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SURVEY AND SUMMARY

Understanding microRNA-mediated gene regulatory networks through mathematical modelling

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ABSTRACT

The discovery of microRNAs (miRNAs) has added a new player to the regulation of gene expression. With the increasing number of molecular species involved in gene regulatory networks, it is hard to obtain an intuitive understanding of network dynamics. Mathematical modelling can help dissecting the role of miRNAs in gene regulatory networks, and we shall here review the most recent developments that utilise different mathematical modelling approaches to provide quantitative insights into the function of miRNAs in the regulation of gene expression. Key miRNA regulation features that have been elucidated via modelling include: (i) the role of miRNA-mediated feedback and feedforward loops in fine-tuning of gene expression; (ii) the miRNA–target interaction properties determining the effectiveness of miRNA-mediated gene repression; and (iii) the competition for shared miRNAs leading to the cross-regulation of genes. However, there is still lack of mechanistic understanding of many other properties of miRNA regulation like unconventional miRNA–target interactions, miRNA regulation at different sub-cellular locations and functional miRNA variant, which will need future modelling efforts to deal with. This review provides an overview of recent developments and challenges in this field.

INTRODUCTION

MicroRNAs (miRNAs) are a class of small endogenous non-coding RNAs (ncRNAs) with a length of ∼22 nt (1,2). MiRNAs function as evolutionarily conserved post-transcriptional gene regulators that, in most cases, decrease the stability or inhibit translation of messenger RNAs (mRNAs) through binding to complementary sequences. These sequences are found in different regions of mRNAs, mainly in their three prime untranslated regions (3′-UTRs; (3)), and also in their 5'-UTRs (4) and coding sequences (5). In addition to their well-studied repressive function, miRNAs can act in a context-dependent fashion to increase translation of targets by both transcriptional and post-transcriptional mechanisms (6). So far, 2588 mature miRNAs have been identified in humans, and the genome location, sequence and annotation of these transcripts can be found in the public data repository miRBase v21 (7). Estimates based on computational and experimental analyses suggest that more than half of protein-coding genes are targets of miRNAs in *Homo sapiens* (8). In addition, recent experimental studies have shown that miRNAs can also interact with long ncRNAs (9). The broad interaction of miRNAs with other molecular species indicates their pervasive roles in the regulation of key cellular processes, including proliferation, differentiation and apoptosis (10,11). In addition to exerting critical function during normal development and cellular homeostasis, miRNAs have been found deregulated in many multifactorial and highly prevalent human diseases such as cancer (12–15).

Computational methods that utilise the canonical seed-match model, evolutionary conservation, miRNA–target binding energy as well as miRNA and mRNA expression data have been developed to identify putative miRNA targets. This has fostered the discovery and experimental validation of miRNA targets. The implementation and application of these methods have already been reviewed and discussed elsewhere (16–18). Despite the relative ease in identification of putative miRNA–target interactions using com-

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putational algorithms, experimentation is essential to identify *bona fide* miRNA targets. Analyses using sequencing technologies, such as high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS–CLIP also known as CLIP-seq), can provide a transcriptome-wide view of miRNA–target interactions (19). The database starBase is established for identifying miRNA targets from large scale CLIP-seq data (20).

On the other hand, under the systems biology paradigm the integration of quantitative experimental data with mathematical modelling has been used to investigate the regulation of gene expression by miRNAs as dynamical systems. The key idea is that miRNA regulation embedded in gene regulatory networks can be represented with mathematical models that encode molecular species and interactions that make up these networks. The general procedure for creating mathematical models accounting for miRNA-mediated gene regulatory networks includes four key steps (21). Firstly, a miRNA-mediated gene regulatory network can be reconstructed by establishing molecular interactions, such as miRNA–target interactions and the interactions between miRNAs and their transcriptional factors (TFs). Secondly, the network can be translated into a mathematical model using a particular framework, such as ordinary differential equations (**ODEs**) that can be used to describe biochemical reactions that make up the network. Thirdly, model parameter values can be characterised using information from the literature, databases and/or estimated by fitting model simulations to experimental data. Finally, the model can be used to study properties and behaviours of the dynamic system represented by the regulatory network. The available tools for constructing and simulating such kind of models have been reviewed and summarised by Alves *et al.* (22). Data-driven modelling provides the means for integrating quantitative data into the model equations, thereby making the model a tool for predicting the features of miRNA regulation in these networks (23–25). Mathematical modelling has proven to be useful at elucidating the fine-tuning of biological processes underlying cell and tissue function both at temporal and spatial resolution (26). It has also been used to develop hypothesis on the structure and regulation of biochemical networks, to integrate multiple sources of quantitative data into a coherent analysis framework, or to pave the way towards biomarker discovery, a new drug or a novel therapy (24,25,27–29).

We shall here focus on a review of those studies that make use of mathematical modelling to describe the molecular activity and biological function of miRNAs in the context of gene regulatory networks. These studies illustrate how mathematical modelling can advance our understanding of miRNA function at both cellular and disease levels. This review article includes four sections. In the first section, we show mathematical modelling helps to unravel the role of miRNA-mediated network motifs, such as feedback loops (FBLs), feedforward loops (FFLs) and target hubs, in fine-tuning gene expression. In the second section, we discuss the quantitative description of molecular mechanisms underlying miRNA-mediated gene regulation through mathematical modelling. In the third section, we demonstrate the utility of mathematical modelling in elaborating the role that miRNA played in determining the cross-regulation of competing endogenous RNAs (ceRNAs). In the last section, we enumerate modelling studies that characterise the role miRNAs in orchestrating gene regulatory networks that are essential to the initiation, progression and treatment of cancer.

MiRNA-mediated network motifs fine-tune gene expression

Network motifs are small recurring regulatory circuits embedded in complex gene regulatory networks (30). The small network motif composed by two interacting components can induce complex regulatory patterns, which are critical for the emergence of given phenotypes (30). Intracellular networks are specially enriched by network motifs integrating TFs and their targets, and these motifs are well known to enable regulatory features like homeostasis, oscillatory behaviour and all-or-nothing gene expression pattern (31). In recent times, it has been found that miRNAs can play a role in these circuits, and they act either as a targets or repressors of TFs (32). The involvement of miRNAs in TF network motifs adds an additional layer of complexity by providing target-specific repression mechanisms at the post-transcriptional level, thus allowing unique features for these TF-miRNA motifs. For example, in comparison to TFs, miRNAs can quickly turn off or resume protein translation by binding to or disassociating from an already transcribed mRNA, thus leading to rapid and adaptive changes in gene expression (33). The evolutionary advantage of combining TF and miRNA target regulation in gene circuits is still an open debate, but one promising hypothesis is that the combination of miRNA- and TF-mediated gene regulation allows for defining tightly controlled gene expression programs at both temporal and spatial scales (33). In addition, these circuits are crucial for controlling cell fate, including cell proliferation and apoptosis (34). For example, cell differentiation can be associated with the existence of miRNA-mediated positive FBLs governing the occurrence of **bistability**, a sophisticated regulatory condition in which the network switches to a new state upon a transient perturbation (Figure 1). These complex, non-linear dynamical properties such as bistability can only be fully understood by integrating experimental data into mathematical modelling and analysing the properties of the network motifs using tools and methods from theoretical biology. In the following, we show some remarkable examples that integrate mathematical modelling with experimental data to advance our understanding of the dynamics and regulation of network motifs involving miRNAs (35,36).

Nested TF-miRNA feedback loops govern cell cycle. In recent literature, an increasing number of TF-miRNA circuits have been identified to have the structure of miRNA-mediated FBLs. In these circuits, a TF positively or negatively regulates the expression of a miRNA, which subsequently suppresses the TF in a post-transcriptional manner (Table 1). These kinds of FBLs can give rise to bistability in gene expression (31), and they can also confer robustness to biological processes by resisting intrinsic and extrinsic noise (37–39). Intrinsic noise stems from the stochasticity of transcription, translation and decay of molecular species (40), while extrinsic one refers to fluctuations propagating

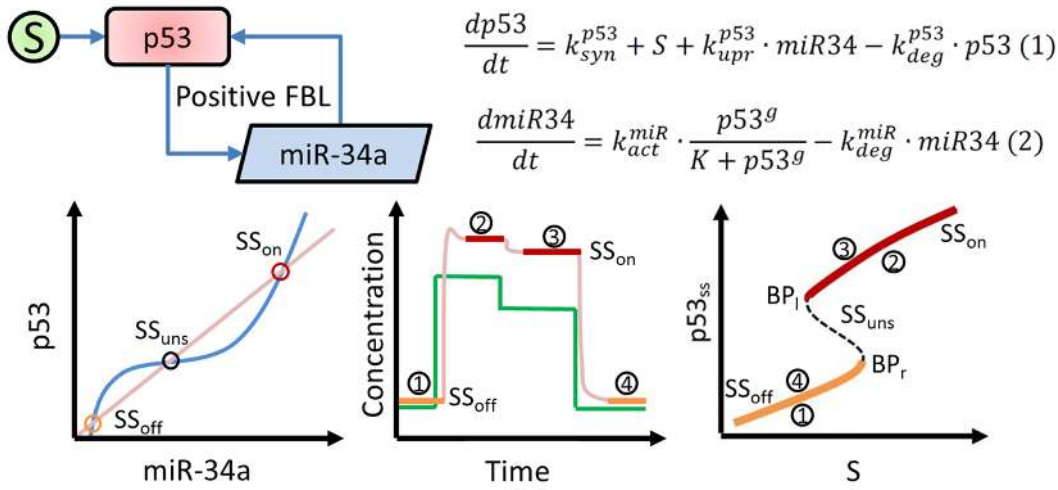


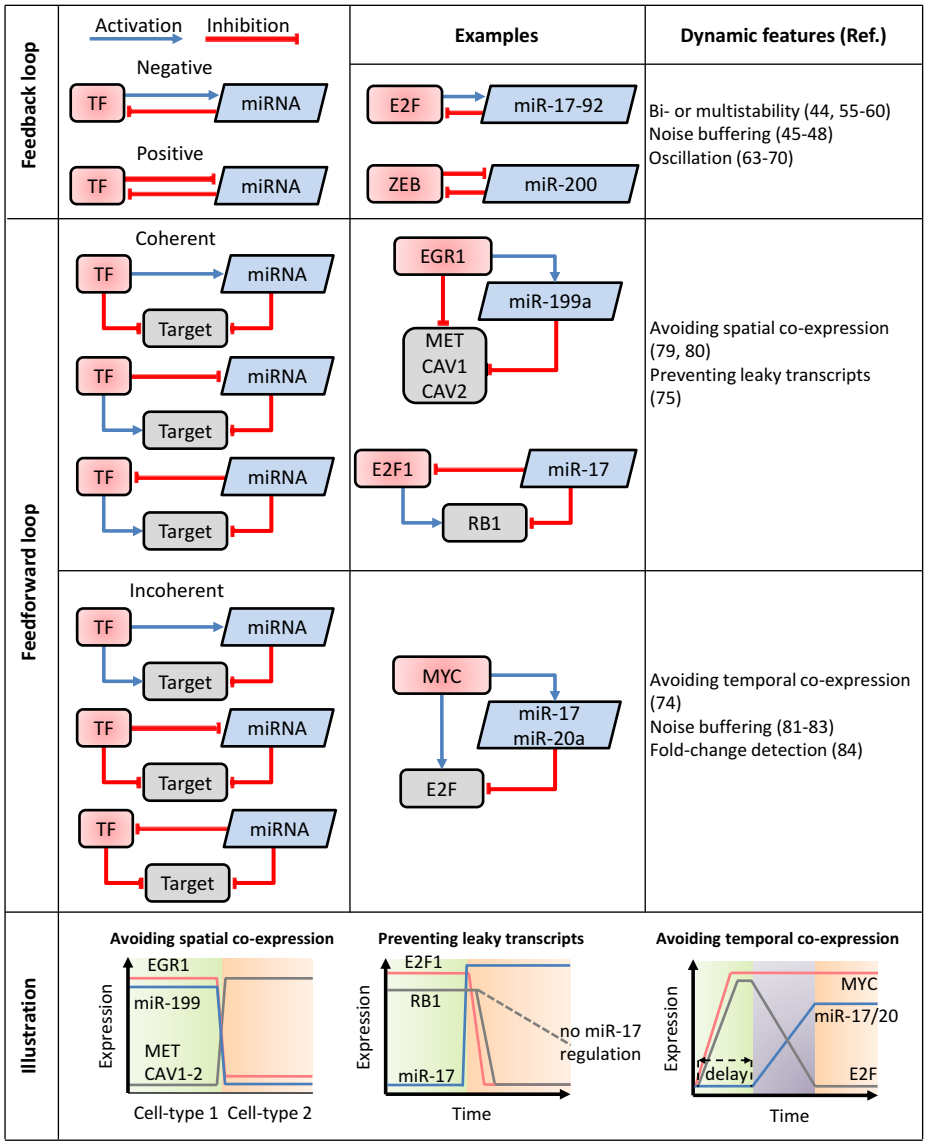
Figure 1. Bistability in miRNA-mediated feedback loops. Here, we used a model that accounts for a positive FBL composed of the TF p53 and miR-34a to explain bistability in p53 steady states. In the FBL, p53 upregulates the transcription of miR-34a, and in turn the miRNA indirectly upregulates p53 expression via repressing SIRT1, a negative regulator of p53 (36). We also included upstream signals (S) such as DNA damage signalling that can upregulate p53 expression. In Equation 1, the four terms correspond to the synthesis of p53, the upregulation of p53 by upstream signals, the upregulation of p53 by miR-34a and the degradation of p53. In Equation 2, the Hill function represents the transcriptional activation of miR-34 by p53 and the second term corresponds to the degradation of the miRNA. To identify bistability, we drew the trajectories of p53 (the red line) and miR-34a (the blue line) at their equilibrium states (i.e. $dp53/dt = 0$ and $dmiR34/dt = 0$). We obtained three intersections (the circles) of the trajectories that stand for three steady states of p53. One of them is unstable (the black circle; S_{uns}), and the other two are stable, corresponding to 'on' (the red circle; S_{on}) and 'off' (the orange circle; S_{off}) steady states of p53, respectively. Biologically, the 'off' steady state of p53 can be associated with cell proliferation, and the 'on' steady state can be associated with cell cycle arrest as a result of sudden upregulation of p53 expression by DNA damage signalling. The middle plot shows the evolution of p53 (the red line) and S (the green line) over time, and p53 can rest in S_{off} or S_{on} depending on the intensity of S. The bifurcation plot shows different steady states of p53 ($p53_{ss}$) against different intensities of S. The intersections of the stable steady states and unstable ones represent bifurcation points (BP_l and BP_r). When the value of S crosses these points, the steady state of p53 switches between the two stable states (the solid lines) but cannot stay on the unstable one (the dashed line). The numbers correspond to the steady states of p53 as shown in the middle plot. Similarly, bistability can also be found in oscillatory behaviours: stable oscillation attracts neighbouring oscillations of a model variable, and unstable one drives them away. More examples of bistability were reviewed by Tyson and Novák (31), and for fundamental mathematical explanation, the interested reader is referred to (35).

from external factors (e.g. environment) to gene regulatory networks (41).

A remarkable case of multiple TF-miRNA FBLs appears in the regulation of the E2F family, which is involved in the regulation of cancer-associated phenotypes like malignant proliferation, apoptosis evasion, angiogenesis and chemoresistance (42). The E2F activity can be regulated by multiple miRNAs adding a new layer to the regulation of the intricate E2F network (42). A well-known case is the regulation of E2F family by the miR-17-92 cluster. The cluster is encoded within about 1 kilo base on chromosome 13 and contains six miRNAs. The transcription of the miRNA cluster can be induced by E2F while some members of the cluster inhibit E2F at the post-transcriptional level, thereby forming a negative FBL (43). In addition, E2F can promote its own transcription forming a positive FBL. The two FBLs compose the E2F/miR-17-92 network whose complex regulatory dynamics can be studied through mathematical modelling (Figure 2). ODE modelling of the network in the context of glioma showed that the miRNA cluster can function alternatively as an oncogene or a tumour suppressor (44). Such a dual role of the miR-17-92 cluster could result from the bistable steady states that E2F possesses in the circuit. The switch between the two states is controlled by the values of two key model parameters. The two parameters correspond to the intensity of growth factor signalling and the inhibition of E2F translation by the miRNA cluster, respectively. Model simulations showed that the switch of the E2F steady states can make cells transit in four cell states: resting cell state (quiescent), normal (cell cycle) and abnormal (cancer) cell proliferation and cell death (apoptosis). As a result, the transitions endow the miRNA cluster with the opposite function in glioma (Figure 2).

In follow-up studies, the introduction of noise into external signalling (i.e. extrinsic noise) or into E2F expression (i.e. intrinsic noise) showed that the regulation by the miRNA cluster confers robustness to the E2F network by enabling cells to resist extrinsic noise (45,46). Interestingly, the ability of miRNAs in **noise buffering** was recently demonstrated in a synthetic motif, in which an artificial negative FBL formed by a TF and miR-223 was constructed in murine cells (47). Similar properties were also found in a theoretical study focusing on a TF self-regulation loop mediated by a miRNA (48). Moreover, **stochastic modelling** of the E2F/miR-17-92 FBL, under the assumptions that the number of molecules involved in these reactions is small and thus intrinsic noise should be considered, recovered most of the features identified in the ODE model. In addition, this stochastic model showed substantial differences regarding the number of stable states exhibited by the system (49). This result provided us with complementary insights into the regulatory circuit. Of note, the biogenesis of the miRNA cluster members can be selectively controlled by the progenitor miRNA, an intermediate product between primary and precursor miRNA, thus yielding a potential mechanism for differential production of miRNAs in the cluster (50). This

Table 1. MiRNA-mediated feedback loops and feedforward loops



Feedback loops are classified into positive and negative loops. In positive loops, both the miRNA and the TF have the same overall effect (activation or inhibition) on each other, and this effect can be direct or indirect. In negative loops, the overall effect of the miRNA and the TF on each other is opposite. Feedforward loops are classified into coherent and incoherent loops. In coherent feedforward loops, the miRNA and the TF have the same effect on their common target. In incoherent feedforward loops, the miRNA and the TF have opposite effect on their common target. Some biological examples for these loops are presented, and their possible dynamic features are illustrated (see main text for detailed descriptions).

factor may increase the complexity of the interactions between the miRNA cluster and its targets and should be considered in future modelling efforts investigating regulatory role of the miRNA-17-92 cluster in the E2F network.

Beside the miR-17-92 cluster, the E2F network can also be regulated by other miRNAs, and mathematical modelling has been used to explore their function in the E2F network (51,52). By simulating the role of the miR-449 and miR-34 families in the crosstalk between p53 and E2F1, Yan *et al.* (52) showed the coordination of the two miRNA families in regulating cell cycle progression after response to DNA damage. Modelling-based analyses indicated that the miR-449 family can induce apoptosis after cells respond to DNA damage, while the miR-34 family can help avoiding transmission of DNA damage to daughter cells by promoting transient cell cycle arrest. In addition, recent experimental studies have shown the existence of several other regulatory loops involving miRNAs in the regulation of the E2F family in cancer (42). The fact that these loops are untangled and their regulation is cancer type-specific suggests that more modelling efforts will be necessary to advance our understanding of the E2F regulation.

The mutually inhibitory TF-miRNA feedback loops regulate epithelial to mesenchymal transition in cancer metastasis. The switch between epithelial and mesenchymal phenotypes is a hallmark of cancer metastasis. Epithelial to mesenchymal transition (EMT) makes cells gain migrating ability

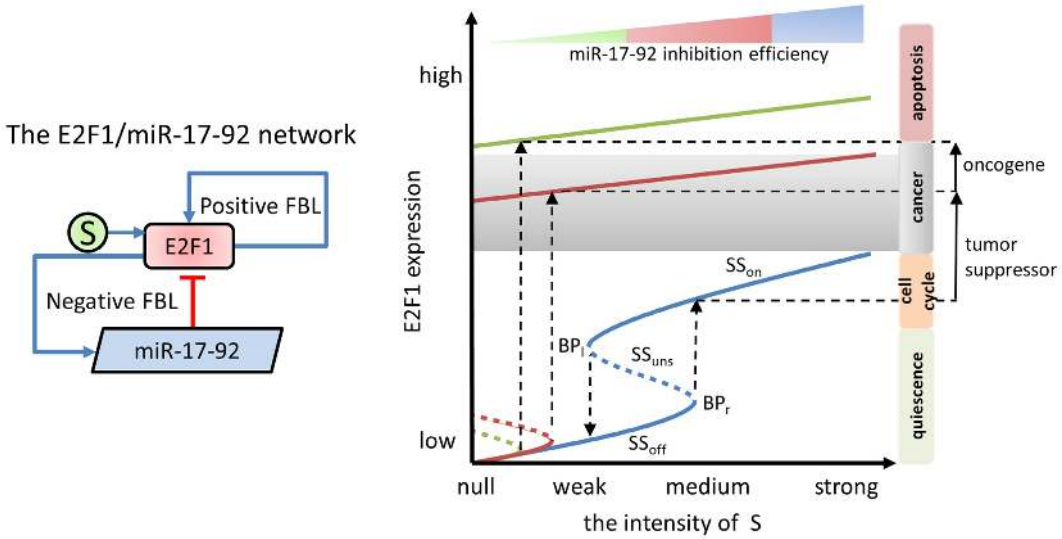


Figure 2. Oncogenic and tumour suppressor property of the miR-17-92 cluster in the E2F network. The diagram illustrates an abstract model of the interactions between the miR-17-92 cluster and the E2F family. With the increasing intensity of the growth factor signal (S), the expression of E2F can switch between the 'off' and 'on' steady states (S_{off} and S_{on}; the blue solid lines), but cannot stay on the unstable one (S_{uns}; the blue dashed line). When the inhibition efficiency of the miRNA cluster decreases (the red and green lines), the switch of E2F from S_{on} to S_{off} becomes irreversible, as the left bifurcation points (B_l) locate outside of the intensity interval of S, which contains biologically reasonable values. When cells express low E2F, decreasing miRNA inhibition efficiency results in a transition from the cell cycle zone to the cancer zone (i.e. shift from the blue line to the red line), showing the tumour suppressor property of the miRNA cluster. In contrast, when cells are already in the cancer zone, unchanged miRNA inhibition efficiency prevents them from exiting the cancer zone (i.e. shift from the red line to the green line), showing the oncogenic property of the miRNA cluster. The bifurcation plot is modified from (44).

and is therefore involved in the initiation of the invasion-metastasis cascade. Mesenchymal to epithelial transition (MET) makes cells regain epithelial characteristics, thus allowing colonisation and outgrowth of metastases (53). It has been found that miRNAs belonging to the miR-200 and miR-34 families can inhibit metastasis, presumably by reversing EMT and promoting MET (54). Mathematical modelling has been used in combination with experimental work to establish the basis of the miR-34/SNAIL and miR-200/ZEB loops underlying EMT and MET in cancer metastasis and aggressiveness (55–58). Tian *et al.* (55) proposed that the two TF-miRNA FBLs function together as a bistable switch to control EMT. In particular, model simulations showed that the epithelial phenotype corresponds to high levels of the miRNAs (miR-34 and miR-200) and low levels of the TFs (SNAIL and ZEB), whereas the mesenchymal phenotype corresponds to high levels of the TFs and low levels of the miRNAs. In addition, based on the model analysis the authors proposed the existence of a hybrid epithelial-mesenchymal phenotype (also known as partial EMT) corresponding to low levels of miR-34 and ZEB and high levels of SNAIL and miR-200. Such a phenotype endows cells with both adhesion and migratory properties, thus leading to collective cell migration (59). On the other hand, Lu *et al.* showed distinctive roles of both FBLs and proposed that the ZEB/miR-200 loop is accountable for the initiation and completion of EMT, whereas the SNAIL/miR-34 loop acts as a noise-buffering integrator of EMT-inducing signals like Wnt, Notch and p53, thereby preventing aberrant activation of EMT due to undesired signals (56,57). The ZEB/miR-200 loop allows for the existence of three phenotypes (i.e. three stable states; Figure 3). These phenotypes are the epithelial phenotype (high miR-200, low ZEB), the mesenchymal phenotype (low miR-200, high ZEB) and the hybrid phenotype (medium miR-200, medium ZEB).

Although both models have different assumptions for the EMT network, they both can exhibit multistability that is in agreement with the following experimental observations (58): (i) with the help of ZEB, SNAIL can suppress its downstream transcriptional targets associated with cell adherence such as E-cadherin; (ii) upon withdrawal of EMT-inducing signals, cells with high levels of ZEB can undergo EMT, a transition that is not possible for cells expressing low ZEB; and (iii) reverting EMT requires the suppression of both the EMT-inducing signal and ZEB, whereas knockdown of SNAIL does not suffice to revert EMT. Interestingly, further experimental results concerning the features of partial EMT are consistent with the tristability results, suggesting that medium levels of miR-200 and ZEB correspond to partial EMT (58). However, this experimental evidence may be cell-line specific, so more experiments will be required to further characterise the features of partial EMT.

In a follow-up study, Huang *et al.* (60) connected the EMT network with the Rac1/RhoA circuit that controls the transitions between the mesenchymal phenotype and the amoeboid phenotype. As mentioned before, when migrating cancer cells show the partial EMT phenotype they move collectively; while when cancer cells migrate individually they show the mesenchymal or the amoeboid phenotype. The resulting model was used to investigate the transitions between collective and individual migration phenotypes during carcinoma metastasis. Model simulations showed that the transitions between the two migration phe-

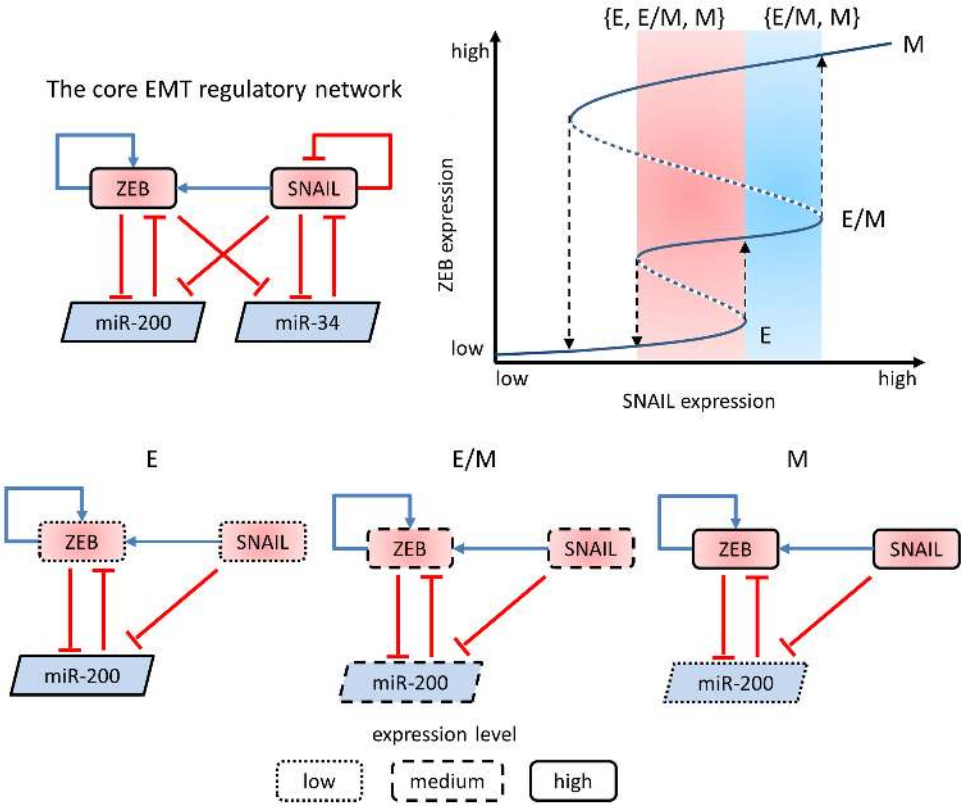


Figure 3. The EMT regulatory network. The EMT network is composed of two TFs (SNAIL and ZEB) and two miRNAs (miR-200 and miR-34). The inputs of the network are EMT-inducing signals such that Wnt and Notch activate ZEB and SNAIL, and p53 activates miR-200 and miR-34. With the change of SNAIL expression, the ZEB/miR-200 loop functions as a switch that makes ZEB jump among the three stable steady states (the solid blue lines), but ZEB cannot stay on the unstable steady states (the dashed blue lines). The three stable states correspond to three phenotypes: the epithelial phenotype (E), the mesenchymal phenotype (M) and the hybrid phenotype (E/M). The cartoons illustrate the corresponding gene expression profile for each phenotype. The colour areas in the bifurcation plot represent the possible combinations of phenotypes for different expression levels of ZEB and SNAIL. The bifurcation plot is modified from (58).

notypes are governed by miR-200 and miR-34. According to their results, high levels of the two miRNAs can restrict the transition of metastatic cancer cells towards the individual migration phenotype, thereby suggesting the role of the miRNAs in suppressing plasticity of tumour cell movement that can favour carcinoma metastasis. This continued work is a good example showing the reuse of an early mathematical model to investigate new features of a biological system that is associated with the addition of extra components and the introduction of new hypotheses. In this case, the upgraded model provides a precise understanding of the molecular mechanism underlying distinctive migration phenotypes of cancer cells.

MiRNA feedback loops regulate oscillatory gene expression. Negative FBLs are ubiquitous in gene regulatory networks as their existences provide cells with abilities to maintain homeostasis and to adjust signals that are not desirable (61). However, under some conditions negative FBLs including TFs, such as p53 and NF-κB, can induce sustained **oscillations** in gene expression, a regulatory pattern in which the FBL components are alternatively expressed or activated over time (62). MiRNAs can also make contribution to oscillatory FBLs, in which a TF slowly activates the expression of a miRNA, while the miRNA quickly inhibits the TF by translation repression or mRNA degradation, thereby satisfying the criterion to give rise to oscillations (62).

A series of models included in theoretical works and biological case studies have been developed to characterise the role of miRNAs in regulating gene oscillations. Overall, the theoretical works showed not only the determinant role of miRNAs in provoking oscillatory gene expression, but also their abilities to control the amplitude and frequency of gene oscillations (63–67). Further efforts combining modelling with quantitative data have been made to investigate the role of miRNAs in regulating oscillatory gene expression in different biological contexts, such as inflammation, neuron differentiation and cellular stress response. Xue *et al.* (68) investigated the role of miR-21 and miR-146a in shaping the oscillations of NF-κB, a key TF involved in triggering inflammatory response. Their work indicated that the negative FBL involving miR-21 (NF-κB→IL-6→miR-21-|NF-κB) can stimulate the NF-κB oscillations, while the FBL involving miR-146a that can indirectly repress IL-6 through IRAK1 (IL-6→NF-κB→miR-146a-|IRAK1→IL-6) has the ability to dampen the oscillations. These results pointed to the possibility that the balance between alternative miRNA-embedded FBLs may play a role in fine-tuning the NF-κB oscillations. In the context of neuron differentiation, Goodfellow *et al.* (69) showed

that miR-9 modulates the transient oscillatory behaviour of HES1 expression through a negative FBL (HES1→miR-9-|HES1), thereby contributing to adjusting the timing of neuron differentiation. In addition, Moore *et al.* (70) showed that inhibition of the p53-promoted miR-192, miR-34a or miR-29a can significantly reduce the number of breast cancer cells showing p53 oscillations upon DNA damage induction. These miRNAs are further involved in the feedback regulation of p53 by repressing its known regulators (e.g. p53→miR-34a-|SIRT1-|p53). This suggests that the p53 oscillations emerge out of a complex network of nested FBLs, in which miRNAs play a crucial role in conferring robustness to the oscillations.

The most important oscillatory gene expression program is the circadian rhythm, a conserved gene regulatory system which provides a mechanism to synchronise cell and organism activity to the periodic oscillation of sunlight. Interestingly, recent experimental studies have shown that multiple miRNA-mediated FBLs are important for sustaining and shaping circadian rhythms (71). As mentioned before, miRNA-mediated FBLs can buffer noise in gene expression, and this feature may play an important role when these FBLs confer robustness to circadian rhythms. In line with this, a data-driven mathematical modelling focusing on miRNA regulation of circadian rhythms will be necessary sooner or later.

MiRNA feedforward loops confer robustness to gene regulatory networks. Recent computational studies and experimental evidence suggest that miRNA-mediated FFLs are also prominent network motifs (72,73). Such motifs often contain a TF that promotes the expression of a miRNA, and both of them have a common target gene in the regulatory circuit. Thus, the TF can exert a direct action on the target gene expression but also an indirect one through the miRNA. The other configuration is also possible that a miRNA represses a TF and their common target, and the transcription of the target is simultaneously regulated by the TF (Table 1). Genome-wide surveys of recurring interaction patterns between TFs and miRNAs showed that TF-miRNA FFLs occur frequently in gene regulatory networks. In mammalian genomes, these network motifs can provide additional robustness to key gene circuits associated with cell development and differentiation (72–74). For example, the involvement of miRNAs in the E2F1/RB1 circuit results in miRNA-mediated FFLs, and model simulations showed that these FFLs can reinforce the stability of the two genes' concentrations against intrinsic noise, thus ensuring correct transition from G0/G1 to S phase in the cell (75).

Depending on the nature of the interactions between their components, miRNA-mediated FFLs can be classified into two classes (76–78). In coherent FFLs, the direct and indirect actions on the targets are consistent, while in incoherent ones the two actions are opposite (Table 1). From a theoretical point of view, miRNA-mediated coherent FFLs can serve to avoid spatial co-expression of miRNAs and their targets (74). For example, high or low expression levels of EGR1 in different types of cells can up- or down-regulate miR-199, resulting in opposite expression levels to their mutual targets MET, CAV1 and CAV2 in different types of cells (79) (Table 1 avoiding spatial co-expression). This feature could explain the observation that targets of a miRNA are usually at lower levels in a tissue/cell expressing the miRNA than in other tissues/cells (80). Besides, this kind of FFLs can provide an exquisite mechanism to prevent undesired leaky transcripts of a gene targeted by a TF and a miRNA (74). For example, upregulation of miR-17 can quickly turn off RB1 through inhibiting its transcriptional activator E2F1 and degrading the already transcribed mRNAs (i.e. leaky transcripts) of RB1. If there is no miR-17 regulation, the leaky transcripts of RB1 will degrade slower and the degradation rate is based only on the natural half-life of the transcripts (Table 1 preventing leaky transcription). In contrast, miRNA-mediated incoherent FFLs can exert their function in a dynamic fashion: when a TF gets activated, a delay between the transcription of a miRNA and that of their common target can create a temporal shutdown mechanism that avoids temporal co-expression of the miRNA and its target (74). For example, upregulation of MYC can increase the expression levels of E2F and E2F-targeting miRNAs. Due to the delay of miRNA upregulation (e.g. caused by miRNA biogenesis), E2F and its targeting miRNAs can avoid co-expressing at the same time (Table 1 avoiding temporal co-expression). Furthermore, a number of modelling studies indicated that incoherent FFLs including miRNAs have the capability to buffer extrinsic noise in target gene regulation, thus uncoupling target gene expression from noise in TF concentration or activity (81–83). As we mentioned before, the ability of miRNA-mediated FBLs in buffering gene expression noise is also demonstrated. The fact that both miRNA-mediated FFLs and FBLs can reduce noise in gene expression shows the non-uniqueness solutions for the same biological consequence, and we think that investigating the rationality for two distinctive miRNA-mediated network motifs providing a similar advantage is worth a theoretical analysis.

Deeping into the features of FFLs, mathematical modelling has also shown that incoherent FFLs can induce fold-change detection in gene regulation, that is, the intensity and duration of the transcription for the mutual target gene depends on the fold-change in the expression level of the TF and not on its absolute expression level (Figure 4). This fold-change detection property can be explained using the so-called Weber's Law, which states that the change in a stimulus (e.g. a signal that changes the expression of a TF) must meet a minimum threshold based on the ratio to its original magnitude to make a noticeable effect in the downstream targets (84). This property may allow cells to have an identical response to external signals of different magnitudes but showing the same fold-change despite cell-to-cell variation in the basal level of the TF expression (84). So far, both theoretical analysis and data-driven modelling of the fold-change detection in miRNA-mediated incoherent FFLs are missing, and they are necessary to unravel the role of this interesting property in different biological contexts.

MiRNA target hubs: concurring gene regulation by multiple miRNAs. Another key motif in gene regulatory networks is target hub genes that can interact with many network components, and therefore their deregulation can affect major parts of the network (85). It has been shown that some

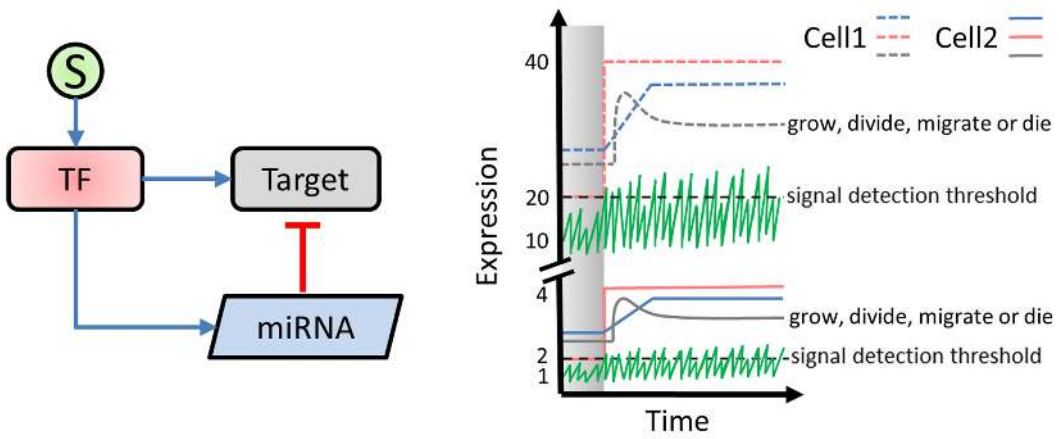


Figure 4. Fold-change detection in miRNA-mediated incoherent FFLs. Fold-change detection allows heterogeneous cells to tolerate signal noise by relative change in their intensities (the shaded area). In other words, the two cells receive external signals (S; the green lines) with different absolute intensities but the expression of the TF and the miRNA stays at their initial levels (the red and blue lines). When S exceeds a detection threshold (the non-shaded area), which is determined by the relative change of its intensity, the TF responds to S leading to the upregulation of the miRNA and its target (the grey lines). Such a mechanism may allow for the same response of the cells when the upregulation of the TF expression differs in absolute magnitude but shows the same relative change (2-fold-changes).

genes are especially prone to miRNA regulation and can be targeted by dozens of miRNAs, making these genes become miRNA target hubs (86). For example, CDKN1A is an experimentally verified miRNA target hub, and its coding protein p21 is a cell cycle regulator that plays an important role in cancer progression (87). So, it is interesting to ask how this miRNA target hub is regulated by multiple miRNAs and what biological consequences of this regulation are. Two experimental groups have proven that the close proximity of two miRNA binding sites on a common target (i.e. cooperative miRNA regulation) can induce stronger repression of the gene (88,89). Based on this evidence, we developed an ODE model to investigate the regulation of CDKN1A by multiple and potentially cooperative miRNAs (90,91). Model simulations showed that selective expression of CDKN1A-targeting cooperative miRNAs can fine-tune its protein expression level, thereby resulting in tightly regulated p21 expression in various cellular processes, such as cell cycle and apoptosis. Based on these results on the relevance of miRNA cooperativity, we launched a systematic search for human genes that could be regulated by cooperative miRNAs and made these information available for the public using an online database (92). Interestingly, we found that such genes are enriched in the human genome, so miRNA cooperativity should be considered when future modelling and experimental efforts are made to investigate their regulation.

Mechanistic modelling of miRNA-mediated post-transcriptional gene repression

MiRNA-mediated post-transcriptional gene repression is a complicated process with a variety of possible mechanisms, including initiation block, post-initiation block, deadenylation of target mRNAs to induce quick decay and translocation of target mRNAs to P-bodies followed by degradation (93). In addition to these distinctive mechanisms, the features of miRNA regulation can also play a role in determining the repression ability of given miRNAs for specific target genes. For example, the accessibility to miRNA binding sites can be characterised by their locations (3′ UTR, 5′ UTR or coding region), abundance and their affinities to miRNAs (94–96). MiRNA turnovers can differ in different biological contexts (97–99). The production of mature miRNAs is subjected to upstream processing proteins, such as Dgcr8 and Dicer, whose expression and activity can be context-specific (100,101), while the efficiency of the formation of miRNA-induced silencing complexes (MIRISCs) can be modulated by the availability of Argonaute proteins (AGO) (102,103). A miRNA–mRNA interaction can be catalytic if the miRNA molecules are completely recycled after interacting with the target, or stoichiometric if they are not (104,105). Interestingly, the computational algorithm miRBooking, which considers the stoichiometric mode of miRNA action, showed significant improvement in the accuracy of miRNA target predictions in comparison to other algorithms that do not consider this feature of miRNA regulation (106). Taken together, the various mechanisms combined with different features of miRNA regulation make the post-transcriptional gene repression an intricate process, which requires the efforts from both experimental and computational researchers to understand it. Herein, we review publications that utilise mathematical models to analyse distinct regulatory properties associated with gene repression by miRNAs.

The role of miRNA-mediated gene repression mechanisms in shaping context-specific gene expression. Some seminal modelling studies revealed that the effectiveness and kinetics of miRNA-mediated gene repression is strongly associated with the number of miRNA binding sites, the location of the binding sites and their binding affinities to miRNAs as well as the turnover of miRNAs and their targets (107–111). For example, by modelling miRNA-mediated gene repression through binding to coding regions or to 3′ UTRs, Brümmer and Hausser (111) showed that miRNAs can

significantly speed up repression of specific genes through binding to their coding regions, and these genes, which produce long half-life mRNAs and short half-life proteins, are typically involved in cell proliferation. It is also worth mentioning that a kinetic model considering the complete post-transcriptional repression process has been developed in a series of publications (112–114). The model was used for discriminating those mentioned distinct mechanisms that can lead to the same effect (i.e. gene repression), and also for simulating gene expression dynamics when several mechanisms occur at the same time. Interestingly, model simulations have provided plausible explanation for a number of experimental observations, namely: (i) the same miRNA can use distinct mechanisms for different target genes; (ii) several mechanisms can co-occur to repress the expression of given genes; and (iii) the effectiveness of gene repression by a miRNA can differ in different biological contexts (114). Besides, such a model provides a template for investigating the detailed miRNA repression mechanism of given genes.

The role of AGO and MIRISC in determining the effectiveness of miRNA-mediated gene repression. With the increasing understanding of miRNA repression mechanism, AGO and MIRISCs have been introduced into mathematical models. Klironomos and Berg (115) showed that the kinetics of the post-transcriptional gene repression is determined by the catalytic or stoichiometric interaction type between miRNAs and their targets, the efficiency of MIRISC formation and degradation, and the availability of AGO. Hausser *et al.* (116) found that mild repression commonly observed for many target genes upon miRNA transfection may be caused by two factors: the delay in the loading of miRNA into AGO and the higher stability of proteins compared to mRNAs for given miRNA target genes. More interestingly, it has been also observed that miRNA can upregulate gene expression under certain biological contexts (117). Model-based analyses indicated that this observation can be explained by a mechanism that allows reversible mRNA–miRNA binding, protein translation from MIRISC, and selective return of RNAs from the complex (118,119).

Furthermore, Barad *et al.* (101) showed that a self-regulatory negative feedback on the miRNA processing protein Dgcr8 allows for efficient miRNA production, thus ensuring effective gene repression by miRNA. Except for such self-regulation, the biogenesis of some miRNAs can be regulated by other miRNAs, and consequently this can affect their repressive abilities on target genes (120,121). For example, by modelling the interactions between hypoxia-response miRNAs, Zhao and Popel (122) showed that in hypoxia upregulated let-7 expression leads to downregulated AGO1 expression and miRISC formation, therefore resulting in the derepression of VEGF targeted by miR-15 due to the reduced miRISC activity. This example also demonstrates the role of stress signals in modulating the repression effectiveness of specific miRNAs, hence affecting their function in stress response. Consequently, this may change cellular stress environments in which cancer cells evolve (34).

MiRNA-mediated gene regulation at the single-cell level. The previous studies have shown the breadth and importance of gene regulation by miRNA in models that account for the dynamics in cell populations. However, population averages often mask properties that show cell-to-cell variations (123). To obtain an accurate representation of miRNA-mediated gene repression in individual cells, Mukherji *et al.* (124) used a two-colour fluorescent reporter system to measure both transcription and translation following regulation by miRNA in single mammalian cells. By integrating single-cell data with mathematical modelling, the authors found that gene repression by miRNA can vary dramatically among individual cells, and this variation can lead to the modest gene repression on average level, which is in agreement with the results from population-based studies (Figure 5). Besides, the authors showed that miRNAs can establish expression thresholds for their target genes, and these thresholds can determine how these miRNA target genes transit from repression to derepression. Namely, if the target mRNA abundance is below the threshold, the gene is highly repressed; while if the target mRNA abundance is above the threshold, the gene is relieved from miRNA repression. Further analyses indicated that when the miRNA abundance is stable, the increasing interaction strength between the miRNA and its target mRNA sharps the transition from repression to escaping from miRNA repression (Figure 5, left); and when the interaction strength remains unchanged, the increasing miRNA abundance increases the sharpness of the transition and also the level of the threshold (Figure 5, right). Moreover, a follow-up study showed that miRNA regulation can result in distinctive noise profiles of protein expression in mouse embryonic stem cells. In particular, the authors found that miRNA regulation can decrease noise in protein expression for lowly expressed genes, while increasing noise for highly expressed genes (125). In line with our previous discussion, this result demonstrates the ability of miRNA in conferring robustness to gene expression at the single-cell level.

Competing endogenous RNAs and miRNAs

It is not controversial that miRNAs can target protein-coding and ncRNAs that have specific binding sites for given miRNAs (126–128). These RNA transcripts can act as competitive endogenous RNAs (ceRNAs), which compete for common miRNAs and thus cause diluted gene repression by the miRNAs. In other words, the more binding sites available in ceRNA candidates, the lower are the effective concentration of their targeting miRNAs (39). In turn, it is also hypothesized that miRNA binding sites in RNA transcripts have evolved to become crosstalk hubs of gene interactions, thereby affecting the expression levels and activities of ceRNAs (129). This means that competition and depletion of shared miRNAs by ceRNAs is a mechanism for indirect interaction and cross-regulation of RNA species. This suggests a complex network of interacting RNA species linked by their abilities to bind to and to deplete miRNAs. Under these circumstances, mathematical modelling becomes a useful tool to provide a precise and quantitative understanding of ceRNA cross-regulation through shared miRNAs, thus allowing addressing questions like which kinetic parameters control the emergence of the effective miRNA-mediated crosstalk between ceRNAs

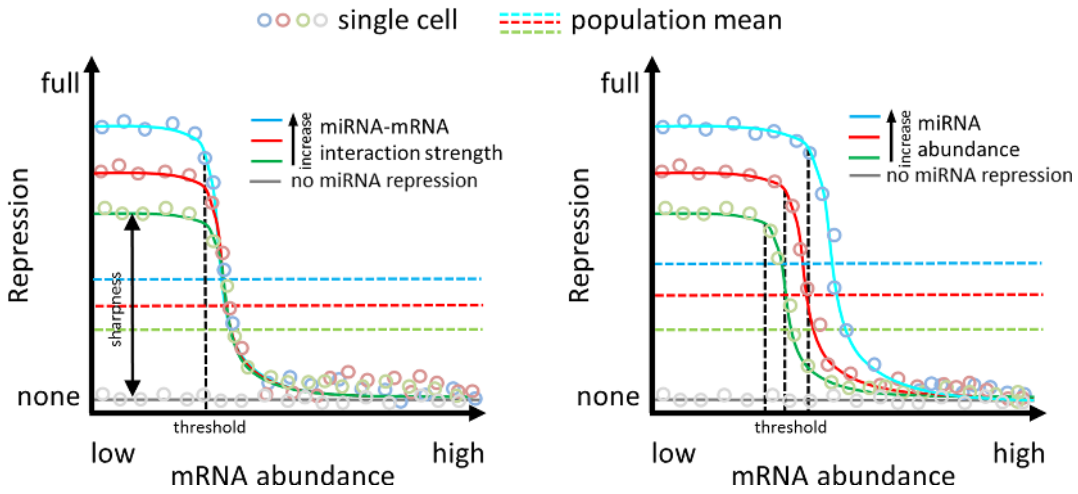


Figure 5. MiRNA-mediated gene repression at the single-cell level. Due to the existence of the gene expression threshold created by miRNA (the vertical black dashed lines), the repression of a gene by its targeting miRNA can vary dramatically at the single-cell level (the circles), resulting in mild gene repression at the population level (the horizontal colour dashed lines). The varying abundance of the target mRNA (x-axis) results in distinctive miRNA-mediated repression profiles (y-axis) in individual cells. The solid lines represent the model predictions that are validated by the single-cell data. The interaction strength between the miRNA and its target mRNA can be characterised by the number of the miRNA binding sites on the 3′ UTR of the mRNA and the affinities of these binding sites to the miRNA. When miRNA abundance is stable, the increasing interaction strength leads to shaper transition of gene repression but does not affect the level of the threshold (Left). The sharpness of the transition is defined by the distance from the maximum repression of the gene to non-repression. When the interaction strength remains unchanged, modulating miRNA abundance alters the sharpness of the transition and also the level of the threshold (Right).

and what type of effective interaction networks may result from such a simple titration mechanism (130–139).

Kinetic modelling of a minimal ceRNA network, in which one miRNA interacts with two competing RNAs, showed that ceRNA activity is determined by the relative abundance of ceRNAs and miRNAs as well by the type of their interaction (stoichiometric or catalytic) (130). Further extension of the minimal model by considering interactions between multiple miRNAs and ceRNAs allowed for the characterisation of mean and noise profiles of ceRNA network components and the response time of the network components required to resume their steady states upon perturbation (130,131). Several examples accounting for dynamics of the crosstalk between ceRNAs through miRNAs are shown in Figure 6.

By integrating miRNA-mediated ceRNA crosstalk with TF regulation, Martirosyan *et al.* (139) showed that miRNA regulation of a gene through the ceRNA network can outperform its regulation by a TF, suggesting the possible role of miRNAs as major regulators rather than fine-tuners of gene expression. To study noise characteristics within a ceRNA network composed by a miRNA, a protein-coding RNA and a ncRNA, Noorbakhsh *et al.* (134) defined the intrinsic noise as the variance in protein level divided by mean protein level squared. By simulating the noise profile, the author showed that the noise is dramatically high when the combined transcription rate of the ceRNAs approximates the transcription rate of the miRNA (i.e. the cross-regulation of the two ceRNA happens). This property makes this noise quantity a possible measure for detecting the miRNA-mediated interactions between the two ceRNAs (134). The ability of co-regulated ceRNAs and miRNAs to propagate oscillatory behaviour was demonstrated with a mathematical model accounting for the ceRNA network equipped with an oscillator that drives the circadian clock in diverse organisms (135).

Furthermore, mathematical models have also been proposed to study specific ceRNA cross-regulation via shared miRNAs (137,140,141). Yuan *et al.* (137) studied the cross-regulation of mKate and EYFP in a synthetic circuit, in which their mRNAs were engineered to have partial and perfect complementarity to miR-21. The results showed that the repression of EYFP by miR-21 can be significantly relieved when the expression of mKate mRNA increases due to its strong binding sites to miR-21. This derepression effect can be compensated by increasing the concentration of miR-21, suggesting a strategy to reduce the off-target effect of miR-21 in *in vivo* experiments. Interestingly, not only endogenous ceRNAs can compete with miRNAs for targets, but also exogenous ceRNAs from virus can inhibit miRNA activity (142). Mathematical modelling of miR-122 sequestering by hepatitis C virus RNAs showed the sponge effect of the virus RNAs on diluting the inhibition activity of host miR-122. As a consequence, global derepression of host miR-122 targets was observed, suggesting a mechanism that can facilitate the long-term oncogenic potential of the virus (143).

In summary, although the above reports have described ceRNA interactions via shared miRNAs in diverse biological settings, certain criteria have to be met to observe the cross-regulation under physiological conditions. Factors, such as the expression levels of miRNAs and ceRNAs, the number of miRNA binding sites and their binding affinities, have been suggested to modulate the effectiveness of ceRNA crosstalk (129,144). Indeed, it has also been shown that under physiological conditions a significant relief of a gene from miRNA repression may happen only when the ratio of miRNA molecules to their binding

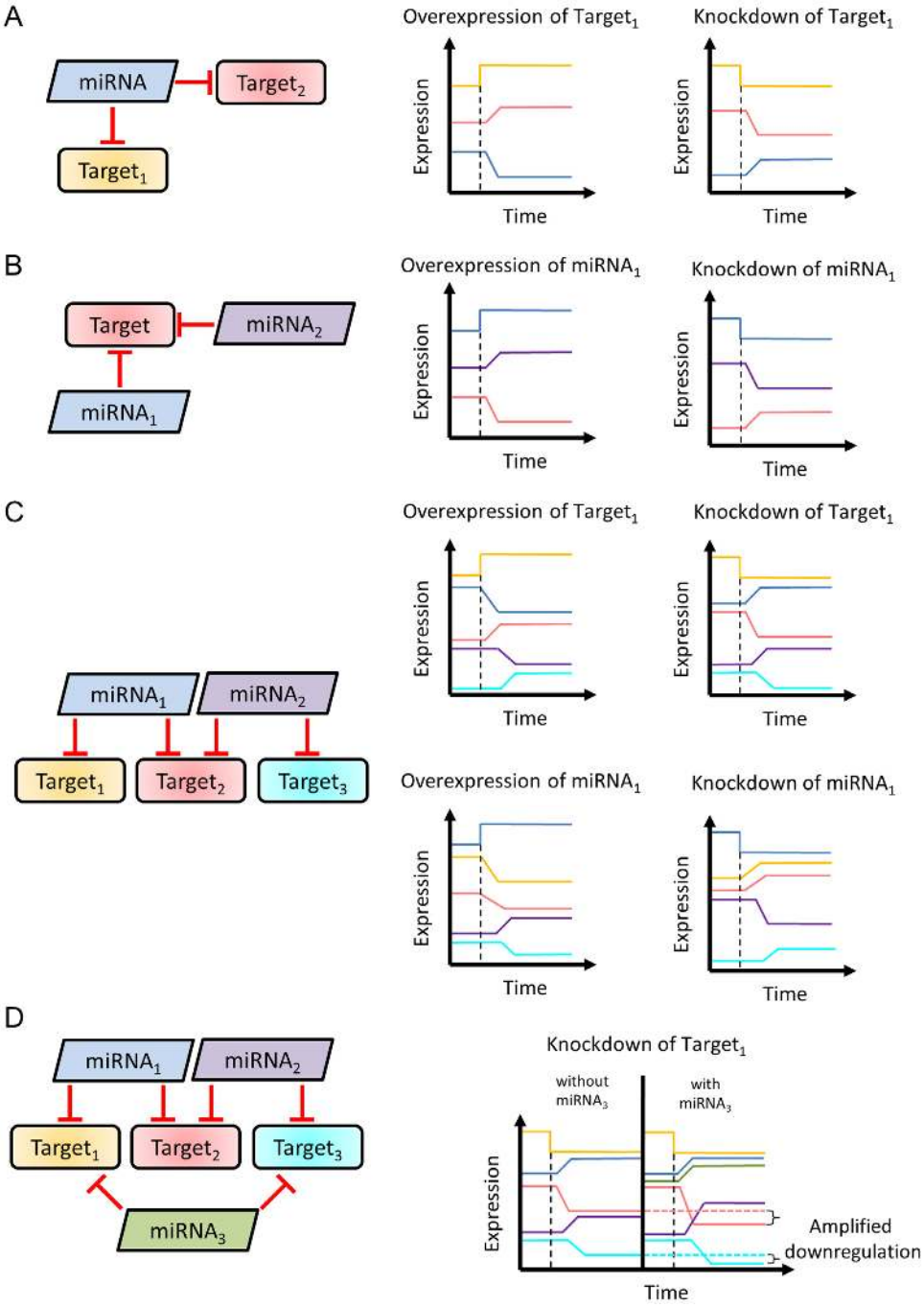


Figure 6. MiRNA-ceRNA networks. (**A**) The cross-regulation two ceRNAs through one miRNA. Up- or downregulation of one ceRNA ($Target_1$) can result in the same change in the expression of the other ceRNA ($Target_2$) through competing their common miRNA. (**B**) The cross-regulation two miRNAs through one ceRNA. Up- or downregulation of $miRNA_1$ can result in the same change in the expression of $miRNA_2$ through competing their common ceRNA (Target). (**C**) The interactions of multiple miRNAs and ceRNAs. Up- or downregulation of $Target_1$ leads to the same change of $Target_2$ expression but opposite expression change of $Target_3$ through competing two miRNAs that share $Target_2$ and regulate $Target_1$ and $Target_3$, respectively. Such modulations in $Target_1$ expression result in the opposite change in miRNA expression. Similar dynamics of $Target_3$ expression can also be achieved by modulating the expression of $miRNA_1$. (**D**) In comparison to (C), the involvement of an additional miRNA ($miRNA_3$) can result in amplified effect on ceRNAs. The knockdown of $Target_1$ results in upregulation of free $miRNA_3$. Due to the participation of $miRNA_3$ in repressing $Target_3$, the expression of $Target_3$ is downregulated to a lower level compared to the ceRNA network without $miRNA_3$ (the extended dashed lines). Similar effect can be transmitted to $Target_2$, due to the increased $miRNA_2$ level as a result of lower $Target_3$. The line colours in the plots are corresponding to the node colours in the cartoons. The black dashed line indicates the time point at which the sudden change (up- or downregulation) in the expression of network components happens.

sites lies in a reasonable range (144,145). Furthermore, by using CLIP-seq Bosson *et al.* (146) revealed that high abundance miRNAs are not susceptible to ceRNA competition. This result is supported by the experimental evidence that miR–122, a highly expressed miRNA in liver, is not sensitive to physiological expression of individual competing transcripts (147). On the other hand, Bosson *et al.* (146) showed that high affinity targets of low abundance miRNAs, such as the miR-92-25 family, can create a scenario of physiological RNA competition. These findings suggest that the ceRNA hypothesis may not be a general mechanism underlying regulatory functions of miRNAs but only explain exceptional circumstances. Besides, we have foreseen a trend to expand gene regulatory networks by adding the interactions between miRNAs and other non-coding transcripts such as circular RNAs and long ncRNAs. In this context, mathematical modelling will be a necessary methodology to provide us with a comprehensive and systematic understanding of gene regulation by ncRNAs.

MiRNA regulation of genes in biomedicine: case studies in cancer

Temporal and spatial control of gene expression is essential for the correct functioning of cellular processes such as cell cycle, cell differentiation and apoptosis whose dysregulation underlies the emergence of many diseases. Protein-coding genes are major regulators of these processes, and therefore their regulation by miRNAs plays an important role in the disease-associated dysregulation of these cellular events. On the other hand, miRNA regulation allows specific manipulation of undruggable protein-coding genes, thus making miRNAs great potential for therapeutic application (148). All of this explains why miRNAs draw great interest from a biomedical perspective, especially for cancers.

By targeting tens to hundreds of genes, the so-called oncomiRs contribute to cancer progression by regulating gene networks that underlie tumour cellular responses such as impaired cell-death, abnormal proliferation and metastatic migration (15,149). In contrast, miRNAs can function as tumour suppressors through impeding tumour progression or sensitising tumour cells to intrinsic or extrinsic apoptosis. Mathematical modelling has been applied to advance our understanding how miRNAs regulate intracellular cancer signalling pathways, and thereby identifying potential miRNA targets for therapeutic intervention, such as let-7 and miR-15 for suppressing angiogenesis in tumour growth (122), downregulating miR-9 for reducing lung metastasis (150), inhibiting miRNAs that favour colon cancer (151) and utilising a combined therapy composed of targeted inhibitors and *BCR.ABL*-targeting miRNAs for treating chronic myeloid leukemia (152). To illustrate this idea, we here discuss in detail some examples. Schuetz *et al.* (153) developed a hybrid model for glioma regulation by coupling a key signalling pathway with cell phenotypes. In this model, the phenotype (either proliferation or migration) of a tumour cell was determined by ODEs accounting for the LKB1/AMPK/miR-145 pathway (154). The phenotype switching of tumour cells were simulated using an **agent-based model**. The simulation results supported the experimental finding that sufficient amount of glucose can increase miR-415 expression, leading to the repression of AMPK via LKB1 which consequently makes tumour cells proliferative; however, glucose deprivation can cause upregulation of AMPK through downregulating miR-451, as a result tumour cells separate from each other and start migrating. These modelling results suggest miR-415 as a potential target for glioblastoma therapy.

It has been shown that deregulation of miRNAs can lead to drug resistance in cancer (155). Reversal of deregulated miRNAs can normalise intracellular signalling pathways that are consistently dysregulated in cancer and hence sensitise tumour cells to chemotherapies (155–158). We developed a multi-level ODE model of the E2F1/p73/DNp73 signalling pathway to investigate the role that miR-205 plays in resisting conventional genotoxic and cytostatic drugs (153–160). In the model, given genes of the pathway were defined to regulate the proliferation and apoptosis of tumour populations represented by ODEs. By systematic perturbation of parameter values accounting for tumour-associated genetic variation in the pathway, we identified a number of *in silico* gene expression signatures associated with chemoresistance, and the most prominent one showed high expression levels of E2F1 and ERBB3 and low expression level of miR-205. Further model simulations and experimental validation showed that among the tumour cells exhibiting genetic heterogeneity in the E2F1/p73/miR-205 signalling pathway, the ones with high E2F1 and low miR-205 are resistant to the conventional chemotherapies and can even relapse after the therapy.

The modelling of miRNAs regulation in the context of biomedicine, especially in cancer, has tremendous future perspectives. On one hand, one can expect more crucial miRNA-mRNA interactions associated with phenotypes to be found in different cancers and other multifactorial diseases. On the other hand, a number of pharmaceutical companies and translational research institutes are currently essaying miRNA vectors and antagomiRs as potential anticancer therapies or adjuvant therapies (148,160). In this context, mathematical modelling will be necessary to assess the bio-distribution and the dose-dependent effects of these miRNA therapies. Modelling strategies already used in pharmacokinetics and -dynamics will have to be adapted to the speciality of miRNAs.

CONCLUSION

Complex networks enriched with non-linear regulatory motifs require mathematical modelling for the integration of multi-level quantitative data, for their mechanistic understanding and for their therapy-oriented application. Mathematical modelling has shown that miRNA-enriched circuits display complex regulatory patterns and non-linear dynamics, even for small network motifs with only a few components. Key miRNA repression features that have been elucidated via modelling include: (i) the ability of miRNA-mediated FBLs to enable bistability or multistability in gene expression; (ii) the possible ability of miRNA-mediated FFLs to allow fold-change detection of gene expression; (iii) the fact that the effectiveness of miRNA-mediated gene repression can be determined by the molecular properties of the miRNA–target interaction; (iv) the fact that the mild

gene repression by miRNAs at the cell population level can be a result of diverse repression degree at the single-cell level; and (v) the existence of cross-regulation of ceRNAs via shared miRNAs. From a biomedical perspective, mathematical modelling in combination with experimentation has shown its merit in elucidating the contribution of miRNAs to dysregulated signalling pathways in cancer and has also provided a promising approach to deliver novel miRNA-based therapies for cancer.

So far, as most experimental studies focused on direct miRNA–target interactions, modellers included these interactions into gene regulatory networks associated with certain phenotypes. Mathematical modelling of these biological systems allows systematic simulations to unravel the role of miRNAs within the systems and hence provides a system-level understanding of miRNA-mediated gene repression. However, beside those conventional miRNA–target interactions, recent experiments have shown that primary or precursor miRNAs produced during miRNA biogenesis can also compete with mature miRNAs for their binding sites on target mRNAs. For example, mouse primary and precursor miR-151 are in competition with the mature form for binding sites situated within 3′-UTR of the E2F6 mRNA, leading to derepression of E2F6 (161). Thus, it will be of interest to model how such unconventional miRNA regulation can affect the repression of corresponding target genes. It is also worth noting that except for acting as inhibitors of gene expression miRNAs can influence the local structure of targeting mRNAs, and subsequently the availability of RNA-binding motifs that can be recognized by RNA-binding proteins (RBPs) (162). For example, the unconventional binding of miRNAs could lead to the formation of hairpins in their targeting mRNAs that can serve as nucleation sites for RBPs, thus provoking simultaneous and/or sequential RBP-mediated regulation (163). This kind of interaction suggests new regulatory functions of miRNAs, and thus there is great interest to investigate the output of those miRNA functions using mathematical models.

On the other hand, published models mainly focus on temporal dynamics of gene regulation by miRNAs, but future models of **partial differential equations** will be needed to consider spatial information of miRNAs within cells, as different subcellular locations (such as RNA granules, endomembrane, mitochondria and the nucleus) are required for the processing and degradation of miRNA itself, or for silencing or activation of miRNA targets (164). Furthermore, it has been shown that miRNAs loaded into extracellular vesicles can circulate in body fluids (165–167), so it will be interesting to model intercellular communication by means of the circulating miRNAs, even though the small number of miRNAs in extracellular vesicles like exosome may undermine the practical ability of the miRNA-based intercellular communication (168). Moreover, it is worth noting that individual miRNAs have variants, also called miRNA isoforms, and they are usually transcribed from a single locus or homologous loci but heterogeneous in length, sequence or both (169,170). These heterogeneities may affect target selection, miRNA stability or loading efficacy into MIRISCs, and thus special attention should be paid when modellers scrutinise them. Besides, a very recent experimental study has shown that fever caused by infection can increase the expression of miR-142–5p and miR-143, and in turn both miRNAs attenuate body temperature by targeting several cytokines that act as endogenous pyrogens (171). This novel finding is worthy a modelling effort to provide a quantitative understanding of the negative feedback mechanism that mediates fever.

In summary, we foresee numerous future challenges faced by the modelling community, aiming at improving our understanding of miRNA regulation in gene regulatory networks.

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GLOSSARY

ODE model	Biochemical reaction networks can be described using ordinary differential equations (ODEs). In ODE models, model variables, such as concentrations of proteins and RNAs, depend on the time. Given a specific model configuration (e.g. parameter values for kinetic rates and initial conditions for model variables), a ODE model always yields the same output (i.e. the model is deterministic). Non-linear dynamics, such as bistability and oscillation, can be an output of an ODE model accounting for specific network motifs such as feedback loops.
PDE model	In comparison to ODEs, partial differential equations (PDEs) are used to describe a dynamic system in which model variables depend both on time and space.
Stochastic model	A class of mathematical models which is used to describe the time evolution of a biochemical reaction network in a way that takes account of random variations in model variables. In contrast to deterministic models, the same model configuration can yield different outputs.
Agent-based model	A computational approach that models tissue dynamics as a result of interplay of individual cells. In these models, cells are represented by 'agents', and their behaviours are determined by rules for their movements and phenotypes. In a hybrid model, the rules for determining cell phenotypes, such as proliferation, apoptosis and metastasis, can be an output of an ODE model.
Bi- or multistability	Bi- or multistability is the co-existence of two or multiple stable equilibria for molecular species. Bistability creates two distinct cell states or phenotypes in genetically identical cells.
Noise buffering	Noise buffering refers to mechanisms that keep gene expression stable and hence decrease the variation in gene expression
Oscillation	If the concentration of a molecular species oscillates sustainably, its concentration cannot reach an expected equilibrium but shows repetitive variation around the equilibrium over time.