Neural fate decisions mediated by combinatorial regulation of Hes1 and miR-9

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Abstract In the nervous system, Hes1 shows an oscillatory manner in neural progenitors but a persistent one in neurons. Many models involving Hes1 have been provided for the study of neural differentiation but few of them take the role of microRNA into account. It is known that a microRNA, miR-9, plays crucial roles in modulating Hes1 oscillations. However, the roles of miR-9 in controlling Hes1 oscillations and inducing transition between different cell fates still need to be further explored. Here we provide a mathematical model to show the interaction between miR-9 and Hes1, with the aim of understanding how the Hes1 oscillations are produced, how they are controlled, and further, how they are terminated. Based on the experimental findings, the model demonstrates the essential roles of Hes1 and miR-9 in regulating the dynamics of the system. In particular, the model suggests that the balance between miR-9 and Hes1 plays important roles in the choice between progenitor maintenance and neural differentiation. In addition, the synergistic (or antagonistic) effects of several important regulations are investigated so as to elucidate the effects of combinatorial regulation in neural decision-making. Our model provides a qualitative mechanism for understanding the process in neural fate decisions regulated by Hes1 and miR-9.

Keywords Neural fate decision · Hes1 · miR-9 · Oscillation · Bifurcation

1 Introduction

During nervous system development, neural progenitors give rise to distinct types of cells by changing their competency over time [1]. Maintenance of neural progenitors is significant for generating cells in correct numbers and cell types. It has been shown that oscillatory

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expression of *Hes1*, a Notch signaling effector, is necessary for keeping a population of cells in an efficiently proliferating progenitor state. Experimental observations show that *Hes1* displays a short-period (ultradian) oscillatory expression in neural progenitors with a period of about 2–3 h [2]. In addition, *Hes1* expression is repressed before cell division in those neural progenitors that underwent neuronal differentiation and the suppression is maintained in daughter neurons [3, 4]. The oscillatory versus persistent manner of *Hes1* is important for the transition from proliferation to differentiation [5]. Though *Hes1* is necessary for cell-fate decisions, the molecular mechanisms of *Hes1* regulation are still not well understood. It is therefore important to understand how *Hes1* oscillations are produced, how they are controlled, and further, how they are terminated for neural progenitor cells to differentiate.

The oscillatory behavior of *Hes1* has been the focus of many studies. It has been reported that *Hes1* oscillations are driven by negative auto-regulation in transcription and ubiquitin-proteasome-mediated degradation [6]. In addition, the period of the oscillations is mainly determined by the delay of transcription and translation [7] and different binding sites in the *Hes1* gene promoter have also been reported to influence the expression of *Hes1* [8]. In addition, some studies have attempted to integrate other pathways, e.g., the Notch signaling pathway, to analyze *Hes1* oscillations. A lateral inhibition is proposed to explore the roles of *Hes1* oscillations in the Notch signaling pathway during neural fate assignment [9, 10]. Actually, a simple *Hes1* auto-repression alone is not adequate to produce, control, and even terminate oscillations [6]. Recently, several studies have incorporated the effects of microRNAs (miRNAs) into *Hes1* oscillations [11, 12]. However, the fundamental principle that *Hes1* is regulated by miRNAs is not well understood and still needs to be explored.

MicroRNAs, the small noncoding RNAs, regulate gene expression mainly at the RNA level and are vital components of cellular processes [13]. Recent studies have presented a new proposal that targets of miRNAs can also modulate the levels and functions of miRNAs in turn, thus forming various feedback loops [14]. Several studies have shown that miR-9, expressed in both embryonic and adult brains [15], might function as a regulatory switch at different stages in neurogenesis by interacting with its target mRNAs, e.g., miR-9 can form a feedback loop with nuclear receptor TLX in neural fate determination [16]. Besides TLX, several other targets of miR-9 have been identified, such as *Hes1*, REST, and FoxG1 [17–19]. Understanding these novel functions has just begun, and only a small subset of miRNAs have been characterized. Specifically, microRNA-mediated feedback loops in gene networks play crucial roles in neural fate decisions and need to be well understood. Recently, experimental observations indicated that miR-9 can target *Hes1* mRNA to form a double-negative feedback loop in order to modulate *Hes1* oscillations and fate decisions [11]. These observations lead us to consider how the *Hes1* oscillations are controlled and terminated so as to facilitate cell-fate decisions when the regulation of miR-9 is incorporated.

Here, we present a mathematical model involving *Hes1* and miR-9 to analyze the switch related to neural proliferation and differentiation. By incorporating the effect of miR-9 into the *Hes1* oscillator, we demonstrate that both mRNA stability and miR-9 can affect the oscillatory behavior of *Hes1* and they seem to have similar effects in regulation. To scrutinize the regulation by miR-9 of *Hes1*, we explore the dynamics of the system to show the influence of miR-9 on cell-state choice. Furthermore, we investigate the synergistic (or antagonistic) effects of several important elements in order to elucidate the effects of combinatorial regulation in decision-making. Finally, we investigate the stability of the system by performing a sensitivity analysis for both the steady stable state and oscillatory state, thus giving an explanation for the mechanisms of neurogenesis.

2 Methods

2.1 Hes1 and miR-9 form a self-limiting oscillator

A mathematical model of Hes1 oscillations integrating Notch signaling has previously been constructed for the study of neural fate decisions [10]. This model mainly focuses on proteins including Hes1, Notch, and Delta1. Here, we develop a mathematical model by incorporating miR-9 into the oscillatory model but neglecting the intercellular Notch signaling. A network that includes three negative regulatory processes between Hes1 and miR-9 is schematized in Fig. 1. The network consists of *Hes1* mRNA, Hes1 protein in the cytoplasm, Hes1 protein in the nucleus, pri-miR-9, and mature miR-9. The model presented in our work involves several aspects: (i) the Hes1 protein in the cytoplasm can be transported into the nucleus and then represses its own transcription [6]; (ii) the Hes1 protein in the nucleus represses the expression of pri-miR-9 by binding to its gene promoter [11]; (iii) pri-miR-9 is transcribed from its gene and converted into pre-miR-9 and subsequently to mature miR-9 [20]; and (iv) mature miR-9 regulates translation of *Hes1* mRNA by binding to its 3' untranslated region through base-pairing between the two RNA molecules [17].

2.2 Model formulation

The regulatory relationship of our model can be summarized in a set of differential equations, which describe the behavior of *Hes1* mRNA, Hes1 protein in the cytoplasm, Hes1 protein in the nucleus, pri-miR-9 and mature miR-9 with concentration levels M , H_C , H_N , $M_{\text{pri-miR-9}}$ and $M_{\text{miR-9}}$, respectively:

$$\frac{dM}{dt} = v_1 \frac{K_1^n}{K_1^n + H_N^n} - v_2 \frac{M}{K_2 + M} - dMM_{\text{miR-9}}, \quad (1)$$

Fig. 1 Oscillator system formed by miR-9 and Hes1. The Hes1 protein in the cytoplasm can be transported into the nucleus and then represses its own transcription. In addition, Hes1 and miR-9 can form a double-negative feedback loop. The Hes1 protein in the nucleus represses the expression of pri-miR-9 by binding to its gene promoter, while mature miR-9 regulates translation of *Hes1* mRNA by binding to its 3' untranslated region through base-pairing between the two RNA molecules

$$\frac{dH_C}{dt} = v_3M - v_4 \frac{H_C}{K_4 + H_C} - v_5H_C, \quad (2)$$

$$\frac{dH_N}{dt} = v_5H_C - v_6 \frac{H_N}{K_6 + H_N}, \quad (3)$$

$$\frac{dM_{miR-9}}{dt} = k_5M_{pri-miR-9} - v_7 \frac{M_{miR-9}}{K_7 + M_{miR-9}} - dMM_{miR-9}, \quad (4)$$

$$\frac{dM_{pri-miR-9}}{dt} = p + v_8 \frac{K_8^n}{K_8^n + H_N^n} - v_9 \frac{M_{pri-miR-9}}{K_9 + M_{pri-miR-9}} - k_5M_{pri-miR-9}. \quad (5)$$

The degradation rates of all components are assumed to obey Michaelis-Menten kinetics. Equation (1) describes the change of *Hes1* mRNA, which is negatively regulated by the Hes1 protein in the nucleus and repressed by miR-9 through base-pairing between the two RNA molecules. Both the Hes1 translation in the cytoplasm and its transportation to the nucleus are assumed to be linear in Eqs. (2) and (3) [10]. Equations (4) and (5) describe the production of miR-9 and pri-miR-9. The conversion from pri-miR-9 to miR-9 is also assumed to be linear, which is the same as the base-pairing between Hes1 and mature miR-9. The production of pri-miR-9 is inhibited by the Hes1 protein in the nucleus, as shown in Eq. (5). The basal transcription activity of pri-miR-9 is also considered, denoted as p , assuming that naked DNA has a low transcription activity [21].

Because the parameters cannot be reliably obtained from experimental data, we take parameter values from published papers. For Hes1 auto-regulation, we adopt the parameter values used in [10, 22]. These parameters are chosen to make the period of Hes1 oscillations in neural progenitors close to 2–3 h. For parameters related to miR-9, K_7 , K_8 , and K_9 , we adopt the parameter values used in [23]. The parameters related to the linear form of miR-9 synthesis are chosen according to [24], while the Michaelis-Menten form is adopted according to [10, 22]. The parameter d related to the degradation of the RNA complex is chosen according to [25], which is experimentally validated. All these parameters related to miR-9 are chosen to make the period of Hes1 oscillations close to 2–3 h. The standard parameter values are shown in Table 1, unless otherwise indicated.

3 Results

3.1 The generation of Hes1 oscillations

It is known that the negative auto-regulation in which Hes1 protein inhibits its own transcription may generate oscillations. Besides the negative feedback loop, *Hes1* mRNA degradation is also important for Hes1 to generate oscillations [26, 27]. In addition, miR-9 regulates *Hes1* mRNA expression by directly promoting its degradation [17]. To further elucidate the effects of the regulatory processes at the transcriptional and post-transcriptional levels, we focus on

Table 1 Parameter values in the model

Parameters	Definitions	Values	References
K_1 (nM)	Inhibition constant for repression of <i>Hes1</i> expression by nuclear <i>Hes1</i>	0.157	[10]
K_2 (nM)	Michaelis constant for degradation of <i>Hes1</i> mRNA	0.104	[10]
K_4 (nM)	Michaelis constant for degradation of <i>Hes1</i> protein in the cytoplasm	0.142	[10]
K_6 (nM)	Michaelis constant for degradation of <i>Hes1</i> protein in the nucleus	0.13	[10]
K_7 (nM)	Michaelis constant for degradation of miR-9	0.1	[23]
K_8 (nM)	Inhibition constant for repression of pri-miR-9 by nuclear <i>Hes1</i>	0.15	[23]
K_9 (nM)	Michaelis constant for degradation of pri-miR-9	0.1	[23]
n	Hill coefficient	2	[10]
k_5 (min^{-1})	Synthesis rate constant of miR-9	0.1	[24]
d ($\text{nM}^{-1} \text{min}^{-1}$)	Association rate of two RNAs	0.015	[25]
v_1 (nM min^{-1})	Synthesis rate constant of <i>Hes1</i> mRNA	1.0	[10]
v_2 (nM min^{-1})	Maximum degradation rate of <i>Hes1</i> mRNA	0.2	[10]
v_3 (min^{-1})	Synthesis rate constant of <i>Hes1</i> protein in the cytoplasm	0.575	[10]
v_4 (nM min^{-1})	Maximum degradation rate of <i>Hes1</i> protein in the cytoplasm	0.851	[10]
v_5 (min^{-1})	Cytoplasm-nucleus transportation rate of <i>Hes1</i> protein	0.021	[10]
v_6 (nM min^{-1})	Maximum degradation rate of <i>Hes1</i> protein in the nucleus	0.162	[10]
v_7 (nM min^{-1})	Maximum degradation rate of miR-9	0.08	[10]
v_8 (nM min^{-1})	Synthesis rate constant of pri-miR-9	0.5	[10]
v_9 (nM min^{-1})	Maximum degradation rate of pri-miR-9	0.06	[10]
p (nM min^{-1})	Basal transcription rate of pri-miR-9	0.03	[24]

the effects of *Hes1* mRNA synthesis, its degradation, and its base-pairing with miR-9 on the system dynamics. To give a visual depiction of how *Hes1* oscillations occur, we perform bifurcation analysis with v_1 , the synthesis rate of *Hes1* mRNA, as a control parameter, which represents the influence of *Hes1* mRNA synthesis in producing oscillations. A supercritical Hopf bifurcation occurs at $v_1=0.6098$. Notably, we find two saddle-node bifurcation points of limit cycles, causing the coexistence of two simultaneously stable oscillations, i.e., birhythmicity, as shown in Fig. 2a. To further exploit the relationship between *Hes1* mRNA synthesis and its degradation (including the degradation of *Hes1* mRNA v_2 and the association rate of two RNAs, d , in generating oscillations), we perform codimension two bifurcation analysis in the parameter spaces of (v_1, v_2) and (v_1, d) , respectively. The results show a similar tendency that the larger v_2 or d becomes, the larger v_1 is needed to generate oscillations, as shown in Fig. 2b and c, respectively. In the parameter spaces of (v_1, v_2) and (v_1, d) , we can gain insight into the various domains of the complex dynamics. The diagrams can be divided into four parts, showing the different dynamics in the parameter spaces: (I) one stable steady state, (II) coexistence of a stable oscillation and a sustained state, (III) birhythmicity, and (IV) one stable oscillatory state. Such complex dynamics have also been observed in other biological models [28–30].

To investigate further the effects of *Hes1* mRNA degradation and the base-pairing of *Hes1* mRNA and miR-9 on the system dynamics, we choose v_2 and d as key parameters and study their effects on *Hes1* oscillations. Figure 3a demonstrates that the *Hes1* mRNA degradation rate v_2 causes significant changes in *Hes1* both in expression level and periodicity. When v_2 is

Fig. 2 Bifurcation properties. **a** Bifurcation diagram with v_1 as a control parameter. **a** Hopf bifurcation occurs at $\bar{v}_1 = 0.6098$. (Stable steady states: red line; unstable steady states: black line; stable limit cycles: green empty circles; unstable limit cycles: blue empty circles). **b** Bifurcation diagram in the parameter space of v_1 and v_2 . **c** Bifurcation diagram with v_1 and d as control parameters. The blue curve represents the saddle node bifurcation of limit cycles and the red curve represents Hopf bifurcation. The dynamics of the system can be divided into four classes: (I) one stable steady state, (II) coexistence of a stable oscillation and a sustained state, (III) birhythmicity, and (IV) one stable oscillatory state

small, the expression of Hes1 is relatively high and the period of the oscillations is relatively longer. As v_2 increases, Hes1 oscillations appear to have a lower expression level and a shorter periodicity. These results show that the dynamics of the model is in agreement with the experimentally observed oscillations induced by stabilization of *Hes1* mRNA [6, 27, 31]. Similarly, as the association rate of miR-9 and *Hes1* mRNA d increases, we also find a tendency similar to the case of increasing v_2 . However, the influence of d in inducing oscillations is much weaker than v_2 . However, as d varies, the period of Hes1 oscillations changes significantly, which will be discussed in detail in the next section.

Fig. 3 Modulation of Hes1 oscillations by *Hes1* mRNA self-degradation and its co-degradation with miR-9. **a** An increase in the degradation rate of *Hes1* mRNA, v_2 , results in the down-regulation of Hes1. **b** An increase in the co-degradation rate of *Hes1* mRNA and miR-9, d , down-regulates Hes1

These results indicate that both *Hes1* mRNA self-degradation and its binding with miR-9 play important roles in Hes1 oscillations. Although the core requirement for Hes1 oscillations is the negative feedback loop, the generation of Hes1 oscillations also depends on the balance between *Hes1* mRNA synthesis and its degradation. In other words, the generation of Hes1 oscillations needs a strong negative feedback loop, i.e., large v_1 and weak degradation (small v_2 and d). In addition, the *Hes1* mRNA self-degradation and its co-degradation with miR-9 play similar roles in determining Hes1 oscillations. However, which one is more important and the relationship between them need further analysis.

3.2 miR-9 negatively regulates Hes1 oscillations

We now discuss the effects of miR-9 on regulating Hes1 oscillations. There is much experimental evidence for mutual exclusion of Hes1 and miR-9 [11, 17]. In addition, high expression of miR-9 promotes neural differentiation [20, 32, 33], while Hes1 expression is down-regulated in this process [34, 35]. Actually, primary miR-9-2 transcripts are more abundant in Hes1-negative cells than in positive ones [11]. To demonstrate the effects of miR-9 on Hes1 oscillations, we change the value of parameter p , which is related to pri-miR-9 synthesis, so as to change the expression level of miR-9. Time evolution of the Hes1 protein in the nucleus, pri-miR-9, and miR-9 in the presence of low and high levels of p are shown in Fig. 4a and b, respectively. At a low basal transcription rate of pri-miR-9, represented by p , a high level of Hes1 together with low levels of pri-miR-9 and miR-9 are observed and they are all in oscillatory states, as shown in Fig. 4a. The miR-9 oscillations are inversely related to Hes1 levels. However, at a high level of p , the system stops oscillations and remains in a stable steady state, as shown in Fig. 4b. Indeed, the level of miR-9 is increased, while the level of Hes1 is reduced. Although a precise regulatory mechanism remains to be determined, the model can account for the experimental phenomenon that raising miR-9 may induce dampening of the oscillations and neural differentiation. When miR-9 is over-expressed, dampening of Hes1 oscillations and gradual accumulation of mature miR-9 occur, leading to increased probability of differentiation. This is entirely consistent with a previous observation that the burst of pri-miR-9 transcription induced by a stimulation of serum leads to gradual accumulation of miR-9. Over-expression of miR-9 represses the expression of Hes1, with the majority of cells displaying a number of cycles of oscillations, which finally induces the termination of oscillations and occurrence of neuronal differentiation [11, 17].

Experimental observations indicate that a lack of miR-9 repression also leads to dampening of Hes1 oscillations [11]. To explain the phenomenon, we compare Hes1 activity under conditions of lack, normal, and over-expression of miR-9. We therefore vary the synthesis rate of pri-miR-9, v_8 , while the basal transcription rate of pri-miR-9, p , is set to zero (Fig. 5). In agreement with the experimental findings, both lack and over-expression of miR-9 can decrease the cycles of Hes1 oscillations. Differently, the Hes1 expression level is decreased by over-expression of miR-9 but increased by lack of miR-9.

To examine the relationship between the expression of miR-9 transcripts and Hes1 oscillations, we next analyze how the period of Hes1 oscillations changes with the pri-miR-9 synthesis rate. The period of Hes1 decreases as v_8 increases, as shown in Fig. 6a. In order to determine whether miR-9 regulates the period of Hes1 oscillations at the post-transcriptional level, we also examine how the period changes with the association rate of the two RNAs, d . When d is small, the period of Hes1 decreases sharply; while as d increases, the period tends to be stable, as shown in Fig. 6b.

Fig. 4 Time evolution of Hes1 in the nucleus, miR-9 and pri-miR-9. **a** A high level of Hes1 together with low levels of pri-miR-9 and miR-9 are observed at $p=0.03$ and they are all in oscillatory states. **b** The system stops oscillations and evolves toward a stable steady state at $p=0.3$

These results indicate that a certain level of miR-9 can negatively regulate Hes1 in an oscillatory manner, while its over-expression can also dampen the oscillations to induce differentiation. How the regulation of miR-9 is employed to terminate the oscillations and permanently down-regulate Hes1 in order to initiate the differentiation still needs to be investigated. To determine the miR-9 expression that may trigger cell differentiation, we enhance p in order to increase the transcription rate of pri-miR-9. A Hopf bifurcation occurs at the bifurcation point of $\bar{p} = 0.2375$, which governs the switch from proliferation to differentiation, as illustrated in Fig. 7. When $p < \bar{p}$, Hes1 oscillations occur and cells remain in the progenitor state. On the contrary, when $p > \bar{p}$, cells exit oscillations so as to differentiate.

Fig. 5 miR-9 modulates Hes1 oscillations. Compared with the set of standard parameters (red curve), lack of miR-9 repression reduces the cycles of Hes1 oscillations (blue curve). Over-expression of miR-9 also decreases the number of Hes1 oscillation cycles (green curve) and finally terminates Hes1 oscillations

Fig. 6 The period of Hes1 oscillations influenced by miR-9. **a** The period decreases with the pri-miR-9 synthesis rate v_8 . **b** The period decreases sharply when the association rate of two RNAs, d , is slow. As d increases, the period tends to be stable

In summary, besides the auto-repression of Hes1, miR-9 is another important element to modulate oscillatory patterns of Hes1 and is therefore essential in making fate choice. Hes1 oscillations can be maintained only within a certain range of miR-9. Both lack and over-expression of miR-9 can dampen Hes1 oscillations. In addition, over-expression of miR-9 can even terminate Hes1 oscillations.

3.3 Synergistic and feedback signaling mechanisms

During the regulatory process, it seems that some regulations, i.e., parameters, play similar roles in regulating the dynamics of Hes1 and miR-9, e.g., an increase in d or v_2 or both results in down-regulation of Hes1. In addition, an increase in p or v_8 or both induces dampened Hes1

Fig. 7 Bifurcation diagram with p , the basal transcription rate of pri-miR-9, as a control parameter. A Hopf bifurcation occurs at $\bar{p} = 0.2375$, which shows the boundary between stable steady states (red line) and limit cycles (green circles)

oscillations. The correlation of these parameters still needs to be further examined. We therefore focus on how variations in pairs of parameters affect the system dynamics.

Both the *Hes1* mRNA degradation and the base-pairing between *Hes1* mRNA and miR-9 control the stability of *Hes1* mRNA. We therefore choose the *Hes1* mRNA degradation rate, v_2 , and the association rate of the two RNAs, d , as the control parameters to examine their combinatorial effects on regulating the system dynamics. It is known that the *Hes1* mRNA degradation rate is one of the key factors for Hes1 oscillations [26] and miR-9 negatively regulates the dynamics of Hes1 expression by the binding of mRNA and miRNA, which enhances the degradation of both RNAs. As v_2 and d increase, Hes1 proteins both in the cytoplasm and nucleus are expressed in a low level. To test the mutual effects, we analyze the bifurcation properties with v_2 and d as control parameters. The bifurcation diagram reflects the relationship between *Hes1* mRNA degradation and the binding of two RNAs in regulating the system dynamics, as shown in Fig. 8a. It indicates that: (i) either *Hes1* mRNA degradation or the association can induce oscillations solely; (ii) large *Hes1* mRNA degradation or large *Hes1* mRNA degradation and the association can terminate oscillations so as to induce cell differentiation, while only a large association rate cannot; (iii) when $d > 0.62$, the two sources

Fig. 8 Bifurcation properties. **a** Bifurcation diagram in the parameter space of d and v_2 . When $d > 0.62$, they could co-compensate the inefficiency of each other in order to generate oscillations. When $d < 0.62$, they act antagonistically to induce a cell-state transition. **b** Bifurcation diagram in a parameter space of p and v_8 . The functions of p and v_8 are complementary in generating oscillations. When $p > 0.5$ or $v_8 > 1$ or both are large enough, oscillations cannot occur. **c** Bifurcation diagram in the parameter space of v_1 and v_8 . For a larger v_8 , a larger v_1 is needed to produce oscillations. **d** Bifurcation diagram in the parameter space of v_5 and d

of oscillations could co-compensate the inefficiency in order to generate oscillations, i.e., they act synergistically to generate oscillations; and (iv) on the other hand, when $d < 0.62$, they act antagonistically to induce a cell-state transition. These results suggest that *Hes1* oscillates only within certain values of mRNA stability and the association. Values above or below certain ranges render oscillations unsustainable. They also suggest that *Hes1* mRNA degradation and the binding of two RNAs may act synergistically or antagonistically so as to alter cell-fate decisions although both of the two mechanisms control the stability of *Hes1* mRNA.

Similarly, to examine the relationship between the basal transcription rate p and the synthesis rate v_8 of pri-miR-9, we analyze the bifurcation set in the parameter space of p and v_8 . It is found that the functions of p and v_8 in generating oscillations are complementary, as shown in Fig. 8b. It seems that they play similar roles in generating oscillations. When p or $v_8 > 1$ or both are large enough, oscillations do not occur. These results are consistent with the experimental observation that miR-9 over-expression can lead to terminating of *Hes1* oscillations [11].

Besides the auto-repression of *Hes1*, *Hes1* mRNA and miR-9 also form a double-negative feedback loop. To further explore the synergistic mechanisms of these feedback loops, we perform bifurcation analysis in the parameter spaces of (v_1, v_8) and (v_5, d) . For a larger synthesis rate of pri-miR-9 v_8 , a larger synthesis rate of *Hes1* mRNA v_1 is needed to produce oscillations, as shown in Fig. 8c. A small synthesis rate of *Hes1* mRNA cannot keep cells in an oscillatory state. The function of high *Hes1* expression is to generate oscillations, while miR-9 acts as an inhibitor to regulate *Hes1*. The bifurcation diagram in the parameter space of v_5 and d shows combinatorial properties of the two negative regulations. They act synergistically to generate oscillations, as shown in Fig. 8d. In other words, *Hes1* oscillations can be realized by decreasing the association rate, d , or increasing the *Hes1* level by decreasing the *Hes1* cytoplasm-nucleus transportation rate v_5 , which further inhibits the production of pri-miR-9 and then decreases the miR-9 level. These results indicate that the balance between miR-9 and *Hes1* tunes the system dynamics and controls the time to differentiation.

To better understand the role of the different feedback loops shown in Fig. 1, we observe the alternation by abrogating them individually. We first remove H_N in Eq. (1) in order to delete the *Hes1* auto-repression and find that *Hes1* oscillations disappear though miR-9 still inhibits *Hes1* mRNA. When there is no *Hes1* binding to its own gene promoter, its expression is up-regulated and its oscillations are disrupted. These results suggest that inhibition by *Hes1* of its own transcription is necessary for *Hes1* oscillations. We then test the effects of miR-9 on the system dynamics. We let $d=0$ to denote a zero binding rate of miR-9 to *Hes1* mRNA so as to disrupt the association of the two RNAs. When the post-transcriptional regulation does not exist, *Hes1* oscillations always occur and the system cannot evolve toward a stable steady state, which indicates that miR-9 down-regulates *Hes1* by reducing the stability of *Hes1* mRNA and eventually induces cell differentiation. We also remove H_N in Eq. (5), which denotes the transcriptional repression of pri-miR-9 by the *Hes1* protein in the nucleus and study its effects on system dynamics. In the absence of inhibition by *Hes1*, pri-miR-9 is up-regulated and its oscillations almost cannot be observed although the entrainment of *Hes1* mRNA oscillates due to the co-degradation of *Hes1* mRNA and miR-9, as shown in Fig. 9. Knockout of *Hes1*-negative regulation to pri-miR-9 also leads to a significant increase in the level of mature miR-9. After a short increase, miR-9 eventually evolves toward an up-regulated level. The reason for the first increase of miR-9 may be the accumulation due to its low degradation rate and it becomes stable after a long run.

Overall, these findings show how the *Hes1* oscillations are controlled. *Hes1* auto-repression, its self-degradation, and its co-degradation with miR-9 are predominant components to

Fig. 9 Expression of miR-9 and pri-miR-9 with and without the inhibition of miR-9 by Hes1. *Red and green solid curves* represent the expression of miR-9 and pri-miR-9 in a normal system, respectively. *Red and green dashed curves* represent the expression of miR-9 and pri-miR-9 after removing the inhibition of pri-miR-9 by Hes1, respectively

generate, control, and terminate Hes1 oscillations. Hes1 and miR-9 combinatorially modulate the system dynamics, which is essential for cells to make fate decisions.

3.4 Parameter sensitivity analysis

It is important for biological systems to adapt to both internal and external variations and also react precisely to the strength of perturbations. Parameter sensitivity analysis illustrates how system behavior depends on parameter variations. In order to investigate the parameters crucial in impacting a whole system, we perform sensitivity analysis for all parameters. Here, we use a relative sensitivity analysis to describe the comparative effects of a steady regime. The relative sensitivity is defined as $S_R = \frac{ds^{ss}/s^{ss}}{dp/p}$ [36], which is frequently used to provide a description of model robustness characteristics. To perform sensitivity analysis, each parameter is increased 10% from its base value while other parameters are kept unchanged. We choose the basal transcription rate of pri-miR-9 $p=0.3$ at which the system is in the steady regime. The system is also stable when each parameter is increased. The relative changes in the steady states are shown in Fig. 10. Sensitivity analysis shows that the parameters can be divided into two groups. In fact, with one group of parameters, e.g., v_1 and K_1 , increasing them results in an increase in Hes1 and a decrease in miR-9, which can be observed in boundary regions of the central nervous system [5, 15]. Another group of parameters, e.g., v_8 and p , play opposite roles, which is important to determine the differentiated state. The effects of perturbing some parameters, e.g., K_7 and K_9 , are negligible, indicating a good robustness of the system. These results suggest the essential factors that determine the formation of non-oscillatory states of Hes1 after neural differentiation.

To study the essential factors influencing Hes1 oscillations, we performed a period sensitivity analysis. Each parameter is increased and decreased 10% from its base value, while other parameters are kept unchanged. In order to keep cells in oscillatory states, we set the value of the basal transcription rate of pri-miR-9, represented by p , equal to 0.03. The influences of each parameter to the period of oscillations are shown in Fig. 11. These observations indicate

Fig. 10 The relative sensitivity of each variable with parameter variations at $p=0.3$. Each column represents a parameter and each row represents a variable. Each parameter is increased 10% from its base value, while other parameters are kept unchanged

that the most essential factors influencing the period of oscillations are related to *Hes1* mRNA, as indicated in its synthesis rate v_1 and degradation rate v_2 . The two factors influence the period in an opposite way. An increase in v_1 makes the period of oscillations longer, which can maintain cells in a progenitor state. In addition, the *Hes1* self-inhibition constant, K_1 , is also sensitive and plays a similar role as v_1 . The maximum synthesis rate of pri-miR-9, v_8 , is also

Fig. 11 Dependence of the period on parameter variations at $p=0.03$. Each parameter is increased and decreased by 10% while other parameters are kept unchanged

sensitive to perturbations and an increase in v_8 makes the period of oscillations shorter, which is consistent with the crucial roles of miR-9 in modulating Hes1 oscillations. The system is insensitive to the perturbations of all other parameters. These results highlight the essential roles of Hes1 auto-regulation and miR-9 transcription in modulating oscillations.

4 Discussion

Recently, studies aimed at understanding the functions of biological circuits comprising positive and negative feedback loops have had promising results. There are many important models that explore the dynamics of complex systems, such as interlinked positive versus negative feedback loops [37] and paradoxical components [38]. Previous theoretical studies mainly focused on Hes1 but had a lack of knowledge in miRNA regulating Hes1. Our work provides theoretical analysis by integrating miR-9 into a Hes1 oscillator to form a double-negative feedback loop so as to provide a tunable mechanism governing progenitor maintenance and neural differentiation. Different from the delay differential equation model represented by Goodfellow [12], in which the changes in *Hes1* mRNA, Hes1 protein and miR-9 are tracked, while the pri-miR-9 is not incorporated, we developed an ordinary differential equation model that is relatively easier to analyze. In our model, we use ODEs and perform the analysis of the regulation both at transcriptional and post-transcriptional levels by involving pri-miR-9 and miR-9 to reveal possible mechanisms of experimental observations. We consider the effects of miR-9 on *Hes1* mRNA stability alone because the attenuation of protein translation by miR-9 is still not clear. In addition, the two mechanisms play similar roles in reducing Hes1 production and therefore we just consider the co-degradation of two RNAs. If it is necessary to study the attenuation of protein translation a similar analysis to Goodfellow [12] can be performed, which we omitted here. The principal finding in Goodfellow's work is that miR-9 has two major functions, one being to provide the Hes1 oscillator the ability to attain two stable states, either a high or a low and the other one being to measure lapsed time. However, there are still several questions that remain to be answered. The effects of combinatorial regulation in controlling cell states have been less investigated. The correlation of important regulations still needs to be examined. To further confirm the effects of miR-9 on Hes1 oscillations, we still need to observe the effects on the Hes1 expression level and its period. Here, we showed that both miR-9 and Hes1 are required to generate, control and terminate Hes1 oscillations.

Consistent with known properties of Hes1 and miR-9, the model shows that: (i) oscillations of Hes1 are mainly caused by Hes1 auto-repression and instability of its mRNA [6]; (ii) miR-9 can also regulate the stability of *Hes1* mRNA by base-pairing [17]. However, it is unclear how *Hes1* mRNA degradation and miR-9 are coordinated to result in an exit from oscillations; (iii) miR-9 and Hes1 are inversely related and miR-9 at a certain level is required for Hes1 oscillations [11]. Over-expression of miR-9 can disrupt and dampen Hes1 oscillations, which promotes neural differentiation, but it seems paradoxical for experimental findings to indicate that miR-9 knockdown can also dampen Hes1 oscillations. Besides the results obtained from the model, which are consistent with experimental observations, a number of predictions can also be made: (i) the self-degradation of *Hes1* mRNA and co-degradation of the two RNAs may act synergistically or antagonistically so as to alter cell-fate decisions, depending on the choices of parameter values, although both of them control the stability of *Hes1* mRNA; (ii) for the biological phenomena found in experiments that both over-

expression and down-regulation of miR-9 can dampen Hes1 oscillations, our model predicts that lack of miR-9 indeed makes the period of Hes1 oscillations longer but results in a slight increase in its expression, which gives reasonable explanations of the paradoxical experimental findings; (iii) since cell differentiation can be influenced by both intrinsic and extrinsic factors, modifying them can act in a number of ways. In order to observe the interaction between them, we modify them simultaneously to see how cell states are controlled by tuning intrinsic (synthesis rate and cytoplasm-nucleus transportation rate of Hes1) and extrinsic factors (synthesis rate of miR-9 and association rate of two RNAs). We find that the balance between Hes1 and miR-9 is essential for the transition from cell proliferation to neural differentiation. It gives cells the innate ability to regulate the time for differentiation and also endows cells with the elasticity to adapt to external perturbation; and (iv) sensitivity analysis for all parameters is performed to demonstrate their influences on neural fate decisions and make clear the most sensitive factors in regulating them.

Although the model given here is simple, it highlights the dynamics tuned by Hes1 and miR-9 during cell-fate progression. The model will be further improved as more information is available in the future.

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References

1. Kohwi, M., Doe, C.Q.: Temporal fate specification and neural progenitor competence during development. *Nat. Rev. Neurosci.* **14**, 823–838 (2013)
2. Shimojo, H., Ohtsuka, T., Kageyama, R.: Oscillations in notch signaling regulate maintenance of neural progenitors. *Neuron* **58**, 52–64 (2008)
3. Kabos, P., Kabosova, A., Neuman, T.: Blocking HES1 expression initiates GABAergic differentiation and induces the expression of p21(CIP1/WAF1) in human neural stem cells. *J. Biol. Chem.* **277**, 8763–8766 (2002)
4. Imayoshi, I., Isomura, A., Harima, Y., Kawaguchi, K., Kori, H., Miyachi, H., Fujiwara, T., Ishidate, F., Kageyama, R.: Oscillatory control of factors determining multipotency and fate in mouse neural progenitors. *Science* **342**, 1203–1208 (2013)
5. Baek, J.H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T., Kageyama, R.: Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. *Development* **133**, 2467–2476 (2006)
6. Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., Kageyama, R.: Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. *Science* **298**, 840–843 (2002)
7. Monk, N.A.: Oscillatory expression of Hes1, p53, and NF-kappaB driven by transcriptional time delays. *Curr. Biol.* **13**, 1409–1413 (2003)
8. Zeiser, S., Müller, J., Liebscher, V.: Modeling the Hes1 oscillator. *J. Comput. Biol.* **14**, 984–1000 (2007)
9. Momiji, H., Monk, N.A.: Oscillatory Notch-pathway activity in a delay model of neuronal differentiation. *Phys. Rev. E* **80**, 021930 (2009)
10. Wang, R., Liu, K., Chen, L., Aihara, K.: Neural fate decisions mediated by *trans*-activation and *cis*-inhibition in Notch signaling. *Bioinformatics* **27**, 3158–3165 (2011)
11. Bonev, B., Stanley, P., Papalopulu, N.: MicroRNA-9 Modulates Hes1 ultradian oscillations by forming a double-negative feedback loop. *Cell Rep.* **2**, 10–18 (2012)
12. Goodfellow, M., Phillips, N.E., Manning, C., Galla, T., Papalopulu, N.: microRNA input into a neural ultradian oscillator controls emergence and timing of alternative cell states. *Nat. Commun.* **5**, 3399 (2014)
13. Pillai, R.S.: MicroRNA function: multiple mechanisms for a tiny RNA? *RNA* **11**, 1753–1761 (2005)
14. Pasquinelli, A.E.: MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nat. Rev. Genet.* **13**, 271–282 (2012)

15. Coolen, M., Katz, S., Bally-Cuif, L.: miR-9: a versatile regulator of neurogenesis. *Front. Cell. Neurosci.* **7**, 220 (2013)
16. Zhao, C., Sun, G., Li, S., Shi, Y.: A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. *Nat. Struct. Mol. Biol.* **16**, 365–371 (2009)
17. Tan, S.L., Ohtsuka, T., González, A., Kageyama, R.: MicroRNA9 regulates neural stem cell differentiation by controlling Hes1 expression dynamics in the developing brain. *Genes Cells* **17**, 952–961 (2012)
18. Conaco, C., Otto, S., Han, J.J., Mandel, G.: Reciprocal actions of REST and a microRNA promote neuronal identity. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 2422–2427 (2006)
19. Bredenkamp, N., Seoighe, C., Illing, N.: Comparative evolutionary analysis of the FoxG1 transcription factor from diverse vertebrates identifies conserved recognition sites for microRNA regulation. *Dev. Genes Evol.* **217**, 227–233 (2007)
20. Shibata, M., Nakao, H., Kiyonari, H., Abe, T., Aizawa, S.: MicroRNA-9 regulates neurogenesis in mouse telencephalon by targeting multiple transcription factors. *J. Neurosci.* **31**, 3407–3422 (2011)
21. Widder, S., Schicho, J., Schuster, P.: Dynamic patterns of gene regulation I: simple two-gene systems. *J. Theor. Biol.* **246**, 395–419 (2007)
22. Uriu, K., Morishita, Y., Iwasa, Y.: Random cell movement promotes synchronization of the segmentation clock. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 4979–4984 (2010)
23. Zhou, W., Li, Y., Wang, X., Wu, L., Wang, Y.: MiR-206-mediated dynamic mechanism of the mammalian circadian clock. *BMC Syst. Biol.* **5**, 141 (2011)
24. Zhou, P.P., Cai, S.M., Liu, Z.R., Wang, R.Q.: Mechanisms generating bistability and oscillations in microRNA-mediated motifs. *Phys. Rev. E.* **85**, 041916 (2012)
25. Lai, X., Bhattacharya, A., Schmitz, U., Kunz, M., Vera, J., Wolkenhauer, O.: A systems' biology approach to study microRNA-mediated gene regulatory networks. *Biomed. Res. Int.* **2013**, 703849 (2013)
26. Kiparissides, A., Koutinas, M., Moss, T., Newman, J., Pistikopoulos, E.N., Mantalaris, A.: Modelling the Delta1/Notch1 pathway: in search of the mediator(s) of neural stem cell differentiation. *PLoS ONE* **6**, e14668 (2011)
27. Jensen, M.H., Sneppen, K., Tiana, G.: Sustained oscillations and time delays in gene expression of protein Hes1. *FEBS Lett.* **541**, 176–177 (2003)
28. Decroly, O., Goldbeter, A.: Birhythmicity, chaos, and other patterns of temporal self-organization in a multiply regulated biochemical system. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6917–6921 (1982)
29. Leloup, J.C., Goldbeter, A.: Chaos and birhythmicity in a model for circadian oscillations of the PER and TIM proteins in *Drosophila*. *J. Theor. Biol.* **198**, 445–459 (1999)
30. Pendergast, J.S., Niswender, K.D., Yamazaki, S.: The complex relationship between the light-entrainable and methamphetamine-sensitive circadian oscillators: evidence from behavioral studies of Period-mutant mice. *Eur. J. Neurosci.* **38**, 3044–3053 (2013)
31. Kobayashi, T., Mizuno, H., Imayoshi, I., Furusawa, C., Shirahige, K., Kageyama, R.: The cyclic gene Hes1 contributes to diverse differentiation responses of embryonic stem cells. *Genes Dev.* **23**, 1870–1875 (2009)
32. Hafizi, M., Atashi, A., Bakhshandeh, B., Kabiri, M., Nadri, S., Hosseini, R.H., Soleimani, M.: MicroRNAs as markers for neurally committed CD133+/CD34+ stem cells derived from human umbilical cord blood. *Biochem. Genet.* **51**, 175–188 (2013)
33. Annibali, D., Gioia, U., Savino, M., Laneve, P., Caffarelli, E., Nasi, S.: A new module in neural differentiation control: two microRNAs upregulated by retinoic acid, miR-9 and -103, target the differentiation inhibitor ID2. *PLoS ONE* **7**, e40269 (2012)
34. Indulekha, C.L., Divya, T.S., Divya, M.S., Sanalkumar, R., Rasheed, V.A., Dhanesh, S.B., Sebin, A., George, A., James, J.: Hes-1 regulates the excitatory fate of neural progenitors through modulation of Tlx3 (HOX11L2) expression. *Cell. Mol. Life Sci.* **69**, 611–627 (2012)
35. Maddodi, N., Bhat, K.M., Devi, S., Zhang, S.C., Setaluri, V.: Oncogenic BRAFV600E induces expression of neuronal differentiation marker MAP2 in melanoma cells by promoter demethylation and down-regulation of transcription repressor HES1. *J. Biol. Chem.* **285**, 242–254 (2010)
36. Wolf, J., Becker-Weimann, S., Heinrich, R.: Analysing the robustness of cellular rhythms. *Syst. Biol. (Stevenage)* **2**, 35–41 (2005)
37. Tian, X.J., Zhang, X.P., Liu, F., Wang, W.: Interlinking positive and negative feedback loops creates a tunable motif in gene regulatory networks. *Phys. Rev. E.* **80**, 011926 (2009)
38. Hart, Y., Alon, U.: The utility of paradoxical components in biological circuits. *Mol. Cell* **49**, 213–221 (2013)