Stem Cell Research 17 (2016) 238–247

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

miR-410 controls adult SVZ neurogenesis by targeting neurogenic genes☆

Yao-chang Tsan, Maria H. Morell [1], K. Sue O'Shea *

Department of Cell and Developmental Biology, School of Medicine, University of Michigan, Ann Arbor, MI 48109, United States

ARTICLE INFO

Article history:
Received 29 April 2014
Received in revised form 14 June 2016
Accepted 11 July 2016
Available online 17 July 2016

Keywords:
Astrocyte
Embryonic stem cell
MicroRNA
Neural stem cell
Neuron
Oligodendrocyte

ABSTRACT

Over-expression of the early neural inducer, Noggin, in nestin positive subventricular zone (SVZ), neural stem cells (NSC) promotes proliferation and neuronal differentiation of neural progenitors and inhibits the expression of a CNS-enriched microRNA-410 (miR-410) (Morell et al., 2015). When expressed in neurospheres derived from the adult SVZ, miR-410 inhibits neuronal and oligodendrocyte differentiation, and promotes astrocyte differentiation. miR-410 also reverses the increase in neuronal differentiation and decreased astroglial differentiation caused by Noggin over-expression. Conversely, inhibition of miR-410 activity promotes neuronal and decreases astroglial differentiation of NSC. Using computer prediction algorithms and luciferase reporter assays we identified multiple neurogenic genes including Elavl4 as downstream targets of miR-410 via the canonical miRNA-3′ UTR interaction. Over-expression of Elavl4 transcripts without the endogenous 3′UTR rescued the decrease in neuronal differentiation caused by miR-410 overexpression. Interestingly, we also observed that miR-410 affected neurite morphology; over-expression of miR-410 resulted in the formation of short, unbranched neurites. We conclude that miR-410 expression provides a new link between BMP signaling and the crucial lineage choice of adult neural stem cells via its ability to bind and control the expression of neurogenic gene transcripts.

1. Introduction

Intracellular and extracellular mechanisms combine to maintain the neurogenic niches in the adult brain (review, (Ming and Song, 2011)). In fact, considerable work has demonstrated that dynamic interactions between these compartments regulate cell behaviors within the germinal niche and ultimately its neuronal vs glial output. In addition to transcriptional regulators, intracellular modulators including non-coding microRNAs have been shown to control proliferation, self-renewal and cell fate choice in the nervous system via their ability to bind and inhibit translation or to promote degradation of critical target genes (Gangaraju and Lin, 2009).

Cell type-specific microRNAs (He et al., 2012; Jovicic et al., 2013) which bind and suppress lineage specifying genes or groups of genes, are thought to play a critical role in maintaining the niche and controlling differentiation. For example, one of the most abundant microRNAs in the CNS, miR-124, is up-regulated at the transition from subventricular zone (SVZ) transit amplifying C cells to neuroblasts (A cells), thereby promoting neurogenesis (Bian and Sun, 2011; Bian et al., 2013b; Bian et al., 2013a). Regulatory networks are beginning to be identified—i.e., miR-25 can promote proliferation of neural stem cells (NSC) via its ability to regulate the IGF signaling pathway (Brett et al., 2011), while Sox2 regulates miR-137, which in turn targets Ezh2 to inhibit differentiation and promote NSC proliferation (Szulwach et al., 2010). Although many genes and epigenetic mechanisms that regulate miRNAs have been identified (Ji et al., 2013), the extracellular regulation of miRNAs, particularly within the CNS, has been less well studied.

Despite the potential importance of microRNAs in understanding CNS development (Lang and Shi, 2012), disease (Eacker et al., 2009), in identifying molecular markers (Di Leva and Croce, 2013), and potentially in therapeutic approaches (Pers and Jorgensen, 2013), the critical link between the extracellular compartment and microRNA expression and function is poorly understood (Bian et al., 2013a). In a previous paper (Morell et al., 2015), we describe a transgenic mouse in which expression of the BMP signaling pathway inhibitor, Noggin, in SVZ NSC promotes neuronal and oligodendroglial differentiation, while decreasing astrocyte differentiation both in vivo and in vitro. Microarray analysis of RNAs from Noggin over-expressing and control SVZs identified a microRNA, miR-410, which was down-regulated with BMP pathway inhibition. We have determined that miR-410 is expressed in the SVZ NSC niche, and transiently in mESC as they differentiate into neurons, where it inhibits neuronal differentiation and can reverse the noggin-

☆ The authors declare no competing financial interests.

* Corresponding author at: Department of Cell and Developmental Biology, University of Michigan, 109 Zina Pitcher Pl, 3051 BSRB, Ann Arbor, MI 48109-2200, United States.
E-mail address: oshea@umich.edu (K.S. O'Shea).
[1] Current address: Centro Pfizer de Genomica e Investigacion Oncologica, Avda. de la Ilustracion, 114 18007 Granada, Spain.

http://dx.doi.org/10.1016/j.scr.2016.07.003

stimulated increase in neuronal differentiation. Predicted targets of miR-410: *Elavl4*, *Sox1*, *Smad7*, *Tcf4* and *Fgf7* were validated in luciferase assays, and expression of *Elavl4* rescued the inhibitory effects of miR-410 on neuronal differentiation, providing an additional mechanistic link between BMP signaling and neurogenesis. Unexpectedly, miR-410 also affected process outgrowth and neuronal morphologies.

2. Materials and methods

2.1. Animals

Noggin inducible transgenic mice were generated as described (Morell et al., 2015), and are available at the Jackson Laboratories, Stock Number 23410, FVB-Tg(tetO-Nog,-EGFP)53Hda/J. Additional wildtype FVB/N animals were purchased from Taconic. All animals were handled according to protocols approved by University of Michigan Committee on the Use and Care of Animals (UCUCA).

2.2. Embryonic stem (ES) cell culture

The mouse embryonic stem cell line D3 was cultured in 10% FBS (Atlanta Biologicals) in DMEM (LifeTechnologies) containing 4.5 mM HEPES (LifeTechnologies), 1.5 mM L-glutamine (LifeTechnologies), 5 ng/ml LIF (GlobalStem) and 0.00038% (v/v) β-mercaptoethanol (Sigma). Cells were grown on 0.1% gelatin (Sigma) coated tissue culture flasks and passaged one to three every other day. For neural differentiation, 1×10^5 cells per well were plated on gelatin coated 6-well plates in 80% N2 medium: 20% B27 medium (LifeTechnologies) with 1 μM retinoic acid (Sigma). The medium was change every other day. After 6 days of differentiation, the cells were fixed for immunocytochemistry or RNAs extracted.

2.3. Neurosphere culture

8 to 10 week old mice were sacrificed by cervical dislocation ($n \geq 5$ per group). The subventricular zones were microdissected, tissue pooled and dissociated in 0.133% (w/v) trypsin (LifeTechnologies), 0.067% (w/v) hyaluronidase (Sigma) and 0.69 mM kynurenic acid (Sigma) in artificial cerebral spinal fluid (ACSF) (124 mM NaCl, 5 mM KCl, 3.2 mM MgCl, 26.2 mM $NaHCO_3$, 10 mM glucose, and 0.098 mM $CaCl_2$) at 37 °C for 30 min. Trypsin was stopped by adding 1:1 trypsin inhibitor solution (0.022% w/v) (LifeTechnologies) and 0.001% (w/v) DNaseI (LifeTechnologies) in N2 medium. Cell clumps were broken to single cells by trituration using polished glass pippettes. Cells were then cultured in N2 medium supplemented with 10 ng/ml FGF2 (Sigma), 20 ng/ml EGF (Sigma), and 2 μg/ml heparin (Sigma). Medium was changed twice each week.

2.4. Plasmid transfection

Two weeks after isolation, neurospheres were disaggregated by trituration using polished Pasteur glass pipettes, transfected with the miR-410 over-expression, miR-410 sponge, with the Elavl4 over-expression, or control plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. They were grown in N2 medium supplemented with FGF2, EGF, and heparin for an additional week. For differentiation, neurospheres were disaggregated in 0.25% trypsin/1 mM EDTA at 37 °C for 90 s followed by addition of trypsin inhibitor solution and trituration, then 1.5×10^4 cells per well plated in N2 medium supplemented with 1% FBS to 48-well tissue culture plates pre-coated with 6.67 μg/ml poly-ornithine/water solution for 2 h at room temperature. Medium was changed on day 4 and cells were fixed on day 7 for further analysis. To generate ES cell lines over-expressing miR-410, D3 ES cells were transfected with pPUS2-miR-410, as described below. Selection in 10 μM puromycin was started 24 h after transfection. Four days after transfection the cells were passaged 1:10 and single colonies were picked and expanded to derive cell lines.

2.5. Lentiviral transduction

Seven days after isolation, primary neurospheres were pooled, dissociated by gentle trituration, then transduced with 1×10^6 moi lentiviral particles per group. Three days post-transduction the neurospheres were plated for differentiation as described above.

2.6. Luciferase reporter assays

HEK293T cells were cultured in 10% FBS/DMEM and passaged 1:5 every other day. 0.4 μg of either the miR-410 over-expression or control vector and 0.4 μg of either pmirGLO-wt-3′UTR or pmirGLO-mutated-3′ UTR were transfected into 5×10^4 HEK293 cells. Cell lysates were harvested 48 h after transfection for analysis. Luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's protocol in a Lumat LB 9507 luminometer (Berthold Technologies).

2.7. DNA constructs

The miRNA expression vector pPUS2C was constructed by inserting the mCherry coding sequence (R.Y. Tsien, UCSD) into the pUS2 plasmid (D. Turner, University of Michigan) between the *Bam*HI and *Eco*RI sites. A separate puromycin resistance cassette was cloned into the second multiple cloning site. To develop the miR-410 over-expression vector, 360 bp of mouse genomic sequence containing the full pre-miR-410 was cloned by PCR (FW: TAGAATTCGTGCTGCCTGTGTCAACCCTACTC; REV: TATCTAGAATCTGGCCAATGCTTCGTG) into pPUS2C between the *Eco*RI and *Xba*I sites. A miRNA sponge targeting miR-410 was made as described previously (Ebert et al., 2007) and inserted into the pPUS2C plasmid between the *Eco*RI and *Xba*I sties. The efficacy of the miR-410 over-expression vector, pPUS2C-miR-410, and the miR-410 sponge vector, pPUS2C-410SP, was tested in D3 mESC. The full length mouse *Elavl4* coding region with either the full length 3′UTR, a truncated 3′UTR, or lacking the entire 3′UTR, was cloned by PCR and inserted into the pCIG plasmid (Ben Allen, University of Michigan). Lentivirus: miR-410 over-expression, miR-410 sponge, or scrambled control sequences were cloned into the pLentilox-eGFP backbone vector (U of M Vector Core). Functional virus particles were packaged in 293FT cells (Invitrogen) by co-transfecting the expression vectors with packaging plasmid pMD2.G and psPAX2 (Addgene) as previously described (Barde et al., 2010). For luciferase assays: the full length or truncated 3′UTR of miR-410 target candidate genes was amplified by PCR and cloned into the pmirGLO plasmid (Promega) between *Nhe*I and *Sal*I sites according to the manufacturer's protocol. For genes with a 3′UTR shorter than 800 bp, the full length 3′UTR was cloned into the vector. For genes with a 3′UTR longer than 800 bp, a region at least 800 bp long containing the miR-410 site in the center was cloned. Vectors containing a 3′UTR with a mutated miR-410 site (TTAATTAA) were made by PCR based site-directed mutagenesis.

2.8. Immunohistochemistry

For immunohistochemical localization of cell type-restricted proteins, cells were fixed in 2% PFA at room temperature for 15 min, permeabilized in 0.1% Triton X-100/0.1% sodium citrate in PBS for 10 min, blocked with 10% normal donkey serum/0.5% Triton X-100/0.1% sodium azide in PBS. The cells were then incubated with primary antibodies at 4 °C overnight. The following day the cells were washed in PBS and incubated with secondary antibodies for 30 min at room temperature. Nuclear staining was done by incubating the cells in 1 μM Hoechst 22358 at room temperature for 5 min. Antibodies included: βIII tubulin (Tuj1 antibody; 1:1000, Covance); GFAP (1:500, Santa Cruz); and Olig2 (1:1000,

Millipore). Secondary antibodies were obtained from Jackson labs and used at 1:400–1:1000.

2.9. Protein extraction and western blotting

Total cell lysates were collected in RIPA buffer from neurospheres 7 days post transfection with either control or miR-410 over-expression plasmids as described (Sambrook et al., 1989). After electrophoresis, β-actin (1:1000, Sigma) and Elavl4 (1:200, Millipore) were blotted with primary antibodies, scanned and quantified.

2.10. RNA extraction and RT-PCR

Total RNA was extracted from microdissected SVZ or cells in culture in Trizol (Invitrogen) following the manufacturer's protocol. 1 μg of DNase-treated RNA was used for RT-PCR either with the miScript Reverse Transcription kit (Qiagen) for miR-410 or the Verso cDNA kit (Thermo Scientific) for mRNA transcripts.

2.11. Quantitative PCR and primer design

Quantification of miR-410 expression was done using the miScript quantitative PCR system (Qiagen) following the manufacturer's protocol. For qPCR on other genes, primers were designed using Lasergene software (DNASTAR) and verified by the NCBI primer-BLAST program (http://blast.ncbi.nlm.nih.gov). qPCR was done in triplicate using the Abgene SYBR system (Thermo Scientific) on a Bio-rad iCycler qPCR system (Bio-rad). Data were then analyzed by the ΔΔCt method. Primers and conditions are available on request.

2.12. Image quantification

To assess the number of neurons and astrocytes present following neurosphere and ESC differentiation, photomicrographs (20×) were taken along two perpendicular lines bisecting the culture dish: from 0 to 180° and along a second line from 90 to 270°. Numbers of neurons, astrocytes and total cell numbers were counted from at least three wells each from three independent experiments ($n \geq 9$ wells) and analyzed using Students *t*-test. The nuclear expression of the oligodendrocyte precursor maker Olig2 was quantified using a custom written MATLAB (MathWorks Inc.,) script. Images of Hoechst stained cells were loaded and a binary image of nuclear regions was created by thresholding (Otsu, 1979). The binary image was processed to create a mask image. The average intensity of Olig2 fluorescence was measured over each nuclear mask. Total numbers of Olig2-positive oligodendrocyte precursors were counted from 2 to 3 wells each from three independent experiments ($n \geq 6$) and the percentage of Olig2+ nuclei were analyzed as described. Scholl analysis was carried out to quantify alterations in neurite morphology. A concentric ring template with an inter-ring distance of 25 μm was overlaid on images of neurons stained with βIII tubulin antibody. Processes were analyzed only if it was possible to clearly distinguish processes from those of adjacent cells; i.e., they were clearly separated. The template was centered on the neuronal cell soma, and the number of crossings at each of the Scholl rings counted (Brunette et al., 2010). The maximal process length was considered to be the furthest Scholl ring crossed. A process that branched on a ring (Fig. 6.B) was counted as two crossings. At least 50 neurons were counted from each of three replicate wells from: Scrambled control; miR-410 over-expression; Elavl4 over-expression; over-expression of both miR-410 and Elavl4. The mean number of Scholl ring crossings were computed, plotted in Excel, and analyzed using Students *t*-test.

2.13. Computational analyses

The enhancer region sequence of the *Mirg* gene was identified and downloaded from Transcriptional Regulatory Element Database (TRED, http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home). Potential miR-410 targets were identified by searching four databases: TargetScan (http://www.targetscan.org), ElMMo (http://www.mirz.unibas.ch/ElMMo2), PicTar (http://pictar.mdc-berlin.de) and MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5).

3. Results

3.1. Noggin controls miR-410 expression

We identified a previously uncharacterized microRNA, miR-410, in the SVZ germinal zone of a transgenic mouse in which Noggin expression can be inducibly driven to Nestin positive NSCs (Morell et al., 2015). Over-expression of Noggin in NSC significantly increased neuronal and oligodendrocyte differentiation while down-regulating the expression of miR-410. miR-410 is a 21 nt microRNA embedded in the miRNA-encoding gene *Mirg*. miR-410 was originally described as restricted to the developing central nervous system (Wheeler et al., 2006; Han et al., 2012), suggesting it may have important roles in neurogenesis. Since miR-410 was down-regulated by Noggin, we reasoned that BMP signaling may directly regulate its expression. We analyzed the 1.3 kb enhancer region from 1000 bp upstream to 200 bp downstream of the *Mirg* transcription starting site, and identified three GG-C/A-GCC GC-rich BMP-specific Smad binding elements (Morikawa et al., 2011), in addition to 10 Smad consensus binding motifs (GTCT/AGAC) (Massague et al., 2005). When we compared the expression levels of miR-410 in Noggin induced and un-induced control adult SVZ using quantitative-PCR against the mature form of miR-410, we found that miR-410 was down-regulated 16-fold in the Noggin induced SVZ (not shown), suggesting miR-410 expression may be regulated by BMP signaling, and validating our microarray analyses.

3.2. Over-expression of miR-410 reduces neuronal differentiation and the number of Olig2 positive cells in neurosphere differentiation assays

To test the function of miR-410 in neuronal and glial differentiation, we over-expressed and inhibited miR-410 function in primary neurospheres derived from adult SVZ. On day 7 of culture, the neurospheres were transduced with lentivirus as described above; 72 h later >90% of the cells expressed eGFP (not shown), increasing their mature miR-410 expression 28.8-fold compared with cells transfected with control lentivirus as measured by qPCR. In addition, we observed a 37% reduction in mature miR-410 in neurospheres transduced with miR-410 sponge. Transduced neurospheres were differentiated and stained using GFAP or β-III tubulin (Tuj1), (Fig. 1.A) antibodies. Neurospheres transduced with lentivirus carrying either eGFP alone or the miR-Scrambled did not show significant differences in neuronal differentiation (23.6 ± 3.5% vs 21.5 ± 1.1%, respectively, $p \leq 0.24$), and were combined as a single control group. Control neurospheres yielded 22.5 ± 2.8% neurons (Fig. 1.B.A), and over-expression (OE) of miR-410 significantly reduced that percentage to 14.9 ± 1.3% ($p \leq 7.5 \times 10^{-4}$, control vs OE). Exposure to the miR-410 sponge significantly increased the number of Tuj1+ neurons to 33.8 ± 1.8% ($p \leq 4.9 \times 10^{-5}$ compared to the control group; Fig. 1.B.B). As expected, the majority of the cells formed in the neurosphere assay in control conditions were astrocytes (77.5 ± 3.5% (eGFP alone: 76.4 ± 3.5%; scramble: 78.5 ± 1.1%, $p \leq 0.24$). Over-expression of miR-410 significantly increased the percentage of astrocytes to 85.1 ± 1.3% ($p \leq 7.5 \times 10^{-4}$, OE compared to control), while expression of the miR-410 sponge decreased the percentage of astrocytes to 66.2 ± 1.8% ($p \leq 4.9 \times 10^{-5}$, sponge compared to control) (Fig. 1.B.C).

We also examined the role of miR-410 in oligodendrocyte differentiation. Over-expression of miR-410 in NSC significantly reduced the Olig2+ cell number from 7.7 ± 1.1% in the control group to 4.9 ± 2.5% ($p \leq 0.023$). However, miR-410 loss of function did not significantly

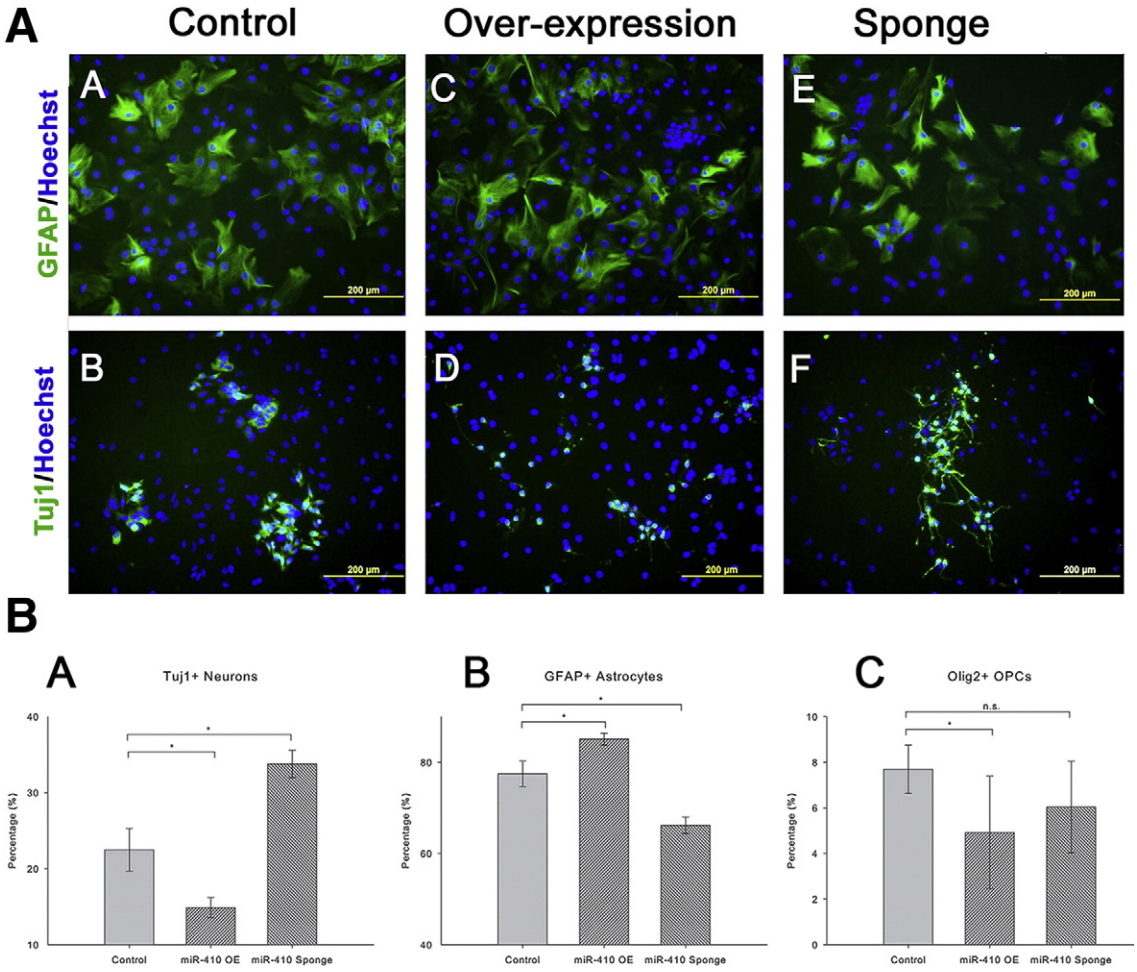

Fig. 1. Effects of miR-410 over-expression and inhibition on neuron, astrocyte and oligodendrocyte differentiation. A. Primary neurospheres isolated from 8 week-old mice were transduced with vectors carrying either eGFP alone, a scrambled miRNA control, miR-410 (Over-expression), or the miR-410 sponge. After 72 h, the neurospheres were dissociated and plated for 7 days of differentiation. Cells were fixed and immunohistochemical localization of GFAP (astrocytes) or β-III tubulin (Tuj1 antibody, neurons) carried out. There was widespread differentiation of GFAP positive astrocytes in all culture conditions (A,C,E), and differentiation of β-III tubulin/Tuj1 + neurons in control cultures (B) and robust differentiation in the presence of the microRNA sponge (F), but few neurons differentiated when miR-410 was over-expressed (D). Hoechst 22358 identifies nuclei. Scale bars = 200 μM. B. Quantitative analysis indicated that compared with controls, over-expression (OE) of miR-410 significantly inhibited neuronal differentiation (A), promoted astrocyte differentiation (B), and reduced the number of Olig2-positive cells (C). Conversely, miR-410 loss of function via the miR-410 sponge increased neuronal differentiation (A) and reduced astrocyte differentiation (B), but did not affect OPC commitment (C). Percentages of positive cells are expressed as mean ± SD, * = $p \le 0.01$. n.s. = not significantly different.

affect oligodendrocyte precursor cell (OPC) commitment (6.0 ± 2.0% vs 7.7 ± 1.1%, $p \le 0.08$) (Fig. 1.B.C), suggesting that any regulation of oligodendrocyte differentiation by miR-410 may be asserted through another mechanism or with different kinetics.

3.3. Over-expression of miR-410 inhibits neural differentiation of ESC

To examine the function of miR-410 in a pluripotent stem cell model, we generated two mouse embryonic stem cell lines (#20 and #24) that over-express miR-410 (20- and 6-fold respectively, data not shown). miR-410 is expressed at very low levels in pluripotent ESC, but is transiently up-regulated during early neural differentiation (data not shown). The miR-410 over-expressing cells and the parental D3 cells were plated and differentiated in defined neural induction media for 6 days before fixation and staining for β-III-tubulin (Tuj1). Expression of miR-410 inhibited the differentiation of both SOX3 + neural precursor cells and of β-III-tubulin (Tuj1) positive neurons after 6 days of neural differentiation compared with the control cell line D3 (Fig. 2.A) suggesting that neuronal differentiation is not simply delayed. To obtain additional quantitative data from the dense cultures, neuronal differentiation was quantified using q-RT-PCR. Over-expression of miR-410 in line 20 reduced the expression of the early neuronal marker, *β-III-tubulin*, 4.3 fold and of the neural progenitor cell marker, *Sox3*, 15.3 fold compared to the parental cell line D3, while expression of the astroglial marker *Gfap* was increased 2.5 fold in the miR-410 over-expressing ESC lines (Fig. 2.B). Similar results were obtained with line 24 (data not shown). The two cell lines showed no difference in the expression of markers of pluripotency (*Oct4*), of endoderm (*FoxA2*), or mesoderm (*T*, Brachyury). The epiblast marker *Fgf5* was down-regulated 7-fold, while the trophectoderm marker transcript *Cdx2* was not detected in either cell line.

3.4. miR-410 acts downstream of Noggin/BMP

To determine the relationship of miR-410 to Noggin and BMP signaling, we transfected mouse primary neurospheres with miR-410 expression vectors with 0.5 μg/ml noggin or without exogenous Noggin protein added to the culture media (Fig. 3). As expected (Morell et al., 2015), Noggin alone promoted neuronal differentiation (control: 27.3 ± 1.5%; Noggin exposure: 37.5 ± 1.1%; $p \le 3.3 \times 10^{-4}$), while co-overexpression of miR-410 with Noggin treatment largely reduced the increase in β-III-tubulin (Tuj1) positive neurons observed in the Noggin alone group (+Noggin, control) (+Noggin, miR-410 OE: 29.6 ± 0.9%, $p \le 2.9 \times 10^{-4}$) (Fig. 3.A). Noggin treatment alone decreased astrocyte

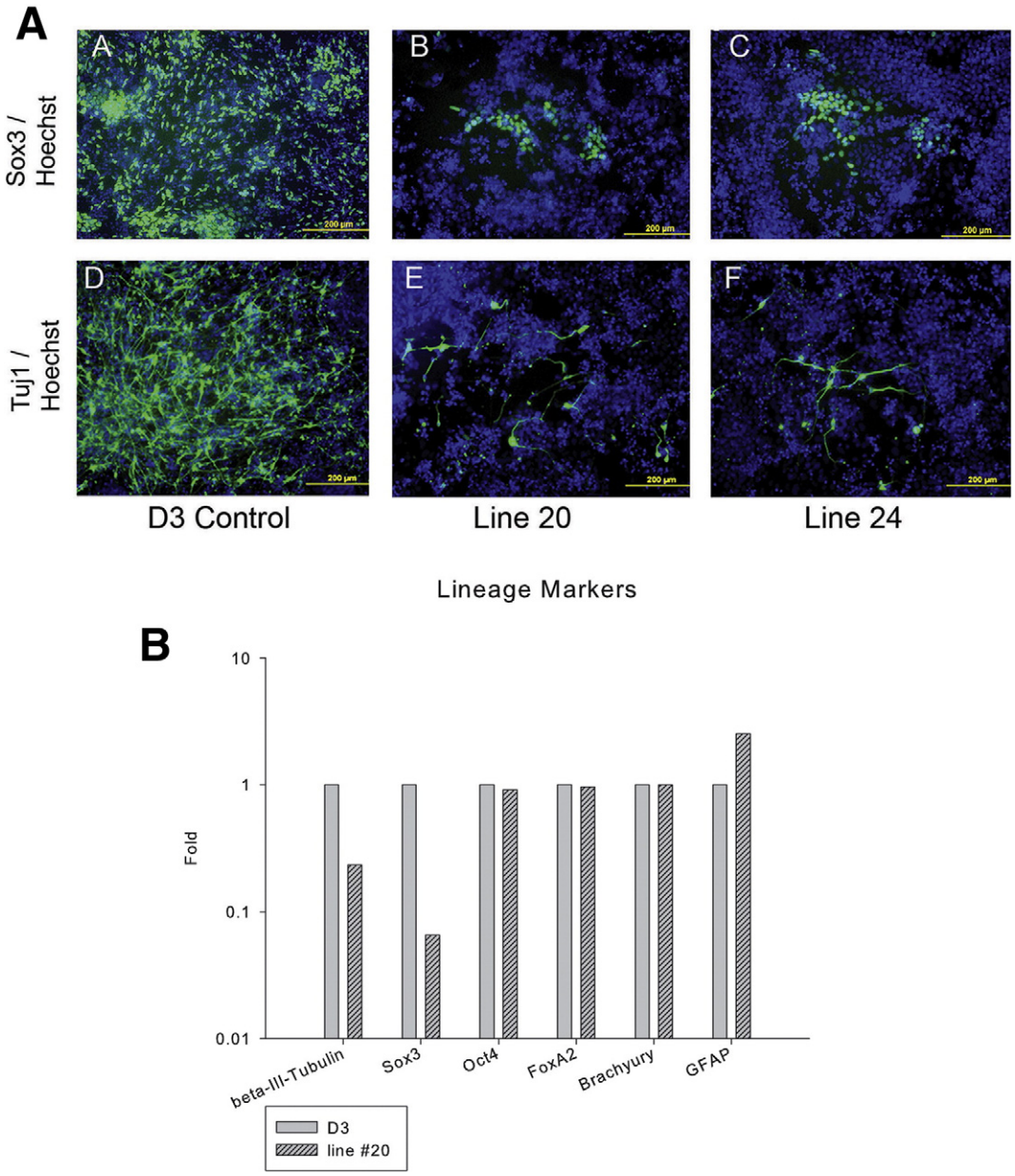

Fig. 2. miR-410 over-expression inhibits neuronal differentiation of ESC. A. Immunohistochemical analysis. Two mouse embryonic cell lines (#20 and #24) that over-express miR-410 (20- and 6-fold respectively) were generated as described. The miR-410 over-expressing ES cells and the parental D3 control ES cells were plated and differentiated in defined neural media for 6 days before fixation and localization of Sox3 (A-C) or β-III-tubulin (Tuj1 antibody, D–F). Over-expression of miR-410 inhibited both the differentiation of Sox3 + neural precursors (B,C) and of Tuj1 + neurons (E,F) compared with the control D3 cell line (A, D). FITC – secondary antibody). Hoechst 22358 = DNA. B. q-RT-PCR analysis. Over-expression of miR-410 reduced expression of the early neuron marker, *β-III-tubulin* by 4.3-fold and reduced the expression of the neural precursor marker *Sox3* by 15.3-fold, compared to the parental cell line D3. There was no difference in the expression of the pluripotency marker *Oct4*, the endoderm marker *FoxA2*, or the mesoderm marker *T*, Brachyury, while the astrocyte marker *Gfap* was increased 2.5 fold by miR-410 over-expression in line 20. The trophoblast marker *Cdx2* was not detected in either cell line.

number (−Noggin, control: 72.6 ± 1.6%, +Noggin, control: 62.5 ± 1.1%; $p \le 4.3 \times 10^{-4}$), and over-expression of miR-410 partially reversed the decrease in astrocyte cell numbers (+Noggin, miR-410 OE: 70.3 ± 1.0%, $p \le 3.7 \times 10^{-4}$) (Fig. 3.B). These results suggest that miR-410 functions downstream of BMP signaling. However, since over-expression of miR-410 only partially rescued the phenotypes caused by Noggin, other factors, or timing, may play a role in miR-410′s ability to modulate Noggin's effects on differentiation.

3.5. miR-410 down-regulates expression of Elavl4

To examine the mechanism(s) underlying the ability of miR-410 to affect NSC lineage differentiation, we analyzed potential target candidates of miR-410 using four databases (TargetScan, PicTar, ElMMo, and MicroCosm). Of the predicted candidates, we identified seven genes with high predicted affinity to miR-410 and known to function in neurogenesis or in self-renewal and tested them in luciferase assays. The 3′UTR of the candidate targets was cloned after a luciferase coding region into a test plasmid. As an unbiased control, the predicted seed-binding regions of the 3′UTRs were mutated to a *Pac*I restriction enzyme site. The luciferase test plasmids were then co-transfected with a miR-410 expression vector into HEK293T cells, which do not express miR-410 (data not shown). Over-expressed miR-410 should target the wildtype 3′UTR and down-regulate luciferase expression, sparing the mutated 3′UTR. The efficiency of miR-410 induced gene down-regulation was quantified by luciferase activity. Among the

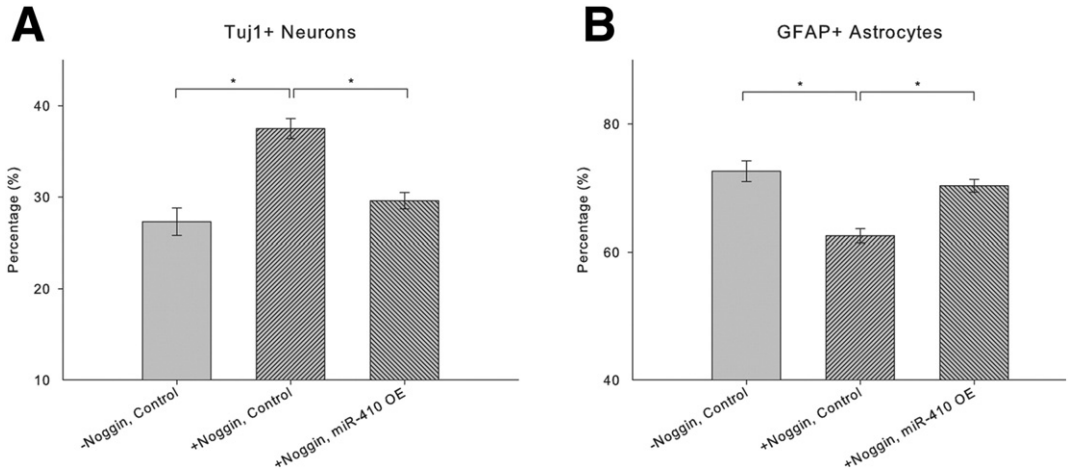

Fig. 3. Effects of exogenous Noggin protein on neuronal differentiation of miR-410 over-expressing NSC. Neurospheres were transfected with miR-410 over-expression vectors and grown in suspension for 7 days ± 0.5 μg/ml Noggin protein. The neurospheres were then dissociated and plated for 7 days of differentiation, then fixed and stained with antibodies to β-III tubulin (Tuj1 antibody, neurons) and GFAP (astrocytes) and cell numbers counted. Percentages of positive cells are expressed as mean ± SD, * = $p \le 0.01$. A. Noggin treatment strongly promoted neuronal differentiation compared with controls, while miR-410 over-expression largely rescued the increase in Tuj1-positive neurons produced by Noggin, suggesting that miR-410 functions downstream of BMP signaling. B. Noggin exposure alone reduced astrocyte differentiation, and miR-410 over-expression partially rescued the decrease in GFAP-positive astrocytes caused by Noggin.

genes tested, the 3′UTR of *Zfx* was not targeted by miR-410, the 3′UTR of *Musashi2* actually up-regulated luciferase expression, while miR-410 down-regulated luciferase expression from *Elavl4*, *Sox1*, *Tcf4*, *Fgf7*, and *Smad7* (Fig. 4.A).

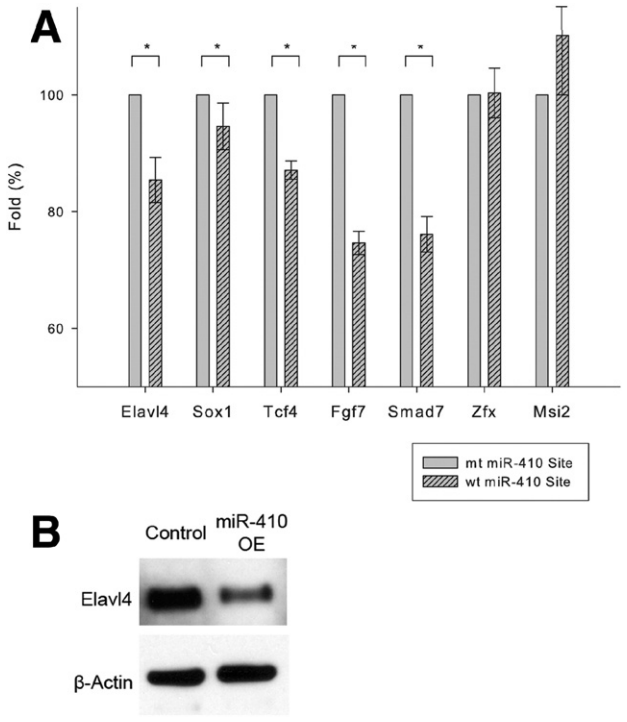

Fig. 4. Validation of miR-410 candidate targets. A. Computer predicted miR-410 candidate targets were examined using luciferase reporter assays. The 3′UTR of the candidate genes was cloned and inserted after a luciferase reporter. The predicted seed sequence region in the 3′UTR was mutated to create the mutant control (mt miR-410). Among the genes tested, *Elavl4*, *Sox1*, *Tcf4*, *Fgf7*, and *Smad7* were verified as targets of miR-410, while *Zfx* and *Msi2* were not. Values are expressed as mean ± SD, *: $p \le 0.01$. B. Neurospheres over-expressing miR-410 or a control vector were lysed and the whole cell lysate was blotted against Elavl4. Elavl4 protein expression was down-regulated approximately 45% compared to the control, supporting Elavl4 as a downstream target of miR-410.

3.6. miR-410 controls neuronal differentiation via Elavl4

Given its striking role in regulating neurogenesis (Kasashima et al., 1999; Akamatsu et al., 2005; Mansfield and Keene, 2012), and our luciferase results, we next determined if Elavl4 was functionally targeted by miR-410 in neurosphere assays. In western blot, Elavl4 protein levels were decreased approximately 45% in neurospheres over-expressing miR-410 compared to control neurospheres (Fig. 4.B). To determine if miR-410 affects NSC lineage decision by regulating Elavl4 expression, we co-transfected neurospheres with the miR-410 expression vectors and Elavl4 vectors which contain the Elavl4 coding sequence without the wildtype 3′UTR, the Elavl4 coding sequence and full-length 3′UTR, or Elavl4 with a truncated 800 bp 3′UTR containing the miR-410 binding site used in the luciferase assays. Transfected neurospheres were then differentiated as described above and stained to identify βIII-tubulin + neurons (Fig. 5.A). Neurospheres transfected with control vectors generated 26.1 ± 1.4% neurons (Fig. 5.B). Neurospheres transfected with Elavl4 with either the full-length 3′UTR (24.3 ± 1.7%) or with the truncated 3′UTR (25.7 ± 1.6%) showed no significant difference in neuronal differentiation. However, over-expression of Elavl4 without the 3′UTR alone drastically increased the percentage of neurons to 38.7 ± 2.0%. This suggests that the pro-neuronal differentiation function of Elavl4 may be tightly regulated by miRNAs, and particularly by miR-410, as the presence of the truncated 3′UTR containing the miR-410 binding site alone was sufficient to abrogate its protein activity. Over-expression of miR-410 decreased the percentage of neurons to 16.5 ± 1.9% ($p \le 5.1 \times 10^{-3}$). Over-expression of both miR-410 and Elavl4 without the 3′UTR restored neuronal differentiation to near control levels (28.8 ± 1.2%; $p \le 0.1$) (Fig. 5.B). These data suggest that miR-410 inhibits neuronal fate by down-regulating the expression of a crucial neuronal gene *Elavl4*, as over-expression of a miR-410-resistant Elavl4 rescued the reduction in neuronal fate produced by miR-410. However, as co-expression of resistant Elavl4 and miR-410 did not lead to the same level of neuronal differentiation as Elavl4 alone, miR-410 likely asserts its neuronal-inhibition through additional targets.

3.7. miR-410 alters neuronal morphology

Neurons differentiated from adult neurospheres typically have multiple long, highly branched cell processes (Fig. 6.C), while neurons exposed to miR-410 often had very short, unbranched processes. We

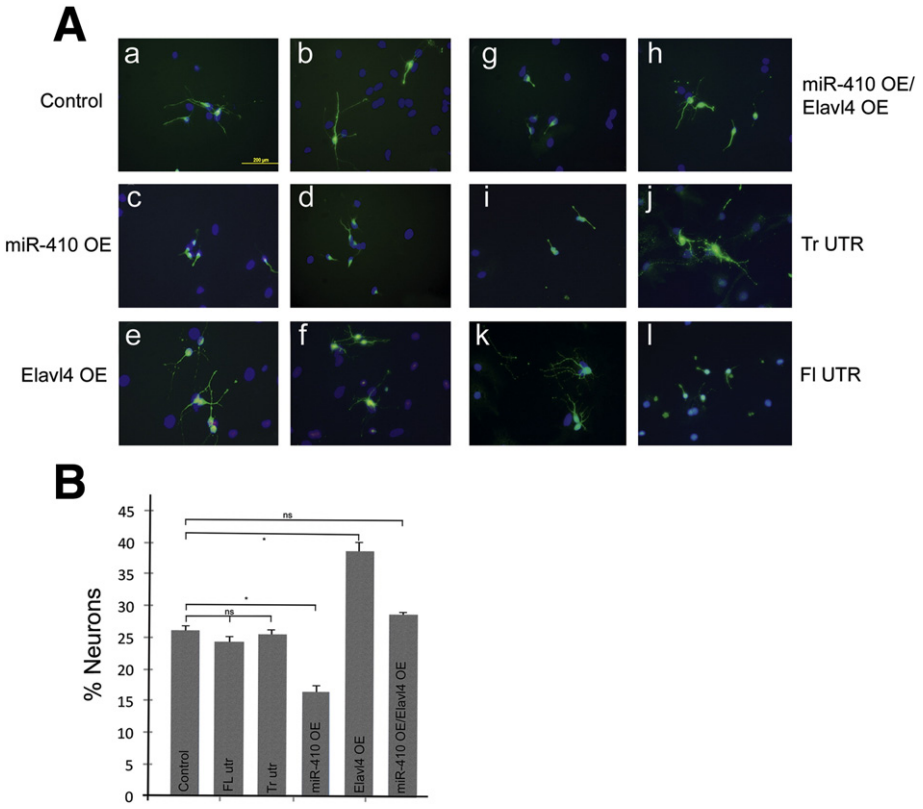

Fig. 5. Elavl4 rescues the alterations in neuronal differentiation caused by miR-410 over-expression. A. Neuronal differentiation in control neurospheres (a, b), following over-expression of miR-410 (c, d), of Elavl4 (e, f), of miR-410 and Elavl4 (g, h), expression of the truncated 3′UTR (i, j) or of the full length 3′UTR (k, l). Neurospheres were transfected with expression vectors, then after 7 days, they were dissociated and plated for differentiation, then after an additional seven days in culture the cells were fixed and localization of β-III tubulin/Tuj1 (neurons) and DNA (Hoechst) carried out and positive cells counted. B. Neurospheres transfected with Elavl4 and either the full-length 3′UTR (FL utr; 24.3 ± 1.7%) or with a truncated 3′UTR (Tr utr; 25.7 ± 1.6%) showed no significant difference in neuronal differentiation compared to neurospheres transfected with empty control vectors (Control; 26.1 ± 1.4%). However, over-expression of Elavl4 without the 3′UTR alone (Elavl4 OE) increased the percentage of neurons to 38.7 ± 2.0%. This suggests that the pro-neuronal differentiation function of Elavl4 is tightly regulated by miRNAs, presumably by miR-410 dominantly, as the presence of the truncated 3′UTR containing the miR-410 binding site alone was sufficient to abrogate its protein activity. Over-expression of miR-410 (miR-410 OE) decreased the percentage of neurons to 16.5 ± 1.9% ($p \leq 5.1 \times 10^{-3}$). Over-expression of Elavl4 without its 3′UTR in the presence of miR-410 restored neuronal differentiation to near control levels (miR-410 OE/Elavl4 OE; 28.8 ± 1.2%; $p \leq 0.1$). These data suggest that miR-410 inhibits neuronal fate by down-regulating expression of a crucial neuronal determination factor *Elavl4*, as over-expression of a miR-410-resistant Elavl4 without the 3′UTR rescued the reduction in neuronal differentiation produced by miR-410. However as co-expression of resistant Elavl4 and miR-410 did not produce the same level of neuronal differentiation as Elavl4 alone, miR-410 likely asserts its neuronal inhibition through additional targets.

employed Scholl analysis to quantify the length and the branching complexity of the neurites from each treatment, with mean neurite crossings per ring plotted against the distance from the soma to the ring (Fig. 6.A). Processes from the miR-410 over-expression group were short and largely unbranched, while those of Elavl4 over-expressing cells were the longest and most complex, followed by control neurons and those that expressed both Elavl4 and miR-410. The mean longest processes in each group were: Elavl4 OE: 8 ± 0.4 Scholl crossings, control: 6.56 ± 0.33 crossings, Elavl4 OE + MiR-410 OE: 5.25 ± 0.41 crossings and miR-410 OE: 3.67 ± 0.26 crossings. Our data confirm previous reports that Elavl4 promotes neurite outgrowth and branching (Abdelmohsen et al., 2010). In contrast, miR-410 over-expression severely reduced neurite outgrowth, while over-expression of miR-410 resistant Elavl4 slightly rescued the neurite phenotype. This suggests that the neurite outgrowth inhibitory property of miR-410 is likely asserted through Elavl4 and other downstream targets.

4. Discussion

We have identified and begun to characterize a novel miRNA, miR-410, which on inhibition of BMP signaling by Noggin, is down-regulated in the adult SVZ. Over-expression of miR-410 in SVZ neurospheres inhibited neuronal differentiation and increased the number of astrocytes produced. Loss of function of miR-410 had the opposite effect – promoting neuronal differentiation at the expense of astrocyte formation, while co-expression of miR-410 with Noggin reduced the increase in neuronal differentiation caused by Noggin to control levels, suggesting that miR-410 functions downstream of BMP signaling. To understand the mechanisms underlying these effects, we tested multiple candidate targets of miR-410: *Elavl4*, *Sox1*, *Smad7*, *Tcf4*, and *Fgf7*, which were down-regulated by miR-410. In fact, co-expression of miR-410 resistant *Elavl4* also reversed the decrease in neuronal differentiation caused by miR-410, suggesting Elavl4 acts downstream of miR-410. We also observed that while over-expression of Elavl4 without the 3′UTR drastically increased neuronal differentiation, over-expression of Elavl4 with either the full-length or truncated 3′UTR containing the miR-410 site did not alter neuronal differentiation. This suggests that endogenous miR-410, and other miRNAs that target the short truncated 3′UTR are sufficient to abrogate the pro-neuronal differentiation function of Elavl4. Surprisingly, we also observed that over-expression of miR-410 had an impact on neuronal morphology, with miR-410 over-expressing neurons characterized by processes that were short and less branched–which may be a product of Elavl4's ability to regulate neurite formation (Abdelmohsen et al., 2010). Interestingly, one downstream effector of the miR-379-410 cluster is Pumilio2 (Fiore et al., 2009), an important regulator of dendritogenesis, so that miR-410 could also affect Pumilio2 proteins to regulate process outgrowth.

Although the functions of miRNAs in neurogenesis have been widely studied (De Pietri Tonelli et al., 2008; Cheng et al., 2009; Zhao et al.,

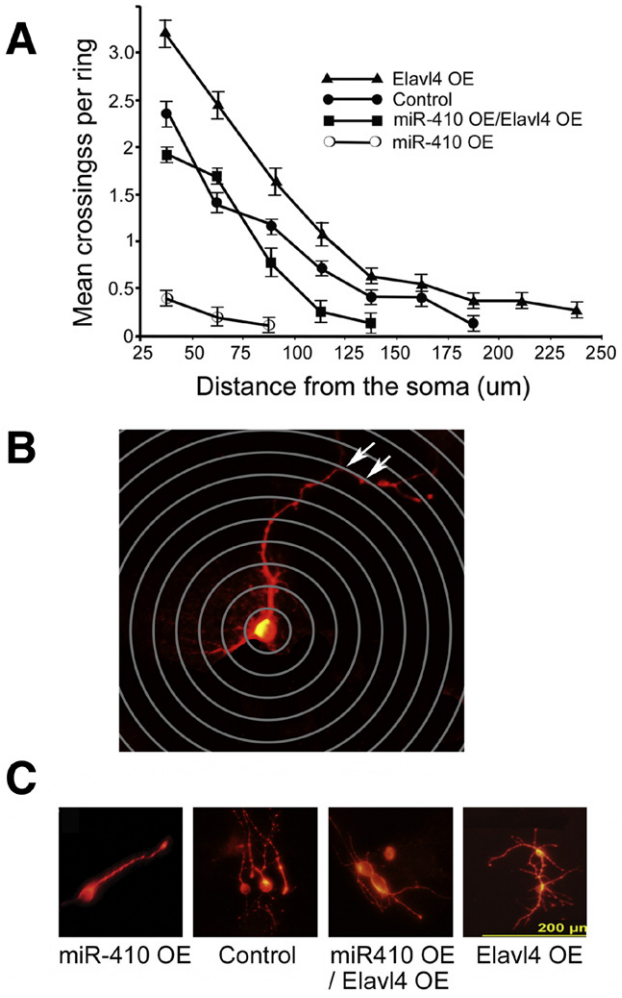

Fig. 6. Scholl analysis. A. To examine neurite branching complexity, mean total neurite crossings per ring was plotted against the distance from the soma to the ring. *Elavl4* over-expressing neurons exhibited the greatest neurite length/complexity compared to other groups. miR-410 over-expression severely impeded neurite outgrowth, while over-expression of miR-410 resistant Elavl4 largely rescued the neurite phenotype. B. Scholl analysis. A 25 μm inter-ring distance grid was centered over the soma and crossings counted. If a neurite branched on a grid (arrows) it was scored as two crossings. C. Representative images of neurons from the treatment groups.

2009; Szulwach et al., 2010), there is little information regarding crosstalk between exogenous growth factors and miRNA expression or function (Terao et al., 2011; Wang et al., 2012; Kao et al., 2013). Here we demonstrate that the BMP signaling pathway plays a role in NSC lineage differentiation by regulating miR-410 and its target genes. miR-410 is encoded in the miRNA containing gene *Mirg*, a member of the maternally imprinted *Dlk2-Gtl2* gene cluster, which is enriched in the brain (Tierling et al., 2006; Han et al., 2012). We have identified multiple BMP/Smad responsive elements in the enhancer region of the *Mirg* gene. Interestingly, expression of two other miRNAs from the same cluster, miR-382 and miR-377, are regulated by TGF-β signaling (Lan and Chung, 2011; Milosevic et al., 2012), suggesting that Smad signaling may play a crucial role in the regulation of the entire miRNA cluster. In addition, Smad 1 and 5 have been reported to bind the pri-miR-21 hairpin and promote miR-21 biogenesis (Davis et al., 2008; Blahna and Hata, 2012). BMP signaling and downstream Smad proteins may similarly regulate the expression of miR-410 and it will be of interest to determine if BMP-specific Smad 1/5/8 signaling directly regulates the expression of *Mirg* and miR-410.

While the astrocyte is predominant fate of adult SVZ neurospheres (Li et al., 2010), few miRNAs have been shown to regulate astrocyte differentiation (Zhang et al., 2013), unlike the many miRNAs that have been reported to regulate neuronal cell fate (Cheng et al., 2009; Aranha et al., 2011). BMP signaling also plays an important role in oligodendrocyte differentiation, as interfering with Smad signal transduction (Colak et al., 2008) or application of another BMP inhibitor, Chordin, can redirect neuronal precursors to an oligodendrocyte fate (Jablonska et al., 2010). We have observed that miR-410 over-expression leads to a reduction in OPC numbers; however, miR-410 loss of function did not significantly affect OPC differentiation. This suggests that miR-410 may inhibit oligodendrocyte lineage commitment through a second mechanism, and/or there are additional controls on OPC differentiation.

miR-410 expression was strikingly down-regulated in the adult SVZ following Noggin over-expression in our inducible transgenic mice (Morell et al., 2015). Noggin was initially characterized by its ability to rescue the phenotype of UV light-dorsalized embryos (Smith and Harland, 1992; Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995), and inactivation of BMP signaling by small molecule inhibitors such as SB-431542 and Dorsomorphin is widely used to generate neural progenitor cells from ESCs and hiPSC (Vogt et al., 2011; Mak et al., 2012). In the adult SVZ, Noggin is expressed by ependymal cells where it has been suggested to act as a "brake" on neurogenesis (Lim et al., 2000; Colak et al., 2008), and to control lineage progression in the developing and adult SVZ (Bonaguidi et al., 2008), following injury (Cate et al., 2010), and in aging (Bonaguidi et al., 2008). One mechanism by which Noggin regulates lineage differentiation may be via its ability to control miR-410 expression, and thereby neurogenic genes.

Elavl4/*HuD* is a member of the Elav-like RNA binding protein family. *Elav*, or embryonic lethal abnormal vision, was originally identified in *Drosophila* (Campos et al., 1985). Of the four mammalian Elav homologs, *Elavl1*, or *HuA*/*R*, is ubiquitously expressed and has been proposed to play a role in mRNA stability (Brennan and Steitz, 2001). The other three family members, *Elavl2* (*HuB*/*Hel-N1*), *Elavl3* (*HuC*), and *Elavl4* (*HuD*) are restricted in their expression to neurons (Okano and Darnell, 1997); we show that *Elavl4* is expressed in NSC isolated from adult SVZ (Fig. 4.B). Neuron-specific Elav-like proteins recognize and bind GU and AU rich sequences in the 3′UTR and intronic regions of target mRNA transcripts (Ince-Dunn et al., 2012). These proteins are also crucial in the regulation of mRNA stability and alternative splicing. AU-rich sequences are common in the 3′UTR; 10% of total cellular mRNAs are estimated to have AU-rich elements in the 3′UTR (Halees et al., 2008). *Elavl4* has previously been reported to stabilize several genes crucial in proliferation and neuronal differentiation including: *p21* (Joseph et al., 1998), *N-Myc* (Manohar et al., 2002), *Musashi1* (Ratti et al., 2006), as well as *NGF*, *Neurotrophin 3*, and *BDNF* (Lim and Alkon, 2012). Several groups have employed CLIP/microarray approaches to identify other downstream targets of *Elavl4* (Bolognani et al., 2010; Perrone-Bizzozero et al., 2011; Ince-Dunn et al., 2012). Interestingly, in addition to transcripts involved in neural development, *Elavl4* regulates a wide range of genes that control RNA processing, cell signaling, vesicle transport and neurotransmitter biosynthesis, suggesting that miR-410 may be involved in many other crucial cellular functions. A preliminary FACS sort of hGFAP-EGFP+ cells from uninduced SVZ indicated that miR-410 expression was highest in hGFAP+ (B cells) (data not shown); understanding the miR-410 expression kinetics in different cell types of the SVZ will inform in vivo approaches to expand the NSC pool and may suggest novel therapeutic strategies.

Elavl4 is not the only downstream target of miR-410. In addition to *Elavl4*, we also identified *Sox1*, *Smad7*, *Tcf4*, and *Fgf7* as targets of miR-410, and a recent report identified *PAX6* as well (Choi et al., 2015). All have been implicated in neurogenesis. We have previously reported that inhibition of *Tcf4* prevents the terminal differentiation of mESC to β-III tubulin+ neurons (Slawny and O'Shea, 2011), suggesting miR-410 may also control NSC lineage decisions through the Wnt signaling pathway. The fibroblast growth factor family has long been known to regulate adult neurogenesis (Gage et al., 1995; Palmer et al., 1995; Tao

et al., 1997; Jin et al., 2003). Although *Fgf7*-null mice are viable, *Fgf7* is essential for proper inhibitory synapse formation in hippocampal CA3 neurons (Terauchi et al., 2010), and *Fgf7*-null animals exhibit increased dentate neurogenesis (Lee et al., 2012), but its function in the adult SVZ is not known. Smad7 is one of the two inhibitory Smad proteins (Massague et al., 2005); NSC isolated from adult *Smad7*-null animals exhibited decreased neuronal differentiation (Krampert et al., 2010), consistent with our results. *Sox1*, a member of the SoxB1 family of HMG-box DNA binding proteins, is one of the earliest markers of the neural ectoderm (Pevny et al., 1998). NPCs isolated from *Sox1*-null mice form neurospheres, but are deficient in neuronal differentiation (Kan et al., 2007), while over-expression of *Sox1* in E17 neurospheres promotes neuronal differentiation (Kan et al., 2004). These data suggest that miR-410 may also control NSC neuronal fate through the regulation of *Sox1* or other critical transcripts. The detailed interaction of these factors with miR-410 will require further clarification.

Thus, it appears that in the adult SVZ, in the absence or low levels of Noggin, BMP proteins expressed by astrocytes and NSC bind and activate BMPR, presumably BMPR-1A, activating its downstream SMAD proteins. Activated SMADs up-regulate the expression of the miRNA coding gene *Mirg* which encodes miR-410. Higher levels of miR-410 then bind the transcripts of its targets: *Elavl4*, *Sox1*, *Tcf4*, *Fgf7* and *Smad7* decreasing their expression levels. Attenuated expression of *Elavl4*, and likely *Sox1*, then inhibit neuronal lineage differentiation. Down-regulation of *Smad7* may further enhance Smad signal transduction, forming a positive feedback loop and strengthening lineage choice. Noggin, produced by the ependymal cells in the SVZ, binds BMP proteins, inhibiting downstream signaling, and reducing the expression of miR-410. The reduction in miR-410 may de-repress expression of its target genes, increases levels of Elavl4, which promotes neuronal differentiation of NSC. At the same time higher levels of Smad7 may further tune down BMP signaling through a positive feedback loop enhancing/reinforcing the neuronal fate decision.

Our results (this work and our previous report (Morell et al., 2015)) support a model in which the majority of SVZ-derived oligodendrocytes form from cells at the transition between activated B cells and C cells themselves (Parras et al., 2004; Menn et al., 2006). BMP signaling inhibits oligodendroglial differentiation, while antagonizing BMP signaling may recruit cells from the more committed Dlx2-positive C/A cell population (Colak et al., 2008) to the oligodendroglial lineage. However, in our previous report, over-expression of Noggin in the SVZ increased the numbers of both Mash1+ C cells and Olig2+ OPCs while the number of Dlx2/Oli2 double positive cells remained similar. Thus, it is unlikely the C cells are the major source of the OPCs. miR-410 acts directly downstream of BMP signaling to inhibit neurogenesis and oligodendrogenesis, although the molecular mechanisms controlling oligodendrogenesis requires further clarification.

miR-410 has recently been suggested to be a prognostic marker in neuroblastoma (Gattolliat et al., 2011); patients with high miR-410 expression levels having higher survival rates. miR-410 also targets the hepatocyte growth factor receptor MET to regulate proliferation and invasion of glioma cells (Chen et al., 2012). We have observed that primary human glioblastoma cells express low levels of miR-410 (unpublished data), suggesting that miR-410, in addition to regulating lineage commitment, may also control proliferation and cancer stem cell behavior. However opposite functions have been reported in non-small cell lung cancer, as miR-410 appears to promote cell proliferation by targeting BRD7 in these cells (Li et al., 2015). Further clarification of miR-410 functions in different tissues and cells is of great importance and crucial to shed light on the multiple functions of miR-410. Overall, miR-410 may provide a new mechanism involved in the essential choice by NSC between self-renewal and differentiation. Further elucidation of miR-410 function may also identify novel approaches to the treatment of CNS injury and cancers.

Acknowledgements

The authors gratefully acknowledge: Helen Zhang, Jack Parent, Dan Goldman and Roman Giger for advice and reagents, Martin Engelke for help with programming, Einor Jacobsen for Scholl analysis assistance, Ben Allen, Roger Tsien, and David Turner for plasmids. The work was supported by NIH grant NS-048187.

References

Abdelmohsen, K., Hutchison, E.R., Lee, E.K., Kuwano, Y., Kim, M.M., Masuda, K., Srikantan, S., Subaran, S.S., Marasa, B.S., Mattson, M.P., Gorospe, M., 2010. miR-375 inhibits differentiation of neurites by lowering HuD levels. Mol. Cell. Biol. 30, 4197–4210.
Akamatsu, W., Fujihara, H., Mitsuhashi, T., Yano, M., Shibata, S., Hayakawa, Y., Okano, H.J., Sakakibara, S., Takano, H., Takano, T., Takahashi, T., Noda, T., Okano, H., 2005. The RNA-binding protein HuD regulates neuronal cell identity and maturation. Proc. Natl. Acad. Sci. U. S. A. 102, 4625–4630.
Aranha, M.M., Santos, D.M., Sola, S., Steer, C.J., Rodrigues, C.M., 2011. miR-34a regulates mouse neural stem cell differentiation. PLoS One 6, e21396.
Barde, I., Salmon, P., Trono, D., 2010. Production and titration of lentiviral vectors. Curr. Protoc. Neurosci. 21 (Chapter 4:Unit 4).
Bian, S., Sun, T., 2011. Functions of noncoding RNAs in neural development and neurological diseases. Mol. Neurobiol. 44, 359–373.
Bian, S., Hong, J., Li, Q., Schebelle, L., Pollock, A., Knauss, J.L., Garg, V., Sun, T., 2013a. MicroRNA cluster miR-17-92 regulates neural stem cell expansion and transition to intermediate progenitors in the developing mouse neocortex. Cell Rep. 3, 1398–1406.
Bian, S., Xu, T.L., Sun, T., 2013b. Tuning the cell fate of neurons and glia by microRNAs. Curr. Opin. Neurobiol. 23, 928–934.
Blahna, M.T., Hata, A., 2012. Smad-mediated regulation of microRNA biosynthesis. FEBS Lett. 586, 1906–1912.
Bolognani, F., Contente-Cuomo, T., Perrone-Bizzozero, N.I., 2010. Novel recognition motifs and biological functions of the RNA-binding protein HuD revealed by genome-wide identification of its targets. Nucleic Acids Res. 38, 117–130.
Bonaguidi, M.A., Peng, C.Y., McGuire, T., Falciglia, G., Gobeske, K.T., Czeisler, C., Kessler, J.A., 2008. Noggin expands neural stem cells in the adult hippocampus. J. Neurosci. 28, 9194–9204.
Brennan, C.M., Steitz, J.A., 2001. HuR and mRNA stability. Cell. Mol. Life Sci. 58, 266–277.
Brett, J.O., Renault, V.M., Rafalski, V.A., Webb, A.E., Brunet, A., 2011. The microRNA cluster miR-106b~25 regulates adult neural stem/progenitor cell proliferation and neuronal differentiation. Aging (Albany NY) 3, 108–124.
Brunette, K.E., Tran, P.V., Wobken, J.D., Carlson, E.S., Gorgieff, M.K., 2010. Gestational and neonatal iron deficiency alters apical dendrite structure of CA1 pyramidal neurons in adult rat hippocampus. Dev. Neurosci. 32, 238–248.
Campos, A.R., Grossman, D., White, K., 1985. Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects. A developmental-genetic analysis. J. Neurogenet. 2, 197–218.
Cate, H.S., Sabo, J.K., Merlo, D., Kemper, D., Aumann, T.D., Robinson, J., Merson, T.D., Emery, B., Perreau, V.M., Kilpatrick, T.J., 2010. Modulation of bone morphogenic protein signalling alters numbers of astrocytes and oligodendroglia in the subventricular zone during cuprizone-induced demyelination. J. Neurochem. 115, 11–22.
Chen, L., Zhang, J., Feng, Y., Li, R., Sun, X., Du, W., Piao, X., Wang, H., Yang, D., Sun, Y., Li, X., Jiang, T., Kang, C., Li, Y., Jiang, C., 2012. MiR-410 regulates MET to influence the proliferation and invasion of glioma. Int. J. Biochem. Cell Biol. 44, 1711–1717.
Cheng, L.C., Pastrana, E., Tavazoie, M., Doetsch, F., 2009. miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. Nat. Neurosci. 12, 399–408.
Choi, S.W., Kim, J.J., Seo, M.S., Park, S.B., Kang, T.W., Lee, J.Y., Lee, B.C., Kang, I., Shin, T.H., Kim, H.S., Yu, K.R., Kang, K.S., 2015. miR-410 inhibition induces RPE differentiation of amniotic epithelial stem cells via overexpression of OTX2 and RPE65. Stem Cell Rev. Rep. 11, 376–386.
Colak, D., Mori, T., Brill, M.S., Pfeifer, A., Falk, S., Deng, C., Monteiro, R., Mummery, C., Sommer, L., Gotz, M., 2008. Adult neurogenesis requires Smad4-mediated bone morphogenic protein signaling in stem cells. J. Neurosci. 28, 434–446.
Davis, B.N., Hilyard, A.C., Lagna, G., Hata, A., 2008. SMAD proteins control DROSHA-mediated microRNA maturation. Nature 454, 56–61.
De Pietri Tonelli, D., Pulvers, J.N., Haffner, C., Murchison, E.P., Hannon, G.J., Huttner, W.B., 2008. miRNAs are essential for survival and differentiation of newborn neurons but not for expansion of neural progenitors during early neurogenesis in the mouse embryonic neocortex. Development 135, 3911–3921.
Di Leva, G., Croce, C.M., 2013. miRNA profiling of cancer. Curr. Opin. Genet. Dev. 23, 3–11.
Eacker, S.M., Dawson, T.M., Dawson, V.L., 2009. Understanding microRNAs in neurodegeneration. Nat. Rev. Neurosci. 10, 837–841.
Ebert, M.S., Neilson, J.R., Sharp, P.A., 2007. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat. Methods 4, 721–726.
Fiore, R., Khudayberdiev, S., Christensen, M., Siegel, G., Flavell, S.W., Kim, T.K., Greenberg, M.E., Schratt, G., 2009. Mef2-mediated transcription of the miR379-410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels. EMBO J. 28, 697–710.
Gage, F.H., Coates, P.W., Palmer, T.D., Kuhn, H.G., Fisher, L.J., Suhonen, J.O., Peterson, D.A., Suhr, S.T., Ray, J., 1995. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc. Natl. Acad. Sci. U. S. A. 92, 11879–11883.
Gangaraju, V.K., Lin, H., 2009. MicroRNAs: key regulators of stem cells. Nat. Rev. Mol. Cell Biol. 10, 116–125.

Gattolliat, C.H., Thomas, L., Ciafre, S.A., Meurice, G., Le Teuff, G., Job, B., Richon, C., Combaret, V., Dessen, P., Valteau-Couanet, D., May, E., Busson, P., Douc-Rasy, S., Benard, J., 2011. Expression of miR-487b and miR-410 encoded by 14q32.31 locus is a prognostic marker in neuroblastoma. Br. J. Cancer 105, 1352–1361.
Halees, A.S., El-Badrawi, R., Khabar, K.S., 2008. ARED Organism: expansion of ARED reveals AU-rich element cluster variations between human and mouse. Nucleic Acids Res. 36, D137–D140.
Han, Z., He, H., Zhang, F., Huang, Z., Liu, Z., Jiang, H., Wu, Q., 2012. Spatiotemporal expression pattern of Mirg, an imprinted non-coding gene, during mouse embryogenesis. J. Mol. Histol. 43, 1–8.
He, M., Liu, Y., Wang, X., Zhang, M.Q., Hannon, G.J., Huang, Z.J., 2012. Cell-type-based analysis of microRNA profiles in the mouse brain. Neuron 73, 35–48.
Hemmati-Brivanlou, A., Kelly, O.G., Melton, D.A., 1994. Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell 77, 283–295.
Ince-Dunn, G., Okano, H.J., Jensen, K.B., Park, W.Y., Zhong, R., Ule, J., Mele, A., Fak, J.J., Yang, C., Zhang, C., Yoo, J., Herre, M., Okano, H., Noebels, J.L., Darnell, R.B., 2012. Neuronal Elav-like (Hu) proteins regulate RNA splicing and abundance to control glutamate levels and neuronal excitability. Neuron 75, 1067–1080.
Jablonska, B., Aguirre, A., Raymond, M., Szabo, G., Kitabatake, Y., Sailor, K.A., Ming, G.L., Song, H., Gallo, V., 2010. Chordin-induced lineage plasticity of adult SVZ neuroblasts after demyelination. Nat. Neurosci. 13, 541–550.
Ji, F., Lv, X., Jiao, J., 2013. The role of microRNAs in neural stem cells and neurogenesis. J. Genet. Genomics 40, 61–66.
Jin, K., Sun, Y., Xie, L., Batteur, S., Mao, X.O., Smelick, C., Logvinova, A., Greenberg, D.A., 2003. Neurogenesis and aging: FGF-2 and HB-EGF restore neurogenesis in hippocampus and subventricular zone of aged mice. Aging Cell 2, 175–183.
Joseph, B., Orlian, M., Furneaux, H., 1998. p21(waf1) mRNA contains a conserved element in its 3′-untranslated region that is bound by the Elav-like mRNA-stabilizing proteins. J. Biol. Chem. 273, 20511–20516.
Jovicic, A., Roshan, R., Moisoi, N., Pradervand, S., Moser, R., Pillai, B., Luthi-Carter, R., 2013. Comprehensive expression analyses of neural cell-type-specific miRNAs identify new determinants of the specification and maintenance of neuronal phenotypes. J. Neurosci. 33, 5127–5137.
Kan, L., Israsena, N., Zhang, Z., Hu, M., Zhao, L.R., Jalali, A., Sahni, V., Kessler, J.A., 2004. Sox1 acts through multiple independent pathways to promote neurogenesis. Dev. Biol. 269, 580–594.
Kan, L., Jalali, A., Zhao, L.R., Zhou, X., McGuire, T., Kazanis, I., Episkopou, V., Bassuk, A.G., Kessler, J.A., 2007. Dual function of Sox1 in telencephalic progenitor cells. Dev. Biol. 310, 85–98.
Kao, C.J., Martiniez, A., Shi, X.B., Yang, J., Evans, C.P., Dobi, A., de Vere White, R.W., Kung, H.J., 2013. miR-30 as a tumor suppressor connects EGF/Src signal to ERG and EMT. Oncogene 33, 2495–2503.
Kasashima, K., Terashima, K., Yamamoto, K., Sakashita, E., Sakamoto, H., 1999. Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation. Genes Cells 4, 667–683.
Krampert, M., Chirasani, S.R., Wachs, F.P., Aigner, R., Bogdahn, U., Yingling, J.M., Heldin, C.H., Aigner, L., Heuchel, R., 2010. Smad7 regulates the adult neural stem/progenitor cell pool in a transforming growth factor beta- and bone morphogenetic protein-independent manner. Mol. Cell. Biol. 30, 3685–3694.
Lamb, T.M., Knecht, A.K., Smith, W.C., Stachel, S.E., Economides, A.N., Stahl, N., Yancopolous, G.D., Harland, R.M., 1993. Neural induction by the secreted polypeptide noggin. Science 262, 713–718.
Lan, H.Y., Chung, A.C., 2011. Transforming growth factor-beta and Smads. Contrib. Nephrol. 170, 75–82.
Lang, M.F., Shi, Y., 2012. Dynamic Roles of microRNAs in Neurogenesis. Front. Neurosci. 6, 71.
Lee, C.H., Javed, D., Althaus, A.L., Parent, J.M., Umemori, H., 2012. Neurogenesis is enhanced and mossy fiber sprouting arises in FGF7-deficient mice during development. Mol. Cell. Neurosci. 51, 61–67.
Li, L., Harms, K.M., Ventura, P.B., Lagace, D.C., Eisch, A.J., Cunningham, L.A., 2010. Focal cerebral ischemia induces a multilineage cytogenic response from adult subventricular zone that is predominantly gliogenic. Glia 58, 1610–1619.
Li, D., Yang, Y., Zhu, G., Liu, X., Zhao, M., Li, X., Yang, Q., 2015. MicroRNA-410 promotes cell proliferation by targeting BRD7 in non-small cell lung cancer. FEBS Lett. 589, 2218–2223.
Lim, C.S., Alkon, D.L., 2012. Protein kinase C stimulates HuD-mediated mRNA stability and protein expression of neurotrophic factors and enhances dendritic maturation of hippocampal neurons in culture. Hippocampus 22, 2303–2319.
Lim, D.A., Tramontin, A.D., Trevejo, J.M., Herrera, D.G., Garcia-Verdugo, J.M., Alvarez-Buylla, A., 2000. Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. Neuron 28, 713–726.
Mak, S.K., Huang, Y.A., Iranmanesh, S., Vangipuram, M., Sundararajan, R., Nguyen, L., Langston, J.W., Schule, B., 2012. Small molecules greatly improve conversion of human-induced pluripotent stem cells to the neuronal lineage. Stem Cells Int. 2012, 140427.
Manohar, C.F., Short, M.L., Nguyen, A., Nguyen, N.N., Chagnovich, D., Yang, Q., Cohn, S.L., 2002. HuD, a neuronal-specific RNA-binding protein, increases the in vivo stability of MYCN RNA. J. Biol. Chem. 277, 1967–1973.
Mansfield, K.D., Keene, J.D., 2012. Neuron-specific ELAV/Hu proteins suppress HuR mRNA during neuronal differentiation by alternative polyadenylation. Nucleic Acids Res. 40, 2734–2746.
Massague, J., Seoane, J., Wotton, D., 2005. Smad transcription factors. Genes Dev. 19, 2783–2810.
Menn, B., Garcia-Verdugo, J.M., Yaschine, C., Gonzalez-Perez, O., Rowitch, D., Alvarez-Buylla, A., 2006. Origin of oligodendrocytes in the subventricular zone of the adult brain. J. Neurosci. 26, 7907–7918.
Milosevic, J., Pandit, K., Magister, M., Rabinovich, E., Ellwanger, D.C., Yu, G., Vuga, L.J., Weksler, B., Benos, P.V., Gibson, K.F., McMillan, M., Kahn, M., Kaminski, N., 2012. Profibrotic role of miR-154 in pulmonary fibrosis. Am. J. Respir. Cell Mol. Biol. 47, 879–887.
Ming, G.L., Song, H., 2011. Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70, 687–702.
Morell, M., Tsan, Y.C., O'Shea, K.S., 2015. Inducible expression of noggin selectively expands neural progenitors in the adult SVZ. Stem Cell Res. 14, 79–94.
Morikawa, M., Koinuma, D., Tsutsumi, S., Vasilaki, E., Kanki, Y., Heldin, C.H., Aburatani, H., Miyazono, K., 2011. ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif. Nucleic Acids Res. 39, 8712–8727.
Okano, H.J., Darnell, R.B., 1997. A hierarchy of Hu RNA binding proteins in developing and adult neurons. J. Neurosci. 17, 3024–3037.
Otsu, N., 1979. A threshold selection method from gray level histograms. IEEE Transactions on Systems, Man and Cybernetics 9, 5.
Palmer, T.D., Ray, J., Gage, F.H., 1995. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol. Cell. Neurosci. 6, 474–486.
Parras, C.M., Galli, R., Britz, O., Soares, S., Galichet, C., Battiste, J., Johnson, J.E., Nakafuku, M., Vescovi, A., Guillemot, F., 2004. Mash1 specifies neurons and oligodendrocytes in the postnatal brain. EMBO J. 23, 4495–4505.
Perrone-Bizzozero, N.I., Tanner, D.C., Mounce, J., Bolognani, F., 2011. Increased expression of axogenesis-related genes and mossy fibre length in dentate granule cells from adult HuD overexpressor mice. ASN Neuro. 3, 259–270.
Pers, Y.M., Jorgensen, C., 2013. MicroRNA in 2012: biotherapeutic potential of microRNAs in rheumatic diseases. Nat. Rev. Rheumatol. 9, 76–78.
Pevny, L.H., Sockanathan, S., Placzek, M., Lovell-Badge, R., 1998. A role for SOX1 in neural determination. Development 125, 1967–1978.
Ratti, A., Fallini, C., Cova, L., Fantozzi, R., Calzarossa, C., Zennaro, E., Pascale, A., Quattrone, A., Silani, V., 2006. A role for the ELAV RNA-binding proteins in neural stem cells: stabilization of Msi1 mRNA. J. Cell Sci. 119, 1442–1452.
Sambrook J, Fritsch EF, Maniatis T, Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Pr, ed. 2, 1989.
Sasai, Y., Lu, B., Steinbeisser, H., De Robertis, E.M., 1995. Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. Nature 376, 333–336.
Slawny, N.A., O'Shea, K.S., 2011. Dynamic changes in Wnt signaling are required for neuronal differentiation of mouse embryonic stem cells. Mol. Cell. Neurosci. 48, 205–216.
Smith, W.C., Harland, R.M., 1992. Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. Cell 70, 829–840.
Szulwach, K.E., Li, X., Smrt, R.D., Li, Y., Luo, Y., Lin, L., Santistevan, N.J., Li, W., Zhao, X., Jin, P., 2010. Cross talk between microRNA and epigenetic regulation in adult neurogenesis. J. Cell Biol. 189, 127–141.
Tao, Y., Black, I.B., DiCicco-Bloom, E., 1997. In vivo neurogenesis is inhibited by neutralizing antibodies to basic fibroblast growth factor. J. Neurobiol. 33, 289–296.
Terao, M., Fratelli, M., Kurosaki, M., Zanetti, A., Guarnaccia, V., Paroni, G., Tsykin, A., Lupi, M., Gianni, M., Goodall, G.J., Garattini, E., 2011. Induction of miR-21 by retinoic acid in estrogen receptor-positive breast carcinoma cells: biological correlates and molecular targets. J. Biol. Chem. 286, 4027–4042.
Terauchi, A., Johnson-Venkatesh, E.M., Toth, A.B., Javed, D., Sutton, M.A., Umemori, H., 2010. Distinct FGFs promote differentiation of excitatory and inhibitory synapses. Nature 465, 783–787.
Tierling, S., Dalbert, S., Schoppenhorst, S., Tsai, C.E., Oliger, S., Ferguson-Smith, A.C., Paulsen, M., Walter, J., 2006. High-resolution map and imprinting analysis of the Gtl2-Dnchc1 domain on mouse chromosome 12. Genomics 87, 225–235.
Vogt, J., Traynor, R., Sapkota, G.P., 2011. The specificities of small molecule inhibitors of the TGFss and BMP pathways. Cell. Signal. 23, 1831–1842.
Wang, D.J., Legesse-Miller, A., Johnson, E.L., Coller, H.A., 2012. Regulation of the let-7a-3 promoter by NF-kappaB. PLoS One 7, e31240.
Wheeler, G., Ntounia-Fousara, S., Granda, B., Rathjen, T., Dalmay, T., 2006. Identification of new central nervous system specific mouse microRNAs. FEBS Lett. 580, 2195–2200.
Zhang, J., Zhou, Y., Wu, Y.J., Ma, L., Wang, R.J., Huang, S.Q., Gao, R.R., Liu, L.H., Shao, Z.H., Shi, H.J., Cheng, L.M., Yu, L., 2013. Novel cerebellum-enriched miR-592 may play a role in neural progenitor cell differentiation and neuronal maturation through regulating Lrrc4c and Nfasc in rat. Curr. Mol. Med. 13, 1432–1445.
Zhao, C., Sun, G., Li, S., Shi, Y., 2009. A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. Nat. Struct. Mol. Biol. 16, 365–371.