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Chemical Biology Approach for Dissection of RNAi/miRNA Pathway

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Abstract

RNA interference (RNAi)/miRNA has been recognized as one of the most important regulation mechanisms of gene expression at post-transcriptional? levels in eukaryotic cells. Although the main components within the RNAi/miRNA pathway have been identified and characterized, studies on the molecular mechanisms regulating the activity of the RNAi/miRNA pathway have begun to emerge in recent years. High-throughput reporter assays have been developed to monitor the activity of the RNAi/miRNA pathway and applied for proof-of-concept pilot screening, leading to identification of some inhibitors and activators that generally or specifically regulate activity of the RNAi/miRNA pathway. Additionally, in combination with multidisciplinary approaches such as proteomics, biochemistry and genetics, to identify the targeting components, some protein co-factors that play significant roles in the regulation of the activity of the RNAi/miRNA pathway have been identified. Herein we highlight the high throughput reporter assays developed in recent years and the resulting discovery of the RNAi/miRNA enhancers and inhibitors, with attention on developing novel RNAi- or miRNA-based therapeutic interventions.

Keywords: Cell cloning; MicroRNA/RNAi; RNAi activity reporter system; High throughput screening; Small molecule drug library; Chemical biology approach

Introduction

RNA interference (RNAi) has been recognized as one of the most important mechanisms at post transcriptional level in eukaryotic cells, mediated by small non-coding RNA, including in particular, interference RNA (siRNAs), microRNAs (miRNAs) and piwi-interacting RNA (piRNAs) with length of 18-30 nt, to regulate diverse metabolic pathways involved in chromosome architecture and segregation behavior, transcription, and RNA processing and stability, and thereby to phenoltypically coordinate development, growth control, apoptosis, self defense, and stem cell [1-6]. Significant evidences demonstrated that alteration of miRNAs expression profile could contribute to the pathogenesis of a wide range of human diseases as well as phenotypic abnormality among the eukaryotes [7], suggesting the essential roles of miRNAs in maintenance of normal metabolism. Small interference RNAs (siRNAs) based knockdown has been technically adopted in a wide range as a powerful tool to elucidate gene functions, to discover and validate the drug targets and to develop therapeutic intervention. Given the pivotal roles of siRNA/miRNA in diverse biological pathways as well as the broad application of RNAi, it is essential to understand the regulation mechanism of the siRNA/miRNA pathway. Although main components of the RNAi/miRNA pathway have been identified and characterized [2], studies on the regulation mechanism of the pathway itself have received high attention in recent years by using the chemical biology approach, which offers a more dynamic way to monitor the activity of specific pathways compared to traditional forward or reverse genetic approach. Although the emergence of the chemical biology approach to the RNAi/miRNA pathway is still in its infancy, the approaches led to further understanding on the regulation mechanisms by identification of a number of small molecule modulators targeting to the specific steps in the RNAi/miRNA pathway. Herein we highlight the recent discovery of the small molecule modulators on the basis of high throughput reporter assays combined with chemical biology approaches.

RNAi/miRNA Pathway

Generally, mature form of microRNAs are generated by two sequential biochemical processing steps starting from the pri-miRNAs, which are transcribed initially by RNA polymerase II from 5'-capped, spliced and polyadenylated [5,8,9], range from hundreds to thousands of nucleotides in length that bear one or more hairpin structures [10] in mammals. In most cases, the first processing step takes place inside the nucleus where the pri-miRNAs were cropped by Drosha, a RNA endonuclease III in mammals and its partner DGCR8 [9] to produce a ~ 70 nt stem-loop-structured precursor (pre-miRNAs) that harbors the miRNA in the 5' or 3' half of the stem [11,10]. The pre-miRNAs are transported into cytoplasm by exportin—5/RanGTP for the secondary processing [9,10,12,13] to further dice the pre-miRNAs into mature form of miRNAs with length of 18-25 nt by Dicer, another member of RNase III family in collaboration with its cofactors TRBP and PACT [10,14,15]. Besides the general processing steps for generation of mature miRNAs, alternative ways to generate some pre-miRNAs have been proved to bypass the Drosha processing step. One example is "mirtrons", the pre-miRNA-like hairpins that are generated by splicing and debranching of the short hairpin introns [16-18]. Besides "mirtron", some small nucleolar RNAs (snoRNAs), tRNAs, and endogenous short hairpin RNAs (shRNAs) are processed into miRNA-like molecules without relying on Drosha processing either [19-22]. More recently another source of miRNAs, terminal hairpins of endogenous siRNA long-stem-loop precursors, has been identified as being able to bypass the Drosha processing step as well [23].

The mature form of miRNAs must be loaded into RISC complex to functionally regulate gene expression at post-transcriptional levels. It is generally believed that the guide strand is sorted to load into RISC complex to guide the RISC to the right positions, while the passenger strand is finally 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 [24-28].

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Chemical Biological Approaches in Dissection of RNAi/miRNA Pathway

For the purpose of basic research tools to further identify the regulators of RNAi pathway and therapeutic application, the chemical biology approach has been applied on the basis of screening large amount of small molecule drugs libraries? in recent years. Basically, large number of clinical small molecule compounds approved by FDA have been collected and the generated libraries have been available commercially in the format of 96-well plates, such as the NINDS and Prestwick libraries, providing access to mostly FDA-approved and marketed drugs as the priceless source for small molecule screening with focus of new uses for old drugs [29]. Since a single clinical compound may target multiple components of the metabolic pathways different from the known one(s), identification of the new targets will help us further dissect and elucidate new regulation mechanisms of the specific metabolic pathway(s) such as RNAi/miRNA pathway. In addition, given that it takes too long and costs too much to bring new drugs to market and that different targets of the compounds may bear unknown therapeutic functions, it is plausible to speculate screening existing drugs for new uses [29] as an example detailed below that a general antibiotic enoxacin has been proven to function as cancer cell growth inhibition. For screening of such a large number of clinical drugs to dissect the RNAi/miRNA pathway, the reliable and robust assays particularly fluorescence-based reporter system is indispensable. In recent years, several report assay systems have been developed as summarized below, rendering the high throughput screening possible.

In vitro reporter assays to monitor activity of pre-miRNA processing

The first established *in vitro* fluorescence assay monitors the activity of Dicer [30]. In this reporter assay, the 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. By contrast, the Dicer-mediated cleavage of pre-let-7 would emit fluorescence and the cleavage activity of Dicer would be proportional to the increase of fluorescence signal. However, so far no report is available for the application of this assay to small molecule screenings, indicating the limited application of this assay strategy.

In vivo fluorescence-based report assay to monitor the activity downstream maturation of RNAi pathway

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 its targets EGFP mRNA for degradation [31]. The co-transfected Hela cells were 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 assay strategy, two compounds named ATPA-18 and ATPA-21 were identified to specifically inhibit ATP-dependent events occurring during RNAi [31]. 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 to be elusive [31]. While these compounds were the first inhibitors for the 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 quick detection of the fluorescence alterations by drug treatments. However, this strategy can't be applied for identification of the regulatory components upstream of the maturation of the miRNAs. Additionally, the transient co-transfection of two plasmids combined with siRNA followed by drug treatments not only lead to efficiency variation but also make the assay system too complicated for high-throughput screening.

GFP report assay to dissect the RNAi/miRNA pathway in living cells

To further improve the assay efficiency for high throughput screening of small molecule library, lenti-EGFP stable expression 293 cells were infected 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 EGFP level, exposing to the compound(s) inhibiting the RNAi activity, and therefore enabled us to identify inhibitors of the RNAi pathway robustly. However, due to its limited potential for further decrease of EGFP levels, it would nevertheless offer little chance to find any small molecules that could enhance RNAi activity. Thus, we instead chose to use a specific clone named Clone#3 with a modest EGFP knockdown that bears considerable potential for both further elevation and decrease of the EGFP levels, thereby making it possible to identify both inhibitors and enhancers. Using this assay, we performed proof-of-principal pilot screen with a collection of 2000 FDA-approved compounds and natural products. Several compounds were identified to enhance RNAi activity (Figure 1). Of the candidate RNAi enhancer compounds, enoxacin was characterized to dramatically enhance the processing of shRNA into siRNAs and promote loading of siRNAs/miRNAs into the RISC, significantly reducing the dosage required to achieve equal knockdown efficiency in mammalian cells [32]. Enoxacin was identified as an RNAi enhancer in an independent chemical screening as well by another group [33].

In addition to enhancing RNAi, enoxacin could also promote the biogenesis of endogenous miRNAs as well. Further *in vitro* and *in vivo* analyses indicated that the enoxacin-mediated RNAi-enhancing activity is TRBP-dependent, most likely through facilitating the interaction between TRBP and RNAs [32]. Furthermore, we found that enoxacin has no effect on 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. [34] shows 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 with tumor suppressor functions by binding to the miRNA biosynthesis protein TAR RNA-binding protein 2 (TRBP), further highlighting the key role of disrupted miRNA expression patterns in tumorigenesis and suggesting a unique strategy for restoring the distorted microRNAome of cancer cells to a more physiological setting. Enoxacin, clinically used for anti-bacterial therapy, has turned out to have a novel function as a anti-cancer agent, serving as example that old drugs might have new uses.

This assay is not biased towards or against any specific components of miRNA pathway and could confer high efficiency for high throughput screening of the small molecule library. However, it is still not a perfect report assay system in a way that it is unable to distinguish whether the decrease of EGFP levels is due to the drug toxicity-caused

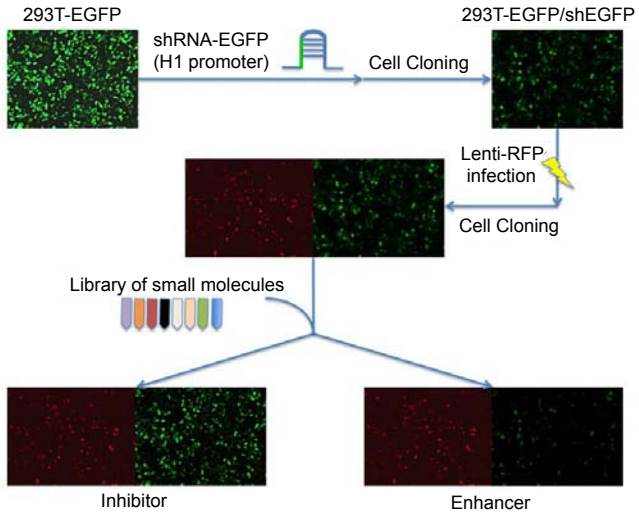

Figure 1: GFP report assay system in living cells for high throughput screening of small molecule drugs to dissect the RNAi/miRNA pathway. 293 cells were infected with lenti-EGFP and cell cloning was applied to isolate EGFP stable expression line (293-EGFP). The 293-EGFP cell line was infected with lenti-short 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. The clone#3 is the proper reporter clone. When treat the clone#3 cells with small molecule drugs, if the drug(s) enhance the 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 clone#3 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 alteration of RNAi activity caused by small molecule drugs.

cell death, growth inhibition, or due to RNAi activity enhancement, and vice versa for elevation of EGFP levels: the drug – conferring - cell division enhancement of inhibition of RNAi activity. To make the reporter system more reliable and efficient, the stable cell clone, Clone#3 mentioned above, was infected with lenti-RFP to select the cell clones named as Clone#3/RFP with appropriate expression of RFP in order to avoid significant toxicity to cells caused by the high level of RFP. With this assay system, well-to-well GFP/RFP ratio could be employed as criterion to evaluate whether the GFP level alterations was really caused by enhanced RNAi activity instead of cell death caused by compound toxicity, and vice versa for the elevated EGFP levels [35] (Figure 2).

Using this new cell-based reporter assay, we continued screening more than 3000 FDA approved small molecule drug and it turned out that iron chelators are a class of new activators that significantly enhance the activity of RNAi/miRNA pathway in a way that cytosolic iron could regulate the activity of the miRNA pathway through poly(C)-binding protein 2 (PCBP2), which is associated with Dicer to promote the processing of miRNA precursors. Further study shows 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 the alteration of miRNA precursors processing into mature form of miRNAs, and suggesting a role of iron homeostasis in the regulation of miRNA biogenesis (Figure 2).

Luciferase-based reporter assay system to monitor the activity of RNAi/miRNAs

This is an alternative for the Lenti-EGFP/lenti-shEGFP/lenti-RFP reporter assay system in monitoring the activity of RNAi/miRNAs. Cells co-transfected with plasmids harboring the fire fly luciferase (FL), renilla luciferase (RL), and short hairpin against FF (shFF), respectively, were treated with small molecule compound library to monitor if the FL levels were elevated or decreased due to the enhanced or inhibited processing of shFL into siFL [36]. Using this assay, two compounds were identified and characterized as inhibitors to suppress pre-miRNA processing due to impaired Dicer activity or to affect the function of miRNAs by blocking small RNAs-loading into RISC [36]. Furthermore, it was found that treatments with either of the two compounds effectively neutralized tumor growth [36]. More easily, 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 si CHECK harboring the FL and RL driven by different promoters, and the transfected cells were treated with small molecules for luciferase assays [35]. Like the EGFP-shEGFP-RFP reporter assay system, this assay is not biased towards or against any specific components of miRNA pathway either because in the report cells, the RFL serves as internal control to distinguish the false alteration of FFL/RL caused by drug-conferred cell growth inhibition or death as well as promotion of cell growth. To our experiences, this assay is more reliable but more expensive and more time-consuming than GFP/RFP-based assays.

Small molecules modulating the activity of specific miRNAs

For RNAi/miRNA-mediated therapeutic research, alteration of some specific miRNAs may be of more importance relative to regulation of general RNAi/miRNAs pathway [37]. The reporter assay systems mentioned above could also be employed for identification of modifiers for specific individual miRNAs. In luciferase-based screening assay, the psiCHECK was reengineered to insert the complementary sequence(s) to some specific miRNA at the downstream of the firefly luciferase coding region, and renilla luciferase driven by another promoter serve as sensors to detect the presence of specific mature miRNA [38,39]. Extensive studies have focused on the identification of miR-21-specific inhibitor for the purpose of discovering cancer therapeutics [37] due to its uniqueness in liver cells and its direct link to several human malignancies. The primary screening using this strategy, a compound with the diazobenzene core structure from among >1200 compounds, was identified to inhibit the level of miR-21 [39]. Further molecular studying indicated that this compound could down-regulate transcription of miR-21 gene by targeting unknown factor(s), but doesn't affect downstream microRNA pathway. Thus, this compound is not a general inhibitor of the miRNA pathway but displays specificity of miR-21.

Perspectives on the Chemical Biology Approach to Tnai/Mirna Pathway

Although some small molecule modulators to RNAi/miRNA pathway have been identified through the pilot screening of the small molecule library, these modifiers alone are not sufficient to further understand the regulation mechanisms. Logically, the regulations of such an essential pathway involved in most critical biological processes must be involved in many co-factors from varied signaling pathways. To further exploit the potential of the chemical biology approach in understanding the mechanisms of the regulation, the screenings must be expanded for more comprehensive and larger-scale compound libraries containing hundreds of thousands of compounds. The expansion of larger scale screening and post-screening studies faces

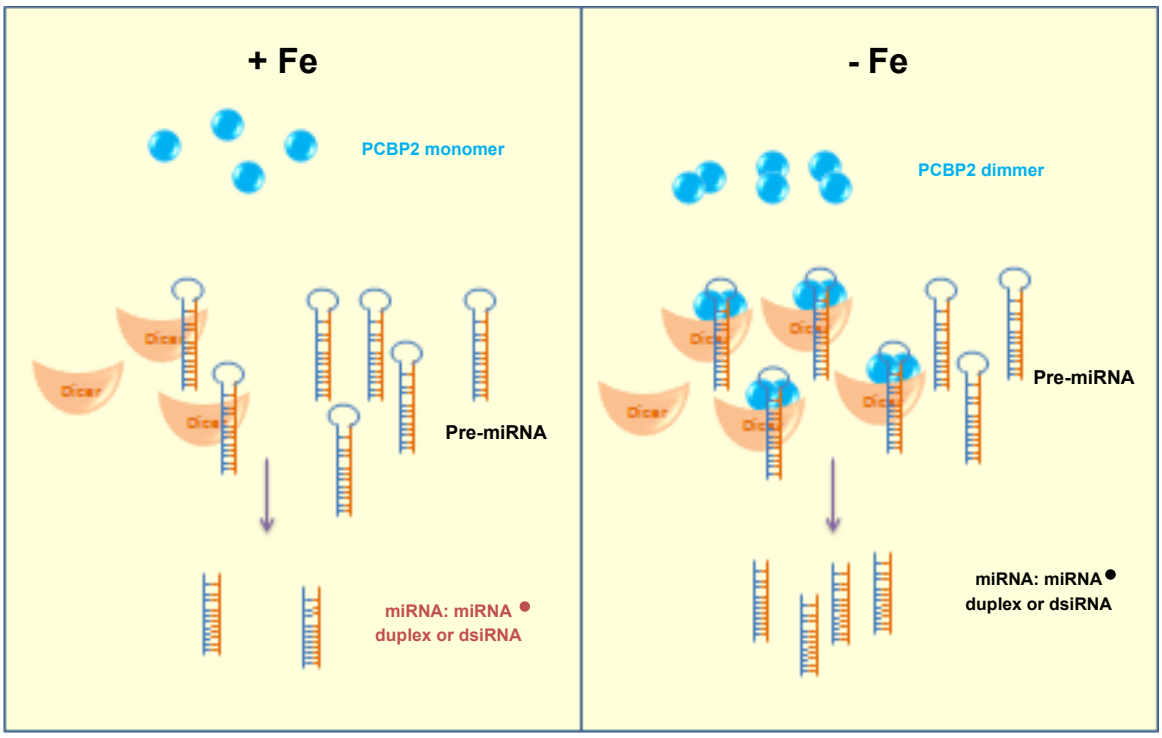

Figure 2: Graphical abstract for mechanism how cellular iron homeostasis regulate RNAi/miRNA pathway. Cellular iron levels affect dimerization of poly(C) binding protein 2 (PCBP2), association of PCBP2 and DICER, binding of PCBP2 and pre-miRNAs, and processing of DICER-conferred processing of pre-miRNAs 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.

several technical issues. The first challenge is further improvement of the existing reporter assay systems to make them more reliable, more robust, cheaper, and more timesaving. The second challenge is a multidisciplinary approach for post-screening study that integrates chemical synthesis, proteomics, biochemistry and genetics to identify the targeting components, including the known components as well as the novel components within RNAi/miRNA pathway. Although the dramatic improvements have been made in proteomics, it is still quite technically challenging to identify the proteins specifically targeted by novel small molecules. Since small molecules, even well characterized ones, might have multiple targets that could give rise to the observed modulation, it is hard to identify all the targets by proteomics approach due to technical limitations. After the identification of candidate protein targets, additional studies using other molecules targeting the same protein(s), if available, and genetic experiments are necessary.

It is acknowledged that the Drosha-Dicer mediated processing from pri-miRNAs to generate functional mature miRNAs is the major pathway for most miRNAs in the genome. However, the processing of some specific miRNAs could be regulated in the cell type or physiological condition dependent manners [40,41], while the underlying mechanism of regulating the processing of miRNA has yet been elucidated. The specific regulation of some miRNAs, particularly those that have been linked to human diseases, is of more importance in terms of mechanism studying and the development of new therapeutic strategy. Thus, it would be essential to identify small molecules modifiers that could regulate the processing and activity of specific miRNAs. While technically challenged, the success in identification of miR-21-specific inhibitor could shed light on the feasibility of the chemical biology approach in this regard [39]. Besides potentially targeting the protein(s) involved in the regulation of selective miRNAs processing, several small molecules could directly bind to RNAs, including miRNAs [42-45]. Given the diversity of RNA secondary structures among miRNA precursor population, it is plausible to speculate that small molecules targeting specific pri-, pre- or mature miRNA could be identified.

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

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