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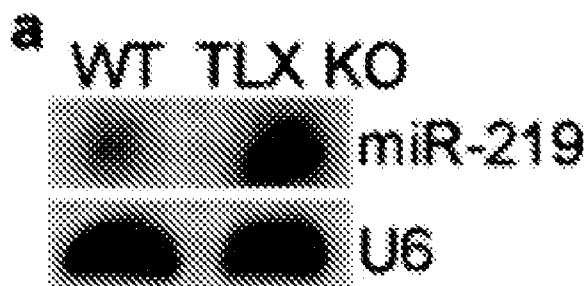
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(54) **Title:** TLX AND MIR-219 AS POTENTIAL THERAPEUTIC TARGETS FOR NEURODEVELOPMENTAL DISORDERS

**Figure 1**



(57) **Abstract:** Disclosed herein are methods of treating neurodevelopmental disorders such as schizophrenia (SCZ), bipolar disorder or depression. The methods entail inhibiting expression of miR-219 or overexpressing TLX thereby promoting proliferation of neural stem cells (NSCs) in the subjects.

## **TLX AND MIR-219 AS POTENTIAL THERAPEUTIC TARGETS FOR NEURODEVELOPMENTAL DISORDERS**

### **PRIORITY CLAIM**

**[0001]** This application claims priority to U.S. Provisional Application No. 62/306,631, filed March 10, 2016, and U.S. Provisional Application No. 62/307,367, filed March 11, 2016, both of which are incorporated by reference herein, including drawings.

### **STATEMENT OF GOVERNMENT FUNDING**

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### **BACKGROUND**

**[0003]** TLX is a nuclear receptor that plays a critical role in vertebrate brain function [1-3]. It is an essential regulator of adult neural stem cell (NSC) self-renewal [3-5] and plays an important role in enhancing learning and memory by regulating adult hippocampal neurogenesis [6,7]. It also plays a role in neurodevelopment through regulation of cell cycle progression in embryonic NSCs [4,8-10]. TLX is a well-characterized transcriptional regulator. It controls target gene expression partly by recruiting transcriptional corepressors, such as HDACs and LSD1 [11-13]. TLX represses the transcription of GFAP, p21, pten, and microRNAs miR-9 and miR-137, but activates Wnt signaling, SIRT1 and MASH1 in NSCs [3,5,11,12,14-16]. However, the function of TLX in regulating gene expression beyond transcriptional regulation has not been reported.

**[0004]** microRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through translational inhibition or RNA degradation [17]. The biogenesis of miRNAs starts from primary transcripts (pri-miRNAs), which are processed by the

nuclear RNaseIII Drosha into precursor miRNAs (pre-miRNAs) that contain hairpin loop structures. The pre-miRNAs are exported to the cytoplasm and further processed into mature miRNAs by the cytoplasmic RNaseIII Dicer. Mature miRNAs are incorporated into an RNA-induced silencing complex (RISC) to repress target mRNAs. The Drosha complex consists of Drosha, DiGeorge syndrome critical gene 8 (DGCR8), RNA helicase p68 (DDX5) and p72 (DDX17) [18-20]. Recent studies reported that SMAD, p53, and BRCA1 bind to Drosha and promote the processing activity of Drosha [21-23]. However, little is known about the physiological effect of miRNA processing.

**[0005]** miR-219 is an miRNA that is specifically expressed in the brain [24,25]. It promotes oligodendrocyte differentiation by repressing negative regulators of oligodendrocyte differentiation [26,27]. In a recent study, miR-219 was shown to promote neural precursor cell differentiation in zebrafish by inhibiting apical polarity proteins, par-3 family cell polarity regulator (PAR3) and protein kinase C iota (PRKCI) [28]. However, whether miR-219 regulates the phenotypes of neural stem/progenitor cells (collectively referred to as NSCs) in mammalian brains remains unknown.

**[0006]** Several studies analyzing miRNA levels in postmortem brains from schizophrenia (SCZ) patients reported an increase in the expression of a set of miRNAs in SCZ patients [29-31]. miR-219 is among the most highly up-regulated miRNAs in brain regions of SCZ patients [29,30,32]. However, the functional relevance of elevated miR-219 expression in SCZ brain cells remains unknown.

## SUMMARY

**[0007]** In one aspect, this disclosure relates to a method for treating a neurodevelopmental disorder in a subject. The method entails inhibiting, repressing, or down-regulating expression of miR-219, overexpressing or up-regulating the expression of TLX, or a combination thereof. In some embodiments, the expression of miR-219 is inhibited, repressed or down-regulated by administering to the subject a therapeutically effective amount of one or more antagonists of miR-219. In some embodiments, expression of TLX is up-regulated by administering to the subject a

therapeutically effective amount of one or more TLX agonists or a vector expressing a gene encoding TLX.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**[0008]** Figures 1a-1g illustrate that TLX inhibits miR-219 processing in NSCs. Figure 1a shows elevated expression of mature miR-219 in TLX KO mouse brains, compared to WT mouse brains, revealed by Northern blot analysis. U6 is included as a loading control. Figure 1b shows the levels of the two primary forms of miR-219, pri-miR-219-1 and pri-miR-219-2, exhibited minimal change in WT and TLX KO mouse brains, as analyzed by RT-PCR. Figure 1c shows the levels of pre-miR-219 and mature miR-219, but not pri-miR-219, increased significantly in TLX KO mouse brains. n=3. Figure 1d shows the levels of pre-miR-219 and mature miR-219, but not pri-miR-219, increased significantly in TLX knockdown NSCs independent of actinomycin D treatment. siC: control RNA; siTLX: TLX siRNA. n=6. Figure 1e shows a scheme for monitoring miRNA processing using a luciferase reporter. The miRNA processing activity is inversely correlated with the luciferase activity. Figure 1f shows overexpression of TLX inhibits miR-219 processing from pri-miR-219 to pre-miR-219. miR-1224-Glo was included as a negative control. The firefly luciferase activity was normalized with the Renilla luciferase internal control. The relative luciferase activity is shown. C: control vector. n=4. Figure 1g shows knockdown of TLX promotes miR-219 processing from pri-miR-219 to pre-miR-219. The relative luciferase activity in NSCs transfected with a vector expressing siC or siTLX, together with the control-Glo, miR-219-Glo reporter, or miR-1224 Glo reporter control. n=4. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 by student's t-test, and n represents experimental repeats in panels c, d, f & g. Error bars are sd of the mean for all the quantification in this study. For each representative image, the experiments were repeated three times or more.

**[0009]** Figures 2a-2f illustrate that TLX interacts with the miRNA processing machinery. Figure 2a shows a scheme for identifying TLX-interacting proteins using mass spectrometry (MS) analysis. Figure 2b shows differentially represented proteins in the HA immunoprecipitates of control HA or HA-TLX-expressing HeLa cells. Arrow indicates a protein band of 68 kD that is specifically detected in the HA

immunoprecipitates of HA-TLX-expressing HeLa cells. Figure 2c shows interaction of TLX with p68, Drosha and DGCR8. Lysates of HA-TLX transfected HEK293T cells were treated with or without DNase and RNase, then immunoprecipitated with HA antibody or IgG control. The immunoprecipitates were blotted with p68 antibody. In parallel, lysates of Flag-Drosha and HA-TLX or Flag-DGCR8 and HA-TLX co-transfected HEK293T cells were treated with or without DNase and RNase. Cell lysates were immunoprecipitated with anti-Flag antibody, then blotted with anti-HA antibody. Figure 2d shows interaction of TLX with Drosha and DGCR8 in mouse brains. Lysates of embryonic mouse brains were immunoprecipitated with TLX antibody, then blotted with anti-Drosha, anti-DGCR8 or anti-TLX antibody. Figure 2e shows a scheme for RNA immunoprecipitation. Lysates of NSCs transduced with TLX siRNA were immunoprecipitated with anti-Drosha, anti-DGCR8, or anti-TLX antibody. RNAs were extracted from the immunoprecipitates, and subjected to RT-PCR for pri-miR-219. Figure 2f shows TLX knockdown promoted the binding of Drosha and DGCR8 to pri-miR-219. Lysates of NSCs transduced with siC or siTLX were immunoprecipitated with IgG control or indicated antibodies. pri-miR-219 RNA associated with Drosha (indicated by solid arrows) or DGCR8 (indicated by open arrows) was determined by RT-PCR.

**[0010]** Figures 3a-3j illustrate that miR-219 inhibits NSC proliferation & promotes neuronal differentiation. Figures 3a and 3b show overexpressing miR-219 in NSCs inhibited cell proliferation (Figure 3a) and promoted neuronal differentiation (Figure 3b). BrdU or Tuj1 staining is shown in red and Dapi counterstaining is shown in blue. Figures 3c and 3d show quantification of BrdU+ cells (Figure 3c) and Tuj1+ cells (Figure 3d) in control RNA (C) and miR-219-treated NSCs. n=5, \*p< 0.001 by student's t-test for both panels. N represents experimental repeats. Figure 3e. shows in utero electroporation of miR-219 decreased NSC proliferation in the VZ/SVZ of embryonic brains. Electroporated cells were labeled by RFP and proliferating cells were labeled by Ki67. Figure 3f shows the percentage of RFP+Ki67+ cells out of total RFP+ cells in control RNA or miR-219-electroporated brains. n=3 mice per group. \*p< 0.01 by student's t-test. Figure 3g shows electroporation of miR-219 induced precocious outward cell migration. The electroporated brains were stained

for neuronal marker doublecortin (DCX). Transfected cells were labeled by RFP. Figure 3h shows the percentage of electroporated cells (RFP+) that migrated to the CP. n=3 mice per group. \*p< 0.01 by student's t-test. Figure 3i shows higher magnification images of RFP+DCX+ cells at the CP of brains electroporated with control RNA or miR-219. Scale bar: 50  $\mu$ m for panels a, b, & e; 100  $\mu$ m for panel g; 25  $\mu$ m for panel i. Figure 3j shows the percentage of Tbr1+RFP+ cells out of total RFP+ cells in control RNA or miR-219-electroporated brains. n=3 mice per group. \*p< 0.01 by student's t-test.

**[0011]** Figures 4a-4d illustrate that an miR-219 inhibitor reversed NSC phenotypes induced by TLX siRNA *in vivo*. Figure 4a shows co-electroporation of TLX siRNA with an miR-219 inhibitor rescued the decrease in NSC proliferation induced by TLX siRNA. E13.5 mouse brains were electroporated *in utero* with 1) a control RNA and the RFP reporter (siC-RFP), 2) TLX siRNA and the RFP reporter (siTLX-RFP), 3) an miR-219 inhibitor with siC-RFP, or 4) an miR-219 inhibitor with siTLX-RFP. The electroporated cells were labeled by RFP and proliferating cells were labeled by Ki67. Figure 4b shows the percentage of RFP+Ki67+ cells out of total RFP+ cells in electroporated brains described in panel e is shown. n=3 mice per group. \*p< 0.001 by student's t-test. Figure 4c shows electroporation was performed as described in panel e and brain sections were stained for neuronal marker DCX. Migration of the electroporated cells was tracked by RFP fluorescence. Figure 4d shows the percentage of electroporated cells (RFP+) that migrated to the CP in electroporated brains described in panel g is shown. n=3 mice per group. \*p< 0.05 and \*\* p< 0.01 by student's t test. Scale bar: 50  $\mu$ m for panel a; 200  $\mu$ m for panel c.

**[0012]** Figures 5a-5i illustrate that a TLX peptide promotes miR-219 processing. Figure 5a shows mapping p68 and Drosha-interacting domain in TLX. A schematic of TLX deletion mutants and the Drosha/p68 interacting domain (Dpi) is shown on the left. A summary of p68 and Drosha binding results is shown on the right. Figure 5b shows deletion of TLX residues 340 to 359 reduced the interaction of TLX with p68 substantially. HEK293T cells were transfected with HA-tagged full length TLX (residues 1-385) or its deletion mutants (residues 1-306, 1-340, or 1-359). Lysates were immunoprecipitated (IP) with HA antibody ( $\alpha$ HA), then probed with p68

antibody ( $\alpha$ p68) in Western blot analysis (WB). Figure 5c shows deletion of TLX residues 340 to 359 reduced TLX interaction with Drosha. HEK293T cells were transfected with Flag-tagged Drosha and HA-tagged full length or deletion mutants of TLX. Lysates were IP with Flag antibody ( $\alpha$ Flag), then probed with HA antibody ( $\alpha$ HA). A non-specific (ns) band in the Western blot was indicated. Figures 5d and 5e show expressing the Dpi peptide abolished the interaction of TLX with Drosha (Figure 5d), but not the interaction of TLX with HDAC5 (Figure 5e), as revealed by co-IP analysis. An empty vector (-) and a control peptide (C) were included as negative controls for the Dpi peptide. Cell lysates were IP with anti-Flag antibody, then blotted with anti-HA or anti-Flag antibody. The expression of individual proteins in the transfected cells was shown by immunoblotting as input. Figure 5f shows expression of the Dpi peptide promotes miR-219 processing. miR-219 processing was monitored using the miR-219-Glo reporter. Expressing the Dpi peptide decreased miR-219-Glo activity compared to expressing the empty vector (-) or a control peptide (C).  $n=3$ . \*\*\* $p < 0.001$  by student's t-test. Figures 5g-5i show the levels of pre-miR-219 (h) and mature miR-219 (Figure 5i), but not pri-miR-219 (Figure 5g), were increased by expressing the Dpi peptide, as revealed by RT-PCR.  $n=4$  (g);  $n=4$  (h);  $n=3$  (i). \* $p < 0.05$ , \*\* $p < 0.01$  by student's t-test in panels h & i. N represents experimental repeats in panels f-i.

**[0013]** Figures 6a-6g illustrate that the Dpi peptide regulates NSC proliferation and differentiation. Figures 6a-6c show expression of the Dpi peptide inhibits NSC proliferation and promotes neuronal differentiation, and this effect could be reversed by the miR-219 inhibitor, TuD-miR-219. Mouse embryonic NSCs were transduced with virus expressing the Dpi peptide or a control peptide (C), in the absence or presence of TuD-miR-219. The virus transduced cells were labeled by a GFP reporter. Cell proliferation was determined by the percentage of GFP+BrdU+ cells (BrdU+GFP+/GFP+) (Figure 6b) and neuronal differentiation was determined by the percentage of GFP+Tuj1+ cells (Tuj1+GFP+/GFP+) (c).  $n=7$  (b);  $n=5$  (c). N represents experimental repeats. Figures 6d-6g show expression of Dpi inhibited NSC proliferation (Figures 6d and 6e), but promoted neuronal differentiation (Figures 6f and 6g) in mouse brains. E13.5 mouse brains were electroporated *in utero* with

vectors expressing: 1) a control peptide and RFP reporter (C); 2) Dpi peptide and RFP reporter (Dpi); 3) TuD-miR-219 plus control peptide and RFP reporter (TuD-miR-219+C); or 4) TuD-miR-219 plus Dpi and RFP reporter (TuD-miR-219 + Dpi). The electroporated cells were labeled by RFP, proliferating cells were labeled by Ki67 (Figure 6e), and neuronal cells were labeled by DCX (Figure 6f). The percentage of RFP+Ki67+ cells (Figure 6e) or RFP+ cells that migrated to the CP (Figure 6g) out of total RFP+ cells is shown. n=3 mice per group for panels e & g. \*p<0.05, \*\*p<0.01, \*\*\*p< 0.001 by student's t-test for panels b, c, e & g. Scale bar: 100  $\mu$ m for panel a; 50  $\mu$ m for panel d; 200  $\mu$ m for panel f.

**[0014]** Figures 7a-7f illustrate that the DISC1-mutant NSCs exhibit increased miR-219 expression and reduced proliferation. Figure 7a shows a schematic diagram showing the pedigree for iPSC generation. iPSCs from a wild type (WT) individual outside of the pedigree (C1) were included as a control. The + and – signs represent the presence and absence of the 4 bp deletion in the DISC1 gene, respectively. The squares represent male, while the circles represent female. Figure 7b shows NSCs derived from both WT (C1, C2, & C3) and DISC1-mutant iPSCs (D1, D2, C1M and C3M) expressed neural precursor markers SOX1 and NESTIN. Scale bar: 50  $\mu$ m. Figures 7c and 7d show RT-PCR showing elevated expression of miR-219 (Figure 7c) and reduced expression of TLX (Figure 7d) in DISC-mutant NSCs (D1, D2, C1M and C3M), compared to that in WT NSCs (C1, C2, & C3). Figures 7e and 7f show the DISC1-mutant NSCs (D1, D2, C1M and C3M) exhibited reduced cell proliferation (Figure 7e) and precocious neuronal differentiation (Figure 7f). NSC proliferation rate was determined by the percentage of BrdU+SOX1+ cells. Neuronal differentiation rate was determined by the percentage of Tuj1+ cells. n=4 for panels c-f. N represents experimental repeats. ANOVA test result was shown below each graph.

**[0015]** Figures 8a-8d illustrate that inhibition of miR-219 or overexpression of TLX rescues reduced cell proliferation in SCZ NSCs. Figure 8a shows overexpression of miR-219 inhibited cell proliferation in WT NSCs. WT (C1, C2, C3) and DISC1-mutant NSCs (D1, D2, C1M, C3M) were transduced with virus expressing a control vector (-miR-219) or miR-219-expressing vector (+miR-219).

NSC proliferation rate was determined by the percentage of BrdU+SOX1+ cells. Figure 8b shows TuD-miR-219 rescued the proliferative defect in DISC1 mutant NSCs. WT and DISC1-mutant NSCs were transduced with a control vector (-miR-219-TuD) or TuD-miR-219-expressing vector (+miR-219-TuD). NSC proliferation rate was determined by the percentage of BrdU+SOX1+ cells. Figure 8c shows knockdown of TLX inhibited cell proliferation in WT NSCs. WT (C1, C3) and DISC1-mutant NSCs (D1, D2, C3M) were transduced with virus expressing a control RNA (-siTLX) or TLX siRNA (+siTLX). NSC proliferation rate was determined by the percentage of BrdU+SOX1+ cells. Figure 8d shows overexpression of TLX rescued the proliferative defect in DISC1 mutant NSCs. WT and DISC1-mutant NSCs were transduced with control vector (-TLX) or TLX-expressing vector (+TLX). NSC proliferation rate was determined by the percentage of BrdU+SOX1+ cells. n=5 for panels a & b, and n=4 for panels c & d. N represents experimental repeats. ANOVA test result was shown below each graph.

**[0016]** Figure 9 shows the knockdown of TLX expression in NSCs. Mouse NSCs were transduced with lentivirus expressing a scrambled control RNA (siC) or TLX siRNA (siTLX). The expression of TLX was determined by RT-PCR. Error bars are sd of the mean for all the quantification in this study. n=5. \*\*p< 0.01 by student's t-test.

**[0017]** Figures 10a-10c illustrate the lack of toxicity and gliogenic induction in miR-219-transfected cells. Figure 10a shows minimal cytotoxicity in miR-219-transfected NSCs. Cytotoxicity was expressed as the percent of lactate dehydrogenase (LDH) release into the medium out of the total LDH activity. n=5. Figures 10b and 10c show no induction of GFAP and MBP expression in miR-219-electroporated mouse brains at E15.5. Scale bar: 100  $\mu$ m.

**[0018]** Figures 11a-11g illustrate that TLX-miR-219 regulates the expression of PDGFR $\alpha$  in NSCs. Figure 11a shows PDGFR $\alpha$  expression was reduced in TLX KO mouse brains. The expression of PDGFR $\alpha$  in WT and TLX KO mouse brains was examined by RT-PCR. GAPDH was included as a loading control. Figure 11b shows miR-219 represses PDGFR $\alpha$  3' UTR reporter with wild type (WT), but not mutant

(MT) miR-219 recognition sites.  $n=3$ .  $*p < 0.01$  by student's  $t$  test. Figures 11c-11e show the expression pattern of miR-219 in NSCs and neurons inversely correlates with that of PDGFR $\alpha$  and TLX. The expression levels of PDGFR $\alpha$  (Figure 11c), miR-219 (Figure 11d) and TLX (Figure 11e) in NSCs and cortical neurons (N) derived from embryonic mouse brains were determined by RT-PCR.  $n=3$  and  $*p < 0.001$  by student's  $t$  test for panels c-e. Figure 11f shows the expression of PDGFR $\alpha$  is decreased in NSCs transfected with miR-219 or TLX siRNA as shown by RT-PCR analysis.  $n=5$ .  $*p < 0.01$ ,  $**p < 0.001$  by student's  $t$  test. Figure 11g shows inhibition of PDGFR $\alpha$  expression by TLX siRNA could be rescued by the miR-219 decoy inhibitor, TuD-miR-219. The expression of PDGFR $\alpha$  in NSCs transduced with scramble control RNA (siC) or TLX siRNA (si-TLX), in the absence or presence of TuD-miR-219, was examined by RT-PCR.  $n=5$ .  $*p < 0.01$  by student's  $t$  test.

**[0019]** Figures 12a-12d illustrate that knockdown of PDGFR $\alpha$  inhibits NSC proliferation & promotes neuronal differentiation and migration in embryonic mouse brains. Figure 12a shows electroporation of PDGFR $\alpha$  siRNA decreased NSC proliferation in the VZ/SVZ of embryonic mouse brains. The electroporated cells were labeled with RFP and proliferating cells were labeled with Ki67. Figure 12b shows the percentage of RFP+Ki67+ cells out of total RFP+ cells in control RNA (siC) or PDGFR $\alpha$  siRNA (siPDGFR $\alpha$ )-electroporated brains is shown.  $n=3$ .  $*p < 0.05$  by student's  $t$ -test. Figure 12c shows electroporation of PDGFR $\alpha$  siRNA led to precocious outward cell migration. The electroporated brains were labeled by RFP and stained for the neuronal marker doublecortin (DCX). Figure 12d shows the percentage of electroporated cells (RFP+) that migrated to the CP in siC and siPDGFR $\alpha$ -electroporated brains.  $n=3$ .  $*p < 0.05$  by student's  $t$  test. Scale bar: 50  $\mu$ m for panel a; 200  $\mu$ m for panel c.

**[0020]** Figures 13a-13d illustrate that PDGFR $\alpha$  functions downstream of miR-219 in embryonic mouse brains. Figure 13a shows co-electroporation with PDGFR $\alpha$  and miR-219 reversed the decrease in NSC proliferation induced by miR-219 in the VZ/SVZ of embryonic mouse brains. The electroporated cells were labeled with RFP and proliferating cells were labeled with Ki67. Figure 13b shows co-electroporation with PDGFR $\alpha$  and miR-219 reversed precocious outward cell migration induced by

electroporation with miR-219 alone. Figures 13c and 13d show the percentage of RFP+Ki67+ cells (Figure 13c) or cells migrated to the CP (Figure 13d) out of total RFP+ cells in miR-219 or miR-219 and PDGFR $\alpha$ -electroporated brains is shown. n=3 for panels c & d. \*p< 0.05, \*\*p< 0.01 by student's t-test. Scale bar: 50  $\mu$ m for panel a; 200  $\mu$ m for panel b.

**[0021]** Figures 14a-14d illustrate that PDGFR $\alpha$  functions downstream of TLX in embryonic mouse brains. Figure 14a shows co-electroporation with PDGFR $\alpha$  and TLX siRNA (siTLX + PDGFR $\alpha$ ) reversed the decrease in NSC proliferation in the VZ/SVZ induced by TLX siRNA alone. A control RNA (siC) was included as a negative control for TLX siRNA. Figure 14b shows the percentage of RFP+Ki67+ cells out of total RFP+ cells is shown. n=3. Figure 14c shows co-electroporation with PDGFR $\alpha$  and TLX siRNA reversed the outward cell migration induced by TLX siRNA alone. The electroporated cells were labeled by RFP. Figure 14d shows the percentage of RFP+ cells that migrated to the CP out of total RFP+ cells is shown. n=3. \*p< 0.05, \*\*p< 0.01, \*\*\*p< 0.001 by student's t test. Scale bar: 50  $\mu$ m for panel a; 200  $\mu$ m for panel b.

**[0022]** Figures 15a-15b show that miR-219 regulates neuronal differentiation in SCZ NSCs. Figure 15a shows overexpression of miR-219 promotes neuronal differentiation in WT NSCs. WT (C1, C2, C3) and DISC1-mutant NSCs (D1, D2, C1M, C3M) were transduced with virus expressing a control vector (-miR-219) or miR-219-expressing vector (+miR-219). Neuronal differentiation rate was determined by the percentage of Tuj1+ cells. Figure 15b shows inhibition of miR-219 reverses precocious neuronal differentiation in SCZ NSCs. The DISC1-mutant NSCs exhibited precocious differentiation, which was reversed by TuD-miR-219. Neuronal differentiation rate was determined by the percentage of Tuj1+ cells. n=5 for panels a & b. N represents experimental repeats. ANOVA test result was shown below the graph.

**[0023]** Figure 16 shows the list of Northern blot probes and RT-PCR primers (SEQ ID NOS:1-34).

## DETAILED DESCRIPTION

**[0024]** The following description of the invention is merely intended to illustrate various embodiments of the invention. As such, the specific modifications discussed are not to be construed as limitations on the scope of the invention. It will be apparent to one skilled in the art that various equivalents, changes, and modifications may be made without departing from the scope of the invention, and it is understood that such equivalent embodiments are to be included herein.

**[0025]** In one embodiment, a method of correcting a defective rate of proliferation in a population of neural stem cells (NSCs) is provided. This embodiment may include a step of contacting the population of NSCs with an effective amount of an miR-219 inhibitor, an agent to increase expression or activity of TLX, or both.

**[0026]** The miR-219 inhibitor used in the embodiments described herein may be any suitable agent that inhibits the expression or activity of miR-219 including, but not limited to, a tough decoy RNA, an RNAi molecule (e.g., shRNA, siRNA, or any other RNA interference molecule), or an aptamer. In certain embodiments, the miR-219 inhibitor is an miR-219-5p hairpin inhibitor (Dharmacon) or TuD-miR-219 (a tough decoy RNA). TuD-miR-219 has the following sequence (SEQ ID NO:35):

5' GAC GGC GCT AGG ATC ATC AAC CTC GAG CGC TAG CAA GTA TTC  
TGG TCA CAG AAT ACA ACG TCG ACC ACT AGT CAA GAT GAT CCT  
AGC GCC GTC TTT TTT 3'

**[0027]** The agent to increase expression or activity of TLX may be any suitable agent including, but not limited to, an agent to chemically modify TLX or a vector expressing a gene encoding TLX. In certain embodiments, the vector expressing TLX may be a plasmid or any suitable recombinant viral vector capable of delivering a nucleotide sequence that is expressed in a cell including, but not limited to, a lentiviral vector, an adenoviral vector, an AAV vector, or any other suitable recombinant viral vector. The vectors described herein may be designed to include the nucleotide sequence of TLX, reproduced below:

**Human NR2E1 (TLX) nucleotide sequence (SEQ ID NO:36):**

ATGAGCAAGCCAGCCGGATCAACAAGCCGCATTTTAGATATCCCCTGCA  
 AAGTGTGTGGCGACCGCAGCTCGGGGAAGCACTACGGGGTCTACGCCT  
 GCGACGGCTGCTCAGGTTTTTCAAACGGAGCATCCGAAGGAATAGGAC  
 CTATGTCTGCAAATCTGGAAACCAGGGAGGCTGTCCGGTGGACAAGACG  
 CACAGAAACCAGTGCAGGGCGTGTCCGGCTGAAGAAGTGTGGGAAGTCA  
 ACATGAACAAAGACGCCGTGCAGCACGAGCGGGGGCCTCGGACGTCCA  
 CCATCCGCAAGCAAGTGGCCCTCTACTTCCGTGGACACAAGGAGGAGAA  
 CGGGGCCGCCGCGCACTTTCCCTCGGCGGCGCTCCCTGCGCCGGCCTT  
 CTTACCCGCGGTACGCAGCTGGAGCCGCACGGCCTGGAGCTGGCCGC  
 GGTGTCCACCACTCCAGAGCGGCAGACCCTCGTGAGCCTGGCTCAGCC  
 CACGCCCCAAGTACCCCCATGAAGTGAATGGGACCCCAATGTATCTCTAT  
 GAAGTGGCCACGGAGTCGGTGTGTGAATCAGCTGCCAGACTTCTCTTCA  
 TGAGCATCAAGTGGGCTAAGAGTGTGCCAGCCTTCTCCACGCTGTCTTT  
 GCAAGACCAGCTGATGCTTTTGAAGATGCTTGGAGAGAACTGTTTGTTT  
 TAGGAATAGCACAAATGGGCCATTCCGGTTGATGCTAACACTCTACTGGCT  
 GTATCTGGCATGAACGGTGACAACACAGATTCCCAGAAGCTGAACAAGA  
 TCATATCTGAAATACAGGCTTTACAAGAGGTGGTGGCTCGATTTAGACAA  
 CTCCGGTTAGATGCTACTGAATTTGCCTGTCTAAATGCATCGTCACTTT  
 CAAAGCCGTTTCTACACATAGTGGTTCTGAACTGAGAAGTTTCCGGAATG  
 CTGCCGCCATTGCAGCCCTTCAAGATGAGGCTCAGCTAACGCTCAACAG  
 CTACATCCATACCAGATATCCCACTCAACCCTGTCGCTTTGGAAAACCTCC  
 TGTTGCTTTTGCCAGCTTTACGTTCTATTAGCCCATCAACTATAGAAGAAG  
 TGTTTTTCAAAAAAACCATCGGCAATGTGCCAATTACAAGACTGCTTTCA  
 GATATGTACAAATCCAGTGATATCTAA

**[0028]** When delivered to a target cell such as a neural stem cell using the vector, the nucleotide sequence above is translated to express the TLX protein, the amino acid sequence of which is shown below:

**Human NR2E1 (TLX) amino acid sequence (SEQ ID NO:37):**

MSKPAGSTSRILDIPCKVCGDRSSGKHVGVYACDGC SGFFKRSIRRNRTYV  
 CKSGNQGGCPVDKTHRNQCRACRLKKCLEVNMNKDAVQHERGPRTSTIRK  
 QVALYFRGHKEENGAAAHFPSAALPAPAFFTA VTQLEPHGLELA AVSTTPER  
 QTLVSLAQPTPKYPHEVNGTPMYLYEVATESVCESAARLLFMSIKWAKSVP  
 AFSTLSLQDQLMLLED AWRELFVLGIAQWAIPVDANTLLAVSGMNGDNTDS  
 QKLNKIISEIQALQEVARFRQLRLDATEFAC LKCI VTFKAVPTHSGSELRSFR  
 NAAAIALQDEAQLTLNSYIHTRYPTQPCRFGKLLLLLPALRSISPSTIEEVFF  
 KKTIGNVPITRLLSDMYKSSDI

**[0029]** In another embodiment, a portion of TLX may be used as the agent to increase expression or activity of TLX, such as the Dpi domain of TLX. The Dpi domain may be expressed by the vector or delivered to the population of NSCs. The nucleotide and amino acid sequences of Dpi are shown below:

**Human TLX DPI nucleotide coding sequence (1021 to 1077nt) (SEQ ID NO:38):**

GGAAAACTCCTGTTGCTTTTGCCAGCTTTACGTTCTATTAGCCCATCAAC  
TATAGAA

**Human TLX DPI amino acid sequence (341aa to 359aa) (SEQ ID NO:39):**

GKLLLLLPALRSISPSTIE

**[0030]** The mir-219 inhibitor, the agent to increase expression or activity of TLX, or both may be used to contact the population of NSCs *in vitro*. In this case, the population of *in vitro* cells are derived from a subject suffering from a neurodevelopmental disorder such as schizophrenia, bipolar disorder, or depression.

**[0031]** Alternatively, the agent to increase expression or activity of TLX, or both may be used to contact the population of NSCs *in vivo*. In this case, the population of *in vivo* cells are present in the nervous system of a subject suffering from schizophrenia, bipolar disorder, or depression.

**[0032]** As discussed in the examples below, the mir-219 inhibitor, the agent to increase expression or activity of TLX, or both causes an increase in NSC proliferation rate, and can therefore be used in methods for treating neurodevelopmental disorders that are associated with having a defective proliferation rate of NSCs, such as schizophrenia, bipolar disorder, or depression. The mir-219 inhibitor, the agent to increase expression or activity of TLX may be used separately or in combination to increase proliferation of NSCs in a subject suffering from one of these disorders.

**[0033]** The terms "treat," "treating," or "treatment" as used herein with regards to a condition refers to preventing the condition, slowing the onset or rate of development of the condition, reducing the risk of developing the condition, preventing or delaying the development of symptoms associated with the condition, reducing or ending symptoms associated with the condition, generating a complete or partial regression of the condition, or some combination thereof. In some

embodiments, treating a condition means that the condition is cured without recurrence.

**[0034]** The phrase “a therapeutically effective amount,” “therapeutically effective dose” or “an effective amount” as used herein refers to an amount of an agent, including a nucleic acid, a peptide, or a chemical compound, or a composition that produces a desired therapeutic effect. The precise therapeutically effective amount is an amount of the agent or composition that will yield the most effective results in terms of efficacy in a given subject. This amount will vary depending upon a variety of factors, including but not limited to the characteristics of the agent or composition (including activity, pharmacokinetics, pharmacodynamics, and bioavailability), the physiological condition of the subject (including age, sex, disease type and stage, general physical condition, responsiveness to a given dosage, and type of medication), the nature of the pharmaceutically acceptable carrier or carriers in the formulation, and the route of administration. One skilled in the clinical and pharmacological arts will be able to determine a therapeutically effective amount through routine experimentation, namely by monitoring a subject's response to administration of an agent, population of cells, or composition and adjusting the dosage accordingly. For additional guidance, see Remington: The Science and Practice of Pharmacy (Gennaro ed. 20<sup>th</sup> edition, Williams & Wilkins PA, USA) (2000).

**[0035]** It is within the purview of one of ordinary skill in the art to select a suitable route of administration of the pharmaceutical compositions disclosed herein. For example, these pharmaceutical compositions can be administered by oral administration including sublingual and buccal administration, and parenteral administration including intravenous administration, intramuscular administration, and subcutaneous administration. In certain embodiments the pharmaceutical composition is administered through an intrathecal or intracranial route of administration.

**[0036]** Also disclosed herein are methods of treating a neurodevelopmental disorder in a subject. The subject may be suffering from schizophrenia, bipolar disorder, or depression. According to the embodiments described herein, the

methods may include administering a therapeutically effective dose of a pharmaceutical composition to the subject. In certain embodiments, the pharmaceutical composition may include (i) an agent to increase expression or activity of TLX, (ii) an miR-219 inhibitor, or both (i) and (ii). As disclosed herein, increasing expression or activity of TLX may maintain normal NSC proliferation in the subject. In certain embodiments, the agent may be a vector expressing a gene encoding TLX. In embodiments where the pharmaceutical composition comprises an agent to increase expression or activity of TLX, the pharmaceutical composition may further comprise an miR-219 inhibitor. In embodiments where the pharmaceutical composition comprises an miR-219 inhibitor, the pharmaceutical composition may further comprise an agent to increase expression or activity of TLX. In other embodiments where the pharmaceutical composition comprises an agent to increase expression or activity of TLX, the methods may further include administering a therapeutically effective dose of a second pharmaceutical composition to the subject, the pharmaceutical composition comprising an miR-219 inhibitor. In other embodiments where the pharmaceutical composition comprises an miR-219 inhibitor, the methods may further include administering a therapeutically effective dose of a second pharmaceutical composition to the subject, the pharmaceutical composition comprising an agent to increase expression or activity of TLX. As disclosed herein, the miR-219 inhibitor may be a tough decoy RNA, an RNAi molecule, or an aptamer. In certain embodiments, the miR-219 inhibitor may be an miR-219-5p hairpin inhibitor or TuD-miR-219.

**[0037]** Expression of miR-219 may also be used as a biomarker to detect schizophrenia, bipolar disorder, or depression. In certain embodiments, the miR-219 may be detected in exosomes or cerebrospinal fluid (CSF).

**[0038]** In this disclosure, a role for miR-219 in the regulation of mammalian NSC proliferation and differentiation is demonstrated, and TLX is identified as an upstream regulator of miR-219. Moreover, p68, Drosha and DGCR8 are identified as novel TLX-interacting molecules, and an unexpected role for TLX in regulating miRNA processing at the post-transcriptional level is uncovered. Furthermore, miR-219 expression is up-regulated, whereas TLX expression is down-regulated in SCZ

NSCs. Overexpression of TLX or an miR-219 inhibitor is able to rescue the proliferative defects in SCZ NSCs.

**[0039]** In this disclosure, it has been demonstrated that TLX regulates miRNA processing independent of its well-characterized role in transcriptional regulation, and that miR-219 acts downstream of TLX to regulate NSC proliferation and differentiation in mammalian brains. Moreover, *miR-219* expression is elevated, whereas *TLX* expression is reduced, in *DISC1*-mutant SCZ patient iPSC-derived NSCs. Overexpression of TLX or inhibition of miR-219 could rescue the reduced cell proliferation in *DISC1*-mutant SCZ NSCs.

**[0040]** Disclosed herein is an unexpected role for TLX in miRNA maturation at the post-transcriptional level beyond transcriptional regulation. In an unbiased search for TLX-interacting proteins, the RNA helicase p68, a component of the miRNA processing machinery, was identified as a novel TLX-interacting protein. Further study revealed that TLX also interacts with p68-associated Drosha and DGCR8, the two main components of miRNA processing machinery. It is shown in this disclosure that TLX inhibits miR-219 processing by interacting with the p68/Drosha/DGCR8 complex, which in turn prevents the miRNA processing machinery from binding to miR-219 primary form. Either knockdown of TLX or blocking the interaction between TLX and the miRNA processing machinery resulted in potent induction of pre-miR-219 and mature miR-219 expression, but had minimal effect on pri-miR-219 expression. The concept that a transcription factor like TLX can participate in post-transcriptional regulation of gene expression may serve as a general paradigm for many of these classes of cellular factors to control cell fate determination.

**[0041]** Robust inhibition of cell proliferation and induction of neuronal differentiation were detected when miR-219 was overexpressed in NSCs. However, no obvious change in cell proliferation and differentiation was observed in NSCs treated with miR-219 inhibitor, presumably because the basal miR-219 expression level is low in NSCs. In TLX siRNA-treated NSCs, where miR-219 expression level was elevated, inhibition of miR-219 was able to rescue the proliferative defect and precocious differentiation. It is also possible that the action of other miRNAs, such as

miR-9, miR-124, miR-137, miR-338, or let-7, could compensate for miR-219 inhibition in NSCs. miR-219 has been shown to induce oligodendrocyte differentiation in electroporated mouse brains that were harvested at E17.5 [27]. However, the induction of oligodendrocyte marker expression in miR-219-electroporated mouse brains harvested at E15.5 was not detected, presumably because brains were harvested at an earlier stage that is active for neurogenesis but not for gliogenesis yet. It is possible that miR-219 could play distinct roles at different developmental stages.

**[0042]** miR-219 is dysregulated in neurodevelopmental disorders, including SCZ, bipolar disorder and depression [29,32,42,43]. Understanding the regulation of *miR-219* expression in mammalian brains will not only broaden the knowledge about neurodevelopment, but also provide insights into the pathogenesis of neurological disorders. It is shown in this disclosure that TLX represses *miR-219* biogenesis in NSCs during mouse brain development. *PDGFR $\alpha$*  was also identified as a downstream target of the TLX-miR-219 cascade in NSCs. PDGFR $\alpha$  has been shown to be expressed in oligodendrocyte progenitor cells [44] and play a role in oligodendrocyte differentiation downstream of miR-219 [26]. It is shown in this disclosure that knockdown of *PDGFR $\alpha$*  expression induced NSC phenotypes similar to that induced by *miR-219* overexpression, whereas overexpression of *PDGFR $\alpha$*  restored NSC phenotypes induced by miR-219 overexpression or TLX siRNA treatment.

**[0043]** DISC1 is required for mouse NSC proliferation [40]. However, little is known about its function in human NSCs. In this disclosure, DISC1 has been found to play a role in regulating human NSC proliferation by studying NSCs derived from *DISC1*-mutant SCZ patient iPSCs and genetically engineering isogenic iPSCs with an introduced *DISC1* mutation. The observation that *miR-219* expression is up-regulated, whereas *TLX* expression is down-regulated, in *DISC1*-mutant NSCs provides a direct link between TLX and miR-219 expression and DISC1 function. Previous studies have shown that *TLX* KO mice exhibit neuroanatomical and behavioral abnormalities similar to that in *DISC1*-mutant mice and SCZ patients, including increased lateral ventricles, reduced cerebral cortex, reduced neurogenesis

and memory, and increased anxiety and hyperactivity [2,3,6,7,45-56]. The finding of altered expression of TLX in *DISC1*-mutant NSCs suggests that mutant DISC1 could regulate TLX expression, which in turn induces abnormal miR-219 expression and inhibition of NSC proliferation.

**[0044]** SCZ is a neurodevelopmental disorder for which the pathological mechanism remains elusive. Increasing evidence suggests that miRNAs may play important roles in the etiology of SCZ [57]. miRNA-219 is highly up-regulated in the prefrontal cortex of SCZ patients [29,32] and mediates the behavioral effects of the NMDA receptor antagonist Dizocilpine [58]. However, whether miR-219 plays a role in SCZ pathogenesis remained unknown. This disclosure has identified a novel role for miR-219 in SCZ NSCs; elevated miR-219 expression reduces SCZ NSC proliferation.

**[0045]** Multiple studies provide evidence that NSC proliferation and neurogenesis are tightly linked to SCZ pathogenesis [39,59,60]. Recent studies using patient iPSCs have identified phenotypic differences in human iPSC-derived neural progenitor cells [61] and provided insights into how risk factors for SCZ regulate NSC phenotypes and neurodevelopment [62]. In this disclosure, a direct link between *DISC1* mutation and altered *TLX* and *miR-219* expression, and a causative link between dysregulated *TLX* and *miR-219* expression and proliferative defects in *DISC1*-mutant SCZ NSCs are identified. Disclosed herein is a molecular mechanism underlying defective NSC proliferation in SCZ. Moreover, both TLX and miR-219 could be potential therapeutic targets for SCZ and that TLX inducers or miR-219 inhibitors may serve as potential therapeutic tools to maintain normal NSC proliferation in SCZ patients.

**[0046]** The working examples below further illustrate various embodiments of this disclosure. By no means the working examples limit the scope of this invention.

#### **Example 1 Materials and Methods**

**[0047] Animals.** Female ICR or Swiss Webster mice at gestation 13.5 were used for in utero electroporation experiments. All mice were produced in the Animal Resource Core of City of Hope. All animal-related work was performed under the

IACUC protocol 03038 approved by City of Hope Institutional Animal Care and Use Committee. Mice were maintained in a 12 hr light:12 hr dark light cycle at 4 mice per cage.

**[0048] Antibodies and immunostaining.** Antibodies were used to Flag epitope tag M2 (Sigma, F2426 for IP), HA (1: 500, Santa Cruz, sc-805), p68 (1:1000, Abcam, ab10261), Drosha (1: 1000, cell signaling, #3364), DGCR8 (1:500, Protein Tech Group, Inc, 10996-1-AP), BrdU (1: 5000, Accurate, OBT0030CX), DCX (1: 300, Santa Cruz, sc-8806) and Ki67 (1: 200, GeneTex, GTX16667). Immunostaining of embryonic mouse brains was performed using antibodies for DCX and Ki67. For Ki67 staining, antigen retrieval was performed by incubating slides in sodium citrate buffer (10 mM sodium citrate, pH 6.0 and 0.1% Triton X) at 80°C for 10 min before staining.

**[0049] Mouse NSC culture.** Embryonic mouse NSCs were prepared using an established protocol [63] as follows. E14.5 mouse brains were dissociated by gentle pipetting. The dissociated cells were seeded on polyornithine- and fibronectin-coated plates and cultured in N2 medium (DMEM F12, 25 µg per ml insulin, 100 ng per ml apo-transferrin, 30 nM sodium selenite, 20 nM progesterone and 100 µM putrescine) supplemented with 10 ng per ml FGF2. Cells were maintained as mycoplasma-free culture as revealed by routine mycoplasma screen using MycoAlert Mycoplasma Detection Kit. For differentiation, NSCs were dissociated into single cells and cultured in N2 medium supplemented with 0.5% fetal bovine serum and 10 µM Forskolin for 5 days. For BrdU labeling, 10 µM BrdU was added to NSCs and pulsed for 30 min. Cells were then fixed and acid treated, followed by immunostaining with anti-BrdU antibody. Transfection of NSCs with reporter plasmid DNA, miRNA or siRNA was performed using TransFectin (BioRad), following manufacturer's instructions. For actinomycin D treatment, control or TLX siRNA-transduced NSCs were treated with 1 µM actinomycin D for 3 hr, followed by cell harvesting and RNA isolation.

**[0050] Plasmid DNAs.** pCK-Flag-Drosha [64] and pCK-Flag-DGCR8 [64] were gifts from Dr. V.N. Kim. To prepare the PDGFRα 3' UTR reporter construct, DNA

fragments containing mouse PDGFR $\alpha$  3' UTR were subcloned into psiCHECK vector (Promega). The miR-219-5P target site 5'-GACAATCA-3' (SEQ ID NO: 40) in PDGFR $\alpha$  3' UTR was mutated into 5'-GATCGTCA-3' (SEQ ID NO: 41) by site-directed mutagenesis. The cDNA of mouse PDGFR $\alpha$  was purchased from ATCC and subcloned into pEF-pUb-RFP vector [4]. To make TLX siRNA or scrambled control RNA-expressing lentiviral vector, DNA fragments containing TLX siRNA or scrambled control siRNA hairpin sequences were subcloned into pHIV-GFP vector [65]. To prepare the Dpi peptide or control peptide-expressing vector, DNA fragment containing the Dpi (amino acid residues 341-359) (SEQ ID NO: 42) or control peptide (amino acid residues 201-223) (SEQ ID NO: 43) of TLX was fused in frame to three copies of nuclear localization signals and cloned into the CMX-HA or CSC-GFP vector [3]. To make miR-219-expressing retroviral vector, DNA oligos of miR-219 were annealed and cloned into the UEG vector [66]. To prepare the construct of TuD-miR-219, DNA oligos of TuD-miR-219, 5'-TCG AAG AAT TGC GTT CTG ATG GAC AAT CA-3' (SEQ ID NO: 44) and 5'-CTA GTG ATT GTC CAT CAG AAC GCA ATT CT-3' (SEQ ID NO: 45) were annealed and cloned into the U6-TuD vector. The DNA fragment containing the U6 promoter and TuD-miR-219 was then subcloned into pHIV-GFP vector or CMVLV lentiviral vector containing a puromycin-resistant gene [65]. To prepare miR-219-Glo vector, 392 bp fragment of pri-miR-219 including the pre-miR-219 hairpin loop was PCR amplified using the following primers: 5'-TTC ATA GAG CTC ACA CCG GCT TGT CCA CCT TAC-3' (SEQ ID NO: 46) and 5'-TTC ATA CTC GAG GAG GAT ACG GAA AGA GGC GAG-3' (SEQ ID NO: 47). The PCR product was digested with SacI and XhoI site and cloned into the pmirGLO vector (Promega). To prepare miR-1224-Glo vector, 398 bp fragment of pri-miR-1224 was PCR amplified using the following primers: 5'-GAT AGC TAG CAA TGG CAA CTC CAA GCG TGC T-3' (SEQ ID NO: 48) and 5'-ATG AGG CCG AGG TGG GGC TGA GTC TAG AGA TC-3' (SEQ ID NO: 49). The PCR product was digested with NheI and XbaI and cloned into the pmirGLO vector (Promega).

**[0051] siRNAs and miRNAs.** All synthetic siRNAs, miRNAs and their controls were purchased from Dharmacon. The ON-TARGET plus siRNA for TLX (J-065577-12-0005) and non-targeting control siRNA (D-001810-01-05) were used for the

experiments of TLX knockdown; the ON-TARGET plus SMARTpool siRNA for PDGFR $\alpha$  (L-048730-00-0005) and control siRNA pool (D-001810-10-05) were used for the experiments of PDGFR $\alpha$  knockdown. The miRIDIAN miRNA mimic for miR-219-5p (c-310578-05-0005), negative control (CN-001000-01-05), and miR-219-5p hairpin inhibitor (IH-310578-07-0005) were used for overexpressing miR-219 or inhibiting miR-219 action in mouse NSCs.

**[0052] Northern blot analysis and RT-PCR.** Total RNAs from tissue cultured cells or 6 to 8-week-old WT or TLX KO mouse brains were isolated using TRizol (Invitrogen) in accordance with manufacturer's instructions. Oligonucleotides complementary to miRNA sequences were end-labeled with  $\gamma^{32}\text{P}$ -ATP and used as probes for Northern blot analysis. The sequences for the probes are listed in Figure 16. RT-PCR was performed to detect the levels of primary, precursor and mature miR-219, or TLX and PDGFR $\alpha$  mRNAs. Reverse transcription was performed using Tetro cDNA synthesis kit (Bioline) and the expression levels of pri-miRNA and pre-miRNA of miR-219, and TLX and PDGFR $\alpha$  mRNAs were determined using DyNAmo Flash SYBR Green qPCR mix (Thermoscientific) and StepOnePlus Real-Time PCR system (Applied Biosystems). For detection of the mature miR-219, TaqMan MicroRNA assay kit (Applied Biosystems) was used according to manufacturer's protocol. Data analysis was done by Comparative Ct method. Results were normalized to  $\beta$ -actin for pri-miRNA, pre-miRNA, TLX and PDGFR $\alpha$  mRNAs, and snoRNA or U6 for mature miRNA. The primers are listed in Figure 16.

**[0053] *In vivo* monitoring of pri-miRNA processing.** The miR-219-Glo, miR-1224-Glo or control-Glo (pmirGLO, Promega) vector was transfected together with TLX-expressing vector or TLX siRNA-expressing vector. The firefly luciferase activity was measured 48 hr after transfection and normalized with the Renilla luciferase internal control. The results were then normalized with the luciferase activity in cells transfected with the control vector(s).

**[0054] Nuclear extract preparation and immunoprecipitation.** To make stable HeLa cell line that express HA or HA-TLX, HeLa cells were transduced with lentivirus expressing HA or HA-TLX and a GFP reporter, and plated the transduced

cells at 1 cell per well in a 96-well plate. The GFP-positive clones derived from single GFP-positive cells were expanded to make stable cell lines. The expression of HA-TLX in cells transduced with the HA-TLX-expressing virus was confirmed by TLX Western blot analysis. Nuclear extracts were prepared from the stable cell line expressing HA or HA-TLX following a published method [67]. Every ml of nuclear extract was pre-cleared using 20  $\mu$ l of protein G and 20  $\mu$ l of IgG-AC (Santa Cruz, sc-2345) for 5 hr, and then incubated with HA beads (Santa Cruz, sc-805 AC) at 4°C for overnight. Proteins pulled down by the HA beads were collected at 8,200 g for 1 min, then washed with 500  $\mu$ l TBS for 20 min twice, and re-suspended in protein loading buffer for protein gel electrophoresis and subsequent mass spectrometry analysis.

**[0055] Mass spectrometry.** Proteins were separated on a 4-12% Bis-Tris NuPAGE gel with MES running buffer (Novex, life technology) and stained with SimplyBlue SafeStain solution (Life Technology) to visualize the differentially expressed proteins. Corresponding protein gel bands were excised and destained in ammonium bicarbonate (100 mM) / acetonitrile (45%) followed by in-gel processing, which included reduction with tris(carboxyethyl) phosphine (10 mM), alkylation with iodoacetamide (50 mM), and digestion with sequencing grade trypsin (300 ng per band, Promega); all steps were performed in 100 mM ammonium bicarbonate, pH 7.9. Extracted peptides were acidified with formic acid (1%) and injected straightly into the liquid chromatography (LC) mass spectrometry (MS) system, consisting of a binary pump Agilent 1200 HPLC, a 6520 quadrupole time-of-flight mass spectrometer (Agilent), equipped with a chip cube ion source, utilizing a high capacity LC/MS chip (Agilent) with a 150 mm  $\times$  75  $\mu$ m Zorbax 300SB-C18 on-board analytical reverse phase column and a 160 nl trapping column. 10  $\mu$ l sized peptide samples were loaded at 4  $\mu$ l per min. LC was performed with a gradient mobile phase system containing buffer A (0.1% aqueous formic acid) and B (100% acetonitrile, 0.1% formic acid). A 50-minute gradient elution from the analytical column was conducted from 7 to 85% buffer B at 300 nl per min. MS and tandem MS analysis of peptide ions with  $z > 2^+$  was performed in data-dependent mode. Automated collision energy settings were set by the acquisition software,

MassHunter (Agilent). The resulting data was analyzed using the GPM X! Tandem search engine (The Global Proteome Machine Organization) with the human protein database and Scaffold (Proteome Software) at a 1% false discovery rate setting.

**[0056] Immunoprecipitation and Western blotting.** Cells were lysed with lysis buffer containing 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% NP40, 0.1% deoxycholate and protease inhibitor cocktails (Roche). For DNase and RNase treatment, cell lysates were treated with 40 U per ml DNase and 10 µg per ml RNase at 37°C for 30 min. Lysates were immunoprecipitated using anti-Flag (Sigma, F2426) or anti-HA (Santa Cruz, sc-805 AC) antibodies. For co-immunoprecipitation of endogenous proteins, E13.5 mouse brains were homogenized in the above lysis buffer. Lysates were immunoprecipitated using TLX antibody, followed by immunoblotting using indicated antibodies. To determine whether Dpi disrupts the TLX-Drosha interaction, constructs expressing Dpi (TLX residues 341-359) or a control peptide (TLX residues 201-223), together with HA-TLX and Flag-Drosha were transfected into HEK293T cells. Cell lysates were immunoprecipitated with Flag antibody (Sigma, F2426), followed by immunoblotting with anti-HA (1:500, Santa Cruz, sc-805) or anti-Flag antibody (1:500, Sigma, F1804). Images in Figs. 1a and 1b, Figs. 2c, 2d and 2f, and Figs. 5b-5e have been cropped for presentation.

**[0057] RNA immunoprecipitation (RIP).** RIP was performed under native condition [68]. NSCs were transduced with lentivirus expressing TLX siRNA or scrambled control RNA. Cell pellets were resuspended in ice cold lysis buffer containing 100 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.0), 0.5% NP40, 1 mM DTT, 100 U per ml RNase Inhibitor (Promega), and protease inhibitor cocktail (Roche), and lysates were passed through a 27.5 gauge needle 4 times to promote nuclear lysis. Eighty units of DNase (Ambion) was added to the lysates, which were then incubated on ice for 30 min. Cell lysates were diluted in NT2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.05% NP40, 1 mM DTT, RNase Inhibitor and protease inhibitor cocktail) and pre-cleared with protein G agarose. One tenth volume of supernatant was saved as input. The rest of the supernatant was incubated with 5 µg of antibodies at 4°C for overnight. After incubating with protein G agarose, the RNA-antibody complex was precipitated and washed with NT2 buffer 4

times. RNA was extracted using Trizol (Invitrogen) according to manufacturer's instructions. Reverse transcription was performed using Tetro cDNA synthesis kit (Bioline), followed by PCR reaction.

**[0058] *In utero electroporation.*** A solution including 100  $\mu$ M miRNA, siRNA or miRNA inhibitor with 5  $\mu$ g per  $\mu$ l plasmid DNA expressing RFP only or RFP and PDGFR $\alpha$  was electroporated into E13.5 wild type ICR mouse brains. Both male and female mice were used. For electroporation of the Dpi peptide-expressing vector, plasmids expressing Dpi and RFP or Dpi, RFP and TuD-miR-219, at 2.5  $\mu$ g per  $\mu$ l each, were electroporated into E13.5 mouse brains. Two days later, the electroporated brains were dissected and sectioned at 20  $\mu$ m thickness, followed by immunostaining.

**[0059] Human iPSC culture and differentiation.** Human iPSCs were maintained and cultured in Essential 8 (E8) medium (Gibco, A15169-01). For NSC differentiation, iPSCs were detached using 0.5 mM EDTA and cultured in E8 medium for 6 days in suspension for embryoid body (EB) formation, then switched to neuronal induction medium (50% DMEM/F12, 50% Neurobasal, 0.5 X N2, 0.5 X B27, 2 mM L-Glutamine, 0.1 mM NEAA, and 100 units penicillin/streptomycin) supplemented with 5  $\mu$ M SB431542 and 0.25  $\mu$ M LDN for 3 days. The EB spheres were transferred into matrigel-coated plates and cultured in neuronal induction medium for 7 days. Rosette structures were mechanically lifted and cultured in neuronal induction medium supplemented with basic FGF (5 ng per ml) and EGF (20 ng per ml) for expansion. Neurospheres were stained for NSC markers using antibodies for SOX1 (1:500, Millipore, AB15766) and NESTIN (1:1000, BD, 611659). All the cells used in this study were maintained as mycoplasma-free culture as revealed by routine mycoplasma screen using MycoAlert Mycoplasma Detection Kit.

**[0060] Human NSC proliferation and differentiation.** Human iPSC-derived NSCs were seeded on matrigel coated 24-well plates in proliferation media and cultured for 24 hr. Lentivirus expressing miR-219 or TuD-miR-219 and a GFP reporter was added to human NSCs in 24-well plates for 16 hr. The virus-transduced cells were labeled by GFP. For proliferation assay, cells were allowed to recover for

2 days and then treated with 10  $\mu$ M BrdU for 1 hr, followed by immunostaining for BrdU and SOX1. Nuclei were counter-stained using DAPI. NSC proliferation rate was determined using the percentage of BrdU+SOX1+ cells, which was calculated as BrdU+SOX1+/DAPI+ cells for non-virus-transduced cells and BrdU+SOX1+GFP+/GFP+ cells for GFP-expressing virus-transduced cells. For differentiation, NSCs were switched to differentiation medium containing N2 and B27 (1:1) with 1  $\mu$ M retinoic acid and 0.5% FBS in DMEM F12 media. Cells were allowed to differentiate for 2 weeks, followed by immunostaining for Tuj1. The neuronal differentiation rate was determined using the percentage of Tuj1+ cells, which was calculated as Tuj1+/DAPI+ cells for non-virus-transduced cells and Tuj1+GFP+/GFP+ cells for GFP-expressing virus-transduced cells.

**[0061] Statistical analysis.** Student's t-test and ANOVA were used for statistical analyses for comparison of experimental results as reported in each figure and legend. All results were expressed as mean  $\pm$  s.d. The sample size was chosen based on our preliminary studies. Statistical significance was defined as  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$  as specified in the figure legend. No samples, mice or data points were excluded from the reported analyses. No randomization was used for sample assignment and data collection, and no blinding was performed.

## **Example 2 TLX represses miR-219 processing**

**[0062]** When comparing gene expression in wild type (WT) and *TLX* knockout (KO) mouse brains, dramatically elevated expression of miR-219 were detected in *TLX* KO brains (Fig. 1a). Because *TLX* is a transcription factor, the level of primary transcripts of *miR-219* was examined next. miR-219 is derived from two primary transcripts, pri-miR-219-1 and pri-miR-219-2. No expression of pri-miR-219-1 was detected in both WT and *TLX* KO brains, and not much change in the expression of pri-miR-219-2 was observed in WT and *TLX* KO brains either (Fig. 1b & 1c). Because pri-miR-219-2 was only detected in the brain, pri-miR-219-2 is referred to as pri-miR-219 hereafter.

**[0063]** The expression levels were determined for the precursor form of miR-219 (pre-miR-219) in *TLX* KO brains. The level of pre-miR-219 increased

substantially in *TLX* KO brains, compared to WT brains, similar to the change in mature miR-219 level, whereas no dramatic change was observed in pri-miR-219 level (Fig. 1c). The levels of all three forms of miR-219 in *TLX* knockdown NSCs were examined. Knockdown of *TLX* by siRNA was confirmed by RT-PCR (Fig. 9). Consistent with observation in *TLX* KO brains, considerable increase in the levels of pre-miR-219 and mature miR-219 was seen in *TLX* knockdown NSCs, compared to control NSCs, whereas minimal change was detected in the level of pri-miR-219 (Fig. 1d). The up-regulation of pre-miR-219 and mature miR-219 by *TLX* knockdown was not affected by the treatment of the transcriptional inhibitor actinomycin D (Fig. 1d). These results suggest that *TLX* regulates the expression level of miR-219 at the post-transcriptional level, presumably through inhibiting the processing of miR-219 from the primary form to the precursor form.

**[0064]** To confirm that *TLX* plays a role in miR-219 processing, a luciferase-based processing assay was performed. HEK293T cells were transfected with a luciferase reporter construct containing pri-miR-219 sequences that include the Drosha/DGCR8 binding sites. The pri-miR-219 sequences were placed between the coding region of the luciferase gene and its polyadenylation signal. Cleavage of polyadenylation tails from the luciferase transcripts by Drosha/DGCR8 would induce degradation of the luciferase transcripts and reduce luciferase activity (Fig. 1e). It was found that ectopic expression of *TLX* in HEK293T cells reduced miR-219 processing, as revealed by increased luciferase activity of miR-219-Glo (Fig. 1f). Expression of *TLX* had no effect on luciferase activity of miR-1224-Glo, a reporter that contains part of miR-1224, an miRtron that is processed into pre-miRNA independent of Drosha cleavage<sup>33</sup> (Fig. 1f). In contrast to overexpression of *TLX*, knockdown of *TLX* in NSCs promoted miR-219 processing, as shown by reduced luciferase activity of miR-219-Glo, compared to control RNA-treated cells (Fig. 1g), but had no effect on luciferase activity of miR-1224-Glo (Fig. 1g). These results indicate that *TLX* negatively regulates miR-219 processing from the primary form to the precursor form.

**Example 3 TLX interacts with the miRNA processing machinery**

**[0065]** In a parallel effort, novel TLX-interacting proteins were identified. Nuclear extracts of HA-TLX-expressing HeLa cells were immunoprecipitated with an HA antibody. Proteins specifically pulled down in HA-TLX-expressing cells, but not in control cells, were subjected to mass spectrometry analysis to determine their identity (Fig. 2a, b). The RNA helicase p68 is among the proteins that were uniquely represented in the pull-downs of HA-TLX-expressing cells. Seventeen peptides of p68 were detected in the HA immunoprecipitates of HA-TLX-expressing cells, but not in that of control HA-expressing cells.

**[0066]** To confirm the interaction of TLX with p68, HEK293T cells were transfected with HA-TLX. p68 was detected in the HA-TLX immunocomplex and the interaction was not affected by the treatment with DNase and RNase (Fig. 2c). Because p68 is a component of the Drosha complex that processes pri-miRNAs into pre-miRNAs [18,19], it was hypothesized that TLX could interact with the miRNA processing machinery via its interaction with p68. To test whether TLX interacts with Drosha and DGCR8, HEK293T cells were transfected with Flag-Drosha or Flag-DGCR8 and HA-TLX. HA-TLX was detected in the immunocomplexes of both Flag-Drosha and Flag-DGCR8, independently of DNase and RNase treatment (Fig. 2c).

**[0067]** To confirm the interaction of endogenous TLX with Drosha/DGCR8, E13.5 mouse brains were harvested, where TLX is highly expressed [4]. Brain lysates were immunoprecipitated with a TLX-specific antibody. Both Drosha and DGCR8 were detected in the TLX immunocomplex (Fig. 2d). These results indicate that TLX interacts with components of the miRNA processing machinery.

**[0068]** The interaction of TLX with Drosha and DGCR8 led us to hypothesize that TLX could inhibit miR-219 processing by preventing the miRNA processing machinery from binding to pri-miR-219. Cell lysates were made from NSCs transduced with lentivirus expressing TLX siRNA and performed RNA immunoprecipitation to determine if knockdown of TLX would affect the binding of Drosha and DGCR8 to pri-miR-219 (Fig. 2e). Weak binding of Drosha and DGCR8

to pri-miR-219 was detected in NSCs transduced with lentivirus expressing a control RNA, and no binding of TLX to pri-miR-219 was detected (Fig. 2f). Knockdown of TLX increased the binding of both Drosha and DGCR8 to pri-miR-219 substantially. These results indicated that TLX inhibits Drosha and DGCR8 from binding to pri-miR-219.

#### **Example 4 miR-219 inhibits mammalian NSC proliferation**

**[0069]** Because TLX plays an important role in regulating mammalian NSC proliferation and differentiation [3,4], the observation that TLX regulates miR-219 processing in mouse NSCs led to a hypothesis that miR-219 could be involved in regulating mammalian NSC phenotypes. To test whether miR-219 affects mammalian NSC proliferation, NSCs were isolated from E14.5 mouse brains and treated with the miR-219 RNA duplex. BrdU labeling was performed to monitor cell proliferation. Treatment with miR-219 reduced cell proliferation substantially (Fig. 3a, c), but had minimal cytotoxicity (Fig. 10a). These results indicate that miR-219 inhibits mammalian NSC proliferation. The effect of miR-219 was then tested on NSC differentiation. E14.5 mouse NSCs were treated with the miR-219 RNA duplex and cultured in differentiation medium. Treatment with miR-219 increased the percentage of  $\beta$ III tubulin (Tuj1)-positive neurons substantially, compared to treatment with a control RNA (Fig. 3b, d). These results indicate that miR-219 promotes mammalian NSC differentiation into neurons.

**[0070]** To determine the effect of miR-219 on NSC regulation *in vivo*, miR-219 RNA duplex was electroporated together with an RFP-expressing vector into NSCs of E13.5 embryonic brains *in utero*. The brains were dissected at E15.5 and analyzed by immunohistochemistry. Immunostaining with Ki67, a proliferation marker, revealed that overexpression of miR-219 decreased cell proliferation in the ventricular zone and subventricular zone (VZ/SVZ) of mouse brains, where NSCs reside (Fig. 3e, f). To determine the effect of miR-219 overexpression on neuronal differentiation, immunostaining with doublecortin (DCX), a neuronal marker, was performed. Compared to control RNA-transfected cells, substantially more miR-219-electroporated (RFP+) cells migrated from VZ/SVZ to the cortical plate (CP), where

neurons are located (Fig. 3g, h). Moreover, the RFP+ cells migrated to the CP were positive for DCX (Fig. 3i), confirming their neuronal identity. Immunostaining with Tbr1, another neuronal marker, was also performed. Quantification of the RFP+ cells that expressed Tbr1 revealed a much higher percentage of Tbr1+RFP+ cells in miR-219-electroporated brains than that in control RNA-electroporated brains (Fig. 3j). miR-219 has been shown to induce oligodendrocyte differentiation in *in utero* electroporated mouse brains harvested at E17.5 [27]. However, the induction of either astrocyte marker GFAP or oligodendrocyte marker MBP expression was not detected in miR-219-electroporated brains harvested at E15.5 (Figs. 10b and 10c), presumably because brains were analyzed at an early cortical developmental stage when neurogenesis is active but gliogenesis is not yet. These results indicate that miR-219 inhibits mammalian NSC proliferation and promotes their neuronal differentiation during early brain development.

#### **Example 5 miR-219 acts downstream of TLX to regulate NSC phenotypes**

**[0071]** To confirm that miR-219 acts downstream of TLX in NSCs, the effect of TLX knockdown on NSC proliferation and differentiation was tested to see if they could be rescued by an miRNA hairpin inhibitor that is designed specifically to inhibit miR-219 action by interfering with miR-219 binding to downstream targets. When TLX siRNA was electroporated into the VZ/SVZ of E13.5 mouse brains, the percentage of RFP+Ki67+ cells was decreased dramatically, compared to that in control RNA-electroporated brains. However, when TLX siRNA and the miR-219 inhibitor RNA were co-electroporated, the percentage of RFP+Ki67+ cells was recovered substantially (Fig. 4a, b). On the other hand, an increasing number of TLX siRNA-electroporated cells migrated to the CP, compared to control cells (Fig. 4c, d). This phenotype is similar to the effect induced by electroporation of miR-219 into the VZ/SVZ of mouse brains (Fig. 3g, h). In brains co-electroporated with TLX siRNA and the miR-219 inhibitor, the percentage of transfected cells that migrated to the CP was also restored towards the control level (Fig. 4c, d). These results indicate that miR-219 is an important TLX downstream target in regulating mammalian NSC proliferation and differentiation *in vivo*.

**Example 6 PDGFR $\alpha$  is a target gene of miR-219 and TLX in NSCs**

**[0072]** To uncover mechanisms underlying miR-219-mediated regulation of NSC phenotypes, potential miR-219 target genes were identified using TargetScan, which revealed a set of candidate miR-219 targets, the 3' UTR of which can base pair with miR-219. Among the candidate targets, *PDGFR $\alpha$*  is a confirmed miR-219 target [26] and is expressed in NSCs [34]. RT-PCR showed that the expression of *PDGFR $\alpha$*  was dramatically decreased in *TLX* KO brains (Fig. 11a). Moreover, miR-219 repressed the activity of the luciferase reporter with the wild type *PDGFR $\alpha$*  3' UTR, but not the reporter that with the mutant 3' UTR, in which the base pairing with miR-219 was destroyed (Fig. 11b), suggesting that miR-219 inhibits *PDGFR $\alpha$*  expression.

**[0073]** Moreover, the expression of *PDGFR $\alpha$* , similar to *TLX*, was relatively high in NSCs but low in neurons, whereas the expression of *miR-219* was relatively low in NSCs but increased in neurons, inversely correlating to the expression of *PDGFR $\alpha$*  and *TLX* (Figs. 11c-e), supporting the concept that *TLX* represses the expression of *miR-219*, while miR-219 inhibits the expression of *PDGFR $\alpha$* .

**[0074]** Whether overexpressing *miR-219* or knockdown of *TLX* regulates *PDGFR $\alpha$*  expression was tested next. Repression of *PDGFR $\alpha$*  expression was detected in *miR-219*-transfected NSCs and *TLX* siRNA-transfected NSCs, respectively (Fig. 11f), indicating that *PDGFR $\alpha$*  indeed acts downstream of miR-219 and *TLX*. To determine if inhibition of *PDGFR $\alpha$*  by *TLX* is mediated through up-regulating *miR-219*, NSCs were co-transduced with lentivirus expressing *TLX* siRNA and lentivirus expressing TuD-miR-219, a tough decoy inhibitor [35] of miR-219. Co-expressing TuD-miR-219 with *TLX* siRNA rescued the inhibition of *PDGFR $\alpha$*  expression by *TLX* siRNA substantially (Fig. 11g), suggesting that *TLX* regulates *PDGFR $\alpha$*  expression through miR-219. Taken together, these results demonstrate that *PDGFR $\alpha$*  is a downstream target gene of the *TLX*-miR-219 regulatory cascade.

**[0075]** How *PDGFR $\alpha$*  regulates NSC proliferation and differentiation *in vivo* was studied. *In utero* electroporation of *PDGFR $\alpha$*  siRNA into E13.5 mouse brains reduced

NSC proliferation as shown by decreased Ki67+RFP+ cells in the VZ/SVZ, whereas the number of RFP+ cells migrated to the CP was increased (Figs. 12a-d). To determine if PDGFR $\alpha$  is a critical downstream target gene of miR-219 in NSC regulation, if the effect of miR-219 on NSC proliferation and differentiation could be reversed by overexpressing PDGFR $\alpha$  was tested. When PDGFR $\alpha$  and an RFP reporter were electroporated together with miR-219, the number of RFP+Ki67+ cells in the VZ/SVZ increased substantially (Figs. 13a and 13c), whereas the RFP+ cells that migrated to the CP reduced considerably, compared to that in brains electroporated with RFP and miR-219 only (Figs. 13b and 13d). These results indicate that *PDGFR $\alpha$*  is an important downstream target of miR-219 in regulating NSC proliferation and differentiation.

**[0076]** Because the expression of *PDGFR $\alpha$*  was reduced in *TLX* knockdown NSCs (Figs. 11f and 11g), if overexpression of *PDGFR $\alpha$*  could rescue *TLX* siRNA-induced inhibition of NSC proliferation was determined. Co-electroporation of PDGFR $\alpha$  with *TLX* siRNA to E13.5 mouse brains showed an increase in the number of Ki67+RFP+ cells in the VZ/SVZ and a decrease in the number of RFP+ cells migrated to the CP, compared to electroporation with *TLX* siRNA alone (Figs. 14a-14d). These results indicate that PDGFR $\alpha$  acts downstream of *TLX* to regulate NSC proliferation and differentiation in mammalian brains.

#### **Example 7 A *TLX* peptide promotes miR-219 processing**

**[0077]** To determine the region in *TLX* that is critical for the interaction with the miRNA processing machinery, the minimal domain of *TLX* was mapped for p68 and Drosha interactions by serial deletion (Fig. 5a). Co-immunoprecipitation analysis revealed that the *TLX* residues 340-359 were critical for the *TLX*-p68 interaction (Fig. 5a, b). Deletion of *TLX* residues 340-359 also reduced the interaction of *TLX* with Drosha dramatically (Fig. 5a, c). The *TLX* region spanning residues 340 to 359 was determined to be the Drosha/p68-interaction (Dpi) domain (Fig. 5a).

**[0078]** To determine whether the Dpi domain interferes with the *TLX*-Drosha interaction, an HA-tagged *TLX* peptide containing the Dpi domain (Dpi) was co-expressed with HA-tagged full-length *TLX* (HA-*TLX*) and Flag-Drosha in HEK293T

cells. The interaction of TLX with Drosha, as determined by co-immunoprecipitation, was substantially reduced when Dpi was co-expressed, compared to that when an empty vector (-) or a control peptide (C) was expressed (Fig. 5d). To determine the specificity of Dpi on the interaction of TLX with its interacting partners, Dpi was expressed together with HA-TLX and Flag-HDAC5, a known transcriptional corepressor of TLX [11]. In contrast to the interaction of TLX with Drosha, the interaction of TLX with HDAC5 was not blocked by Dpi (Fig. 5e). These results indicate that Dpi specifically interferes with the interaction of TLX with Drosha, but not the interaction of TLX with a transcriptional coregulator.

**[0079]** To determine if the interaction between TLX and the miRNA processing machinery is critical for regulation of miR-219 processing by TLX, Dpi was used to block the interaction of TLX with the miRNA processing machinery and miR-219-Glo, a luciferase reporter containing the pri-miR-219 sequence in its 3' UTR to monitor miR-219 processing. Co-transfection of TLX with Dpi reduced the luciferase activity substantially, compared to transfection with TLX alone (Fig. 5f), suggesting that expression of Dpi promoted miR-219 processing from pri-miR-219. In contrast, a control peptide that contains TLX residues outside the Dpi domain failed to boost miR-219 processing (Fig. 5f).

**[0080]** The Dpi peptide was electroporated into NSCs and determined miR-219 processing by evaluating the levels of the three forms of miR-219, pri-miR-219, pre-miR-219 and mature miR-219. The levels of both pre-miR-219 and mature miR-219 forms increased considerably, whereas no significant change was detected in the level of pri-miR-219 (Figs. 5g-5i). These results further demonstrate that expressing the Dpi peptide promotes miR-219 processing but has no effect on the transcription of pri-miR-219.

**[0081]** Whether expressing Dpi could affect NSC proliferation and differentiation was tested. NSCs from E14.5 mouse brains were transduced with lentivirus expressing Dpi or a control peptide and a GFP reporter. Compared to the control peptide, expression of Dpi reduced cell proliferation substantially (Figs. 6a and 6b). To determine if inhibition of NSC proliferation by Dpi is mediated by modulating miR-

219, NSCs were co-transduced with Dpi and the miR-219 decoy inhibitor, TuD-miR-219. Expressing TuD-miR-219 rescued the Dpi-mediated inhibition of NSC proliferation substantially (Figs. 6a and 6b), suggesting that Dpi regulates NSC proliferation through modulating miR-219. Treatment with Dpi also increased the percentage of Tuj1-positive neurons significantly, compared to treatment with the control peptide, and co-expressing TuD-miR-219 reversed this effect largely (Fig. 6c). These results together indicate that the Dpi peptide inhibits NSC proliferation and promotes neuronal differentiation by modulating miR-219 expression.

**[0082]** To determine the effect of Dpi on NSC proliferation and differentiation *in vivo*, a vector expressing a control peptide or Dpi was electroporated together with an RFP reporter into E13.5 mouse brains *in utero*. Expression of Dpi decreased Ki67+RFP+ cells in the VZ/SVZ, compared to expression of the control peptide. Co-electroporating Dpi with TuD-miR-219 rescued the reduced cell proliferation induced by Dpi (Figs. 6d and 6e). These results indicate that Dpi inhibits NSC proliferation *in vivo*, presumably through regulating miR-219 expression. On the other hand, more RFP+ cells that had been electroporated with the Dpi-expressing vector migrated from the VZ/SVZ to the CP. Co-electroporating Dpi with TuD-miR-219 reversed the precocious migration largely (Figs. 6f and 6g). These results indicate that Dpi promotes neuronal differentiation *in vivo* through modulating miR-219 action. Together with the observation that Dpi promotes miR-219 processing (Figs. 5f-5i), these results suggest that modulation of miR-219 processing by TLX regulates NSC proliferation and differentiation.

#### **Example 8 Elevated miR-219 expression inhibits SCZ NSC proliferation**

**[0083]** Elevated expression of *miR-219* has been observed in various brain regions of SCZ patients [29,30,32]. Whether *miR-219* expression is altered in SCZ NSCs was tested by obtaining induced pluripotent stem cells (iPSCs) derived from SCZ patients (D1 & D2, Fig. 7a) of pedigree H [36] with a 4 bp deletion in the coding sequence of the *DISC1* gene [37,38]. This deletion causes a frameshift and premature termination of translation in *DISC1* [37,38]. The SCZ iPSCs were differentiated into NSCs. Wild type (WT) NSCs derived from iPSCs of unaffected

individuals (C1, C2 and C3) were used as controls [37,38]. Both SCZ and WT iPSC-derived NSCs expressed human NSC markers SOX1 and NESTIN (Fig. 7b).

**[0084]** RT-PCR revealed that the level of miR-219 increased substantially in *DISC1*-mutant SCZ NSCs, compared to WT control NSCs (Fig. 7c). To determine if the *DISC1* mutation is sufficient to induce elevated miR-219 expression in NSCs, the isogenic iPSC lines C1M and C3M were used, in which the 4 bp deletion seen in the SCZ patients was introduced into the *DISC1* gene in the WT control iPSC lines C1 and C3 [38]. Similar to what was seen in the SCZ NSCs, a considerable increase was observed in miR-219 level in C1M and C3M NSCs, compared to that in their isogenic WT controls (Fig. 7c). These results together indicate that miR-219 expression is elevated in NSCs of *DISC1*-mutant SCZ patients and that a *DISC1* mutation is sufficient to induce miR-219 up-regulation in NSCs. In contrast to elevated *miR-219* expression, reduced expression of *TLX* was observed in both the *DISC1*-mutant SCZ NSCs (D1 & D2) and the genetically engineered C1M and C3M NSCs that contain the *DISC1* mutation (Fig. 7d). The inverse correlation between *TLX* and miR-219 expression in SCZ NSCs further supports the hypothesis that *TLX* negatively regulates miR-219 level in NSCs.

**[0085]** Reduced NSC proliferation has been observed in post-mortem brain specimens from SCZ patients [39] and *DISC1* has been shown to regulate NSC proliferation in the developing mouse cortex [40,41]. However, whether *DISC1* regulates cell proliferation in human NSCs and whether mutant *DISC1* induces abnormal NSC proliferation remained unknown. The results showing that mutant *DISC1* induces elevated expression of miR-219 in SCZ NSCs led to a hypothesis that increased expression of miR-219 induced by mutant *DISC1* could result in abnormal cell proliferation in SCZ NSCs. To test this hypothesis, cell proliferation in WT and *DISC1*-mutant SCZ NSCs was compared by BrdU and SOX1 double labeling. The percentage of BrdU+SOX1+ cells was significantly reduced in *DISC1*-mutant SCZ NSCs, compared to that in WT control NSCs (Fig. 7e). To test whether the *DISC1* mutation is sufficient for the observed proliferative defects, cell proliferation in NSCs derived from the isogenic iPSC lines C1, C1M, and C3, C3M was compared. The decrease in NSC proliferation observed in SCZ NSCs was

recapitulated in C1M and C3M NSCs (Fig. 7e). To determine if abnormal expression of miR-219 in *DISC1*-mutant NSCs tips the balance between human NSC proliferation and differentiation, neuronal differentiation in WT and *DISC1*-mutant NSCs under a spontaneous differentiation condition was compared. Quantification of Tuj1+ neuronal cells revealed increased neuronal differentiation in *DISC1*-mutant NSCs, compared to WT NSCs (Fig. 7f), consistent with the observation of reduced NSC proliferation in *DISC1*-mutant NSCs (Fig. 7e).

**[0086]** To determine if elevated miR-219 expression is sufficient to reduce cell proliferation in human NSCs, miR-219 was overexpressed in WT NSCs using an miR-219-expressing retroviral vector and NSC proliferation was determined by BrdU and SOX1 double labeling. Reduced cell proliferation was observed in miR-219-overexpressing WT NSCs compared to control vector-expressing WT NSCs, in a manner similar to the reduced cell proliferation observed in *DISC1*-mutant NSCs when compared to WT NSCs (Fig. 8a). In parallel, the miR-219-overexpressing WT NSCs were induced for neuronal differentiation. The rate of neuronal differentiation was determined by the percentage of Tuj1+ cells. Overexpression of miR-219 in WT NSCs also increased neuronal differentiation rate, compared to that in control vector-treated WT NSCs (Fig. 15a). These results indicate that elevated miR-219 expression is sufficient to inhibit cell proliferation in human NSCs. Together with the observation that miR-219 expression is abnormally up-regulated in SCZ NSCs, these results suggest that the abnormally elevated miR-219 expression could be an underlying factor for reduced NSC proliferation observed in SCZ patients.

**[0087]** To determine if the proliferative defect in *DISC1*-mutant NSCs indeed resulted from abnormally elevated miR-219 expression, miR-219 in *DISC1*-mutant NSCs was inhibited using TuD-miR-219. NSC proliferation was monitored by BrdU and SOX1 double labeling. Treating the *DISC1*-mutant NSCs (D1, D2, C1M and C3M) with TuD-miR-219 increased the proliferative rate in these cells substantially, largely rescuing the proliferative defects of the *DISC1*-mutant NSCs (Fig. 8b). Treatment with TuD-miR-219 also reversed the elevated differentiation in *DISC1*-mutant NSCs considerably, as revealed by the reduced percentage of Tuj1+ cells, compared to that in cells treated with a control vector (Fig. 15b). These results

indicate that miR-219 plays an important role in regulating cell proliferation in *DISC1*-mutant SCZ NSCs and that an miR-219 inhibitor could rescue the proliferative defect in these cells.

**[0088]** In addition to elevated *miR-219* expression in *DISC1*-mutant SCZ NSCs, reduced expression of *TLX* in *DISC1*-mutant SCZ NSCs was also detected (Fig. 7). To test if *TLX* knockdown is able to reduce cell proliferation in human NSCs, WT NSCs were transduced with a *TLX* siRNA-expressing lentiviral vector. Reduced cell proliferation was observed in *TLX* knockdown WT NSCs, compared to control vector-transduced WT NSCs (Fig. 8c). To test if increase of *TLX* expression could rescue the proliferative defect in SCZ NSCs, *TLX* in *DISC1*-mutant NSCs was overexpressed. Elevated expression of *TLX* in *DISC1*-mutant NSCs increased cell proliferation (Fig. 8d), consistent with the observation in *DISC1*-mutant NSCs treated with TuD-miR-219 (Fig. 8b). These results together indicate that the *TLX*-miR-219 cascade is important in regulating cell proliferation in *DISC1*-mutant SCZ NSCs.

**[0089]** All publications and patent documents cited herein are incorporated by reference.

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## CLAIMS

I/We claim:

1. A method of correcting a defective rate of proliferation in a population of neural stem cells (NSCs) comprising:  
contacting the population of NSCs with an effective amount of an miR-219 inhibitor, wherein the miR-219 inhibitor causes an increase in NSC proliferation rate.
2. The method of claim 1, wherein contact of the population of NSCs with the miR-219 inhibitor takes place *in vitro*.
3. The method of claim 2, wherein the population of NSCs is derived from a subject suffering from schizophrenia, bipolar disorder, or depression.
4. The method of claim 1, wherein contact of the population of NSCs with the miR-219 inhibitor takes place *in vivo*.
5. The method of claim 4, wherein the population of NSCs is present in the nervous system of a subject suffering from schizophrenia, bipolar disorder, or depression.
6. The method of claim 5, wherein the increase in NSC proliferation rate maintains normal NSC proliferation of the subject.
7. The method of claim 1, wherein the miR-219 inhibitor is a tough decoy RNA, an RNAi molecule, or an aptamer.
8. The method of claim 7, wherein the miR-219 inhibitor is an miR-219-5p hairpin inhibitor or TuD-miR-219.
9. The method of claim 1, further comprising contacting the population of NSCs with an effective amount of an agent to increase expression or activity of TLX.
10. The method of claim 9, wherein the agent is a vector expressing a gene encoding TLX.

11. A method of correcting a defective rate of proliferation in a population of neural stem cells (NSCs) comprising:  
contacting the population of NSCs with an effective amount of an agent to increase expression or activity of TLX, wherein the agent causes an increase in NSC proliferation rate.
12. The method of claim 11, wherein contact of the population of NSCs with the agent takes place *in vitro*.
13. The method of claim 12, wherein the population of NSCs is derived from a subject suffering from schizophrenia, bipolar disorder, or depression.
14. The method of claim 11, wherein contact of the population of NSCs with the agent takes place *in vivo*.
15. The method of claim 14, wherein the population of NSCs is present in the nervous system of a subject suffering from schizophrenia, bipolar disorder, or depression.
16. The method of claim 15, wherein the increase in NSC proliferation rate maintains normal NSC proliferation of the subject.
17. The method of claim 11, wherein the agent is a vector expressing a gene encoding TLX.
18. The method of claim 11, further comprising contacting the population of NSCs with an effective amount of an miR-219 inhibitor.
19. The method of claim 18, wherein the miR-219 inhibitor is a tough decoy RNA, an RNAi molecule, or an aptamer.
20. The method of claim 19, wherein the miR-219 inhibitor is an miR-219-5p hairpin inhibitor or TuD-miR-219.
21. A method of treating a neurodevelopmental disorder in a subject comprising:  
administering a therapeutically effective dose of a pharmaceutical composition

to the subject, the pharmaceutical composition comprising an miR-219 inhibitor, wherein the miR-219 inhibitor maintains normal NSC proliferation in the subject.

22. The method of claim 21, wherein the subject is suffering from schizophrenia, bipolar disorder, or depression.

23. The method of claim 21, wherein the miR-219 inhibitor is a tough decoy RNA, an RNAi molecule, or an aptamer.

24. The method of claim 22, wherein the miR-219 inhibitor is an miR-219-5p hairpin inhibitor or TuD-miR-219.

25. The method of claim 21, wherein the pharmaceutical composition further comprises an agent to increase expression or activity of TLX.

26. The method of claim 25, further comprising administering a therapeutically effective dose of a second pharmaceutical composition to the subject, the pharmaceutical composition comprising an agent to increase expression or activity of TLX.

27. The method of claim 25 or 26, wherein the agent is a vector expressing a gene encoding TLX.

28. The method of claim 21, wherein administration of the pharmaceutical composition is an oral, intravenous, intrathecal, or intracranial route of administration.

29. A method of treating a neurodevelopmental disorder in a subject comprising:  
administering a therapeutically effective dose of a pharmaceutical composition to the subject, the pharmaceutical composition comprising an agent to increase expression or activity of TLX, wherein the agent maintains normal NSC proliferation in the subject.

30. The method of claim 29, wherein the subject is suffering from schizophrenia, bipolar disorder, or depression.

31. The method of claim 29, wherein the agent is a vector expressing a gene encoding TLX.
32. The method of claim 29, wherein the pharmaceutical composition further comprises an miR-219 inhibitor.
33. The method of claim 29, further comprising administering a therapeutically effective dose of a second pharmaceutical composition to the subject, the pharmaceutical composition comprising an miR-219 inhibitor.
34. The method of claim 32 or 33, wherein the miR-219 inhibitor is a tough decoy RNA, an RNAi molecule, or an aptamer.
35. The method of claim 34, wherein the miR-219 inhibitor is an miR-219-5p hairpin inhibitor or TuD-miR-219.

Figure 1

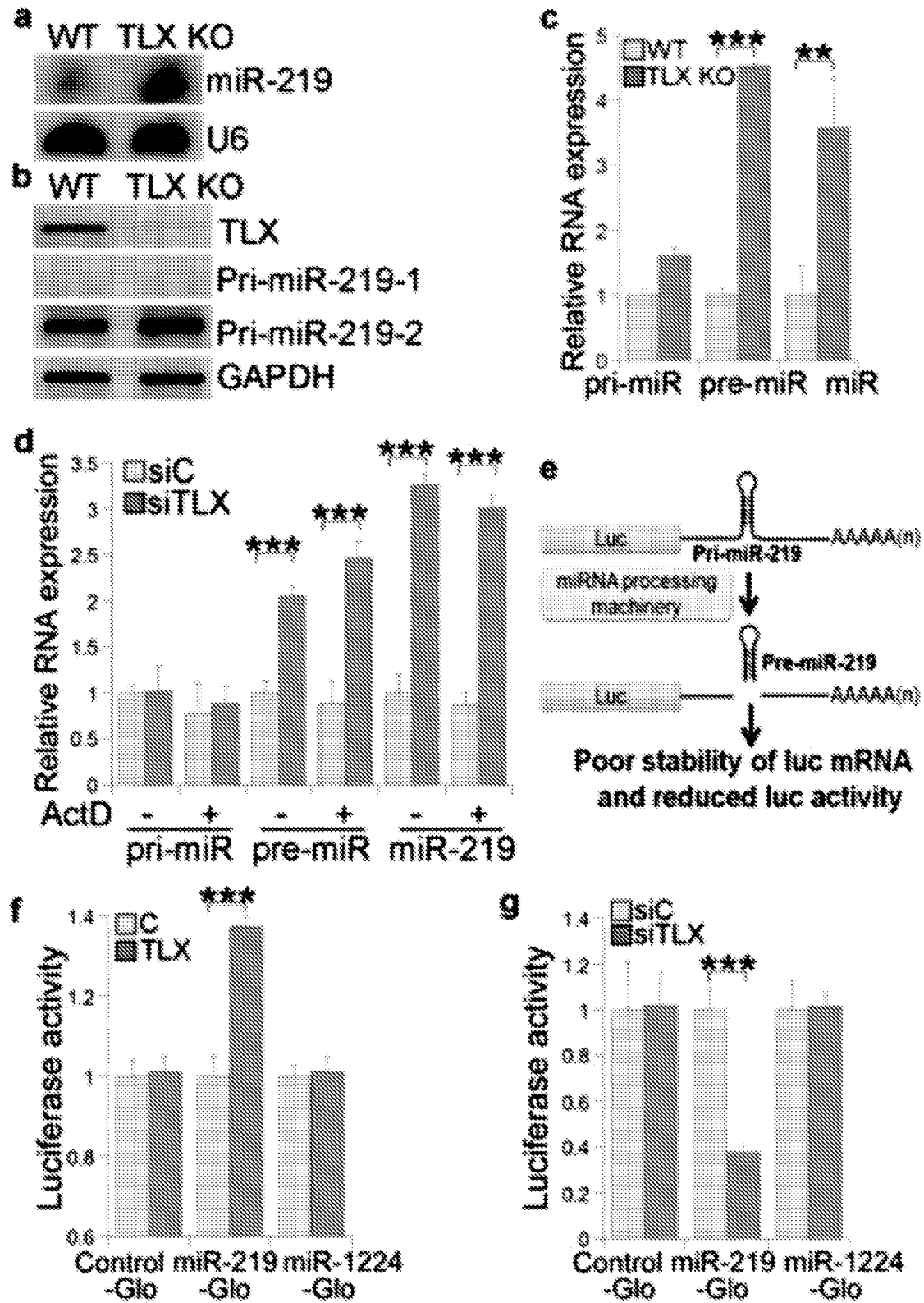


Figure 2

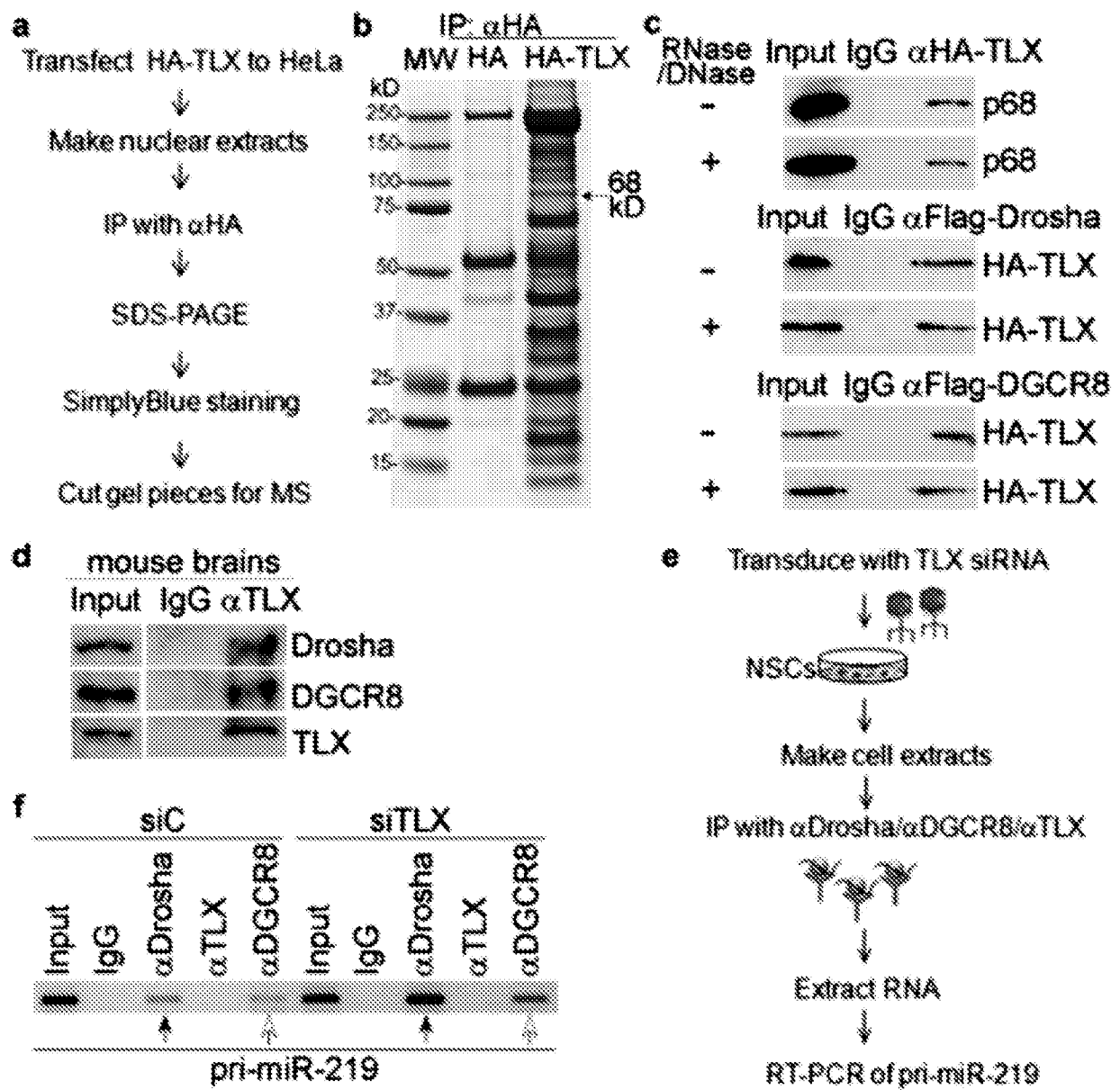


Figure 3

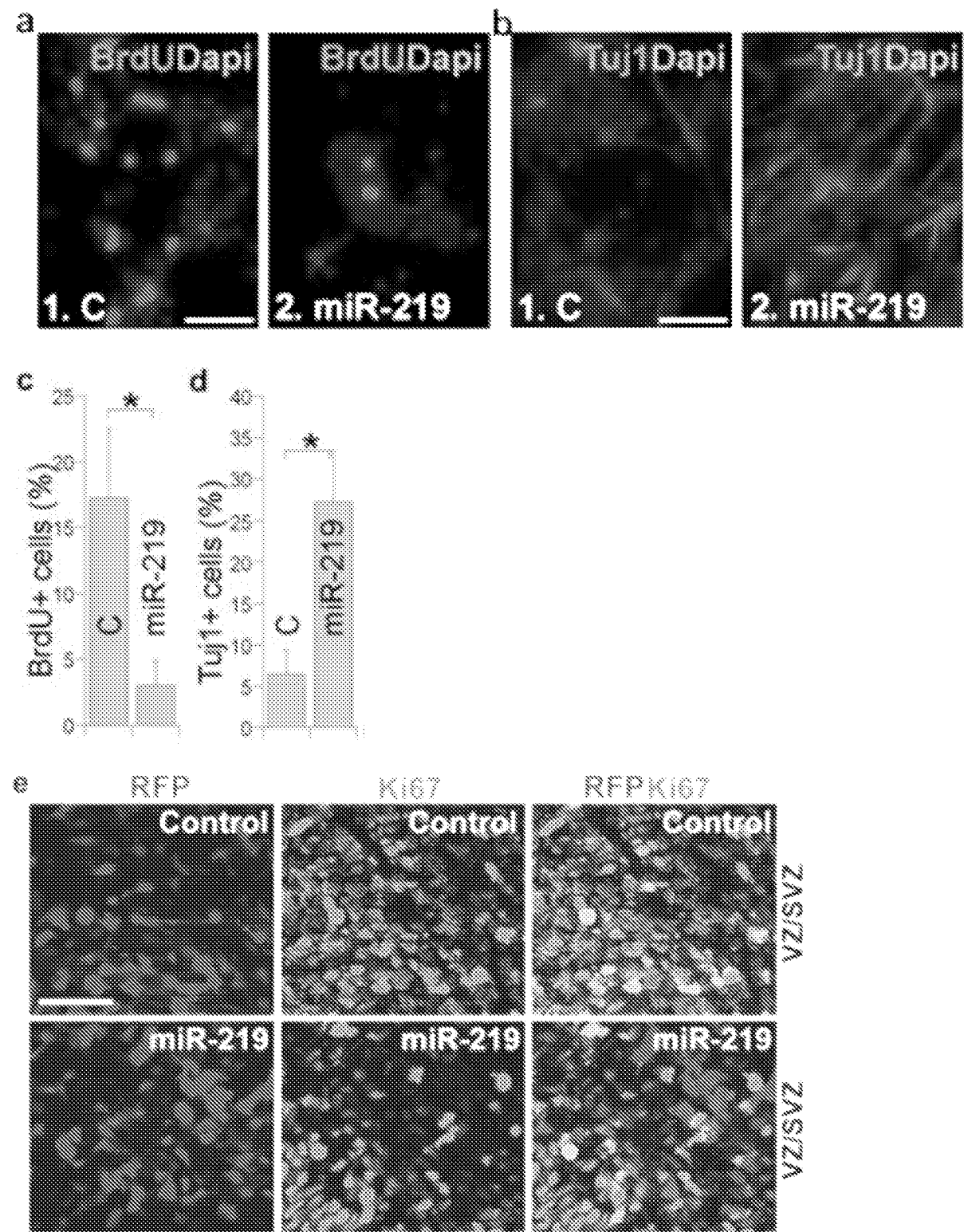


Figure 3 (continued)

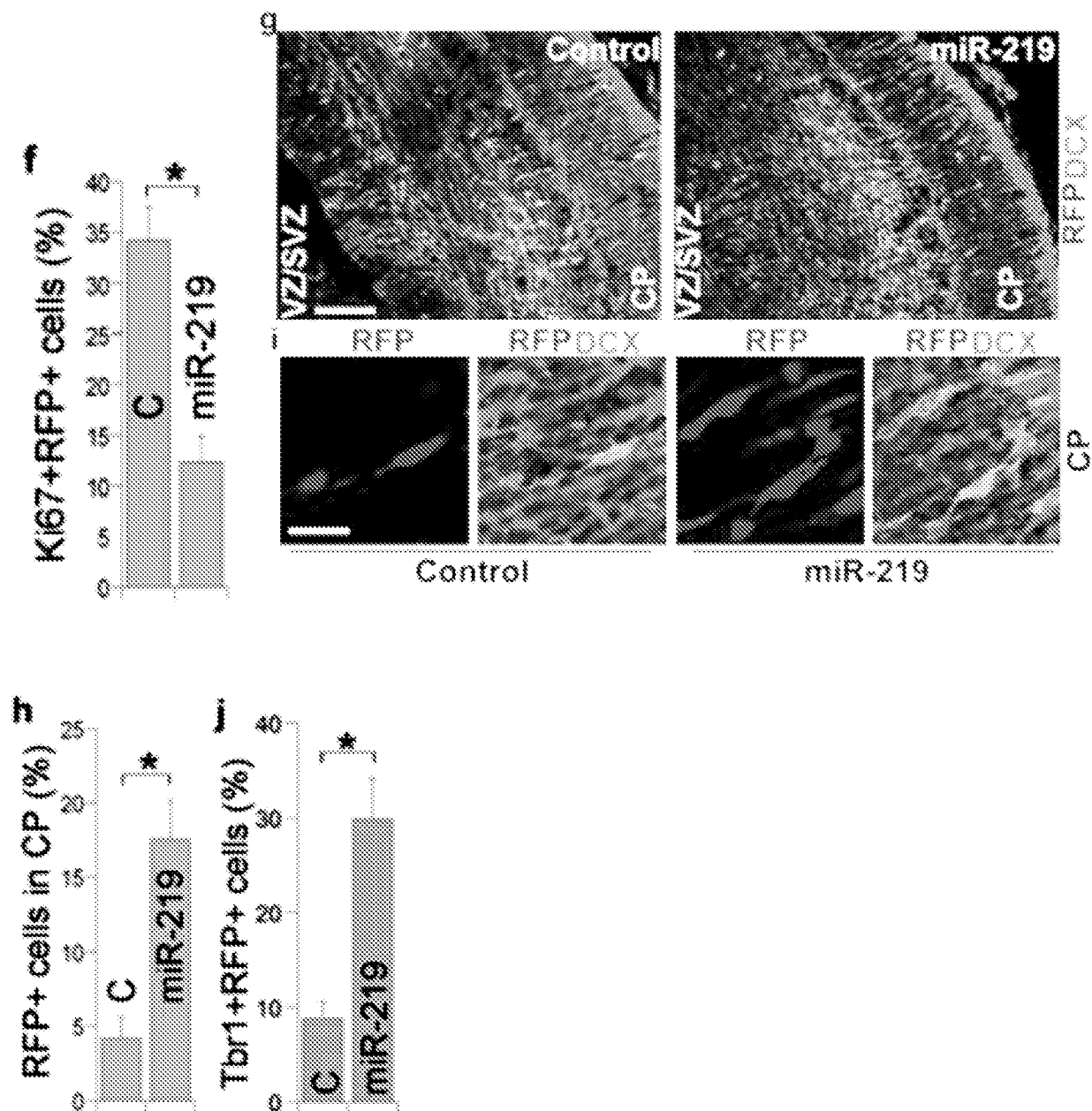


Figure 4

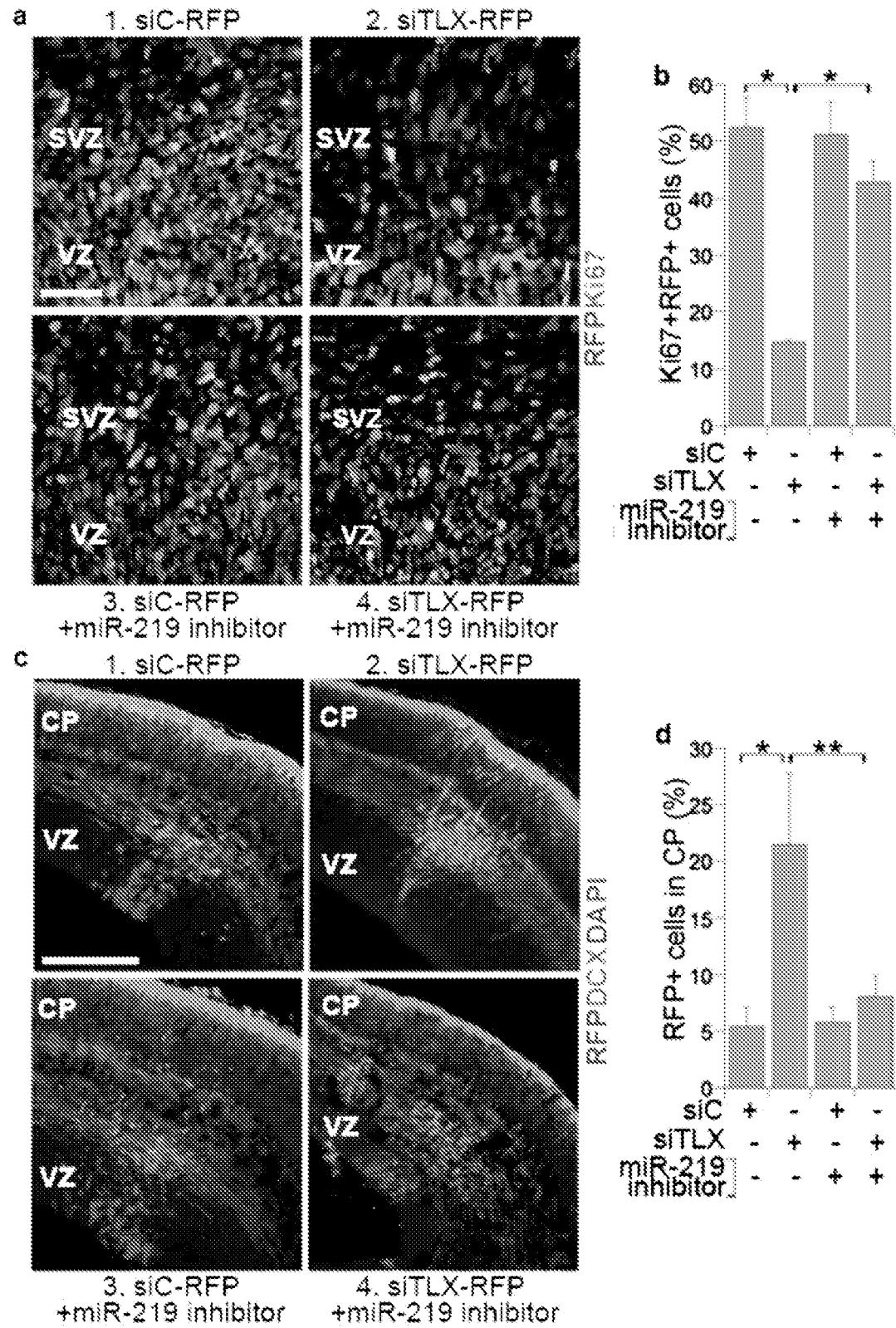


Figure 5

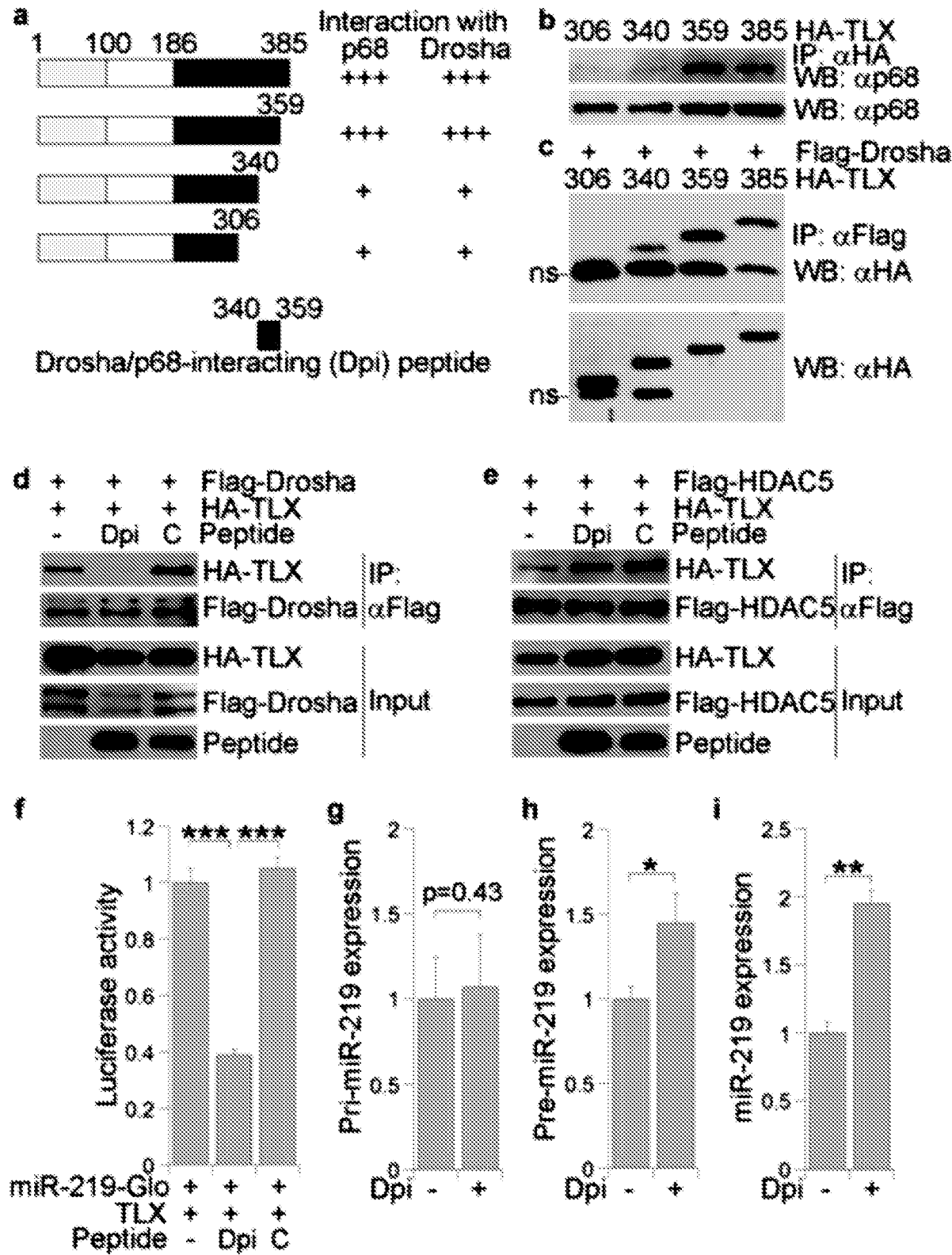


Figure 6

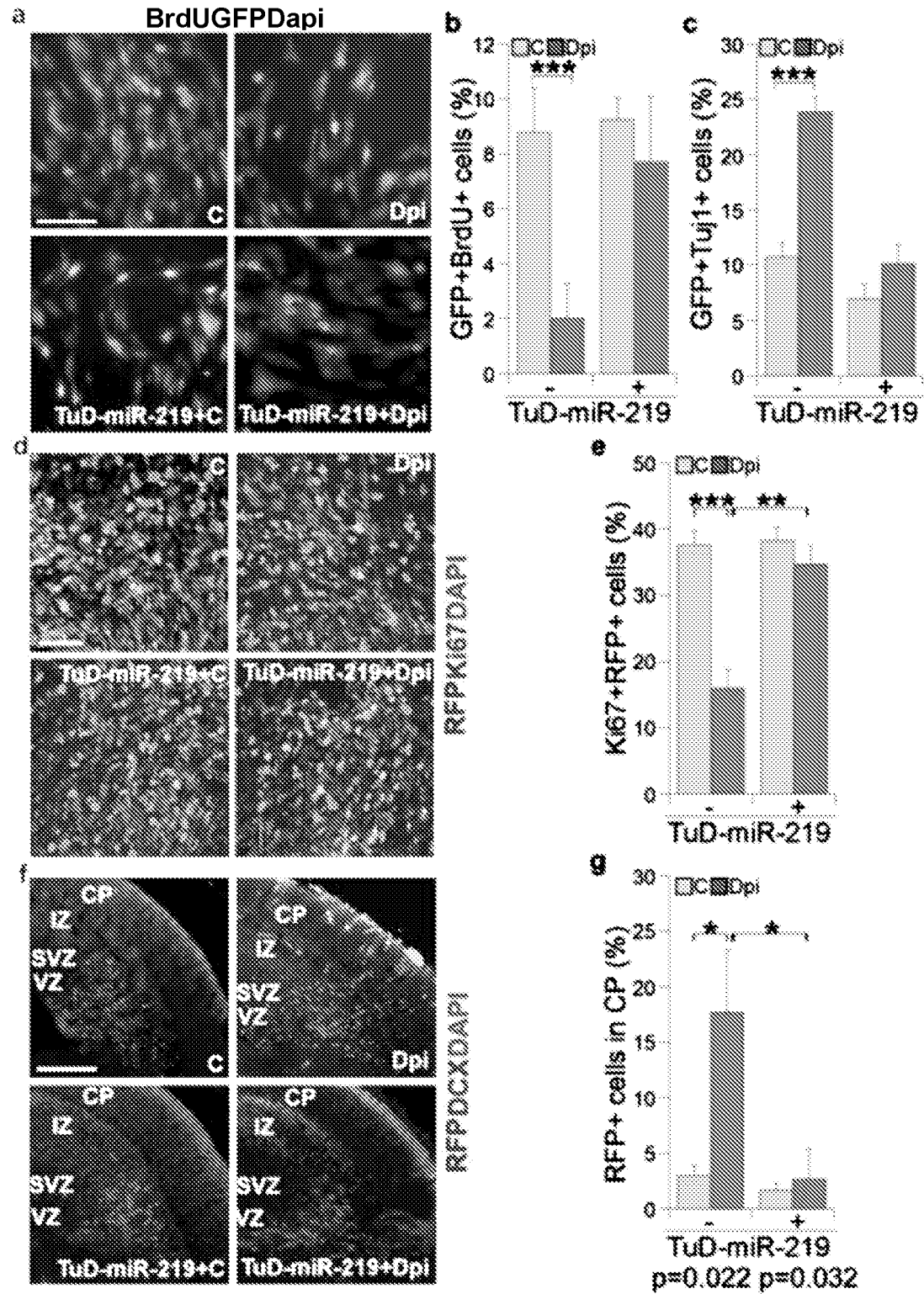


Figure 7

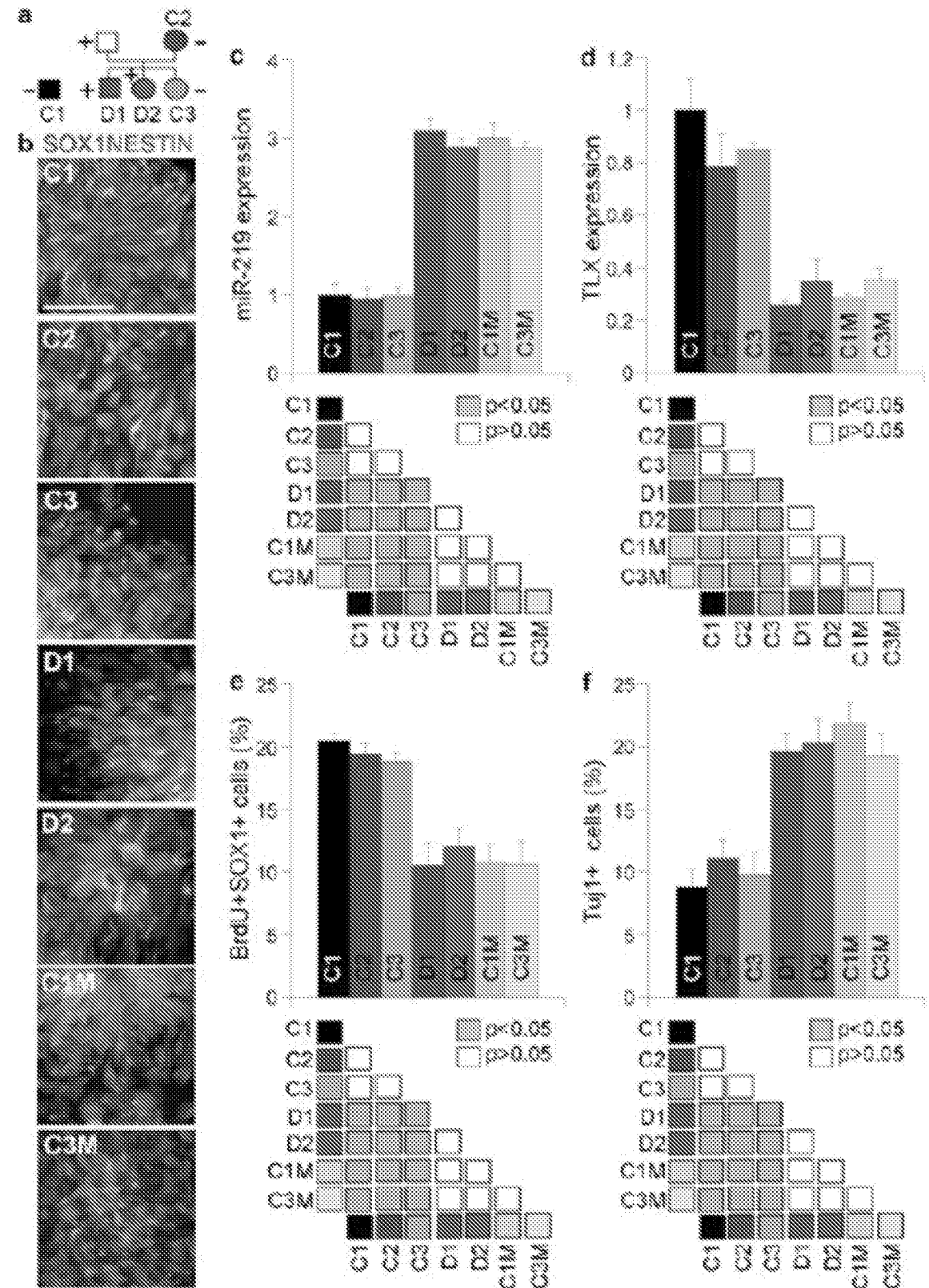


Figure 8

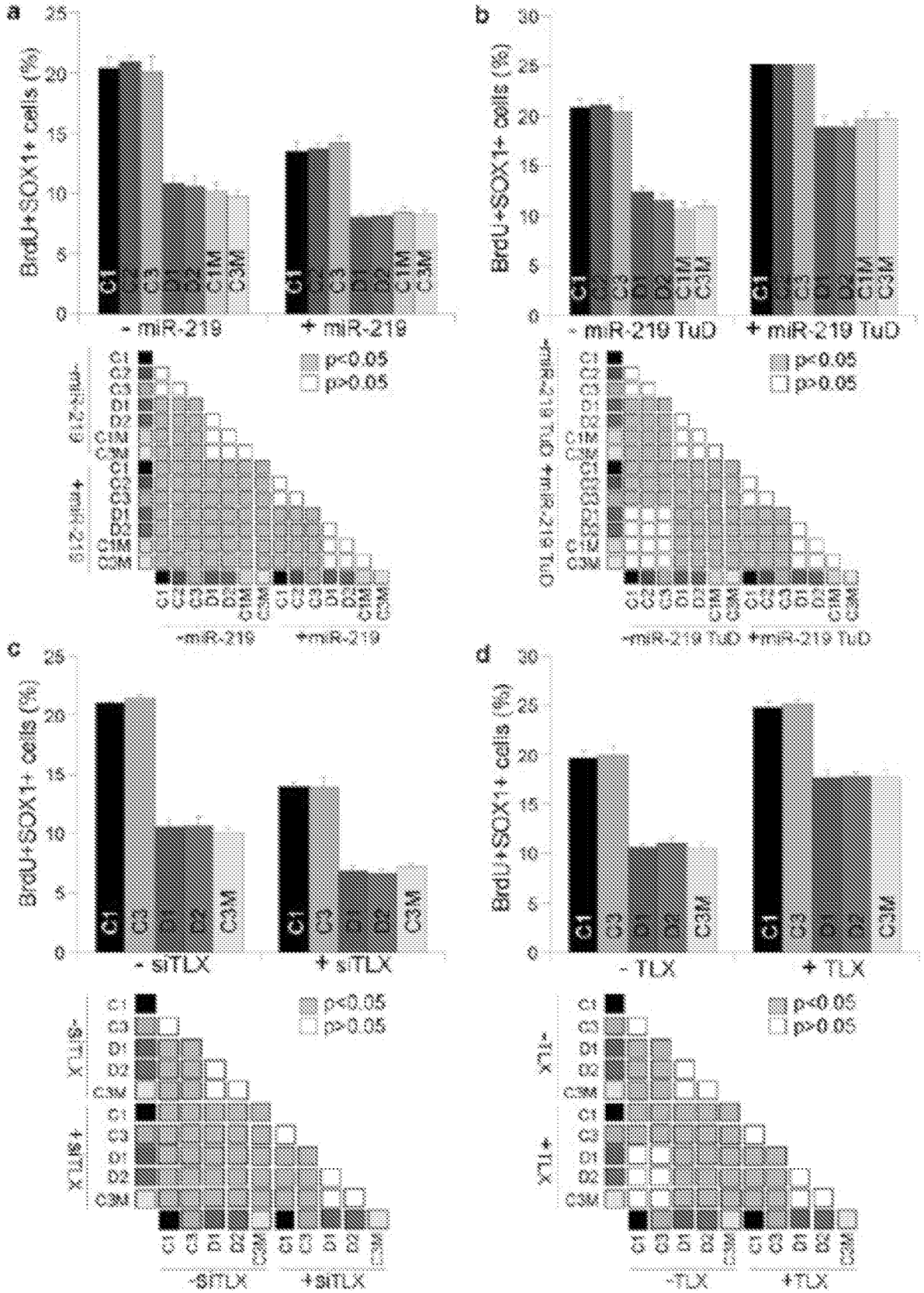


Figure 9

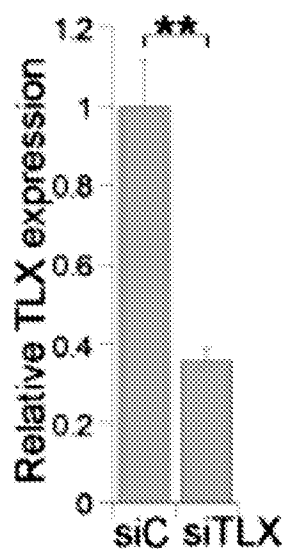


Figure 10

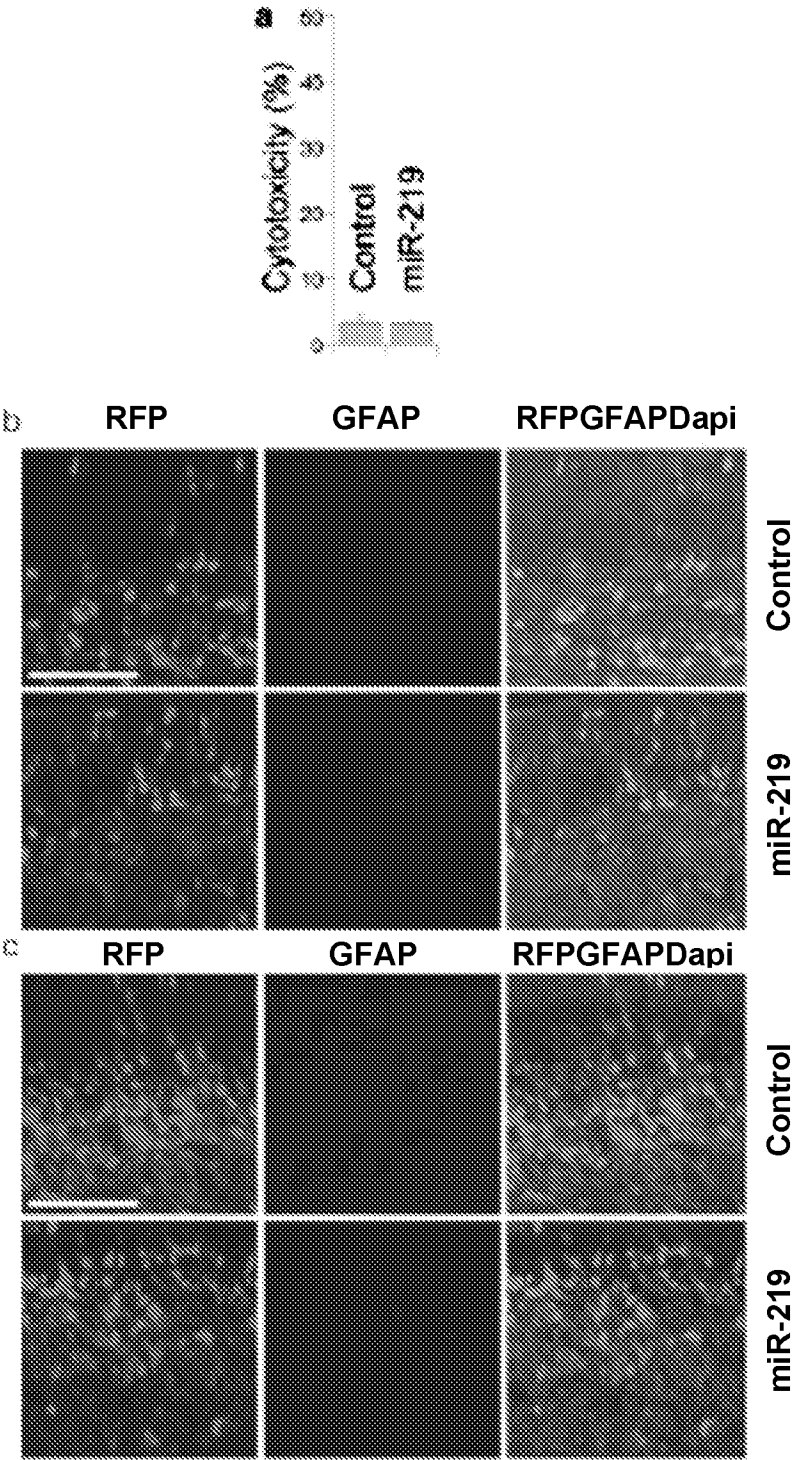


Figure 11

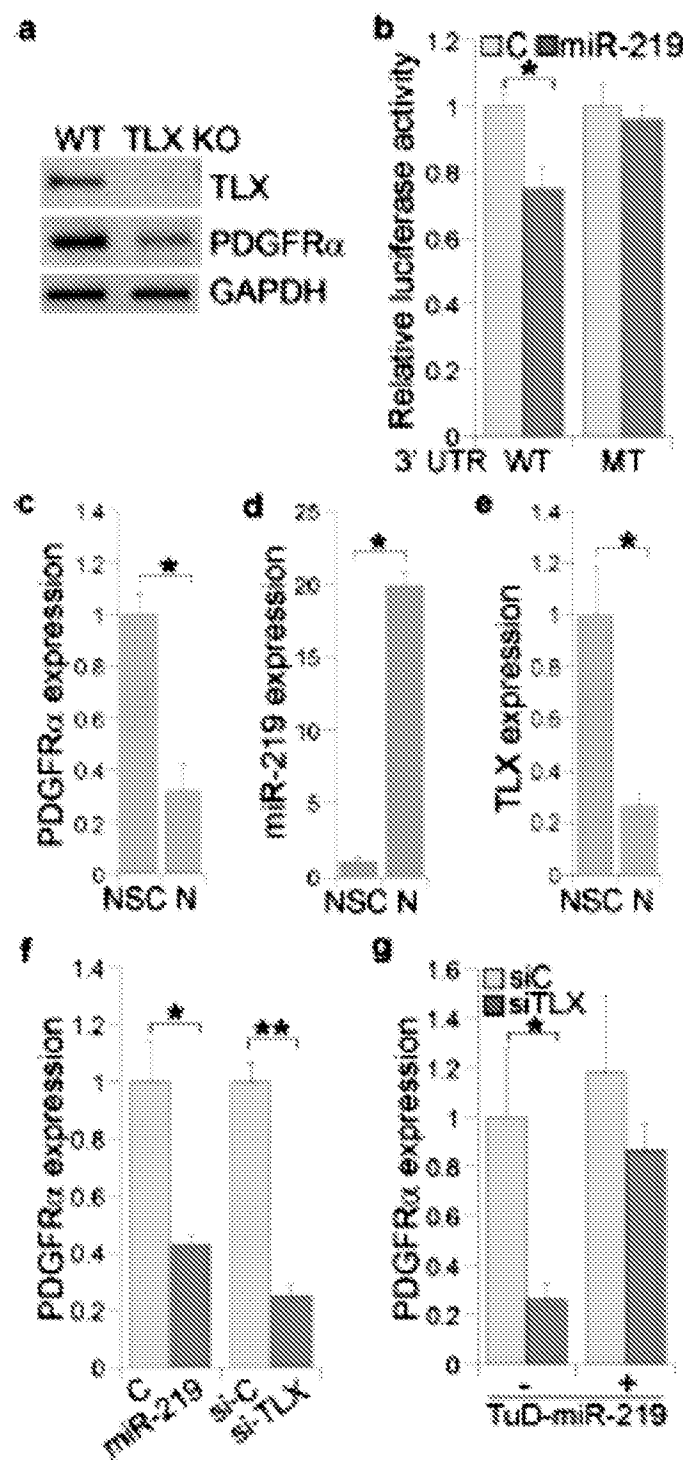


Figure 12

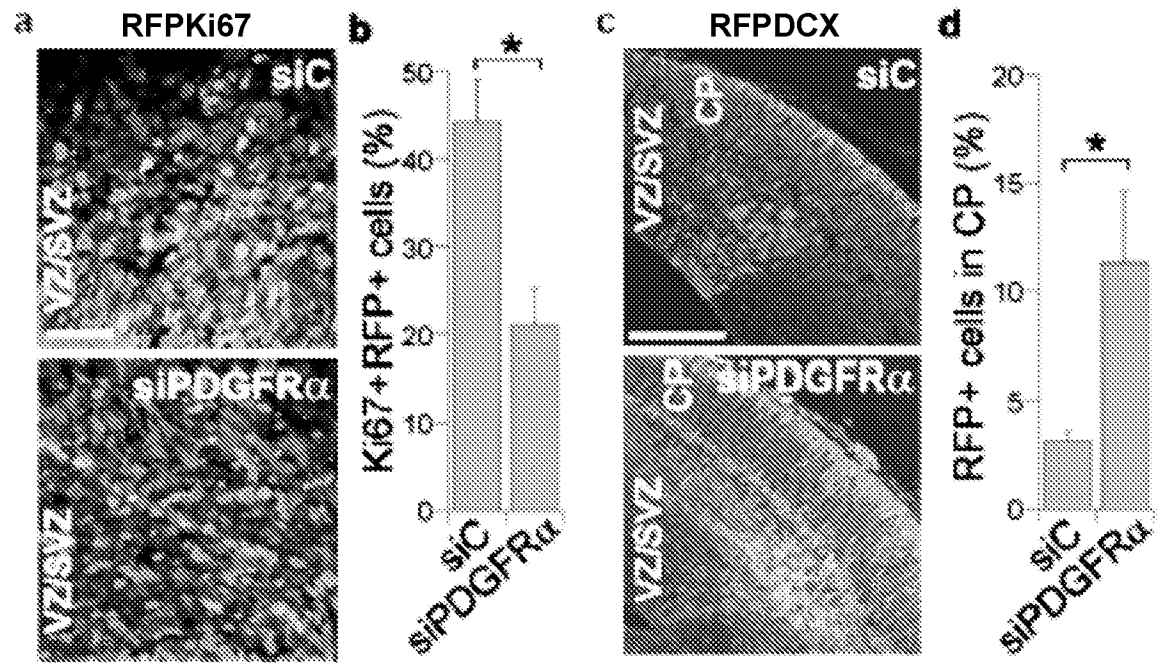


Figure 13

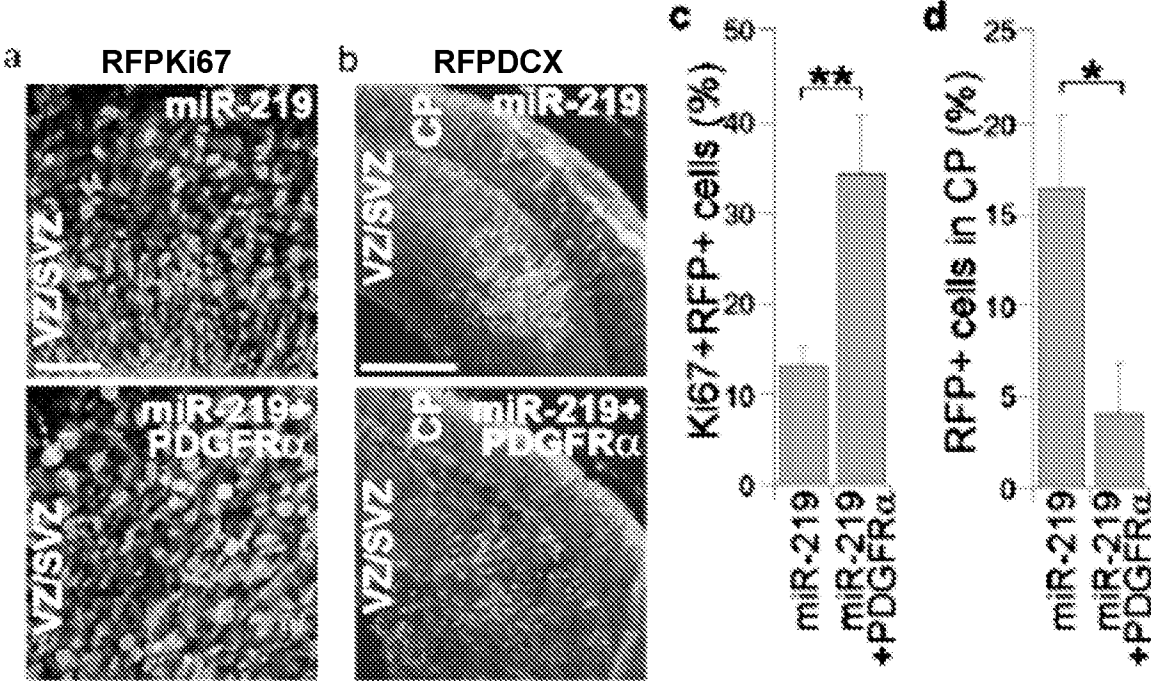


Figure 14

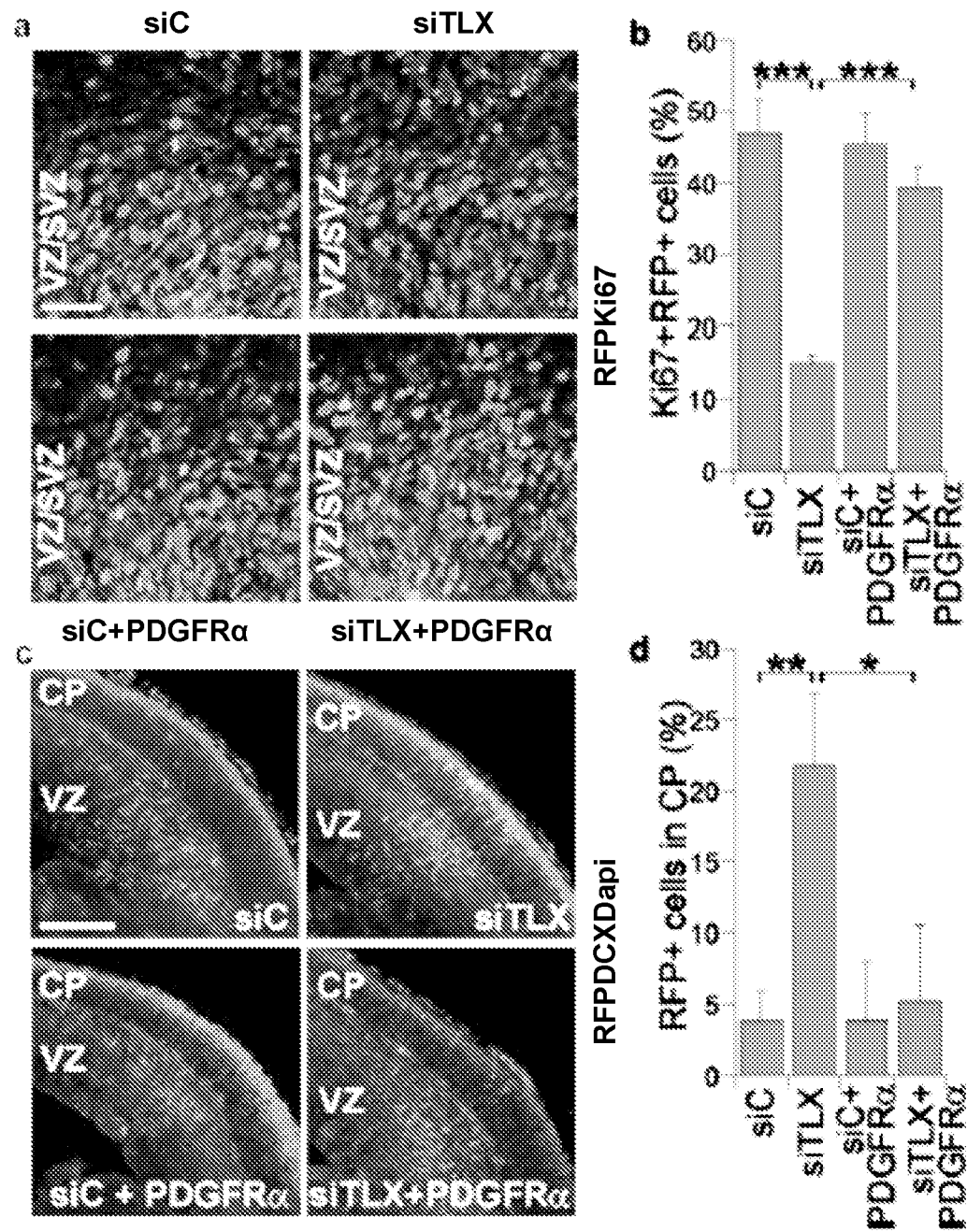
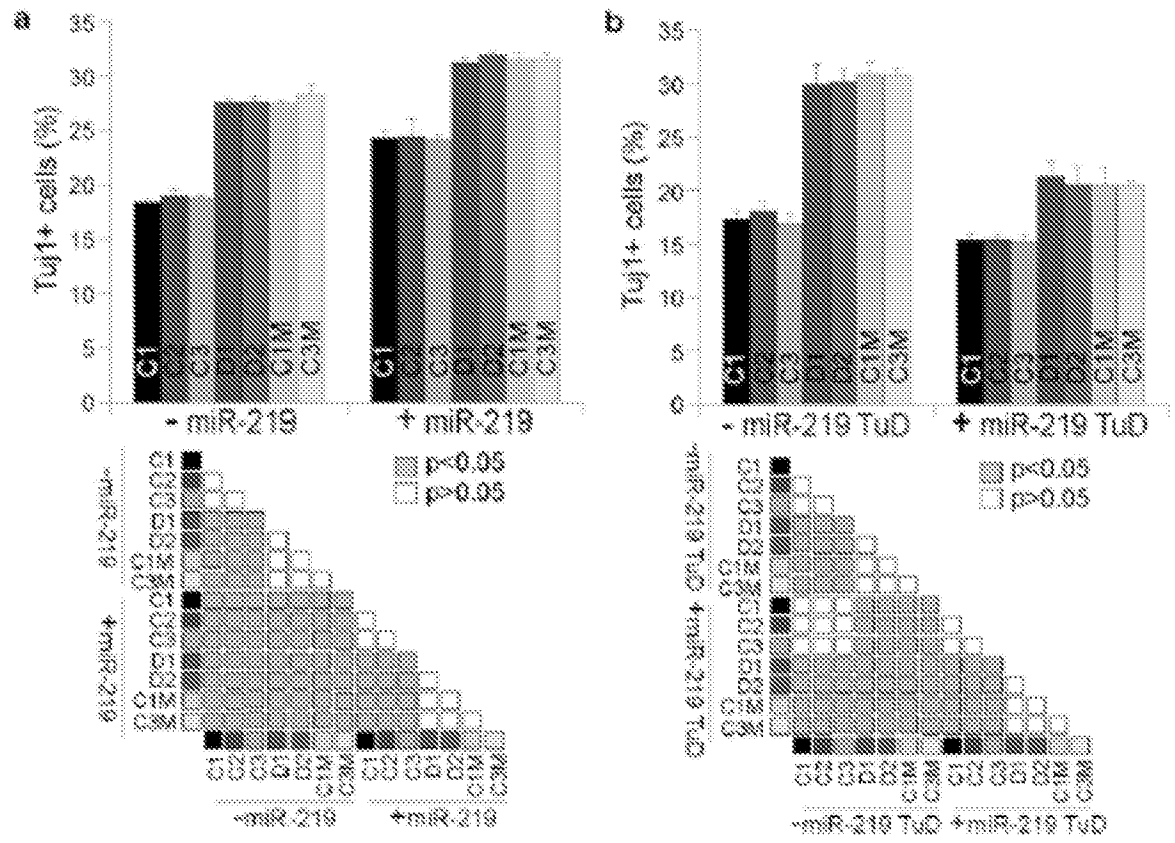


Figure 15



**Figure 16**

| Gene           | Strand    | Sequence                       | Assay         | SEQ ID NO: |
|----------------|-----------|--------------------------------|---------------|------------|
| miR-219        | Antisense | 5'-AGAATTGCGTTTGGACAATCA-3'    | Northern blot | 1          |
| U6             | Antisense | 5'-TATGGAACGCTTCTCGAATT-3'     | Northern blot | 2          |
| mTLX           | Forward   | 5'-GTCTTTACAAGATCAGCTGATG-3'   | RT-PCR        | 3          |
|                | Reverse   | 5'-ATGTCACCTGGATTGTACATATC-3'  | RT-PCR        | 4          |
| pri-miR-219-1  | Forward   | 5'-TTTCCCACGCCAGACATTAC-3'     | RT-PCR        | 5          |
|                | Reverse   | 5'-GATCCCCAACTTCTCTCAAGC-3'    | RT-PCR        | 6          |
| pri-miR-219-2  | Forward   | 5'-TTGCCGAGCTTCTGCGAGGTA-3'    | RT-PCR        | 7          |
|                | Reverse   | 5'-TGTCCTCTCTTTCATGCCAG-3'     | RT-PCR        | 8          |
| PDGFR $\alpha$ | Forward   | 5'-CAAACCTGACCATGCCACCAG-3'    | RT-PCR        | 9          |
|                | Reverse   | 5'-TCTCGATGGCACTCTCTTCCG-3'    | RT-PCR        | 10         |
| ROR $\beta$    | Forward   | 5'-TACGTGGTGGAGTTCGCCAAG-3'    | RT-PCR        | 11         |
|                | Reverse   | 5'-CCCATGCAAGTTGCAGACTGC-3'    | RT-PCR        | 12         |
| LMO3           | Forward   | 5'-GTTTGGTGTAAACGGGAACTGCG-3'  | RT-PCR        | 13         |
|                | Reverse   | 5'-TCCTCGTAGTCTGTCTGGCAAAG-3'  | RT-PCR        | 14         |
| HMGA2          | Forward   | 5'-ACATCAGCCCAGGGACAACCT-3'    | RT-PCR        | 15         |
|                | Reverse   | 5'-CAAGAGTCCGCAGAGGAGGAT-3'    | RT-PCR        | 16         |
| EphrinB2       | Forward   | 5'-TTCAGCCCTAACCTCTGGGGT-3'    | RT-PCR        | 17         |
|                | Reverse   | 5'-AACCCAGGAGATTGTTCCCGG-3'    | RT-PCR        | 18         |
| mGAPDH         | Forward   | 5'-CATCACCATCTTCCAGGAGC-3'     | RT-PCR        | 19         |
|                | Reverse   | 5'-GCTGTAGCCGTATTCATTGTC-3'    | RT-PCR        | 20         |
| pri-miR-219-2  | Forward   | 5'-TACGCAGCTCCCGAGATCTGGTG-3'  | RT-PCR        | 21         |
|                | Reverse   | 5'-CAGCGTGGACCTCGTCTCTGTAG-3'  | RT-PCR        | 22         |
| pre-miR-219-2  | Forward   | 5'-CTGATTGTCCAAACGCAATTCTTG-3' | RT-PCR        | 23         |
|                | Reverse   | 5'-CAGATGTCCAGCCACAATTCTC-3'   | RT-PCR        | 24         |
| PDGFR $\alpha$ | Forward   | 5'-GTGGCCTGGACGAACAGAGACT-3'   | RT-PCR        | 25         |
|                | Reverse   | 5'-GGAACCTGTCTCGATGGCACTC-3'   | RT-PCR        | 26         |
| m TLX          | Forward   | 5'-GGTTCAGACAGCTCCGATTAGAC-3'  | RT-PCR        | 27         |
|                | Reverse   | 5'-TGGAGAGCGGCAATGGCGGCAGC-3'  | RT-PCR        | 28         |
| $\beta$ -Actin | Forward   | 5'-CCGAGCGTGGCTACAGCTTC-3'     | RT-PCR        | 29         |
|                | Reverse   | 5'-ACCTGGCCGTCAGGCAGCTC-3'     | RT-PCR        | 30         |
| hTLX           | Forward   | 5'-CTAAGAGTGTGCCAGCCTTC-3'     | RT-PCR        | 31         |
|                | Reverse   | 5'-TGTTAGCATCAACCGGAATGG-3'    | RT-PCR        | 32         |
| hGAPDH         | Forward   | 5'-CCTGTTTCGACAGTCAGCCG-3'     | RT-PCR        | 33         |
|                | Reverse   | 5'-CGACCAAATCCGTTGACTC-3'      | RT-PCR        | 34         |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/21962

## A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 5/0797, 15/113; C07K 14/705 (2017.01)

CPC - C12N 5/0623, 15/113; C07K 14/70567

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category*    | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                      | Relevant to claim No.                                                                                   |
|--------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|
| X<br>--<br>Y | US 2006/0040321 A1 (SHI et al.) February 23, 2006; abstract; paragraphs [0012], [0014], [0030], [0070]-[0071], [0098], [0106]                                                                                                                                                                                                           | 11, 12, 14, 17, 29, 31<br>-----<br>6, 9-10, 13, 15, 16, 18-26,<br>27/25-26, 28, 30, 32, 33,<br>34/32-33 |
| Y            | ZHAO, C et al., MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling. PNAS. 02 February 2010. Vol. 107, No. 5, pp. 1876-1881, DOI: 10.1073/pnas.0908750107; abstract; page 1878, first column, second paragraph; page 1880, second column, fourth paragraph         | 1-7, 9, 10                                                                                              |
| Y            | KOCERHA, J et al., MicroRNA-219 modulates NMDA receptor-mediated neurobehavioral dysfunction. PNAS. 03 March 2009. Vol. 106, No. 9, pp. 3507-3512, DOI: 10.1073/pnas.0805854106; abstract; page 3509, first column, second paragraph; page 3509, second column, second and fourth paragraphs; page 3510, first column, second paragraph | 1-7, 9, 10, 18-26,<br>27/25-26, 28, 32, 33,<br>34/32-33                                                 |
| Y            | US 2010/0227908 A1 (CAIRNS) September 9, 2010; abstract; paragraphs [0021], [0078]                                                                                                                                                                                                                                                      | 3, 5, 6, 13, 15, 16, 22, 30                                                                             |
| A            | HUDISH, LI et al., miR-219 Regulates Neural Precursor Differentiation by Direct Inhibition of Apical Par Polarity Proteins. Developmental Cell. 25 November 2013. Vol. 27, pp. 387-398, DOI: 10.1016/j.devcel.2013.10.015; page 388, first column, third paragraph                                                                      | 1-10, 21-26, 27/25-26, 28                                                                               |
| A            | THOMAS, M et al., PEI-complexed LNA antiseeds as miRNA inhibitors. RNA Biology. August 2012. Vol. 9, Issue 8, pp. 1088-1098, DOI: 10.4161/ma.21165; abstract                                                                                                                                                                            | 8, 20, 35                                                                                               |



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&amp;" document member of the same patent family

Date of the actual completion of the international search

29 May 2017 (29.05.2017)

Date of mailing of the international search report

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Name and mailing address of the ISA/

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/21962

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                              |                                     |
|-------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|
| Category*                                             | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                           | Relevant to claim No.               |
| A                                                     | HARAGUCHI, T et al., Vectors expressing efficient RNA decoys achieve the long-term suppression of specific microRNA activity in mammalian cells. Nucleic Acids Research. 17 February 2009; Vol. 37, No. 6, DOI: 10.1093/nar/gkp040; abstract | 8, 20, 24, 35                       |
| P, X                                                  | MURAI, K et al., The TLX-miR-219 cascade regulates neural stem cell proliferation in neurodevelopment and schizophrenia iPSC model. Nature Communications. 11 March 2016. Vol. 7, No. 10965, DOI: 10.1038/ncomms10965                        | 1-26, 27/25-26, 28-33, 34/32-33, 35 |