splicing in nonneuronal cells (Makeyev et al., 2007). miR-124, thus, plays an important role in the differentiation of NSCs to mature neurons by increasing neuron-specific transcript levels.

Another brain-enriched miRNA, miR-137, plays an important role in regulating fate determination of NSCs (Sun et al., 2011b) (Fig. 1). Overexpression of miR-137 suppresses proliferation and accelerates neuronal differentiation of NSCs. In this regard, it has been suggested that miR-137 makes a negative feedback loop with the histone H3K4 demethylase lysine-specific demethylase 1 (LSD1) and the orphan nuclear receptor TLX, which is an essential regulator of NSC self-renewal. miR-137 targets LSD1 and inhibits its function. On the other hand, TLX represses the expression of miR-137 by recruiting LSD1 to the genomic region of miR-137. This feedback loop of miR-137 with LSD1 and TLX may regulate the balance between proliferation and differentiation of embryonic NSCs during neural development.

1.5. Epigenetic regulation in adult neurogenesis

Even after brain development is completed, a limited number of NSCs is maintained in the adult brain. These NSCs contribute to brain functions through daily production of neurons. Adult neurogenesis occurs in 2 restricted brain regions (Bond et al., 2015; Christian et al., 2014; Lledo et al., 2006; Ma et al., 2009a; Ming and Song, 2011). The subventricular zone (SVZ) of the lateral ventricle is one of the restricted regions in which neurogenesis persists. NSCs in the SVZ, named type B cells, proliferate infrequently and mostly display a quiescent state in the cell cycle. In the process of neurogenesis, they first give rise to transit-amplifying NPCs (type C cells), which then proliferate and differentiate into neuroblasts (type A cells). Neuroblasts migrate into the olfactory bulb (OB) along the rostral migratory stream and differentiate into mature granule and periglomerular neurons there (Fig. 2).

The subgranular zone (SGZ) of the adult hippocampal dentate gyrus (DG) also sustains NSCs into adulthood. Similar to the adult SVZ, relatively quiescent radial glia–like cells (type 1 cells) serve as NSCs in the SGZ. Type 1 cells give rise to transit-amplifying NPCs (type 2a/b cells), and they eventually differentiate into neurons through the neuroblast stage (type 3 cells) (Fig. 3). Newly generated neurons migrate into the granule cell layer along blood vessels and integrate in preexisting neural circuits (Sun et al., 2015). These newly generated neurons have been implicated in various hippocampal functions, such as hippocampus-dependent learning and memory.

For adult neurogenesis to occur, NSCs must go through several steps including maintenance and proliferation of Neural Stem/Precursor Cell (NS/PC) and neuronal commitment, migration, and maturation (Christian et al., 2014; Duan et al., 2008; Sun et al., 2011a) (Figs. 2, 3). A growing number of studies have shown that these processes in adult neurogenesis are governed by both cell extrinsic and intrinsic factors including extracellular signals from the stem cell niche, transcription factors, and epigenetic regulators (Bond et al., 2015; Fuentealba et al., 2012; Jobe et al., 2012; Ming and Song, 2011; Sun et al., 2011a). Moreover, to ensure neurogenesis in the adult brain, NSCs need to be maintained from embryonic to adult stages. Thus, the mechanism that sustains NSCs into adulthood is also pivotal for adult neurogenesis to occur. For instance, recent studies have elucidated the importance of histone modifications in regulating these processes to warrant neurogenesis in the adult brain (Lim et al., 2009; Molofsky et al., 2003). In this section, we introduce examples of epigenetic mechanisms contributing to the maintenance of adult neurogenesis.

1.6. Histone modifications in adult NS/PCs

The PcG and Trithorax (TrxG) complexes have been implicated in regulating adult neurogenesis. The PcG complex is responsible for gene repression, which is mediated by H3K27 methylation, whereas the TrxG complex catalyzes trimethylation of H3K4 (H3K4me3) in promoter-proximal nucleosomes to activate expression of their target genes (Blackledge et al., 2015; Ng and Gurdon, 2008; Ringrose and Paro, 2007). PRC1 consists of multiple subunits, and one of its components, B cell–specific Moloney murine leukemia virus integration site 1 (Bmi1), contributes to modulating the proliferation capacity of NSCs and maintaining them to adulthood (Fig. 2). Deletion of *Bmi1* attenuates the proliferation capacity of NSCs, resulting in the depletion of NSCs during development (Molofsky et al., 2003). This occurs through up-regulation of the cell cycle–dependent kinase inhibitor $p16^{Ink4a}$, whose deletion partially rescues the phenotype of *Bmi1* KO mice.

The TrxG complex component mixed-lineage leukemia 1 (Mll1) histone methyltransferase is required for neuronal differentiation in the SVZ (Lim et al., 2009) (Fig. 2). Deletion of *Mll1* in NSCs caused no defects in NSC proliferation, survival, and glial differentiation but severely impaired neuronal differentiation. Mll1 is responsible for H3K4me3, and its recruitment to gene promoters has been associated with gene activation. Mll1 directly targets distal-less homeobox 2 (Dlx2), a homeodomain-containing transcription factor important for neurogenesis in the OB. When *Dlx2* expression is active, its promoter shows high level of H3K4me3. However, in the absence of Mll1, the *Dlx2* promoter enters the bivalent state, i.e., a posed state for transcription harboring both repressive (H3K27me3) and active (H3K4me3) histone modifications. These findings indicate that Mll1-mediated resolution of the poised state (H3K4me3 + H3K27me3) to the active state (H3K4me3 only) of the *Dlx2* promoter is crucial for neuronal differentiation of NSCs.

One model to explain how bivalency is resolved by Mll1 is the cooperation of Mll1 with histone demethylase against H3K27me3 to activate target gene expression. A Jumonji domain containing 3 (Jmjd3; also called KDM6B), which is an H3K27me3-specific demethylase belonging to the family of JmjC domain–containing proteins, also acts as a critical activator of neuronal differentiation of adult SVZ NSCs (Fig. 2). Jmjd3 activates expression of a number of neuronal genes, including *doublecortin*, *Nkx2.2*, *Dlx2*, and *Dlx5* (Jepsen et al., 2007; Park et al., 2014). Upon neuronal differentiation, Jmjd3 is enriched in both the promoter and enhancer of *Dlx2*. This is coupled with decreased H3K27me3 level and increased *Dlx2* expression. Interestingly, this enrichment of Jmjd3 is impaired by deletion of *Mll1*, suggesting that Mll1 is required for the recruitment of Jmjd3 to the gene expression regulatory region. These findings highlight the importance of crosstalk between histone methylation enzymes in adult neurogenesis.

Adult neurogenesis is known to be regulated by many physiological and pathologic conditions. One of the pathologic conditions, kainic acid–induced seizure, leads to aberrant neurogenesis in the adult hippocampus (Jessberger et al., 2005, 2007a, 2007b; Matsuda et al., 2015). The HDAC inhibitor valproic acid, an antiepileptic agent that is widely used in human clinical treatment, suppresses kainic acid–induced aberrant proliferation of NS/PCs in the adult DG, contributing to the alleviation of cognitive impairment in hippocampus-dependent learning (Jessberger et al., 2007a,b). In addition, valproic acid also functions to induce neuronal differentiation of adult hippocampal NSCs by up-regulating the proneural gene *NeuroD*, whereas it inhibits astrocyte and oligodendrocyte differentiation (Hsieh et al., 2004). In the HDAC family, HDAC2 plays a critical role in neuronal maturation in both adult hippocampal DG and SVZ (Figs. 2, 3). It has been shown that specific ablation of HDAC2 in NSCs impairs neuronal differentiation and induces abnormal maturation of newborn neurons and cell death in both SGZ and SVZ, although the proliferation rate of transit-amplifying NPCs is increased by HDAC2 deletion. This combination of increased proliferation and defective neuron generation in HDAC2-deficient mice may result from the prolonged expression of stem cell–specific genes during neuronal differentiation. Sox2, an important transcription factor for proliferation and stemness

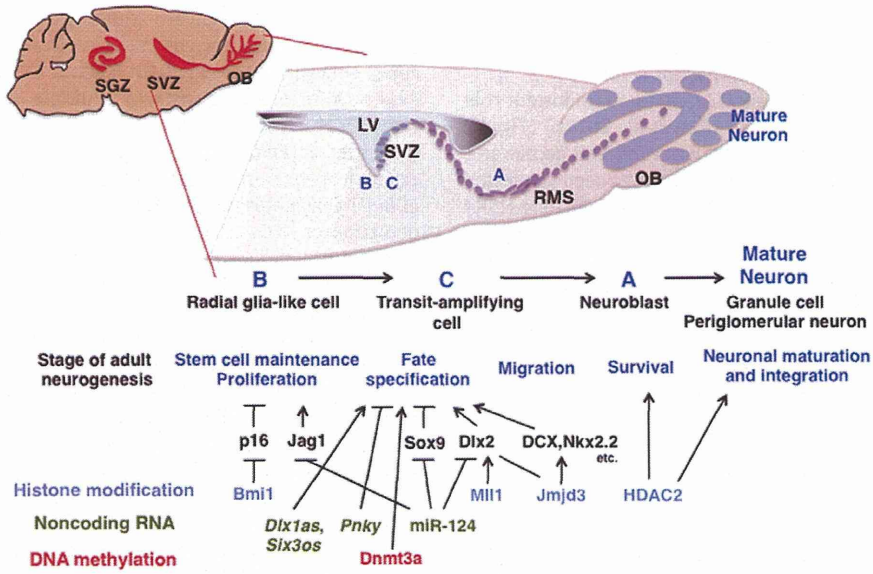

Fig. 2. Schematic representation of adult neurogenesis and factors relevant to epigenetic regulation in the SVZ and OB. Adult neurogenesis in the SVZ is composed of 5 stages: (1) stem cell maintenance and proliferation, (2) fate specification, (3) migration, (4) survival, and (5) neuronal maturation and integration. Briefly, radial glia-like cells (type B) become activated and produce transit-amplifying NPCs (type C), which frequently proliferate and generate neuroblasts (type A). Newly generated neuroblasts migrate along the rostral migratory stream and differentiate into immature neurons in the OB. Finally, synaptic integration and maturation of granule cells and periglomerular neurons are induced in the OB. Representative epigenetic factors that are relevant in regulating each stage of adult SVZ neurogenesis are also shown. Abbreviations: LV, lateral ventricle; OB, olfactory bulb; RMS, rostral migratory stream. →, promotion; ⊣, inhibition.

of NSCs, is not normally expressed in neuroblasts, which are already committed to differentiate into neurons. However, Sox2 expression is sustained even in the neuroblasts in the DGs of HDAC2-deficient mice, suggesting that HDAC2 is essential to terminate Sox2 expression as NS/PCs differentiate into neurons and that Sox2 expression should be repressed in neuroblasts and neurons to ensure proper neuronal differentiation and maturation (Jawerka et al., 2010).

Sox2 limits the activity of the PRC2 complex to suppress excessive acquisition of H3K27me3 at the regulatory regions of proneural and neurogenic genes, such as *Neurog2*, *NeuroD1*, and *brain-derived*

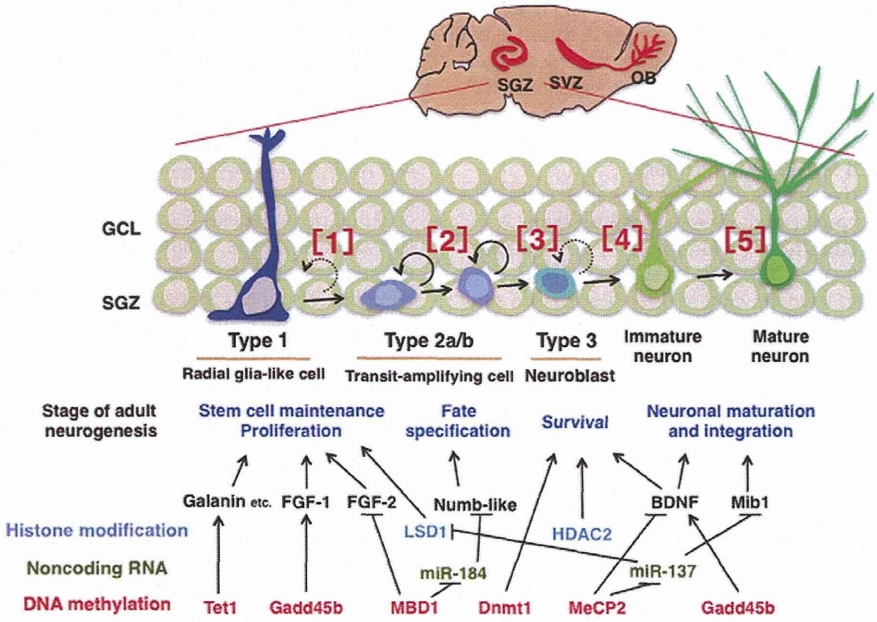

Fig. 3. Schematic diagram of the current view of lineage relationships and epigenetic regulation during adult hippocampal neurogenesis. Adult hippocampal neurogenesis mainly proceeds through 4 stages: (1) stem cell maintenance and proliferation, (2) fate specification, (3) survival, and (4) neuronal maturation and integration into preexisting circuits. The numbers on the figure indicate the detailed developmental steps of adult hippocampal neurogenesis: [1] activation of quiescent radial glia-like cells (type 1) in the SGZ, [2] proliferation of precursor and intermediate progenitors (type 2a, 2b, transit-amplifying cells), [3] generation of neuroblasts (type 3), [4] differentiation into immature neurons, and [5] maturation of immature neurons. Epigenetic factors, including DNA methylation, histone modification, and noncoding RNAs, regulate adult hippocampal neurogenesis by means of crosstalk between each pathway. Solid (frequent) and dashed (slow or rare) semicircular arrows indicate the cell division frequency of each cell type. Abbreviations: GCL, granule cell layer; SGZ, subgranular zone. →, promotion; ⊣, inhibition.

neurotrophic factor (*Bdnf*) (Amador-Arjona et al., 2015). These genes have bivalency in their promoters, whereby their expression is poised to be activated once NSCs initiate neuronal differentiation in response to the stimuli of neurogenic cues. Sox2 interacts with these gene promoters and precludes the recruitment of the PRC2 component Ezh2. In the absence of Sox2, H3K27me3, but not H3K4me3, increases in the poised proneural and neurogenic gene promoters, correlating with the increased accumulation of Ezh2. Moreover, Wnt signaling, a neurogenic cue that induces neurogenesis in adult as well as embryonic stages, activates the expression of poised proneural and neurogenic genes. Sox2-deficient NSCs, however, fail to express these genes upon Wnt stimulation. Specific ablation of *Sox2* in adult hippocampal NSCs impairs both proneural and neurogenic gene expression, resulting in decreased neurogenesis, accompanied by increased cell death and functionally aberrant newborn neurons. These findings suggest the important role of Sox2 in maintaining permissive epigenetic states of neurogenesis-related genes in the adult hippocampal NSCs, allowing these genes to respond to neurogenic cues immediately.

1.7. DNA methylation in adult neurogenesis

The role of DNA methylation in adult neurogenesis is still unclear compared with other epigenetic regulation characters such as histone modification or noncoding RNAs. Expression of Dnmt1 and of the de novo DNA methyltranseferase Dnmt3a, but not 3b, is observed in the adult brain. Dnmt1 is particularly highly expressed in proliferative NS/PCs compared with quiescent NSCs and postmitotic neurons and astrocytes in the adult hippocampus. Although deletion of *Dnmt1* in adult NSCs does not affect their proliferation or differentiation, it decreases the survival of newly generated neurons in the adult hippocampal DG (Noguchi et al., 2015). Interestingly, *Dnmt1* KO in postmitotic neurons does not affect their survival, suggesting that DNA methylation maintained by Dnmt1 in the NSC stage plays an important role for the survival of newly generated neurons only after NSCs differentiate into neurons in the adult hippocampus (Fig. 3). On the contrary, Dnmt3a is reportedly associated with adult neurogenesis (Wu et al., 2010) (Fig. 2). Deletion of *Dnmt3a* decreased neuronal differentiation of NSCs in the SVZ, resulting in a reduction of neuroblasts migrating into the OB. Dnmt3a targets nonpromoter regions of neurogenic genes and induces their expression. DNA methylation and Dnmt3a occupancy in nonpromoter regions are negatively correlated with elevation of H3K27me3 and enrichment of the PRC2 components Ezh2 and suppressor of zeste 12. This suggests that Dnmt3a-dependent methylation promotes neuronal differentiation by inhibiting PRC2-mediated repression of gene expression.

In addition to DNA methyltransferase, Tet1, an important factor for active demethylation via oxidation of 5mC, also plays an important role in regulating adult hippocampal neurogenesis (Zhang et al., 2013) (Fig. 3). *Tet1* deficiency impairs the proliferation of adult NSCs and causes DNA hypermethylation of genes involved in proliferation and neuronal protection, including *Galanin*, *Ng2*, and *Neirglobin* (*Ngb*), resulting in decreased neurogenesis. Moreover, *Tet1* KO mice display impaired spatial learning and memory, suggesting that Tet1-mediated DNA demethylation is important for cognition.

Active DNA demethylation occurs not only in NS/PCs but also in the cells providing the niche for NS/PCs, which contributes to regulating the expression of neurogenic cues. A member of the growth arrest and DNA damage 45 (Gadd45) gene family, Gadd45b, is induced in mature hippocampal neurons by neuronal activity, such as electroconvulsive treatment and voluntary running. Gadd45b expression in mature hippocampal neurons is dependent on depolarization-induced calcium influx and calmodulin kinase activity (Greer and Greenberg, 2008; Ma et al., 2009b). Gadd45b has been implicated in the DNA excision repair–based DNA demethylation mechanism and induces active DNA demethylation in vertebrates. In the adult mouse hippocampus, Gadd45b promotes activity-induced DNA demethylation within the regulatory region IX of *Bdnf* and brain-specific promoter B of *fibroblast growth factor 1* (*Fgf1*). These 2 genes play crucial roles in adult neurogenesis as well as neuronal survival and maturation. Thus, Gadd45-dependent DNA demethylation and expression of secreted factors, such as BDNF and FGF1, influence the neurogenic niche, and these proteins serve as key regulators of homeostasis in adult neurogenesis (Fig. 3).

DNA methylation normally leads to repression of gene expression through the recruitment of methylated DNA-binding protein family members such as methyl-CpG-binding protein 1 (MBD1) and methyl-CpG-binding protein 2 (MeCP2). These 2 factors are also involved in controlling the expression of neurogenic cues. NSC-specific *MBD1* KO decreases adult neurogenesis and impairs spatial learning. MBD1 directly interacts with the promoter region of *Fgf2*, an essential growth factor for NSCs. In the absence of *MBD1*, the *Fgf2* promoter region becomes hypomethylated, resulting in increased expression of *Fgf2* in NSCs and further leading to neuronal differentiation arrest (Li et al., 2008a) (Fig. 3). *MeCP2*, the causal gene of Rett syndrome, is highly expressed in postmitotic neurons and functions as a major regulator for neuronal gene expression in the CNS (Amir et al., 1999; Lyst and Bird, 2015; Shahbazian et al., 2002). *MeCP2* KO mice display no deficiencies in early postnatal neurogenesis, but neuronal maturation of newly generated neurons is impaired in the adult hippocampal DG (Smrt et al., 2007) (Fig. 3). One of the best-known targets of MeCP2 is *Bdnf*, which regulates several aspects of neurogenesis, including proliferation, differentiation of NSCs, and development and survival of newborn neurons (Li et al., 2008b; Murray and Holmes, 2011). MeCP2 physically occupies the hypermethylated *Bdnf* promoter and suppresses its expression. This interaction between MeCP2 and the *Bdnf* promoter is disrupted by the induction of DNA demethylation in response to neuronal activity, which increases *Bdnf* transcription. In addition, neuronal activity–induced calcium influx causes posttranscriptional modification of MeCP2, such as phosphorylation at Ser421, which decreases the affinity of MeCP2 for the *Bdnf* promoter and facilitates its transcription (Chen et al., 2003; Zhou et al., 2006b). MeCP2 Ser421 phosphorylation is induced in response to neuronal stimulation and plays a significant role in synapse development and behavior (Cohen et al., 2011).

1.8. Noncoding RNAs in adult neurogenesis

The roles of miRNA in NSCs and in adult neurogenesis have been extensively studied as well. Accumulating evidence indicates that miRNA plays a substantial role in fine tuning the progression of adult neurogenesis (Figs. 2, 3).

In the SVZ of the adult mammalian brain, brain-specific miR-124 is highly expressed during the transition from transit-amplifying NPCs to neuroblasts and immature neurons (Cheng et al., 2009). miR-124 promotes the differentiation of the NPCs into neuroblasts and controls the timing of lineage progression. miR-124 has several known direct targets in the SVZ including *Jag1*, *Dlx2*, and *Sox9*. Because these genes are known to contribute to distinct steps in adult neurogenesis, such as self-renewal and neuronal differentiation of NSCs, it is conceivable that miR-124 affects multiple aspects of adult neurogenesis in the SVZ (Cheng et al., 2009; Doetsch et al., 2002; Nyfeler et al., 2005) (Fig. 2).

Another representative miRNA that plays an important role in NSC proliferation and differentiation is miR-137, which is a direct target of Sox2 and MeCP2 in adult SGZ (Szulwach et al., 2010). Overexpression of miR-137 promotes proliferation of NSCs in the hippocampal DG, whereas its repression enhances the differentiation of NSCs into both neurons and astrocytes. One of the targets of miR-137 is *Lsd1*, which is known to suppress the proliferation of NSCs in the embryonic brain and adult hippocampus (Sun et al., 2010) (Fig. 3). In addition, miR-137 also regulates neuronal maturation, dendritic elaboration, and spine development by suppressing mind bomb 1 expression

(Smrt et al., 2010) (Fig. 3). Mind bomb 1 is a ubiquitin ligase that is important for neurodevelopment, and its overexpression partially rescues the neuronal maturation impairments caused by miR-137 overexpression. MBD1 targets miR-184 and represses its expression in adult NSCs (Liu et al., 2010). miR-184 targets mRNA for *Numb-like* (*Numbl*), an inhibitor of Notch signaling, for maintenance of adult NSCs. High levels of miR-184 expression promote proliferation at the expense of differentiation of adult hippocampal NSCs. Thus, MBD1, miR-184 and Numbl form a regulatory network to maintain the balance between proliferation and differentiation of NSCs in the adult hippocampal DG (Fig. 3).

Recently, the roles of lncRNA in adult neurogenesis have just begun to be studied. The lncRNAs *Dlx1as* and *Six3os* are expressed in NSCs in the SVZ and play key roles in the glial-neuronal lineage specification of adult NSCs (Ramos et al., 2013) (Fig. 2). *Dlx1as* is transcribed from the *Dlx1/2* gene cluster, whereas *Six3os* is transcribed from a site proximal to the *Six3* homeobox gene (Dinger et al., 2008; Liu et al., 1997). Loss-of-function analysis in the SVZ revealed that both *Dlx1as* and *Six3os* affect fate determination of adult NSCs into neurons, but *Six3os* has an additional function in regulating NSC differentiation into oligodendrocytes. Another example of functional lncRNA is the *rhabdomyosarcoma 2-associated transcript* (*RMST*), which is specifically expressed in the brain and whose expression is repressed by REST/neuron-restrictive silencer factor. *RMST* directly interacts with Sox2 and tethers it to the promoter regions of neurogenic transcription factors such as *Ascl1*, *Dlx1*, and *Neurog2* to induce their expression and the neuronal differentiation of NSCs (Ng et al., 2012, 2013). *Pinky* (*Pnky*) has been identified by means of RNA-seq as a neural-specific lncRNA, expressed in both embryonic and adult NSCs (Ramos et al., 2015). *Pnky* interacts with the splicing regulator polypyrimidine tract binding protein 1 and suppresses neuronal differentiation of NSCs by inhibiting the precise expression and alternative splicing of key genes related to neuronal differentiation (Fig. 2).

2. Closing remarks and perspective

In the last 2 decades, the existence of NSCs even in human brain has been clearly proven, and NSC research is currently progressing with dramatic speed and nurturing the hope that NSCs can be used for the clinical treatment of various neural disorders and diseases. For example, transplantation of NSCs into the lesion site of injured spinal cord has been shown to have great potential to recover locomotion ability in patients affected with spinal cord injury (Abematsu et al., 2010; Fujimoto et al., 2012; Iwanami et al., 2005; Ogawa et al., 2002). In addition, implanting cells that are genetically engineered to become dopaminergic neurons, one of the neuronal subtypes specifically lost in Parkinson's disease, into the brain of Parkinson's disease model animals has been undertaken to ask if such a strategy could be a clinical treatment for Parkinson's disease (Kim et al., 2011; Liu et al., 2012). For these and other neuronal disorders, establishing a treatment to recover abilities of the CNS that are lost in diseases and injury is now becoming one of the most encouraging and worthwhile challenges in NSC research. In terms of producing desirable cells from NSCs for such treatments, a deep understanding of the mechanisms regulating NSC proliferation and differentiation is essential. Development of an in vitro culture system for NSCs triggered research to verify the signaling molecules that enable them to proliferate and differentiate into specific cell types in the CNS. These approaches have already identified many neurogenic and gliogenic molecules, but they have also uncovered the existence of intrinsic mechanisms in NSCs that influence their responsiveness to these cues. As we have discussed in this review, epigenetic regulation constitutes these intrinsic mechanisms, and recent researches have shown its significant contribution to brain development and adult neurogenesis. Nevertheless, there are still many questions to be addressed regarding the mechanisms by which epigenetic modifications regulate the maintenance and differentiation of NSCs. The current revolution of next-generation sequencing technology allows us to analyze genome-wide expression patterns and epigenetic modifications in each cell type. These analyses have demonstrated that different types of cells have unique patterns of epigenetic modifications and that these patterns are established as stem cells differentiate into each lineage (Jepsen et al., 2007; Park et al., 2014; Ramos et al., 2015; Wapinski et al., 2013; Wu et al., 2010). Although these studies have revealed significant epigenetic modifications regulating differentiation of NSCs, we do not yet know how these unique patterns are established in specific gene promoters or indeed how much they are involved in stem cell differentiation.

We introduced above several examples of epigenetic modification changes in specific gene promoters that are induced by transcription factors via blocking and recruiting epigenetic modification enzymes to their cognate loci. However, the affinity of transcription factors for the loci themselves is also influenced by epigenetic modifications (Takizawa et al., 2001; Wapinski et al., 2013). Thus, it is plausible that the unique epigenetic modification patterns established in each gene locus or cell type are governed by the interplay between multiple transcription factors and epigenetic modification enzymes. Recently, advanced genome-editing systems, such as the TALEN and CRISPR-Cas9 systems, have been developed (Doudna and Charpentier, 2014; Hsu et al., 2014; Vasileva et al., 2015). We believe that, by using these systems, we will be able to alter the epigenetic states of specific sites in the genome, without changing DNA sequences to manipulate the cell's behavior (Kearns et al., 2015; Mendenhall et al., 2013).

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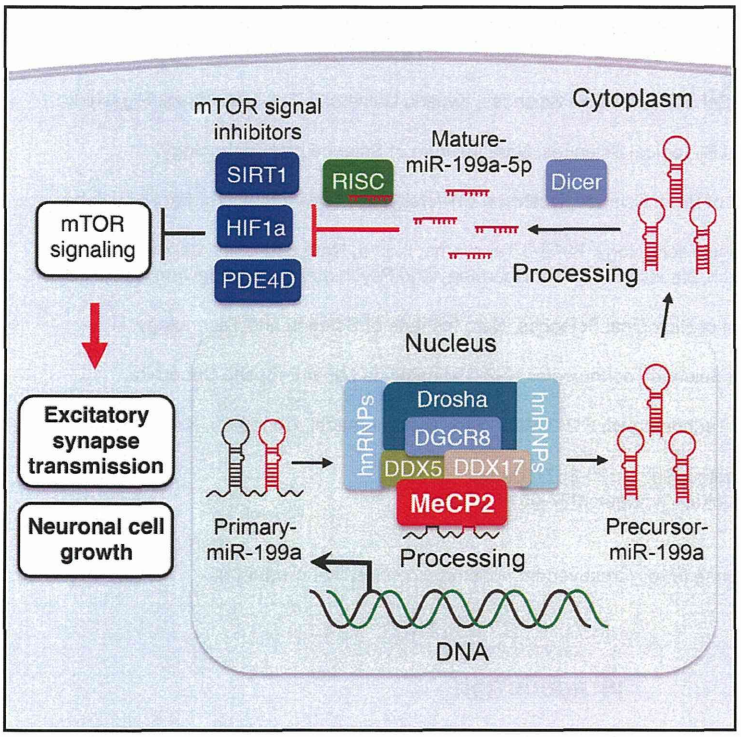
miR-199a Links MeCP2 with mTOR Signaling and Its Dysregulation Leads to Rett Syndrome Phenotypes

Graphical Abstract



Authors

Keita Tsujimura, Koichiro Irie, Hideyuki Nakashima, ..., Takaya Abe, Shigeo Takamori, Kinichi Nakashima

Correspondence

kin1@scb.med.kyushu-u.ac.jp

In Brief

Tsujimura et al. find that MeCP2 facilitates processing of miR-199a, which, in turn, leads to upregulation of mTOR signaling. Genetic deletion of *miR-199a-2* recapitulates RTT phenotypes, suggesting that the MeCP2/miR-199a/mTOR axis may contribute to RTT pathophysiology.

Highlights

- MeCP2 facilitates the processing of miR-199a as a component of the Drosha complex
- miR-199a ameliorates RTT neuronal phenotypes and its inhibition blocks MeCP2 function
- miR-199a positively controls mTOR signaling by targeting mTOR signaling inhibitors
- Genetic deletion of *miR-199a-2* recapitulates numerous RTT phenotypes

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miR-199a Links MeCP2 with mTOR Signaling and Its Dysregulation Leads to Rett Syndrome Phenotypes

Keita Tsujimura,[1,2] Koichiro Irie,[1] Hideyuki Nakashima,[1] Yoshihiro Egashira,[3] Yoichiro Fukao,[4,9] Masayuki Fujiwara,[4,10] Masayuki Itoh,[5] Masahiro Uesaka,[1] Takuya Imamura,[1] Yasukazu Nakahata,[6] Yui Yamashita,[7] Takaya Abe,[8] Shigeo Takamori,[3] and Kinichi Nakashima[1,2,*]

[1]Department of Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan
[2]Laboratory of Molecular Neuroscience, Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan
[3]Laboratory of Neural Membrane Biology, Graduate School of Brain Science, Doshisha University, 1-3 Miyakodani, Tatara, Kyotanabe, Kyoto 610-0394, Japan
[4]Plant Global Education Project, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan
[5]Department of Mental Retardation and Birth Defect Research, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
[6]Laboratory of Gene Regulation Research, Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101, Japan
[7]Animal Resource Development Unit, RIKEN Center for Life Science Technologies, 2-2-3 Minatojima Minami-machi, Chuou-ku, Kobe 650-0047, Japan
[8]Genetic Engineering Team, RIKEN Center for Life Science Technologies, 2-2-3 Minatojima Minami-machi, Chuou-ku, Kobe 650-0047, Japan
[9]Department of Bioinformatics, Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan
[10]Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata 997-0017, Japan
*Correspondence: kin1@scb.med.kyushu-u.ac.jp
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SUMMARY

Rett syndrome (RTT) is a neurodevelopmental disorder caused by *MECP2* mutations. Although emerging evidence suggests that MeCP2 deficiency is associated with dysregulation of mechanistic target of rapamycin (mTOR), which functions as a hub for various signaling pathways, the mechanism underlying this association and the molecular pathophysiology of RTT remain elusive. We show here that MeCP2 promotes the posttranscriptional processing of particular microRNAs (miRNAs) as a component of the microprocessor Drosha complex. Among the MeCP2-regulated miRNAs, we found that miR-199a positively controls mTOR signaling by targeting inhibitors for mTOR signaling. miR-199a and its targets have opposite effects on mTOR activity, ameliorating and inducing RTT neuronal phenotypes, respectively. Furthermore, genetic deletion of *miR-199a-2* led to a reduction of mTOR activity in the brain and recapitulated numerous RTT phenotypes in mice. Together, these findings establish miR-199a as a critical downstream target of MeCP2 in RTT pathogenesis by linking MeCP2 with mTOR signaling.

INTRODUCTION

Rett syndrome (RTT) is a severe progressive neurodevelopmental disorder that affects approximately one in 10,000 females. Afflicted individuals appear to develop normally for the first 6–18 months but then regress, with the onset of various neurological symptoms including impaired motor function, mental retardation, seizure, autistic features, and stereotyped behaviors (Bienvenu and Chelly, 2006). RTT is predominantly caused by mutations in the X-linked gene encoding methyl-CpG binding protein 2 (MeCP2) (Amir et al., 1999). Mutations in *MECP2* are also associated with other neurodevelopmental diseases including psychiatric disorders, cognitive disorders, and some cases of autism (Cohen et al., 2002; Klauck et al., 2002; Lam et al., 2000; Orrico et al., 2000).

MeCP2 was originally identified as a methylated-DNA-binding transcriptional repressor (Jones et al., 1998; Nan et al., 1997). MeCP2 induces repression of gene expression by recruiting a corepressor complex (Harikrishnan et al., 2005; Jones et al., 1998). However, MeCP2 is becoming recognized as a pleiotropic protein, which can also mediate transcriptional activation and mRNA splicing (Chahrour et al., 2008; Young et al., 2005). Although MeCP2-deficient neurons clearly underlie the RTT phenotype, other studies have shown that the loss of MeCP2 in glia negatively influences normal neuronal functions in a non-cell-autonomous fashion in vitro and in vivo (Ballas et al., 2009; Lioy et al., 2011; Maezawa and Jin, 2010).

Several mouse models of RTT have been reported (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002). These models recapitulate many characteristic features of RTT, including the delayed-onset neurological phenotype and early mortality. Conditional deletion of MeCP2 in the brain causes symptoms that are indistinguishable from those observed in conventional MeCP2-knockout (KO) mice. Moreover, re-expression of MeCP2 in the MeCP2-deficient brain is sufficient to prevent the onset of the neurological phenotype (Guy et al., 2007; Luikenhuis et al., 2004).

In addition to genetic studies, several lines of evidence further support the effects of MeCP2 dysfunction on neuronal properties in RTT patients and model mice. Although both exhibit profound neurological abnormalities, the major neuropathological changes in the CNS are characterized by smaller neuronal soma and an overall decrease of brain size (Armstrong, 2005; Bauman et al., 1995; Chahrour and Zoghbi, 2007; Chen et al., 2001; Kaufmann et al., 2000). Subtle alterations of neuronal density, dendritic arborization, and spine formation in some specific brain regions and stages are also found in both patients and model mice in which *MeCP2* is mutated (Armstrong, 2005; Belichenko et al., 1994; Chapleau et al., 2009; Landi et al., 2011). It has been proposed that synaptic alterations constitute a major substrate of the disease symptoms (Boggio et al., 2010; Zoghbi, 2003). Neurophysiological studies of RTT patients and model mice have revealed alterations in excitatory synaptic functions (Asaka et al., 2006; Dani et al., 2005; Glaze, 2005). In particular, synaptic properties of MeCP2-deficient neurons are well studied in in vitro culture systems. Primary cultured hippocampal neurons from MeCP2-KO mice show a decrease in the frequency of spontaneous excitatory synaptic transmission (Nelson et al., 2006), and loss and overexpression of MeCP2 have opposite effects, respectively decreasing and increasing the number of excitatory synapses in individual neurons (Chao et al., 2007). The molecular mechanisms underlying these morphological and physiological alterations are unknown.

The mechanistic target of rapamycin (mTOR) protein kinase acts as a critical sensor/integrator of diverse environmental signals that are converted to neuronal activity and synaptic inputs. mTOR function is mediated through two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 phosphorylates a range of substrates to control major cell processes such as mRNA translation, lipid synthesis, and autophagy. mTORC2 is implicated in the activation of Akt and in the regulation of the cytoskeleton (Laplante and Sabatini, 2012; Shimobayashi and Hall, 2014). The evolutionarily conserved mTOR signaling pathway has been shown to play a critical role as a key determinant of cellular size (Lloyd, 2013). Further crucial roles of mTOR signaling in brain functions are highlighted by the fact that mutations of genes in the mTOR signaling pathway occur in various neurological diseases such as neurodevelopmental and psychiatric disorders (Costa-Mattioli and Monteggia, 2013).

The most distinctive phenotypes caused by MeCP2 deficiency, i.e., smaller neuronal soma and an overall decrease of brain size, offer a clue to unveil RTT molecular pathology. As described above, because mTOR signaling is a critical regulator of cell/tissue size, it has been assumed that mTOR signaling is involved in RTT pathogenesis. In support of this hypothesis, MeCP2-KO brain and MeCP2-mutated human neurons were recently reported to exhibit reduced activation of the mTOR signaling pathway (Li et al., 2013; Ricciardi et al., 2011). Furthermore, several works suggest that the activation of mTOR ameliorates abnormal phenotypes in MeCP2 mutant mice, RTT patient-derived neurons, and human embryonic stem (ES) cell-derived neurons with *MECP2* mutation (Li et al., 2013; Marchetto et al., 2010; Tropea et al., 2009). This raises the possibility that mTOR plays a crucial role downstream of MeCP2, which may be a key to elucidate the molecular basis of RTT pathogenesis. Nevertheless, the molecular mechanism linking MeCP2 function and mTOR signaling is obscure.

In this study, we found that MeCP2 associates with the microRNA microprocessor Drosha complex and identified miR-199a as an mTOR-regulating downstream target of the MeCP2-Drosha complex. We further demonstrated that genetic deletion of *miR-199a-2* led to a reduction of mTOR activity in the brain and recapitulated numerous RTT phenotypes in mice. These findings therefore suggest that MeCP2/miR-199a/mTOR axis contribute to RTT pathophysiology.

RESULTS

MeCP2 Associates with miRNA Microprocessor Drosha Complex

To obtain a clue to elucidate the molecular mechanism that underlies RTT pathogenesis and links MeCP2 to the mTOR signaling pathway, we performed comprehensive proteomic screening of mouse brain cells to identify protein partners of MeCP2 in highly enriched populations of the four major CNS cell types: neural stem cells (NSCs), neurons, astrocytes, and oligodendrocytes (Figure S1A). We then categorized the MeCP2-interacting candidate molecules in each cell type according to the Gene Ontology (GO) biological processes in which they are involved (Figure 1A). Because MeCP2 is a well-known transcriptional repressor/activator, it was expected that transcription-related molecules would appear among the MeCP2 interactors; surprisingly, however, we also observed numerous members of other functional categories such as nucleosome assembly, translation, and RNA processing (Figure 1A). Strikingly, five components of the miRNA microprocessor Drosha complex (Gregory et al., 2004)—the DEAD-box RNA helicases DDX5 and DDX17, and heterogeneous nuclear ribonucleoproteins G/M/U (hnRNPG/M/U)—were common to at least three or all four of the CNS cell types (Figures 1B and 1C). These same Drosha complex components were also detected, by LC-MS/MS, in endogenous MeCP2 complexes obtained from postnatal day (P)1 mouse brains (Figure 1D). These findings prompted us to assume that MeCP2 plays hitherto-unknown roles in miRNA processing.

The miRNA microprocessor Drosha complex consists of Drosha, DiGeorge syndrome critical region gene 8 (DGCR8), and multiple classes of RNA-associated proteins, including DDX5 and DDX17, that are required for the processing of some, but not all, miRNAs (Gregory et al., 2004). The appearance of Drosha complex proteins among MeCP2 co-immunoprecipitates prompted us to examine whether MeCP2 might function unexpectedly as a facilitator of miRNA processing.

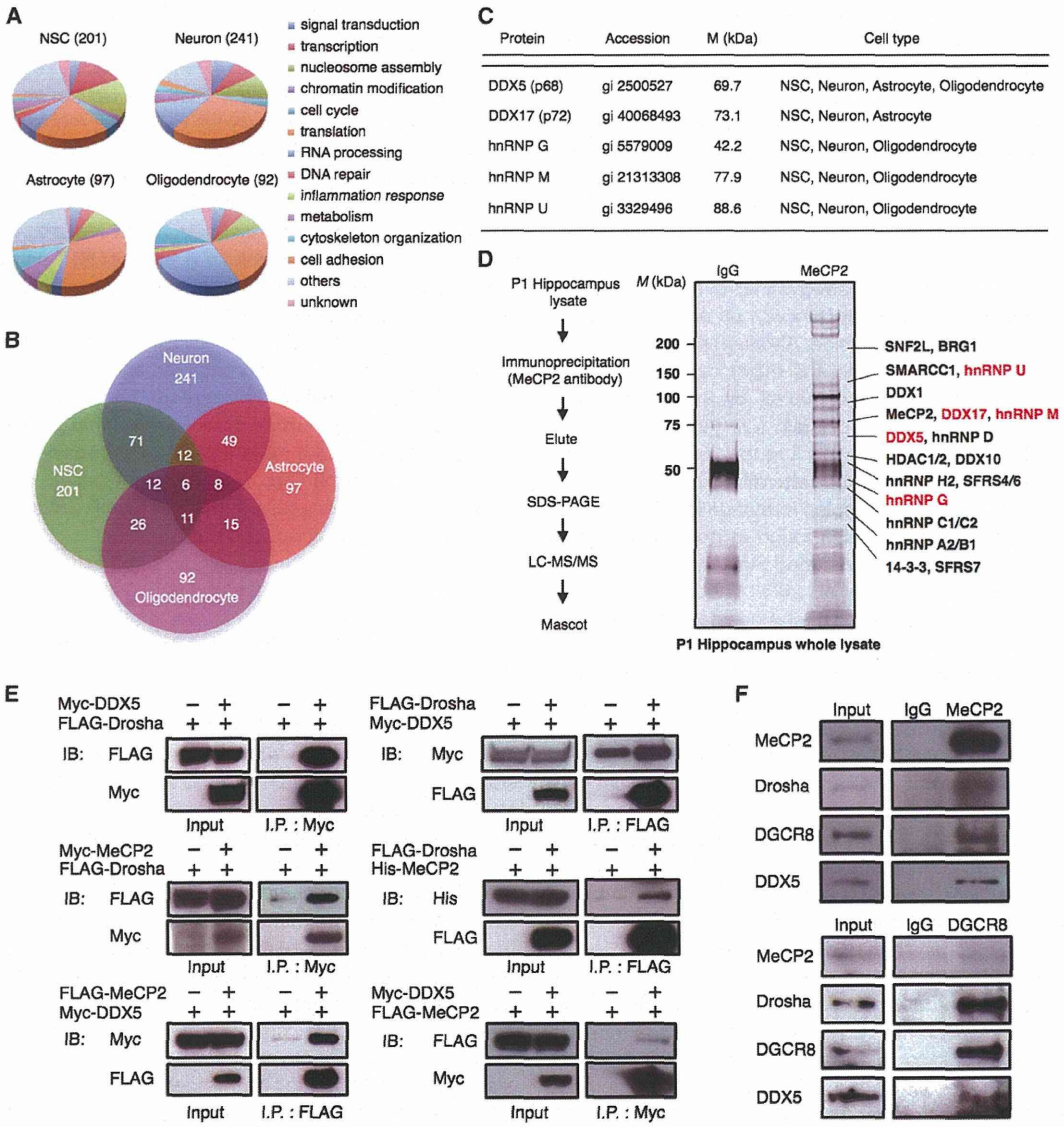

Figure 1. MeCP2 Associates with the Microprocessor Drosha Complex

(A) Pie charts representing MeCP2 interactors identified in each CNS cell type. Proteins identified in either or both MeCP2α and MeCP2β immunoprecipitates were considered as MeCP2 binding partners and are categorized.

(B) Venn diagram of common MeCP2-binding proteins identified in the four CNS cell types.

(C) Components of the Drosha complex identified by mass spectrometry as MeCP2-binding proteins. Cell types in which each protein was identified are indicated in the rightmost column. M, molecular mass.

(D) Validation of MeCP2 protein partners identified in the proteomic screen by LC-MS/MS analysis of the immunoprecipitate (using anti-MeCP2 antibody) from P1 mouse brain lysate. Components of the Drosha complex are indicated in red. Molecular masses of marker proteins are indicated. M, molecular mass.

(E and F) Immunoprecipitation assays were performed to examine the association between exogenous or endogenous MeCP2 and components of the Drosha complex in HEK293T cells (E) and P2 mouse brain (F).

IB, immunoblot; I.P., immunoprecipitation.

We first investigated molecular interactions between MeCP2 and components of the Drosha complex. Exogenous MeCP2 associated with DDX5 and Drosha in human HEK293T cells (Figure 1E). Using a reciprocal co-immunoprecipitation assay, we also confirmed that endogenous MeCP2 associated with core components of the Drosha complex including Drosha, DGCR8, and DDX5 in a mouse brain lysate (Figure 1F). Biochemical analysis also revealed that exogenous and endogenous MeCP2 complex contains the components of Drosha complex (Figures S1B–S1D). These results indicate that MeCP2 associates with