MicroRNA-9 Modulates Cajal–Retzius Cell Differentiation by Suppressing Foxg1 Expression in Mouse Medial Pallium

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Vertebrate brain hosts a diverse collection of microRNAs, but little is known about their functions *in vivo*. Here we propose that mouse *microRNA-9* (*miR-9*) targets *Foxg1* mRNAs for proper generation of Cajal–Retzius cells in the medial pallium. *miR-9* expression is mediolaterally graded, being most intense in the cortical hem; it contrasts with the *Foxg1* expression in a reciprocal gradient. The 3′ untranslated regions of tetrapod, but not of teleost, *Foxg1* mRNAs conserve *miR-9* target sequences and are regulated by *miR-9*. Gain- and loss-of-function analyses of *miR-9* showed that *miR-9* negatively regulates endogenous *Foxg1* protein level. Moreover, *miR-9* overexpression in developing telencephalon at E11.5 by electroporation resulted in ectopic Reelin-positive cells over the cortex beyond the marginal zone. In addition, inhibition of endogenous *miR-9* function by antisense oligonucleotides caused the regression of *Wnt3a*-positive cortical hem and reduction of *reelin*-, *p73*-, and *NeuroD1*-positive cells.

Key words: *microRNA-9*; *Foxg1*; telencephalon; medial pallium; Reelin; Cajal–Retzius cells

Introduction

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that have been identified in the diverse range of eukaryotic genomes (for review, see Krutzfeldt et al., 2006). Most animal miRNAs form an imperfect base pair with 3′ untranslated regions (UTRs) of their target mRNAs; the second through seventh nucleotides (the seed region) are most critical for target recognition and suppression (Doench and Sharp, 2004; Lewis et al., 2005). The base pairing results in either the degradation of the target mRNAs or translational repression; miRNAs are thought to function in fine tuning of gene expression that may be essential in many biological processes (for review, see Kloosterman and Plasterk, 2006). The brain hosts a diverse collection of miRNAs, many being expressed in specific brain regions or neurons, suggesting their roles in brain development (Kapsimali et al., 2007). However, little is known of miRNA functions in brain development (Giraldez et al., 2005; Choi et al., 2008), although several studies *in vitro* have indicated miRNA's roles in neurogenesis (Vo et al., 2005; Conaco et al., 2006; Schratt et al., 2006). *microRNA-9* (*miR-9*) is one such microRNA present in both *Drosophila* and vertebrates (Krichevsky et al., 2003; Li et al., 2006). *miR-9* is not expressed in midbrain–hindbrain boundary (MHB) region, and

the reason was recently postulated as being because *miR-9* inhibits several components of the Fgf signaling pathway, thereby delimiting the MHB organizer activity (Leucht et al., 2008). The *miR-9* expression is most apparent in cortex, and in this report we propose that *miR-9* targets *Foxg1* for proper Cajal–Retzius cell differentiation in the medial pallium of developing telencephalon.

Materials and Methods

Plasmid construction. Approximately 200 bp genomic fragments encoding *miR-9-1*, *miR-9-2*, and *miR-9-3* were amplified by PCR and inserted into either the pEF-1α vector (Invitrogen) to generate *miR-9-1WT*, *miR-9-2WT*, and *miR-9-3WT*, respectively, for transfection into P19 cells, or the pCMS-EGFP vector (SV40-EGFP; Clontech) to generate *CMV-miR-9-2WT-SV40-EGFP* for electroporation into telencephalon. Primer sets used to amplify the fragments are described in supplemental material (available at www.jneurosci.org). Site-directed mutagenesis in the mature nucleotide *miR-9* sequences was performed as described previously (Mochizuki et al., 2000). Four-base substitution was introduced into the seed sequence (CTTTGGT to CAAACGT) of either *miR-9-1* or *miR-9-2* to generate *miR-9-1MT* and *miR-9-2MT*, respectively. Luciferase reporters conjugated with each 3′UTR were constructed as described previously (Zhao et al., 2005); concatemeric oligonucleotides for each 3′UTR are given in the supplemental data.

Luciferase assay. Luciferase assay was performed with P19 cells as described previously (Zhao et al., 2005); for the details, see supplemental material (available at www.jneurosci.org).

In situ hybridization, immunohistochemistry, and immunoblot. Whole-mount and section *in situ* hybridization were performed as described previously (Wilkinson and Nieto, 1993). Antisense digoxigenin (DIG)- or fluorescein isothiocyanate-labeled RNA probes were synthesized using DIG or Fluorescein RNA Labeling Mix (Roche). DIG-labeled LNA-oligonucleotides were purchased from Exiqon. Immunohistochemistry, immunocytochemistry, and immunoblot were performed as described previously (Hevner et al., 2003; Boutz et al., 2007); brains were sectioned

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at 14 μm . Immunoblot experiments were repeated at least two times. Quantification of band intensity was carried out by ImageJ (Abramoff et al., 2004). The sources of probes and antibodies used are described in the supplemental material (available at www.jneurosci.org).

Reverse transcription-PCR. cDNAs were synthesized and reverse transcription (RT)-PCR was performed as described previously (Uno and Ueda, 2007). Primers used are described in the supplemental material (available at www.jneurosci.org). The quantification was normalized by the amount of TATA binding protein amplification. For all primer sets tested, correlation (R^2) was >0.98 , and the slope was -3.1 to -3.6 in each standard curve.

Electroporation. One microgram of plasmid or 100 μM antisense oligonucleotide solution was electroporated as described previously (Borrell et al., 2005). Antisense oligonucleotide for *miR-9* (*miR-9AS*; CAUACAGCUAGAUAAACCAAGA) was purchased from Dharmacon; control is scrambled oligonucleotide (CAGAUGACAGAUCAAUAAACCA). Embryonic day 13.5 (E13.5) neocortex cells were dissociated and cultured as described previously (Meberg and Miller, 2003). Electroporation of *miR-9-2WT* into these cells was conducted using MicroPorator (Digital Bio).

Results

miR-9 expression is intense in developing medial pallium

In mouse, *miR-9* is transcribed as three primary miRNAs (*pri-miRNAs*), *miR-9-1*, *miR-9-2*, and *miR-9-3* (Griffiths-Jones, 2006). Their mature forms have 23 identical nucleotides (see Fig. 3C), which are perfectly conserved not only in vertebrates but also in *Drosophila*. Quantitative RT-PCR demonstrated that the expressions of all three *pri-miR-9* members were not apparent at E8.5, and *miR-9-2* was most and *miR-9-1* least abundant in E10.5 brain (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). By whole-mount *in situ* hybridization, *miR-9* was first detected in the telencephalic primordium at E9.0, when the primordium is to be formed (Fig. 1Aa). At E9.5, the expression took place in the preoptic area and infundibular area (Fig. 1Ab,Ac,Ac', arrows) and in the hindbrain (Fig. 1Ab,Ac, arrowheads). At E10.5, the transcripts were also found in the otic vesicles and spinal cord (Fig. 1Ac). The *in situ* hybridization in sections at E10.5 demonstrated that the expression is dorsolaterally graded in the cortex, being dorsomedially most intense (Fig. 1Ba,Bb, arrowheads), although it was not apparent in the choroidal roof. The subcortex scarcely expressed *miR-9*, but it was found in the caudal pallidum (Fig. 1Bb, arrow). At E11.5, in the medial pallium the *miR-9*-positive area abutted the *transthyletin* (*TTR*)-positive choroid plexus region (Fig. 1Bc,Bc'). The *miR-9* expression was low in the lateral cortex, but was apparent in the ventricular zone of ganglionic eminences at this stage. In E12.5 cortex, *miR-9*-positive domain was restricted to the most medial pallium (Fig. 1Bd,Bd'), but the expression was retained in the marginal zone over the entire cortex, including the ventral pallium. *miR-9* was not expressed in the *Wnt3a/TTR*-double-negative junctional epithelia (Fig. 1Bd',Be', arrowheads) (Grove et al., 1998). The medial end of *miR-9* expression coincided with *Wnt3a*-positive cortical hem

Figure 1. *miR-9* expression in developing telencephalon. **A**, Whole-mount *in situ* hybridization at E9.0 (**Aa**), E9.5 (**Ab**), and E10.5 (**Ac**, **Ac'**). Arrows indicate the expression in preoptic area, and arrowheads indicate the expression in hindbrain. **Aa–Ac**, Lateral views; **Ac'**, a frontal view. **B**, Section *in situ* hybridization with probes at days indicated. All are frontal views. **Ba**, Anterior view; **Bb**, posterior view. **Bc–Be'**, Magnified views of medial regions in **Bc–Be**. Arrowheads in **Ba** and **Bb** indicate the expression in medial pallium, and arrows indicate the expression in pallidum. **Bc**, **Bc'**, *miR-9* expression (blue) abuts the *TTR*-positive (orange) choroid plexus. **Bd–Be'**, It is found in *Wnt3a*-positive cortical hem but not in the junctional epithelium (arrowheads) between the choroid plexus and cortical hem. Scale bars: **A**, 250 μm ; **Ba–Be**, 200 μm ; **Bc'–Be'**, 100 μm .

(Fig. 1Bd–Be'). At E12.5, *miR-9* expression was also apparent in the septum, cortex/subcortex boundary, and differentiated cell zone of the subcortex.

miR-9 regulates *Foxg1* expression *in vitro*

Several computational algorithms have been developed to predict miRNA targets; target candidates of *miR-9* were first sought using PicTar (Krek et al., 2005), Target scan (Lewis et al., 2003), and miRanda (John et al., 2004) algorithms. The candidates were further selected by two criteria: (1) conservation of the recognition sequences in the 3'UTRs of the candidate genes among human, mouse, and chick orthologs; and (2) expression and/or functional data reported in brain development. The criteria selected four genes: *Lhx5*, *Id4*, *Nr2e1*, and *Foxg1* (supplemental Fig. 2A, available at www.jneurosci.org as supplemental material). It was then determined by a Luciferase assay with P19 cells whether *miR-9* regulates the expression of these genes at the protein level. Wild-type (WT) *miR-9-1*, *miR-9-2*, and *miR-9-3* all suppressed the expression of Luciferase from a luciferase reporter conjugated with *Foxg1* 3'UTR (*luc-Mm-Foxg1WT*), whereas mutant (MT) *miR-9-1* or *miR-9-2*, in which the seed sequence was transversely mutated, did not (Fig. 2A). Furthermore, the luciferase reporter conjugated with mutant *Foxg1* 3'UTR (*luc-Mm-Foxg1MT*), in which the target sequence was transversely mutated, was not effectively suppressed by *miR-9-1WT*, *miR-9-2WT*, or *miR-9-3WT* (Fig. 2A). On the other hand, *miR-9-2WT* had no effect on the Luciferase expression from luciferase reporters conjugated with

Figure 2. *miR-9* targets *Foxg1* 3' UTR. **A**, Essential roles of seed sequence and target sequence in suppression of *Foxg1* 3' UTR by *miR-9*. Effects of WT or MT *miR-9-1*, *miR-9-2*, or *miR-9-3* on Luciferase expression from *luciferase* reporters conjugated with WT or MT *Foxg1* 3' UTR are shown (for the details, see supplemental material, available at www.jneurosci.org). In all the assays, the *luciferase* expression was nearly the same at the mRNA level. **B**, *Foxg1* spatial expression pattern in the medial pallium. **Ba**, *miR-9* (purple) and *Foxg1* (brown) mRNA expression in E12.0 telencephalon; **Bb–Bd**, *Foxg1* protein, *Foxg1* mRNA, and *Wnt3a* mRNA expression boundaries. Arrows in **Bb–Bd** indicate the limits of cortical hem marked by *Wnt3a* (**Bd**). Scale bars: **Ba**, 200 μ m; **Bb–Bd**, 100 μ m. **C**, Conservation of *miR-9* target sequences in vertebrate *Foxg1* 3' UTRs. **D**, Luciferase assay of *miR-9* suppression of mouse (*Mm*), chick (*Gg*), *Xenopus* (*Xt*), and zebrafish (*Dr*) *Foxg1* 3' UTRs. **E**, Two-base mutations in *miR-9* as indicated in red (MT1–10). Sequences assumed to make base pairing in the wild-type *miR-9* with *Foxg1* target sequence are boxed. **F**, Luciferase assay of the *Foxg1*-suppressing activities of *miR-9-2* mutants as shown in **E**.

Lhx5, *Id4*, *Nr2e1*, or *Foxg1* 3' UTR (supplemental Fig. 2B, available at www.jneurosci.org as supplemental material). In these assays, the *luciferase* expression was nearly the same at the mRNA level (data not shown).

At E12, *Foxg1* is expressed in the entire telencephalon, but it is graded in the medial pallium and absent in the most medial pallium, including cortical hem, where *miR-9* is intensively expressed (Fig. 2Ba). In addition, the medial boundary of *Foxg1* protein expression would be more lateral than that of the *Foxg1* mRNA expression (Fig. 2Bb–Bd).

The *miR-9* target sequence in *Foxg1* 3' UTR is highly conserved among vertebrates (Fig. 2C). Indeed, Luciferase expres-

sion from *luciferase* reporters conjugated with chick or *Xenopus tropicalis* *Foxg1* 3' UTR (*luc-Gg-Foxg1WT* and *luc-Xt-Foxg1WT*, respectively) were effectively suppressed by mouse *miR-9-2WT*, and not by *miR-9-2MT* (Fig. 2D). In contrast, *miR-9-2WT* had no effect on the Luciferase expression from a *luciferase* reporter conjugated with zebrafish *Foxg1* 3' UTR (*luc-Dr-Foxg1WT*). *miR-9* target sequences are somewhat and their 5' and 3' flanking sequences are greatly distorted in the zebrafish *Foxg1* 3' UTR (Fig. 2C).

Core sequences in *miR-9* function

To determine the sequences essential to the *Foxg1*-suppressing activity in mature *miR-9*, mutations were introduced at each of

the two nucleotides above the 23 nt (*MT1–10*) (Fig. 2*E*); their activities were examined by cotransfecting either of the mutated forms with the *luc-Mm-Foxg1* reporter into the P19 cells (Fig. 2*F*). The luciferase assay demonstrated that mutations not only in the seed sequence (8 nt from the 5' end: *MT1–4*) but also at the 13th and 14th nucleotides (*MT6*) extinguished the repressive activity; mutations at the 15th through 23rd nucleotides (*MT7–10*) had no significant effect. Thus, for the *miR-9* activity, not only the seed sequence but also the 13th through 15th CTA are essential, whereas 18th through 20th GTA are not; both are predicted to make base pairing with *Foxg1* 3'UTR target sequence.

miR-9 suppresses *Foxg1* protein expression

We found that P19 cells efficiently differentiate into cells of dorsal telencephalic character when treated with 1 nM retinoic acid, expressing *Foxg1* and *miR-9-2* (supplemental Fig. 3*A,B*, available at www.jneurosci.org as supplemental material). In these cells, the *miR-9-2WT*, but not *miR-9-2MT*, inhibited the *Foxg1*, but not Nr2E1, protein expression (supplemental Fig. 3*C*, available at www.jneurosci.org as supplemental material). Expression of *Foxg1* mRNA was not significantly affected (supplemental Fig. 3*D*, available at www.jneurosci.org as supplemental material). Furthermore, *miR-9* knockdown with LNA antisense oligonucleotide (Ørom et al., 2006) (*LNA-miR-9AS*) enhanced the *Foxg1* protein expression (supplemental Fig. 3*E*, available at www.jneurosci.org as supplemental material). The *miR-9* effects on *Foxg1* protein expression were also examined in neocortex cells. The *miR-9-2WT* overexpression in E12.5 cortex by electroporation repressed the *Foxg1* protein expression (Fig. 3*A*); the repression was also demonstrated in the cells dissociated in culture (Fig. 3*B*). In addition, conversely, *miR-9-2* knockdown by the electroporation of 100 μ M *miR-9* antisense oligonucleotides caused the upregulation of *Foxg1* protein expression (Fig. 3*C*).

miR-9 regulates the differentiation of Cajal–Retzius cells

Foxg1 has been indicated to function in early corticogenesis by promoting the proliferation of cortical progenitor cells and inhibiting their premature differentiation into Cajal–Retzius cells, restricting their differentiation in the *Foxg1*-negative cortical hem region (Hanashima et al., 2007). Therefore, *miR-9* function in Cajal–Retzius cell differentiation was examined by electroporating a *miR-9-2/EGFP* expression vector into the E11.5 cerebral cortex. E11.5 is the stage when the initial neurogenesis occurs with the emergence of preplate harboring Cajal–Retzius cells (Takiguchi-Hayashi et al., 2004; Campbell, 2005); p73, p21, and Reelin are the markers of these cells. At 24 or 48 h after electroporation, p73-, p21-, and Reelin-positive cells were abundantly found over the entire cortex beyond the marginal zone (Fig.

Figure 3. Effects of *miR-9* on *Foxg1* protein expression in the cortex cells. ***A***, *miR-9-2WT* or control vector together with *SV40-EGFP* was electroporated into the neocortices at E12.5, and the EGFP-positive regions were dissected out from six cerebral hemispheres at E13.5. *Foxg1* expression level was examined by immunoblotting; EGFP and GAPDH3 served as loading control. Experiments were conducted two times; quantification of the bands indicated 69% and 42% reduction of *Foxg1* protein by *miR-9-2 WT* in each experiment. ***B***, E13.5 neocortex cells were dissociated, transfected with *CMV-miR-9-2WT-SV40-EGFP* or *SV40-EGFP* by electroporation, and cultured for 24 h. *Foxg1* expression was examined by immunostaining. Scale bar, 10 μ m. ***C***, The effect of *miR-9-2* knockdown. *miR-9AS* or control oligonucleotide was electroporated with *SV40-EGFP* into the neocortices. *Foxg1* expression level in the neocortices was examined by immunoblotting. Experiments were conducted two times; quantification of the bands indicated 2.3- and 1.8-fold increase of *Foxg1* protein expression by *miR-9AS* in each experiment.

4*Aa,Ab,Ad,Ad'*; supplemental Fig. 4*A*, available at www.jneurosci.org as supplemental material); they were the cells that expressed EGFP strongly (Fig. 4*Ad'*). Loss of *Foxg1* in mouse cortex also causes premature neural differentiation in the ventricular zone as identified by the emergence of an early neural marker, Tuj1-positive cells (Muzio and Mallamaci, 2005); normally, Tuj1-positive cells are scarcely found in the ventricular zone. The *miR-9-2* electroporation enhanced Tuj1-positive cells in the ventricular zone as well (Fig. 4*Ac,Ac'*). In addition, migrating neurons in the cortex appeared disorganized by *miR-9-2* overexpression, but its details remain for future studies. Of note is that when neocortex was electroporated with *miR-9* at E14.5 and examined at E15.5, Reelin-positive cells were not induced ectopically (supplemental Fig. 4*B*, available at www.jneurosci.org as supplemental material), suggesting that only young neocortical cells are sensitive to either *miR-9* or *Foxg1*.

The role of *miR-9* in Cajal–Retzius cell differentiation in the medial pallium was further examined by electroporating *miR-9* antisense oligonucleotide (*miR-9AS*); *miR-9* expression is intensively found in the medial pallium at E11.5 (Fig. 1*Bc*). At 12 h after electroporation, no obvious change was observed in the expression of *reelin*, *p73*, *NeuroD1*, or *Wnt3a*; *NeuroD1* is a marker for early differentiating neurons, and *Wnt3a* a cortical hem marker. At 24 h after electroporation, however, some de-

crease was observed in the expression of these markers, and at 48 h significant decrease was observed. Regression of the *Wnt3a*-positive cortical hem was distinct, and the reduction of *reelin*- and *p73*-positive Cajal–Retzius cells was apparent (Fig. 4*B*); *reelin*-positive cells remained reduced at 72 h. *miR-9AS* also reduced the *NeuroD1*-positive cells, whereas the expression of *Ngn2*, a cortical progenitor cell marker, was largely unaffected (data not shown).

Discussion

In mammals, *miR-9* is most abundantly expressed in cortex during the development (Fig. 1). However, *miR-9* targets have not yet been identified, and their functions in telencephalon development remain to be clarified. During the course of this study, a comparative analysis of the 3'UTR among amniote *Foxg1* orthologs anticipated that *miR-9* and *miR-33* may regulate *Foxg1* posttranscriptionally (Brendenkamp et al., 2007). Here we propose that *miR-9* indeed targets *Foxg1* mRNAs and regulates in the most medial pallium for proper generation of Cajal–Retzius cells that play essential roles in mammalian corticogenesis (for review, see Rice and Curran, 2001).

miR-9 represses *Foxg1* expression

We demonstrated that *miR-9* represses endogenous *Foxg1* protein expression not only in P19 cells (supplemental Fig. 3, available at www.jneurosci.org as supplemental material) but also in cortex cells (Fig. 3). The base pairing between the seed sequence of miRNAs and the target sequence in the 3'UTR of target mRNAs is essential for the target recognition and suppression (Doench and Sharp, 2004; Lewis et al., 2005). In the *miR-9* suppression of *Foxg1* 3'UTR, the seed sequence is indeed essential (Fig. 2*E,F*), but the 12th

Figure 4. Effects of *miR-9* on Cajal–Retzius cell differentiation *in vivo*. **A**, The effect of *miR-9-2* overexpression. CMV-*miR-9-2WT*-SV40-EGFP or SV40-EGFP was electroporated at E11.5, and the neocortices were immunostained for p73 (**Aa**), p21 (**Ab**), Tuj1 (**Ac**, **Ac'**), Reelin (**Ad**, **Ad'**), and EGFP at E13.5. **Ac'**, **Ad'**, Magnified views of the ventricular regions. **B**, The effect of *miR-9* knockdown. *miR-9AS* or control oligonucleotide was electroporated with SV40-EGFP into the medial pallium, and expressions of markers indicated were examined at indicated hours after the electroporation by *in situ* hybridization. In each panel, the left (–) indicates the control unelectroporated side, and the right (+) indicates the electroporated side. Scale bars: **A**, 20 μ m; **B**, 200 μ m (scale bars in **B**, from top to bottom, indicate 12 and 24 h, 48 h, and 72 h, respectively).

through 14th CTA, which were predicted to make base pairing with *Foxg1* 3'UTR, were also critical for the *miR-9* activity. Not only *Foxg1* but also *Lhx5*, *Id4*, and *Nr2e1* mRNAs have similar target sequences for *miR-9* seed sequence in their 3'UTR, but only *Foxg1* 3'UTR is sensitive to *miR-9* suppression. The 5' and 3' sequences flanking the target sequence are different among these mRNAs. Zhao et al. (2005) proposed the role of sequences flanking the target sequence; miRNA is less accessible to the target sites of which flanking sequences have complex secondary structures. ΔG values of the expected secondary structures of 5' and 3' 70 bp sequences flanking the *miR-9* target sequence of *Foxg1* 3'UTR are -5.3 kcal/mol and -1.2 kcal/mol (Bredenkamp et al., 2007), respectively; the secondary structures of these sequences are particularly unstable. In contrast, ΔG values of the secondary structures of those sequences in *Lhx5* 3'UTR are -19.3 and -12.2 kcal/mol; in *Id4* 3'UTR, -5.1 and -7.9 kcal/mol; in *Nr2e1* 3'UTR, -19.3 and -8.6 kcal/mol, respectively. The secondary structures of these sequences are stable, and Zhao's model well explains why *Foxg1* mRNA is, but *Lhx5*, *Id4*, and *Nr2e1* are not, the *miR-9* target.

miR-9 regulates Cajal–Retzius cell differentiation

We demonstrated by both overexpression and knockdown experiments that *miR-9* regulates Reelin-, p73-, and p21-positive Cajal–Retzius cell differentiation in cortex (Fig. 4; supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Studies with *Foxg1* mutants have suggested that neural progenitor cells in the cortex by midgestation are prone to differentiate into Cajal–Retzius cells, and *Foxg1* suppresses this differentiation, sustaining the undifferentiated state in the progenitor cells (Hanashima et al., 2004; Martynoga et al., 2005; Shen et al., 2006). The major source of Cajal–Retzius cells is the cortical hem with the peak of their generation at E10.5–E11.5 (Takiguchi-Hayashi et al., 2004; Yoshida et al., 2006). *Foxg1* expression takes place at E8.5–E9.0 before the *miR-9* expression (Tao and Lai, 1992); *Foxg1* is graded in the medial pallium and absent in the most medial pallium or the cortical hem. *miR-9* expression takes place at E9.5 and by E11.5 is graded in the most medial pallium reciprocally to the *Foxg1* expression. Subsequently, *miR-9* expression becomes more restricted; at E12.5, it is found in the cortical hem region and marginal zone, being reminiscent of the distribution of the *Wnt3a*-positive cell streak from cortical hem (Fig. 1*Bd,Bd'*) (Yoshida et al., 2006). The medial boundary of *Foxg1* protein expression would terminate more laterally than that of the *Foxg1* mRNA expression (Fig. 2*B*). We propose that *miR-9* modulates *Foxg1* protein expression in the medial pallium for the proper differentiation of Cajal–Retzius cells.

In *Foxg1* heterozygous mutants, the *Foxg1* protein level is reduced to approximately one-half, but the cortical hem is not expanded, and production of Cajal–Retzius cells is not elevated (Hanashima et al., 2004; Muzio and Mallamaci, 2005; Eagleson et al., 2007; Hanashima et al., 2007). This raises the question of whether *miR-9* indeed modulates the *Foxg1* protein level in medial pallium for Cajal–Retzius cell production. However, reduction of *Foxg1* expression to approximately one-half with small interfering RNA increases the differentiation of cultured E12 cortical cells into Reelin-positive cells (Shen et al., 2006). *Foxg1* would have a critical threshold around a haploid amount to suppress Cajal–Retzius cell differentiation. At the same time, we cannot exclude the possibility that *miR-9* targets not only *Foxg1* but also other genes for Cajal–Retzius cell differentiation; miRNAs are known to target multiple mRNAs (Lim et al., 2005).

Cortical Cajal–Retzius cells have several sources other than

cortical hem at the borders of the developing pallium: the septum and pallial–subpallial boundary (PSB) (Bielle et al., 2005). *Foxg1* is probably involved in the Cajal–Retzius cell production at these sites (Hanashima et al., 2007). *miR-9* is also expressed in PSB and septum (Fig. 1*B*), where it might also counteract *Foxg1* in the generation of Cajal–Retzius cells. *miR-9* is also expressed in preoptic area and ganglionic eminences (Fig. 1*A,B*); *Foxg1* is essential to subcortex development (Xuan et al., 1995; Martynoga et al., 2005). There are also several sites other than telencephalon at which *Foxg1* and *miR-9* are coexpressed. One such site is the otic vesicle (Fig. 1*A*), where *Foxg1* is reported to be essential to inner ear development (Pauley et al., 2006).

Reelin-positive cells are present in the telencephalon of all vertebrates, including lamprey (Molnár et al., 2006). These cells in tetrapods are considered mammalian Cajal–Retzius-like, whereas in zebrafish the majority of the cells in dorsal telencephalon are Reelin-positive but unlikely to be homologous to Cajal–Retzius cells in mammals (Costagli et al., 2002). Tetrapod *Foxg1* 3'UTRs were regulated by *miR-9*, whereas the zebrafish one was not. In zebrafish embryo, *miR-9* is evenly expressed in proliferative zones as well as in neurons of almost the entire dorsal telencephalon (Kapsimali et al., 2007). Moreover, Medaka *Foxg1* 3'UTR does not conserve the *miR-9* target sequence, and *Foxg1* gene has not yet been identified in the fugu genome.

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