

REVIEW

Probing microRNA network with small molecules

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MicroRNAs (miRNAs) are emerging as novel regulators in gene expression. Due to the large population of miRNAs and their important function inside cells, miRNA network is essential for normal physiology but still remains unclear. Recent results also showed miRNAs are closely related with the occurrence and development of various human diseases. Thus, revealing miRNA network is urgently needed for not only biological studies but also medical treatment. Small molecules that can regulate miRNA pathway hold great potential in probing miRNA network. With developed cellular reporter system, we have found two small molecules from photoreaction products that can universally activate miRNA and specifically inhibit myomiRs. With the latter small molecule, a novel miRNA regulatory pathway, miR-221/222-myod-myomiRs, was successfully unveiled.

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MicroRNAs (miRNAs) are tiny non-coding RNAs, which consist of ~22 nucleotides and play pivotal roles in regulation of gene expression [1]. Generally, miRNAs interact with target mRNAs through binding to their 3' untranslated regions (UTR) in a manner of sequence complementarity, resulting in degradation or suppressed translation of target mRNAs. Previous researches have pointed out that over 30% of the human genome are under the regulation of miRNAs [2]. Together with the evidence that there are many miRNAs in human cells and one miRNA could target multiple mRNAs [3, 4], the network formed among miRNAs, their transcriptional factors, target mRNAs, and other unknown biomolecules is thus extremely complicated but also fundamental in cells. As a result, abnormal miRNAs have found to be highly related with many human diseases including cancers [5, 6]. It is therefore urgent and important to explore miRNA network, which is also challenging due to lack of appropriate tools.

Chemical genetic studies using small molecules with

specific activity in biological systems have successfully dissected a considerable number of unknown signaling transduction pathways inside cells [7-9], indicating miRNA network may also be unveiled by small molecules targeting the miRNA pathway. Recently, several small molecules that can adjust miRNA transcription, maturation or function have been reported by us and others [10-16], including specific or universal modifiers of miRNAs. Note worthily, cellular iron homeostasis has been successfully connected with the miRNA pathway through a small-molecular activator of miRNA [17]. Therefore, small molecules that can regulate miRNA pathway hold great promise in probing unknown miRNA network.

Our group aims to identify small molecules with regulatory activity toward miRNA pathway from a special compound library based on organic reactions and then employ these small molecules as tools to dig the miRNA network. To find out such small molecules, cellular reporter systems that can report changes in miRNA pathway were

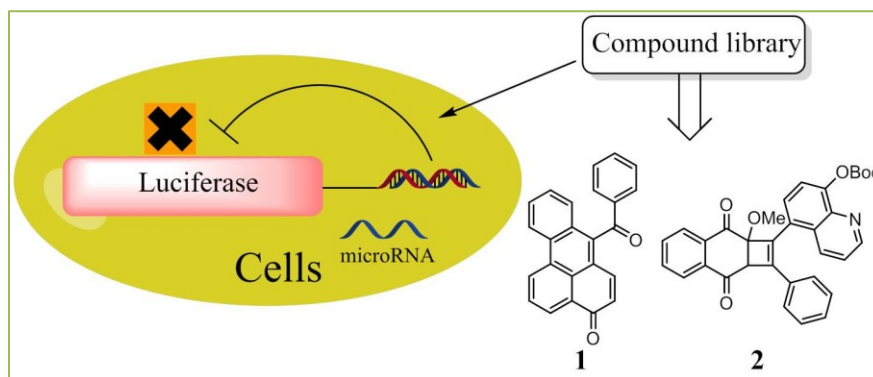


Figure 1. Cellular reporter systems for identification of small molecules that can regulate miRNA pathway and identified small molecular modifiers of miRNAs.

developed by us at first. In specificity, luciferase reporter genes that contain complementary sequences of miRNAs at their 3' UTR were transfected into cell lines with high expression levels of these miRNAs, resulting in the cellular reporter systems (Figure 1). Activity of candidate small molecules can then be read out by measuring luciferase signals.

With the cellular reporter systems, we first identified a small molecule (**1**, Figure 1) as a universal activator of miRNAs, which were screened from photoreaction products^[11]. Small molecule **1** was found to promote maturation of miRNAs and stimulate protein expression levels of trans-activation-responsive region RNA-binding protein (TRBP) that is critical in miRNA processing. In the meanwhile, another small molecule (**2**, Figure 1) also from photoreaction products came out as an inhibitor of muscle-specific miR-1^[14]. Small molecule **2** inhibited miR-1 without effects on other cancer-related miRNAs, indicating it may have specific activity toward myogenic miRNAs (myomiRs).

MyomiRs including miR-1, miR-133a, and miR-206 are overexpressed in muscle and cardiac cells, and are involved in both development and disease of heart or muscle^[18, 19]. Recent investigations also showed miR-133a is related with brown fat differentiation^[20], indicating miR-133a may be involved in metabolic diseases. The discovery of unknown myomiR network shall benefit related studies and treatment. And we then checked the effect of small molecule **2** toward miR-133a and miR-206, showing both these two myomiRs could be inhibited by small molecule **2**^[15]. These results confirmed the specific inhibitory activity of small molecule **2** toward myomiRs. The expression levels of primary and mature myomiRs obtained from quantitative RT-PCR analysis showed both of them were decreased after treatment. At the same time, we observed that treatment with small molecule **2** led to inhibited differentiation of muscle cells,

which was validated by quantitative RT-PCR analysis of differentiation-related genes. These results indicated factors in the transcriptional pathway that control myomiR expression and cell differentiation may be regulated by small molecule **2**. Previous studies have demonstrated myoD is the major transcriptional factor of myomiR and also an important regulatory factor of muscle cell differentiation^[21, 22]. We thus then checked expression levels of myoD, leading to the discovery that protein expression was decreased by small molecule **2** while mRNA expression remained consistent. The unique expression patterns of myoD mRNA and protein inspired us that there may exist other miRNAs in the upstream pathway of myoD. After performing bioinformatic calculation, miR-221/222 that were already known to be related with the differentiation of skeletal muscle appeared^[23, 24]. MiR-221/222 could target the position 383-389 of myoD 3' UTR and the free energies of miR-221/222-myoD hybrid were comparable to that of authentic miRNA-target pairs. Then, miR-221/222 expressions in muscle cells were investigated after treatment with small molecule **2**, revealing their expression levels were up-regulated. To confirm the direct regulation of miR-221/222 on myoD, miR-221/222 mimics or inhibitors were transfected into muscle cells, leading to repressed or enhanced protein expression of myoD respectively. And myoD 3' UTR was inserted into the 3' UTR of luciferase gene, luciferase signals were also found to be regulated by miR-221/222, demonstrating direct regulation of miR-221/222 on myoD. Importantly, mutation at position 383-389 of myoD 3' UTR caused no interaction between miR-221/222 and luciferase gene, validating the result of bioinformatic calculation. The indirect regulation of miR-221/222 on myomiRs was also confirmed by measuring myomiR expression levels after overexpression or knockdown of miR-221/222. In summary, small molecule **2** successfully revealed a novel miRNA regulatory pathway in muscle cells, which is the miR-221/222-myoD-myomiRs pathway (Figure 2).

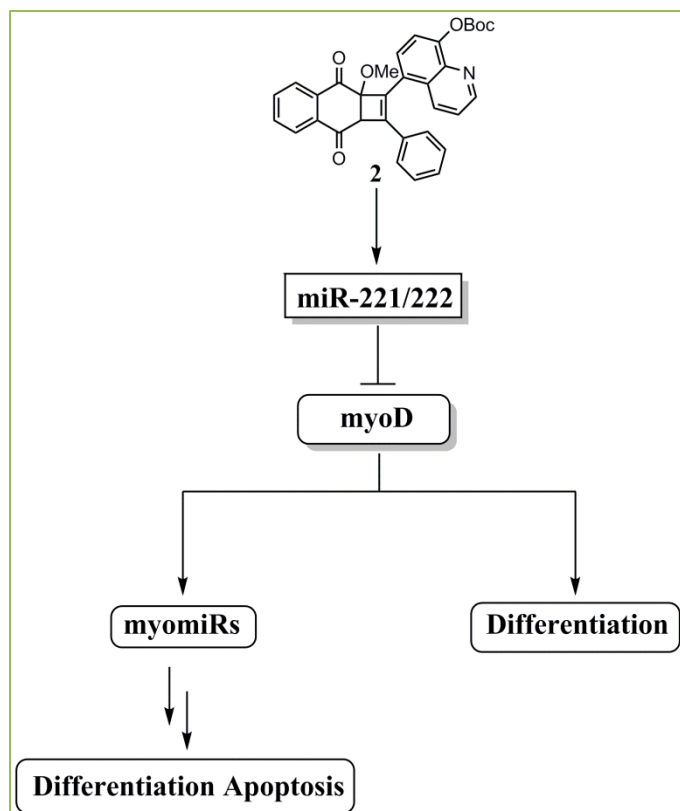


Figure 2. MiR-221/222-myoD-myomiR regulatory pathway revealed by small molecule 2.

Beside these two small molecules, we have also discovered some other small molecules with unique regulatory activity toward miRNA pathway and are now trying to explore relative miRNA network. In addition, functionalization of active small molecules with bioorthogonal group has recently shown as an efficient way for target identification and exploring unknown signaling pathways [25-27]. And we are now trying to modify small molecule 1 and 2 with bioorthogonal groups to identify their direct targets. The discovery of their direct targets is expected to reveal more unknown factors in miRNA processing or myomiR network. With the identification of more active small molecules that can regulate miRNA pathway and further chemical modification, miRNA network is believed to get clear in the future.

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