

**ABSTRACT:** MicroRNAs (miRNAs) are a class of small regulatory RNAs involved in gene regulation. The regulation is effected by either translational inhibition or transcriptional silencing. In vertebrates, the importance of miRNA in development was discovered from mice and zebrafish dicer knockouts. The miRNA-9 (miR-9) is one of the most highly expressed miRNAs in the early and adult vertebrate brain. It has diverse functions within the developing vertebrate brain. In this article, the role of miR-9 in the developing forebrain (telencephalon and diencephalon), midbrain, hindbrain, and spinal cord of vertebrate species is highlighted. In the forebrain, miR-9 is necessary for the proper development of dorsoventral telencephalon by targeting marker genes expressed in the telencephalon. It regulates proliferation in telencephalon by regulating *Foxg1*, *Pax6*, *Gsh2*, and *Meis2* genes. The feedback loop regulation between miR-9 and *Nr2e1/Tlx* helps in neuronal migration and differentiation. Targeting *Foxp1* and *Foxp2*, and *Map1b* by miR-9 regulates the radial migration of neurons and axonal development. In the organizers, miR-9 is inversely regulated by *hairy1* and *Fgf8* to maintain zona limitans interthalamica and midbrain–hindbrain boundary (MHB). It maintains the MHB by inhibiting Fgf signaling genes and is involved in the neurogenesis of the midbrain–hindbrain by regulating *Her* genes. In the hindbrain, miR-9 modulates progenitor proliferation and differentiation by regulating *Her* genes and *Elav3*. In the spinal cord, miR-9 modulates the regulation of *Foxp1* and *Onecut1* for motor neuron development. In the forebrain, midbrain, and hindbrain, miR-9 is necessary for proper neuronal progenitor maintenance, neurogenesis, and differentiation. In vertebrate brain development, miR-9 is involved in regulating several region-specific genes in a spatiotemporal pattern.

**KEYWORDS:** miR-9, telencephalon, diencephalon, midbrain, hindbrain, spinal cord

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## Introduction

MicroRNAs (miRNAs) are endogenously expressed 20–25 nucleotide-long RNA molecules whose identification began with *lin-4* in *Caenorhabditis elegans*, where it was shown to be involved in gene regulation at the larval developmental stage.<sup>1</sup> Since then, miRNAs have been reported to regulate multiple biological processes from development to cell proliferation, growth regulation, and apoptosis.<sup>2</sup>

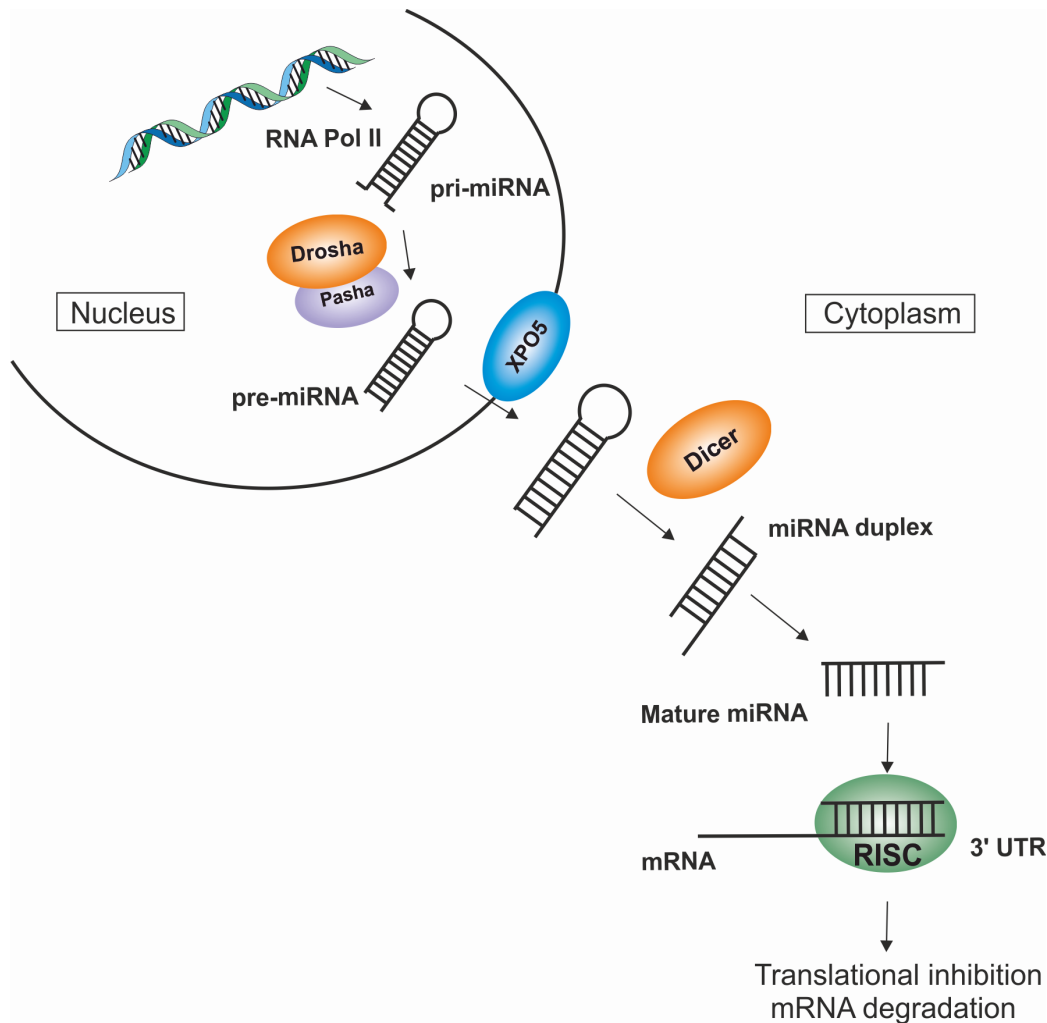
MiRNAs are transcribed by RNA Pol II as primary transcripts (primary miRNA, pri-miRNAs), which are usually several kilobases long and contain local stem-loop structures.<sup>3</sup> The maturation of the miRNA begins with the cleavage of larger hairpins into smaller hairpins called the precursor miRNA (pre-miRNA) by Drosha.<sup>4</sup> In the case of *Drosophila*, an additional protein Pasha was identified and was shown to have pri-miRNA-processing capability. Pasha knockouts (KOs) showed the accumulation of pri-miRNA suggesting that Drosha works together with Pasha.<sup>5</sup> Post nuclear processing, the pre-miRNAs are transported into the cytoplasm where they are processed again by the enzyme dicer, generating the mature miRNA.<sup>6</sup> The miRNA duplexes are incorporated into a protein complex consisting of Argonaute and

accessory proteins called the pre-miRNA-induced silencing complex (pre-RISC).<sup>7</sup> The removal of the passenger strand from the miRNA duplex results in the formation of the mature RISC complex, which then targets suitable mRNAs resulting in posttranscriptional repression or the destruction of the mRNA target (Fig. 1).<sup>8–10</sup>

## miRNAs in Development

The miRNAs are involved in numerous cellular and biological events.<sup>11–13</sup> They are ideal candidates for the fine-tuning of gene expression due to their nature of having specific spatiotemporal expression patterns.<sup>14</sup> The importance of miRNAs in development is revealed by the loss of miRNA-processing enzymes and its machinery proteins (dicer, Drosha, dgcr8, Argonaute).<sup>15–19</sup>

**Dicer significance in development.** Dicer is essential for the processing of mammalian miRNA. Dicer knockout (KO) studies in mice and zebrafish<sup>16,17</sup> have provided insights into the role of miRNAs from a developmental perspective. Dicer ablation results in embryonic lethality in mice<sup>16</sup> and zebrafish;<sup>17</sup> thus, conditional KO (CKO) serves as a tool to study the role of dicer in the vertebrate system.



**Figure 1.** miRNA biogenesis.

**Notes:** The schematic figure shows miRNA biogenesis, processing, maturation, and regulation. The miRNAs are transcribed within the nucleus by RNA polymerase II from the noncoding region of the genome. The transcribed pri-miRNA is processed by Drosha/Pasha microprocessor complex into pre-miRNA. The pre-miRNA is exported from the nucleus into the cytoplasm through the nuclear membrane protein, XPO5. In the cytoplasm, the endonuclease dicer processes the pre-miRNA into miRNA duplex. The miRNA duplex is unwound by a helicase and forms the mature miRNA (~18–25 nucleotides). The mature miRNA is incorporated into the RISC with Argonaute protein as a catalytic component. The miRNA–RISC complex binds at the 3'UTR of the mature mRNA and regulates posttranslational gene expression by translation inhibition or mRNA degradation (transcriptional silencing). **Abbreviations:** miRNA, microRNA; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; 3'UTR, 3' untranslated region; XPO5, Exportin 5.

**Conditional dicer KO in development.** The functional implication for the necessity of miRNA in development comes from the zebrafish maternal–zygotic (MZy) dicer KO embryo, a CKO. The MZy dicer shows abnormalities in brain development with the reduction in the formation of brain vesicles.<sup>20</sup> In mice, the dicer CKOs were generated using cre–lox mechanism. The normal expression patterns of the brain-specific genes in which the CKOs were created are as follows: *Foxg1-cre*<sup>21</sup> at E8.0, *Wnt1-cre*<sup>22</sup> at E8.5, *Emx1-cre* at E9.5,<sup>23–26</sup> *Nestin-cre* at E10.5,<sup>27</sup> *Nex-cre*<sup>28,29</sup> and *hGFAP-cre*<sup>30,31</sup> at E13.5, and *CaMKII- $\alpha$ -cre* at E15.5<sup>32</sup> (Table 1, Fig. 2A and B).

The dicer CKOs from *Foxg1-cre*, *Emx1-cre*, and *Nestin-cre* show that miRNAs are necessary in the early forebrain development and *Nex-cre*, *CAMKII-cre*, and *GFAP-cre* in the

postnatal forebrain development.<sup>28,33–36</sup> The *Wnt1-cre* dicer demonstrates the necessity of the miRNAs in midbrain and hindbrain development.<sup>37</sup> Finally, *Wnt1-cre* and *hGFAP-cre* dicer shows the necessity of miRNAs in hindbrain development.<sup>30,37</sup> Some of the CKO embryos died early or postnatal.<sup>28,35,38</sup> The dicer CKOs provide valuable information on the role of miRNAs in development which include cell proliferation, neurogenesis, migration, maturation, and differentiation. The dicer KO studies are described in detail in the following sections in context with the role of miRNA-9 (miR-9) in brain development.

**Role of miR-9 in development.** The miR-9 is one of the highly expressed miRNAs in the developing vertebrate brain and involved in several cellular functions and development.<sup>39–41</sup>

**Table 1.** Summary of dicer Knockout studies.

SPECIES	KNOCKOUTS/ MUTANT	GENE EXPRESSION DURING EMBRYONIC STAGE	EXPRESSION STUDIED STAGE	PHENOTYPE OF THE DICER MUTANTS	DOWNREGULATED miRNA	REFERENCES
Zebrafish	<i>Dicer1</i> KO	–	–	Homozygous and trans-heterozygous appeared normal till 8 dpf (day post fertilization). Later showed severe developmental growth arrest and died after 14–15 days.	NS	Wienholds et al <sup>17</sup>
	MZy (maternal and zygotic) <i>dicer1</i> Knockout	–	–	Normal axis formation and differentiate multiple cell types, but shows abnormal morphogenesis during gastrulation, brain formation, somatogenesis and heart development.	NS	Giraldez et al <sup>20</sup>
	<i>Dicer1</i> KO	Dicer expression—E4 <sup>171</sup>	E7.5–11.5	At 7.5, embryo appeared small with morphological abnormalities and lacks primitive streak marker, <i>brachyury</i> . Dicer mutant is arrested before the body plan is configured during gastrulation. Lacks Oct 4—a marker for ES cell maintenance and proliferation. E8.5—necrotic embryo. E9.5–11.5—empty and necrotic decidua.	NS	Bernstein et al <sup>16</sup>
Mice	<i>Foxg1-cre-Dicer1</i> conditional KO	<i>Foxg1/BF-1</i> expression—E8 <sup>21</sup>	E10.5–12.5	1) At E11.5, the neuroepithelial markers: Prominin1, Notch signaling inhibitor Numb, Sox2 (sex determination region Y box 2) and Numb inhibitor Musashi have not been altered; and the genes involved in forebrain patterning; <i>Foxg1</i> , <i>Pax6</i> , <i>Ngn1</i> , <i>Emx2</i> , <i>Olig2</i> , <i>Dlx2</i> have also not been altered. 2) The Nestin, Sox9, Erb2 from the radial glial cells of the VZ have been reduced in the dorsal telencephalon by E11.5 and E12.5. 3) E11.5–12.5: The neurons and basal progenitors are greatly affected in dicer KO. 4) There is an increase in apoptosis from E11.5 onwards. The size of the telencephalon is greatly reduced by E14.5.	miR-9 and miR-124 is depleted by E11.5 and undetected in the dorsal telencephalon.	Nowakowski et al <sup>33</sup>
	<i>Wnt1-cre Dicer</i> conditional KO	<i>Wnt1</i> expression—E8.5 <sup>22</sup>	E12.5, E14.5 and E18.5	1) Dicer mutant fetuses exhibited dramatic malformation of the tectum and cerebellum and the eyelids were open. 2) The skeletal structures that are derived from the cranial NC were lost or mostly ablated in Dicer mutant mice. 3) Deletion of Dicer in the NC cells resulted in the malformation of the dorsal root ganglia, enteric nervous system and sympathetic ganglia. 4) Dicer is required for the specification of dopaminergic neurons.	The expression of miR-9, miR-124 and miR-218 in the midbrain and rostral hind-brain area was mostly eliminated.	Huang et al <sup>37</sup>
Mice	<i>Emx1-cre-Dicer</i> conditional KO	<i>Emx1</i> expression—E9.5 <sup>26</sup>	E10.5, E12.5, E13.5, E14.5, E15.5	1) The neural progenitor markers have been reduced from E15.5 onwards in the VZ. 2) They have normal karyotype, but have enlarged nuclei. 3) Increased apoptosis.	The miR-181b and miR-125b expressed in NSCs have been absent in dicer mutant.	Kawase-Koga et al <sup>72</sup>
	<i>Emx1-cre-Dicer</i> conditional KO	<i>Emx1</i> expression—E9.5 <sup>26</sup>	E13.5, E15.5, E16.5, E17.5, E18.5	1) Survived till P30. 2) The cortex was significantly smaller and the hippocampus was not detectable due to the lack of neurogenesis. 3) At E17.5, the CP was slightly enlarged and the VZ/SVZ and IZ were greatly reduced. 4) The early born neurons were reduced at E15.5. 5) The Tbr1 cells were observed in the CP and IZ.	The miR-17, miR-181 was undetectable in the cortex of E13.5, E15.5 and P1.	Kawase-Koga et al <sup>36</sup>

(Continued)



Table 1. (Continued)

SPECIES	KNOCKOUTS/ MUTANT	GENE EXPRESSION DURING EMBRYONIC STAGE	EXPRESSION STUDIED STAGE	PHENOTYPE OF THE DICER MUTANTS	DOWNREGULATED miRNA	REFERENCES
			E13.5, E14.5, E15.5, E16.5, E18.5	<ol style="list-style-type: none"> <li>Has smaller cortex and not distinguishable by P0 showing dicer ablation affecting hippocampal progenitor development.</li> <li>The transcription factor <i>Lhx2</i> reduced in the hippocampus by E13.5 onwards.</li> <li>The proliferative cells were reduced at E15.5 in hippocampal neuroepithelial cells and DG progenitors.</li> <li>The hippocampal markers <i>Wnt7b</i>, <i>Lef1</i> have been reduced and <i>Prox1</i> was absent in the hippocampus by E16.5.</li> <li>Ectopic <i>Reelin</i> was observed in cortex at E18.5 suggesting abnormal CR neuron migration and mature neurons were reduced in the hippocampus at E18.5.</li> <li>Ectopic <i>Tbr2</i> was observed in the cortical progenitor zone and in hippocampal region at E18.5.</li> </ol>	miR-9 and let-7a was undetectable by E16.5.	Li et al <sup>28</sup>
			E18.5	The dorsal and lateral cortical wall was thinner and the ventricles were larger. The proliferating cells from VZ and SVZ were reduced. The post-mitotic neurons were thinner in the CP, in the IZ was expanded in cortex at E18.5 indicating the late born neurons were reduced.	-	Kawase-Koga et al <sup>36</sup>
	<i>Nestin-cre-Dicer</i> conditional KO	Nestin expression—E10.5 <sup>27</sup>	E13.5, E14.5, E15.5, E16.5, E18.5	<ol style="list-style-type: none"> <li>Died after birth.</li> <li>Hippocampal morphogenesis occurs normally but has reduced hippocampal size.</li> <li>The proliferative cells were reduced at E15.5 in hippocampal neuroepithelial cells, DG progenitors and increased apoptosis observed.</li> <li><i>Reelin</i> expression was normal in E15.5 and E18.5.</li> <li>The transcription factor <i>Lhx2</i> was reduced in the hippocampus from E18.5 onwards.</li> <li>The hippocampal marker <i>Prox1</i> has been reduced in the hippocampus by E18.5. Neurogenesis was affected.</li> <li>Ectopic <i>Tbr2</i> was observed in the cortical progenitor zone and in hippocampal region at E18.5.</li> </ol>	miR-9 and let-7a was undetectable by E14.5.	Li et al <sup>28</sup>
			E15.5, E16.5	<ol style="list-style-type: none"> <li>Microcephaly, gross enlargement of the lateral ventricles and marked cortical thinning in the embryos.</li> <li>Progenitors were distributed throughout the cortex, instead of confined to the VZ and SVZ.</li> <li>CR neurons are numerous and their distribution is dysmorphic.</li> </ol>	NS	Mcloughlin et al <sup>111</sup>
	<i>Nex-cre-Dicer</i> conditional KO	Nex expression—E13.5 <sup>29</sup>	P1, P22	<ol style="list-style-type: none"> <li>Died by P23.<sup>38</sup></li> <li>The hippocampal morphology was not significantly affected, but has smaller brain.</li> <li>The hippocampal markers <i>Wnt7b</i> have been reduced in the hippocampus by P1.</li> <li>The NeuN was greatly reduced in CA3 pointing to out defects in neurogenesis.</li> <li>Apoptosis was seen in CA3 and DG cells of the hippocampus at P22.</li> </ol>	miR-9 and let-7a was undetectable by P22.	Li et al <sup>28</sup>



<i>hGFAP-cre</i> KO	E13.5 <sup>31</sup>	E15.5, P1	<p>The brain lipid binding protein (BLBP) is distributed in the VZ/SVZ and CP, rather than in MZ. The RG is translocated in the pia at P1–P5. The retraction of radial process is reduced in the postnatal cerebral cortex. Notch and its ligand Jag2 signaling are enhanced.</p> <p>1) Phenotype of Bergmann glia (BG) cells was studied. 2) Smaller and underdeveloped cerebellum with aberrant BG morphology. 3) Notch 1 target gene expression affected.</p>	miR-9 and miR-124 reduced by P1	Zhang et al <sup>34</sup>
<i>CamKII-cre</i> conditional KO	CamKII expression—E15.5 <sup>32</sup>	P0, P15, P21	<p>1) Microcephaly, increased ventricle size and reduction of size in white matter. 2) Motor impairments.</p>	miR-9 levels decreased.  miR-132 (dendritic outgrowth) reduced in the cortex and hippocampus. miR-124 was reduced in the cortex.	Kuang et al <sup>30</sup>  Davis et al <sup>35</sup>

**Abbreviations:** NS, not specified; E, embryonic day; P, postnatal day; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; MZ, mantle zone; LGE, lateral ganglionic eminence; DG, dentate gyrus; RG, radial glia.

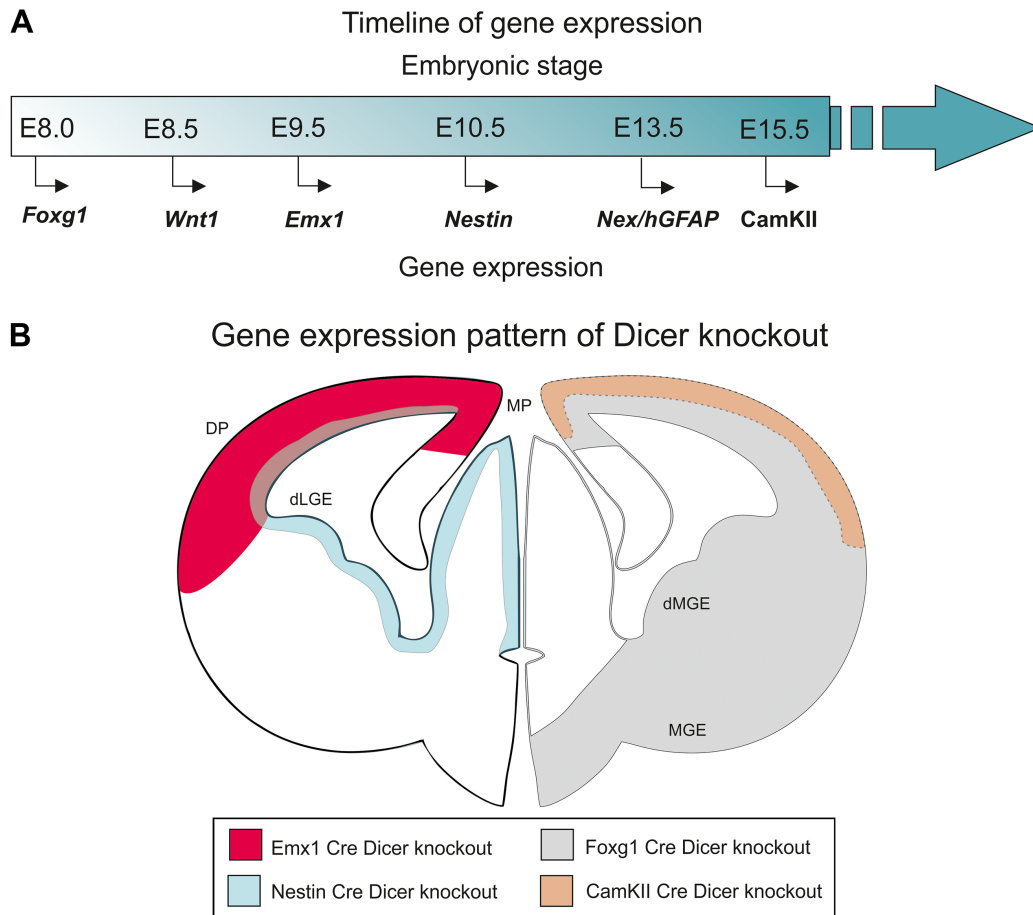
The miR-9 is conserved among the vertebrates and specifically expressed in the central and peripheral nervous system;<sup>39–42</sup> it has also been reported to be present in multiple copies (Table 2).<sup>41,43</sup> The retention of multiple copies has also been linked to the differential expression of its paralogs and specialization in their function.<sup>44</sup> The first evidence of its function in the nervous system comes from mouse,<sup>45</sup> where it has been reported to be involved in the patterning of brain structures and in cellular events like proliferation, maturation, and differentiation of neurons in the brain.<sup>46–49</sup> The expression pattern of miR-9 in the vertebrates is summarized in Table 3.

Apart from its prominent role in development, miR-9 has been correlated with several other cellular roles and pathology.<sup>2,50</sup> The most significant of these roles is its association with cancer, where aberrant levels of the miRNA have been identified in tumor formation or progression.<sup>51</sup> In the case of gastric cancer, miR-9 knockdowns have shown an inhibitory role in the proliferation of tumor cells.<sup>52</sup> Contrastingly, in the case of ovarian cancer cells, the overexpression of miR-9 was shown to be useful in the suppression of tumor proliferation.<sup>53</sup> The miR-9 has also been shown to regulate interferon-regulated genes and MHC class I molecules.<sup>54</sup> In this review, the role of miR-9 in early brain development is summarized.

## Brain Development

The developing central nervous system (CNS) is regionalized into four distinct regions, namely prosencephalon (forebrain), mesencephalon (midbrain), rhombencephalon (hindbrain), and spinal cord. The vertebrate forebrain is further divided anteriorly into telencephalon (prospective cerebral hemisphere) and posteriorly into diencephalon (prospective thalamus and hypothalamus). The rhombencephalon is subdivided into metencephalon (prospective pons and cerebellum) and myelencephalon (prospective medulla oblongata).<sup>55,56</sup> The organizers mediate the anterior–posterior (AP) axis and dorsoventral (DV) axis development. The organizers are special group of cells that produce secreted molecules and act as long-lasting coordinators of many cellular events like cell fate, survival, proliferation, maturation, and differentiation. The organizers along the AP axis are anterior neural ridge (ANR)/anterior neural border (ANB), zona limitans interthalamica (ZLI), and isthmus or midbrain–hindbrain boundary (MHB).<sup>57,58</sup>

**Cell biology of neurogenesis.** In the early phase of development, the neural plate and neural tube are made of a single layer of neuroepithelial cells. The neuroepithelial cells are primary neural stem cells (NSCs) in the developing neural tube. These NSCs divide symmetrically resulting in massive expansion prior to neurogenesis.<sup>59</sup> The telencephalic neurogenesis in mice takes place between E10 and E17.<sup>33,60</sup> In chicken, the telencephalic neurogenesis takes place between E4 (HH24) and E10,<sup>61</sup> while the neurogenesis of the optic tectum takes place between E3 (HH18) and E20 (HH45).<sup>62,63</sup> In zebrafish, the telencephalic formation and the morphogenetic movement take place between 18-hour postfertilization



**Figure 2.** Time line of gene expression and gene expression pattern in the developing brain.

**Notes:** (A) The time line of gene expression in mouse embryo. (B) The outline of the schematic sketch is adapted from Grant et al.<sup>169</sup> Modified and reused under the terms of a CC-BY-NC 3.0 license. This shows that the area will be devoid of dicer in the CKO with the representative genes in the cre/lox system.

**Abbreviation:** CKOs, conditional knockouts.

**Table 2.** miR-9 and their location in chromosomes of different species.

SPECIES	miR-9 VARIANTS	CHROMOSOMES	EXPRESSION PATTERN	REFERENCES
Mouse	miR-9-1	3	Proliferative zone in telencephalon	Shibata et al <sup>180</sup>
	miR-9-2	13	Telencephalon	
	miR-9-3	7	Proliferative zone in telencephalon	
Xenopus	miR-9a-1	GL173063.1	Forebrain, eye, midbrain and hindbrain	Bonev et al, <sup>48</sup> Walker and Harland <sup>126</sup>
	miR-9a-2	GL172712.1		
	miR-9b/miR-9-3	GL172838.1/GL172658.1		
Zebrafish	miR-9-1	16	Telencephalon	Nepal et al <sup>130</sup>
	miR-9-2	10	Retina, telencephalon	
	miR-9-3	25	Telencephalon	
	miR-9-4	22	Posterior brain regions, telencephalon	
	miR-9-5	5	Telencephalon	
	miR-9-6	7	Hypothalamus, telencephalon	
	miR-9-7	2	Telencephalon	
Chicken	miR-9-1	28	NS	
	miR-9-2	Z	NS	

**Abbreviation:** NS, not specified.

**Table 3.** MiR-9 expression in the developing brain of different species.

SPECIES	BRAIN VESICLE	miR-9 EXPRESSION	DEVELOPMENTAL STAGE	REFERENCES
Mice	Telencephalon	+	E9, E9.5, E10, E11.5, E12.5, E14.5, E15.5, E16.5, E18.5	33, 47, 80, 173
	Diencephalon	–	E9, E9.5, E10, E11.5	
	Midbrain	NS		
	MHB	NS		
	Hindbrain	+	E9.5, E10	
	Spinal cord	+	E9.5, E10	
Xenopus	Forebrain	+	Stage 19, 24, 28, 30, 36	48, 126
	Midbrain	+		
	MHB/ZLI	–		
	Hindbrain	+		
	Spinal cord	–		
Zebrafish	Telencephalon	+	20–30 hpf	39, 43, 46, 128
	Diencephalon	+		
	Midbrain	+	30–35 hpf	
	MHB	–		
	Hindbrain	+	20–30 hpf	
	Spinal cord	+		
Chick	Telencephalon	+	HH18, 20, 22–25	42, 83, 132, 167
	Diencephalon	+		
	Midbrain	+		
	MHB	NS	HH11–29	
	Hindbrain	+		
	Spinal cord	+		

**Abbreviation:** NS, not specified.

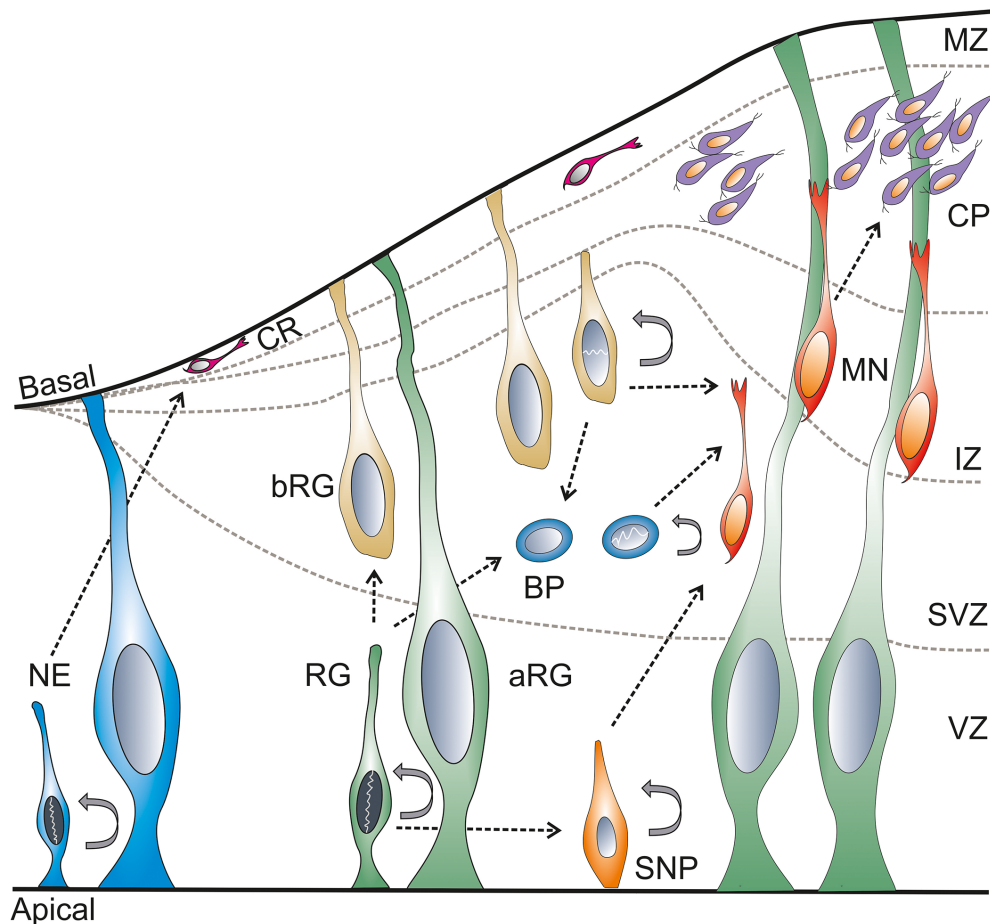
(hpf) and 5 day postfertilization (dpf),<sup>64</sup> and the tectal neurogenesis starts by 24 hpf.<sup>65</sup> Cortical neurogenesis takes place between stage 22 and 36,<sup>66,67</sup> and tectal neurogenesis between stage 35 and 58 in *Xenopus*.<sup>68</sup> In the early phase, the NSCs divide asymmetrically and form the NSC and Cajal–Retzius (CR) neurons. The generation of CR neurons is modulated by miR-9.<sup>47,60</sup> The CR neurons<sup>69,70</sup> migrate from the ventricular zone (VZ) to the basal lamina that forms the preplate. As the development progresses, the preplate forms the subventricular zone (SVZ), intermediate zone (IZ), the cortical plate (CP), and the marginal/mantle zone (MZ).<sup>71</sup> In later stages, the NSCs give rise to the neuronal progenitor cell types: the apical (cells from the VZ) and basal progenitors (cells from the SVZ) and neurons (reside in the MZ). The apical and basal progenitors consist of radial glia and intermediate progenitors.<sup>72</sup> These progenitors mature and form the neurons in the MZ (Fig. 3).<sup>59,73,74</sup>

The miR-9 is expressed throughout the cellular development of neurons, ie, proliferation, neurogenesis, migration, maturation, and differentiation.<sup>45,74–78</sup> The miR-9 is also one of the specific markers for neuronal cells.<sup>79</sup> It is expressed in the proliferating and differentiating cells in the telencephalon, diencephalon, and optic tectum.<sup>39</sup> The miR-9 regulates

proliferation by targeting several transcription factors (*Foxg1*, *Meis2*, *hairy1*, *hes1*, *her* genes, etc.).<sup>46–49,80</sup> The miR-9 suppresses TLX (human homolog of the *tailless* gene; also known as nuclear receptor subfamily 2, group E member 1 [Nr2e1]) expression to negatively regulate proliferation and accelerate neural differentiation.<sup>75</sup> Along with miR-124, miR-9 acts as a key factor in regulating neuronal differentiation.<sup>77</sup> Axon development in the developing cortex is regulated by miR-9 via targeting Map1b.<sup>81</sup> Neurite outgrowth/migration is also controlled by miR-9 through its regulation of *Foxp1* and *Foxp2*.<sup>82,83</sup>

**Telencephalon development.** Telencephalon is derived from the rostral margin of the neural plate.<sup>84</sup> During early gastrulation, the formation of the neural plate (neural induction) is promoted by fibroblast growth factor (Fgf) and repressed by transforming growth factor- $\beta$  and Wnt signaling. Following neural induction, the rostral margin neural plate cells known as ANR in mouse or the ANB in zebrafish which express Fgf8 is essential for the development of telencephalon.<sup>85–87</sup>

During the early telencephalon development, at the closing end of the neural tube, the telencephalic midline is formed. Within the midline, there are three signaling centers: the dorsal midline, the rostral midline, and the ventral midline. The dorsal midline expresses several bone morphogenetic protein



**Figure 3.** Cell biology of neurogenesis.

**Notes:** During development, at the early stage, the NE divides asymmetrically and forms NE and CR neurons. These CR neurons migrate to the basal region and form the preplate; where at later stages the preplate forms the SVZ, IZ, and CP. At later stages, the RG divides and gives rise to different types of cells, namely SNP and BP. According to the residence of the RG, the cells are known as aRG and bRG cells. The bRG mostly resides in the SVZ. The SNP matures and migrates to CP and MZ. The MNs might also arise from RG and BP. The NE, RG, bRG, BP, and SNP are capable of self-dividing.

**Abbreviations:** NE, neuroepithelial; CR, Cajal–Retzius; RG, radial glial; aRG, apical radial glia; bRG, basal radial glia; BP, basal/intermediate progenitors; SNP, small neuronal progenitor; SVZ, subventricular zone; MZ, mantle zone; CP, cortical plate; IZ, intermediate zone; MNs, motor neurons.

(BMP) genes.<sup>88</sup> The ANR/ANB and the nearby area becomes the rostral midline that expresses several *Fgf* genes including *Fgf8*.<sup>89</sup> The ventral midline is defined by the expression of sonic hedgehog (SHH) signaling.<sup>90</sup>

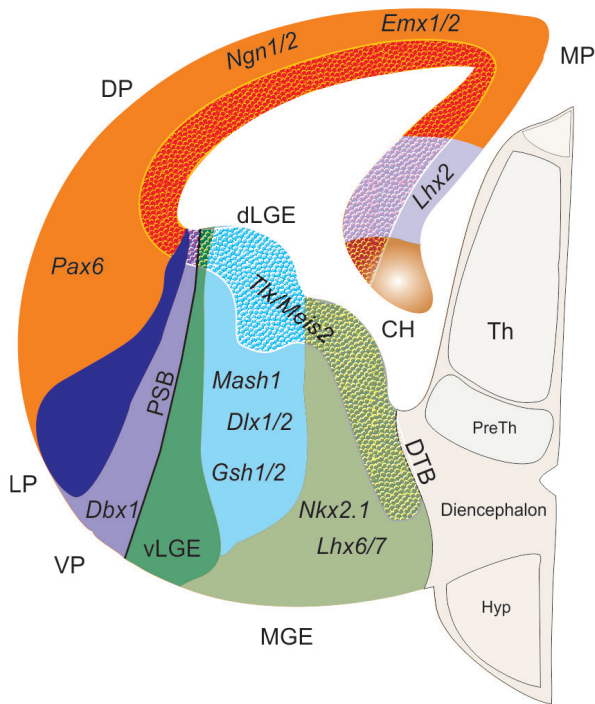
The DV patterning of telencephalon is the first step in the formation of the neocortex. The dorsal half forms the pallium, later develops into the cortex, and the ventral half forms the subpallium that later develops into the basal ganglia.<sup>57</sup> The pallium is divided into four main subregions: dorsal, medial, lateral, and ventral pallium. The DV patterning is defined by the generation of different cell types from the dorsal and ventral precursor cells; dorsally, they give rise to glutamatergic neurons and form the neocortex and hippocampus, and ventrally they give rise to GABAergic neurons and form the medial ganglionic eminence (MGE) and lateral ganglionic eminence (LGE), which form the striatum and globus pallidus.<sup>91</sup> The transcription factor, *Pax6*, is expressed in the dorsal telencephalon, whereas *Gsh2* is expressed in the ventral

telencephalon. The counteraction between *Pax6* and *Gsh2* forms the pallio-subpallial boundary (PSB; Fig. 4).<sup>92,93</sup>

Overall, the telencephalon is induced by the repression of Wnt signaling. The formation of the telencephalic midline involves the interaction of *Shh* ventrally, *Fgf* genes rostrally, *Bmp* laterally, and *Wnt* genes dorsally. The signaling centers regulate the expression of *Emx2*, *Pax6*, and *Gsh2*, which is necessary for the regulation of region-specific neurogenic genes such as *Ngn1* and *Mash1* on dorsal and ventral telencephalon, respectively (Fig. 4).<sup>91,94</sup>

*Role of miR-9 in telencephalon development of mice.* In mice, miR-9 has three variants such as miR-9-1, miR-9-2, and miR-9-3 that express the same mature product, miR-9.<sup>95</sup> The miR-9-2 is abundantly expressed in the telencephalon, while miR-9-1 and miR-9-3 are observed in the proliferative zone (Table 2).<sup>80</sup> The miR-9 expression is first observed at E9.0 stage in the telencephalic primordium. At E10.5, miR-9 is observed in optic vesicles and spinal cord, where they are dorsolaterally graded in





**Figure 4.** Gene expression in the developing telencephalon.

**Notes:** The schematic sketch is adapted from Grant et al.<sup>169</sup> Modified and reused under the terms of a CC-BY-NC 3.0 license. The telencephalon is divided into dorsal (pallium) and ventral (subpallium) telencephalon. The pallium expresses Pax6 and the subpallium expresses Gsh2. The repression of Pax6 and Gsh2 expression leads to the formation of PSB. The DP progenitor expresses Pax6, Emx1/2, and Ngn1/2. The MP expresses Lhx2, while the VP expresses Dbx1. The ventral subpallium is subdivided into MGE and LGE, where the MGE progenitors express Gsh2, Mash1, and Dlx1/2, and LGE expresses Nkx2.1 and Lhx6/7. The textured area shows Tlx and Meis2 expression in the MP, DP, dLGE and dMGE.

**Abbreviations:** CH, cortical hem; MP, medial pallium; DP, dorsal pallium; VP, ventral pallium; LP, lateral pallium; dLGE, dorsal lateral ganglionic eminence; vLGE, ventral lateral ganglionic eminence; MGE, medial ganglionic eminence; PSB, pallial-subpallial boundary; DTB, diencephalon–telencephalon boundary; Th, thalamus; preTh, prethalamus; Hyp, hypothalamus.

the cortex with the subcortex sparsely expressing miR-9. The caudal pallium expresses miR-9 at E10.5; later at E11.5, in the medial pallium, and at E12.5, the expression was restricted to the medial pallium and retained in the MZ of the entire cortex including ventral pallium.<sup>47</sup> The hippocampus that is derived from the medial pallium expresses miR-9.<sup>28</sup> Histology shows that miR-9 is expressed throughout the proliferative zone of the developing telencephalon<sup>80,96</sup> and in some differentiating neurons of the neocortex.<sup>49,96,97</sup>

The forkhead box protein G1 (Foxg1) is the earliest expression marker in the telencephalon and is expressed at E8.0.<sup>98</sup> The *Foxg1-cre* dicer KO shows that there are no obvious changes in the telencephalic markers: the pan-telencephalic marker such as FoxG1 and dorsal telencephalic markers such as Pax6, Emx2, and Ngn2, and ventral telencephalic markers such as Olig2 and Dlx2. The miR-9 was reduced and undetectable in the telencephalon at E11.5 stage, when

neurogenesis occurs. The Nestin (a progenitor marker) in the VZ radial progenitor cells was reduced, with a loss of transcription factor, Sox9 (a co-enhancer of *Nestin* gene)<sup>99,100</sup> and Erb2 receptor (mediator for the formation of radial glial cells via neuregulin1 signaling).<sup>101</sup> The postmitotic cells were markedly reduced in E12.5 telencephalon. The CR cells have been depleted in this dicer KO. The proportion of basal progenitor *Tbr2* cells has been increased in E11.5 ventral telencephalon and appears at the telencephalic wall, where these cells are not usually found. The telencephalon size was markedly reduced due to apoptosis from E12.5 onward.<sup>33</sup>

Interestingly, Foxg1 is upregulated and miR-9 is down-regulated in the olfactory and GnRH neurons in *Dlx5* (*distal-less* homeobox gene) mutant mice. Thus, proving that miR-9 regulates Foxg1 and *Dlx5* facilitates the production of miR-9.<sup>102</sup> *Dlx5* is one of the earliest markers for the rostral ectoderm. The *Dlx5* is expressed in the ANR at E7.5, ventral cephalic epithelium at E8.5 and from E9.5 onward in the telencephalon.<sup>103,104</sup> However, in the telencephalon, the interaction between *Dlx5* and miR-9 is yet to be established.

The Emx1 (empty spiracles homolog 1) is specifically expressed in the dorsal telencephalon and observed at E9.5.<sup>23–25</sup> The *Emx1-cre* dicer KO shows that the miR-9 is barely detectable from E10.5 telencephalon in the VZ neural progenitor cells. Detailed study indicates that the miR-9 expression is abrogated in the preplate (E11.5), CP (E12.5), and VZ of the telencephalon in comparison to the normal phenotype.<sup>96</sup> There is no difference in the apical and basal progenitor population, but there is a reduction in the cortical neuronal layer, which was attributed to apoptosis via depletion of miRNAs. Until E13.5, the apical (Pax6-expressing cells) and basal (*Tbr2*-expressing cells) progenitor cell division and progression were not affected;<sup>96</sup> however, at E14.5, there was a decrease in the mitotic apical and basal progenitors.<sup>36,96</sup> At E15, the cortices had higher *Tbr1* and *Tuj1* expression indicating that early neurogenesis is altered. At E17.5, the cerebral cortices were smaller, and the VZ and IZ were reduced with slightly enlarged CP. The *Emx1-cre* dicer KO mice survived until postnatal day 30 (P30).<sup>36</sup>

The *Emx1-cre* dicer KO also shows severe defects in the hippocampus with a reduction in miR-9 at E16.5. The *Emx1-cre* has reduced hippocampal neuroepithelial cells and dentate gyrus (DG) granular progenitors.<sup>28</sup> The early hippocampal marker, *Lhx2*, which helps in the expansion of the cortical hem<sup>105</sup> where CR neurons are formed,<sup>106</sup> was reduced in the hippocampus from E13.5 onward. At E16, the hippocampal marker such as *Wnt7b* and *Lef1* had been reduced in the hippocampus and fimbria and *Prox1* in the DG.<sup>28</sup> The CA1, CA3, and DG regions of hippocampus are formed at E18.5.<sup>107,108</sup> The *Tbr2* (progenitor marker) and *Prox1* (neuronal marker) expression in the DG was absent. Ectopic expression of *Tbr2* was observed in cortical progenitor zone of *Emx1-cre* dicer KOs. However, mature neurons were reduced in all the three regions.<sup>28</sup>



The *Emx1-cre* dicer KO has increased *Foxp2* expression in the dorsal telencephalon as well as in some neural progenitors of the VZ,<sup>82</sup> which was not observed in the wildtype.<sup>109</sup> Similarly in miR-9-2/3 double KO at E18.5, the *Foxp2* was increased in the layer VI concluding that *Foxp2* expression was increased in the absence of miR-9.<sup>80</sup> *Foxp2* is a member of forkhead family of transcription factors. *Foxp2* is expressed in the ganglionic eminence as early as E12.5, in the CP at E14.5, and from E16.5 onwards as a gradient from lateral<sup>high</sup> to medial<sup>low</sup> cortex. They are expressed in the migratory or postmigratory neurons of the striatum.<sup>109</sup> The *Foxp2* has a 3' untranslated region (UTR) target site for miR-9. Experimental evidence proved that the increased *Foxp2* in the dorsal telencephalon of *Emx1-cre* dicer is due to the lack of miR-9,<sup>82</sup> which is similar to *miR-9-2/3* mutant.<sup>80</sup> Ectopic *Foxp2* expression in the embryonic neocortex impairs radial migration of neurons.<sup>82</sup> In the dorsal telencephalon, miR-9 represses *Foxp2* expression for proper dorsal telencephalon development that includes radial migration of neurons.

The Nestin expression starts at E9.5 in the developing cerebral cortex,<sup>110,111</sup> The *Nestin-cre* dicer mice did not survive postnatally. At E15, the Tuj1 (a neurofilament marker) was found in IZ, early-born *Tbr1* neurons in the CP, and the neurofilament was observed in the axons of IZ. This denotes early neurogenesis being unaffected. No difference in the expression of neuronal progenitor markers such as Pax6 and *Tbr2* was observed. At E18, the brain size of the mice appears to be normal. However, histological sections show larger ventricles, reduced dividing cells in the VZ, SVZ and thinning of postmitotic neurons in the CP. This shows that the late-born neurons are affected, indicating the need for miRNA for proper function.<sup>36</sup>

The *Nestin-cre* dicer KO has reduced hippocampal size, and miR-9 was undetectable in the cortex and hippocampus at E14.5. Similar to *Emx1-cre*, *Nestin-cre* also has reduced hippocampal neuroepithelial cells and DG granular progenitors. The early hippocampal marker *Lbx2* was reduced from E18.5. The DG *Tbr2* (progenitor marker) and *Prox1* (neuronal marker) expression was reduced. Similar to *Emx-cre* dicer KO, *Tbr2* was ectopically expressed. Early-born neurons expressing *Tbr1* were increased in CA1 and DG regions, although mature neurons were reduced in all the three regions of the hippocampus.<sup>28</sup>

McLoughlin et al<sup>111</sup> reported that *Nestin-cre* dicer mutant has cortical thinning and enlargement of lateral ventricles at E16. At E15.5, the nestin expression was distributed throughout the cortical parenchyma. The neural precursor cell proliferation was greatly reduced in the VZ and SVZ of E15.5 and E16 cortex due to delayed cell division. The thinning of cortex is also due to apoptosis. The CR neuron migration and differentiation was defective, which was observed as more CR neurons in the CP than on the MZ. The premature maturation of neurons was observed by the overproduction of DCX and Rnd2 (a maturation marker). Precocious astrocyte differentiation was also observed in the *Nestin-cre* dicer KO cortex.<sup>111</sup>

The *Nex* expression starts at E13.5 in the hippocampus.<sup>28,29</sup> The *Nex-cre* dicer KO showed smaller brains and died at P23.<sup>38</sup> The miR-9 was reduced at P22 in the hippocampus. The CA3 region is severely affected in *Nex-cre* dicer KO. The mature neurons were reduced in all the three regions.<sup>28</sup> The *CaMKII-cre* dicer KO resulted in microcephaly and reduction in the size of white matter tracts.<sup>35</sup> The *CaMKII-cre* was specific to forebrain structures such as the thalamus, cortex, and hippocampus with little emphasis on cerebellum, medulla, and pons.<sup>32,36</sup> The hGFAP starts at E13.5.<sup>31</sup> The *hGFAP-cre* dicer KO shows that miR-9 is necessary for proper cerebral cortex development via Notch1 signaling.<sup>34</sup> However, there are no significant abnormalities during embryonic stages in these KOs (*Nex-cre*, *CaMKII-cre*, and *hGFAP-cre*). This might be due to the delayed dicer deletion. These studies indicate that dicer is necessary for the survival of postnatal neurons.

The conditional dicer KOs, namely *Foxg1*, *Emx1*, *Nestin*, *Nex*, *GFAP*, and *CaMKII*, show loss of miR-9 expression in the telencephalon.<sup>28,33,34,112</sup> The conditional dicer KOs point out the influence of dicer in early and late neurogenesis in the developing telencephalon. However, it also provides indirect evidence for the necessity of miR-9 in telencephalic development; the role of miR-9 in the development of telencephalon comes from miR-9 KO study. The miR-9-2/3 double KO exhibits severe growth retardation with small cerebral hemispheres and olfactory bulbs, and they died within one week postnatal. Histology of postnatal miR-9-2/3 double KO showed reduction in cortical and VZ, with an expansion of lateral ventricles. The proliferating zone in subpallium is hyperplastic with reduced differentiation. The miR-9-2 or miR-9-3 mutant does not show any defect in neurogenesis in pallium and subpallium; however, the miR-9-2/3 double mutant does.<sup>80</sup> This miR-9-2/3 KO shows that miR-9 is necessary for proper telencephalic development by controlling neural progenitor proliferation and differentiation. Overall, *Foxg1-cre*, *Emx1-cre*, *Nestin-cre*, *Nex-cre*, and *hGFAP-cre* dicer KOs point out that miR-9 is necessary for the early and late neurogenesis of the developing cortex and hippocampus.

The *miR-9-2/3* double mutant shows the direct role of miR-9 in telencephalon development. The telencephalic markers such as *Foxg1*, *Nr2e1/Tlx*, *Pax6*, *Meis2* (myeloid ecotropic viral integration site 2), and *Gsh2* have miR-9 target binding sites in their 3'UTR.<sup>80</sup> The miR-9 targets *Foxg1* for the generation of CR neurons in the medial pallium. The *miR-9* and *Foxg1* genes are expressed in reciprocal gradient along the medial pallium at E11.5.<sup>47</sup> The *Foxg1/BF1* is necessary for the early development of telencephalon.<sup>87,113</sup> *Foxg1* mutants displayed delayed cell cycle progression, reduction in proliferative progenitors and increased neurogenesis. *Foxg1* controls proliferation through the regulation of Fgf signaling and differentiation through the regulation of BMP signaling.<sup>114</sup> The *Foxg1*-driven dicer mutant also shows that miR-9 is depleted and undetected at E11.5 in the cortex.<sup>33</sup> In the early stages of *miR-9-2/3* double mutant, *Foxg1* was increased



in the pallium at E12.5–E14.5, but at E16.5 there was no significant increase. This signifies that miR-9 is necessary for *Foxg1* regulation at the early stage for the differentiation of CR and early-born neurons. However, in the later stage, Elavl2 (RNA-binding protein) counteracts the suppression of miR-9 by binding to the U-rich region of *Foxg1* 3'UTR in the pallium. Both the *Foxg1* dicer mutant and miR-9-2/3 double KO studies indicate that miR-9 and *Foxg1* negatively regulate each other for proper pallial progenitor proliferation and CR cell differentiation (Fig. 5A).

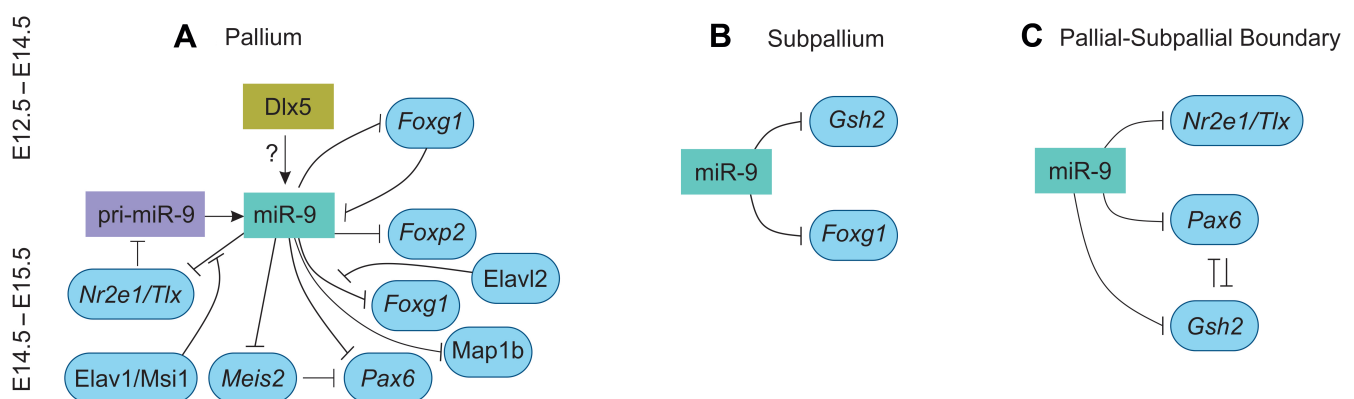
The *Foxg1* and *Pax6* are expressed in the progenitors of the developing telencephalon.<sup>87,115</sup> *Foxg1* regulates telencephalic progenitor proliferation by an autonomous mechanism as well as region-specific progenitor proliferation through the regulation of *Pax6*.<sup>116</sup> In E14.5, miR-9-2/3 double mutant, *Pax6* is reduced in the pallium with an increase in *Meis2*.<sup>80</sup> *Meis2* belongs to three amino acid loop extension superclass family of homeobox genes.<sup>117</sup> *Meis2* is first observed at E10.5 in the ventrolateral telencephalon (ie, LGE and MGE), and at E12.5, it is observed in the VZ of the entire telencephalon. At E16.5, *Meis2* is observed in the striatum and CP.<sup>118</sup> *Meis2* acts as an essential *Pax6* cofactor in adult olfactory bulb neurogenesis<sup>119</sup> and is known to regulate *Pax6* expression.<sup>120</sup> It can be postulated that at later stages, miR-9 regulates *Pax6* via *Meis2* for progenitor proliferation in VZ and SVZ of the developing pallium.

However, *Nr2e1* has 3'UTR binding site for miR-9; at E13.5, the *Nr2e1* mRNA was upregulated in miR-9-2/3 double KO.<sup>80</sup> The introduction of miR-9 or let-7d into the VZ of E13.5 embryonic cortex results in the reduction in proliferative cells, and they migrate into the CP from the VZ at E15.5. The *Tlx* null mutant has upregulated pri-miR-9-1 and pri-miR-9-2.<sup>75,76</sup> The let-7d inhibits TLX and promotes

miR-9 expression.<sup>76</sup> This suggests that miR-9 helps in neuronal migration and differentiation through loop regulation of *Tlx* and miR-9 at early stages (E12.5–15.5). At E16.5, there is no reduction of *Nr2e1* in the miR-9-2/3 double mutant. The presence of RNA-binding protein, Elavl1 and Msi1, in the pallium may attenuate the suppression of *Nr2e1* by miR-9.<sup>80</sup> Thus, miR-9 plays a role in the development of pallium by regulating *Foxg1*, *Pax6*, *Nr2e1*, and *Meis2* (Fig. 5A).

In the E15.5 miR-9-2/3 mutant subpallium, *Gsh2* and *Foxg1* are increased.<sup>80</sup> The mechanistic role of *Foxg1* is not clearly understood in subpallial progenitor proliferation and differentiation.<sup>114,121</sup> *Gsh2* is necessary for the identification of early LGE progenitor cells.<sup>122,123</sup> The increase in *Gsh2* and *Foxg1* results in hyperplastic, where the proliferating cells expand into differentiating areas of the subpallium. This shows that miR-9 is necessary for proper subpallial development by regulating *Gsh2* and *Foxg1* expression (Fig. 5B).

*Pax6* and *Gsh2* have a role in specifying dorsal and ventral telencephalon,<sup>92</sup> and these cells by counteraction form the PSB.<sup>93,124</sup> The *Tlx/Nr2e1* is expressed in the VZ and spans along the PSB, with the exception of the dorsomedial and ventromedial regions. The *Tlx* mutant is similar to *Pax6* mutants. The *Tlx/Nr2e1* along with *Pax6* regulates the establishment of PSB.<sup>125</sup> Similar to *Tlx/Nr2e1* mutant,<sup>125</sup> the miR-9-2/3 double-mutant subpallium<sup>80</sup> shows that the development of basal ganglia including striatum and globus pallidus was suppressed, the PSB shifted dorsally, and the ventral pallidum was lost. This was substantiated by the inhibition of *Pax6* via *Meis2* and upregulation of *Gsh2* and *Nr2e1*. Overall, this suggests that miR-9 is necessary for proper PSB formation by regulating *Pax6*, *Gsh2*, and *Nr2e1* as well as for normal DV telencephalic development (Fig. 5C).



**Figure 5.** Role of miR-9 in mice brain development.

**Notes:** The schematic figure shows the mechanism of gene regulation in telencephalon by miR-9. (A) In pallium, miR-9 regulates several genes, namely *Foxg1*, *Foxp2*, *Pax6*, *Meis2*, *Nr2e1/Tlx*, and *Map1b*. At E12.5–E14.5, the *Foxg1* is inhibited by miR-9 for the differentiation of CR neurons. However, at E14.5–15.5, there is no reduction in *Foxg1* in the pallium of miR-9-2/3 KO mice. This is due to the Elavl2, an RNA-binding protein inhibit the binding of miR-9 to the 3'UTR of *Foxg1*. Likewise, Elavl1/Msi1 inhibits *Nr2e1/Tlx* regulation by miR-9. The axonal development is regulated by miR-9 by targeting Map1b. The interaction between *Pax6*, *Meis2*, *Nr2e1*, and miR-9 leads to proper neuronal proliferation in the pallium. The migration of CR and early-born neurons is facilitated by miR-9 interaction with *Foxp2*. (B) The miR-9 regulates *Foxg1* and *Gsh2* for proper subpallium development. (C) The miR-9 regulates *Pax6*, *Gsh2*, and *Nr2e1/Tlx* to form the proper PSB.

**Abbreviations:** CR, Cajal–Retzius; KO, knockout; miR-9, miRNA-9; PSB, pallial-subpallial boundary; 3'UTR, 3' untranslated region.



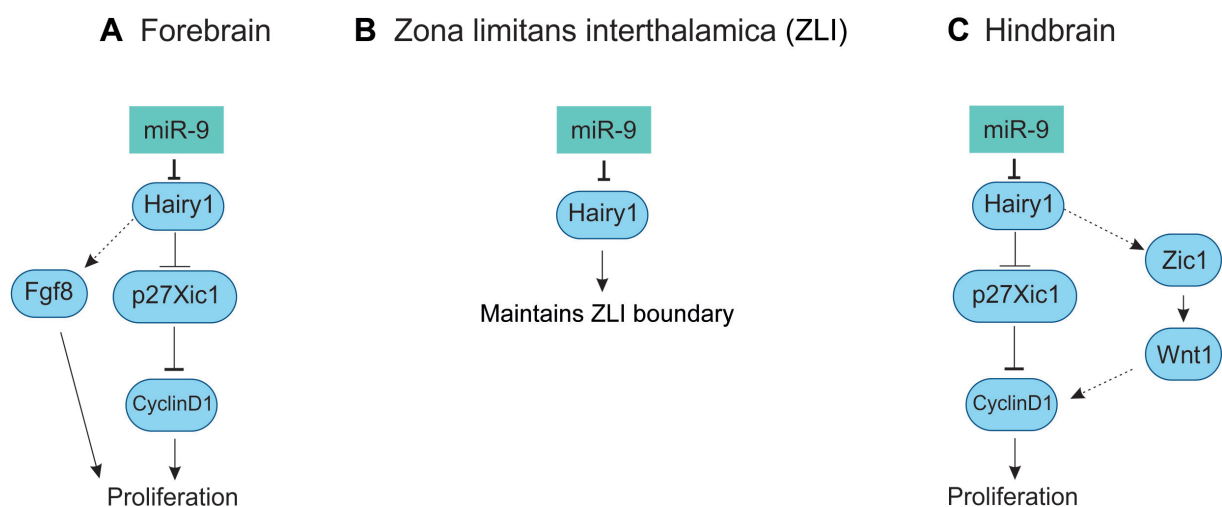
**Role of miR-9 in telencephalon development of *Xenopus*.** In *Xenopus tropicalis*, miR-9 is expressed from four variants that produce mature miR-9. The pre-miR-9 expression is similar in all the transcripts in comparison to the mature miR-9 expression. At stages 19, 30, and 36, the pre-miR-9 transcripts were observed in the forebrain, eye, midbrain, and hindbrain, but not observed in MHB (Table 2). The miR-9 expression is evident from stage 18/19 in the anterior neural plate stage of the prospective forebrain. At stage 23/24, mature miR-9 was observed in the forebrain, eye and retina, midbrain, and hindbrain with the exception of MHB.<sup>48,126</sup> In the forebrain, miR-9 expression was widespread, in both neural progenitors (VZ) and postmitotic neurons (MZ). Morpholino (MO) miR-9 studies indicate the miR-9 was necessary for the survival of progenitors and neuronal differentiation. The miR-9 knockdown results in the inhibition of neurogenesis via apoptosis. It has been proposed that *hairy1* is the main target for miR-9, where it affects proliferation via Fgf8 in the forebrain (Fig. 6A).<sup>48</sup> Zebrafish *Her6*, mammalian *Hes1*, *Xenopus hairy1*, and chicken *hairy1* belong to the family of transcription factor, hairy/enhancer of split. The hairy/enhancer of split belongs to basic helix-loop-helix proteins of DNA-binding transcription factors.<sup>127</sup>

**Role of miR-9 in telencephalon development of zebrafish.** In zebrafish, miR-9 expression is observed in the telencephalon by 24 hpf.<sup>46,128</sup> The zebrafish have seven miR-9 variants that express the same mature product, miR-9.<sup>41</sup> Each pri-miR-9 variant shows differential expression pattern (Table 2). At Prim6 stage<sup>129</sup> (25 hpf), the mature miR-9 is expressed only in the telencephalon. However, the pri-miR-9 variants 1–7 are expressed in the telencephalon with subtle differences; pri-miR-9-2 is expressed in the developing retina, pri-miR-9-4 in the posterior brain regions, and pri-miR-9-6 in the hypothalamus.<sup>130</sup> At 30 hpf, the miR-9 is expressed in the entire CNS, but

spared MHB.<sup>46,128</sup> At 48 hpf, all pri-miR-9 variants expressed throughout the CNS, but always spared MHB. Similarly, the mature miR-9 is expressed throughout the entire CNS.<sup>130</sup> In the telencephalon, miR-9 is regulated by *her6* via feedback mechanism in the proliferative cells, but not in the differentiated cells.<sup>128</sup> The miR-9 regulates Fgf target genes (*dusp6* and *Pea3*) in the telencephalon<sup>46</sup> (Fig. 7A). Limited information is available on the role of miR-9 in zebrafish telencephalon development; however, studies are needed to know how miR-9 is regulated, influences gene regulation and thereby promotes cellular changes in the zebrafish telencephalon.

**Role of miR-9 in telencephalon development of chick embryo.** The early expression of miR-9 is observed in the area opaca at HH (Hamburger Hamilton stage)<sup>131</sup> 4–5. At HH18, miR-9 is diversely expressed in all the developing brain vesicles and in the spinal cord. At HH20, it is expressed in the telencephalic vesicle, hindbrain, and spinal cord. From HH22–25 onward, miR-9 is expressed in the telencephalic vesicle, diencephalon, midbrain, hindbrain, and spinal cord (for detailed information, see GEISHA website).<sup>42,132</sup> The chick has two pre-miRs (miR-9-1 and miR-9-2), which are processed by the internal dicer to produce the same mature product, miR-9.<sup>83</sup> So far no research reports are available on the role of miR-9 in chick telencephalon.

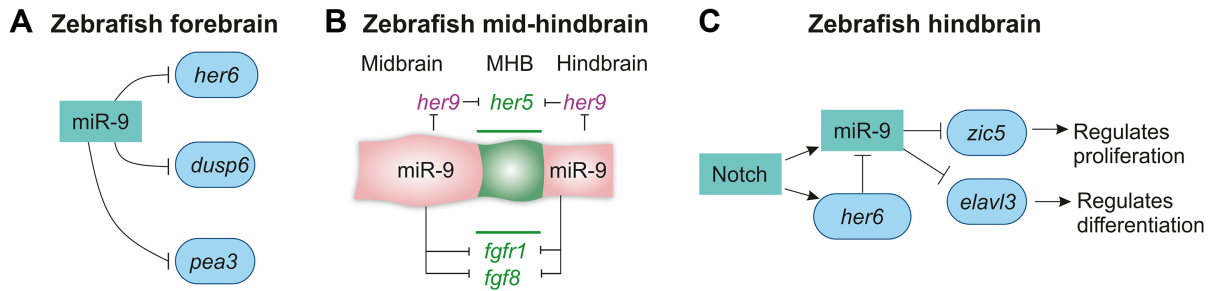
**Diencephalon development.** The diencephalon is the posterior part of the developing forebrain and develops into the thalamus and hypothalamus.<sup>56–58</sup> The ZLI acts as an organizer for diencephalic development.<sup>133–136</sup> It is a narrow strip of *Shb*-expressing cells in the diencephalon, transecting between prethalamus and thalamus (Fig. 8).<sup>21</sup> The neural tissues anterior to ZLI and in between MHB and ZLI can induce *Shb*.<sup>135</sup> The ZLI is formed at the interface between the anterior Zinc finger proteins of the Fez family and posterior homeodomain protein of Iroquois (*Irx*) family.<sup>137,138</sup> Like *Gbx2* in the



**Figure 6.** Role of miR-9 in *Xenopus* brain development.

**Notes:** (A) This shows the mechanism of miR-9 in forebrain development. In the forebrain, miR-9 inhibits hairy1 and mediates the effects through Fgf8 signaling for the proliferation of cells. (B) In ZLI, miR-9 inhibits hairy1 for the proper maintenance of the boundary. (C) In the hindbrain, the miR-9 inhibits hairy1 for the proliferation of cells and mediates the effects through Wnt1 signaling.

**Abbreviation:** miR-9, miRNA-9.



**Figure 7.** Role of miR-9 in zebrafish brain development.

**Notes:** (A) Zebrafish forebrain: In the forebrain, miR-9 inhibits proneural gene *her6* and targets Fgf signaling pathway genes (*dusp6*, *pea3*). However, detailed study is not available on this account. (B) Zebrafish midbrain and hindbrain: The miR-9 regulates MHB by limiting Fgf8 and its receptor Fgfr1 expression within the MHB. The miR-9 regulates the progenitor state in the MH by regulating *her5* and *her9*. (C) Zebrafish hindbrain: The miR-9 is regulated by Notch signaling and its target gene, *her6*. The miR-9 regulates proliferation by inhibiting *Zic5* and differentiation by inhibiting *elavl3*.

**Abbreviations:** MH, midbrain–hindbrain; MHB, MH boundary; miR-9, miRNA-9.

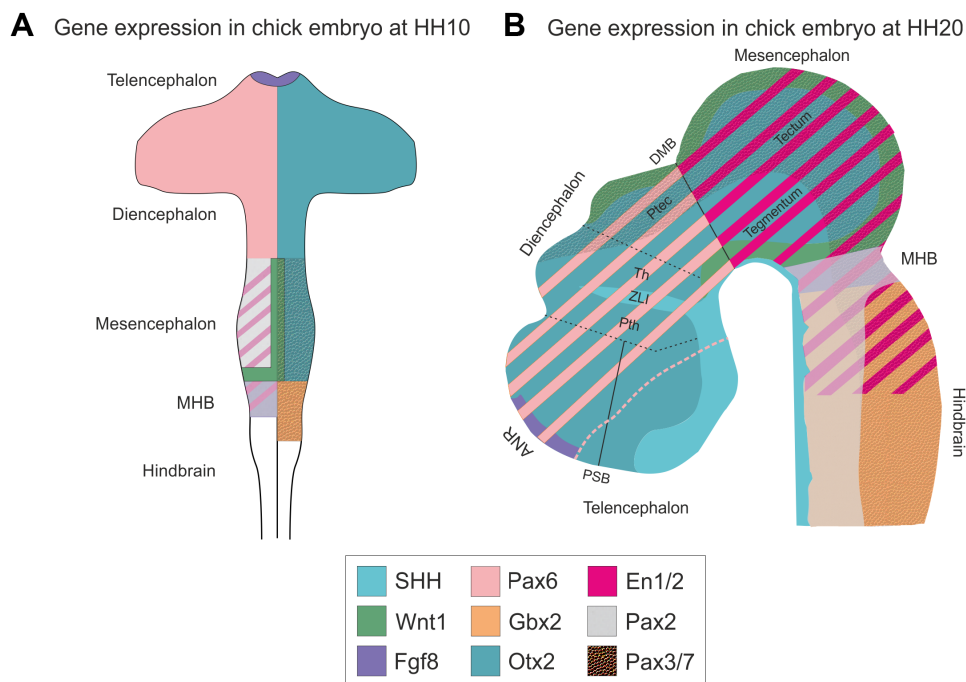
MHB, *Irx3* positions the ZLI in the posterior forebrain.<sup>139</sup> The *Six3* and *Irx3* mutually repress each other and form the ZLI boundary.<sup>140</sup>

*Role of miR-9 in the diencephalon development of mice.* The miR-9 is not expressed in the diencephalon of E11.5 mouse.<sup>33</sup>

No reports are available with regard to gene regulation by miR-9 expression in diencephalon.

*Role of miR-9 in the diencephalon development of Xenopus.*

In *Xenopus tropicalis*, miR-9 is not expressed in the MHB and ZLI, where hairy1 and Fgf8 are expressed. The miR-9



**Figure 8.** Gene expression in developing chick embryo.

**Notes:** The schematic figure shows the gene expression in developing chick embryo at HH10 (A) and at HH20 (B). (A) This figure is adapted from Nakamura et al, with permission from the author.<sup>170</sup> At HH10, the MHB is formed by the repressive action of *Otx2* and *Gbx2*. *Fgf8* is expressed in the ANR and MHB. DMB is formed by the repressive action between *Pax2/En1* and *Pax6*. *Pax3/7* expression in the midbrain/mesencephalon tectum ensures the tectal differentiation. The *Pax2*, *En1/2*, and *Fgf8* overlap with each other. (B) *Shh* is secreted in the entire floor plate in developing CNS. Cell lineage restrictions are observed in the PSB, regions anterior and posterior to the ZLI and DMB. The *Pax2*, *En1/2*, *Wnt1*, and *Fgf8* overlap each other in the MHB and are necessary for the maintenance of MHB. The two signaling centers are ANR and MHB, where ANR is necessary for the maintenance of forebrain identity and MHB is necessary for the development of midbrain and hindbrain structures. The gene expressions shown are *Shh* (turquoise), *Otx2* (dark turquoise), *Gbx2* (orange), *Wnt1* (Spring green), *Fgf8* (purple), *En1/2* (magenta), *Pax6* (pink), *Pax2* (pale white), and *Pax3/7* (textured).

**Abbreviations:** Pth, prethalamus; ZLI, zona limitans interthalamica; Th, thalamus; ptec, pretectum; ANR, anterior neural ridge; PSB, pallial-subpallial boundary; DMB, di-mesencephalic boundary; MHB, midbrain–hindbrain boundary; CNS, central nervous system.



MO and hairy1 target protection assay leads to the expansion of hairy1- and Fgf8-positive domain,<sup>48</sup> which indicates that miR-9 inversely regulates hairy1 expression via non-cell autonomous signaling pathway in ZLI (Fig. 6B). Another study has shown that *hes1* in mouse, similar to hairy1 in *Xenopus*, inversely regulates miR-9 biogenesis.<sup>49</sup>

**Role of miR-9 in the diencephalon development of zebrafish.** The miR-9 expression is observed in the diencephalon.<sup>46,128,141</sup> However, there are no research reports available on gene regulation by miR-9.

**Role of miR-9 in the diencephalon development of chick.** There are no reports on the role of miR-9 on chick embryonic diencephalon. However, the miR-9 expression is observed in the diencephalon<sup>42</sup> from HH22 onward.

**Mesencephalon/midbrain development.** The MHB plays an important role for the AP development of midbrain. The midbrain consists of two main components along the DV axis: the dorsal tectum and the ventral tegmentum. The tectum develops from the alar plate, and the tegmentum develops from the basal plate.<sup>142</sup> The MHB arises from the morphological constriction between the midbrain and hindbrain. The organization of MHB has been divided into four stages: (1) positioning and establishment, (2) induction, (3) maintenance,<sup>55</sup> and (4) morphogenesis.<sup>143</sup> The *Otx2* and *Gbx2* are the critical factors for position and establishment of the MHB.<sup>144,145</sup> These signals refine the position of MHB.<sup>146–148</sup> The *Otx2* and *Gbx2* repress each other, from where the *Fgf8* is expressed.<sup>149</sup> In zebrafish, *her5* has been postulated to establish the midbrain and hindbrain cells,<sup>150</sup> and *Pou2* is necessary for the establishment of MHB.<sup>151</sup> The induction of MHB is regulated by the members of Pax, Fgf, Wnt, and En families, where *Fgf8* plays a crucial role.<sup>152</sup> In *Xenopus*, *Xiro* along with *Otx2/Gbx2* is necessary for the induction of *Fgf8* at MHB.<sup>153</sup> The maintenance of MHB is achieved by interdependent regulation of genes, namely *Pax2/5*, *Wnt1*, *En1/2*, and *Fgf8* (Fig. 5A and B).<sup>55,143</sup> Recently, it was shown that the maintenance and morphogenesis of MHB in zebrafish was governed by the transcription factor *Grhl2b* (grainy head-like 2b).<sup>154</sup>

**Role of miR-9 in the mesencephalon/midbrain development of mice.** *Foxg1* is also expressed in the MHB at E10.5.<sup>98</sup> However, there is no report on the role of miRNAs in the MHB of *Foxg1*-cre dicer KO.

The observation in *Emx1*-cre dicer KO shows that there is no change in midbrain structure and miR-9 expression in the mesencephalon,<sup>96</sup> which is obvious since *Emx1* expression is observed only in the telencephalon.<sup>26</sup>

The *Pax2*-cre dicer KO and *Wnt1*-cre dicer KO have similar impaired growth in the midbrain and hindbrain structures.<sup>37,155</sup> These *Pax2*-cre mice died at E18.5. *Pax2* is expressed in the MHB<sup>156</sup> and *Wnt1* is expressed in the midbrain at E8.<sup>157</sup> The *Wnt1*-cre dicer KO shows several face malformation. The size of the tectum, rostral (superior colliculus), and caudal (inferior colliculus) tectum was clearly reduced, and the cerebellum was ablated in these KOs. The

AP pattern was unaffected. The expression of genes such as *Otx2*, *Gbx2*, *Wnt1*, *Fgf8*, and *Lmx1b* was not affected, and the MHB was correctly positioned. In comparison to the wild type, the miR-9, miR-124, and miR-218 were greatly eliminated in tectum, ventral midbrain, and rostral hindbrain of the *Wnt1*-cre dicer mutant. The dicer mutant points out that the *TH* expression was markedly reduced in the ventral midbrain. The *TH* and *Nurr1*-positive cells failed to properly differentiate into mDA (mesencephalic dopaminergic) neurons.<sup>37</sup> This shows that dicer mutant have mDA precursor cells, but needs miRNAs to differentiate into proper mDA neurons. It appears that the miRNAs (miR-9, miR-124, miR-218) eliminated in *Wnt1*-cre dicer KOs are necessary for the proper development of mDA neurons.

**Role of miR-9 in the mesencephalon/midbrain development in Xenopus.** At stages 30–36, the miR-9 is expressed in the midbrain as well as in the hindbrain with the exception of MHB. The miR-9 expression in the midbrain and hindbrain is restricted to the proliferating progenitor cells of the VZ. The primary miR-9 (miR-9a-1, miR-9a-2, miR-9-3) is expressed in the midbrain (Table 2).<sup>48,126</sup> The *hairy1* is expressed in the ventral midbrain that coincides with the expression of miR-9a-1.<sup>48</sup> It might be possible that miR-9 regulates *hairy1* for neurogenesis and differentiation in the *Xenopus* midbrain.

**Role of miR-9 in the mesencephalon/midbrain development in zebrafish.** In zebrafish, miR-9 is not expressed in the MHB.<sup>46,128</sup> The gain of function (GOF) of miR-9 experiment shows that overexpression leads to the loss of MHB and MHB marker gene expression. The GOF and loss of function (LOF) studies show that miR-9 has influence on the Fgf signaling pathway genes (*Fgf8*, *Fgfr1*, and *canopy1*) and its target genes such as *Pea3* and *dusp6* are downregulated. Experimental evidence proves that *Her5*, *her9*, *canopy1*, *fgf8*, and *fgfr1* are *in vivo* targets of miR-9. Independent of Fgf signaling interaction, miR-9 also promotes midbrain–hindbrain neurogenesis and neuronal differentiation *in vivo* via regulating *her* (*her5* and *her9*) genes (Fig. 7B).<sup>46</sup> Overall, miR-9 is involved in neurogenesis, Fgf signaling, and maintenance of MHB.

**Role of miR-9 in the mesencephalon/midbrain development in chick.** The miR-9 is expressed in the midbrain;<sup>42</sup> however, no research reports are available on the role of miR-9 in gene regulation of midbrain and MHB genes.

**Hindbrain development.** The developing hindbrain is formed from the rudimentary segments of rhombomeres, where the cells move freely between rhombomeres before the boundary formation and after the boundary formation they expands within the boundaries.<sup>158</sup> The development of hindbrain depends on the retinoic acid gradient signals, tyrosine kinases, and several transcription factors along the rhombomere boundaries.<sup>159–161</sup> In zebrafish, several *Wnt* genes are expressed in the boundaries between the rhombomeres.<sup>162,163</sup> In chick, *Hox* genes play a main role in the formation of rhombomere boundaries.<sup>159,160</sup>



*Role of miR-9 in the hindbrain development of mice.* In *Wnt1-cre* dicer mutant, notable defects were observed in the hindbrain that included the ablation of cerebellum. The miR-9 along with miR-124 and miR-218 expression was ablated in rostral hindbrain of the *Wnt1-cre* dicer mutant. There was no difference in the expression of *Gbx2* expression in the hindbrain. The mitotic progenitors in the hindbrain were similar to the control,<sup>37</sup> indicating dicer KO does not affect proliferation in mice hindbrain. Further research is needed to pinpoint the role of miR-9 in the developing hindbrain.

In *bGFAP-cre* dicer mutant, the activity was observed as early as E13.5 at the rhombic lip of the cerebellar plate and in postnatal cerebellar cells including the Bergmann glia. Defective cerebellar foliation was observed at E16.5. In the Bergmann glia, miR-9 is needed for the maintenance of these cells via Notch1 signaling.<sup>30</sup>

*Role of miR-9 in the hindbrain development of Xenopus.* Bonev et al reported that in *Xenopus*, miR-9 expression was restricted to the *Sox3* (neural progenitor marker)-positive domain, ie, VZ cells of the developing hindbrain. The miR-9 is involved in the hindbrain development through *hairy1* on proliferation via Wnt signaling (Fig. 6C). The MO experiment also shows that miR-9 is necessary for the differentiation of neuron. In comparison to forebrain, miR-9 is necessary for limiting progenitor proliferation in hindbrain and promotes the onset of neurogenesis.<sup>48</sup>

*Role of miR-9 in the hindbrain development in zebrafish.* In zebrafish, at 30 hpf, the miR-9 expression is observed in the hindbrain with the absence of MHB and rhombomere boundary.<sup>41,46,128</sup> In the hindbrain, radial glial cells in the VZ express miR-9, where the expression is segmentally pattern and stronger near the rhombomere boundaries. The miR-9 expression is also observed in the neurons of the MZ. The miR-9 regulates hindbrain neurogenesis by maintaining neural progenitor cells through *zic5* and *her6* and neuronal differentiation by targeting *elavl3/HuC* (Fig. 7C). The miR-9 was positively regulated by Notch signaling and negatively regulated by Notch target *Her6*.<sup>128</sup> The miR-9 inhibits *Her9*, thereby regulates neuronal progenitor maintenance.<sup>46</sup> Overall, miR-9 is involved in proper neuronal progenitor maintenance and neurogenesis in zebrafish.

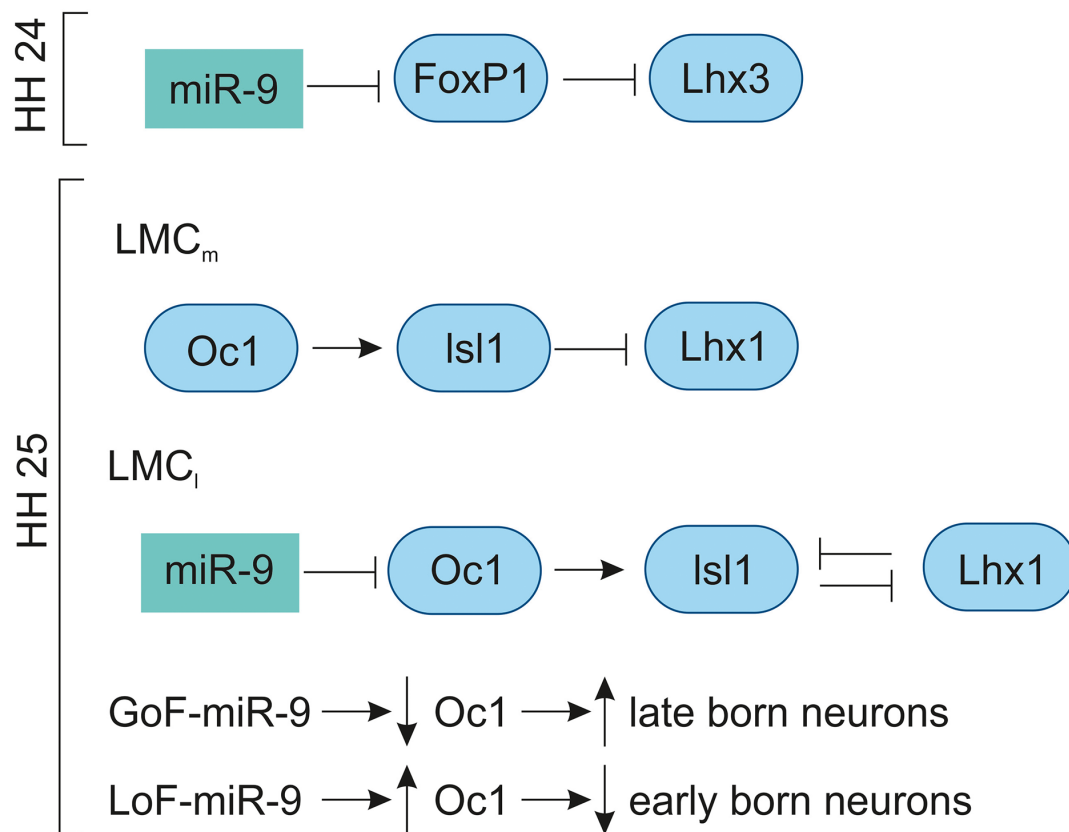
*Role of miR-9 in the hindbrain development of chick.* From HH20 onward, the miR-9 expression is observed in the hindbrain. At HH24, it is strongly expressed in the margin of the roof plate and absent in the floor plate.<sup>42</sup> However, there are no research reports on gene regulation by miR-9 in the hindbrain.

**Spinal cord development.** The spinal cord develops from the posterior neural tube by the induction of cues from the dorsal ectoderm. The regional differentiation occurs along the DV and rostrocaudal axis. The retinoid synthesis by RALDH2 (retinaldehyde dehydrogenase 2) is critical for establishing the distinction of spinal cord neural cells.<sup>164</sup> The dorsally positioned neurons are induced by BMP signaling,

and the ventrally positioned neurons are induced by the graded expression of SHH. The *Hox* genes govern the patterning of spinal cord.<sup>165</sup> The motor neurons (MNs) and interneurons (IN) are generated by the graded action of SHH. The graded SHH repress the class I homeodomain proteins (*Pax6/7*, *Irx3*, *DBX1/2*) and class II proteins (*Nkx2.2* and *Nkx6.1*) for the generation of five distinct class of neurons (ventral IN—V0-3 and MN) from the ventral progenitor cells along DV axis of the ventral neural tube.<sup>166</sup> The combinatorial action of *Nkx6.1*, *Nkx2.2*, and *Irx3* restricts the generation of MN to a single progenitor domain.<sup>164,166</sup> The median motor column (MMC) neurons innervate axial muscles and lateral motor column (LMC) innervate limb muscles.<sup>164</sup>

*Role of miR-9 in the spinal cord development of chick.* The miR-9 is expressed in the VZ of developing chick spinal cord from HH11 to HH29.<sup>42,83</sup> In the MN, the miR-9 expression was low at HH20, increased at HH24 and undetectable at HH29. At HH24, miR-9 overlaps with the MN markers (*FoxP1* and *Isl1/2*) in the LMC; however, they do not overlap with MN markers (*Lhx3*, *HB9*) of the MMC neurons. The overexpression of miR-9 results in switching of LMC into MMC neurons by repressing the production of LMC neurons by downregulating *FoxP1* and *Isl1/2* and promoting the differentiation of MMC neurons by upregulating *Lhx3* and *HB9*. The overlapping expression of miR-9 and *FoxP1* along with experimental evidence shows that miR-9 regulates *FoxP1* expression in LMC in a dose-dependent manner. In chick, preganglionic motor column (PGC) is positioned along the thoracic level innervate axons to the sympathetic target. The overexpression of miR-9 shows that there is a reduced expression of PGC MN marker, *Smad* and *Isl1/2*.<sup>83</sup> In the LMC, the GoF-miR-9 shows that *FoxP1* is reduced and vice versa in the LoF-miR-9. Likewise, the reduction and increase in *FoxP1* are inversely proportional to the *Lhx3* MN marker in the LMC.<sup>167</sup> Overall, miR-9 finely tunes *FoxP1* expression for normal LMC (Fig. 9) and PGC neuron specification.

At HH25, the miR-9 expression is increased in the intermediate and VZs of the spinal cord. At HH28, miR-9 is expressed in specific subpopulations of the MNs; the MN migration is completed by this stage, where the MMC (marker: *Lhx3*), medial LMC (marker: *Isl1/FoxP1*), and lateral LMC (marker: *Lhx1/Hb9*) can be clearly distinguished. The transcription factor OC1 (*Onecut 1*) is expressed in the lateral aspect of the ventral horn. At HH25, the OC1 is co-localized with *Isl1/Lhx3* of early-born neurons of MMC, in the medial LMC with *Isl1/FoxP1* and in the lateral LMC with *Lhx1/Hb9*. At HH27, the lateral MMC shows less OC1 compared to the medial MMC, while the miR-9 is reversed in strength. The medial LMC has strong OC1 expression with no miR-9, and lateral LMC has strong miR-9 with reduced OC1. The GoF-miR-9 results in the reduction in lateral MMC and medial LMC, while the medial MMC and lateral LMC are increased. The opposite reactions are observed in LoF-miR-9. The GoF-miR-9 results in the



**Figure 9.** Role of miR-9 in chick spinal cord development.

**Notes:** At HH24, miR-9 fine-tunes Foxp1 for LMC MN specification via inhibiting Lhx3. At HH25, miR-9 regulates OC1 protein for the proper development of early- and late-born MN neurons in the LMC. The GOF and LOF with miR-9 indicate that miR-9 inversely regulates OC1 for early- and late-born MNs. The OC1 acts upstream of Isl1, controls the specific neuronal subtypes within LMC. OC1 has stronger expression with no miR-9 expression in the LMC<sub>m</sub>, thereby regulates LMC<sub>m</sub> MNs by promoting Isl1 expression and inhibiting Lhx1 expression. However, in the LMC<sub>i</sub> the expression of miR-9 expression inhibits OC1 expression and promotes Lhx1 expression in the LMC<sub>i</sub> MNs.

**Abbreviations:** GOF, gain of function; LMC, lateral motor column; LOF, loss of function; miR-9, miRNA-9; MN, motor neuron; OC1, Onecut 1.

downregulation of OC1 and an increase in late-born neurons. Likewise, LOF-miR-9 results in the upregulation of OC1 and an increase in early-born neurons.<sup>168</sup> Overall, miR-9 regulates Onecut 1 to promote the switching of early-born to late-born MN population (Fig. 9).

### Conclusion

In the developing brain, miR-9 influences gene expression, proliferation, neurogenesis, maturation, migration, and differentiation in a spatiotemporal pattern. The studies point out that miR-9 is expressed in neuronal cells which are proliferative and differentiated cells. The endogenous miR-9 expression pattern also differs from species to species; in zebrafish and *Xenopus*, miR-9 is excluded from the MHB, but in mice and chicken there is no such distinction in the expression pattern. The miR-9 is necessary for the maintenance of boundaries: PSB, ZLI, and MHB.

Although the studies on dicer KO mice show the lack of miR-9, one cannot ignore the plethora of other miRNAs involvement in brain development. At the same time, we could not reject the experimental evidence showing the necessity of

miR-9 and its role in vertebrate brain development. The other dicer KOs have similar developmental defects in telencephalon in comparison to miR-9-2/3 double mutant. Combining these facts with other experimental evidence like GOF/LOF, we can conclude that miR-9 plays a role in the vertebrate telencephalon development in mice. One among such facts is the regulation of CR neurons by miR-9. The miR-9 modulates the generation, maturation, differentiation, and migration of CR neurons in the developing telencephalon. The miR-9 is also governed by several genes, like *Notch*, *Hes/hairy1*, *Tlx*, *Dlx5*, etc. There are also feedback loop mechanisms for *Tlx*, *Foxg1*, and *Foxp1* with miR-9 that regulates cellular events within the developing vertebrate brain.

Several hairy/enhancer of split genes (*Xenopus hairy1*, zebrafish *her5*, *her9*, *her6*) are regulated by miR-9. Most of these are proneural genes involve in the regulation of progenitor pools. In these studies, we found that miR-9 regulates proliferation and differentiation via targeting *her* genes. Even within the species, the miR-9 is differentially regulated. For example, *hairy1* mediates the effects of miR-9 on proliferation through *Fgf8* signaling in the forebrain and via *Wnt* signaling in the hindbrain.



Further studies are required to completely understand the role of miR-9 in vertebrate brain development. For instance, miR-9 expression has been observed in the chick diencephalon from HH22, and reports on the role of miR-9 in the embryonic diencephalon are lacking. Similarly, miR-9 expression has been reported in the zebrafish diencephalon, but data on its gene regulatory role are not yet available. In the case of the chick midbrain and hindbrain, no data are available on the role of miR-9 in both these regions. This situation is also prevalent in the case of the chick telencephalon, allowing much more scope for understanding the role of miR-9. This may also provide an insight into the role played by miR-9 from an evolutionary standpoint. To conclude, miR-9 is necessary for the development of the embryonic brain.

### Author Contributions

Conceived and designed the review, analyzed the data, contributed equally in writing the manuscript: BR and AAPA. Agree with manuscript results and conclusions: BR and AAPA. Drawn figures for the manuscript using graphic software: BR. Jointly developed the structure and arguments for the paper: BR and AAPA. Made critical revisions and approved final version: AAPA. All authors reviewed and approved of the final manuscript.

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