WT reporter gene was co-transfected with miR-29b (Figure 13). To determine whether the predicted miR-29b target site in the *ICAT* 3'-UTR is

required for repression of ICAT expression, multiple base-pair mutations were introduced into the *ICAT* 3' UTR. These mutations of miR-29b target sites abolished miR-29b binding to the MUT construct. Activity of the reporter gene containing the mutant 3'-UTR was not affected by miR-29b. These results indicate that miR-29b represses ICAT protein expression by direct binding to the predicted target sites in the *ICAT* 3'-UTR.

**5. miR-29b antisense induces a profound proliferative defect during brain development**

To determine whether miR-29b plays a pivotal role in neuronal differentiation and migration *in vivo*, 2'-O-methyl modified anti-miR-29b inhibitor was microinjected into the left ventricle of mouse embryos at day E13.5. Inhibition of miR-29b by *in utero* electroporation of anti-miR-29b inhibitor into embryonic mouse brains led to a proliferative defect and premature outward

cortical migration (Figure 14). In contrast, the loss of function of ICAT induced by RNAi-mediated gene silencing *in utero* results in proper cortical migration. The miR-29b antisense inhibits TBR-1, a member of the brachyury (T-box) family of transcription factors. TBR-1 activates the RELN protein, which is secreted by Cajal-Retzius cells in cortical development (Hevner et al., 2001).

Cells transfected with control siRNA radially migrated at the cortical marginal zone and showed normal distribution of RELN expression. In contrast, a decrease in RELN-positive Cajal-Retzius neurons in the marginal zone was observed in brains with inhibition of miR-29b, presumably because TBR-1 expression in the cortical plate is suppressed in the presence of anti-miR29 (Figure 15). TBR-1 is an early pyramidal neuron marker that is expressed toward the pial surface and in the cortical plate (Hevner et al., 2001).

Malformations of cortical development can arise when preferentially labels projection neurons.

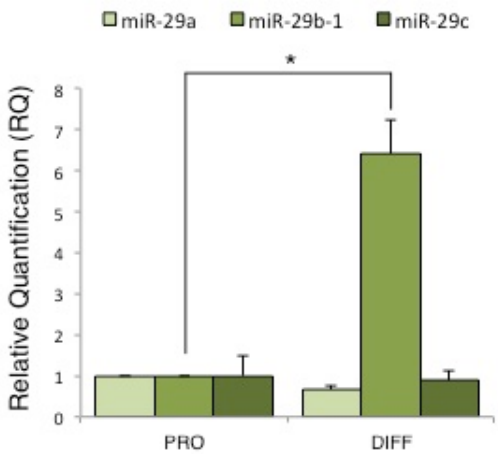


**Figure 7 Expression profiles of miR-29 subtypes in NSCs.** NSCs were harvested and total RNA was subjected to qRT-PCR to quantify expression of miR-29 subtypes a, b, and c. miR-29b, but not miR-29a or 29c, is significantly upregulated in 3D cultured NSCs, whereas ICAT is reduced as aged.

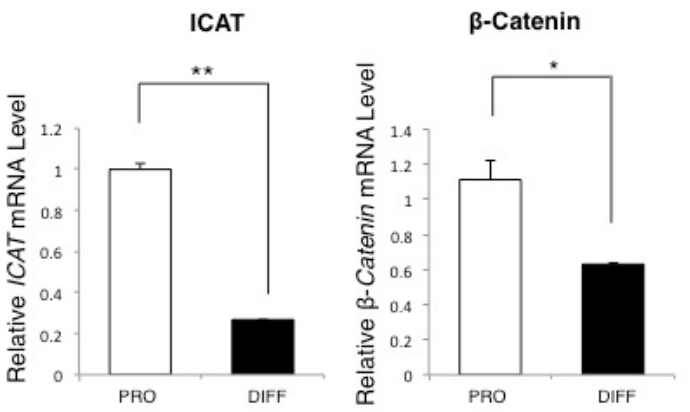


**Figure 8 Wnt signaling components expression pattern in NSCs** ICAT decrease results in an increase in the expression of miR-29b. ICAT and its binding partner β-catenin are highly expressed in fetal NSCs but expression declines with age (day 1, 3, 5). This decrease correlates with the increased expression of miR-29 family members, which are known to target and repress ICAT protein expression.

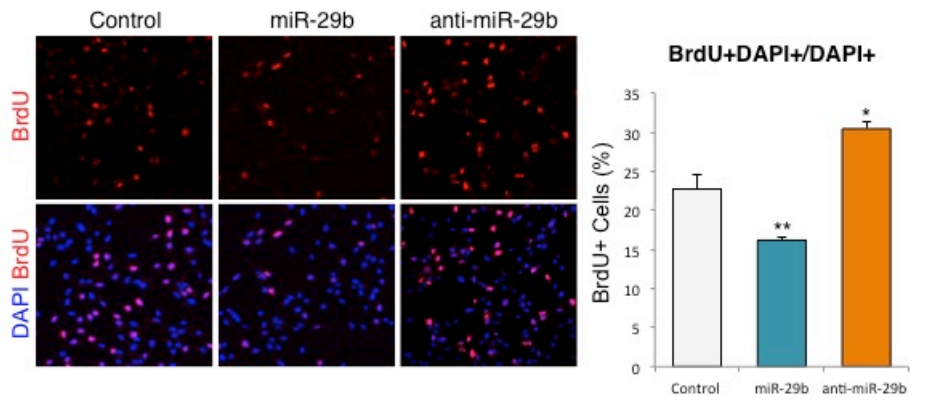


**Figure 9 miR-29b reduces NSCs proliferation**. NSCs from fetuses transduced with miR-29b or anti-miR-29b were plated on laminin-coated dishes, cultured in medium supplemented with EGF and basic FGF for 24 h, and labeled with a 1-h pulse of BrdU. Cells harboring a nuclear BrdU signal with DAPI (4,6-diamidino-2-phenylindole) were counted as positive. Quantification of BrdU incorporation was analyzed using Student's *t*-test. Approximately 1,000 cells were quantified for sections.

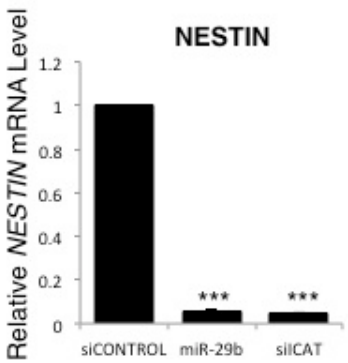


**Figure 10 miR-29b diminishes NSCs self-renewal**. ICAT-deficient NSCs showed a 90% reduction in expression of Nestin. Nestin is a type VI intermediate filament (IF) protein expressed in stem cells. Error bars show SEM of a triplicate experiment.

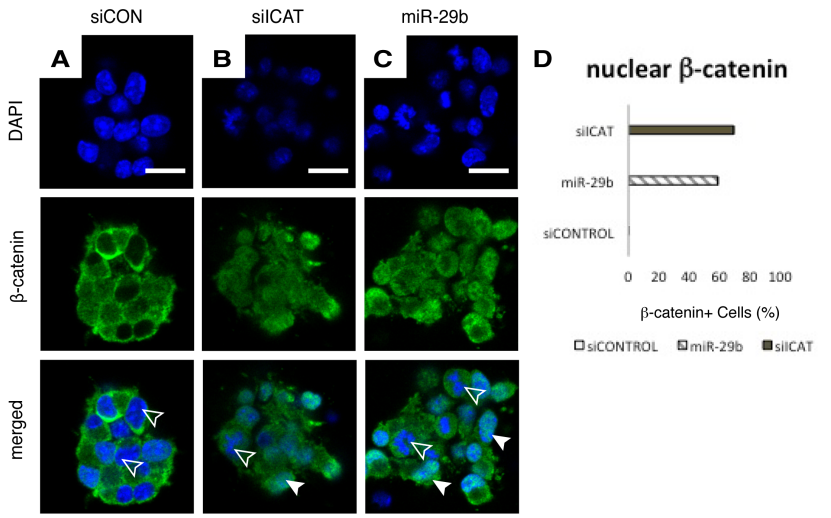


**Figure 11 Nuclear β–catenin is necessary for NSC differentiation** (A-C) β-catenin was enriched in the nuclei of differentiated NSCs. Scale bars indicate 15 μm Microscopy was performed on a Zeiss LSM 700 (Carl Zeiss, Germany) confocal laser scanning microscope or an Olympus FV1000-MPE confocal/multiphoton microscope. 3D reconstruction was performed using Imaris (custom software developed by Bitplane scientific software). (D) β-catenin is accumulated in the nuclei while miR-29b or siICAT was transfected in sphere-cultured NSCs. 60~70% of total cell population showed nuclear β-catenin.

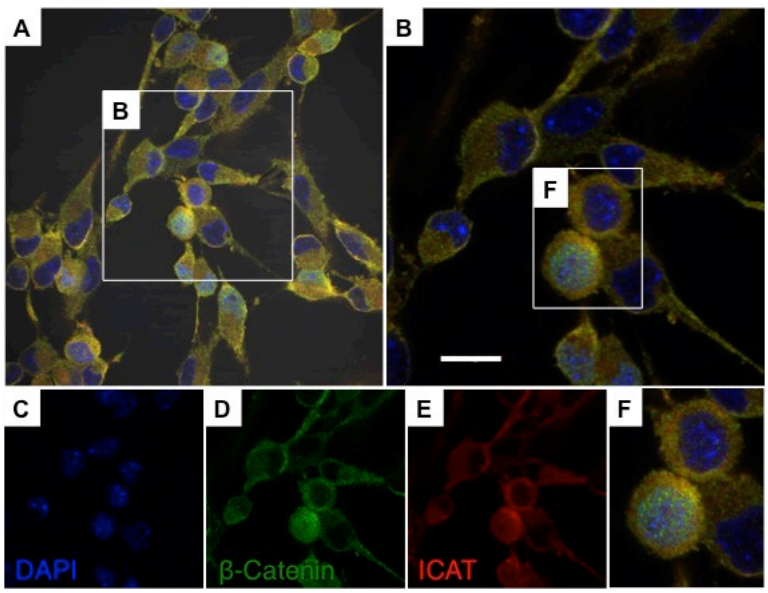


**Figure 12 ICAT co-localized with β–catenin in the nuclei in response to FGF-2, EGF** ICAT co-localized with β-catenin in the cytosol while basic FGF, EGF present. Scale bars indicate 10 μm (B). 100X magnification

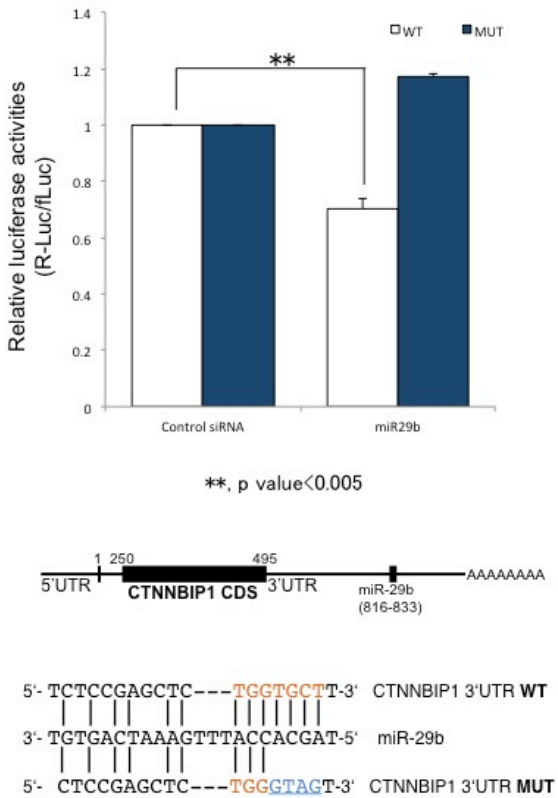


**Figure 13 miR-29b directly targets the 3'-UTR of ICAT.** HEK293T cells were transfected with luciferase reporters containing either wild-type 3'-UTR or the multiple-base mutant (TGCT to GTAG) 3'-UTR. Co-transfection of these reporters with miR-29b resulted in a decrease in the luciferase reporter activity of the wild-type 3'-UTR, but not the mutant 3'-UTR, indicating that miR-29b binds directly to ICAT 3' UTR. Firefly reporter luciferase activity was measured and normalized to *renilla* activity. complementary to seed sequences at 5'-end of miRNAs

miR-29b binding sites (5'-TGGTGCT-3') at 3'-UTR are indicated by red letters, and mutated bases are shown in blue.

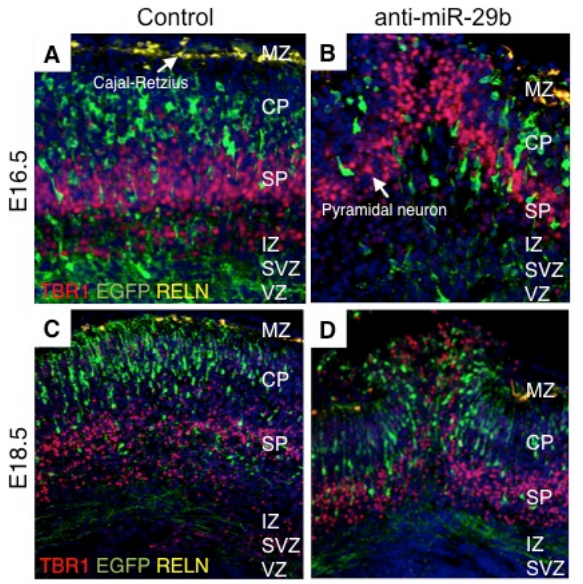


**Figure 14 Inhibition of miR-29b by *in utero* electroporation results in impaired corticogenesis.** TBR1 activates the RELN protein secreted by Cajal-Retzius cells during cortical development. The cortical plate at E16.5 shows TBR1-positive early pyramidal neuronal cells in red and RELN-expressing cells in the marginal zone in yellow. Inhibition of miR-29b by *in utero* electroporation of anti-miR-29b into embryonic mouse brains led to premature outward cortical migration. GFP-positive cells, transfected with control siRNA showed a normal distribution of RELN expression. In contrast, RELN-positive Cajal-Retzius neurons were decreased in brains microinjected with anti-miR-29b. MZ, marginal zone; CP, cortical plate; SC, subplate cells; IZ, intermediate zone, SVZ, subventricular zone; VZ, ventricular zone. Data are expressed as means ± SEM values (error bars) or assays performed in triplicate (*$p$<0.05; independent Student's $t$-test).

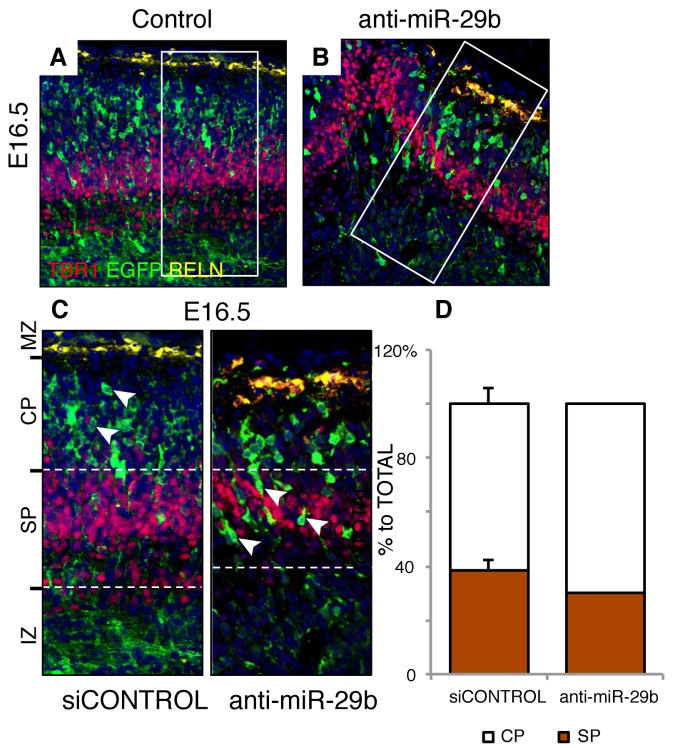


**Figure 15 Inhibition of miR-29b by *in utero* electroporation induced a profound defect in corticogenesis during mouse development.** During normal development, suppression of ICAT expression by miR-29 enhances neurogenesis *in vivo*. The cortical plate at E16.5 shows TBR1-positive early pyramidal neuronal cells (red) and RELN-expressing cells in the marginal zone (yellow). 2'-O-Me-modified anti-miR-29b oligonucleotides were microinjected into the left ventricle of mouse embryos at E13.5. TBR1 expression in the CP is suppressed when miR29 is inhibited. Genetic background: C57BL/6. Error bars show SEM; assays were performed in triplicate.

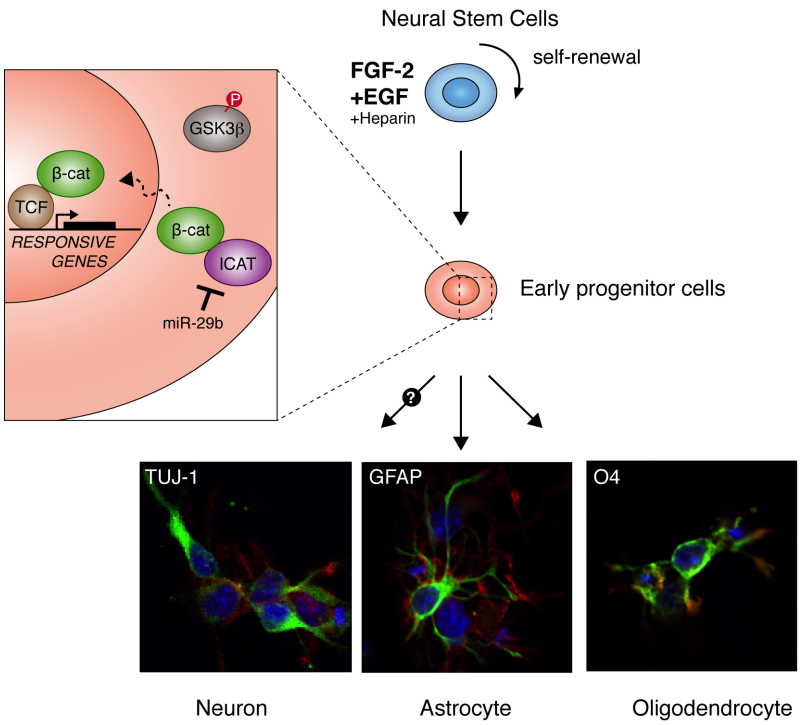


**Figure 16. Schematics of putative Wnt/β-catenin-mediated neurogenesis in mammals.** A potential schematic model of ICAT-dependent neurogenesis shows that miR-29b targets ICAT, thereby facilitating proliferation of neural stem cells in the developing brain.

# DISCUSSION

β-catenin is a well-known multifunctional protein involved in the regulation of mammalian CNS development. However, the mechanisms by which Wnt/β-catenin signaling components, such as ICAT regulate developmental disabilities in the CNS remain uncertain. Phosphorylated GSK-3β leads to β-catenin accumulation in the nucleus, where it couples with TCF. ICAT interferes with the association between β-catenin and TCF. *ICAT*$^{-/-}$ mouse embryos unveil severe malformation of the forebrain and craniofacial bones. This research aimed to investigate whether the miR-29 families regulate the expression of ICAT and, if so, whether dysregulation of ICAT by miR-29 results in defective neurogenesis and these pathologies are due to impaired β-catenin-mediated signaling events.

Here I demonstrate that miR-29b regulate ICAT-mediated Wnt/β-catenin signaling during fetal neurogenesis. The molecular nature of the downstream components of β-catenin is not well defined. Given the observations that: (1) miR-29 family members bind directly to ICAT; (2) β-catenin that is not bound to ICAT translocates to the nucleus; and (3) anti-sense against miR-29b alters the functional properties of ICAT, it seems likely that ICAT is a component of β-catenin-induced signal pathways (Figure 16). Although the genetic targets regulated by β-catenin and ICAT remain to be characterized, our data provide a link between miR-29b regulation and corticogenesis (Monuki and Walsh, 2001). Furthermore, these results may contribute to understanding the role of miR-29 miRNAs in the progression of many types of cancer (Park et al., 2009), especially brain cancer. miRNA molecules might also be considered as a putative therapeutic approach in human

patients with malignant glioblastoma multiforme (GBM), which represents the most common type of primary brain tumor.

NSCs and cancer stem-like cells share the morphological and functional similarities such as, self-renewal, extensive proliferation and invasiveness (Lee et al., 2006). These research findings may identify innovative therapeutic strategies to treat or prevent neurological diseases in humans, such as malignant GBM.

**Conclusions and perspectives**

There are diverse tumor types that arise from different cell types in the CNS, including neurons, glias, meninges and ependymal cells. The World Health Organization (WHO) has developed a scoring pattern in gliomas ranged from grade I to IV. GBM, categorized as grade IV tumors, is highly malignant and pro-mitotically active, and shows angiogenic pathologies. Gliomas

represent heterogeneity harboring multiple cell types including microglial cells originated from macrophages, ECs, pericytes, and smooth muscles cells (Charles et al., 2011).

The cancerous cells are thought to originate from multiple routes. First, they may arise from NSCs that undergo genetic mutations in tumor suppressor genes (p53, PTEN) (Duerr et al., 1998; Maintz et al., 1997) and oncogenes to alter “stem cell-like” properties through genomic instability, epigenetic dysregulation. Second, dedifferentiated non-neoplastic cells transform and produce astrocytic- and oligodendrocytic-based gliomas or neuroblastomas in adults.

**FGF- or EGF -related molecular pathologies in glioma**

It is observed overexpression of a variety of growth factors (i.e. PDGF (Guha et al., 1995), FGF2 (Takahashi et al., 1992), EGF) and their cognate

receptors in cancer. Epidermal growth factor receptor (EGFR) is amplified or hyperactivated via mutations in 30-50% of GBMs, it leads to signal transduction, such as the RAS-ERK, PI3K-AKT pathways, involved in cell proliferation and survival. To date, missense mutated EGFRs, known as EGFRvIII induced gliomas-like pathologies are well characterized, but FGFRs' are poorly defined.

**Pathological defects led by molecular alterations**

Many gene expression profiles and chromosomes are commonly altered in cancer. 81.8% of malignant gliomas (MGs) such as, anaplastic astrocytomas and GBMs (WHO grade III and IV) show mutation of the p53 gene (31.8%) or $p14^{ARF}$ deletion (54.5%). MDM2 overexpression (9.1%), EGFR amplification (50%), PTEN (phosphatase and tensin) mutations

(27.3%) and loss of heterozygosity (LOH) at the chromosomal regions 10q23.3 (86.4%) and 10q25-26 (100%) (Fulci et al., 2000).

Furthermore, $INK4A^{ARF}$ locus (encodes $p16^{INK4A}$ and $p14^{ARF}$) (Ichimura et al., 1996; Jen et al., 1994) deletion results in loss of inhibition of cyclin-dependent kinase 4 (CDK4) and MDM2. $p16^{INK4A}$ inhibits G1 transit to S-phase in the cell cycle. It causes an inhibition of retinoblastoma (Rb) and p53. p53 protein is often mutated in cancer, it altered cell-cycle arrest in G1.

In addition, PTEN, a tumor suppressor gene at 10q23 (*MMAC1*) (Myers et al., 1997) dephosphorylates phosphatidylinositol-3,4,5-trisphosphate (PIP3) to phosphatidylinositol-4,5-bisphosphate (PIP2), it results in the inhibition of AKT transactivation (Maehama and Dixon, 1998). To date, a majority of the changes in cell signal transduction become important in the progress to higher-grade gliomas.

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# 국문 초록

**서론:** 신경 줄기 세포는 중추신경계 발달 초기 미성숙, 미분화된 상태로 계속 증식할 수 있는 self-renewal 성격을 가지며, neuron 및 astrocyte 와 oligodendrocyte 로 분화가능한 다분화능을 지닌다. 본 학위논문에서는 FGF receptor 를 통한 신호전달 매개체 중 하나인 β-catenin 이 초기 뇌발생 과정 중 신경 줄기 세포 증식과 생존에 필수적인 역할을 한다는 가설 하에 실험을 진행하였다. FGF2 에 의해 활성화되어질 것으로 예상되는 intermediates 중 하나인 β-catenin 이 binding partner 인 ICAT (inhibitor of β-catenin and T-cell factor)에 의해 기능적으로 조절되어질 것이라고 예상하였으며 이 때 non-coding small RNA 인 microRNA 중 miR-29b 에 의해 조절되는 ICAT 의 발현에 미치는 영향을 연구, 그 생리적 의미를 파악하고자 하였다.

**방법:** 생체환경과 유사한 미세환경의 구현을 위해 최적화된 3D microfluidics platform 을 사용하여 qRT-PCR, immunofluorescence 및 luciferase 활성 측정

등의 방법으로 신경 줄기 세포의 분화와 증식에 관하여 평가하였다. 또한 한림대학교 약리학교실 연구팀과의 공동 연구로 13 일자 임신 쥐의 fetus 에 miR-29b 를 *in utero* electroporation 을 통해 주입한 후 뇌 형성에 미치는 영향을 조사하였다.

**결과:** 신경 줄기 세포의 3D 배양시 분화가 유도됨에 따라 miR-29b 의 발현은 통계적으로 유의하게 증가하였으나 miR-29a 또는 -29c 는 유의적인 변화가 없었으며 반면 ICAT 은 감소하였다. ICAT 3'-UTR construct 의 luciferase reporter 활성은 miR-29b 의 처리에 의하여 감소하였으나 scrambled control siRNA 는 reporter 의 활성에 영향을 미치지 않았다. 이 결과는 miR-29b 가 직접적으로 ICAT 의 3'-UTR 을 타겟한다는 것을 의미한다. 또한 miR-29b 는 신경 줄기 세포의 self-renewal 과 증식을 감소시키고 어떠한 세포 lineage 로 분화하는 지를 결정하였다. 또한, *in utero* electroporation 에 의한 생체 내 실험에서 miR-29b 의 저해는 마우스의 발생과정 중의 corticogenesis 에 심각한 결함을 초래한다는 사실을 발견하였다.

**결론:** 뇌발생 초기 miR-29b 는 ICAT 발현을 억제하여 Wnt/β-catenin 신호전달을 조절함으로써 발생과정 중의 설치류 마우스 신경세포 신생에 중요한 역할을 한다. 또한 이 결과는 miR-29b 가 ICAT 에 의한 Wnt/β-catenin 신호 전달을 조절함으로써 신경계질환을 치료 혹은 예방할 수 있는 새로운 치료전략으로서 활용될 수 있음을 시사한다.

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**주요어 :** 신경 줄기 세포, β-catenin, ICAT, **microRNA**-29b

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