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Transcription factor and microRNA co-regulatory loops: important regulatory motifs in biological processes and diseases

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Abstract

Transcription factors (TFs) and microRNAs (miRNAs) can jointly regulate target gene expression in the forms of feed-forward loops (FFLs) or feedback loops (FBLs). These regulatory loops serve as important motifs in gene regulatory networks and play critical roles in multiple biological processes and different diseases. Major progress has been made in bioinformatics and experimental study for the TF and miRNA co-regulation in recent years. To further speed up its identification and functional study, it is indispensable to make a comprehensive review. In this article, we summarize the types of FFLs and FBLs and their identified methods. Then, we review the behaviors and functions for the experimentally identified loops according to biological processes and diseases. Future improvements and challenges are also discussed, which includes more powerful bioinformatics approaches and high-throughput technologies in TF and miRNA target prediction, and the integration of networks of multiple levels.

Keywords: *FFLs; FBLs; TF-miRNA co-regulatory network; target prediction; cell proliferation*

INTRODUCTION

Transcription factors (TFs) are modular proteins that regulate gene transcription through binding to the promoter region of target genes by their DNA-binding domain [1]. MicroRNAs (miRNAs) are a kind of small noncoding RNAs ~22 nucleotides in length. They are able to silence the expression of target genes by mRNA degradation or translational inhibition [2]. Both TFs and miRNAs are involved in the regulation of various biological processes, including cell proliferation, differentiation and apoptosis. Thus, the dysregulation of TFs and miRNAs is associated with many diseases.

Gene expression regulation is a complex biological process, which is controlled by various factors including proteins and RNAs in multiple levels. As key regulators of gene expression, TFs and miRNAs are able to co-regulate the expression of targets in forms of feed-forward loops (FFLs) and feedback loops (FBLs) [3,4]. These two kinds of loops are important motifs in gene regulatory networks, which were initially proposed to describe the co-regulation between different TFs on the same target [5,6]. However, TFs and miRNAs can also co-regulate gene expression in the same ways. Several studies reported that the TF-miRNA co-regulation was

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prevalent in animal genomes [3,4,7]. The TF-miRNA FFLs influence many aspects of normal cells and diseases. TF-miRNA FFL has a specific function in noise buffering effect. It can minimize the response to stochastic signaling noise and homeostatically maintain steady-state levels of the target protein by tuning the translation of its target in a direction opposite to that of the signal [8]. This robustness imbued by FFL can protect cells against rapidly flipping between alternative cell decisions in the development process [8]. The TF-miRNA FFL may also facilitate the 'miRNA-target spatiotemporal avoidance' phenomenon, in which a miRNA and its targets are expressed in adjacent tissues or in consecutive developmental stages for some cases [9]. FBLs also play critical roles in cell differentiation and development. Inui *et al*. [8] elucidated that double-negative FBLs could generate mutually exclusive or bistable expression of the miRNA and TF, and convert a transient signal into a longer lasting cellular response.

Much progress has been made in the study for TF and miRNA co-regulation in recent years. To further speed up its identification and functional study, it is necessary to summarize the methods and review its influences on cellular processes and diseases. In this review, we summarize the types of FFLs/FBLs and their identified methods, as well as the functions in biological processes and diseases. The future challenges and perspectives on TF-miRNA co-regulation are also discussed.

TYPES OF FFLs AND FBLs

In this part, we briefly describe the types and potential functions of FFLs and FBLs. The FFL is a motif in which a TF regulates a miRNA or a miRNA represses a TF, and both of them co-regulate a joint target. FFLs can be divided into three types according to the master regulator: TF-FFL, miRNA-FFL and composite FFL (Figure 1a) [3]. In a TF-FFL, TF is the master regulator, which regulates its partner miRNA and their mutual target, while in a miRNA-FFL, miRNA is the master regulator. TF-FFL and miRNA-FFL can combine into a composite FFL, in which TF and miRNA regulate each other.

In another way, FFLs also can be classified into coherent and incoherent loops based on the effects of miRNAs and TFs on their mutual targets. In a coherent FFL, the regulatory paths have the same effects on the target (either activation or repression) (Figure 1b), whereas in an incoherent loop the expression of the target is controlled by two reverse paths (Figure 1c). Thus, coherent and incoherent FFLs could lead to different effects on gene regulation. The left graph in Figure 1b shows a typical coherent loop, in which the miRNA transcription is induced by a TF and they jointly repress the expression of their mutual target. This kind of circuit serves as a surveillance mechanism to suppress the expression of 'leaky' genes that should not be expressed in specific cells [4,10]. For example, YAN is repressed by Pnt-p1 and miR-7 in the differentiation of photoreceptors [11]. The Figure 1c shows the types of incoherent FFLs and the left one is a typical FFL that has two advantages on regulating gene expression. Firstly, in this case miRNAs have a fine-tuning function to keep the expression level of targets in correct ranges, and the co-expression of miRNAs with their targets allow miRNAs to better fine-tune the stable status of targets [4,10]. Secondly, it prevents undesired activation of targets from stochastic signaling. Such noise buffering helps to maintain the homeostasis of target proteins and the uniformity in their expression profiles within cell populations [12].

The FBLs are not abundant in pure transcriptional networks [13], whereas they are recurrent between miRNAs and TFs in a variety of organisms [7]. The TF-miRNA FBL is a motif in which TF and miRNA regulate each other, and each of them regulates their targets separately. There are two types of FBLs according to the regulation of TFs on miRNAs: single-negative and double-negative loop (Figure 1d). The double-negative loop, in which a miRNA and a TF repress each other, has two steady states: either TF is on and miRNA is off, or the opposite [14]. This kind of FBL can act as a toggle switch between two different fates and therefore play key roles in cell differentiation [15,16]. For instance, the double-negative FBL composed of miR-200 family and ZEB1/SIP1 controls the epithelial to mesenchymal transition (EMT) [15]. In the single-negative loop, a TF activates the transcription of a miRNA, which in turn inhibits the translation of TF. This kind of FBL allows a tightly control for the protein level of TF. For example, the single-negative FBL consisting of miR-17–92 cluster and TF E2F1 is critical for balancing the protein level of E2F1 and controlling the progress of cell cycle [17,18].

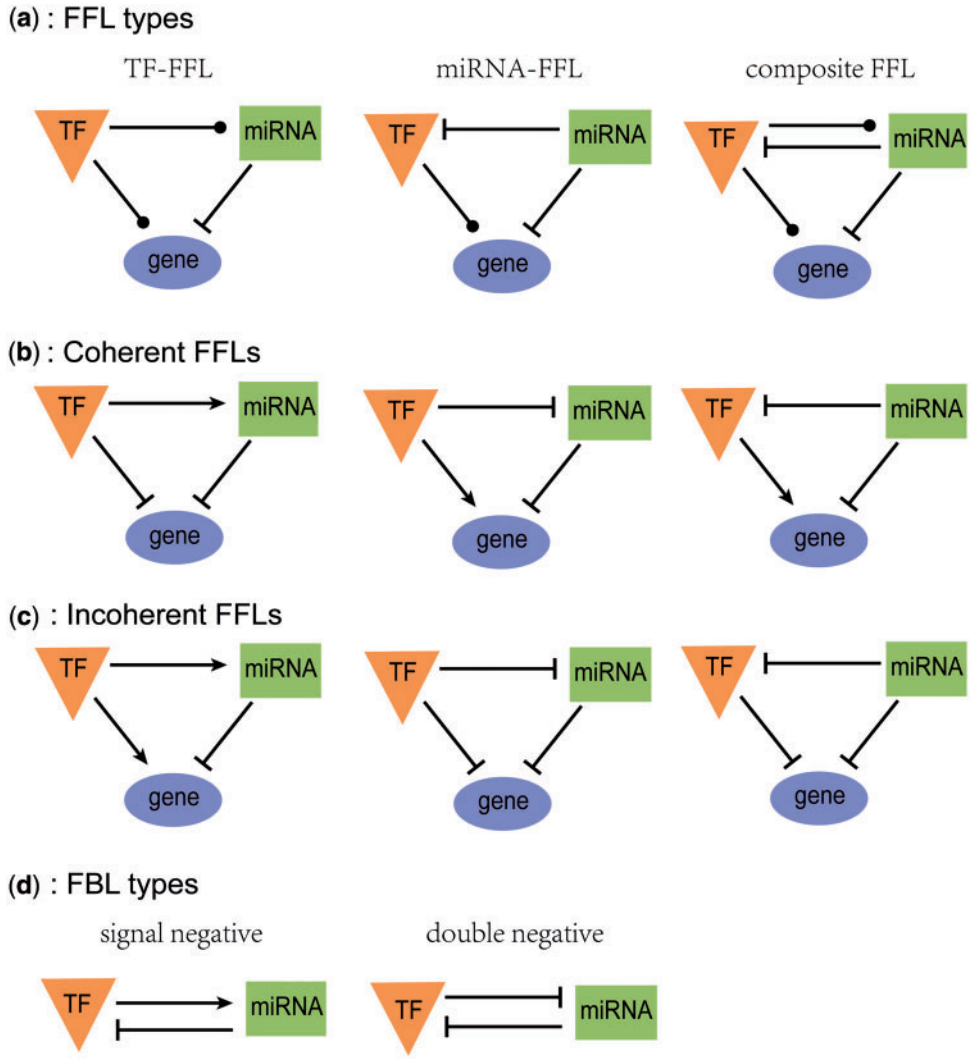

Figure 1: FFL and FBL types. (**a**) Three types of FFLs classified by the master regulator. Blunt arrows with dot end represent transcriptional activation or repression. (**b**) Coherent FFLs. In this kind of FFLs, two paths that regulate target gene have the same effects (either activation or repression). (**c**) Incoherent FFLs. The target gene is regulated by two opposite paths. (**d**) FBL types. Nodes: triangles are TFs; rectangles are miRNAs; ovals are genes; Edges: sharp arrow means activation; T-shaped arrow represents repression.

CONSTRUCTION OF TF-miRNA CO-REGULATORY NETWORKS

As the importance of TF-miRNA FFLs and FBLs was realized, several groups including us employed different strategies to identify these motifs and construct TF-miRNA co-regulatory networks [19,20].

Web servers and databases

Several web servers were developed by other groups to construct TF-miRNA regulatory networks through integrating gene and miRNA expression profiles with target prediction, such as dChip-GemiNI (http://www.canevolve.org/dChip-GemiNi/), MAGIA (http://gencomp.bio.unipd.it/magia2/) and mir ConnX (http://www.benoslab.pitt.edu/mirconnx) [21–23]. The dChip-GemiNI ranks the predicted FFLs by their explanatory power to account for the differential expression of genes and miRNAs between two biological conditions [21]. MAGIA performs an integrative approach to detect TF-FFLs and miRNA-FFLs [22]. MirConnX combines sequence information with gene expression data to identify all kinds of FFLs [23]. Both of MAGIA and mirConnX can accept the control/disease and time-series data, which expands their application range. MIR@NT@N (http://mironton.uni.lu) is a framework that predicts regulatory networks and subnetworks, including FFLs and FBLs,

based on interactions among TFs, miRNAs and genes obtained from a large-scale database [24]. In addition, several databases were constructed for TF-miRNA co-regulation. For example, CircuitsDB is a database devoted to the study of human and mouse TF-miRNA FFLs by predictions [25]. This database also characterized MYC-specific FFLs through collecting experimentally validated interactions of MYC, miRNA and their joint targets from independent databases [26]. TransmiR curated >700 TF-miRNA regulatory entries in 16 organisms from several hundred publications [27].

Each of these web tools or frameworks has their unique characteristics, but they share many common elements in the process of identifying FFLs and constructing network. According to these methods, we summarized a universal and comprehensive pipeline for constructing the TF-miRNA co-regulatory networks. The key steps are identifying regulatory relationships among TFs, miRNAs and targets.

TF–gene regulation

There are ~2000 TF genes in the human genome [28,29]. However, only partial targets of a small group of TFs have been detected based on the results of individual and high-throughput experiments. Chromatin immunoprecipitation (ChIP) followed by sequencing (ChIP-seq) or by microarray hybridization (ChIP-chip) are two high-throughput technologies to detect TF binding regions in genome-wide and have greatly accelerated the identification of TF targets. However, they also generate a large number of false positives, which can be reduced by integrating the expression profile of TF perturbation [30].

To date, there are several strategies to obtain TF targets by experiments and predictions. (i) We can collect the experimentally verified TF targets from publications or databases such as TRANSFAC [31]. (ii) The huge amount of ChIP-seq and ChIP-chip data generated by ENCODE or other projects are useful resources for TF targets identification. These data are available at NCBI-GEO, UCSC genome browser, CHEA (http://amp.pharm.mssm.edu/lib/chea.jsp) [32] and Factorbook.org (http://factorbook.org/) [33]. (iii) The TF binding sites (TFBSs) of several hundreds of human TFs have been characterized by experiments and these TFBS matrices are available in the JASPAR and TRANSFAC databases [31,34]. We can predict TF targets through searching for these TFBS matrices in gene promoters. UCSC genome browser also provides predicted TFBS data across the genome. In practice, we could integrate several of these methods and data to obtain comprehensive and accurate TF targets.

TF–miRNA regulation

Currently, most miRNAs are believed to be transcribed by RNA Polymerase II [35]. Therefore, TFs will bind to the miRNA promoter regions to either activate or repress their transcription. All of those methods for predicting TF target genes can also be applied to predict TF target miRNAs. However, there are some differences on the promoter identification between protein-coding genes and miRNAs. For miRNAs in a cluster, which are transcribed as a single unit [36], it prefers to use the upstream region of the first pre-miRNA as the promoter of the miRNA cluster. In another way, there are intronic and intergenic miRNAs according to their locations relative to protein coding genes. Several studies reported that some intronic miRNAs may be transcribed with their host genes and some are not co-expressed [37–39]. For the intronic miRNAs transcribed with their host genes, the promoters of the host genes should be used. For the intronic miRNAs that transcribe independently and the intergenic miRNAs, the upstream regions of their pre-miRNAs are used as the promoters. Thus, the promoter regions of different kinds of miRNAs should be obtained separately when we plan to predict the TFs regulation on miRNAs.

miRNA–gene/TF regulation

Up to now, there are more than a dozen of miRNA target prediction algorithms, such as TargetScan [40], miRanda [41], PITA [42], Pictar [43] and RNAhybrid [44]. However, different prediction algorithms are not consistent with each other. Thus, users often combine the results of several prediction algorithms to reduce false positives. In addition, some studies combined target prediction algorithms with miRNA and mRNA expression profiles to detect miRNA targets, such as miRNAMap 2.0 [45] and MiRonTop [46]. MiRNA–target interactions verified by experiments were also collected by several databases. TarBase [47] and miRTarBase [48] manually curated the experimentally verified miRNA targets for nearly 20 species. MiRecords not only curated the validated targets but also predicted miRNA targets with 11

established prediction programs [49]. MiR2Disease provided a resource of miRNA dysregulation in diseases and collected the verified miRNA targets [50]. In practice, we may combine the experimentally verified miRNA targets with predicted targets by different methods.

Construction of TF–miRNA regulatory networks

According to the definition of FFLs and FBLs in Figure 1, they can be easily extracted based on the predicted and/or validated relationships among TFs, miRNAs and genes. Fisher's exact test and randomization processes are usually used to evaluate whether the FFLs and FBLs are significantly enriched from genome background. The detail of these two test methods can be found in the previous studies [3,19,51]. These FFLs and FBLs would be merged together to form a comprehensive TF-miRNA regulatory network, which can be visualized by software such as Cytoscape [52]. The workflow for constructing TF–miRNA regulatory networks is shown in Figure 2.

TF-miRNA CO-REGULATION IN CELL PROLIFERATION

Cell proliferation is an important process during the growth and development of individuals, which is regulated by a complex network consisting of

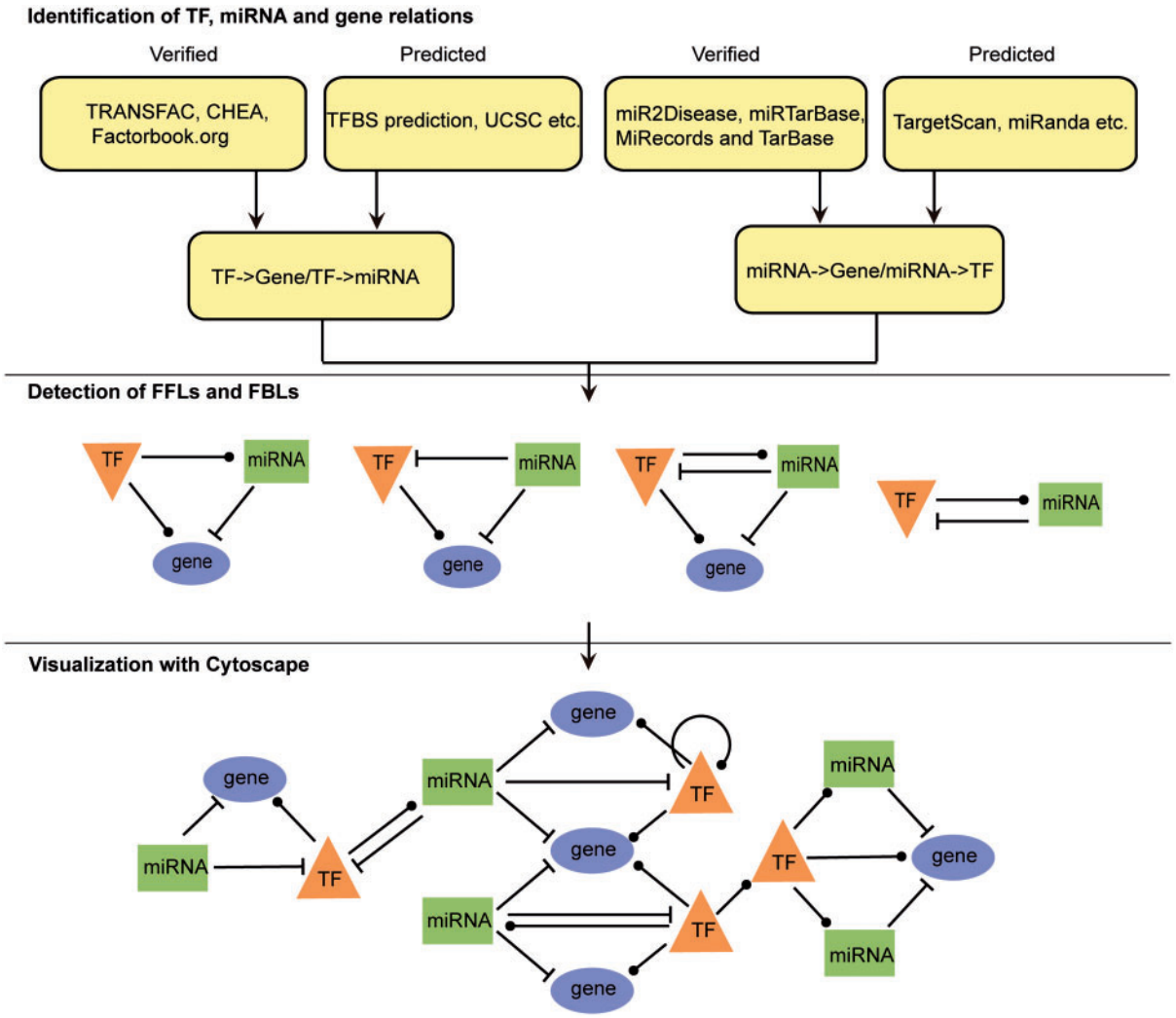

Figure 2: A workflow for constructing the TF-miRNA co-regulatory network. There are three steps in this process. Firstly, obtaining the regulatory relations among TFs, miRNAs and genes based on the experimentally verified or predicted data. Secondly, the FFLs and FBLs are identified by the relations obtained in the first step. Finally, the TF-miRNA co-regulatory network is constructed by merging the FFLs and FBLs together.

miRNAs and TFs. TFs of E2F family are activated at the G1/S phase boundary and play critical roles in controlling cell cycle progression by regulating the timely expression of genes required for DNA synthesis [53]. E2F1, one member of E2F family, can directly activate the transcription of miR-17–92 cluster, which in turn represses its translation [54] (Figure 3). Similar interactions have been proposed for E2F1 and miR-17–92 paralog clusters miR-106b-25 and miR-106a-363 [55,57]. Thus, E2F1 stimulates its own transcription in a positive autoregulatory loop and prevents its excessive accumulation in negative FBLs by activating miR-17–92 and its paralog clusters (Figure 3). E2F1 activity is repressed by pocket proteins (e.g. pRb and p130). This repression can be released by miRNAs in the above clusters through silencing key members of the E2F pathway such as CDK inhibitors (e.g. p21 and p57) and pocket proteins. However, this release is repressed by E2F1 itself through activating these genes (Figure 3) [55]. In summary, E2F1 balances its protein level and activity through forming single-negative FBLs and incoherent FFLs with miRNAs (Fig. 3). These incoherent loops regulate the protein level of E2F1 to achieve optimal activity and ensure the progression of cell cycle. Furthermore, these miRNA clusters also impart a buffering effect, where they protect E2F1 activation against fluctuating signals.

However, the balancing and buffering effect between E2F1 and miRNAs in this network can be broken by oncogene c-Myc or tumor suppressor p53 (Figure 3). Several studies reported that c-Myc could activate the expression of members from the above miRNA clusters, and then lead to the deregulated and hyperactive E2Fs in cancer cells [18,56,58]. For example, c-Myc activates miR-17 and miR-20a to avoid a G1 checkpoint arising from the untimely accumulation of E2F1 [58]. Tagawa *et al.* [59] reported that the enforced expression of the miR-17–92 cluster cooperates with c-Myc in mice to promote formation of B-cell lymphomas. In addition, c-Myc promotes the progress of the cell cycle through forming a positive FBL with E2F1 directly [60]. P53 indirectly inhibits E2F1 through different ways, including activating the expression of CDK inhibitors and activating the miR-34 family members, which target crucial cell-cycle effectors such as Cdk4, Cdk6, cyclin E2 and E2Fs [17,61]. Ran Brosh *et al.* [55] elucidated that p53 repress the expression of miRNAs in these three clusters in an E2F1-mediated manner. P53 inactivation or overexpression of representative miRNAs of these three clusters promotes cell proliferation and delays senescence.

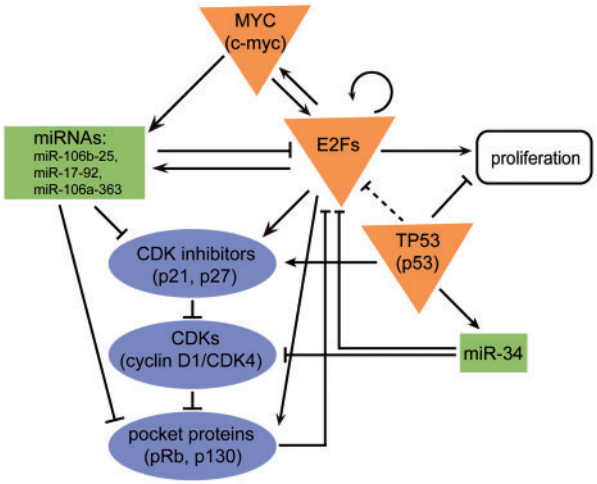

Figure 3: A schematic model for TF-miRNA co-regulatory network in cell proliferation. The E2F family and three miRNA clusters form several composite FFLs with CDK inhibitors and pocket proteins. They corporately control the progression of the cell cycle. The oncogene c-Myc can promote cell cycle progress through directly activating the E2F family and miRNA clusters, while the tumor repressor p53 represses E2Fs activity in an indirect way. The meanings of sharp arrows and T-shaped solid arrows are same as Figure I. T-shaped dotted arrow indicates the indirect repression of P53 to E2Fs. This figure is drawn based on two previous articles [55,56].

TF-miRNA CO-REGULATION IN CELL DIFFERENTIATION AND DEVELOPMENT

The combined TF-miRNA regulatory motifs, especially FBLs, have emerged as popular regulatory mechanisms in cell differentiation and development. In this section, we will introduce the important functions of FBLs and FFLs using the following identified examples.

FBL in epithelial–mesenchymal transition

The EMT occurs during embryologic development and adult tissue remodeling, and is viewed as an essential early step in the metastasis of epithelial-derived tumors [62,63]. MiR-200 family and TFs ZEB1 and SIP1 (also known as ZEB2) induce this transition through establishing a double-negative

FBL (Figure 4a). In epithelial cells, a stable state is maintained by the high levels of miR-200 family, which inhibits the expression of ZEB1/SIP1. Thus, miR-200 family increases the expression of ZEBs-repressed epithelial genes such as E-cadherin, which plays a crucial role in the cell–cell adhesion and the maintenance of tissue architecture. In turn, a mesenchymal state is instigated through high expression of ZEB1/SIP1 in response to the transforming growth factor beta (TGFβ) induction [15,64], which will repress miR-200 family. Once the levels of miR-200 fall below a threshold, the mesenchymal phenotype will be maintained.

FBL in skeletal myogenesis

It is reported that some miRNAs and TFs are involved in muscle development and muscle-related diseases. Among them, TF Yin Yang 1 (YY1) and two miRNAs (miR-1 and miR-29) play critical roles in skeletal myogenesis through forming double-negative FBLs (Figure 4b) [16,65]. In myoblasts, the constitutive activity of NF-κB activates YY1 expression, which subsequently suppresses two inducers of myogenesis miR-1 and miR-29. The downregulation of miR-1 releases Pax7, which is required for satellite cell proliferation and prevents myogenesis (Figure 4b) [66]. Thus, the high expression of YY1 maintains myoblasts in an undifferentiated state. At the onset of myogenesis, YY1 is downregulated by decreased NF-κB signaling, which leads to the upregulation of miR-1 and miR-29. In turn, miR-1 and miR-29 further decreases the expression of YY1. Pax7 also is downregulated by miR-1 (Figure 4b). Thus, the differentiation from myoblasts into myotubes is ensured by the high expression of miR-1 and miR-29 [16].

FBL in neuronal cell fate decision

Taste receptor neurons of *Caenorhabditis elegans* have two alternative fates 'ASE left' (ASEL) and 'ASE right' (ASER), which share many bilaterally symmetric features. These two terminally differentiated states require the activity of a set of regulatory factors that interact with one another in a bistable, double-negative FBL [67,68]. In ASEL neurons, the ASEL-specific inducer genes (TF die-1 and miRNA lsy-6) activate other ASEL-specific effectors (lim-6, flp-4, flp-20 etc.) and repress ASER-inducers (TF cog-1 and mir-273) (Figure 4c), which determines the stable state of ASEL. Similarly, in ASER neurons, ASER-inducer genes (TF cog-1 and mir-273) are expressed and maintain the stable ASER state through activating ASER effectors (gcy-5, gcy-22, and hen-1) and repressing the ASEL-inducer genes (TF die-1 and miRNA lsy-6) (Figure 4c). As a result, the inducer genes of ASEL and ASER jointly form a double-negative FBL, which affects the neuronal cell fates decision with ASEL and ASER effectors [67].

FBL in midbrain dopaminergic neuron maturation

Midbrain dopaminergic neurons (DNs) are the main source of dopamine in the mammalian central nervous system and these cells are lost in Parkinson's disease [69]. Kim *et al*. [70] identified that miR-133b is specifically expressed in midbrain DNs and regulates the maturation and function of midbrain DNs within a single-negative FBL. In this FBL, miR-133b is induced by Pitx3 and in turn downregulates Pitx3 activity (Figure 4d). Such kind of FBL has been shown to speed response time and provide the stability in the context of dynamic changes [71]. Midbrain DN function is dynamic, thereby this FBL limits the fluctuant ranges over which the concentrations of Pitx3 and miR-133b.

FBL in cardiogenesis

Cardiogenesis is regulated by serum response factor (SRF) and miR-133a-2 in a single-negative FBL [72]. The TF SRF is able to enhance the expression of miR-133a-2, which in turn represses the level of SRF (Figure 4e). Niu *et al*. [72] have found that SRF activity is tightly regulated through this miR-133a-2–dependent negative FBL and the delicate balance of miR-133a-2–directed silencing of SRF activity is profound in human heart disease.

FFL in photoreceptor differentiation

The double-negative FBL is prevalent in cell differentiation and development. However, it brings an intrinsic risk that stochastic fluctuations make cells rapidly flip between alternative cell decisions [8]. Robustness is imbued by the coherent FFLs, which has been characterized in photoreceptors differentiation of *Drosophila melanogaster* [11]. The miR-7 and TF Yan control this differentiation process in a FFL way. In progenitor cells, Yan is activated by Notch signaling and then represses miR-7 directly. Yan also repress miR-7 transcription indirectly through Ttk69, a transcription repressor, which is targeted by Phyl ubiquitin ligase (Figure 4f). Thus, the high expression of Yan stabilizes the progenitor cells state. EGF

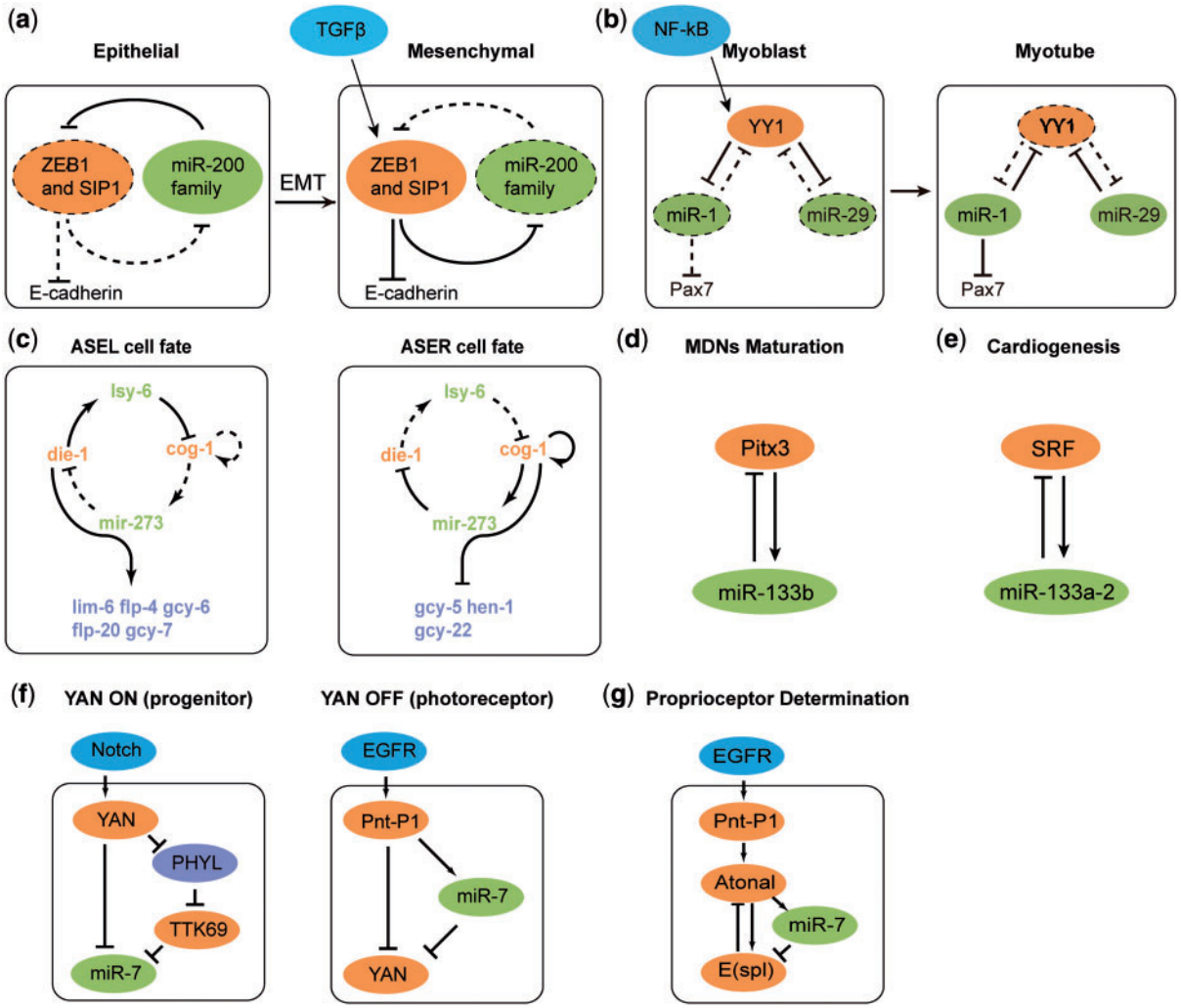

Figure 4: FFLs and FBLs in cell differentiation. Orange ovals are TFs; green ovals are miRNAs; light blue ovals are upstream signals. Dotted line means the activation or repression is inactive; dotted oval means the gene or miRNA is repressed or in a low expression. (**a**) The FBL between TFs ZEBI/SIPI and miR-200 family in EMT. In epithelial cells, ZEBI and SIPI are repressed by miR-200 family. EMT is induced when ZEBI and SIPI are activated by the TGFβ signal and miR-200 family is repressed. (**b**) The FBLs in skeletal myogenesis. The high expression of TF YYI activated by NF-κB signal maintains the undifferentiated states of myoblast cells. At the onset of myogenesis, the down-regulation of the NF-κB-YYI pathway leads to an upregulation of miR-1 and miR-29, which ensures myoblast cells properly differentiate into myotubes. (**c**) The FBL in neuronal cell differentiation. The high expression of TF die-1 and miRNA lsy-6 or TF cog-1 and miR-273 determines the taste receptor neurons of *C. elegans* to differentiate into 'ASE left' or 'ASE right'. (**d**) The FBL in midbrain dopaminergic neurons (MDNs). MiR-133b is induced by TF Pitx3 and in turn down-regulates Pitx3 activity. (**e**) The FBL in cardiogenesis. The activity of TF SRF is tightly regulated through this miR-133a-2 dependent single negative FBL. (**f**) Two FFLs in the differentiation of photoreceptor. The expression level of TF YAN determines this differentiation process. (**g**) The FFL in proprioceptor determination. The sustained activation of EGFR signaling to TF Ato results in sustained repression of TF E(spl) by miR-7 and the stable expression of Ato, and thus induces the proprioceptor determination. A colour version of this figure is available at BIB online: http://bib.oxfordjournals.org.

signaling induces progenitor cells to differentiate into photoreceptors by activating another coherent FFL: Pnt-P1 directly represses Yan and activates miR-7, which in turn represses Yan (Figure 4f). This FFL upregulates miR-7 and promotes Yan degradation, relieving miR-7 from repression. Coherent FFLs of this type, in which X activates Y, and both of them repress Z, generates a delay or persistence that rejects fluctuations in X [73]. This buffering stabilizes cellular responses and ensures that the cell switches from one state (Yan ON) to the other state (Yan OFF) when there is a persistent decrease in Yan [11].

FFL in proprioceptor determination

Precursor cells of proprioceptor transiently express the Atonal (Ato) gene in proneural cluster (PNC), which enables a subset of PNC cells to adopt a sensory organ precursor (SOP) fate, and SOPs then proceed to form the sensory organs. The process of SOP determination is controlled by two inducers miR-7 and Ato and one repressor E(spl) in an incoherent FFL (Figure 4g). In this loop, Ato directly activates the transcription of E(spl), and also indirectly represses E(spl) expression through activating miR-7. In addition, E(spl) feeds back to Ato to create a single-negative FBL and is interconnected with the FFL. In this network, the sustained activation of Ato by EGFR signaling results in a sustained repression of E(spl) by miR-7 and a stable expression of Ato, which induces the SOP determination [11].

TF-miRNA CO-REGULATION IN DISEASES

The abnormal expression of TF and miRNA is usually associated with diseases. In cancers, miRNAs and TFs can function as oncogenes or tumor suppressors in a cooperative way, thus triggering global alteration of gene expression. In this part, we will introduce the functions of TF-miRNA FFLs and FBLs in cancers and other diseases.

Cancers

MiRNA and TF are important regulators in all kinds of cancers. However, their FFLs and FBLs were only analyzed in a few of well-studied cancers. Acute myeloid leukemia (AML) is characterized by the rapid growth of hematopoietic precursors blocked at different differentiation stages, such as erythroid, or granulocytic [74]. Two studies reported that miR-223 and three TFs (C/EBPα, NFI-A and E2F1) play critical roles in granulocyte differentiation and the occurrence of AML (Figure 5a) [75,76]. Fazi *et al.* [76] elucidated granulopoiesis is controlled by miR-223 and two TFs C/EBPα and NFI-A through forming a regulatory circuit. In promyelocytic cells, the NFI-A maintains low miR-223 expression through binding to the miR-223 promoter. During granulopoiesis, C/EBPα is highly expressed, which replaces NFI-A from the miR-223 promoter and upregulates miR-223 expression. In turn, miR-223 represses NFI-A, thus subtracting it from the competition with C/EBPα and maintaining sustained high level of miR-223 expression. On the other hand, miR-223 represses the translation of E2F1 resulting in inhibition of cell-cycle progression and myeloid differentiation [75]. However, C/EBPα is deregulated in AML and the activation of miR-223 is inhibited, which results in accumulation of E2F1. Moreover, overexpressed E2F1 inhibits miR-223 transcription, thus promoting myeloid cell proliferation and blocking differentiation [75]. Thus, miR-223 as a molecular switch is regulated by C/EBPα and E2F1 in granulopoiesis and in AML, respectively. It could be a therapeutic target for AML subtypes in which E2F1 inhibition is dysregulated [75].

T-cell acute lymphoblastic leukemia (T-ALL) is a highly aggressive hematologic malignancy. To study the regulatory mechanism, we constructed a TF-miRNA co-regulatory network specific for T-ALL through collecting T-ALL–related genes, miRNAs and TFs [51]. MiR-19 is an oncogene inhibiting the expression of tumor suppressor genes, such as PTEN and BCL2L11, and is overexpressed in T-ALL patients. MiR-19 and CYLD, as hubs in this network, form an incoherent FFL with NF- κB, in which NF-κB actives the transcription of miR-19 and CYLD, and miR-19 represses the translation of CYLD. In addition, CYLD can reduce the nuclear translocation of NF-κB (Figure 5b). This FFL suggests that miR-19 and CYLD play critical regulatory roles in the NF-κB pathway and provides a new mechanism for the sustained activation of NF-κB in T-ALL. NF-κB pathway has been proposed as a potential target for therapies of T-ALL [77].

Breast cancer is a common disease among women. MiR-34a acts as a tumor suppressor by blocking cell cycle arrest to G1-phase and inducing cell senescence and apoptosis in breast, colon, lung and other cell lines [61,78]. It is highly expressed in normal tissues and is reduced in several cancer types [79,80]. However, Peurala *et al.* [81] found that miR-34a has a high or medium expression in most of the breast cancer patients, which is associated with a lower risk of recurrence or death from breast cancer. The transcription of miR-34a is modulated by two TFs P53 and MAZ, and both of them can form FFLs with miR-34a and target genes (Figure 5c). Many of their targets are cancer-related genes, which are involved in several cellular pathways such as 'cell death' and 'cell cycle'. The target genes co-regulated by miR-34a, P53 and MAZ might be used in predicting clinical outcome [81].

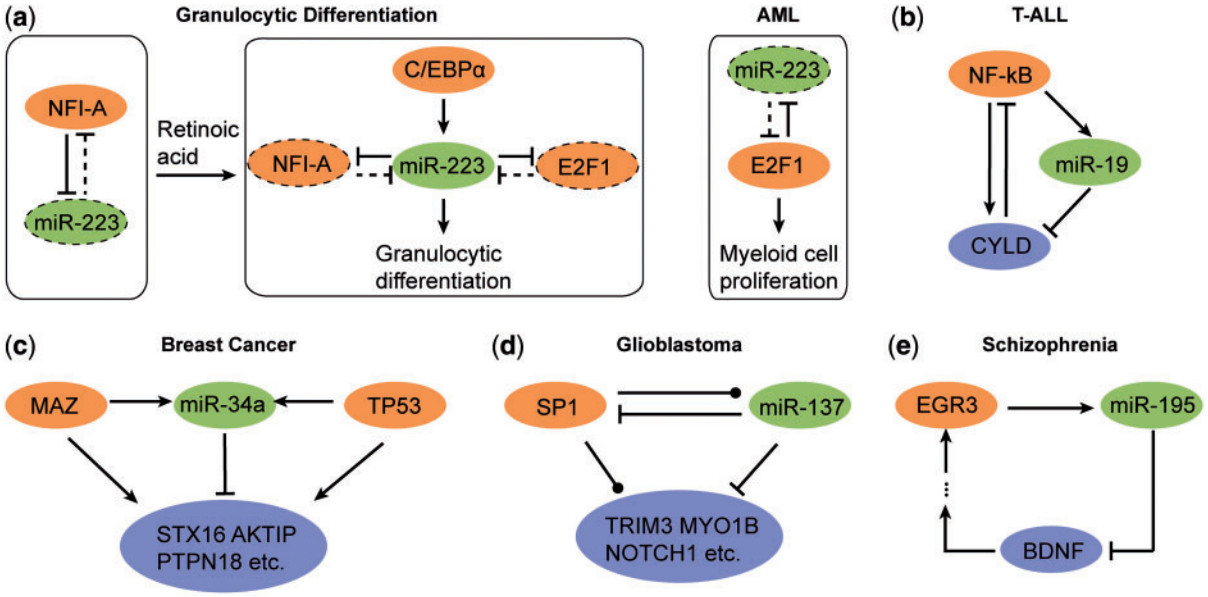

Figure 5: FFLs and FBLs in diseases. (**a**) The FBL in granulocytic differentiation and myeloid cell proliferation. In the undifferentiated cells, TF NFI-A maintains the miR-223 at low level. The TF C/EBPα is activated by retinoic acid and upregulates miR-223 expression, which in turn represses TFs NFI-A and E2FI, resulting in inhibition of cell cycle and advance of granulocytic differentiation (left). C/EBPα is deregulated in AML and overexpressed E2FI inhibits miR-223 transcription, thus promoting myeloid cell proliferation and blocking granulocytic differentiation (right). (**b**) A FFL in T-ALL. (**c**) The predicted FFLs in breast cancer. (**d**) A predicted FFL in glioblastoma. (**e**) A FFL in schizophrenia. TF EGR3 activates the transcription of miR-195, and in turn miR-195 indirectly reduces the expression of EGR3 by repressing gene BDNF.

Ovarian cancer is a malignant gynecologic neoplasm. Researchers have inferred TF-miRNA co-regulatory networks associated with its survival and recurrence through the simultaneous analysis of co-expression profiles and clinical characteristics of patients. In these networks, the aberrant expression of three miRNAs (miR-16, miR-22–5p and ebv-miR-BHRF1–2-5p), four TFs (FOS, EGR2, EGR1 and TGFB1) and many of their targets are associated with the survival and recurrence. Some of these molecules may be important biomarkers for the diagnosis of ovarian cancer [82].

Glioblastoma multiforme (GBM) is the most common and highest-grade brain tumor. Sun *et al.* have constructed a comprehensive GBM-specific TF-miRNA co-regulatory network through compiling GBM-related molecules and identified many 3-node and 4-node FFLs with statistical significance. In addition, they generated a subnetwork for the Notch signaling pathway, in which four TFs (EP300, SP1, TEAD1 and TBX5) and four miRNAs (miR-137, miR-34a, miR-9 and miR-92b) act as hubs. Figure 5d gives an example of the high-order subnetworks that form the GBM-specific TF-miRNA co-regulatory network [83].

Other diseases

Schizophrenia is a complex and severe mental disorder. Its pathophysiology and molecular mechanisms have remained poorly understood [84]. In our previous study, we analyzed the schizophrenia-specific TF-miRNA regulatory network, in which TF EGR3 and miR-195 are core regulators. In our result, we found that EGR3 is induced by BDNF via a PKC/MAPK-dependent pathway, and then EGR3 activates the transcription of miR-195, which in turn inhibits BDNF expression. Thus, EGR3, has-miR-195 and BDNF form a critical feedback regulatory loop (Figure 5e) [19]. This FBL implies that EGR3 negatively regulates its own transcription. The expression of EGR3 is decreased in schizophrenia patients [85] and it is a downstream gene of many other signaling pathways. Thus, EGR3 maybe play important roles in schizophrenia.

Interstitial lung disease (ILD) is a term that describes a broad category of restrictive lung disorders, which is associated with the dysregulation of many regulators and signaling pathways including TGFβ [86]. Cho *et al.* [87] conducted a system analysis of the molecular networks of ILD by identifying FFLs from the differentially expressed miRNAs, TFs

and genes. ZEB1 and miR-23a, as the top hubs of this network, play key roles in promoting disease progression. ZEB1, an inducer of the EMT, activates the transcription of miR-23a, which in turn represses the translation of NEDD4L. NEDD4L, an ubiquitin ligase, downregulates TGFβ pathway activity by triggering Smad2/3 and Tgfbr1 ubiquitination. Thus, ZEB1-mediated EMT stabilizes or enhances TGFβ signaling and promotes disease progression through activating the miR-23a clusters' regulation of Nedd4L protein levels [87].

CONCLUSIONS AND FUTURE CHALLENGES

The TF-miRNA co-regulatory network undoubtedly provides a comprehensive view of gene expression regulation at a systems level. It will be more powerful once reliable and complete data of miRNA and TF targets is incorporated. For the identification of miRNA targets, the high-throughput techniques CLIP-seq (cross-linking immunoprecipitation high-throughput sequencing) and CLASH (cross-linking, ligation and sequencing of hybrids) speed up this process. The CLASH technique developed by Helwak *et al*. [88] ligates and sequences miRNA–target RNA duplexes, thus can detect miRNA targets in a more accurate and high-throughput way. In addition, this work found that about half of the miRNA binding sites are not in mRNA 3′ untranslated regions (UTRs) but in the 5′ UTRs and coding sequence (CDS) regions based on the CLASH technology [88]. Thus, the miRNA target prediction and validation are still an urgent issue. Studies about TF target identification are also insufficient, although ChIP-seq and other methods accelerate the identification of TF targets. Much work has been done to reduce the false positives in ChIP-seq, but an integration of the ChIP-seq result with corresponding perturbed expression profiles of TF will make it more reliable.

To date, computational TF-miRNA regulatory networks are available for some genomes and many diseases [4,19,51,82]. However, experimentally verified regulations are only a small part of all the predicted network motifs. It is critical to speed up the confirmation of TF-miRNA co-regulatory networks to study their functions. Besides that, it is difficult to detect the powers of FFLs or FBLs in regulating gene expression or their impacts in diseases. As a trial to address this problem, the web sever dChip-GemiNI calculates the network motif score and the false discovery rate for each candidate TF-miRNA FFL and ranks them by their explanatory power to account for the differential expression of genes and miRNAs [21].

It is context-dependent for TF and miRNA regulating gene expression. The ChIP-seq results have shown that each TF can target various genes in different cell lines [89]. Neph *et al*. [90] also found that human TF networks are highly cell selective through analyzing the dynamics of 475 TFs across 41 diverse cell and tissue types. To obtain an accurate analysis of gene expression, it is essential to combine all the data from expression profiles of miRNAs and TFs, the high-throughput experiments of TFs, miRNAs target detection and the TF-miRNA co-regulatory network.

It will be crucial to integrate TF-miRNA regulatory networks with other functional networks, such as signaling pathways, metabolic pathways, protein–protein interaction networks and co-expressed signatures [20,91]. This integration will aid in explaining how these networks regulate the biological processes and diseases at the systems level.

Key Points

- TFs and miRNAs can jointly regulate gene expression in the forms of FFLs and FBLs, which influence many aspects of normal cells and diseases.
- FFLs and FBLs can be classified into different types based on the master regulator or the regulation effects of two paths on target. Different types of loops have different mechanisms in gene regulation.
- The identification of TF and miRNA targets is a key step for detecting FFLs and FBLs. It is better to combine the experimentally verified targets with predicted targets by different methods.
- FFLs and FBLs are popular regulatory models and critical for biological processes and diseases. FFL has a specific function in noise buffering effect. It can minimize the cell response to stochastic signaling noise and maintain steady-state levels of targets. FBL can act as a toggle switch between two different fates in cell differentiation.

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