Weak Regulation of Many Targets Is Cumulatively Powerful—An Evolutionary Perspective on microRNA Functionality

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Abstract

Why do microRNAs (miRNAs) weakly repress so many targets such that most targets do not have phenotypic effects? An increasingly accepted view posits that weak targeting has no biological function and each miRNA effectively has only a few functional targets. Here, we review the evolutionary arguments for this postulate and find these arguments seriously flawed. In contrast, from the systems perspective, the power of broad and weak targeting may reside in the cumulative effects of all repressions, which collectively stabilize gene regulatory networks. This view predicts that miRNAs would show little tendency to downsize their target pools. A survey of "twin-miRs" production indeed validates this prediction.

Key words: **microRNA target, expression repression, mRNA degradation, regulatory network, network stability.**

Introduction

Many interactions in the gene regulatory network show strong effects on the expression of the targeted genes, leading to significant phenotypic consequences. Transcription factors are often involved in such interactions. In contrast, microRNAs (miRNAs), a major class of regulatory molecules (Bushati and Cohen 2007; Rottiers and Naar 2012; Garofalo and Croce 2015; Vidigal and Ventura 2015), remain a central conundrum in gene regulation. Individual miRNAs in animals are often shown to represses hundreds of target genes (Bartel 2009; Betel et al. 2010; Reczko et al. 2012; Agarwal et al. 2015). As a result, most repressions are weak, thus raising the question why miRNAs would dilute their regulation strength so broadly. The absence of a convincing explanation may be a reason for the declined interest in miRNAs in recent years, even though they may hold a key to understanding the behavior of the network.

A most straightforward view, which has gained wide acceptance (Flynt and Lai 2008; Ecsedi et al. 2015; Pinzon et al. 2017), asserts that the vast majority of weakly repressed targets are noises. Only a few strongly repressed targets that can exert phenotypic effects are biologically relevant. Whether this hypothesis of "few targets" ($\sim$10?) should replace the standard "many targets" ($>$100) view will be central to our understanding of miRNA functionality. Furthermore, the resolution may shed light on the behavior of gene regulatory networks. Several recent publications have provided empirical support and mathematical reasoning for the function of "many weak targets" at the level of the entire network, rather than at the level of individual genes.

The "Few Targets" Hypothesis—A Critique

There are three main lines of evidence for the "few targets" hypothesis—1) Few targets are conserved over long evolutionary time; 2) Most targets are too weakly repressed to exert a fitness effect; and, most contentious of all, 3) A very small number of miRNA targets govern each phenotype. Each argument will be reviewed below.

Evolutionary Conservation

A most common argument for the functionality of miRNA repression, strong or weak, is target conservation. Between mammalian orders, which should have lost most of the neutral sites in 160 (80×2) My of divergence, the number of conserved target sites for each miRNA is usually $>$100 (Friedman et al. 2009; Wu et al. 2009; Xu et al. 2013; Agarwal et al. 2015). However, there are two objections to the conservation argument. The first objection is "overconservation" referring to target conservation in species without the corresponding miRNA (Pinzon et al. 2017). This phenomenon seems to indicate that the observed target conservation is not due to miRNA functions but something else. Unfortunately, the objection conflates two phenomena—the lack of divergence due to conservation and the apparent conservation due to insufficient time to diverge. For example, since any 8-bp target site would have a $>$90% chance of being identical among apes, the miRNA target sites in chimpanzee would appear conserved for miRNAs that exist only in humans. This "anomaly" in target overconservation vanishes when more distantly related species are compared. Indeed, between vertebrates or between mammalian orders, there is no "overconservation" (Pinzon et al. 2017). In addition, Friedman et al. (2009), taking into account the background level of conservation, still predicted hundreds of conserved targets and thus invalidated the claim of overconservation.

A more compelling objection is "underconservation"—the low degree of target conservation in long term evolution. For

example, between vertebrates and invertebrates there is virtually no shared targets (<1% of the total [Chan et al. 2005; Chen and Rajewsky 2006]). Nevertheless, this lack of long term conservation is likely due to a shift in selective pressure, rather than the absence of it. Xu et al. (2013) found that miRNA target sites are conserved in a taxa-specific manner (Xu et al. 2013). For example, among ten mammals from different orders, there is much more target site conservation than between human and fish, even though the two sets of comparisons have both undergone ~800 My of evolution. It is shown that >80% of miRNA target sites are selectively constrained among mammals but, between mammals and fishes, <10% of the target sites appear selectively constrained (Xu et al. 2013). Therefore, the whole set of predicted miRNA target sites is likely under selective constraint at any given time but the set of conservative genes may shift over hundreds of millions of years.

Fitness Consequence

Second, the repressions by miRNAs (Baek et al. 2008; Selbach et al. 2008; Hausser and Zavolan 2014) are generally weaker than the variations of expression in natural populations (Rockman and Wray 2002; Cheung et al. 2003; Subkhankulova et al. 2008). Such weak repressions are commonly proposed to be biologically irrelevant, presumably on the assumption that natural variations are themselves neutral noises. However, this assumption contradicts population genetic analyses. For example, deleterious genetic variants are very common in natural populations (Fay et al. 2001; Subramanian 2011) and a portion of the expression variation should reduce fitness as well (Keren et al. 2016).

Given that miRNAs often weakly repress the mRNA level, their effects on phenotypes might be inconsequential unless the phenotype is very dose-sensitive. Pinzón et al. (2017) thus use haplo-insufficiency (HI) as the proxy for dose-sensitivity. HI genes are loci that produce a phenotype when one gene copy in the diploid genome is missing (Dang et al. 2008; Huang et al. 2010). Pinzón et al. found that the 299 HI genes curated by Dang et al. (2008) harbor significantly more miRNA target sites than the background. Although the logic is reasonable, the choice of phenotypes is not. Many of the 299 HI genes produce a disease phenotype in old age. Such phenotypes may have little fitness consequence in human's evolution.

Clearly, the relevant phenotype pertaining to miRNA repression should be fitness, or at least a fitness-related phenotype. If there are indeed only 299 HI genes pertaining to fitness, most loci in the human genome should be haplo-sufficient and we should have seen many null alleles in the survey of human polymorphism. But such observations have never been reported. It is premature to suggest that all except ~300 or so genes in the human genome are haplo-sufficient in fitness and their repressions by miRNAs do not have any consequence.

Phenotypic Control

The third, and perhaps the most contentious, argument is that the number of miRNA target genes for any given phenotype is often reported to be small, close to one. The experimentation supporting the argument follows the four steps: 1) The deletion of a miRNA leads to a phenotype in the knockout (KO) background; 2) Many putative target genes are shown to have an elevated expression in the KO line; 3) A target gene is nominated to be the candidate gene responsible for the phenotype; 4) Finally, if restoring the expression of this candidate gene (e.g., by RNAi) in the KO background results in full rescue of the phenotype, then this target gene is said to be principally (or even solely) responsible for the phenotype. Some studies suggest a single target gene for a given phenotype by this sequence of steps (Karres et al. 2007; Smibert and Lai 2010; Varghese et al. 2010; Weng and Cohen 2012; also see discussion in Ecsedi et al. 2015 and Pinzón et al. 2017).

By testing one single gene and reaching the "one major-gene" conclusion, these studies assume the absence of redundancy in controlling the phenotype. Without testing a good sample of many target genes, the assumption seems difficult to justify. Therefore, a particularly interesting approach has been used, which indeed suggests the *let-7* miRNA of *C. elegans* controlling the vulva rupture phenotype through the *lin-41* target gene alone. Mutating both *let-7* and *lin-41* in the same direction, Ecsedi et al. aim to retain the *let-7*/*lin-41* repression while removing all other repressions. By doing so, they did rescue the vulva rupture phenotype.

The *let-7*/*lin-41* experiment of Ecsedi et al. is currently the most widely cited proof of the single-gene hypothesis and the limited available proof might be attributed to the technical demand (of precise gene editing in vivo). However, the technique may not furnish such a proof. By changing only one bp in *let-7*, it is questionable whether all other repressions have been removed, as recently pointed out by Liufu et al. (2017; supplementary text S2). Furthermore, although other *let-7* family members cannot repress *lin-41* which has *let-7* specific compensatory pairing, they can repress other targets of *let-7* (Broughton et al. 2016). In addition, Hunter et al. (2013) reported >20 target genes of the same *let-7* miRNA affecting the same vulva phenotype in *C. elegans* (see Liufu et al. 2017 for further discussions).

Other studies that probe several target genes have also reported redundancy. For example, two out of the seven genes tested (*string* and *wingless*) are responsible for the same cuticular phenotype in miR-965 mutant flies (Verma and Cohen 2015). A systematic study of multiple targets and multiple phenotypes would be necessary. One such study is Liufu et al. (2017) who analyzed five target genes of the miR310s family cluster of two species of Drosophila. Three phenotypes are examined (eggshell morphology, egg hatchability and male fertility). In order to restore the target gene repression to the wild type level, the Gal4-UAS system is used, where the Gal4 is under the control of miR310s' own indigenous promoter. Their observations show that, for each phenotype, four target genes (out of five) control the phenotype. Although these five target genes are selected for possible functional connections, they represent only a fraction of the total target pool. Hence, redundant control seems widespread.

What is particularly surprising is the incoherent phenotypic control. The repression of each target by miR310s drives

the phenotype in opposite directions (Liufu et al. 2017). In two of the three phenotypes, one target gene enhances the phenotype whereas three other genes reduce it. The incoherent control removes a major hurdle in accepting the "many targets" view. If all targets drive the phenotype in the same direction, either the phenotypic value would be very high or the control must be extremely redundant. Multiple incoherent mechanisms that govern the same system would usually suggest stability control (Hornstein and Shomron 2006; Wu et al. 2009), much like the accelerator and the brake "incoherently" governing the speed of an automobile.

The "Many Targets" Hypothesis—A System-Level Solution

The empirical evidence thus supports the view of hundreds of targets being repressed by each miRNA. The repression of each target would naturally be weak and each individual gene's phenotypic effect is difficult to gauge. What then might be the functions of so many target genes?

A system-level hypothesis has been proposed whereby miRNAs function to stabilize gene regulatory networks (GRNs) (Hornstein and Shomron 2006; Wu et al. 2009; Posadas and Carthew 2014). Earlier studies invoke regulatory motifs comprising a few genes (or nodes). Motifs that play a stabilizing role include simple negative feedback (two nodes) and incoherent feed-forward (three nodes) loops (Hornstein and Shomron 2006; Wu et al. 2009; Posadas and Carthew 2014). Recent studies have extended the analysis to the level of GRN containing hundreds of nodes, with the smaller motifs embedded in it. For example, in RNA: RNA cross-talks, many RNAs participate in a common network where most target sites sponge off some miRNAs and indirectly influence the repressions of other targets (Salmena et al. 2011; Tay et al. 2014; Thomson and Dinger 2016). The RNA:RNA network permits competitive sequestration of miRNAs (i.e., sponging) and any target gene would experience the aggregate effects of all its competitors. Repression effects can thus be cumulative among all these competitors.

However, in the literature on the powers and limitations of RNA cross-talks (e.g., Thomson and Dinger 2016), a weakness is rarely addressed. According to the May–Wigner theory (May 1972, 2001), highly connected networks can be easily perturbed, thus departing from the equilibrium. Because RNA:RNA cross-talk networks, as defined, are densely connected, they may be inherently unstable. To build a theoretical framework for stability control, we suggest a lesson from ecology. If we compare GRNs to foodwebs (May 1972, 2001; Allesina and Tang 2012), the underlying dynamics can be similarly described by an interaction matrix, M, of size $n \times n$ where n is the number of nodes, be they species or genes. A node may positively influence another node, for example, a gene up-regulating another gene (or more rabbits leading to more foxes). On the other hand, negative regulation may be at least as common, for example, more foxes leading to fewer rabbits. As May (1972) and subsequent studies (Allesina and Tang 2012; Chen et al. 2017) point out, large and highly interactive networks, especially those dominated by positive interactions, are difficult to stabilize. This may be a hint on why miRNAs always exert downward regulation on their targets.

The role of miRNAs in stabilizing GRNs has now been explicitly modeled (Chen et al. 2017). Let the off-diagonal elements of M, M_{ij}, represent gene interactions and the diagonal elements, M_{ii}, represent degradations (or death). According to the May–Wigner theory, the stability of the network depends on the sum of the diagonal elements, M_{ii}s. Since miRNAs contribute to target degradations, they contribute to this sum, $\Sigma_{i=1,\ N}\ M_{ii}$. Therefore, the effects of miRNA repressions on GRN stability are cumulative. Most important, it can be shown that, the wider the spread of repressions over different nodes is, the more stable the network becomes (Chen et al. 2017).

A Simple Test of the "Many Targets" Hypothesis

From the systems perspective on stability, it would be advantageous for miRNAs to spread the repression effect as widely as possible (When the spread becomes very broad, there would be counteracting forces; see Chen et al. [2017]). A most efficient way for miRNAs to increase the number of targets is to use both strands of the precursor for production (fig. 1A). Since the seed regions from both arms are distinct in sequence, the target pool would be roughly doubled. Here, we use the terms of major versus minor miRs to designate their relative abundance (in lieu of the older nomenclature of miR versus miR* where * may imply functional noise). In many reports, the existence of minor miRs has been noted (Ro et al. 2007; Okamura et al. 2008; Czech et al. 2009; Chiang et al. 2010; Ghildiyal et al. 2010; Kuchenbauer et al. 2011; Yang et al. 2011; Pundhir and Gorodkin 2015). However, because even the major form appears to have too many targets, the extra targets of the minor miR deepen the conundrum.

The conundrum disappears in the systems perspective, which makes it possible to quantify and compare the contributions of miRs across all loci. In such an analysis, the minor miR can be compared with the major miR of another miRNA. If we assume all major miRs are functional, then the more abundantly expressed minor miRs should be functional as well. We then examine a comprehensive data set of small RNA sequences in mammal (Meunier et al. 2013). As noticed before, miRNA abundance is highly uneven. In figure 1B, 18% of miRNA loci (101 of 566) account for 99% of total miR production (red and black dots where the 29 red dots, or 5% of all miRNA loci, account for 90% of miR production). As 82% of miRNA loci collectively contribute to a mere 1% of total miRs, most miRNAs are extremely lowly expressed. Furthermore, many of these miRNA loci are evolutionarily conserved and should have fitness contributions. Among the more highly expressed 101 miRNAs, about 60% of them use both arms for miR production (fig. 1B) and 14% of them express the

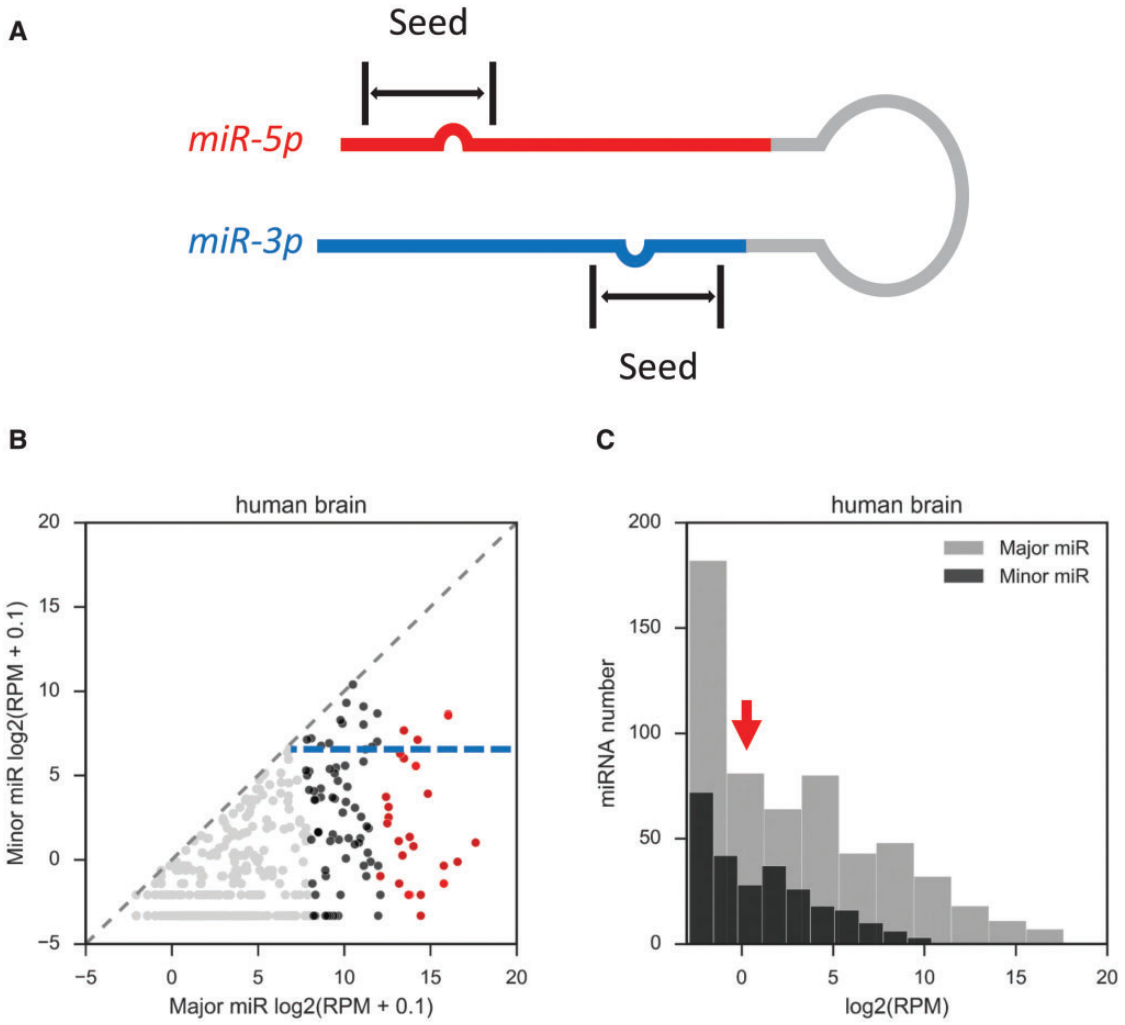

Fig. 1. Comparison of the expressions of major versus minor miRs. (*A*) The hairpin of a precursor miRNA has the potential to produce two mature products from both arms, denoted miR-5p (in red) and miR-3p (in blue), respectively. The seed regions are indicated. The major/minor designation depends on their relative abundance. (*B*) The sequencing data of human brains from a comprehensive data set (Meunier et al. 2013) is used to show the relative expressions of major versus minor miRs. Patterns from other tissues or other mammals are similar (data not shown). The scatter plot shows expressions of major versus minor miRs for each miRNA precursor. Highest expression miRs that account for 90% and 99% of the total reads count are indicated by red and black dots, respectively. The 17 miRNAs above the blue dashed line produce minor miRs that rank in the top 20% of all miRs in expression, both major and minor. (*C*) The distribution of expression abundance for major and minor miRs, respectively. The data are based on 566 miRNA precursors although only 258 of them produce minor miRs. The red arrow indicates the minor miR whose expression ranking is 129 (50% percentile) in all minor miRs. RPM, reads per million.

minor miR at >10% of the major miR in abundance. Some of those minor miRs with nonnegligible expression have been demonstrated to be able to repress their targets (Ro et al. 2007; Okamura et al. 2008; Zhou et al. 2010; Yang et al. 2011; Wu et al. 2013; Pink et al. 2015; Rhodes et al. 2015; Salah et al. 2015; Yonemori et al. 2016), and their sequence conservation also implies their functional importance (Okamura et al. 2008; Yang et al. 2011; Agarwal et al. 2015).

The comparison of all major and minor miRs is given in figure 1C where 50% of the minor miRs are shown to be more abundant than 40% of the major miRs (the red arrow). Otherwise, the distributions of expression of major and minor miRs are similar, albeit with different means. In total, 17 minor miRs are more highly expressed than 80% of the major miRs (dots above the blue dashed line of figure 1*B*). It is therefore fairly common to find miRNAs that produce twin-miRs, both of which being more highly expressed than the major form of many other miRNAs.

Conclusions and Implications

If most miRNAs' target genes are biologically irrelevant, as is often speculated, miRNAs should have been selected to reduce the size of the target pool, for example, by gradually shedding twin-miR production. Since the prediction is not supported, maintaining a large pool of targets is likely a central function of miRNAs. Recent works have suggested that broad repressions of target genes contribute to GRN stabilization via motifs (Hornstein and Shomron 2006; Wu et al. 2009; Ebert and Sharp 2012; Pelaez and Carthew 2012;

Posadas and Carthew 2014) or RNA cross-talks (Salmena et al. 2011; Tay et al. 2014). Chen et al. (2017) explicitly formulate the idea in the framework of the May–Wigner theory (May 1972, 2001). With the systems-level theory, the many weakly repressed targets of miRNAs could act collectively to stabilize the network.

Finally, Waddington (1942) proposed "developmental canalization" to explain stable development under unstable inputs. By stabilizing GRNs, miRNAs have been suggested to be the elusive "canalizing agents" (Hornstein and Shomron 2006; Wu et al. 2009) that ultimately contribute to stabilizing development and phenotype (Ebert and Sharp 2012; Posadas and Carthew 2014).

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