mRNA-miRNA reciprocal regulation generates versatile dynamics

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Abstract

miRNAs serve as crucial post-transcriptional regulators of gene expression. Recent experimental studies report that an miRNA and its target mRNA reciprocally regulate each other and miRNA is recycled with a ratio upon degradation of mRNA-miRNA complex. The functionality of this mutual regulation and dynamic consequences of miRNA recycling are not fully understood. Here, we built a set of mathematical models of mRNA-miRNA interactions and systematically analyzed their dynamical responses under various conditions. First, we found that mRNA-miRNA reciprocal regulation manifests great versatility, such as subsensitive activation, ultrasensitive and subsensitive inhibition, depending on parameters such as the miRNA recycle ratio and the mRNA-miRNA complex degradation rate constant. Second, ultrasensitivity from reciprocal mRNA-miRNA regulation contributes to generation of bistability. Furthermore, the degree of ultrasensitivity is amplified by a stronger competing mRNA (ceRNA). Last, multiple miRNA binding sites on a target mRNA leads to emergence of nonmonotonic dual response (duality) and bistability even in the absence of any imposed feedback regulation. Thus, we demonstrated several novel functionalities that can be generated from simple mRNA-miRNA reciprocal regulation, in addition to canonical miRNA mediated degradation and translational repression of mRNA. Quantitative experiments are suggested to test the model predictions.
Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules containing about 22 nucleotides that exist ubiquitously in many living organisms for post-transcriptional regulation of gene expression. Growing studies reveal that miRNAs are essential in various essential processes such as pluripotency and reprogramming [1], epithelial-to-mesenchymal transition [2] and metastasis [3]. Dysregulation of miRNAs correlates with pathological conditions such as cancer development, cardiovascular and metabolic diseases. During the last decade, studies have been accumulated on the basic molecular mechanisms of miRNA biogenesis, function and degradation [4]. With recent quantitative measurements on miRNA dynamics using techniques such as quantitative fluorescence microscopy [5], it becomes a timely and urgent need to perform systematic mathematical analysis on the mRNA-miRNA mutual regulation and its effect on gene regulatory network dynamics.

Through base-pairing interactions, miRNA inhibits its target mRNA through two modes, translational repression and mRNA degradation [6]. The degree of sequence complementarity between miRNA and mRNA determines the mode of mRNA silencing [7]. Extensive complementarity, which are often formed in plants, induces cleavage and degradation of the target mRNA [8]. Partial complementarity, which occurs between the vast majority of miRNAs and their target mRNA in metazoans, results in translational repression or degradation [6]. In addition, under some circumstance, miRNA can stimulate translation of mRNA through an Argonaute/FMR1-mediated mechanism [10]. Overall miRNA-mediated regulation can establish a threshold level of target mRNA [5].

Reversely, mRNA targets reciprocally control the stability and function of miRNAs [10,12]. Target interaction can stabilize an miRNA by preventing its release from argonaute (Ago) and subsequent destabilization [13]. The miRNAs in a mRNA-miRNA complex may be either degraded together with the mRNA with extensive complementarity [14-16], or be recycled [17]. Furthermore, each miRNA may target tens or hundreds mRNAs [18,19], enabling cross-talk between competing endogenous RNAs (ceRNAs) targeted by the same miRNA [20]. Consequently, the reciprocal regulation between miRNAs and their targets adds a significant level of complexity to the mRNA-miRNA relationships.

There are two major classes of parameters characterizing the regulation dynamics between miRNA and the target mRNA. One is the thermodynamic standard free energy of binding between mRNAs
and miRNAs, $\Delta G^0$, which reflects the strength of the interactions. It is affected by the number of complementary base pairs formed and RNA folding free energy [21]. Another class of kinetic parameters includes the degradation rates of mRNA, miRNA and mRNA-miRNA complexes, mRNA translation rates, and the recycle ratios of miRNAs. We defined the latter as the recycle probability of miRNAs upon degradation of the complex mRNA-miRNA. Extensive efforts have been made to determine $\Delta G^0$, and several computational tools are available for in silico prediction [18]. Figure 1A shows the distribution of standard free energy of binding between miR-34a and 354 mRNAs calculated using PicTar [21]. Notably different mRNAs may have the same $\Delta G^0$. Figure 1B gives four such examples. These mRNAs, each has only one miR-34a binding site, form complexes with miR-34a with drastically different configurations and number of complementary base pairs. It is questionable that these mRNAs, even under the same condition such as concentrations of involved molecular species, undergo the same miR-34a mediated regulation kinetics.

In this work we use mathematical and computational analyses to show that both the thermodynamic $\Delta G^0$ and the less-discussed kinetic parameters determine the kinetics of mRNA-miRNA reciprocal regulation. We found that the reciprocal regulation between mRNA and miRNA is either ultrasensitive or subsensitive, and either inhibitive or protective. Furthermore, the degree of response sensitivity is amplified when a stronger competitor (ceRNA) is involved. Ultrasensitivity from the mRNA-miRNA reciprocal regulation contributes to bistability generation when it is equipped with a positive feedback loop. Alternatively, bistability can be generated from mRNA-miRNA reciprocal interaction when there are more than one miRNA binding sites on the target mRNA.
Model and Methods

Model of mRNA-miRNA reciprocal regulation

Figure 2A summarizes all possible scenarios of mRNA-miRNA reciprocal regulation. miRNAs either suppress an mRNA through translational repression or accelerated degradation, or activate an mRNA through stimulated translation. Reversely, an mRNA either suppresses an miRNA through accelerated degradation, or activates an miRNA through sequestering it from degradation. Figure 2B shows the corresponding kinetic schemes for an mRNA with $N$ miRNA binding sites. There are $C_N^i = N!/(i!(N-i)!)$ different complexes that consist of one mRNA and $i(\leq N)$ copies of miRNA.

Notice that the binding/unbinding events between miRNAs and mRNAs are typically much faster than other processes, such as transcription, translation and degradation, we assume that the binding/unbinding processes can be approximated to be in quasi-equilibrium. With no detailed information on cooperativity of mRNA-miRNA binding, we assume that each miRNA binds to the mRNA independently, and the binding free energy for each binding site is the same ($\Delta G^0$). Then each form of the mRNA-miRNA$_i$ complex ($R_i$) has the same level, denoted as $[R_i]$, and the total level of the mRNA-miRNA$_i$ complex is $C_N^i[R_i]$. Furthermore, the total levels of the miRNA and mRNA under study, $[miR]_t$ and $[mR]_t$, are given below, respectively,

$$[mR]_t = \sum_{i=1}^{N} C_N^i[R_i] + [mR],$$

$$[miR]_t = \sum_{i=1}^{N} iC_N^i[R_i] + [miR].$$

Under the quasi-equilibrium approximation, the following relationship exists between $[R_i]$ and $[R_{i-1}]$:

$$K[miR][R_{i-1}] = [R_i], \quad i = 1...N,$$

where $[miR]$ is the cellular level of the free miRNA under study, $[R_0]$ is defined as the mRNA concentration $[mR]$, and $K = \exp(-\Delta G^0)$ is the binding constant. Here $\Delta G^0$ is in the unit of $k_B T$, the product of Boltzmann’s constant and temperature. The degradation rate constant of $[R_i]$ is $d_{Ri}$,
Figure 2. Mathematical model setup. (A) Reported possible mRNA-miRNA reciprocal regulations. (B) Kinetic schemes for mRNA-miRNA interaction with multiple miRNA binding sites in the sequence of mRNA.

and the translation rate of the \([R_i]\) is \(k_{si}\). Upon degradation of the complex mRNA-miRNA, miRNA molecules can be recycled with a ratio \(\lambda_i\) (0 ≤ \(\lambda_i\) ≤ 1). Thus, the equations of total level of miRNA, mRNA and protein ([P]) are:

\[
\frac{d[miR]_i}{dt} = k_{miR} - d_{miR}[miR] - \sum_{i=1}^{N} (1 - \lambda_i) C_N^i d_{Ri}[R_i],
\]

(4)

\[
\frac{d[mR]_i}{dt} = k_{mR} - d_{mR}[mR] - \sum_{i=1}^{N} C_N^i d_{Ri}[R_i],
\]

(5)

\[
\frac{d[P]}{dt} = k_{s0}[mR] + \sum_{i=1}^{N} k_{si} C_N^i [R_i] - d_p[P].
\]

(6)

The parameters values are used as follows except specified. \(k_{miR} = 0.1, d_{miR} = 0.1, k_{mR} = 0.1, d_{mR} = 0.1, d_{Ri} = 1, k_{s0} = 0.1, k_{si} = 0.01, d_p = 0.01, K_i = 100, \lambda_i = 0.5\). Throughout this work all the variables and parameters are in arbitrary units.

**Sensitivity**

The steady-state response curve is often used to describe how the output of the system (O) depends on the input (I). To quantify the sensitivity of the system, we define the instantaneous sensitivity as the ratio of the fractional changes in response output (\(\Delta O/O\)) and stimulus input (\(\Delta I/I\),
\[ s(I) = \lim_{\Delta I \to 0} \frac{\Delta O/O}{\Delta I/I} = \frac{dO/O}{dI/I} = \frac{d\log(O)}{d\log(I)}. \]

Instantaneous sensitivity is also known as 'logarithmic gain' in biochemical systems theory \[22\] or as 'local sensitivity coefficient' in the local parameter sensitivity analysis \[23\]. The response is ultrasensitive if \(|s| > 1\), subsensitive if \(0 < |s| < 1\), desensitive if \(s = 0\) and linear if \(|s| = 1\). The sign of \(s\) indicates whether \(I\) inhibits or activates \(O\). In general, the instantaneous sensitivity \(s\) is not constant but depends on the input \((I)\). We denote the dependence of instantaneous sensitivity \(s\) on \(I\) as the instantaneous sensitivity curve, and the extremum of this curve as the maximum (in the sense of the absolute value) sensitivity \((s_m)\) of the specific I-O curve. Under this definition, the maximum sensitivity of a Hill function \(\left( \frac{I^n}{K_n + I^n} \right)\) is exactly the Hill coefficient \(n\). Thus, \(s_m\) can be used as the gauge of the degree of sensitivity. The steady-state response curves are performed with PyDSTool \[24\].

**Results**

**miRNA can either inhibit or promote protein production of target mRNA with varying sensitivity**

Given that miRNA and mRNA molecules reciprocally regulate each other, it is important to explore the interplay between them. Let's start with the case that an mRNA has only one binding site for the miRNA, and explore how the miRNA regulates the mRNA. Figure 3A shows that under mode of accelerated degradation of mRNA by miRNA \(d_{R1} > d_{mR}\), the protein product of the mRNA decreases with the miRNA synthesis rate constant \(k_{miR}\), reflecting inhibition of miRNA on mRNA. Most of the response curves are sigmoidal shaped with respect to \(k_{miR}\) in logarithmic scale, exhibiting a progression from weak inhibition that accelerates and approaches total inhibition with increase of \(k_{miR}\). The sharpness of the sigmoidal shape decreases and eventually disappears when the recycle ratio \(\lambda\) increases. Shape change of the response curves are also reflected by the corresponding instantaneous sensitivity curves (Fig. 3B), which are bell-shaped with an extremum in the middle, where the mRNA level is most sensitive to miRNA level change. The maximum sensitivity \(s_m\), which is negative for inhibition, increases with the recycle ratio \(\lambda\) (Fig. 3C). That is, the larger the recycle ratio, and so
the more efficient of miRNA, the less sensitive the inhibition of mRNA by miRNA is. Therefore, ultrasensitivity is generated by sacrificing the efficiency of inhibiting the mRNA. Notedly, when the recycle ratio is near 1, $|s_m|$ is less than 1. That is, regulation of mRNA by miRNA shows subsensitivity instead of ultrasensitivity when the miRNA is almost completely recycled. The threshold dynamics is more transparent in Fig. 3D. When plotted against the mRNA synthesis rate constant, $k_{mR}$, the protein level remains low until $k_{mR}$ exceeds a threshold value so that mRNA molecules escape from miRNA-mediated repression by titrating the miRNA in the system, and the threshold increases with $\lambda$. The threshold behavior is consistent with the quantitative measurements of Mukherji et al [5] (yellow circles in Fig. 3D).

Interestingly, under the mode of accelerated translation of mRNA by miRNA ($k_{s1} > k_{s0}$), which has also been experimentally observed [10], the same model predicts qualitatively different behavior. The protein level increases with $k_{miR}$ (Fig. 3E). The instantaneous sensitivity is again bell-shaped but assumes positive value that is less than 1 (Fig. 3F), indicating subsensitive activation. Its maximum, $s_m$ decrease with increase of the recycle ratio $\lambda$ (Fig. 3G).

Therefore, the sensitivity of miRNA regulated mRNA dynamics depends on the mechanistic details, which can be translational repression stimulation, degradation or of translation. Figure 3H shows the maximum sensitivity $s_m$ in the parameter space spanned by the degradation rate constant $k_{dR1}$ and translation rate constant $k_{s1}$ of the mRNA-miRNA complex. In the blue region located in right-bottom corner, $s_m < -1$, thus regulation of mRNA by miRNA shows different degree of ultrasensitivity. In the green region $-1 < s_m < 0$, thus only subsensitivity can be obtained. In the orange region, $s_m > 0$, the regulation of mRNA by miRNA shows subsensitive stimulation, which is largely from the stimulation of translation of mRNA by forming complex with miRNA. Taken together, the regulation of mRNA by miRNA can be ultrasensitive or subsensitive inhibition, and subsensitive stimulation.

Target mRNA can either inhibit or promote miRNA level with varying sensitivity

Similarly we explored the regulation of miRNA by mRNA. Figure 4A shows a typical result of dependence of the total miRNA level ($[miR]_t$) on the mRNA synthesis rate constant, $k_{mR}$. Depending on $\lambda$, the response curve of $[miR]_t$ on $k_{mR}$ shows different shapes. When $\lambda$ is small, $[miR]_t$ shows a threshold with respect to $k_{mR}$, above which miRNA is highly inhibited. With further increase of $\lambda$, the
Figure 3. Sensitivity analyses of the regulation of mRNA by miRNA, with one miRNA binding site on mRNA. (A) Dependence of the protein level on the miRNA synthesis rate constant $k_{\text{miR}}$ under different values of the recycle ratio $\lambda$. (B) Dependence of the instantaneous sensitivity on $k_{\text{miR}}$ under different values of $\lambda$. (C) Dependence of the maximum sensitivity $s_m$ of the $k_{\text{miR}}$-$[P]$ response curve on the value of $\lambda$. (D) Dependence of the protein level on the expression rate constant of mRNA $k_{\text{mR}}$ under different recycle ratio, also plotted are experimental data (yellow circles) from ref [5]. (E-G) Same as (A-C) but with different values of $k_s$ and $d_{R1}$ to achieve subsensitive activation of mRNA by miRNA. (H) The maximum sensitivity $s_m$ of the $k_{\text{miR}}$-$[P]$ response curve in the parameter space spanned by the mRNA-miRNA complex translation rate constant $k_{sR1}$ and the degradation rate constant $d_{R1}$. $k_s = 0.1$ and $d_{R1} = 0.5$ in (A)-(D); $k_s = 0.2$ and $d_{R1} = 0.1$ in (E)-(G).
extent of inhibition decreases, and eventually the curve bends upward, indicating promotion of miRNA by the target mRNA. The sensitivity curve again is bell-shaped with an extremum at the threshold value of \(k_{mR}\) (Fig. 4B). \(s_m\) increases with \(\lambda\), from \(s_m < -1\) (ultrasensitive inhibition), to \(-1 < s_m < 0\) (subsensitive inhibition), then to \(0 < s_m < 1\) (subsensitive activation) (Fig. 4C).

To systematically explore the three modes of regulation of miRNA by mRNA, Fig. 4D shows the maximum sensitivity \(s_m\) of the \(k_{mR^-[miR]t}\) curve in the space spanned by the degradation rate of mRNA-miRNA complex \(k_{dR1}\) and recycle ratio \(\lambda\). The space is divided into three regions, ultrasensitive inhibition, subsensitive inhibition and subsensitive protection. At \(\lambda = 0\), the system dynamics changes from protection to inhibition when \(d_{R1} = d_{miR}\). A larger \(\lambda\) allows stronger protection of miRNA from degradation through forming the mRNA-miRNA complex, and counteracts the effect of increased \(d_{R1}\).

One can reveal this dependence analytically. Notice that by setting \(d[miR]_t/dt = 0\) in Eqn. 4 one has the steady state expression \([miR]_t = k_{miR}/d_{miR} + [(d_{miR} - (1 - \lambda)d_{R1})/d_{miR}][R1]\). That is, the protection and inhibition regions are separated by \(d_{R1} = 1/\lambda d_{miR}\).

In summary, regulations between mRNA and miRNA are double edged, either activation or inhibition, and also show different level of sensitivity, either ultrasensitive or subsensitive. Various kinetic parameters, such as the recycle ratio of miRNA, affect the features of regulation.

**Nonlinearity from mRNA-miRNA reciprocal interaction equipped with a positive feedback loop can create bistable switch**

Positive feedback loops frequently exist in the regulation between mRNA and miRNA, such as miR-200/zeb, miR-34/snaIl and let-7/lin28 [25-28]. A positive feedback loop with an ultrasensitivitity arm can produce bistability [29], which plays essential roles in cell fate decision. We hypothesize that ultrasensitivity from the mRNA-miRNA reciprocal regulation contributes to the generation of bistability. Therefore we added an inhibition arm in which the protein product of mRNA inhibits the synthesis of miRNA, thus encloses a double-negative feedback loop (Fig. 5A). We multiplied an inhibitory Hill function \(\frac{K^n}{K^n+[P]^n}\) to \(k_{miR}\) in Eqn 4 to describe this inhibition of miRNA synthesis by the protein. If the Hill coefficient \(n \leq 1\), this inhibition arm is not ultrasensitive. Therefore in this model system the required nonlinearity can come either from protein inhibition on miRNA synthesis, or directly from the mRNA-miRNA mutual regulation.
Figure 4. Sensitivity analyses of the regulation of miRNA by mRNA, with one miRNA binding site on mRNA. (A) Dependence of total miRNA level on the mRNA synthesis rate constant $k_{mR}$ under different values of the recycle ratio $\lambda$. (B) Dependence of the instantaneous sensitivity on the miRNA synthesis rate constant $k_{mR}$ under different values of $\lambda$. (C) The maximum sensitivity $s_m$ of the $k_{mR}$-$[miR]_t$ response curve depends on $\lambda$. (D) The maximum sensitivity $s_m$ of the $k_{mR}$-$[miR]_t$ response curve in the space spanned by the mRNA-miRNA complex degradation rate constant $d_{R1}$ and $\lambda$.

First, when the Hill coefficient $n = 1$, the system can generate bistability. As shown in Fig. 5B, for a specific set of parameters the nullclines of $[mR]_t$ and $[miR]_t$ have three intersection points, two of which are stable steady states and the other is an unstable steady state. The bifurcation diagrams in Fig. 5C and D further show existence of bistable regions of the protein level while varying $k_{mR}$ or $k_{miR}$. The two-parameter bifurcation diagrams in Fig. 5E and F show how the parameter region of bistability changes over $n$ and $k_{miR}$, and over $n$ vs. $k_{mR}$, respectively. As expected, with other parameters fixed the value of $n$ needs to exceed a critical value to generate bistability. This critical value has cusp-shaped dependence on $k_{miR}$ and $k_{mR}$, which relates to the bell-shaped sensitivity curves in Fig. 3B and Fig. 4B. Notably this critical value of $n$ can be less than 1 for certain values of $k_{miR}$ and $k_{mR}$. That is, both the mRNA-miRNA mutual regulation and the protein regulation on miRNA synthesis contribute to the generation of bistability, as long as the composite nonlinearity exceeds a threshold value.

Based on results in Fig. 3 and Fig. 4 we hypothesize that the miRNA recycle ratio $\lambda$ also affects the critical value of $n$. Indeed Fig. 5G shows that the space of $\lambda$ and $n$ is divided into monostable and bistable regions. The smaller the value of $\lambda$, the smaller the $n$ that is required for generating bistability.
Figure 5. Bistability is generated with a composite ultrasensitivity from the mRNA-miRNA reciprocal regulation (with one miRNA binding site on mRNA) and a positive feedback loop. (A) A double negative feedback loop is formed by adding an inhibition arm in which protein inhibits the synthesis of miRNA. (B) Nullclines of the total levels of protein and miRNA. (C-D) One-parameter bifurcation diagrams of the protein level v.s. the mRNA synthesis rate constant $k_{mR}$, and v.s. the miRNA synthesis rate constant $k_{miR}$, respectively. (E-F) Two-parameter bifurcation diagrams for $k_{miR}$ v.s. $n$ and $k_{mR}$ v.s. $n$ respectively. (G) The bistability region in the space of Hill coefficient $n$ recycle ratio $\lambda$. $n = 1$ in (B)-(G).

This is consistent with the results that the maximum sensitivity ($s_m$) of the reciprocal regulation between miRNA and mRNA increases with the decrease of recycle ratio (Fig. 3C and Fig. 4C). When the recycle ratio is near 1, $n$ must be larger than 1 to generate bistability. That is, when miRNA is almost completely recycled, a larger nonlinearity from the other source is required to generate bistability. In general when the miRNA is not fully recycled, bistability can come from synergistic effect of the mRNA-miRNA reciprocal regulation and protein-miRNA transcriptional regulation. For example in our previous study shown that a double negative feedback loop between Snail1 and miR-34 functions as a bistable switch to control the transition of epithelial to partial EMT state. In this system snail1 mRNA has only one binding site for miR-34, and the nonlinearity mainly comes from Snail1 inhibition of miR-34 transcription (with $n = 2$) [28].
Competing endogenous RNAs amplify ultrasensitivity of mRNA response to miRNA regulation

In the above analyses, the miRNA has only one target mRNA. Actually, miRNA typically targets multiple mRNAs \[18,30\]. For example, miR-200 targets both zeb2 and pten \[31\]. To examine the effect of ceRNAs on the regulation of mRNA/Protein by miRNA, we expanded the model to include a ceRNA. As shown in Fig. 6A, without ceRNA, \[mR\] \(_t\) first decreases slowly with an increasing \(k_{mR}\), then the decrease accelerates after \(k_{mR}\) exceeds a threshold value so more miRNA molecules are available to effectively inhibit mRNA. The response curve of \[mR\] \(_t\) changes at the presence of ceRNA, and the change depends on the value of \(K_c\), the ceRNA-miRNA binding constant. When \(K_c \ll K_1\), ceRNA only manifests its effect on the level of \[mR\] \(_t\) at large \(k_{mR}\) when its concentration is sufficiently high to compete with the target mRNA. On the other hand, when \(K_c \gg K_1\), at small values of \(k_{mR}\) the target mRNA under interest is largely not affected by the miRNA inhibition since the ceRNA binds most of the miRNA molecules, then its amount drops suddenly after \(k_{mR}\) is sufficiently large so the miRNA molecules titrate out the ceRNA molecules. Consequently, the \(k_{mR}-[mR]_t\) response curve first decreases then increases its sharpness with \(K_c\), which is revealed by the \(s_{m}-K_c\) curve in Fig. 6B. That is, when the ceRNA-miRNA binding constant is small, ceRNA desensitize the regulation of the mRNA by miRNA. However, after the binding constant exceeds a threshold, ceRNA sensitizes the regulation.

The above modulation of sensitivity, however, depends on the recycle ratios \(\lambda\) and \(\lambda_c\). As shown in Fig. 6E, depending on \(K_c\), the pattern of \(s_m\) in the space of \(\lambda\) and \(\lambda_c\) changes qualitatively. With \(K_c < K_1\), for a fixed \(\lambda_c\), \(|s_m|\) decreases with increased value of \(\lambda\), consistent with that in Fig. 3. For a fixed \(\lambda\) though, \(|s_m|\) increases with \(\lambda_c\). Especially when \(\lambda_c \to 1\), the response becomes significantly more sensitive to miRNA inhibition. With \(K_c = K_1\), \(s_m\) shows a linear anti-correlated dependence on \(\lambda\) and \(\lambda_c\). This linear dependence turns out to be specific only for using the same parameter sets for the mRNA and the ceRNA except the recycle ratios, otherwise the results would be qualitatively similar to the case of \(K_c < K_1\) or \(K_c > K_1\). With \(K_c > K_1\), the qualitative feature of the response is roughly the opposite of that with \(K_c < K_1\), while the absolute value of \(s_m\) is much bigger. For a fixed \(\lambda_c\), \(|s_m|\) initially decreases monotonically with an increasing value of \(\lambda\), then has an extremum at an intermediate value of \(\lambda\) when \(\lambda_c \to 1\). For a fixed \(\lambda\), \(|s_m|\) instead decreases with \(\lambda_c\).
its difficulty to perform simple mathematical analyses, these results demonstrate that presence of a ceRNA further complicates the dynamic behavior of the mRNA-miRNA regulation.

Intuitively, a ceRNA provides certain protection of the target mRNA from miRNA inhibition through competitive binding of the latter. Indeed Fig. 6D shows that \([mR]_t\) increases with the ceRNA synthesis rate constant \(k_mRc\), and shows positive sensitivity. Figure 6E shows that the sensitivity of this curve increases with \(K_c\). Regulation of mRNA by ceRNA is subsensitive when ceRNA binding is weak (compared to \(K_1\)), and ultrasensitive when ceRNA binding is strong. The response curve sensitivity also depends on the recycle ratio \(\lambda\). As shown in Fig. S1A-B, \(s_m(k_mRc-[mR]_t)\) increase with \(\lambda\) and \(K_c\). Interesting, the value of \(s_m\) is independent of \(\lambda_c\) although the threshold position does increase (Fig. S1C-D). Therefore, mRNAs can regulate each other indirectly by acting as a ceRNA of the other.

This miRNA-mediated crosstalk tunes the sensitivity of mRNA-miRNA regulation. Several studies exist on the molecular determinants of effective ceRNA crosstalks \([32–36]\), such as miRNA/ceRNA ratio, numbers of total and shared miRNA response elements, and target binding affinity. Here, we propose that the most effective ceRNA regulation occurs at the point of the maximum sensitivity. Take together, ceRNA contributes ultrasensitivity in two ways, either through amplifying the sensitivity of miRNA-mediated regulation of mRNA, or through regulating mRNA though common miRNA mediated crosstalks.

Multiple miRNA binding sites on target mRNA leads to ultrasensitivity and bistability

In the above analyses we modeled mRNAs with one miRNA binding site. Some mRNAs have multiple miRNA binding sites. For example, miR-200 can target to zeb mRNA on 5-6 highly conserved binding sites \([25]\). Thus, we systematically examined how the existence of two miRNA binding sites affect the mRNA-miRNA mutual regulation.

Since there are two binding sites, there are two groups of possible mRNA-miRNA complexes, either with one miRNA bound \((R_1)\) or two miRNAs bound \((R_2)\) on mRNA. We denoted the recycle ratios of two mRNA-miRNA complexes as \(\lambda_1\) and \(\lambda_2\), respectively. Figure 7A shows how the total mRNA level depends on the miRNA synthesis rate \(k_{miR}\) under different combinations of \(\lambda_1\) and \(\lambda_2\). Similar to the case of one binding site, the \(k_{miR}-[mR]_t\) curve also shows ultrasensitivity and subsensitivity (cyan
**Figure 6.** Competitions between different mRNAs for a common type of miRNA affects the mRNA sensitivity to miRNA inhibition. Each mRNA is modeled with one miRNA binding site. (A) Response curves of the mRNA total level ($[mR]_t$) on the miRNA synthesis rate constant $k_{miR}$, under different values of the ceRNA-miRNA binding constant. The dashed line is the case without ceRNA. (B) The maximum sensitivity $s_m$ of the $k_{miR}$-$[mR]_t$ response curve depends on the ceRNA-miRNA binding constant. (C) Dependence of the maximum sensitivity $s_m$ of the $k_{miR}$-$[mR]_t$ response curve on recycle ratios of the miRNA upon degradation of mRNA-miRNA complex ($\lambda$) and the ceRNA-miRNA complex ($\lambda_c$) under different ceRNA-miRNA binding constant. (D) Response curves of the mRNA total level ($[mR]_t$) on the ceRNA synthesis rate constant, $k_{mRc}$. (E) The maximum sensitivity $s_m$ of the $k_{mRc}$-$[mR]_t$ curve depends on the ceRNA-miRNA binding constant. $\lambda_c = 0.5$, $k_{mRc} = 0.1$, $d_{mRc} = 0.1$, $k_{mRc1} = 1.0$. 
line and magenta lines respectively in Fig. 7A). More interestingly, different from the response curve with one binding site, bistability occurs under appropriate combination of $\lambda_1$ and $\lambda_2$ (black line in Fig. 7A). To the best of our knowledge, there is no previous report on the bistability that results from the mutual regulation between miRNA and mRNA without any feedback loop. Future experiments can test this novel prediction by fine tuning the two recycle ratios.

To further explore how to generate specific response by tuning the recycle ratios, we locate bistable, subsensitive and ultrasensitive domains in the space of $\lambda_1$ and $\lambda_2$. As shown in Fig. 7B, when $\lambda_1$ is much larger than $\lambda_2$ (magenta star region), $-1 < s_m < 0$, thus the $k_\text{miR} - [m_R]_t$ curve shows subsensitivity. In the cyan region, $\lambda_1 \sim \lambda_2$, $s_m < -1$, thus ultrasensitivity is obtained. When $\lambda_1$ is much smaller than $\lambda_2$ (black circle region), bistability is generated. The underlying reason is that formation of mRNA-miRNA$_2$ protects miRNA under large $\lambda_2$, while an increased level of miRNA promotes formation of mRNA-miRNA then mRNA-miRNA$_2$ with ultrasensitivity under smaller recycle ratio $\lambda_1$ (referring Fig. 4). This is equivalent to an inherent positive feedback loop with ultrasensitivity, meeting the requirement of bistability [29]. The reason why bistability can not be generated from one-binding site model is that the protective effect and ultrasensitivity can not coexist simultaneously with one recycle ratio.

While the regulation of mRNA by miRNA with two binding sites shows three regions, the regulation of miRNA by mRNA shows five different behaviors (Fig. 7C). Three of them, inhibitory subsensitivity (magenta line), inhibitory ultrasensitivity (cyan line) and protective subsensitivity (green line) are also found in the case with one binding site. Two additional novel regions arise: duality, which is defined as the dual capacity of miRNA to both inhibit and activate mRNA (blue line), and bistability (black line). While duality has not been reported experimentally, we predict that the same type of mRNA inhibits a miRNA under low mRNA level, and protects it under high mRNA level. Previous study shows that an incoherent feed-forward loop leads to this nonmonotonic dose-dependence [37]. Here we demonstrated that it can be generated by another regulatory motif that involves mRNA-miRNA mutual regulation with more than one binding sites.

Figure 7D shows that the $\lambda_1$-$\lambda_2$ space divides into five regions based on the value of $s_m(k_\text{miR} - [mi_R]_t)$. Not surprisingly, the bistable region (black circle) is the same as Fig. 7B. While the subsensitivity (magenta stars) and ultrasensitivity (cyan stars) regions shrink their sizes, the duality region (blue
Figure 7. Ultrasensitivity and bistability from multiple miRNA binding sites on one mRNA molecule. (A) Response curves of $[mR]_t$ on the miRNA synthesis rate constant $k_{miR}$ under different combinations of the miRNA recycle ratios upon degradation of complex mRNA-miRNA ($\lambda_1$) and mRNA-miRNA$_2$ ($\lambda_2$). Dotted line denotes unstable states. (B) The maximum sensitivity $s_m(k_{miR}-[mR]_t)$ in the space of $\lambda_1$ and $\lambda_2$. The space is divided into bistable (black squares), subsensitive (Magenta stars) and ultrasensitive (cyan stars) regions. (C) The response curves of $[miR]_t$ on the mRNA synthesis rate constant $k_{mR}$ under different combinations of $\lambda_1$ and $\lambda_2$. (D) The maximum sensitivity $s_m(k_{mR}-[miR]_t)$ in the space of $\lambda_1$ and $\lambda_2$. The space is divided into six regions, bistable (black circles), subsensitive (Magenta stars), ultrasensitive (cyan stars), duality (dual capacity of miRNA to both inhibit and activate mRNA, blue squares) and protection (green diamonds). $d_{mR2} = 1$ and $K_2 = 100$.

squares) emerges and takes over most area of the right-bottom corner. The protective subsensitivity (green diamonds) region resides where both $\lambda_1$ and $\lambda_2$ are large. Taken together, depending on the recycle ratios, mRNA-miRNA mutual regulation can generate ultrasensitivity, duality, and bistability when two binding sites are present.

In summary, the reciprocal regulation between mRNA and miRNA are more versatile with two binding sites than that with one binding site. It is interesting to generalize similar analysis on the mRNA-miRNA systems with more than two binding sites, such as miR-200/zeb system in which 5-6 binding sites are highly conserved [25].

Discussion

It is generally suggested that a regulatory miRNA serve as rheostats to fine-tune the expression of its targets to accommodate cell responses [38, 39]. Here, we systematically analyzed mutual regulation
between miRNA and mRNA with a class of basic mathematical models. To our surprise, we found that this reciprocal regulation gives rise to rich dynamic features, which may play important roles in regulating cellular processes such as cell phenotype change and maintenance.

**mRNA-miRNA regulation provides a new mechanism for ultrasensitivity and bistability**

The dynamics of a biological system is typically nonlinear. The nonlinearity results from a large variety of sources \[40,41\], such as cooperativity \[42\], homo-multimerization \[43\], zero-order ultrasensitivity \[44\], multisite phosphorylation \[45,46\], substrate competition \[47\], lateral interaction \[48\] and molecular titration \[49\]. Bistable and multi-stable switches are often involved in cell fates decision in biological systems \[50,51\]. Reports showed that miRNA regulation of mRNAs contributes to the robustness of biological system when considering feedback or feedforward loops \[52–54\]. Indeed, our analyses showed that miRNA/mRNA reciprocal regulation is also a source of nonlinearity. It shows ultrasensitive and subsensitive inhibition, and subsensitive protection. This nonlinearity can contribute to the generation of bistable switch and cell fate decision in a biological system.

Positive feedback and ultrasensitivity are two prerequisites for bistability. Here we showed that bistability is generated when ultrasensitivity from the mRNA-miRNA reciprocal regulation is equipped with a positive feedback loop. Furthermore, bistability also can be generated from mRNA-miRNA reciprocal regulation when more than one miRNA binding sites exist in the absence of any imposed feedback regulation. This result is analogous to the bistability from multisite phosphorylation \[55,56\]. However, the underlying mechanisms of bistability are different for multisite mRNA-miRNA reciprocal regulation and multisite phosphorylation. The former results from coexistence of ultrasensitivity and miRNA protection, while the later arises from substrate saturation and competitive inhibition \[55\].

**mRNA-miRNA regulation provides mechanism for pathway crosstalks**

Since there are typically multiple targets for a single miRNA, mRNAs can cross-regulate each other by competing for the shared miRNAs \[31,32,35\]. Thus, miRNAs link individual signaling pathways and form an intertwined ceRNA network (ceRNETs) \[57\]. Taking the ceRNETs into account makes the overall regulatory network more complex and leads us to rethink our view of the design principles.
of biological networks. In addition, design of miRNA sponges should take into consideration of the potential effect of ceRNAs on a gene regulation network with miRNA involved. Here, we found that a larger degree of ultrasensitivity can be achieved when a stronger ceRNA is involved. Thus, one can take advantage of this feature to design more effective miRNA sponges.

**Thermodynamic and kinetic parameters control dynamic features of mRNA-miRNA regulation**

The versatile dynamics of mRNA-miRNA regulation results from interplays among a number of thermodynamic and kinetic parameters. For example, in this work we discussed how varying the miRNA recycle ratio alone can qualitatively change the regulation dynamics. It is already shown that miRNAs can shield from exonucleolytic degradation through interacting with its target [13, 58]. The underlying molecular mechanism is not well understood. It is suggested that target mRNA keeps miRNA bound with the Argonaute protein, protecting it from degradation [13]. Furthermore, it is implied that the extent of protection is positively correlated with the number of available target sites [10, 58]. Here, we showed how this molecular-level detail manifests itself in the context of the network-level mRNA-miRNA regulation dynamics.

**Quantitative measurements can test model predictions**

It is surprising that a simple mRNA-miRNA motif, with only one or two miRNA binding sites and possibly presence of just one ceRNA, can generate such diverse dynamics by varying a few controlling parameters. Further studies may likely reveal even richer dynamics when the module is placed in the context of the global network dynamics and cell regulation, where multiple RNAs with different numbers of miRNA binding sites compete for common miRNAs. Given the varying copy numbers of both miRNAs and mRNAs [30], the consequence of stochasticity, which is not discussed here, is another important topic to be explored. An open question is whether cells have explored and utilized all these theoretical possibilities, or functional requirements have converged the parameters to specific regions of the multi-dimensional parameter space.

To address the above question, it is necessary to have quantitative characterization of both thermodynamic and kinetic parameters of mRNA-miRNA regulation. The task is challenging but has
been carried out at certain extent. For example, it has been experimentally shown that miRNA is multiple-turnover, enabling several rounds of target recognition and cleavage per miRNA [17, 59, 60]. Mathematically, the number of the round of target recognition and cleavage per miRNA, \( N_{\text{round}} \), is related to the recycle ratio \( \lambda \), satisfying \( N_{\text{round}} = 1/(1 - \lambda) \). Therefore one can estimate \( \lambda \) from the measured \( N_{\text{round}} \). For example, each human let-7-containing complex directs on average 10 rounds of mRNA cleavage [59], while each human miR-233 molecule regulates at least 2 target mRNA molecules [17], then the corresponding values of \( \lambda \) are 0.9, and 0.5, respectively. Haley and Zamore showed that a let-7 siRNA-directed ribonucleoprotein complex catalyzes more than 50 target RNA cleavages [60], i.e., \( \lambda > 0.98 \). The extent of the complementary between miRNA and mRNA may also provide a clue on the value of \( \lambda \). That is, the free energy of mRNA-miRNA binding is another factor to control the recycle ratio. Then one can design a specific miRNA with certain recycle ratio according to the degree of complementary to achieve desired sensitivity. Combined computational structural modeling and experimental efforts may further accelerate the process.

The mRNA-miRNA regulation dynamics needs to be explored at the level of network dynamics. Mukherji et al. showed experimentally the ultrasensitivity of miRNA-mediated regulation of mRNA [5], which results from molecular titration [49]. Similar quantitative measurements, together with synthetic designs that can modify various thermodynamic and kinetic parameters, can test the predictions made in this work, such as the tunability of ultrasensitivity by controlling the recycle ratio.

In summary, mRNA-miRNA mutual regulation can generate versatile dynamics. Detailed understanding of the mechanistic details and functional roles of the regulation require concerted efforts from both quantitative measurements and computational analyses at both molecular and system levels. As mRNA-miRNA regulation prevalently exists in various biological systems and some of them function as a 'hub' to coordinately regulates others [61], the versatile effects of mRNA-miRNA reciprocal regulation is evolved to adapt to specific biological context and is robust to intrinsic and extrinsic noise. Thus, understanding the functionalities of mRNA-miRNA in the biological system is critical for learning its underlying design principle.
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Figure S1. Dependence of the sensitivity of ceRNA-mediated regulation of mRNA on the recycle ratios $\lambda$ and $\lambda_c$ under different values of the ceRNA-miRNA binding constant $K_c$. (A) Response curves of the protein level on the ceRNA synthesis rate constant ($k_{mRc}$) and (B) the corresponding instantaneous sensitivity with different values of the recycle ratio $\lambda$ under $K_c = 10$ (left), $K_c = 100$ (middle) and $K_c = 1000$ (right). (C-D) Similar to A and B, except with different values of $\lambda_c$. 