MicroRNA-9 Modulates Hes1 Ultradian Oscillations by Forming a Double-Negative Feedback Loop

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SUMMARY

Short-period (ultradian) oscillations of Hes1, a Notch signaling effector, are essential for maintaining neural progenitors in a proliferative state, while constitutive downregulation of Hes1 leads to neuronal differentiation. Hes1 oscillations are driven by autorepression, coupled with high instability of the protein and mRNA. It is unknown how Hes1 mRNA stability is controlled and furthermore, how cells exit oscillations in order to differentiate. Here, we identify a microRNA, miR-9, as a component of ultradian oscillations. We show that miR-9 controls the stability of Hes1 mRNA and that both miR-9 overexpression and lack of miR-9 dampens Hes1 oscillations. Reciprocally, Hes1 represses the transcription of miR-9, resulting in out-of-phase oscillations. However, unlike the primary transcript, mature miR-9 is very stable and thus accumulates over time. Given that raising miR-9 levels leads to dampening of oscillations, these findings provide support for a self-limiting mechanism whereby cells might terminate Hes1 oscillations and differentiate.

INTRODUCTION

Oscillatory gene expression is a widespread and important phenomenon in living systems, from circadian clocks and the cell cycle (Dunlap, 1999; Tyson et al., 2001), to oscillations of regulatory factors in the immune system (Jin et al., 2004; Paszek et al., 2010), to short period (ultradian) during somitogenesis (Pourqué, 2003). More recently, oscillations in regulatory gene expression have been shown to take place in neural progenitors of the mouse cortex. Specifically, several components of the Notch signaling pathway, essential for regulating progenitor maintenance (Gaiano and Fishell, 2002), including Hes1, Dll1, and Ngn2 exhibit oscillatory behavior (Shimojo et al., 2008). Hes genes are master transcriptional repressors of neuronal differentiation, as shown by the outcome of knockdown and overexpression studies in several vertebrate species (Ishibashi et al., 1994; Ohtsuka et al., 1999).

The observation that Hes1 displays ultradian oscillatory expression in neural progenitors (Shimojo et al., 2008), has transformed our view of Notch signaling, from a linear amplification of small initial stochastic differences in regulatory gene expression, to that of a dynamic, cyclical, and mutual, inhibition of differentiation, based on oscillations of gene expression (Kobayashi et al., 2009). Indeed, this pioneering work led to the proposal that Hes1 oscillations are, in fact, necessary to keep a population of cells in an efficiently proliferating progenitor state. It is now crucial to understand how such oscillations are mechanistically controlled, and how they are terminated for cells to differentiate.

The core requirement for Hes1 oscillations is a negative feedback loop, whereby Hes1 protein represses its own transcription. Hes1 mRNA and Hes1 protein are also extremely unstable, with a half-life in the order of 20 min; the rapid degradation of Hes1 protein and Hes1 mRNA is necessary to release cells from inhibition and the initiation of the next cycle of expression (Hirata et al., 2002). However, while the mechanisms of protein degradation have been largely elucidated (Hirata et al., 2002), how mRNA stability is regulated is not well understood. Furthermore, whether this regulation could be employed to terminate oscillations and permanently downregulate Hes1, in order to enter the neuronal differentiation pathway, is completely unknown.

MicroRNAs are a class of small noncoding RNAs, which regulate gene expression at the posttranscriptional level. Recently, microRNAs have been shown to regulate their target expression primarily at the RNA level through deadenylation and decapping of the message (Giraldez et al., 2006; Guo et al., 2010; Lim et al., 2005), suggesting that they are prime candidates for controlling mRNA stability. MiR-9, a highly conserved microRNA, is expressed predominantly in the central nervous system (CNS) of the developing embryo (Wienholds et al., 2005) and is of particular importance in the development of the CNS, exhibiting a prodifferentiation function, in numerous organisms (Delaloy et al., 2010; Leucht et al., 2008; Shibata et al., 2011; Zhao et al., 2009).

Here, we investigate whether miR-9 has a role in controlling the stability of Hes1 mRNA and whether it is an important player in the Hes1 oscillator. Our findings identify miR-9 as an essential, but previously unknown, component of the Hes1 molecular oscillator and furthermore, provide a plausible mechanism for the elusive problem of how oscillations are terminated.

RESULTS

miR-9 Regulates Mouse Hes1 at the RNA Level

We have previously shown that miR-9 regulates the Xenopus homolog of Hes1, hairy1, and that the miR-9 binding site is highly conserved in its vertebrate homologs with 100% sequence
homology in the seed-complementary region (Bonev et al., 2011). In order to determine whether the mouse Hes1 is also regulated by miR-9 in vitro, we designed a luciferase reporter fused to either the wild-type (lucHes1 3’UTR WT) or mutant Hes1 3’ UTR which has a deletion in the seed-complementary region of the miR-9 binding site (lucHes1 3’UTR Mut), was transfected together with either scrambled (pre-Scr) or miR-9 precursors (pre-miR-9) in HeLa cells. Luciferase expression was normalized and expressed relative to the control levels.

(B) Expression of the wild-type or mutant luciferase reporter transfected in the neural progenitor cell line c17.2.

(C) Mature miR-9 levels in c17.2 cells after transfection with either control LNA inhibitor (Control LNAi) or miR-9 LNA inhibitor (miR-9 LNAi), as measured by qRT-PCR at 48 hr.

(D) Representative western blot analysis for endogenous Hes1 protein levels in c17.2 cells, 48 hr after transfection with scrambled/miR-9 precursors or control LNA/miR-9 LNA inhibitor. Values represent fold change in expression from three independent experiments ±SEM.

(E) qRT-PCR analysis for Hes1 mRNA levels after transfection with control LNA, miR-9 LNA inhibitor, or Hes1 target protector LNA (Hes1 TP). Expression was normalized for the housekeeping gene Gapdh and expressed as a fold change compared to the control.

(F) Hes1 mRNA degradation rate was measured after transcription block at t = 0. Relative Hes1 expression (normalized for Gapdh) was plotted on a log scale and nonlinear regression using exponential fit was used to determine the degradation rate (k) according to the equation y = Ae^{-kt}.

(G) Half-life (t1/2) of Hes1 mRNA in c17.2 cells transfected with control LNAi, miR-9 (pre-miR-9), miR-9 LNAi or Hes1 TP. Half-life was determined by the formula t1/2 = ln(2)/k.

Data are represented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 analyzed by Student’s t test. See Figure S1.
Figure 2. miR-9 Modulates Hes1 Oscillations

(A) Representative examples of live imaging and luminescence quantification of single neural progenitor c17.2 cells expressing a ubiquitinated luciferase under the control of Hes1 promoter and fused to either the wild-type (Hes1Pr-ubqluc-3'UTR WT) with or without miR-9; or the mutant Hes1 3' UTR (Hes1Pr-ubqluc-3'UTR Mut), which cannot bind miR-9.
stem cell marker Nestin and can differentiate into either neurons (TuJ1+) or astrocytes (GFAP+) (Ma and Nowak, 2011; Zang et al., 2008). These progenitor cells express miR-9 (Figure S1A). The expression of Hes1 3' UTR Mut reporter was strongly increased compared to the WT reporter (Figure 1B), suggesting that endogenous miR-9 is able to repress the expression of the WT, but not of the Mut reporter.

A locked nucleic acid (LNA) antisense inhibitor (miR-9 LNA) was used to knock down mature miR-9 levels in the c17.2 cell line (Figure 1C). Inhibiting miR-9 led to an increase in the levels of endogenous Hes1 protein, while overexpression of miR-9 significantly reduced the amount of endogenous Hes1 protein compared to scrambled control (Figure 1D).

To validate the specificity of the repression, we used a target protector LNA-modified oligo (Hes1 TP, based on (Choi et al., 2007)), which was designed to specifically disrupt miR-9/Hes1 3' UTR binding (Figure S1B), but had no effect on miR-9 levels (Figure S1C) or the expression of other miR-9 reporters (Figure S1D). The endogenous Hes1 RNA levels were increased to a similar extend in cells transfected with either miR-9 LNA or Hes1 TP, further confirming that Hes1 is a direct target of miR-9 (Figure 1E).

To determine if this increase is due to RNA stabilization, we examined whether miR-9 promotes Hes1 degradation. We used actinomycin D to block transcription in a serum synchronized c17.2 cells as previously described (Hirata et al., 2002) and examined the degradation rate of Hes1 mRNA over 3 hr (Figure 1F). While in cells transfected with the control LNA inhibitor, Hes1 mRNA had a half-life of 25 ± 2.3 min (similar to reports in other cell lines, Hirata et al., 2002), transfection of either miR-9 LNAi or Hes1 TP led to a significant increase in the stability of the Hes1 mRNA to 32.3 ± 2.25 min and 35 ± 5 min, respectively (Figures 1F and 1G). Conversely, overexpression of miR-9 reduced the half-life of Hes1 mRNA to 20 ± 3.2 min (Figure 1G), while scrambled precursors had no effect on the stability (data not shown).

Overall, these findings show that miR-9 regulates Hes1 mRNA expression directly by promoting its degradation, similar to the mechanisms of repression of hairy1 in X. tropicalis (Bonev et al., 2011). Nevertheless, we cannot fully exclude an additional effect on protein translation, because the overexpression of miR-9 reduces the level of Hes1 protein disproportionately to the reduction of mRNA (50% versus 20%; Figures 1D versus 1G). In fact, recent evidence suggests that both mechanisms may occur on the same targets (Bazzini et al., 2012).

miR-9 Modulates Hes1 Oscillations

Hes1 oscillations are driven by negative feedback, delay and high instability of both the RNA and the protein (Hirata et al., 2002), as schematized in Figure S2A. To image Hes1 oscillations in single c17.2 cells, we used ubiquitinated luciferase reporter driven by Hes1 promoter and containing the Hes1 3' UTR (Hes1Pr-ubqluc-3' UTR WT, Figure S2B) (Shimojo et al., 2008). The population was highly asynchronous, with both the period and the amplitude of the Hes1 oscillations varying from cell to cell (Figure 2A) and similar to Hes1 oscillations in isolated forebrain progenitors (Shimojo et al., 2008). On average, we observed ~75% of the cells displaying two or more cycles of oscillations over a 20 hr imaging period.

To examine the effect of miR-9 on Hes1 oscillations, we compared the distribution and the average number of cycles in cells expressing wild-type (n = 36 cells) or mutant (where miR-9 binding is abolished—Figure 1A and Figure S2B; n = 32) reporter. The average number of oscillation cycles was significantly reduced (Figures 2B and 2C), suggesting that lack of miR-9 repression leads to dampening of Hes1 oscillations. Interestingly, overexpression of miR-9 also decreased the average number of oscillation cycles per cell with the majority of the cells displaying 1 or 2 cycles of oscillations (Figures 2B and 2C; n = 36). However, a quantification of the average levels of the luciferase reporter in the cells imaged showed that the expression of the mutated reporter was increased at the end of the imaging experiments compared to the starting levels, but decreased when miR-9 was overexpressed (Figure S2D). These results were consistent with the expression of endogenous Hes1 protein when miR-9 levels were manipulated (Figure 1D).

To further confirm the effect of miR-9 regulation on Hes1 oscillations, we looked at the effect on endogenous Hes1 mRNA by quantitative reverse transcription (qRT)-PCR, by utilizing serum synchronized populations as described by Hirata et al. (2002) and Yoshiura et al. (2007). Using this method, we were able to observe only two peaks of Hes1 expression, presumably due to the cells becoming asynchronous very quickly. When miR-9 was overexpressed, Hes1 mRNA was transiently induced by serum treatment (albeit to a lower levels than the control), but remained suppressed to basal levels thereafter (Figure 2D, compare with the control). Conversely, when Hes1 TP was introduced, the amplitude of the first peak was increased, prior to dampening (Figure 2E), consistent with an increased amount of Hes1 mRNA before transcription is inhibited.

These results suggest that miR-9 regulation is important for allowing the oscillatory pattern of expression of Hes1, but too much miR-9 could also lead to disrupting and dampening of Hes1 oscillations.

Expression of Hes1 Is Inversely Related to miR-9 Primary Transcripts

The expression of miR-9 and vertebrate Hes1 related genes, tends to be mutually exclusive in many areas of the CNS. For
miR-9 is not expressed in boundary regions such as the mid-hindbrain boundary (MHB) and the zona limitans intrathalamicum (ZLI) (Bonev et al., 2011; Leucht et al., 2008; Shibata et al., 2008), where Hes1/Hairy1 is high (Baek et al., 2006) and Hes1 is downregulated in neurons, where miR-9 is abundant (Bonev et al., 2011; Shibata et al., 2011; Smirnova et al., 2005; Dajas-Bailador et al., 2012). Here, we wanted to examine areas of potential overlap. By fluorescent in situ hybridization (FISH), we found that at E12.5, miR-9 was expressed in some differentiating neurons (arrowheads) and was abundant in the ventricular zone (VZ) of the mouse neocortex, an area containing actively proliferating progenitors, (Figure 3A), which express Hes1 in a salt-and-pepper manner (Shimojo et al., 2008).

To examine the expression of miR-9 transcripts in relation to Hes1 in detail, we used double FISH. In the mouse, miR-9 has been shown to be expressed from three different genomic loci, with pri-miR-9-2 being the most abundant (Shibata et al., 2008, 2011). Consistent with these results we detected pri-miR-9-2 expression in the VZ of the mouse neocortex (Figure 3B), while the pri-miR-9-1 was undetectable at this stage (Figure S3A). Unexpectedly, when we examined the expression of pri-miR-9-2 transcripts in relation to Hes1 mRNA, we found that miR-9 expression is increased in Hes1 negative cells of the VZ, compared to Hes1+ progenitors (Figures 3B and 3C).

To examine this relative pattern in greater resolution, we analyzed miR-9 and Hes1 expression in the c17.2 neural progenitor cells at a single cell level. We verified that miR-9-2 is the most abundant primary transcript in this cell line (Figure S3B), consistent with the data in vivo. Using a double FISH (Figure 3D) or a FISH for miR-9-2 combined with immunostaining for Hes1 (Figure 3E), we confirmed the inverse relationship between the two expression patterns. Although this method is not strictly quantitative, in cells, which were high for Hes1 (Figures 3D and 3E, arrows), miR-9-2 expression was relatively weak, while cells expressing low amounts of Hes1 had increased levels of miR-9-2 transcription (Figures 3D and 3E, representative cells shown by arrowheads).

Inverse expression profiles of miR-9 and Hes1 were also observed in neural progenitor cells under proliferation or differentiation conditions. While the expression of Hes1 was relatively high in neural progenitors and decreased upon differentiation (Figure 3F), consistent with its role as inhibitor of neurogenesis (Ishibashi et al., 1994), miR-9 levels were upregulated upon neuronal differentiation of c17.2 cells; P, proliferative conditions (10% serum); D, differentiation conditions (0.2% serum). (G) miR-9 levels are upregulated upon neuronal differentiation in c17.2 cells. Data are presented as mean ± SEM. **p < 0.01. Scale bars = 20 μm. See Figure S3.
levels over time. Under these conditions, the peak of miR-9, we overexpressed Hes1 and examined pri-miR-9-2, which had been theoretically predicted (Xie et al., 2007). This finding is also consistent with the existence of two nonoscillatory states of Hes1 expression in the developing CNS; one is characterized by high, persistent expression of Hes1 and low levels of miR-9 and is observed in boundary regions of the CNS, where neuronal differentiation is limited (Leucht et al., 2008; Baek et al., 2006). This agrees with our previous findings in the frog where inhibiting miR-9 function increased the expression of hairy1 and blocked neuronal differentiation (Bonev et al., 2011). The other nonoscillatory state is characterized by low or no expression of Hes1 and high levels of miR-9 and is observed in cells undergoing neuronal differentiation (Bonev et al., 2011; Dajas-Bailador et al., 2012).

Finally, while maintaining the oscillatory phase of Hes1 expression is important for progenitor maintenance, exiting oscillations with low Hes1 protein (high miR-9) is equally important, as this is one of the requirements for neuronal differentiation to occur. However, the mechanism of exiting oscillations with low Hes1 RNA and protein was not well understood. Here, we have shown that while miR-9 regulates negatively the stability of Hes1 mRNA, and possibly also the translation of the protein, a converse negative interaction also exists, in that Hes1 protein represses miR-9 transcription. Thus, miR-9 and Hes1 are coupled in a double-negative loop, which leads to pri-miR-9 levels peaking out of phase with Hes1. Our findings suggest that pri-miR-9 would oscillate out of phase with Hes1, consistent with the inverse relation of miR-9 and Hes1 mRNA levels in neural progenitor cells. However, mature miR-9 levels steadily accumulate over time, because unlike the primary transcript, the mature miR-9 is very stable. We have also shown that experimentally raising miR-9 levels reduces Hes1 mRNA stability and decreases protein levels, which would eventually lead to dampening the oscillations of Hes1, with low Hes1 protein. Taking these findings...
Figure 4. Hes1 Negatively Regulates miR-9 Transcription
(A) The expression levels of mature miR-9 in c17.2 cells transfected with either control or Hes1 siRNA as examined by qRT-PCR.
(B) Bioinformatic prediction for the presence of Hes1 binding elements (N-boxes) in the 2 kB regions upstream of the miR-9 precursors. N-boxes in the (+) strand are depicted in red, while N-boxes in the (–) strand are in orange.
(C) qRT-PCR for relative levels of the primary miR-9 transcripts in c17.2 cells transfected with control siRNA or Hes1 siRNA.
together, we propose a model of a “self-limiting oscillator,” whereby the accumulation of miR-9 over time allows the cells to escape the progenitor state by dampening Hes1 oscillations. An intriguing question is why in vivo progenitor cells exit the oscillatory phase and differentiate at different time points. We can only speculate on this at the moment, but we suggest that cells may start with different levels of miR-9 or other factors may modulate the abundance or activity of miR-9 in some cells (such as signaling factors, Dajas-Bailador et al., 2012; or other RNA binding proteins, Shibata et al., 2011). Additionally, the levels of miR-9 may be asymmetrically partitioned in mitosis (B. B. and N. P., unpublished data), imparting the daughter cells with different timer lengths. It will also be important to consider how such an oscillatory mechanism may be integrated with the control of neurogenesis by the stepwise (de)phosphorylation of proneural proteins, such ng2, that has been proposed to take place as the cell cycle lengths during neurogenesis (Ali et al., 2011). Ng2 is also known to oscillate in progenitors (Shimojo et al., 2008), and although it is not subject of the same type of negative feedback loop as Hes1, it is quite possible that post-translational modifications would affect its oscillatory properties, potentially due to changes in protein stability.

While these are important questions for the future, we can conclude here that the double-negative feedback loop of pri-miR-9 and Hes1, coupled with high stability of the mature miR-9 RNA, provides a mechanistically plausible explanation for the controlled exit of cells from the progenitor compartment.

EXPERIMENTAL PROCEDURES

Animals

Mice were housed, bred, and treated according to the guidelines approved by the UK home office under the animal (Scientific Procedures) Act 1986.

Fluorescent In Situ Hybridization

Fluorescent in situ hybridization (FISH) using LNA probes and double FISH to detect miR-9/Hes1 expression in the c17.2 cells were performed as previously described (Dajas-Bailador et al., 2012) with some modifications. For double FISH, miR-9-2 antisense probe (~1 kb) was labeled with digoxigenin (DIG) and detected with Cy3-based tyramide signal amplification (TSA) system (Perkin Elmer), while Hes1 (full-length) was labeled with fluorescein isothiocyanate (FITC) and detected using FITC-based TSA (please see Extended Experimental Procedures for detailed protocols).

ChIP

ChIP analysis to detect binding of endogenous Hes1 to miR-9 promoter loci was performed as described previously (O’Donnell et al., 2008) with minor modifications. Detailed protocol is available in the Extended Experimental Procedures section.

Bioluminescence Imaging of Hes1 Expression in the c17.2 Cell Line

c17.2 cells were plated on glass-based dishes (l惋k) and transfected with Hes1Pr-ubqluc-3’ UTR wild-type or mutant or with WT + 30 nM of miR-9 24 hr before imaging. For measurement of bioluminescence, 1 mM D-luciferin (Sigma) was added to the media, the dish was placed on the stage of inverted microscope and was maintained at 37°C in 5% CO₂. Bioluminescence was collected using the 10 x objective and was transmitted directly to a cooled charge-coupled device camera, as described elsewhere (Masamizu et al., 2006). The signal-to-noise ratio was increased by 4 x 4 binning and 30 min exposure.

Serum Synchronization and Measurement of RNA Half-Life

c17.2 cells were synchronized by serum withdrawal as previously described (Hirata et. al., 2002). For half-life measurements, transcription was blocked using actinomycin D (Sigma) and RNA was extracted at specific time points for 3 hr. The half-life of Hes1 mRNA was determined from three independent experiments. Detailed protocols are available in the Extended Experimental Procedures.

Statistical Analysis

All data groups were expressed as the mean ± SEM and statistical significance was determined using Student’s t test. Statistical analysis was done using Sigma Stat 3.0 (Aspire Software) and significance compared to the respective control is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001. Individual comparisons between experimental groups are indicated with brackets. Experiments were repeated at least three times.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.05.017.

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(D) Recruitment of endogenous Hes1 to miR-9-1 and miR-9-2 promoters as revealed by ChIP-qPCR. IgG, negative control; Gapdh, Gapdh promoter sequence.
(E) Dynamic expression of Hes1 mRNA and the three primary miR-9 transcripts analyzed by qRT-PCR in synchronized c17.2 cells upon serum stimulation at t = 0 (Hes1 peaks are indicated with arrows).
(F) qRT-PCR for relative levels of the primary miR-9 transcripts in c17.2 cells transfected with either control (pCS2) vector or Hes1 (pCS2-Hes1).
(G) The expression levels of mature miR-9 in c17.2 cells transfected with either control or Hes1 vector as examined by qRT-PCR.
(H) qRT-PCR for relative levels of mature miR-9 compared to Hes1 mRNA after blocking transcription with Actinomycin D.
(I) Relative expression of mature miR-9 analyzed by qRT-PCR in synchronized c17.2 cells upon serum stimulation at t = 0.
(J) qRT-PCR for mature miR-9 levels in c17.2 cells grown in proliferating conditions (10% serum) 24, 48, and 72 hr after plating.
(K) Model depicting the oscillations of pri-miR-9 is driven by Hes1, which leads to the gradual accumulation of mature miR-9 and eventual dampening of the oscillations, followed by neural differentiation. On all panels values are represented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 analyzed by Student’s t test.

See Figure S4.
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Extended Experimental Procedures

Cryosectioning and Single Fluorescent In Situ Hybridization on Sections

Mouse embryos (stage E11 and E12.5) were fixed in 4% PFA, sectioned on a LEICA CM3050 S cryostat after embedding in OCT and stained as described previously (Chalmers et al., 2003; Regad et al., 2007). Locked-nucleic-acid modified (LNA), digoxigenin labeled probes were obtained from Exiqon. Fluorescent in situ hybridization (FISH) to detect mature miR-9 on 12 µm cryosections was performed as described previously (Dajas-Bailador et al., 2012). For combining miR-9 FISH and immunostaining, anti-acetylated tubulin (1:1000, Sigma) was added to the anti-DIG antibody during the overnight incubation. MiR-9 expression was detected first using TSA-Cy3 method (Perkin Elmer) while acetylated tubulin expression was detected using anti-mouse Alexa 568 secondary antibody (1:500, Roche).

Double Fluorescent In Situ Hybridization on Sections

Antisense probe for pri-miR-9-2 (~1 kb region) was DIG labeled while Hes1 antisense probe (full length) was FITC labeled. Before hybridization, slides were quickly brought to room temperature and sections were fixed for 15 min in cold 4% paraformaldehyde in 0.1 m phosphate buffered saline (PBS) (pH 7.4). Slides were briefly rinsed in PBTw (PBS+0.05% Tween-20), acetylated for 10 min in 0.25% acetic anhydride/0.1 m triethanolamine/0.9% NaCl, and then rinsed 3 x 2 min in PBTw. Sections were then washed through an increasing ethanol series, followed by 5 min in chloroform. Air-dried sections were incubated with 1 µg/ml antisense riboprobes in hybridization buffer (50% deionized formamide, 4 × SSC pH 7.0, 1 mm EDTA, 20 µg/ml yeast tRNA, 10% dextran sulfate, 1 × Denhardt’s solution and 0.25% SDS) and incubated overnight at 65 °C in a moist chamber. The following day, slides were washed at room temperature for 10 min in 2 × SSC, followed by 2 × 30 min washes at 60 °C, RNase treatment at 37 °C for 30 min and then sequentially washed for 30 min at 60 °C in 2 × SSC/50% formamide, followed by 0.5 × SSC. Slides were then incubated for 30min with 3% H2O2 solution to inactivate endogenous peroxidases, followed by 2x washes with TBSTw (100mM Tris pH7.5, 150mM NaCl, 0.05% Tween-20). For the detection of pri-miR-9 slides were blocked for 1 hr in 0.5% milk in TBSTw and incubated overnight at 4 °C with 1:1000 anti-DIG-POD (1:1000, Roche). Signal was detected using Cy3-TSA (Perkin Elmer) for 10min. Slides were then washed 3x TBSTw, the first antibody was inactivated by incubating for 45min in 3% H2O2. The antibody incubation and signal detection was then repeated using anti-FITC-POD (1:500, Roche) and FITC-TSA (Perkin Elmer) respectively.

Quantification of pri-miR-9 Expression in the Mouse Ventricular Zone

To quantify the expression of the primary miR-9-2 transcripts in relation to Hes1 we used double FISH. The total area of the VZ per section was calculated using the IMARIS software (Bitplane). The combined area of the Hes1 positive cells in the VZ was then calculated based on the signal from Hes1 mRNA FISH (green). Hes1 negative area was estimated as the total area of the VZ - Hes1+ area. To calculate the total number of pri-miR-9-2 transcripts we used IMARIS spot analysis based on the signal from pri-miR-9-2 FISH (red). The number of miR-9-2 spots lying inside or outside of the Hes1+ area was then determined. Finally, the number of these spots was divided by the Hes1+ or the Hes1- area respectively and data was presented as miR-9-2 expression per µm² of area. Analysis was performed on 9 sections from 2 different embryos using the same threshold settings for Hes1+ and miR-9-2+ FISH signal.

Fluorescent In Situ Hybridization on Adherent Cells

C17.2 cells were cultured on coverslips and fixed in 4% paraformaldehyde. Following fixation, the cells were permeabilised with 0.2% Triton X-100 in PBS for 5 min to allow more efficient penetration of the riboprobe during the hybridization step. Mature miR-9 was detected as described previously (Dajas-Bailador et al., 2012). To detect both primary miR-9 transcripts and Hes1 mRNA the following modifications were made to the protocol: EDC fixation was omitted and the temperature of the hybridization + subsequent washes was increased to 65 °C. miR-9-2 expression was detected using tyramide signal amplification – Cy3-TSA (Perkin Elmer) following incubation with anti-DIG-POD overnight at 4 °C. The substrate reaction was inhibited by incubating for 45min in 3% H2O2 buffer and Hes1 mRNA expression was detected using anti-FITC-POD antibody followed by FITC-TSA as described above. When combined with immunostaining for Hes1, primary anti-Hes1 antibody (1:50, Millipore) was incubated together with the anti-DIG-POD antibody and detected using anti-rabbit Alexa 568 secondary (1:500, Molecular Probes), following the tyramide signal amplification reaction.

DNA Constructs and Oligos

The lucHes1 3’ UTR WT plasmid used for luciferase reporter assays was described previously (Bonev et al., 2011). The Hes1 promoter – ubiquitin-ligated luciferase reporter used for live imaging was a generous gift from R. Kageyama (Kyoto University, Japan). To generate the mutant versions of these constructs we deleted 4 nucleotides in the seed complementary region (CCAAAAG) of the Hes1 3’ UTR. Map1b, Tix, Oncel1 reporter constructs were described elsewhere (Dajas-Bailador et al., et al., 2012; Bonev et al., 2011). To generate pCS2-Hes1, the region corresponding to the coding sequence of Hes1 was PCR amplified from mouse brain cDNA, subcloned in pCRII-TOPO (Life Technologies) and transferred to pCS2+ using the Xhol/XbaI restriction sites. Putative miR-9-2 promoter fragment was amplified from mouse genomic DNA using the forward primer (5’-gctagCCACTGGGAAAATAGACA-3’) and the reverse primer (5’-ctcagTTTGTGTAGGAAGCGTA-3’) and cloned into the pGL3-basic vector (Promega) upstream.
of luciferase using the Nhel/Xhol restriction sites. miR-9-2 promoter mutant for Hes1 binding was generated by deleting the following nucleotides (underlined): Nbox2 – aCACTAGcgc; Nbox3 – taGTGCTAgtg).

The LNA control (scrambled seq: GTGTAACACGTCTATAGCCCA; Cat N: 199002), LNA miR-9 inhibitor (seq: TCATAACGCTA GATAACCAAAG; Cat N: 410014), and custom designed Hes1 TP (seq: acatctttgcttggcttgg-3'), miR-9-1 Nbox3_F (5'-acttccagaaaggttg-3'), miR-9-1 Nbox3_R (5'-tcgcgaattcagctgaaatg-3'); miR-9-2 Nbox1_F (5'-catcaggtgtggtatcact-3'), miR-9-2 Nbox1_R (5'-ctgctgtagcaaaatgctga), miR-9-2 Nbox2_F (5'-cccagacgctaatcgc-3'), miR-9-2 Nbox2_R (5'-cgccctttacttcctgctg-3'); miR-9-2 Nbox3_F (5'-ggcttcagaaagggcagcag-3'), miR-9-2 Nbox3_R (5'-tgcttggcagctcctct-3'); miR-9-2 Nbox4_F (5'-aagaccaagaggtcagag-3'), miR-9-2 Nbox4_R (5'-tggaaatcactgctgctc-3'). Sequence from the Gapdh promoter, amplified by the following primers: GapdhPr_F (5'-cttttaagccttcgctgcctg-3'), GapdhPr_R (5'-ctcctgtctgcgctgtgtgg-3') was used as a negative control. The experiment was repeated at least 3 times and results were presented as fold enrichment over the control CHIP reaction using anti-IgG antibody (Sigma).

Immunoblotting

Samples were resolved by SDS-PAGE (10% gels) and transferred to Immobilon-P membranes (Millipore Inc.), which were immunoblotted with the following antibodies: anti-Hes1 (MBL, D134-3), anti-GAPDH (Applied Biosystems, cat# AM4300). Immunocomplexes were detected using HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (Pierce). Results were quantified using Intelligent Quantifier software (Bio Image Systems).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChiP) analysis was performed as described previously [O’Donnell, 2008]. Briefly, for each ChiP experiment, c17.2 cells from 2x10cm dishes were cross-linked with 1% formaldehyde for 10 min at room temperature, harvested, and rinsed with 1 x PBS and pooled. Cell nuclei were isolated, pelleted, and sonicated. DNA fragments were enriched by immunoprecipitation using anti-Hes1 antibody (Milipore). After heat reversal of the cross-links, the enriched DNA was purified and analyzed by real-time PCR using the following primers: miR-9-1 Nbox2_F (5'-ggttgctgatcactgctg-3'), miR-9-1 Nbox2_R (5'-gcactggaagga-3'), miR-9-1 Nbox3_F (5'-acatcttcaagaaaggttg-3'), miR-9-1 Nbox3_R (5'-gcagctgaaatgctga-3'); miR-9-2 Nbox1_F (5'-cctcccagcttcctgctg-3'), miR-9-2 Nbox1_R (5'-aacagatcactgctgctg-3'), miR-9-2 Nbox2_F (5'-ccagacgctaatcgc-3'), miR-9-2 Nbox2_R (5'-ccttcctcagctgctgctg-3'); miR-9-2 Nbox3_F (5'-ggcttcagaaagggcagcag-3'), miR-9-2 Nbox3_R (5'-tgcttggcagctcctct-3'); miR-9-2 Nbox4_F (5'-aagaccaagaggtcagag-3'), miR-9-2 Nbox4_R (5'-tggaaatcactgctgctc-3'). Chromatin Immunoprecipitation (ChIP) analysis was performed as described previously [O’Donnell, 2008]. Briefly, for each ChIP experiment, c17.2 cells from 2x10cm dishes were cross-linked with 1% formaldehyde for 10 min at room temperature, harvested, and rinsed with 1 x PBS and pooled. Cell nuclei were isolated, pelleted, and sonicated. DNA fragments were enriched by immunoprecipitation using anti-Hes1 antibody (Milipore). After heat reversal of the cross-links, the enriched DNA was purified and analyzed by real-time PCR using the following primers: miR-9-1 Nbox2_F (5'-ggttgctgatcactgctg-3'), miR-9-1 Nbox2_R (5'-gcactggaagga-3'), miR-9-1 Nbox3_F (5'-acatcttcaagaaaggttg-3'), miR-9-1 Nbox3_R (5'-gcagctgaaatgctga-3'); miR-9-2 Nbox1_F (5'-cctcccagcttcctgctg-3'), miR-9-2 Nbox1_R (5'-aacagatcactgctgctg-3'), miR-9-2 Nbox2_F (5'-ccagacgctaatcgc-3'), miR-9-2 Nbox2_R (5'-ccttcctcagctgctgctg-3'); miR-9-2 Nbox3_F (5'-ggcttcagaaagggcagcag-3'), miR-9-2 Nbox3_R (5'-tgcttggcagctcctct-3'); miR-9-2 Nbox4_F (5'-aagaccaagaggtcagag-3'), miR-9-2 Nbox4_R (5'-tggaaatcactgctgctc-3'). Sequence from the Gapdh promoter, amplified by the following primers: GapdhPr_F (5'-cttttaagccttcgctgcctg-3'), GapdhPr_R (5'-ctcctgtctgcgctgtgtgg-3') was used as a negative control. The experiment was repeated at least 3 times and results were presented as fold enrichment over the control ChiP reaction using anti-IgG antibody (Sigma).

Cell Culture, Transfection, and Luciferase Reporter Assay

c17.2 mouse neural progenitor cell line was a generous gift from Prof Mike White (University of Manchester). They were maintained in proliferative state in DMEM growth media supplemented with 10% serum and antibiotics. Neuronal differentiation was induced by serum withdrawal to a final concentration of 0.2%. For miR-9 target luciferase reporter assays, cells were plated in a 24 well plate and transfected using Lipofectamine 2000 after 24 hours with 100ng of the luciferase reporter and either 30nM (unless otherwise indicated) of scrambled/miR-9 precursors (Ambion). Luciferase expression was analyzed after 48 hr using Dual Luciferase Assay system (Promega). Renila luciferase activity was normalized by the co-expressed firefly luciferase and expressed as a percentage of the control.

For analysis of Hes1 overexpression 600ng/well control pcS2 vector or pcS2-Hes1 was transfected into a 24 well plate and miR-9 expression was analyzed after 48 hr.

For analysis of miR-9-2 promoter sequences, cells were plated in a 24 well plate and transfected with 400ng pGL3 firefly reporter plasmid and 20nM control/Hes1 siRNA, together with 50ng HSV-TK Renila plasmid to control for transfection efficiency. Cells were lysed after 48h and firefly expression was normalized for the co-expressed renilla. All assays were repeated at least 3 times and results were expressed relative to the control.

Serum Synchronization, RNA Isolation, and Quantitative Real-Time PCR Analysis

Cells were plated in a 24 well plate 24h prior to transfection. They were transfected at =80% density and 24h posttransfection the media was changed to 0.2% serum in order to synchronize the population as previously described (Hirata et al., 2002). After additional 24h serum was added to a final concentration of 10% at t = 0 to induce Hes1 expression.

To analyze the expression of Hes1/primary miR-9 transcripts, RNA was extracted every 30min after serum stimulation using TRIZOL (Invitrogen). 1 µg of total RNA was retrotranscribed using RT-AMV (Invitrogen) according to the manufacturer’s instructions. Quantitative real-time PCR was performed in an ABI StepOnePlus Sequence Detection System (Applied Biosystems) using Taqman Fast RealTime PCR master mix and probes purchased from Applied Biosystems. Expression levels were normalized for Gapdh. To analyze the expression of mature cDNA was reverse transcribed from 50ng total RNA using TaqMan microRNA reverse transcription kit (ABI) and the levels were accessed using Taqman microRNA assay (ABI) according to the manufacturer’s instructions. The expression of mature miR-9 was normalized for the small nuclear RNA U6.
Measurement of RNA Half-Life

C17.2 cells were transfected with control LNA, miR-9 LNA and Hes1 TP at ~80% confluency 24h after plating. After another 24h cells were washed with PBS and transferred to media containing 0.2% serum to synchronize the population. After further 24h Hes1 expression was stimulated by adding serum to the media to a final concentration of 10% for 60min and 10 μM actinomycin D (Sigma) was then added (t = 0) to block transcription. RNA was extracted at t = 15, 30, 45, 60, 90, 120 and 180 min using TRIzol (Introgen). Hes1 levels were analyzed by qRT-PCR and normalized for Gapdh expression, while miR-9 levels were normalized for the small RNA U6. The relative expression levels were plotted on a log scale and the degradation rate k was determined using first-order exponential fit. Half-life of the Hes1 RNA was then calculated as $t_{1/2} = \ln(2)/k_{\text{decay}}$. Values were expressed as the mean of three independent experiments ± sem.

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Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakayama, S., and Kageyama, R. (1994). Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements. J. Biol. Chem. 269, 5150–5156.
Figure S1. miR-9 Expression in the Neural Progenitor Cell Line 17.2, Related to Figure 1

(A) In situ analysis for mature miR-9 expression in c17.2 cells using LNA probes. miR-1 was used a negative control. Scale bar = 20mm (B) Design of the Hes1 target protector oligo directed against Hes1 miR-9 binding site. Seed region is boxed in red. (C) Hes1 TP does not affect mature miR-9 levels in c17.2 cells, as analyzed by qRT-PCR. (D) Relative luciferase expression of different miR-9 target reporters in c17.2 cells transfected with either control LNA or Hes1 TP LNA oligos. Hes1 TP upregulates the expression of only Hes1 luciferase reporter but does not affect other miR-9 targets.
Figure S2. Real-Time Imaging of Hes1 Expression in c17.2 cells, Related to Figure 2
(A) Schematic simplified model of Hes1 oscillator, driven by negative feedback loop, delay (not shown) and high degradation rate of both the protein (mp) and the mRNA (mm). For a detailed model see Momiji et al., 2008.
(B) miR-9 binding site (BS) in the 3’ UTR of the Hes1 ubiquitinated luciferase reporter (Shimojo et al., 2008) was mutated to generate Hes1Pr-ubqluc-3’UTR Mut construct.
(C) Quantification of the bioluminescence of Hes1 reporter expression in individual transfected c17.2 cells over ~20 hr imaging period. Luciferase expression was normalized to 1 at t = 0 for all cells.
(D) Quantification of the average bioluminescence of the Hes1 reporter at the beginning (t = 0) and toward the end of the single cell imaging experiment (t = 18h) (n = 34 cells in each condition).
Figure S3. Expression of Primary miR-9 Transcripts in the Mouse Cortex and c17.2 cells, Related to Figure 3
(A) Double fluorescent in situ hybridization (FISH) for pri-miR-9-1 (red) Hes1 mRNA (green) on cryosections from E11 mouse cortex VZ-ventricular zone.
(B) qRT-PCR for pri-miR-9 levels in c17.2 neural progenitor cells.
**Figure S4. Hes1 Regulates miR-9 Expression, Related to Figure 4**

(A) Hes1 protein levels are reduced when c17.2 cells are transfected with Hes1 siRNA. Gapdh was used as a loading control. NS- non-specific control.

(B) Putative miR-9-2 promoter fragment (~2 kB) was inserted upstream of luciferase in the pGL3 vector to make miR-9-2Pr-WT construct. miR-9-2-Mut was constructed by mutating 2 Hes1 binding sites.

(C) Knockdown of Hes1 using siRNA upregulates the expression of the wild-type miR-9-2 reporter, but has no effect on the mutant reporter.

(D) Relative expression of pri-miR-9-2 in c17.2 cells transfected with either control vector (pCS2) or Hes1 (pCS2-Hes1), analyzed by qRT-PCR. Hes1 overexpression represses the pri-miR-9-2 transcription peak.