Chemistry & Biology
Brief Communication
CellPress

Small Molecule Inhibitor of Myogenic microRNAs Leads to a Discovery of miR-221/222-myoD-myomiRs Regulatory Pathway

Su-Bee Tan,[1,2,3] Jinbo Li,[1,3] Xi Chen,[2] Wenjie Zhang,[1] Dianmu Zhang,[2] Chenyu Zhang,[2,*] Donghai Li,[2,*] and Yan Zhang[1,*]
[1]State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, PRC
[2]School of Life Science, State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, PRC
[3]Co-first author
*Correspondence: cyzhang@nju.edu.cn (C.Z.), donghaili@nju.edu.cn (D.L.), njuzy@nju.edu.cn (Y.Z.)
http://dx.doi.org/10.1016/j.chembiol.2014.06.011

SUMMARY

Myogenic microRNAs (myomiRs) that are specifically expressed in cardiac and skeletal muscle are highly relevant to myogenic development and diseases. Discovery and elucidation of unknown myomiRs-involved regulatory pathways in muscle cells are important, but challenging due to the lack of proper molecular tools. We report here a miR-221/222-myoD-myomiRs regulatory pathway revealed by using a small-molecule probe that selectively inhibits myomiRs including miR-1, miR-133a, and miR-206. The small-molecule inhibitor screened from luciferase assay systems was found to inhibit myomiRs and differentiation of C2C12 cells. Using the small molecule as a probe, we found that the transcriptional factor myoD, which is upstream of myomiRs, was further regulated by miR-221/222. This miR-221/222-myoD-myomiRs regulatory pathway was confirmed by over-expressing or knockdown miR-221/222 in muscle cells, which respectively led to the inhibition or enhancement of myoD protein expression and subsequent downregulation or upregulation of myomiR expression.

INTRODUCTION

MicroRNAs (miRNAs) are a novel class of small noncoding RNAs that recently emerged as important endogenous gene regulators (He and Hannon, 2004). Typically, miRNAs function by binding to the 3′ UTR of target mRNAs, leading to repressed translation or mRNA degradation. Through posttranscriptional regulation, miRNAs were estimated to target more than 30% of human genome (Lewis et al., 2005), and consequently are involved in diverse biological processes, including proliferation, differentiation, apoptosis, and immune responses (Ebert and Sharp, 2012; Hoefig and Heissmeyer, 2008). Aberrant expression or function of miRNAs has been reported to be closely related to the occurrence and development of many human diseases (Croce, 2009; Sullivan and Ganem, 2005).

Myogenic miRNAs (myomiRs) including miR-1, miR-133a, and miR-206 are highly enriched in cardiac and skeletal muscle, which were implicated in muscle and heart development or disease through regulation of key genes controlling myogenesis (Callis et al., 2007; Thum et al., 2008; Yang et al., 2007). Moreover, recent studies also found that miR-133a was involved in brown fat differentiation from myogenic factor 5 (Myf5)-positive myoblastic lineage and white adipose lineage (Kornfeld and Brüning, 2012), indicating myomiRs may also play important roles in obesity and other metabolic diseases. Discovery and elucidation of unknown myomiRs-mediated cellular signal transduction pathways remain to be important, but challenging due to the lack of proper molecular tools.

Chemical biology methods using small molecules that possess specific biological activity as probes have demonstrated great potentials in revealing unknown signaling pathways (Schneider-Poetsch et al., 2010; Schreiber, 2005; Sun et al., 2012). In recent years, several types of small-molecule modifiers of endogenous miRNAs have been identified by us and others (Bose et al., 2012; Chen et al., 2012; Gumireddy et al., 2008; Li et al., 2014; Shan et al., 2008; Tan et al., 2013; Young et al., 2010), including small molecules that selectively inhibit the liver-specific miR-122 (Young et al., 2010) or the cancer-related miR-21 (Bose et al., 2012; Gumireddy et al., 2008). A small molecule discovered by Jin et al. recently successfully identified a novel signaling pathway involved in miRNA biogenesis (Li et al., 2012), highlighting the potential of small-molecule modifiers of miRNAs as molecular tools for the elucidation of miRNA-involved regulatory pathway. From a special selection of small molecules obtained from organic photoreactions, we have found a series of active molecules with significant inhibition on the muscle-specific miR-1 in C2C12 cells (Tan et al., 2013). Here we report the chemical biological work using one of the active compounds as probe to investigate myomiR-involved cellular regulatory pathway in C2C12 cells.

RESULTS AND DISCUSSION

Figure 1A showed the chemical structure of the small-molecule inhibitor **1** that has been found to inhibit miR-1 in differentiated C2C12 cells (Tan et al., 2013). During treatment of C2C12 cells with compound **1**, we observed obvious morphology change of the cells in differentiation medium. Normally C2C12 cells

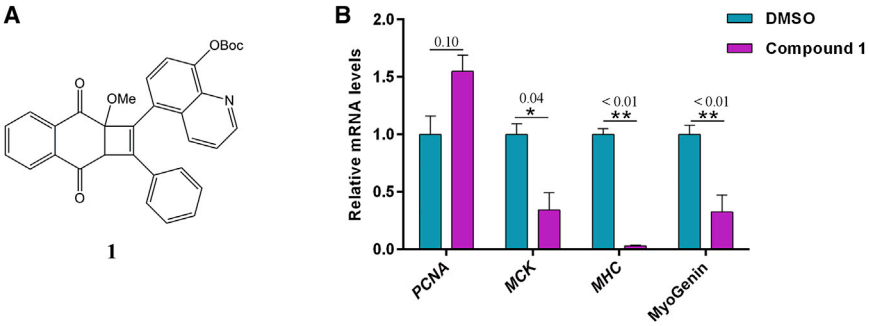

Figure 1. Compound 1 Inhibits Differentiation of C2C12

(A) Chemical structure of compound **1**.

(B) qRT-PCR analysis of mRNA levels of proliferation and differentiation marker genes in C2C12 cells upon treatment of compound **1** (10 μM) for 48 hr in differentiation medium. Data are shown as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$.

appear as small mononucleated and irregular fiber-like cells in culture medium and upon induced differentiation the cells become long multinucleated myotubes. Upon treatment with compound **1**, C2C12 cells stayed in a relative tranquil state without obvious proliferation, differentiation, or apoptosis behavior (Figure S1 available online). Flow cytometry analysis of C2C12 cells treated with or without compound **1** also suggested that compound **1** indeed inhibited the differentiation of C2C12 cells (Figure S2). Further real-time quantitative (qRT)-PCR quantification of proliferation and differentiation markers in C2C12 cells with or without treatment of compound **1** showed that differentiation markers including muscle creatine kinase (*MCK*), myosin heavy chain (*MHC*), and myoGenin were significantly downregulated in cells treated by compound **1**, while the proliferation marker proliferating cell nuclear antigen (*PCNA*) did not respond to compound **1** (Figure 1B).

We checked the effect of compound **1** on other myomiRs including miR-133a and miR-206. Using C2C12 cells transiently transfected with luciferase reporters for miR-133a or miR-206, we were able to tell the effect of compound **1** on endogenous miR-133a or miR-206 through the change of luciferase signal from the reporter cells before and after treatment with the compound. Figure 2A showed the relative luciferase signal from cells transfected with different luciferase reporters upon treatment of compound **1** at the concentration of 10 μM. After treatment with compound **1** for 48 hr, relative luciferase signals increased 7∼10-fold from reporter cells for miR-133a and miR-206 respectively, which is similar to that from the miR-1 reporter cells. In contrast, using compound **1** to treat cells transfected with luciferase gene-containing empty vectors or reporter cells for other miRNAs (miR-150, miR-25, miR-214, miR-21, and miR-9) ended with no significant increase on relative luciferase signal, which indicated that compound **1** was a selective inhibitor of myomiRs (Figure 2A).

Dose-dependence curves of compound **1** on its inhibitory activity toward myomiRs revealed the concentration for 50% of maximal effect values were ∼2.5 μM for miR-1 and ∼5 μM for miR-206 (Figure 2B). To further validate the specificity as well as investigate the mode of action of compound **1**, expression levels of different miRNAs in C2C12 cells with and without treatment of compound **1** for 48 hr were quantified by qRT-PCR. The results showed that expression levels of miR-1, miR-133a, and miR-206 in compound **1** treated C2C12 cells were ∼22%, 8%, and 27% to that in normal C2C12 cells (Figure 2C). In contrast, expression levels of other miRNAs including miR-150, miR-25, miR-214, miR-21, and miR-9 were similar in cells with and without treatment of compound **1**, which showed there was no significant difference. These results again confirmed that compound **1** specifically inhibited myomiRs by downregulating the expression levels of these miRNAs in C2C12 cells. Further investigation on the expression level of primary miR-1 (pri-miR-1), pri-miR-133a, and pri-miR-206 in C2C12 cells upon treatment with compound **1** for 48 hr (Figure 2D) showed that the active compound downregulated myomiRs at transcription level in C2C12 cells.

The simultaneous downregulation of pri-miRs of myomiRs and the differentiation markers in C2C12 cells by compound **1** indicated that the compound might target the upstream regulator that controls the expression of both myomiRs and differentiation-related genes. It has been reported that myomiRs are related to the regulation of muscle development (Chen et al., 2006; McCarthy, 2011) and recent evidence suggested that myoD is the major transcriptional factor of myomiRs (Rao et al., 2006). In addition, myoD is also an important regulator of differentiation (Berkes and Tapscott, 2005). Therefore, our initial hypothesis was that compound **1** might inhibit myoD and further suppress the generation of primary myomiRs and inhibit the expression of differentiation-related genes. To test this hypothesis, we first compared the mRNA level of myoD in C2C12 cells with and without treatment by compound **1**. qRT-PCR analysis showed that there was no significant difference between myoD mRNA levels in C2C12 cells with or without treatment of compound **1**. However, western blot analysis showed that myoD protein expression level in compound **1** treated cells was significantly downregulated compared with that in normal C2C12 cells (Figure 3). The results indicated that compound **1** was able to inhibit the translation of myoD in C2C12 cells without changing the expression level of myoD mRNA. We next tried to investigate how the compound suppressed the translation of myoD protein in C2C12 cells.

MiRNA is known to suppress the translation of its target gene at posttranscriptional level without altering the amount of mRNA of the target gene. Since the inhibition of the small molecule on myoD expression in C2C12 cells was also at posttranscriptional level, we wondered whether any miRNA-involved regulation on myoD expression in C2C12 cells was affected by the small molecule. Then we used bioinformatic calculation that has been commonly used for the estimation of complementarity between miRNA and 3′ UTR region of genes to predict miRNAs with potential binding with myoD (Witkos et al., 2011). Using TargetScan program (Lewis et al., 2005), we calculated both conserved and less conserved miRNA-myoD interactions with full pairing in

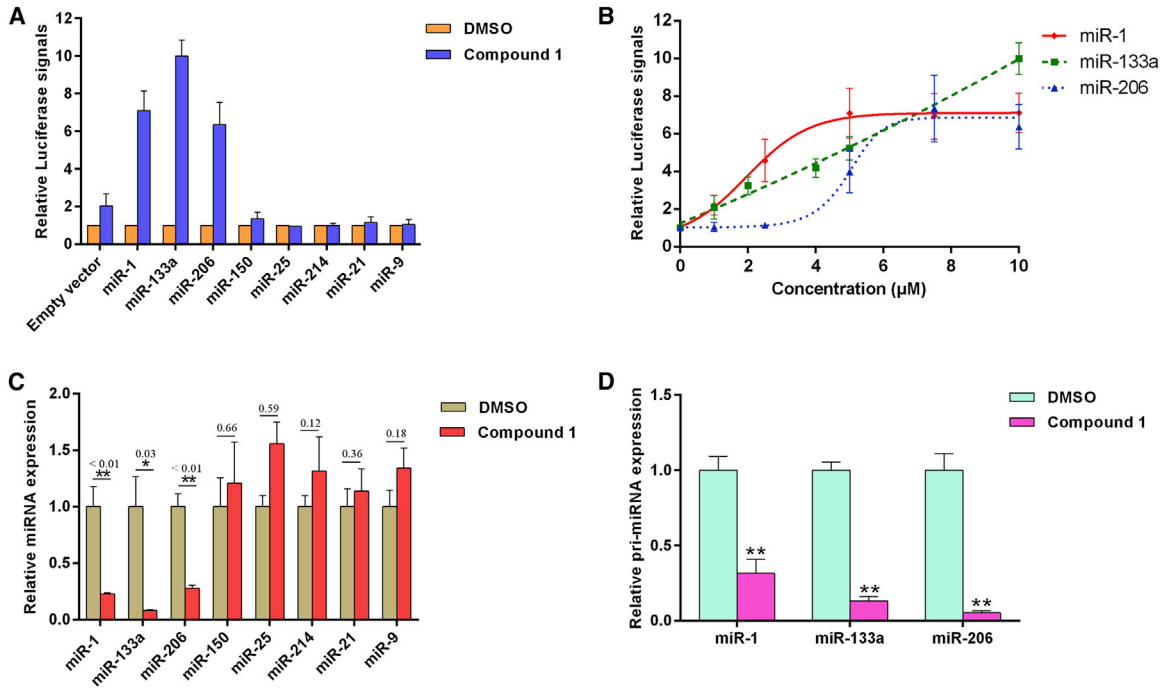

Figure 2. Identification of Compound 1 as a Specific Inhibitor of myomiRs

(A) Relative luciferase signals from cells transfected with empty vector and miRNA reporter vectors upon treatment of compound **1** (10 μM) in differentiation medium.

(B) Luciferase dose-response curves of compound **1** on miR-1, miR-133a, and miR-206 in C2C12 cells. Relative expression levels of (C) mature miRNAs and (D) primary miR-1, miR-133a, miR-206 in cells treated with compound **1** (10 μM) and DMSO for 48 hr. Data are shown as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

miRNA seed sequence (nucleotides 2–7 at 5′). Based on the preliminary prediction on the interaction between different miRNAs with myoD gene, we found that a series of miRNAs with relevance to osteogenesis chondrogenesis, smooth muscle development, muscular size and dystrophy, or heart diseases were able to bind with the 3′ UTR of myoD gene (Figure S3).

The oncogenic miR-221/222 (Chen et al., 2013; Galardi et al., 2011) were predicted to target the position 383–389 of myoD 3′ UTR, which was unexpected and became a topic of interest to us. We then calculated the free energy of RNA hybrid formed by miR-221/222 with myoD. The minimum free energies were around −26.3 kcal/mol for miR-221-myoD hybrid and −28.1 kcal/mol for miR-222-myoD hybrid, which are well within the energy range required for authentic miRNA-target pairs. We also noticed that there are existing evidences that miR-221/222 were related to the differentiation, proliferation, maturation, and regeneration of skeletal muscle (Cardinali et al., 2009; Greco et al., 2009; Togliatto et al., 2013). Therefore, we next investigated whether miR-221/222 in C2C12 cells were upstream regulators of myoD. Using the small molecule **1** as a probe to induce the acute change of myoD protein expression, we found that the upregulation of miR-221/222 level was in accompany with the suppression of myoD protein expression in C2C12 cells treated with compound **1** (Figure 4A). The results suggested the possibility of direct suppression of myoD translation by miR-221/222.

To confirm the regulation of miR-221/222 on myoD protein expression in C2C12 cells, we performed overexpression and knockdown of miR-221, miR-222, and miR-221/222 in C2C12 cells, respectively (Figure S4). As shown in Figure 4B, transfection of C2C12 cells with miRNA mimics of miR-221 and miR-222 separately or together all led to the significantly decreased expression of myoD protein according to western blot analysis. On the other side, myoD protein expression in C2C12 cells transfected with antisense of miR-221 and miR-222 either separately or together was all upregulated compared with that in normal C2C12 cells (Figure 4C). These biochemical evidences further confirmed the regulation of miR-221/222 on myoD gene expression as revealed by the changes of C2C12 cells upon treatment with compound **1**.

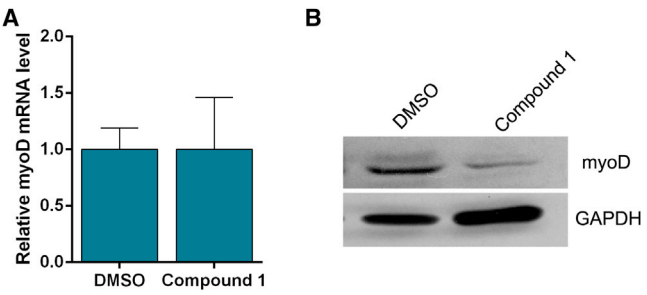

Figure 3. Inhibition of myoD Gene Expression at Posttranscriptional Level in Compound 1 Treated Cells

(A) Quantificative RT-PCR analysis of myoD mRNA expression in C2C12 cells upon treatment of compound **1** (10 μM) for 48 hr in differentiation medium.

(B) Western blotting of myoD protein expression in predifferentiated C2C12 cells after treatment with compound **1** (10 μM) for 48 hr. GAPDH protein was used as loading control. Data are shown as mean ± SD (n = 3).

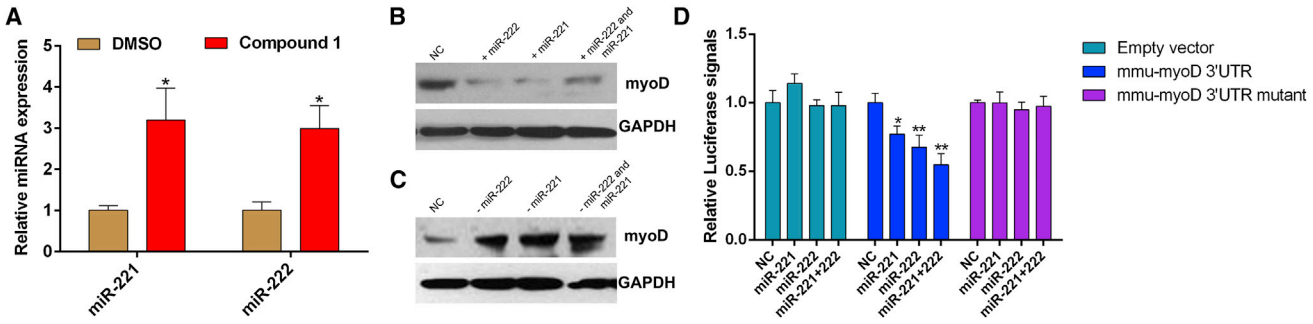

Figure 4. Regulation of miR-221/222 on myoD

(A) qRT-PCR analysis of miR-221/222 in C2C12 cells treated with compound **1** (10 μM) for 48 hr in differentiation medium.

(B) Western blot analysis of myoD protein expressions in C2C12 cells transfected with miR-221/222 mimics and (C) miR-221/222 antisense after incubated under differentiation condition for 48 hr. GAPDH was used as loading control.

(D) Direct recognition of the myoD 3′-UTR by miR-221/222. miR-221/222 or NC were cotransfected with empty vector, reporter vector containing myoD 3′UTR, or mutant myoD 3′UTR into 293T cells. The luciferase signals were determined after 48 hr of transfection. Assay was conducted in triplicates and normalized to NC control. Data are shown as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$.

An additional experiment was performed to determine whether the negative regulation of miR-221/222 on myoD expression was due to their complementary binding to the 383–389 position at 3′ UTR of myoD gene. We constructed a luciferase reporter plasmid by inserting the entire 3′ UTR of myoD segment to the 3′ UTR of luciferase reporter gene. Luciferase reporter plasmid without 3′ UTR of myoD (empty vector) was used as one control. The other control was the luciferase reporter plasmid inserted with 3′ UTR of myoD mutated at position 383–389 to eliminate its potential binding by miR-221/222. The constructed reporter plasmids were cotransfected with miR-221/222 mimics or negative control (NC) oligonucleotide into 293T cells that do not express miR-221/222. Luciferase expression was then used as the readout as the suppression of gene expression by miRNA through binding with the 3′ UTR of myoD. As shown in Figure 4D, in cells transfected with empty vector or the other control plasmid containing mutated binding sites, the presence of miR-221/222 did not show significant influence on luciferase expression. While in cells transfected with the reporter plasmid with myoD 3′UTR, miR-221/222 significantly suppressed the expression of luciferase gene. These results confirmed that miR-221/222 were able to target myoD in C2C12 cells by binding with its 3′ UTR at positions 383–389 and suppress myoD gene expression.

Lastly, to confirm the indirect regulation of miR-221/222 on myomiRs, expression levels of myomiRs were quantified after overexpression and knockdown of miR-221, miR-222, and miR-221/222 in C2C12 cells, respectively. As shown in Figure S5, myomiR expressions were significantly decreased when C2C12 cells were transfected with miR-221 mimic and miR-222 mimic separately or together. While transfection with antisense of miR-221 and miR-222 either separately or together into C2C12 cells induced significant upregulation of myomiR expressions. These results further verified the regulation of miR-221/222 on myomiRs.

SIGNIFICANCE

In summary, we reported a regulatory pathway in cells that was revealed by treatment of C2C12 cells with the selective small-molecule inhibitor of myomiRs. The inhibition of myomiRs by the small molecule was found to be correlated with the downregulated expression of differentiation markers. Transcriptional factor myoD was then found to be suppressed at protein level, but not mRNA level in C2C12 cells treated by the same small molecule, which revealed the possibility of miRNA regulation on myoD expression. Using bioinformatic prediction combined with experimental evidence obtained in the C2C12 cells treated by the small molecule, we discovered that the oncogenic miR-221/222 were able to target myoD gene through binding with its 3′ UTR at positions 383–389 and suppress myoD gene expression. This newly revealed regulatory pathway was further confirmed by biochemical evidences obtained by overexpression or knockdown of miR-221/222 leading to inhibition or enhancement of myoD protein expression and downregulation or upregulation of myomiR expression inside the transfected cells. The work reported here demonstrated the potential applications of small-molecule regulators of endogenous miRNAs as probes to reveal miRNA-involved regulatory pathways inside cells. Further work to modify the small active molecule with bio-orthogonal functionalities for target identification is now underway in our group.

EXPERIMENTAL PROCEDURES

Quantitave RT-PCR Analysis of Mature and Primary miRNA Expression Level

Total RNA from cells treated with compound **1** and DMSO was isolated using TRIzol reagent (Invitrogen) according to manufacturer's protocol. Mature mmu-miR-1, mmu-miR-133a, mmu-miR-206, mmu-miR-221, and mmu-miR-222 in differentiated C2C12 cells; hsa-miR-21 in HeLa cells; hsa-miR-25 and hsa-miR-214 in A549 cells; miR-9 in MCF-7 cells; and miR-150 in RAW267.4 cells were quantified using Taqman miRNA detection assay (Applied Biosystems). In general, 1 μg of RNA extracted from cells was used for each reaction. cDNA of interested miRNA was synthesized from total RNA using specific mature miRNA primers (Applied Biosystems) and cDNA Reverse Transcription Kit (Takara). The reactions were carried out in a thermal cycler for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. Real-time PCR was performed on Applied Biosystems 7300 Fast Real Time PCR system using rTaq polymerase (Takara). The reaction was carried out at 95°C for 10 min followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. For the primary miRNA

expression level analysis, the stem loop structure of primary miRNA was opened by reverse primer in a thermal cycler for 5 min at 85°C and then 5 min at 60°C. The pretreated RNA was synthesized into cDNA and then quantified by qRT-PCR. The thermal procedure for amplification was 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, and 50°C for 60 s. The sequences (5′ to 3′) of the forward and reverse primers for amplification of primary miR-1, miR-133a, and miR-206 were included in Table S1. The relative expression level was calculated using the comparative C_1 method. U6 and β-actin were used as internal controls for quantification of mature and primary miRNAs, respectively.

Quantitative RT-PCR Analysis of Genes mRNA Levels

2 μg of total RNA was reversely transcribed to cDNA using avian myeloblastosis virus reverse transcriptase (Takara) and oligodT (Takara) under thermal procedure of 42°C, 1 hr and 85°C, 5 min. 1 μl of cDNA was subjected to PCR amplification on Applied Biosystem 7300 Real-Time PCR system, using Evagreen dye (Invitrogen) detection assay. The thermal procedure for amplification was 95°C, 5 min, followed by 40 cycles of 95°C, 30 s; 58°C, 30 s; and 72°C, 30 s. The relative abundance of mRNA was determined from the Ct values and beta-actin was used as internal control. The sequences (5′ to 3′) of the forward and reverse primers for amplification of differentiation markers were listed in Table S1.

Overexpression and Knockdown of miR-221 and miR-222

C2C12 cells and 293T cells were seeded on six well plates and transfected the following day with miR-221/miR-222 mimics or miR-221/miR-222 antisense (Ribobio) by using Lipo-fectamine 2000 according to manufacturer's instructions. The efficiencies of the mimics and antisense were confirmed by qRT-PCR analyzing miR-221 and miR-222 levels. For miR-221/miR-222 target validation, the cells were cotransfected with empty vector, luciferase-myoD 3′UTR plasmids, or Luciferase-myoD mutant plasmids, and then harvested for luciferase assay or western blotting after 48 hr incubation in corresponding medium. For analysis of indirect regulation of miR-221/222 on myomiRs, mature miRNA expressions of myomiRs in C2C12 cells were quantified by qRT-PCR 48 hr after transfection of miR-221/miR-222 mimics or miR-221/miR-222 antisense.

Statistical Analysis

All of the data are shown as mean ± SD. For each experiment, we repeated at least three times, and the results are compared using Student's t test. Significant difference was considered if p values are less than 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with article online at http://dx.doi.org/10.1016/j.chembiol.2014.06.011.

AUTHOR CONTRIBUTIONS

S-B.T. conducted biological analysis including qRT-PCR, western blot, and cell cycle analysis; J.L. found the small-molecule inhibitor by luciferase assay, initiated the project, and designed the research together with C.Z. and Y.Z.; X.C. gave assistance in quantifying differentiation markers; W.Z. did chemical synthesis; D.Z. participated in qRT-PCR and micro-array assay; D.L. performed theoretical calculations to predict miRNAs binding with 3′UTR of MyoD; S-B.T., J.L., and Y.Z. wrote the paper.

ACKNOWLEDGMENTS

This work was inspired by the international and interdisciplinary environments of the Asian Core Program, "Asian Chemical Biology Initiative". The authors would like to acknowledge financial support from the National Natural Science Foundation of China (91213306, 21121091, 31470716, and 21372115) and the Natural Science Foundation of Jiangsu Province (BK2012012).

Received: April 11, 2014
Revised: June 19, 2014
Accepted: June 22, 2014
Published: September 18, 2014

REFERENCES

Berkes, C.A., and Tapscott, S.J. (2005). MyoD and the transcriptional control of myogenesis. Semin. Cell Dev. Biol. *16*, 585–595.

Bose, D., Jayaraj, G., Suryawanshi, H., Agarwala, P., Pore, S.K., Banerjee, R., and Maiti, S. (2012). The tuberculosis drug streptomycin as a potential cancer therapeutic: inhibition of miR-21 function by directly targeting its precursor. Angew. Chem. Int. Ed. Engl. *51*, 1019–1023.

Callis, T.E., Chen, J.F., and Wang, D.Z. (2007). MicroRNAs in skeletal and cardiac muscle development. DNA Cell Biol. *26*, 219–225.

Cardinali, B., Castellani, L., Fasanaro, P., Basso, A., Alemà, S., Martelli, F., and Falcone, G. (2009). Microrna-221 and microrna-222 modulate differentiation and maturation of skeletal muscle cells. PLoS ONE *4*, e7607.

Chen, J.F., Mandel, E.M., Thomson, J.M., Wu, Q., Callis, T.E., Hammond, S.M., Conlon, F.L., and Wang, D.Z. (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat. Genet. *38*, 228–233.

Chen, X., Huang, C., Zhang, W., Wu, Y., Chen, X., Zhang, C.Y., and Zhang, Y. (2012). A universal activator of microRNAs identified from photoreaction products. Chem. Commun. (Camb.) *48*, 6432–6434.

Chen, W.X., Hu, Q., Qiu, M.T., Zhong, S.L., Xu, J.J., Tang, J.H., and Zhao, J.H. (2013). miR-221/222: promising biomarkers for breast cancer. Tumour biology *34*, 1361–1370.

Croce, C.M. (2009). Causes and consequences of microRNA dysregulation in cancer. Nat. Rev. Genet. *10*, 704–714.

Ebert, M.S., and Sharp, P.A. (2012). Roles for microRNAs in conferring robustness to biological processes. Cell *149*, 515–524.

Galardi, S., Mercatelli, N., Farace, M.G., and Ciafrè, S.A. (2011). NF-kB and c-Jun induce the expression of the oncogenic miR-221 and miR-222 in prostate carcinoma and glioblastoma cells. Nucleic Acids Res. *39*, 3892–3902.

Greco, S., De Simone, M., Colussi, C., Zaccagnini, G., Fasanaro, P., Pescatori, M., Cardani, R., Perbellini, R., Isaia, E., Sale, P., et al. (2009). Common microRNA signature in skeletal muscle damage and regeneration induced by Duchenne muscular dystrophy and acute ischemia. FASEB journal *23*, 3335–3346.

Gumireddy, K., Young, D.D., Xiong, X., Hogenesch, J.B., Huang, Q., and Deiters, A. (2008). Small-molecule inhibitors of microrna miR-21 function. Angew. Chem. Int. Ed. Engl. *47*, 7482–7484.

He, L., and Hannon, G.J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. *5*, 522–531.

Hoefig, K.P., and Heissmeyer, V. (2008). MicroRNAs grow up in the immune system. Curr. Opin. Immunol. *20*, 281–287.

Kornfeld, J.W., and Brüning, J.C. (2012). MyomiRs-133a/b turn off the heat. Nat. Cell Biol. *14*, 1248–1249.

Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell *120*, 15–20.

Li, Y., Lin, L., Li, Z., Ye, X., Xiong, K., Aryal, B., Xu, Z., Paroo, Z., Liu, Q., He, C., and Jin, P. (2012). Iron homeostasis regulates the activity of the microRNA pathway through poly(C)-binding protein 2. Cell Metab. *15*, 895–904.

Li, J., Tan, S., Kooger, R., Zhang, C., and Zhang, Y. (2014). MicroRNAs as novel biological targets for detection and regulation. Chem. Soc. Rev. *43*, 506–517.

McCarthy, J.J. (2011). The MyomiR network in skeletal muscle plasticity. Exerc. Sport Sci. Rev. *39*, 150–154.

Rao, P.K., Kumar, R.M., Farkhondeh, M., Baskerville, S., and Lodish, H.F. (2006). Myogenic factors that regulate expression of muscle-specific microRNAs. Proc. Natl. Acad. Sci. USA *103*, 8721–8726.

Schneider-Poetsch, T., Ju, J., Eyler, D.E., Dang, Y., Bhat, S., Merrick, W.C., Green, R., Shen, B., and Liu, J.O. (2010). Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. Nat. Chem. Biol. *6*, 209–217.

Schreiber, S.L. (2005). Small molecules: the missing link in the central dogma. Nat. Chem. Biol. *1*, 64–66.

Shan, G., Li, Y., Zhang, J., Li, W., Szulwach, K.E., Duan, R., Faghihi, M.A., Khalil, A.M., Lu, L., Paroo, Z., et al. (2008). A small molecule enhances RNA interference and promotes microRNA processing. Nat. Biotechnol. *26*, 933–940.

Sullivan, C.S., and Ganem, D. (2005). MicroRNAs and viral infection. Mol. Cell *20*, 3–7.

Sun, L., Wang, H., Wang, Z., He, S., Chen, S., Liao, D., Wang, L., Yan, J., Liu, W., Lei, X., and Wang, X. (2012). Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell *148*, 213–227.

Tan, S.B., Huang, C., Chen, X., Wu, Y., Zhou, M., Zhang, C., and Zhang, Y. (2013). Small molecular inhibitors of miR-1 identified from photocycloadducts of acetylenes with 2-methoxy-1,4-naphthalenequinone. Bioorg. Med. Chem. *21*, 6124–6131.

Thum, T., Catalucci, D., and Bauersachs, J. (2008). MicroRNAs: novel regulators in cardiac development and disease. Cardiovasc. Res. *79*, 562–570.

Togliatto, G., Trombetta, A., Dentelli, P., Cotogni, P., Rosso, A., Tschöp, M.H., Granata, R., Ghigo, E., and Brizzi, M.F. (2013). Unacylated ghrelin promotes skeletal muscle regeneration following hindlimb ischemia via SOD-2-mediated miR-221/222 expression. J Am Heart Assoc *2*, e000376.

Witkos, T.M., Koscianska, E., and Krzyzosiak, W.J. (2011). Practical Aspects of microRNA Target Prediction. Curr. Mol. Med. *11*, 93–109.

Yang, B., Lin, H., Xiao, J., Lu, Y., Luo, X., Li, B., Zhang, Y., Xu, C., Bai, Y., Wang, H., et al. (2007). The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat. Med. *13*, 486–491.

Young, D.D., Connelly, C.M., Grohmann, C., and Deiters, A. (2010). Small molecule modifiers of microRNA miR-122 function for the treatment of hepatitis C virus infection and hepatocellular carcinoma. J. Am. Chem. Soc. *132*, 7976–7981.

