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MicroRNAs have been found to be necessary for regulating genes implicated in almost all signaling pathways, and consequently their dysfunction influences many diseases, including cancer. Understanding of the complexity of the microRNA-mediated regulatory network has grown in terms of size, connectivity and dynamics with the development of computational and, more recently, experimental high-throughput approaches for microRNA target identification. Newly developed studies on recurrent microRNA-mediated circuits in regulatory networks, also known as network motifs, have substantially contributed to addressing this complexity, and therefore to helping understand the ways by which microRNAs achieve their regulatory role. This review provides a summarizing view of the state-of-the-art, and perspectives of research efforts on microRNA-mediated regulatory motifs. In this review, we discuss the topological properties characterizing different types of circuits, and the regulatory features theoretically enabled by such properties, with a special emphasis on examples of circuits typifying their biological significance in experimentally validated contexts. Finally, we will consider possible future developments, in particular regarding microRNA-mediated circuits involving long non-coding RNAs and epigenetic regulators.

Abbreviations

3'-UTR	3'-UnTranslated region
AGO	Argonaute protein
ceRNAs	Competing endogenous RNAs
DNFL	Double negative feed-back loop
epi-miRNA	Epigenetic-miRNA
FFL	Feed-forward loop
iMSL	Intronic miRNA self-loop
miRNA	microRNA
MREs	miRNA recognition elements
TF	Transcription factor
NGS	Next generation sequencing
lncRNA	Long non-coding RNA

In a complex organism, cell functioning, behavior and fate are spatially and temporally controlled by the topology and dynamics of gene regulatory networks. The primary regulation of gene expression is thought to be performed by transcription factors (TFs) and microRNAs (miRNAs), acting in composite gene

regulatory networks. The TF- and microRNA-mediated regulatory network in higher eukaryotes is characterized by an impressive degree of complexity. It is by now clear that this complexity can only be addressed by taking into account the interplay between the miRNA and the transcriptional layers of regulation. In the past ten years, studies on recurrent miRNA-mediated circuits in this combined regulatory network, also known as network motifs, have substantially contributed to addressing this complexity, and in particular to better understanding how miRNAs exert their regulatory roles.

Here, we review the current knowledge on miRNA-mediated regulatory circuits describing their occurrence in human gene regulatory networks, and their involvement in diseases and cancer. This review starts with a summary on miRNA biogenesis and functions. Next, miRNA-mediated regulatory networks will be elucidated, and the interplay between TF- and miRNA-mediated regulatory networks will be discussed in detail. In a subsequent section, we will provide some examples of experimentally validated miRNA-mediated

regulatory circuits with reference to diseases. A dedicated section deals with circuits involving miRNA and epigenetic regulators. Finally, to add perspective, we will introduce the role of long non-coding RNAs in the context of the miRNA-mediated gene regulatory network, and discuss some evolutionary aspects of the circuits presented.

Summary of miRNA biogenesis and functions

miRNAs are a class of small endogenous regulatory non-coding RNAs mediating post-transcriptional gene silencing by guiding Argonaute proteins to RNA targets. Originally discovered in *Caenorhabditis elegans* [1, 2], thousands of miRNA genes have been identified in animals, plants and viruses, with the last annotations reporting a total of more than 25 000 entries, around 2500 of which derive from the human genome (miRBase v.21, [3]).

Most miRNA DNA loci are transcribed by RNA polymerase II into long primary miRNA transcripts, called pri-miRNAs. Pri-miRNAs are cleaved by the RNase III enzyme Drosha, to originate ~100 nt long miRNA precursors (pre-miRNAs), which display a hairpin-like secondary structure. Pre-miRNAs are subsequently translocated by Exportin-5 into the cytoplasm, where another RNase III enzyme, Dicer, cleaves off the loop of the pre-miRNAs and generates mature miRNA duplexes. The two strands of the miRNA duplex pair with a few mismatches, and each strand usually has a 2 nt overhang at its 3' end. The duplex then loads onto an RNA-protein complex known as the RNA-induced silencing complex (RISC), where the unwinding of the duplex and the strand selection occur. Mature ~19–25 nucleotide long miRNAs may be generated from the 5' and/or 3' arms of the precursor duplex, and are called miRNA-5p and -3p, respectively. A complementary view on the complexity in genomic origins, biosynthesis pathways and sequence variations is covered in recent reviews [4–7]. The region encompassing the interaction between an miRNA and its target mRNA by Watson–Crick complementarity usually comprises nucleotides 2–7 of the mature miRNA, the so-called miRNA 'seed'. The complementary region is usually located at the 3'-UnTranslated region (3'-UTR) of the target mRNA, although several reports have also mapped miRNA binding sites at the mRNA 5'-UTR [8] and coding sequence region [9, 10].

The primary role of miRNA is in post-transcriptional gene repression through mechanisms of mRNA destabilization or translational inhibition. Since over half of the human transcriptome is predicted to be regulated by miRNAs, miRNAs are expected to be embedded in nearly every signaling pathway and, after more than 20 years of investigations, miRNA-induced mRNA repression has been related to a wide range of phenotypes observed at cellular and organismal levels, in normal and pathological contexts. miRNA expression profiling data sets are publicly available for large panels of cancer cell lines [11] and huge collections of tissues derived from both normal

and tumor samples [12]. miRNA gene expression analysis has collectively contributed to highlighting the complexity associated with miRNA expression regulation. In humans, mounting evidence has pointed at miRNA dysregulation in multiple diseases, including several cancer types, with different functional, tumor suppressor or oncogenic consequences in different contexts [13]. Essential roles have been ascribed to miRNAs in cancer onset, progression [14], and metastasis [15, 16]. Additionally, miRNAs are being regarded as biomarkers as well as RNA-guided therapeutic instruments [17].

miRNA-mediated regulatory networks

The current understanding of miRNA functions is crucially due to the identification of miRNA targets. *In silico* prediction of miRNA-target pairings has been an outstanding problem for bioinformatics research in recent years, since the binding event was shown to be influenced by multiple factors which were challenging to model effectively. As a result, a large body of computational algorithms has been developed [18–22], incorporating diverse experimentally derived criteria in order to reduce the false positive prediction rate. Some of the major features employed in the algorithms developed with this aim have included pairing requirement between miRNA seeds and target regions, even though several types of variants were tolerated (shorter or shifted versions of the seeds, imperfect matching, nucleation bulges, G:U wobble), evolutionary conservation, and physical properties of miRNA-target pairing such as miRNA energy pairing and site accessibility based on the analysis of secondary structure properties in the mRNA. Moreover, several predictive algorithms have exploited correlative expression patterns between miRNAs and their targets, as well as target enrichment in biological signaling pathways or gene ontology categories. Adopting different methodologies can result in different individual miRNA target predictions, but a few general features emerge unambiguously. The interaction network collectively revealed to date shows that single miRNAs are able to regulate a large number of target genes, and that single genes can be under the simultaneous regulation of several miRNAs in a sophisticated combinatorial fashion [23–25]. Careful analysis of functional data suggests the presence of much more complex patterns of interactions than naively expected within the majority of reported miRNA-related datasets, especially in case of detected effects of up-regulation or down-modulation upon miRNA. Several reports, in fact, have highlighted a seeming contradiction between the impact of miRNAs on many aspects of cell physiology and pathology, and the observation of small changes undergone by most predicted miRNA targets, at the mRNA and protein levels, upon miRNA expression perturbation [26–35].

miRNAs with identical seed sequences are usually assembled into families, since—according to the

standard model of gene regulation—they share similar targets, even if they are often encoded from distinct genomic positions, therefore having potentially completely distinct transcriptional regulation [22]. In fact, the majority of miRNAs are grouped in transcriptional units, and coordinately expressed [36]; reports suggest that miRNAs actively co-transcribed, even if belonging to distinct seed families, are more likely to target genes involved in common functions or pathways [37].

Well-established genome-wide approaches, such as RNA immunoprecipitation (RIP) and crosslinking precipitation (CLIP) and variants thereof [38–41], were employed to experimentally identify endogenous mRNAs bound by miRNAs. Unleashing the advantages of next-generation sequencing (NGS) technology for the detection of bound mRNAs, these approaches provided an unprecedented platform for exploring the specificity and spread of miRNA action *in vivo*, even if the amount of false positives and false negatives is still challenging to assess. The number of experimentally validated miRNA-target interactions stored in on-line accessible databases has now reached the order of magnitude of several thousands, encompassing single miRNA-gene interactions (both at the level of RNA down-modulation and protein translation inhibition) or multiple miRNA-gene relationships usually derived from high-throughput experiments [42–45]. Moreover, experimental approaches have been used in studying the transcriptional regulation of miRNAs, and a number of resources made available [46–48]. All these resources should be used with some caution, due to the intrinsic biases originating from any type of literature-based survey.

Even though a number of *in silico* and *in vivo* approaches have been employed in recent years to tackle the essential issue of discovering miRNA targets, some aspects are worthy of mention, such as the variability in the degree of evidence to establish miRNA-target interactions, the obstacles in assessment of accuracy for miRNA target predictions, and the harmonization of context-specific effects derived from *in vivo* approaches. Finally, in line with the network oriented perspective adopted in this review, a crucial point is that deeper analysis of functional data also suggests the presence of a large amount of indirect interaction in computationally or experimentally derived miRNA-target data sets.

Interplay between TF- and miRNA-mediated regulatory networks

In a series of seminal papers [49–51], the interplay between miRNAs and TFs in a mixed regulatory circuit, where transcriptional and post-transcriptional regulatory interactions are connected together, was proposed as a guiding building block in regulatory networks for the fulfillment of developmental genetic programs (see box 1). The inclusion of miRNA-mediated interactions within these regulatory circuits was in particular proposed as

a vehicle evolved to buffer stochastic perturbations in mRNA or protein levels inside cells.

Purely transcriptional networks have been recurrently associated with a scale-free topology, whose distinctive features were identified in the presence of regulatory hubs, and in the presence of several recurrent wiring patterns, called network motifs [52, 53]. The most commonly accepted viewpoint is that network motifs were selected by evolution (and are thus over-represented in the real network) to perform specific elementary regulatory functions [54]. Motifs containing TFs, despite different combinations, show peculiar evolutionary properties in close correspondence with the genomic events that shaped TF repertoire during phylogeny [55].

In mixed regulatory networks comprising transcriptional and post-transcriptional interactions, in particular those mediated by miRNAs, the influence of TFs on miRNA-mediated interactions and vice versa was initially assessed by computational means [23, 56–58], and more recently also confirmed by direct experimental evidence [59]. Analysis of large amounts of experimental data derived from the Encyclopedia of DNA Elements (ENCODE) project, and of *C. elegans* data from the modENCODE project, confirmed that distinct classes of miRNA-controlled circuits are particularly enriched in real networks [60, 61].

We report in figure 1 a few examples of these network motifs. These specific processing units were found to be over-represented in human and mouse regulatory networks, irrespectively of whether regulatory networks were inferred by pure computational analyses or derived from experimentally validated data [23, 56–58, 60–66]. Notably, even if the precise set of circuits can vary according to the network inference algorithm, the observation of such circuits turns out to be highly robust with respect to the algorithm details, with the global number of such regulatory motifs probably reaching the order of magnitude of thousands for the human case.

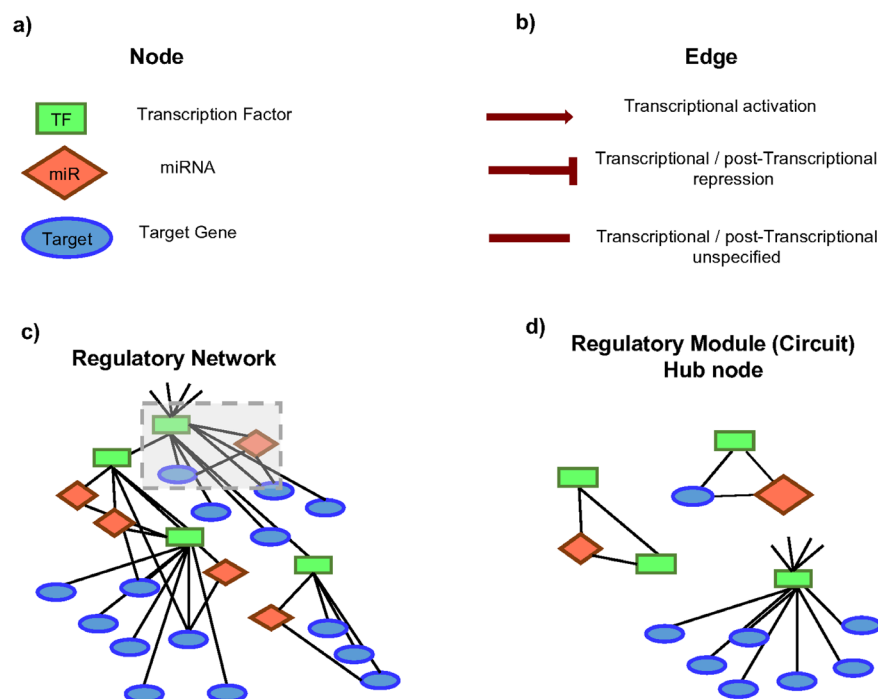
At the level of single miRNAs, single-cell measurements of the levels of proteins encoded by mRNA targets in the presence and absence of modulation by miRNA, highlighted that the miRNA-mediated regulation can establish a threshold level of target mRNA, around which the miRNA can act both as a switch and as a fine-tuner of gene expression [67].

Considering their well-established association with diseases, we now focus on the case of miRNA-mediated feed-forward loops (FFLs, see figure 1(a)), i.e. situations in which a TF regulates an miRNA, and together with it a set of common target genes. The considerable amount of mathematical modeling which followed the identification of FFLs, mainly through bioinformatics methods, contributed to a substantial improvement in our understanding of their functional properties [68]. Two classes of circuit were originally mapped, called coherent and incoherent, depending on the sign of the transcriptional regulations [56]. In the coherent (type II)

Box 1. Regulatory Network basic components and glossary

We summarize here some basic notions of network study. Interested readers can find extensive introductions to network theory in several recent text books, like [103, 104].

- a. Regulatory Network components** A typical regulatory network described in this review can be modeled as a graph composed of three different kinds of constituents (nodes), namely transcription factors (TFs), microRNAs (miRNAs) and Target Genes. TFs are proteins that are able to modulate the rate of transcription of a set of target genes, by binding to a specific cis-DNA sequence usually located upstream of the regulated loci. miRNAs are a class of small endogenous regulatory non-coding RNA molecules that are able to modulate the expression of a set of target genes at the post-transcriptional level, usually by binding to short sequences primarily but not exclusively located at the 3'-UTR of the regulated mRNA. In our scenario, a Target Gene is intended to be a protein-coding gene, whose final gene-expression pattern can be carefully controlled in every cell type and in a time-dependent manner by the mutual influence of a combinatorial pattern of transcriptional and post-transcriptional regulatory interactions. At the same time, both miRNAs and TFs can themselves be regulated at the transcriptional level (indegree > 0, outdegree > 0).
- b. Regulatory Network interactions** Regulatory connections between network nodes can in principle exert both positive and negative effects on target gene expression. A TF can enhance or repress the Target Gene's transcription, with the aid of co-activators or co-repressors. Conversely, the direct regulation exerted by miRNAs is predominately negative, acting through translation inhibition and mRNA degradation. In the scenario considered here, a Target Gene is a node that can be the target of a regulatory pattern both at the transcriptional and post-transcriptional level, but cannot itself act as a further active regulator (indegree > 0, outdegree = 0).
- c. Regulatory Network structure** A typical regulatory network is usually composed of thousands of nodes connected together. Human TFs amount to a few thousand; miRNAs to around two thousand. Each of the approximately ~25 000 annotated protein-coding human genes could be subject to multiple transcriptional or post-transcriptional regulations, thus resulting in putatively many thousands of edges for the global human regulatory network.
- d. Regulatory Network recurrent patterns** Regulatory networks can be particularly studied in terms of smaller functional units and prioritization of most relevant nodes. Of special relevance are 'network motifs', which are circuit topologies frequently recurring in biological systems. In this review, we have focused on miRNA-mediated network motifs, or circuits, i.e. circuits where at least one of the elements is an miRNA. 'Hub nodes' (genes or miRNAs) are nodes with an exceptionally high number of connections and thus neighbors. Both network motifs and hub nodes are usually characterized by important biological functional properties.



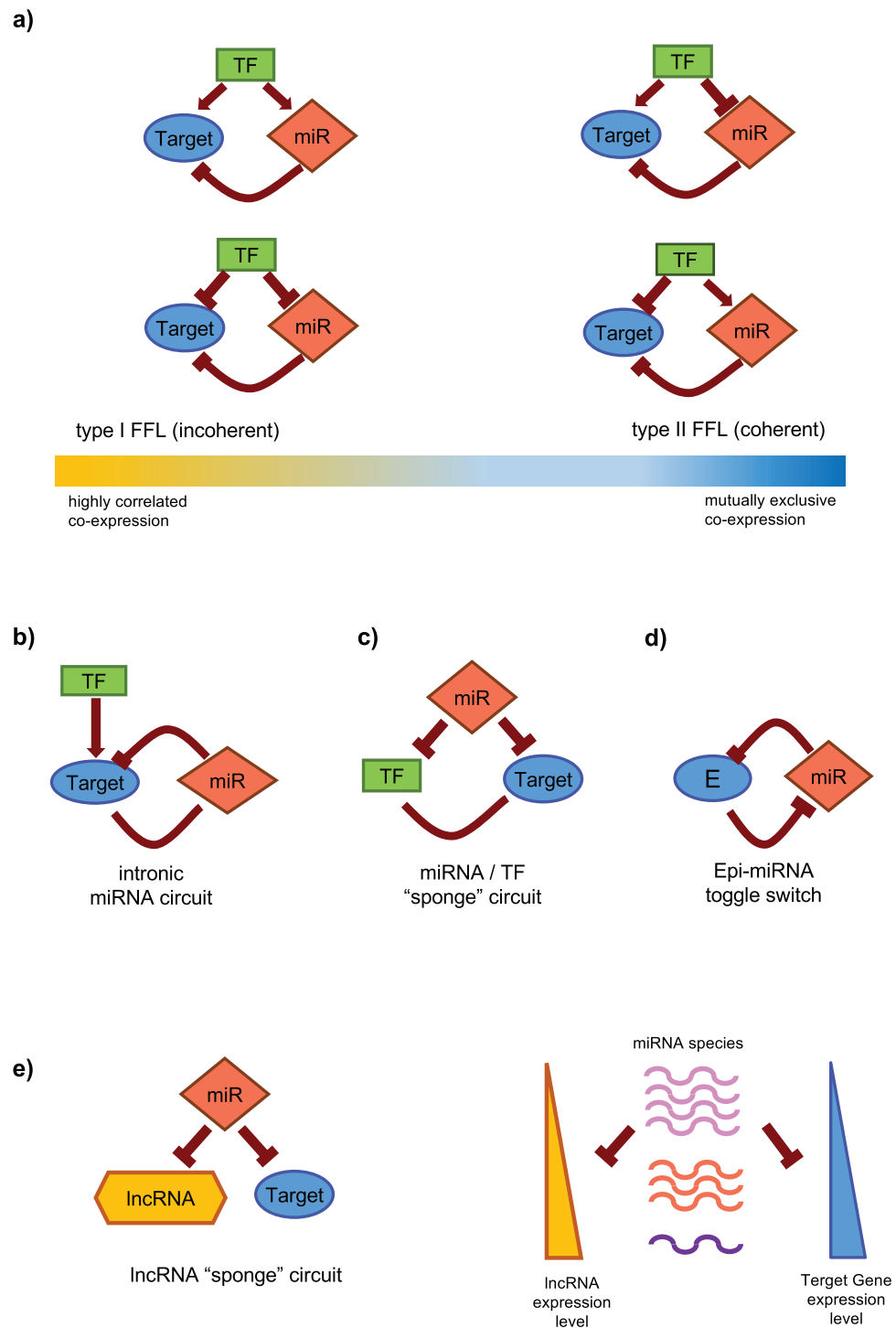


Figure 1. Overview of selected miRNA-mediated regulatory circuits. (a) Schematic representation of a typical miRNA-mediated feed-forward loop (FFL). An miRNA-mediated FFL is composed of a master TF, that regulates an miRNA, and a joint Target Gene. An miRNA usually acts as a negative regulator on its target gene, whilst a TF can activate or repress the target's gene expression. miRNA-mediated FFLs can be classified as type I (incoherent) or type II (coherent) FFLs, depending on whether the transcription of the miRNA and the target is co-regulated or oppositely regulated by the common TF. As a result, the direct and indirect pathways from TF to target exert incoherent regulatory effects on the target in type I circuits, whereas both pathways coherently regulate the target in type II circuits. Different expression patterns emerge in the two cases: co-expression of miRNA and its target for incoherent FFLs, and mutually exclusive expression for coherent ones. (b) Schematic representation of a typical intronic miRNA-mediated self-loop (iMSL). This type of network motif is a variation of the circuit represented in the upper panel, being characterized by a master TF that regulates both an miRNA and a host gene encoded by a single genomic locus. In this configuration, the miRNA is often located inside an intron of the host gene, and is transcribed together with it; therefore, the TF operates the same type of regulation on both the miRNA and the host gene. (c) Schematic representation of a generalized miRNA controlled FFL, in which an miRNA plays the role of master regulator, having a TF as target. In this topology, both activating and repressive TF-target interactions are in principle allowed. (d) Schematic representation of a DNFL involving an epigenetic regulator. Here, an miRNA targets an Epigenetic regulator E , which in turn controls the expression of the same miRNA. In the configuration showed, the regulation of miRNA on target gene and the regulation of E on the miRNA are both negative. (e) Schematic representation of a putative 'sponge' circuit, in which lncRNAs are supposed to influence the level of target genes through an miRNA-dependent mechanism. The miRNA can act on both the mRNA and lncRNA sequence via miRNA recognition elements (MREs) located in the mature messenger sequences. As result of the sponge effect, mRNA-lncRNA interacting species should give rise to correlated patterns of expression levels.

FFLs both the direct and indirect pathways from the TF to the target gene have the same, repressing or activating, effect, while in the incoherent (type I) ones the two pathways exert opposite effects. Correspondingly different expression patterns emerge in the two cases: co-expression of miRNA and its target for incoherent FFLs and mutually exclusive expression for the coherent ones (figure 1(a)). The functionality of coherent circuits is thought to be a post-transcriptional reinforcement of transcriptional regulation by contributing to the elimination of the already transcribed mRNA when transcription is switched off; as such, coherent circuits are apt to avoid spatial co-expression of the miRNAs and their targets [69]. Stochastic modeling and simulations of incoherent circuits showed that this kind of network motif, apart from regulation of spatial co-expression, is appropriately designed to act as a molecular device to control biological noise. Incoherent FFLs can in fact bridge the fine-tuning of a target protein level with an efficient noise control, thus conferring precision and stability to the overall gene expression program, especially in the presence of fluctuations in upstream regulators [70]. It is worth noting that this noise-buffering behavior can be shown to increase in miRNA-mediated FFLs with respect to purely transcriptional FFLs, and is functionally active for a range of model parameters largely in agreement with experimental data [70]. Strikingly, the integrated analysis of *in silico* target prediction, miRNA and gene expression data for the reconstruction of post-transcriptional regulatory networks effectively enable the generation of catalogues of miRNA-mediated FFLs from genomic data [71].

A major lesson emerging from mathematical modeling is that, despite simple topologies, the miRNA-mediated motifs are able to perform fairly complex biological functions. This was confirmed also to be the case for intronic miRNA-mediated self-loops (iMSL, figure 1(b)), where a master TF regulates a single genomic locus, encoding for both an miRNA and a host gene, the host gene itself being a target of the miRNA [72]. Specifically, iMSL modeling indicates that this circuitry can alter the dynamics of the host gene expression, inducing complex responses like adaptation (i.e. the ability of a system to respond to a change in the input and to subsequently return to the original state, even if the stimulus persists) and Weber's law (i.e. the magnitude of the response depends only on the fold change of the input signal). Moreover, they can efficiently filter fluctuations propagating from the upstream network to the host gene [73].

A combination of TF- and miRNA-mediated interactions can be assembled also in FFLs where the master regulator is an miRNA and where one of the targets is a TF (figure 1(c)). In these FFLs, the miRNA concentration can act as a controlling parameter to fine-tune the TF/target ratio to any desired threshold. These circuits are also able to ensure the stability of the TF/target ratio against stochastic fluctuations. It is interesting to note that the peculiar topology of these circuits allows

interpreting the behavior of the TF and target concentrations as the result of TF and target competition for miRNA binding (the so called 'sponge effect'). A genome wide survey of these FFLs in the human regulatory network highlighted a strong enrichment in all the situations in which the TF and its target have to be precisely preserved at the same concentration in presence of external environmental noise [74].

We would like to conclude the brief overview of miRNA-mediated regulatory circuits by mentioning the interactions of miRNAs with regulatory factors other than TFs within circuitry topologies different from the previously discussed FFLs. miRNA-related network motifs have recently emerged in several reports studying the interplay between miRNAs and epigenetic regulators [75–78]. Composite epigenetics and miRNA regulatory circuits (epi-miRNA circuits) consist exclusively of miRNAs and epigenetic regulators. Recent studies showed that epigenetic mechanisms, including DNA methylation and histone modification, commonly regulate the expression not only of protein-coding genes but also of miRNAs. At the same time, epigenetic regulators including DNA methyltransferases, histone deacetylases and Polycomb group proteins can be regulated by a subset of miRNAs. These mutual interactions give rise to complicated feedbacks between miRNA and epigenetic pathways, potentially able to influence the entire profile of gene expression in a given cell. Similarly to considerations previously posed for purely transcriptional regulatory networks, the epi-miRNA network also displays a few recurrent circuits. Among these circuits a special role seems to be played by the double negative feed-back loop (DNFL, figure 1(d)) in which an miRNA (or, in some cases, a set of miRNAs acting in cooperative way) targets an epigenetic regulator, which in turn controls the expression of the same miRNA(s). This network motif, which is usually called 'toggle switch,' can perform diverse types of functions like supporting bi- or multi-stability, noise buffering and oscillation. Indeed, this kind of circuit was shown by mathematical modeling to exhibit a switch-like behavior between two alternative steady states, while being robust to stochastic transitions between these two states [78]. The interplay between miRNAs and epigenetic regulators is currently still relatively unexplored, and can be considered an open field of investigation in the near future.

Examples of experimentally validated miRNA-mediated regulatory circuits involved in diseases

Switching back at an experimental level, the biological role for miRNA-mediated circuits is not fully yet captured. We consider some of the best examples for showing the extent to which the organization of regulatory interactions in feedforward loops underlie genotype-phenotype relationships in diseased conditions.

TF: c-Myc; miR: miR-17-5p|miR-20a; target: E2F1

One of the initial attempts to functionally characterize feedforward loops focused on the proto-oncogene c-MYC, which encodes a transcription factor regulating cell proliferation, growth and apoptosis. Dysregulated expression or function of c-Myc is one of the most common abnormalities in human malignancy. Chromatin immunoprecipitation experiments showed that c-Myc binds to a locus containing a cluster of six miRNAs, thus activating their expression. Two miRNAs in this cluster, miR-17-5p and miR-20a, were shown to negatively regulate the transcription factor E2F1, an additional target of c-Myc promoting cell cycle progression. These findings revealed a mechanism through which c-Myc simultaneously activates E2F1 transcription and limits its translation, allowing a tightly controlled proliferative signal [79].

TF: P53; miR: miR-34; target: MET

An additional example comprises p53, which controls the expression of the proto-oncogene MET by two mechanisms, consisting of suppression of MET at the transcriptional level via promoter repression and on the post-transcriptional level via transactivation of miR-34. The mechanism of p53-dependent suppression of MET is not uniquely identified, since it has been attributed to direct binding of MET promoter by p53 as well as to an indirect mechanism based on inhibition of SP1 binding to DNA through physical interaction between p53 and SP1. Alterations in individual components of the circuit may modulate the course of pathological processes by affecting the extent of cancer invasion, since MET is a crucial regulator of invasive growth [80].

TF: E2F; miR: miR-106b/93/25; target: CDK inhibitors

Another study elucidated a feedforward loop involving E2F1 and a group of cancer-related miRNAs. In this case, E2F1 was found to transcriptionally control the miR-106b/93/25 polycistron and its paralogs. E2F1 and these miRNAs were shown to regulate together a mutual set of target genes. In concordance with the growth acceleration that resulted from the overexpression of these miRNAs, many of their targets are considered anti-proliferative cell-cycle regulators [81].

TF: AP-1; miR: miR-101; target: MMP9

Another feedforward loop, which involves AP-1, miR-101 and common metalloproteinase targets, was associated with migration and invasion of hepatoma cells [82]. AP-1 was found to bind preferentially to the 12-O-tetradecanoylphorbol-13-acetate (TPA) responsive element in the gene promoter or enhancer, and thereby to modulate expression of target genes, like several matrix metalloproteinases (MMPs). This study revealed that AP-1 directly activates miR-101. AP-1 and miR-101 share common targets, namely MMP1, MMP3, MMP9, CD44 and IL-8. The study showed that the dysregulation of this feedforward loop enhances the

activity of MMP9, and thus promotes the migration and invasion of hepatoma cells.

TF: NumB; miR: miR-34a; target: Notch

A recent study [83] identified an incoherent feedforward loop formed by NumB and miR-34a, targeting Notch in early-stage colon cancer stem cells (CCSCs). The analysis showed that this loop enables a robust binary switch between stem and non-stem cell fates robustly. Subversion of this feedforward loop by Numb knockdown degrades Notch bimodality, and gives rise to an intermediate subpopulation of cells with ambiguous and plastic cell fate. Furthermore, this cell fate determination switch plays a role in mouse intestinal stem cells (ISCs). Silencing of the miR-34a-mediated switch was shown to inhibit ISC asymmetric division and contribute to CCSC-like proliferation in stressed tissue. Hence, this study indicated that miRNAs and protein cell fate determinants coordinate to enhance robustness of cell fate decision, and they provide a safeguard mechanism against stem cell proliferation induced by inflammation or oncogenic mutation.

Perspectives for the study of miRNA-mediated regulatory circuits

Summing up, this overview of both theoretical considerations and experimental observations highlighted the substantial contribution of systems biology approaches to decipher the operational functioning properties of miRNA-mediated circuits. Considerations and examples here reported have been focused on the human genome, with human disease and cancer biology as master models. However, similar properties for miRNA-mediated regulatory circuits have also been detected and analyzed in completely independent models, for example in plants [84]. While past studies have usually dealt with individual regulatory interactions, it has become clear that to understand the regulatory activity of a eukaryotic genome is mandatory to directly address the complex nature of the interconnected circuits used as building blocks for the whole systems. Despite their simple topology, essentially all the examples used in this review were shown to be able to generate complex biological functions. In order to fully understand these functions, mathematical modeling approaches based on deterministic as well as stochastic approaches are required [68]. A key application of the constantly growing knowledge on miRNA-mediated circuits is the assessment of the utility of the modeling frameworks proposed to date for the effective interpretation of the extensive molecular profiles of clinical samples [85]. Thinking beyond the simple understanding of the basic mechanisms of operation of a regulatory network, a complete integration of mathematical models with such experimental data could also ultimately generate responses of therapeutic value.

The role of long non-coding RNAs in miRNA-mediated regulatory networks

As a major breakthrough of recent genomic projects, particularly in the case of higher eukaryotes, a vast part of the genomes is reported to apparently encode for so called long non-coding RNAs (lncRNAs), i.e. messenger RNAs with length >200 nts with little or no coding potential [86]. These lncRNAs are localized both in the nucleus and in the cytoplasm, are characterized by lower and very tissue-specific levels of expression compared to protein-coding mRNAs, and have been associated with a large variety of different functions, especially during organism development [87]. For our purposes, it is important to note that a large portion of lncRNAs are known to be transcribed from canonical POLII promoters in 5'-capped and 3'-polyadenylated poly(A), as for conventional mRNAs. Interestingly, lncRNA messengers have been shown to harbor oligonucleotide sequences corresponding to miRNA seeds, and have been proposed to allow Watson–Crick interactions similar to those mediated by miRNA recognition elements (MREs) in the 3'-UTRs of protein coding genes. Albeit controversial [87, 88], these considerations suggest that lncRNAs could act as competing endogenous RNAs (ceRNAs) partners for standard protein coding genes. Computational studies suggest that these RNAs could operate in regulatory miRNA-mediated circuits with a significant portion of protein-coding genes (figure 1(e)).

Studies supporting this idea are mainly based on two types of analysis. First, both *in silico* genomic sequence analysis and sequence data derived from experimentally supported interactions suggest that MREs are generally abundant in lncRNA transcripts [89]. Second, analysis of matched mRNAs, lncRNAs, and miRNAs expression data suggest that, at least phenomenologically, the observed correlation patterns could be associated with miRNA-mediated sponge effects arising between mRNA and lncRNA pairs preferentially located in the cytoplasm [90, 91]. As a further example, computational studies have highlighted that lncRNA–miRNA–gene interactions are active in cancer, and may have prognostic value for predicting clinical outcome in cancer patients [92].

Interestingly, mathematical modeling allowed quantification of the maximal post-transcriptional regulatory power achievable by miRNA-mediated cross-talk in case of ceRNA circuits. Current results suggest that, in addition to its widely-recognized noise-buffering role, miRNA-mediated control may indeed act as a master regulator of gene expression [93, 94].

The role of evolution in miRNA-mediated regulatory networks

Understanding miRNA-mediated regulation in the light of evolution [95] is one of the major open problems in modern regulatory genomics. To date, a thorough analysis of miRNA-mediated regulatory circuits from an evolutionary perspective is still missing, and several aspects could be subject to major further investigations.

As stated above, the original observation that some specific miRNA-mediated regulatory circuits are enriched in real biological networks led to the conjecture that these patterns were *bona fide* selected throughout evolution for their specific functional role. At the system level, the over-representation in real biological networks of small miRNA-mediated regulatory motifs is viewed as indicating the importance of the coordinated activity of TFs and miRNAs. In this context, computational studies unveiled the special role of miRNAs in connecting components of a gene regulatory network with respect to TFs from an evolutionary viewpoint: miRNAs and TFs were shown to have different contributions to the coordinated final networks, with their mutual cooperation proved to shrink the target gene repertoire but at the same time to increase the properties of redundancy and buffering or fine-tuning or shut off leaky expression in the global network [96].

Alternative hypotheses were proposed, even if mainly grounded on purely transcriptional networks: common structural features of transcription networks could appear due to the intrinsic trend of evolution to converge towards certain types of network patterns [55, 97]. Theoretical investigations showed that network motifs, and in particular certain specific types of regulatory circuits, can be seen as the practical realization for a cell of molecular mechanisms capable of achieving specific algorithmic requirements [98]. Recently, analytical results in the case of fold-change detection (FCD) circuits explicitly identified five minimal circuits that optimally trade off speed, noise resistance, and response amplitude, among which the two experimentally observed are present [99], but the role of miRNAs in these contexts needs to be clarified.

Nonetheless, switching back to molecular considerations, it is important to point out that the single network components (nodes) of the regulatory circuits presented in this review are constituted by at least three different molecular species, viz. a TF, an miRNA and a target protein coding gene, each of them characterized, in principle, by specific and completely distinct evolutionary histories. Evolutionary differences between TFs and miRNAs are especially relevant, due to their completely different molecular nature, with a great impact also in the rules determining the corresponding target genes (individual edges in the regulatory network) [100]. In most cases, the evolutionary constraints adopted by miRNA-target and TF-target prediction algorithms force the resulting network to be intrinsically built on conserved elements and interactions. Networks derived from high-throughput experimental data are in this sense somewhat less biased, thus incorporating, at least in principle, species-specific regulatory interactions. Nowadays the complete genomic sequences of tens of living organisms are available, as well as high throughput experimental data for dozens of TFs and miRNAs in a variety of experimental conditions and species. Therefore, in principle, the fulfillment of systematic investigations based on real data, regarding the emergence and evolution of the

cooperation of known molecules in functional units, is conceivable.

Out-of-equilibrium behavior of miRNA-mediated regulatory motifs

As we have seen above, in the last few years there has been a remarkable improvement in our understanding of regulatory motif dynamics. Several different network motifs can now be precisely modeled both at the deterministic and at the stochastic level. What is more interesting, these models are now sophisticated enough to capture not only the equilibrium properties of the various molecular species involved in the motif but also their off-equilibrium dynamical behavior. This makes it possible to address non-equilibrium problems which were once out of reach [101]. A prototypical example is the study of the so called ‘first passage time’, i.e. the time needed for a molecular species (say, a gene activated by a TF) to reach a given threshold [102]. Moreover, it is now possible to study the role of different network motif topologies in controlling the speed and precision of such a process. This class of problems may have relevant biological implications, for instance the gene may be required to reach a threshold level of expression to trigger a specific downstream process, and it could be important to reach such a threshold as fast as possible and with the minimum amount of stochastic timing fluctuations. This problem has been currently addressed only for transcriptional regulatory motifs [101, 102]. It would also be very interesting to extend this type of study to the more complex situation of miRNA-mediated network motifs.

Conclusions

The main purpose of this review was to recapitulate investigations on the interplay between transcriptional and post-transcriptional regulatory layers, with particular interest in the human genome and in the implications for the biology of diseases and cancer. Intensive computational and experimental activities in recent decades have clearly shown how the complexity of the global gene regulatory network can be addressed only in terms of emergent properties resulting from the cooperation of individual molecules within functional units. In conclusion to this short resume focused on the properties of mixed TF- and miRNA-mediated regulatory motifs, we hope that the substantial progress reviewed here will encourage the synergistic development of theoretical and experimental approaches to elucidating the occurrence, composition, topology and function of regulatory circuits other than those here presented.

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