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Probing the microRNA pathway with small molecules

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Abstract

MicroRNA (miRNA)/RNA interference (RNAi) is recognized as one of the most important mechanisms regulating gene expression at the posttranscriptional level in eukaryotic cells. The main components within the miRNA/RNAi pathway are now known and well characterized, but studies on the molecular mechanisms that regulate the activity of the miRNA/RNAi pathway are just beginning to emerge. High-throughput reporter assays have been developed to monitor the activity of the miRNA/RNAi pathway and applied in a proof-of-concept pilot screening, which has led to the identification of some inhibitors and activators that either generally or specifically regulate the activity of the miRNA/RNAi pathway. In addition, combined with multidisciplinary approaches like proteomics, biochemistry, and genetics, some protein co-factors were found to play important roles in the regulation of the miRNA/RNAi pathway. Herein we highlight the high-throughput reporter assays developed in recent years and the resulting discovery of miRNA/RNAi enhancers and inhibitors.

Keywords

microRNA/RNAi pathway; reporter system; high-throughput screening; small molecule; chemical biology approach; enhancer; inhibitor

Introduction

RNA interference (RNAi) is recognized as one of the most important mechanisms regulating gene expression at the posttranscriptional level in eukaryotic cells, mediated by small non-coding RNA, including in particular interference RNA (siRNAs), microRNAs (miRNAs), and piwi-interacting RNA (piRNAs) with a length of 18–30 nucleotides (nt). These small RNAs regulates diverse metabolic pathways involved in chromosome architecture and segregation behavior and transcription and RNA processing and stability, which means it thereby phenotypically coordinates development, growth control, apoptosis, self defense, and stem cell maintenance (Hannon GJ, 2002; Bartel DP, 2004; Plasterk RH, 2006; Hwang HW, Wentzel EA, and Mendell JT, 2007; Carthew RW and Sontheimer EJ, 2009; Lee YS, et al, 2009). Emerging evidence shows that altering the expression profiles of miRNAs can contribute to the pathogenesis of a wide range of human diseases, as well as phenotypic abnormality among the eukaryotes (for reviews, please see Shi XB, et al., 2008), pointing to essential roles for miRNAs in the maintenance of normal metabolism. siRNA-based

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knockdown has been widely adopted as a powerful tool to uncover gene functions, reveal/validate drug targets, and develop therapeutic interventions. Given the pivotal roles of siRNA/miRNA in diverse biological pathways, as well as the broad application of RNAi, it is essential that we understand the regulatory mechanism of the miRNA/RNAi pathway. Although the main components of the miRNA/RNAi pathway have already been identified and characterized, studies into the regulatory mechanisms of the pathway itself have received scant attention until recent years. Although the use of a chemical biology approach to dissect the miRNA/RNAi pathway is still in its infancy, this approach has given us a better understanding of these regulatory mechanisms by uncovering a number of small molecules that could directly or indirectly target to specific steps in the miRNA/RNAi pathway. Herein we highlight the recent discovery of small molecules that could modulate the activity of the miRNA/RNAi pathway as revealed via chemical screen.

The miRNA/RNAi Pathway

In general, mature miRNAs are produced by two sequential biochemical processing events. First the pri-miRNAs, ranging from hundreds to thousands of nucleotides in length and bear one or more hairpin structures, are transcribed initially by RNA polymerase II, which are then 5'-capped, spliced, and polyadenylated in mammals (Kim VN, 2005; Carthew and Sontheimer, 2009; Kim VN et al., 2009; Du and Zamore, 2005). In most cases, the first processing step takes place inside the nucleus, where the pri-miRNAs are cropped by Drosha, an RNA endonuclease III in mammals, along with its partner DGCR8 (Kim VN, et al., 2009) to produce a ~ 70-nt stem-loop-structured precursor (pre-miRNAs) that harbors the miRNA in the 5' or 3' half of the stem (Lee Y, et al., 2003; Du and Zamore, 2005). The pre-miRNAs are then transported into cytoplasm by exportin5/RanGTP for secondary processing (Kim VN, et al., 2009; Okada C, et al., 2009; Du and Zamore, 2005; Bohnsack MT et al., 2004), which further cleaves the pre-miRNAs into mature miRNAs 18–25 nt in length by Dicer, another member of the RNase III family, along with its co-factors TAR RNA-binding protein 2 (TRBP) and PACT (Maniataki and Mourelatos, 2005; Du and Zamore, 2005; Lee Y, et al., 2006). Besides the general processing steps for mature miRNA biogenesis, some pre-miRNAs could be produced alternatively by bypassing the Drosha-mediated processing step. One example is “mirtrons,” the pre-miRNA-like hairpins that are generated by splicing and debranching of the short hairpin introns (Berezikov, et al., 2007; Okamura et al., 2007; Ruby JG et al., 2007). Besides “mirtrons,” some small nucleolar RNAs (snoRNAs), tRNAs, and endogenous short hairpin RNAs (shRNAs) can also be processed into miRNA-like molecules without relying on Drosha processing either (Babiarz JE, et al., 2008; Ender C, et al., 2008; Saraiya AA and Wang CC, 2008; Cole C, et al., 2009). More recently, another source of miRNAs, terminal hairpins of endogenous siRNA long-stem-loop precursors, was found to be capable of bypassing the Drosha processing step, as well (Miyoshi et al., 2010).

The mature form of miRNAs must be loaded into RISC complex to functionally regulate gene expression at posttranscriptional levels. It is generally believed that the guide strand is sorted to load into RISC complex to guide the miRNA to its target mRNA, while the passenger strand is thought to be degraded. Although not common, exceptional cases indeed exist. Recent deep sequencing results in *Drosophila* have indicated that a large number of passenger strands are functional and associated with AGO1 or AGO2, instead of being degraded (Czech B, et al., 2009; Guo Y, et al., 2010; Ghildiyal M, et al., 2010; Okamura K, et al., 2008; Seitz H, et al., 2008).

Dissecting the miRNA/RNAi pathway using a chemical biology approach

Chemical biology is defined as the application of chemical techniques and tools to the study and manipulation of biological systems in ways that use chemical principles to modulate systems to either investigate underlying biology or create a new function. It offers a more dynamic way to monitor the activity of specific pathways than a traditional forward or reverse genetic approach. The combination of chemistry and biology can help identify primary small molecules for drug development, as well as tell us more about the regulation of specific metabolic pathways. To conduct a chemical screen, reliable and robust reporter assays are essential. In recent years, several reporter systems were developed to probe the activity of the miRNA/RNAi pathway.

1. *In vitro* reporter assay monitoring the pre-miRNA processing

The first established *in vitro* fluorescence assay monitors the activity of Dicer (Davies and Arenz, 2006). In this system, pre-let-7 was reengineered to bear an RNA hairpin with a fluorescence emitter at the 5' terminus and a fluorescence quencher at the 3' terminus. In the case of the intact-engineered pre-let-7 without cleavage by Dicer, no fluorescence could be detected due to the close proximity of the fluorophore and the quencher. In contrast, the Dicer-mediated cleavage of pre-let-7 would emit fluorescence, and the cleavage activity of Dicer was proportional to the increase of fluorescence signal.

2. *In vivo* fluorescence-based reporter assay for monitoring the activity of the miRNA/RNAi pathway

An *in vivo* fluorescence-based reporter system was initially developed by transient co-transfection of plasmids harboring enhanced green and red fluorescent proteins (EGFP and RFP, respectively) into HeLa cells together with siEGFP against EGFP mRNA for degradation (Chiu et al., 2005). The co-transfected HeLa cells were then treated with a synthetic small chemical library. The criterion applied for evaluation of the drug-mediated siRNA effects was the ratio of EGFP/RFP fluorescence in the presence of siRNA calculated and normalized to the EGFP/RFP ratio of mock-treated cells. Using this strategy, two compounds, called ATPA-18 and ATPA-21, were found to specifically inhibit ATP-dependent events occurring during RNAi (Chiu et al., 2005). A series of *in vitro* and *in vivo* analyses demonstrated that these compounds specifically affected an early unwinding step in the RNAi pathway, suggesting that its target is an ATP-dependent RNA helicase, although the precise molecular target remains elusive (Chiu et al., 2005). While these compounds were the first inhibitors of siRNA activity, they had no effect on the endogenous miRNA pathway, establishing the timing of siRNAs' unwinding and suggesting that siRNA helicase activity is required for RNAi. The advantage of this assay system is its quick detection of alterations in fluorescence induced by small molecules. However, this strategy cannot be applied to identify the regulatory components upstream of the maturation of the miRNAs. In addition, the transient co-transfection of two plasmids combined with siRNA followed by drug treatments not only leads to variations in efficiency, but also makes the assay system too complicated for high-throughput screening.

3. GFP reporter assay in live cells

To independently develop an assay that could be used for the high-throughput screening of small molecule libraries, we infected HEK-293 cells stably expressing lenti-EGFP with lenti-short hairpin RNA against EGFP (shEGFP) to select the proper cell clones that are critical for the chemical screening. Certainly, a clone with strong knockdown of EGFP may have great potential to elevate the EGFP level when exposed to a compound(s) that inhibits RNAi activity, thereby enabling us to robustly identify inhibitors of the RNAi pathway. However, due to its limited potential to further decrease EGFP levels, it would have little

chance of revealing any small molecules that could enhance RNAi activity. Thus, we chose instead to isolate a clone of cells with modest EGFP knockdown, which has considerable potential to both further elevate and decrease the EGFP levels, making it possible for us to identify both inhibitors and enhancers. Using this assay, we performed a proof-of-principal pilot screen with a collection of 2000 FDA-approved compounds and natural products. Several compounds were found to enhance RNAi activity. Of the candidate RNAi enhancer compounds, enoxacin was chosen for further characterization. We found that it could dramatically enhance the processing of shRNA into siRNAs and promote the loading of siRNAs/miRNAs into the RISC, significantly reducing the dosage required to achieve equal knockdown efficiency in mammalian cells (Shan et al., 2008). Enoxacin was identified as an RNAi enhancer in an independent chemical screening by another group, as well (Zhang et al., 2008).

In addition to enhancing RNAi, enoxacin could also promote the biogenesis of endogenous miRNAs (Shan et al., 2008). Further *in vitro* and *in vivo* analyses indicated that the enoxacin-mediated RNAi-enhancing activity is TRBP-dependent, most likely by facilitating the interaction between TRBP and RNAs (Shan et al., 2008). Furthermore, we found that enoxacin has no effect on a RISC-cleavage *in vitro* assay, arguing against the potential involvement of enoxacin in the step of mRNA-target recognition and RISC-guided cleavage.

A study by Melo et al (2011) showed that enoxacin significantly inhibits cancer growth and leads to cancer cell apoptosis in human cell cultures and xenografted, orthotopic, and metastatic mouse models by enhancing the production of miRNAs functioning as tumor suppressors by binding to the miRNA biosynthesis protein TRBP. This result further emphasizes the key role of disrupted miRNA expression patterns in tumorigenesis and hinting at a unique strategy for restoring the distorted microRNAome of cancer cells to a more physiological setting. Enoxacin, an old drug used clinically as an anti-bacterial therapy, has turned out to have a novel anti-cancer function, a classic example of a new use for an old drug.

Our assay is not biased towards or against any specific components of the miRNA pathway, and could make the high-throughput screening of the small molecule library highly efficient. However, our assay could not distinguish whether the decrease in EGFP levels was due to drug toxicity-caused cell death, growth inhibition, or real enhancement of RNAi activity; there was a similar question about the elevation of EGFP levels and whether that came from drug-conferring cell division enhancement vs. real inhibition of RNAi activity. To address this issue, the clone that we developed was further infected with lenti-RFP to isolate the clone of cells with appropriate expression of RFP. In this reporter system, a well-to-well GFP/RFP ratio could be employed as a criterion to evaluate whether the GFP level alterations were really caused by enhanced RNAi activity rather than by other factors, and vice versa for the elevated EGFP levels (Figure 1).

Using this new cell-based reporter assay, we continued to screen additional small molecules and found that iron chelators are a class of new activators that significantly enhance the activity of the miRNA/RNAi pathway. We showed that cytosolic iron could regulate the activity of the miRNA pathway via poly(C)-binding protein 2 (PCBP2), which is associated with Dicer, to promote the processing of miRNA precursors (Li et al., 2012). Further study revealed that cytosolic iron could modulate the association between PCBP2 and Dicer, as well as the multimerization of PCBP2 and its ability to bind to miRNA precursors, leading to alterations of miRNA precursors processing into mature miRNAs and suggesting a role for iron homeostasis in the regulation of miRNA biogenesis (Figure 2).

4. A luciferase-based reporter assay for monitoring the activity of the miRNA/RNAi pathway

Similar to the GFP reporter system, cells co-transfected with plasmids harboring the fire fly luciferase (FL), renilla luciferase (RL), and short hairpin against FL (shFL) have been developed as a reporter system, as well (Watashi et al., 2010). Using this assay, two compounds were identified and characterized as inhibitors that suppress pre-miRNA processing due to impaired Dicer activity or affect the function of miRNAs by blocking small RNAs loading into RISC (Watashi et al., 2010). Furthermore, treatments with either of the two compounds were found to effectively neutralize tumor growth (Watashi et al., 2010). Using an easier method, HEK-293 cells were transfected with plasmid harboring the short hairpin against fire fly luciferase (shFFL), and single stable clones were isolated to overexpress the shFFL. The shFFL clone cells were transfected with plasmid siCHECK harboring the FL and RL driven by different promoters, and the transfected cells were treated with small molecules for luciferase assays (Li et al., 2012). Like the EGFP-shEGFP-RFP reporter assay system, this assay is not biased towards or against any specific components of the miRNA pathway, either because the RFL serves as an internal control to distinguish the false alteration of FFL/RL caused by drug-conferred cell growth inhibition or death, as well as the promotion of cell growth. In our experience, this assay is more reliable, but more expensive and more time-consuming than a GFP/RFP-based assay. Thus, the GFP/RFP reporter system would be suitable for the initial screen, and the improved shFL/siCHECK reporter assay could be used for validation.

5. Reporter system for monitoring the activity of specific miRNAs

For the development of RNAi/miRNA-mediated therapeutics, alteration of some specific miRNAs may be more important than regulation of the general RNAi/miRNA pathway (Krichevsky and Gabriely, 2009). The reporter assay systems mentioned above could also be employed to identify modifiers for specific individual miRNAs. In a luciferase-based screening assay, the psiCHECK was reengineered to insert the complementary sequence(s) to some specific miRNA downstream of the firefly luciferase coding region, and renilla luciferase driven by another promoter served as a sensor to detect the presence of specific mature miRNA (Young et al., 2010; Gumireddy et al., 2008). Extensive studies have focused on the identification of a miR-21-specific inhibitor as a potential cancer treatment (Krichevsky and Gabriely, 2009) due to its direct link to several human malignancies. The primary screen using this strategy revealed a compound with the diazobenzene core structure from among >1200 compounds that could inhibit the expression of miR-21 (Gumireddy et al., 2008). This compound was found to downregulate transcription of the miR-21 gene by targeting unknown factor(s), but it did not affect downstream microRNA pathway. Thus, this compound is not a general inhibitor of the miRNA pathway, but displays specificity against miR-21 (Gumireddy et al., 2008).

Perspectives on a chemical biology approach to the miRNA/RNAi pathway

Although pilot screens have uncovered several small molecule modulators of the miRNA/RNAi pathway, these modifiers alone are not enough to tell us more about regulatory mechanisms. Logically, the regulation of such an essential pathway involved in most critical biological processes must come from many factors across varied signaling pathways. To further explore the potential of a chemical biology approach for understanding these mechanisms of regulation, we need a more comprehensive and larger-scale screen using hundreds of thousands of compounds. The expansion to larger-scale screens and post-screen studies faces several technical challenges. The first is how to improve the existing reporter assay systems to make them more reliable, robust, cheaper, and faster. The second challenge is to adopt a multidisciplinary approach for a post-screen study that integrates chemical

synthesis, proteomics, biochemistry, and genetics to identify targeting components, including the known components as well as novel components, within the miRNA/RNAi pathway. Although there have been dramatic improvements in proteomics, it is still quite technically challenging to identify the proteins specifically targeted by specific small molecules. Since small molecules, even well-characterized ones, might have multiple targets that could give rise to any observed modulation, identifying all the targets with a proteomics approach is made difficult by technical limitations. After the identification of candidate protein targets, additional studies using other molecules to target the same protein(s), if available, and genetic experiments will be necessary.

We know that the Drosha-Dicer-mediated processing from pri-miRNAs to generate functional mature miRNAs is the major pathway for miRNA biogenesis. However, the processing of some specific miRNAs could be regulated in cell type- or physiological condition-dependent manners (Viswanathan et al., 2008, Thomson et al., 2006), while the underlying mechanism of regulating the processing of miRNA has not yet been elucidated. The specific regulation of some miRNAs, particularly those already linked to human diseases, is particularly important in terms of mechanistic study and the development of new therapeutic strategies. Thus, it is essential to identify small molecule modulators that could regulate the processing and activity of specific miRNAs. While technically challenging, the successful identification of a miR-21-specific inhibitor could shed light on the feasibility of a chemical biology approach in this regard (Gumireddy et al., 2008). Besides potentially targeting the protein(s) involved in the regulation of selective miRNA processing, several small molecules could directly bind to RNAs, including miRNAs (Thomas and Hergenrother, 2008; Tor, 2003; Gooch and Beal, 2004; Henn et al., 2008). Given the diversity of RNA secondary structures among the miRNA precursor population, it is not a stretch to speculate that small molecules targeting specific pri-, pre-, or mature miRNA might be identified.

Because miRNAs are involved in nearly every cellular process, the identification and characterization of small molecule modulators of the miRNA/RNAi pathway will yield novel insights into fundamental pathological mechanisms of human diseases. Furthermore, these RNAi modulators, particularly RNAi enhancers, could foster the development of RNA interference as a tool for biomedical research and therapeutic interventions.

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Figure 1.

GFP report assay system in living cells for high-throughput screening of small molecule drugs to dissect the RNAi/miRNA pathway. 293 cells were infected with lenti-EGFP and cell cloning was applied to isolate EGFP stable expression line (293-EGFP). The 293-EGFP cell line was infected with lenti-short hairpin RNA against EGFP (shEGFP) to select the proper cell clones that have great potential to enhance or inhibit GFP knock down efficiency for screening of small molecule drugs. In this system, if the drug(s) enhances the miRNA/RNAi activity, the GFP level will be further decreased, and vice versa. To further avoid the false enhancement or inhibition of RNAi activity phenomena caused by drug toxicity or enhanced cell growth, the reporter cells were infected with lenti-RFP to select the cell clones with appropriate expression of RFP to avoid significant toxicity caused by strong expression of RFP. Using this assay system, well-to-well GFP/RFP ratio could be employed as criterion to evaluate to evaluate alteration of RNAi activity caused by small molecule drugs.

Figure 2.

Cellular iron homeostasis regulates the activity of the miRNA/RNAi pathway. Cellular iron levels affect dimerization of poly(C) binding protein 2 (PCBP2), association of PCBP2 and DICER, binding of PCBP2 and pre-miRNAs, and processing of DICER-conferred processing of pre-miRNAs to mature miRNAs. The dimerized PCBP2 is the functional form of PCBP2, and the cellular iron level decrease leads to enhanced dimerization of PCBP2, association of DICER and PCBP2, binding of PCBP2 and pre-miRNAs and consequently to promotion of pre-miRNAs processing into mature miRNAs.