SCIENTIFIC REPORTS

OPEN

MicroRNA let-7d regulates the TLX/microRNA-9 cascade to control neural cell fate and neurogenesis

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MicroRNAs have important functions in the nervous system through post-transcriptional regulation of neurogenesis genes. Here we show that microRNA let-7d, which has been implicated in cocaine addiction and other neurological disorders, targets the neural stem cell regulator TLX. Overexpression of let-7d *in vivo* reduced neural stem cell proliferation and promoted premature neuronal differentiation and migration, a phenotype similar to those induced by TLX knockdown or overexpression of its negatively-regulated target, microRNA-9. We found a let-7d binding sequence in the *tlx* 3′ UTR and demonstrated that let-7d reduced TLX expression levels in neural stem cells, which in turn, up-regulated miR-9 expression. Moreover, co-expression of let-7d and TLX lacking its 3′ UTR *in vivo* restored neural stem cell proliferation and reversed the premature neuronal differentiation and migration. Therefore, manipulating let-7d and its downstream targets could be a novel strategy to unravel neurogenic signaling pathways and identify potential interventions for relevant neurological disorders.

Neural stem cells are self-renewing, multipotent precursor cells in the nervous system[1]. Although much effort has been devoted to understanding the mechanisms of neural stem cell proliferation and self-renewal, the mechanisms they use to differentiate and then subsequently mature into neurons are not well characterized. Recently, it has been discovered that microRNAs (miRNAs) can affect multiple processes in the cell including cell proliferation, differentiation, and development[2,3]. On average, miRNAs are 20–22 bp in length and act by binding to the 3′-terminus of their target mRNAs to inhibit gene expression through mRNA degradation or suppression of translation[2,3]. We and others have shown that miRNAs play an important role in neurogenesis; they regulate a diverse number of target genes that have been directly implicated in the neurogenic process[4–6].

One of the founding members of the miRNA family is lethal-7 (let-7), which plays many important roles in normal development and diseases[7–13]. Recently, the functions played by let-7 miRNAs in the nervous system have begun to be identified. During brain development, let-7a can regulate the neuronal differentiation of neural precursors[14]. We and others have shown that in both embryonic and aging brains, the self-renewal of neural stem cells is negatively regulated by let-7b[15,16]. Another member is let-7d, whose expression is highly enriched in the brain and there is evidence to suggest that it plays a role in reward circuit and addiction[17,18]. However, the role that let-7d plays in the control of neural stem cell fate and neurogenesis is unknown.

Previously, we showed that TLX plays a critical role in regulating the maintenance and self-renewal of adult neural stem cells[19,20]. The neural stem cells in the hippocampus that are TLX-positive contribute to learning and memory acquisition in adult mice[21], whereas the TLX-positive neural cells in the subventricular zones of adult brains are the relatively quiescent stem cells[22–24]. TLX also regulates neural development by controlling the cell cycle progression of neural precursor cells in the developing brain[25–27]. Mechanistically, TLX regulates genes that control cell cycle and cell proliferation by recruiting histone modifying enzymes to their promoters[28,29]. In addition, TLX promotes neural stem cell self-renewal by activating canonical Wnt signaling[30]. Furthermore, under hypoxia, TLX maintains the proliferation of adult hippocampal neural progenitors through its regulation of Oct4 expression, and induces Mash1 to activate neuronal lineage commitment[31,32]. Recently, we showed that TLX can regulate the cell fate determination of neural stem cells by repressing the expression of miR-9 and miR-137. These miRNAs in turn regulate their own downstream target genes, including TLX. The expression of TLX can be inhibited by miR-9 and let-7b, both of which can bind to the *tlx* 3′ UTR[15,33,34]. Whether TLX interacts with other miRNAs, including let-7d, and what the functional outcome of these interactions could be, remains unknown.

In this study we show that let-7d functions as a key regulator of neurogenesis through its regulation of the expression of the TLX and miR-9 cascade in the mouse brain. When overexpressed, let-7d inhibited neural stem cell proliferation, accelerated neural differentiation and enhanced neuronal migration, a phenotype similar to those induced by both TLX-knockdown and overexpression of miR-9 in embryonic mouse brains.

Results

Let-7d inhibits neural stem cell proliferation and accelerates differentiation. Although the roles of miRNAs have begun to be appreciated in the nervous system, the expression and function of let-7d in mammalian brains has yet to be studied. We show here that let-7d is highly expressed in the mouse brain, lung and kidney

(Fig. 1a). As shown by Northern blot analysis, let-7d is expressed in neural stem cells (d0) at a very low level. The expression is increased over a 7-day differentiation time course (Fig. 1b). To investigate the role of let-7d in neural stem cell proliferation, we transfected mouse neural stem cells with synthetic let-7d RNA duplexes using concentrations ranging from 0 to 200 nM, and then treated the transfected cells with 5-bromodeoxyuridine (BrdU). The BrdU-treated cells were then immunostained using a BrdU antibody to determine cell proliferation status. Treatment with let-7d suppressed cell proliferation in a dose-dependent manner, as revealed by the dramatically reduced percentage of BrdU-positive cells in response to increasing concentrations of let-7d (Fig. 1c, d). The decreased BrdU labeling in the let-7d-transfected cells was unlikely to have been caused by cell death because similarly low
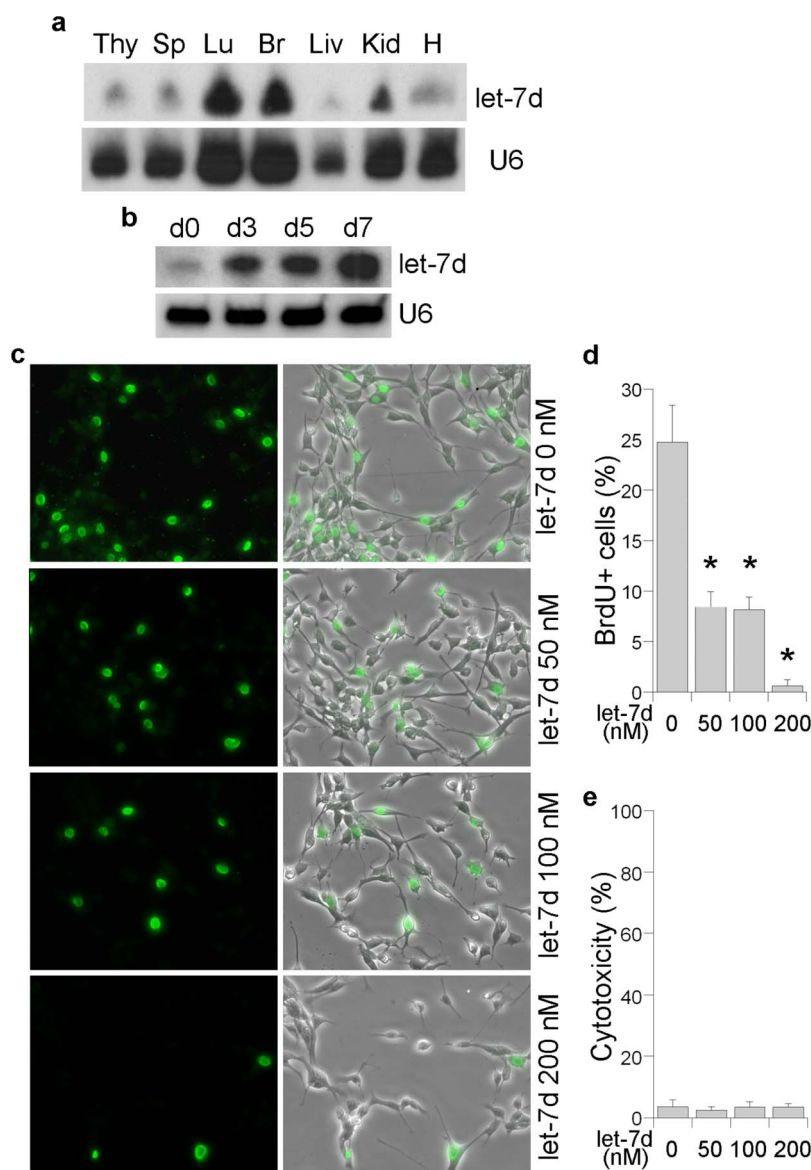

Figure 1 | Let-7d is expressed in neural stem cells and down-regulates neural stem cell proliferation. (a). Northern blot analysis of let-7d expression in different mouse tissues. U6 was used as the loading control. (b). Northern blot analysis of let-7d expression in mouse neural stem cells over 7 days of differentiation. Cells at day (d) 0 are the undifferentiated neural stem cells. U6 was used as the loading control. (c). BrdU labeling of neural stem cells transfected with let-7d. The RNA concentrations (from 0 to 200 nM) were indicated and BrdU labeling is shown in green. The merged images of BrdU staining and phase contrast are shown on the right. (d). Quantification of BrdU labeling in neural stem cells transfected with let-7d. The percentage of BrdU+ cells was scored by dividing the BrdU+ cells with total living cells. Error bars are standard deviation of the mean. * $p < 0.001$ by one-way Anova test. (e). Let-7d transfection did not induce cytotoxicity. Neural stem cells were transfected with increasing concentrations of let-7d RNA duplexes, then analyzed for toxicity using LDH assays.

levels of lactate dehydrogenase (LDH), a marker of cytotoxicity, were detected in both mock and let-7d-treated cells (Fig. 1e). These results indicate that let-7d is expressed in mammalian brains and inhibits neural stem cell proliferation.

To define the role of let-7d in neural differentiation, neural stem cells transfected with let-7d were induced to differentiate into astroglial cells. When neural stem cells were differentiated for 3 days using forskolin and fetal bovine serum (FBS), the let-7d-treated cells exhibited about 2-fold increase in the GFAP-positive astrocyte populations (Fig. 2a, b). In parallel experiments, let-7d induced a 1.6-fold increase in the Tuj1-positive neuronal populations when neural stem cells were induced with retinoic acid and FBS for 3 days (Fig. 2c, d). These results demonstrate a role for let-7d in promoting neural stem cell differentiation into both neurons and astrocytes.

Let-7d mimics TLX siRNA and miR-9 to promote neuronal differentiation and migration in embryonic mouse brains. To determine if let-7d regulates neural stem cell proliferation and
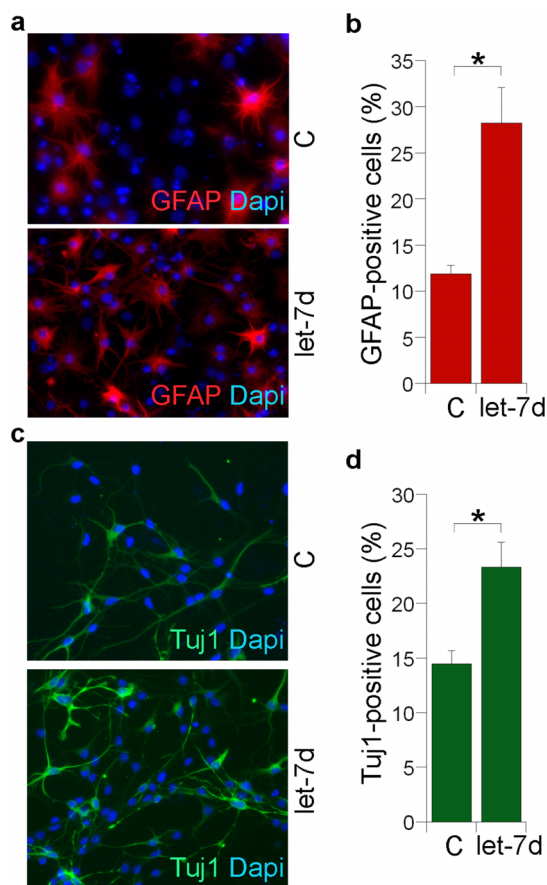

Figure 2 | Let-7d promotes neuronal and astroglial differentiation.
(a). Transfection of let-7d into neural stem cells advanced astroglial differentiation. Let-7d RNA duplexes (100 nM) were transfected into neural stem cells, followed by forskolin and FBS treatment for 3 days. Astrocyte differentiation was evaluated by GFAP staining shown in red. Nuclei dapi staining is shown in blue. (b). Astrocye differentiation was scored as the percentage of GFAP-positive cells over total living cells. (c). Transfection of let-7d into neural stem cells followed by retinoic acid and FBS treatment promoted neuronal differentiation. Neural stem cells were transfected with let-7d and induced to differentiate by treating with retinoic acid and FBS for 3 days. Neuronal differentiation was indicated by Tuj1 staining shown in green. Dapi staining is shown in blue. (d). Neuronal differentiation was quantified as the percentage of Tuj1-positive cells. For both panels (b) and (d), error bars are standard deviation of the mean. * p<0.05 by Student's t-test.

differentiation *in vivo*, let-7d RNA duplexes were transfected into neural stem cells in the ventricular zone of E13.5 mouse brains by *in utero* electroporation. Electroporated brains were analyzed at E15.5. Cells were transfected with let-7d and an RFP reporter. Transfection of let-7d decreased the percent of cells that were labeled by Ki67, a marker of cell proliferation (Fig. 3a, b), but increased the number of transfected cells that migrated to the cortical plate, where differentiated cells reside (Fig. 3c, d). Co-labeling of RFP with the neuronal marker doublecortin (DCX) revealed that the RFP-positive cells that migrated to the cortical plate (CP) are DCX-positive (Fig. 3e), confirming that these cells underwent neuronal differentiation. These results provide *in vivo* evidence that let-7d inhibits neural stem cell proliferation and accelerates neuronal differentiation. Of interest to us were that the phenotypes induced by transfection of let-7d into embryonic mouse brains is similar to that induced by *in utero* knockdown of TLX expression[27] or overexpression of miR-9 in embryonic mouse brains[33]. Introducing either a vector expressing TLX small hairpin interference RNAs or miR-9 RNA duplexes into the ventricular zone of E13.5 mouse brains led to precocious neuronal differentiation and migration[27,33], resembling the phenotypes we observed after *in utero* electroporation of let-7d (Fig. 3c-e). These observations led us to look for a potential link between let-7d and TLX/miR-9.

Let-7d modulates the expression of TLX and miR-9. Using the Miranda (http://www.microrna.org) algorithm[35,36], we identified a let-7d binding site in the 3′ UTR of the *tlx* gene, which is conserved in human, mouse and dog *tlx* sequences (Fig. 4a). To determine if let-7d inhibits TLX expression by targeting the *tlx* 3′ UTR, HEK 293 cells were transfected with the let-7d RNA duplexes together with a luciferase reporter upstream of the mouse *tlx* 3′ UTR, which contains the predicted let-7d recognition site and its surrounding nucleotide sequences. Let-7d repressed the luciferase reporter in a dose-dependent manner (Fig. 4b). Furthermore, disruption of the sequence complementarity between let-7d and the *tlx* 3′ UTR abolished the ability of let-7d to repress the luciferase reporter (Fig. 4c). These results strong imply that let-7d inhibits TLX expression via base-pairing to its 3′ UTR.

To determine if let-7d regulates endogenous TLX expression in neural stem cells, cells were transfected with the let-7d RNA duplexes, then analyzed by RT-PCR to determine the expression levels of TLX (Fig. 4d, e). The levels of TLX mRNA were considerably decreased in let-7d transfected cells when compared to that of control RNA-transfected cells (Fig. 4d, e). Previously, we showed that TLX reduces mature miR-9 expression by repressing the transcription of its primary precursor miR-9-1[33]. Because let-7d inhibited TLX expression (Fig. 4d, e), we next tested if let-7d regulated the levels of the primary and mature miRNA forms of miR-9. In contrast to decreased TLX expression, the level of the miR-9 primary precursor miR-9-1 was elevated in let-7d-transfected cells (Fig. 4e). Accordingly, there was a significant increase in mature miR-9 level in let-7d-treated cells detected by real-time RT-PCR analysis (Fig. 4f). These results demonstrate that let-7d inhibits TLX expression but promotes miR-9 expression, presumably due to reduced expression of TLX, which normally suppresses miR-9-1 transcription.

***In utero* electroporation of TLX rescues let-7d-induced neural phenotypes.** To determine if let-7d promotes neurogenesis by inhibiting TLX expression *in vivo*, we co-transfected let-7d RNA duplexes with a TLX expression vector that lacks its 3′ UTR by *in utero* electroporation into the ventricular zone of E13.5 mouse brains. Because the TLX expression vector carries a RFP reporter, the transfected cells were labeled in red (Fig. 5). Co-transfection of let-7d with an empty RFP vector was included as the control. Co-delivery of the TLX expression vector with let-7d (let-7d + TLX-RFP) into E13.5 mouse brains rescued the reduced levels of neural stem cell proliferation, as revealed by increased Ki67
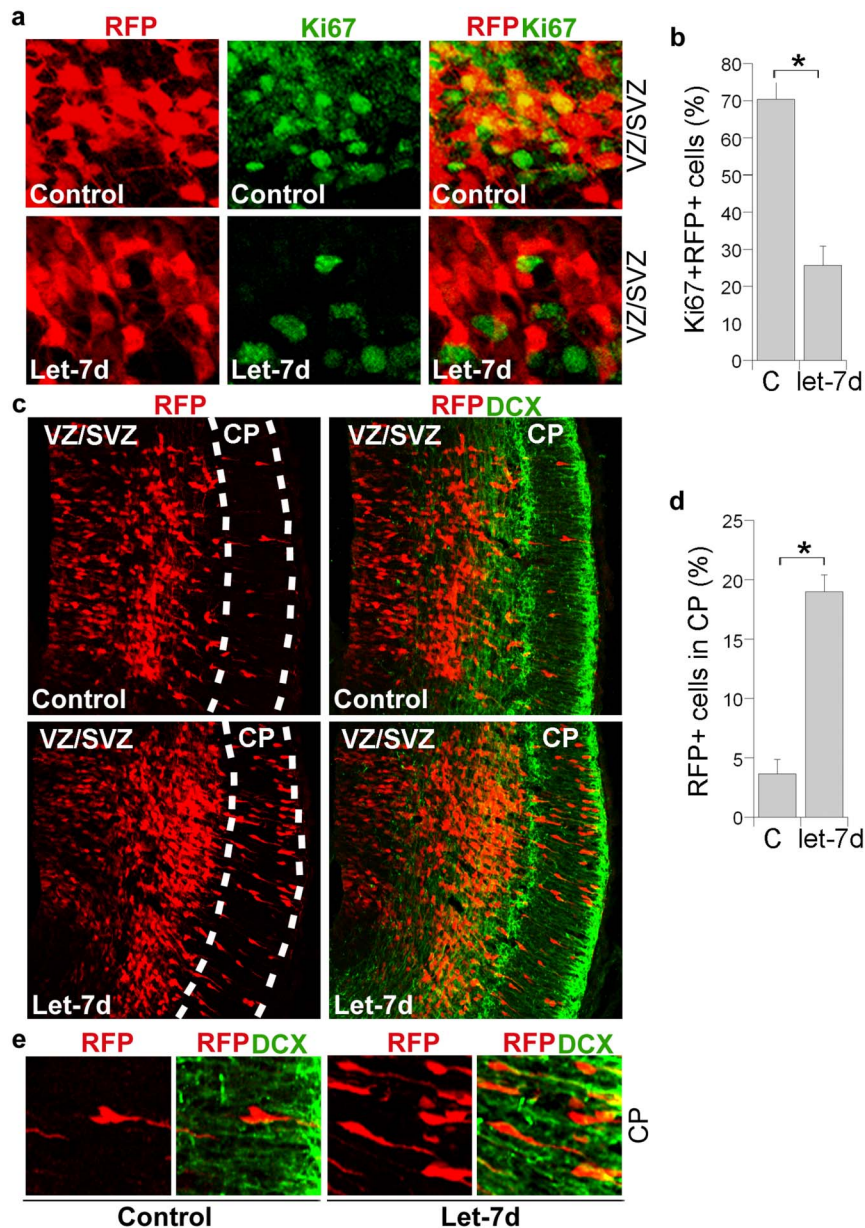
Figure 3 | *In utero* electroporation of let-7d promotes neuronal differentiation and migration in embryonic mouse brains. (a). *In utero* electroporation of let-7d into E13.5 mouse brains reduced the proliferating cell population in the ventricular zone and subventricular zone (VZ/SVZ). The Proliferating cells were labeled by Ki67. (b). Quantification of Ki67+RFP+ cells. The percentage of Ki67+RFP+ cells over total RFP+ cells in the VZ/SVZ of let-7d-electroporated brains were calculated and plotted. Error bars are standard deviation of the mean.* p<0.01 by Student's t-test. (c). Transfection of let-7d into E13.5 mouse brains induced precocious cell migration to the cortical plate. The transfected cells were shown in red due to the expression of the RFP reporter. The let-7d mutant RNA was used as the control RNA. CP stands for cortical plate; DCX stands for doublecortin. (d). Quantification of transfected cells (RFP+ cells) that migrated to the CP. C stands for the let-7d mutant control RNA. Error bars are standard deviation of the mean.* p<0.05 by Student's t-test. (e). Enlarged images of the RFP+DCX+ cells in the CP of control RNA or let-7d-transfected brains. The RFP+DCX+ cells showed yellow processes due to the merge of red and green colors.

staining (Fig. 5a, b). In contrast, the number of RFP+ cells that migrated to the cortical plate was reduced considerably in let-7d + TLX-RFP-transfected brains, when compared to that in the let-7d + RFP-transfected control brains (Fig. 5c, d). These results suggest that TLX is an important target for let-7d to inhibit neural stem cell proliferation and promote neuronal differentiation and migration in the brain.

Discussion

Although we have begun to define the roles played by miRNAs during neurogenesis[4], our understanding of how miRNAs regulate the biology of neural stem cells is still very limited. Specifically, there

have been no studies yet that define the role played by let-7d in neural stem cell maintenance and neurogensis. In this paper, we demonstrated for the first time that let-7d can regulate multiple aspects of neurogenesis, including neural stem cell proliferation, differentiation, and neuronal migration in the mammalian brain. Overexpression of let-7d inhibited the proliferation of neural stem cells and, instead, promoted neuronal differentiation and outward migration. Moreover, we showed that let-7d regulated these effects on neurogenesis by modulating the TLX and miR-9 expression cascade.

We and others have shown that the brain-specific miR-9 and miR-124 miRNAs, the brain-enriched miR-137 miRNA, and the ubiquitously-expressed let7a and let-7b miRNAs, all play roles in
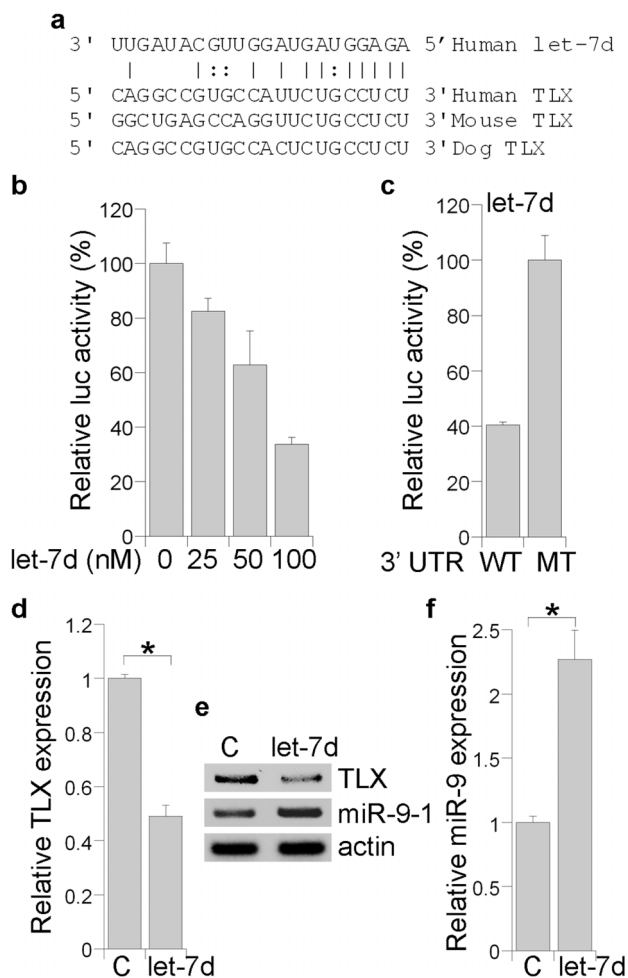
Figure 4 | Let-7d regulates TLX and miR-9 expression. (a). The sequence complementarity between let-7d and the *tlx* 3′ UTR. The vertical lines indicate perfect pairing, whereas the ":" indicates a G-U wobble. (b). Let-7d represses the *tlx* 3′ UTR luciferase reporter in a dose-dependent manner. (c). Mutation in the *tlx* 3′ UTR that disrupts the sequence complementarity with let-7d relieved let-7d-mediated repression of the luciferase reporter. WT and MT represent the wild type and mutant *tlx* 3′ UTR reporter, respectively. (d). Let-7d inhibited TLX mRNA expression, analyzed by real-time RT-PCR analysis. C stands for the control RNA for panels d–f. Error bars are standard error of the mean.* p<0.005 by Student's t-test. (e). Let-7d suppressed TLX expression and induced miR-9-1 expression in neural stem cells, analyzed by RT-PCR analysis. (f). Increased expression of mature miR-9 in let-7d-transfected neural stem cells, measured by real-time RT-PCR. Error bars are standard error of the mean.* p<0.05 by Student's t-test.

neurogenesis during various stages of development[14–16,33,34,37–46]. Their expression is coordinated in the brain to regulate neurogenic events that range from the proliferation and self-renewal of neural stem cells to neuronal differentiation, migration, maturation, and functional integration. Furthermore, since miRNAs usually fine-tune the expression of their target genes, inhibition of the same target genes, such as TLX, by multiple miRNAs, including miR-9 and let-7, may lead to more dramatic effect on target gene expression. Regulation of target gene expression then dictates the roles of these miRNAs in modulating specific processes of cell fate control and function.

In this study, we showed that overexpression of let-7d inhibited neural stem cell proliferation and promoted neuronal differentiation and migration in embryonic mouse brains, a phenotype that is similar to that induced by knockdown of TLX expression. The

phenotypical resemblance between let-7d overexpression and TLX knockdown suggested to us that let-7d might affect TLX expression. Indeed, our results indicate that let-7d can bind to the 3′ UTR of *tlx* mRNA to reduce its expression levels. Moreover, we demonstrated that co-electroporation of let-7d and TLX without its 3′ UTR restored the levels of cell proliferation and reversed the increased neuronal differentiation and migration *in vivo* induced by overexpression of let-7d alone. These results indicate that TLX serves as a key target for let-7d and that let-7d modulates TLX expression to regulate neurogenesis.

The brain-specific miRNA miR-9 has been extensively studied in neurogenesis (see review by Lang and Shi[5]). Electroporation of miR-9 into mouse brains *in utero* decreased neural stem cell proliferation and promoted neuronal differentiation and migration[33]. In a different cellular context, expression of miR-9 enhanced proliferation and inhibited migration of early stage human neural progenitor cells during neural differentiation of human embryonic stem cells *in vitro*[38]. Even more recently, a study of ectopic expression of FoxP2, a miR-9 and miR-132 target gene, in cortical neurons suggested that miR-9 can promote neuronal migration[47]. In this study, we showed that when let-7d was overexpressed, TLX expression was inhibited, which in turn, induced the expression of miR-9, a result consistent with our previous observation that TLX represses miR-9 expression[33]. Indeed, overexpressing let-7d in embryonic mouse brains enhanced neuronal differentiation and migration, which is phenotypically similar to what we see when miR-9 is electroporated *in utero* and overexpressed in embryonic mouse brains[33]. Our observations for these miRNAs are supported by the recent study in which a loss of target gene repression by miR-9 inhibited neuronal migration[47].

Recent studies have suggested a role for let-7d in cocaine addiction and attention deficit hyperactivity disorder (ADHD)[17,18,48]. Defining the role of let-7d in the functions of normal neural stem cells and neurogenesis, and identifying its downstream target genes may help uncover the pathological mechanisms of these neurological disorders. Furthermore, manipulating the expression of let-7d and its downstream targets could identify potential interventions for these disorders in the future.

Methods

Neural stem cell culture, differentiation, immunostaining, cytotoxicity analysis. Mouse neural stem cell isolation, culture and differentiation were performed as described[19]. Immunostaining was performed using antibodies for BrdU (Accurate; diluted 1:10,000), Tuj1 (Covance, 1:2,500), and GFAP (Advance Immuno; 1:1,000), as described[19]. Cytotoxicity of let-7d transfection was assessed using LDH assay as described[15].

DNA construct preparation. The luciferase reporter containing the 1.4 kb mouse TLX 3′ UTR was constructed as described[15]. The pEF-TLX-pUb-RFP (TLX-RFP) expression vector was constructed by placing the TLX coding region without its 3′UTR under the elongation factor EF1α promoter and expressing the RFP reporter under the ubiquitin promoter.

Transfection and reporter assays. Transfection of RNA duplexes alone or together with plasmid DNAs were performed as described[15]. The sequence of the wild type let-7d RNA duplex sense strand is: 5′-AGA GGU AGU AGG UUG CAU AGU-3′. The sequence of the mutant let-7d control RNA duplex sense strand is: 5′-UCU CCU UCU UGG UUG UGU GGU U-3′.

RT-PCR and Northern blotting of let-7d. Mature miR-9 expression was determined by real-time RT-PCR using TaqMan MicroRNA Assay kit (ID 583, Applied Biosystem) and U6 was used as the internal control (ID 001973, Applied Biosystems). Real-time RT-PCR for TLX was performed using SYBR Green PCR master mix (Bio-Rad) and StepOnePlus system (Applied Biosystem) and actin was used as the normalization control. Primers for TLX real-time RT-PCR are TLX forward: 5′-GGT TCA GAC AGC TCC GAT TAG A-3′ and TLX reverse: 5′-TGG AGA GCG GCA ATG GCG GCA GC-3′. Semi-quantitative RT-PCR of TLX and miR-9 primary precursor miR-9-1 was performed as described[33]. Northern blotting of let-7d was performed as described[33] using let-7d antisense probe: 5′-ACT ATG CAA CCT ACT ACC TCT-3′.

In utero electroporation. All animal experiments were performed under the IACUC protocol approved by the City of Hope Institutional Animal Care and Use Committee. *In utero* electroporation was carried out as described[27,33,15]. Brain sections
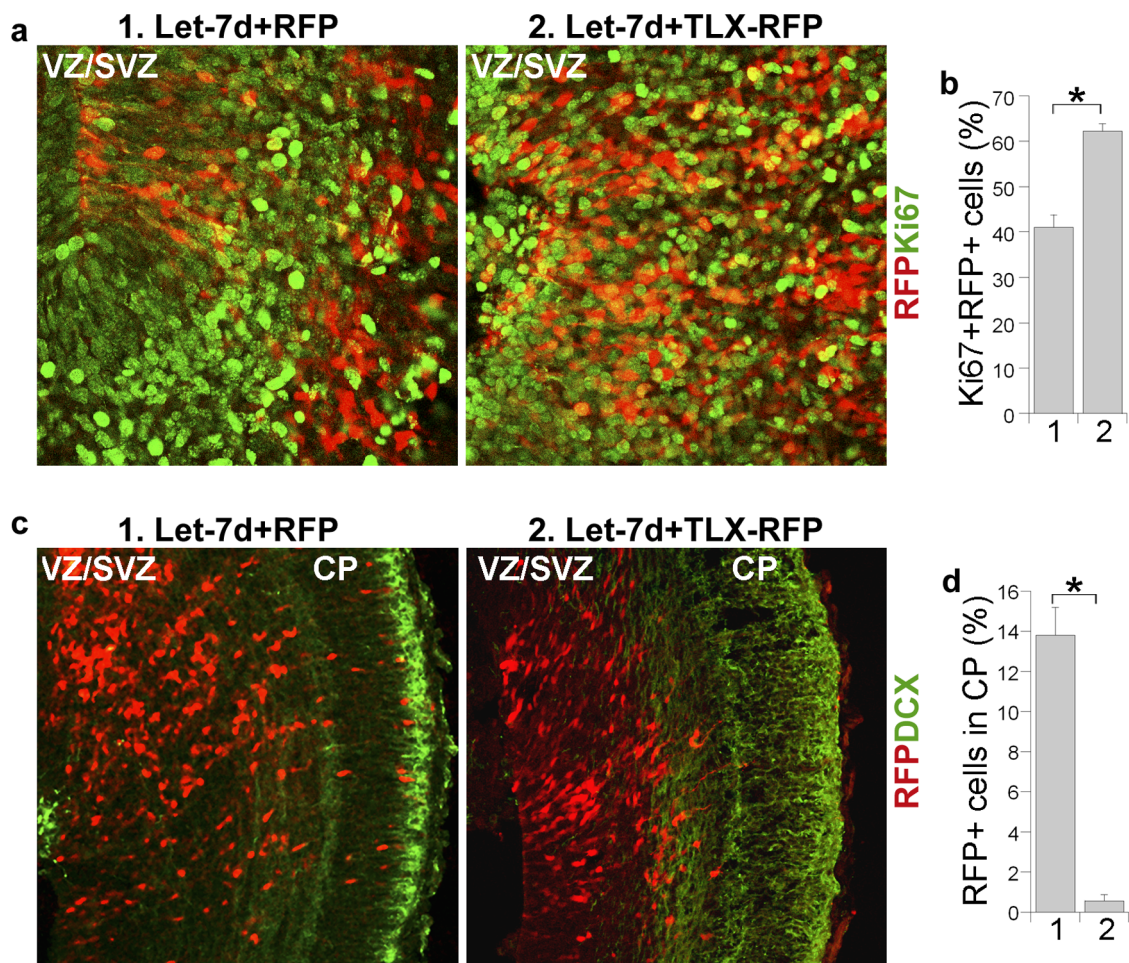
Figure 5 | Suppression of TLX expression is critical for let-7d function in inhibition of neural stem cell proliferation and promotion of neuronal differentiation and migration. (a). Co-expression of TLX lacking its 3′ UTR with let-7d (let-7d + TLX-RFP) restored let-7d-reduced levels of cell proliferation in the germinal zone of embryonic mouse brains. VZ/SVZ stands for ventricular zone and subventricular zone. The transfected cells were RFP+, shown in red. The proliferating cells were Ki67+, shown in green. (b). Percentage of the Ki67+RFP+ cells over total RFP+ cells in let-7d + RFP-transfected brains (1) and let-7d + TLX-RFP-transfected brains (2). Error bars are standard deviation of the mean. * $p < 0.05$ by Student′s t-test. (c). Co-expression of TLX lacking its 3′ UTR reversed let-7d-induced premature cell migration to the cortical plate. The transfected cells were RFP+, shown in red. DCX staining (green) was included to show the brain structure. CP stands for cortical plate. (d). Quantification of transfected cells (RFP+) that migrated to the CP in let-7d + RFP (1) and let-7d + TLX-RFP (2)- electroporated brains. * $p < 0.001$ by Student's t-test. Error bars are standard deviation of the mean for both panels (b) and (d).

were immunostained with antibodies for Ki67 (1:500, Lab Vision) and DCX (1:400, Santa Cruz).

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Author contributions

Y.S. designed research; C.Z., G.S., P.Y., S.L. and Y.S. performed research; C.Z. and Y.S. analyzed data; and Y.S. wrote the paper.

Additional information