Original

miR-27b-3p Was Involved in Retinoic Acid-induced Abnormal Early Myogenic Differentiation of C2C12 Cells via Targeting CaMKIIδ

Bo Liu1, Wei Cong3, Chao Liu3, Yi Tang3, Nan Zhou3, Nan Li3, Ying Zhang3, Yaru Jin2 and Jing Xiao3

1) Institute of Genome Engineered Animal Models for Human Diseases, Dalian Medical University, Dalian, China
2) Department of Oral Pathology, College of Stomatology, Dalian Medical University, Dalian, China
(Accepted for publication, April 3, 2018)

Abstract: Myogenic differentiation is an important stage within the multi-step process of skeletal muscle development. We previously found that excess retinoic acid (RA) could induce derangement of myofilaments in the embryonic tongue by inhibiting differentiation through Wnt5a/CaMKIIδ (calcium/calmodulin-dependent protein kinase II delta) pathway. Furthermore, our recently studies indicated that excess RA could directly induce abnormal expression of series of miRNAs in embryonic tongue, including miR-27b-3p. miR-27b-3p has been reported as a regulator involved in kinds of tumors development. In addition, miR-27 was proved to negatively regulate adipogenesis. It has been indicated that miR-27b could down-regulate the expression of Pax3 and Pax7 and thus inhibits myoblast proliferation. Here, we found that the expression of miR-27b-3p increased at early stage of myogenic differentiation (differentiation day 2, D2) in RA-treated C2C12 cells, but CaMKIIδ expression was reduced. Furthermore, bioinformatics analysis predicted that miR-27b-3p targets the 3’UTR of CaMKIIδ. The direct interaction between of miR-27b-3p and CaMKIIδ was confirmed by luciferase reporter assays. More importantly, rescue experiments indicated that CaMKIIδ mediated miR-27b-3p to regulate the early differentiation defects induced by excess RA, and which was achieved mainly via Myogenin. In conclusion, excess RA disturbed the early differentiation of C2C12 cells by stimulating miR-27b-3p expression which targeted CaMKIIδ, and then controlled the expression of Myogenin, those above implying new mechanism in myogenic differentiation and miR-27b-3p may act as a new biomarker for muscle disease.

Key words: Retinoic acid, miR-27b-3p, CaMKIIδ, Myogenic differentiation

Introduction

Vertebrate skeletal muscle development is a complex and multi-step process involving the myoblasts withdrawal from cell cycle and differentiation into multinucleated myotubes and myofibers terminally. Among which, myoblast differentiation is considered as the most crucial step for determination of muscle cell fate and final maturation of myofiber. Genes govern the myoblast differentiation in a spatio-temporal manner, which mainly including myogenic regulatory factors (MRFs), MEF2 and SRF7. Any variation of the factors involved in this process would lead to muscle related disease.

As an oxidative metabolite of vitamin A, physiological doses all-trans retinoic acid (RA) is essential for normal vertebrate embryonic development. RA deficiency or excess is teratogenic in nearly all vertebrate species, and could results in various degrees of congenital defects2. Our previous study about the palatal development showed that certain intragastic dose of RA on 10th day of pregnancy would result in fetal cleft palate mouse by inhibiting the growth of palatal mesenchymal cells3. Furthermore, we found that the RA induced cleft palate fetal mouse was accompanied by tongue malformation, because of the disruption of proliferation and differentiation of tongue muscle. More importantly, we found that wnt5a/calcium/calmodulin-dependent protein kinase II delta (CaMKIIδ) pathway was closely related with this process.

microRNAs (miRNAs) are about 22 nt single-stranded non-coding RNAs, which function to promote mRNA degradation or inhibit translation by binding the seed sequences of their target mRNAs4. miRNAs express in a tissue-specific manner5 and those miRNAs riched in both human and mouse heart and skeletal muscle6 were known as myomiRs, mainly including miR-1, -133a and -206. These myomiRs were proven to be highly and specifically expressed during the myogenic differentiation process and to be regulated by MFRs and other myogenic transcriptional factors, and which return target commonly differentiation-related genes5. In addition, many non-myomiRs were also proved to be involved in myogenic differentiation process by interacting with the myogenic differentiation-related genes, including miR-31, -34, -22, -145 and so on7. The aberrant expression of these miRNAs could subsequently lead to different muscle malformation or diseases.

miR-27b has been proven to inhibit adipocyte differentiation and be involved in various tumors development8. Actually, the role of miR-27b-3p in myogenesis is controversial. Crist et al proved that miR-27b could inhibit myoblast proliferation and improve differentiation by down-regulating Pax3/7 expression9. The other study indicated that miR-27b could inhibit the expression of myostatin and thus improve the proliferation of myoblasts8. In addition, miR-27b directly targeted Ga2 and then inhibited the proliferation and differentiation of satellite cells10. Taken together, the role of miR-27b in myogenesis needs further exploration and the expression and mechanism of miR-27b in RA induced abnormal myogenic differentiation remain uncovered.

In this paper, we showed the increase of miR-27b-3p expression in RA induced abnormal myogenic differentiation at early stage. Functionally, gain- and loss-of function experiments indicated that miR-27b-3p inhibited the early differentiation of myoblasts. Furthermore, we identified that CaMKIIδ was a downstream target of miR-27b-3p. Finally, rescue experiments determined that CaMKIIδ mediated
Table 1. RNA oligonucleotides’ sequences used in this study

| Oligonucleotide | Sequence (5'-3')                  |
|-----------------|----------------------------------|
| miR-27b-3p mimic| UUCACAGUUCGUAUGUGCCAGAAUU       |
| Duplex NC       | AGCUACUUAGCACUGUGUAAU            |
| miR-27b-3p inhibitor| GCGAGAAAUUGGACAGUUGAAU      |
| Inhibitor NC    | CAGUCAGUACUGCUAGCGUGGAGAAU       |
| siCaMKIIδ       | ACGUGACACUUCGUGUAGGAGAATT       |
| siNC            | AGUGACACGUGUAGGAGAATT           |

Table 2. The oligonucleotides’ sequences used in this study

| Oligonucleotide | Sequence (5'-3')                  |
|-----------------|----------------------------------|
| pmirGLO-       | TATGAGCGCGGTGAAACCTTGCCAATGAG    |
| CaMKIIδ-WT     | ATCTGTTACGTGCAATGAG              |
| pmirGLO-       | GATCTGTTACGTGCAATGAG             |
| CaMKIIδ-Mut    | GATCTGTTACGTGCAATGAG             |

Quantitative realtime PCR

Total RNA extraction from cells was carried out with RNAiso Plus reagent (Takara Bio Inc, Otsu, Japan). To detect the expression of miR-27b-3p reverse transcription was done using a High-Capacity cDNA Reverse Transcription Kit (4366596, Applied Biosystems, Foster City, CA, USA) with specific miRNA primers (000409, ABI), and U6 was used for the internal control (001973, ABI). Q-PCR was followed on ABI 7500 Fast Real-Time PCR System with the TaqMan®Universal Master Mix II Kit (no UNG, 4440040, ABI). cDNA was synthesized using PrimerScriptTM RT reagent Kit (Takara) followed by Q-PCR with the SYBR Premix Ex TaqTM Kit (Takara) on a Thermal Cycler DiceTM Real Time System (TP800, Takara) with GAPDH as an internal control. The sequences of the primers were listed in Table 3.

Immunocytochemistry

Cells cultured in 24 well plates were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature and permeabilized with 0.5% Triton-X100 (Solabio, Beijing, China). After incubated with primary antibody against mouse myosin (1:50, Maixin, Fuzhou, China) or antibody against CaMKIIδ (1:200, Abcam, Cambridge, UK) at 4°C overnight, cells were blocked with fluorescent secondary antibody (1:200, goat anti-mouse, DyLight 488, or goat anti-rabbit, Alexa Fluor 594, 1:200, Abcam), and nuclei were stained by DAPI (D9542, Sigma) for 10 min. Finally, a fluorescence microscope (DP72, Olympus, Tokyo, Japan) was used to capture the pictures from at least three regions in each well.

Western blot

Cells were harvest and lysed in ice-cold RIPA buffer for 30 min on ice. The concentration of total protein was determined by a BCA protein assay kit (Boster, Wuhan, China). Equal amounts of protein were separated by 5% stacking and 12% running SDS-PAGE (Bio-Rad, Hercules, USA) and subsequently transferred to PVDF membranes (Bio-Rad). After the membranes were blocked with Tris-buffered saline-Tween-20 (TBST) containing 5% non-fat milk for 2 hours at room temperature, the membranes were incubated with the primary antibodies against CaMKIIδ (ab105502, Abcam), myosin (05-716, Millipore, Billerica, MA, USA) and GAPDH (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif, USA) at 4°C overnight. After washed three times using TBST, the membranes were incubated with secondary antibodies for 1 hour at room temperature. The protein bands were detected by the enhanced chemiluminescence substrate (Boster).

Statistical analysis

All presented results were collected from at least three independent experiments. The differences between groups were analyzed by two-
Excess RA induced miR-27b-3p expression in early differentiation stage of C2C12

For investigation of RA influence on the expression of miR-27b-
miR-27b-3p directly targets 3’ CaMKIIδ UTR and inhibits CaMKIIδ expression

Our recent studies showed that the expression of miR-27b-3p was up-regulated significantly both in E14.5 mouse tongue and in C2C12 cells cultured in DM from the 2nd day in condition of excess RA then control. In the meantime, the expression of CaMKIIδ was decreased at the same stages. Bioinformation analysis using TargetScan, MiRanda, and MicroCosm predicted that a conserve complementary site in both the 3’ UTR of CamkIIδ and DTNA for the seed sequence of miR-27b-3p. We also found another miRNA, miR-31-5p could also complementary match with the 3’ UTR of CamkIIδ, but the effects of both miR-27b-3p targeted to DTNA and miR-31-5p to CaMKIIδ were mainly displayed in the late stages of C2C12 myogenic differentiation. So, whether miR-27b-3p targeted CaMKIIδ affected the other processes of myogenesis attracted our attention. Combine the above results (Fig. 1 and 2), RA induced the increase of miR-27b-3p and decrease of CaMKIIδ at early stage of C2C12 differentiation. For further confirmation of the predict result between miR-27b-3p and CaMKIIδ by Bioinformation analysis, luciferase reporter assay was conducted in 293T cells. The results indicated that luciferase activity was significantly repressed in the cells co-transfected with the miR-27b-3p mimic and pmirGLO-CaMKIIδ-WT compared with Duplex NC and miR-140-3p groups, while miR-27b-3p mimic displayed no effect on the luciferase activity of pmirGLO-CaMKIIδ-Mut as well as the Duplex NC (Fig. 3B). Moreover, the impact of miR-27b-3p on CaMKIIδ expression was detected at the meantime, and the qRT-PCR revealed that miR-27b-3p suppressed the mRNA levels of CaMKIIδ.
Figure 5. Real-time PCR analyzed the mRNA expression of miR-27b-3p, CaMKIIδ, MyoD and Myogenin in C2C12 cells after transfected with miR-27b-3p mimic for 2 days in DM. Results were presented as means ± SD of triplicate experiments. *P< 0.05, **P< 0.01.

Figure 6. Western blot analyzed Myosin and CaMKIIδ protein expression of C2C12 cells after transfected with miR-27b-3p mimic for 2 days in DM.

Figure 8. Real-time PCR analyzed the knock down efficiency of siCaMKIIδ and the m RNA expression of CaMKIIδ, MyoD and Myogenin in C2C12 cells after transfected with siCaMKIIδ for 2 days in DM. Results were presented as means ± SD of triplicate experiments. *P< 0.05, **P< 0.01.

Figure 7. Immunofluorescence staining analyzed the myosin expression in C2C12 cells transfected with miR-27b-3p mimic for 2 days in DM. Bar = 100 μm.

Figure 9. Western blot analyzed silencing efficiency of siCaMKIIδ (A) and Myosin and CaMKIIδ protein expression of C2C12 cells after transfected with siCaMKIIδ for 2 days in DM (B).
Figure 10. Immunofluorescence staining analyzed the myosin expression in C2C12 cells transfected with siCaMKIIδ for 2 days in DM. Bar = 100 μm.

Figure 11. Real-time PCR analyzed the mRNA expression of CaMKIIδ, MyoD and Myogenin in C2C12 cells transfected with miR-27b-3p inhibitor with or without RA in DM for 2 days. Results were presented as means ± SD of triplicate experiments. *P<0.05, **P<0.01.

**miR-27b-3p over-expression repressed early differentiation of C2C12**

As shown in Fig. 1 and 2, our results indicated that miR-27b-3p and CaMKIIδ expression was up- and down-regulated in RA treated cells on D2 separately. Then we determined whether miR-27b-3p overexpression could suppress the early differentiation of C2C12 significantly. miR-27b-3p mimic and Duplex NC were transfected into C2C12 cells at the beginning of differentiation. As shown in Fig. 5 after 2 days of transfection, the miR-27b-3p expression increased prominently compared with the cells transfected with Duplex NC. However, the CaMKIIδ expression as well as two myogenic regulatory factors MyoD and Myogenin, was repressed significantly (Fig. 5). Western blot results also indicated that the protein level of CaMKIIδ was decreased too (Fig. 6). To confirm the impaired effects of miR-27b-3p on the early differentiation of C2C12 cells, the myosin expression and myotube formation were detected by immunofluorescence staining, results showed that the number and length of myosin positive myotubes were decreased by miR-27b-3p mimic (Fig. 7). Taken together, these results demonstrated that miR-27b-3p mimic decreased CaMKIIδ expression and inhibited early differentiation of C2C12.

**Knockdown of CaMKIIδ impaired the early differentiation of C2C12**

In order to further explore whether the inhibition of CaMKIIδ expression in early differentiation stage of C2C12 was the main cause for the disruption of myogenic differentiation, cells were transfected with siCaMKIIδ to knockdown CaMKIIδ expression during differentiation process. As expected, after CaMKIIδ expression was knockdown (Fig. 8), the transcriptions of CaMKIIδ, MyoD and Myogenin were inhibited significantly after 2 days cultured in DM (Fig. 8). Meanwhile, the remarkably decreased protein levels of myosin and CaMKIIδ indicating by western blot also implicated an inhibition on early differentiation of C2C12 by siCaMKIIδ (Fig. 9). The immunofluorescence staining further confirmed the reduction of myosin in early differentiating C2C12 induced by siCaMKIIδ (Fig. 10). These results showed that interference on CaMKIIδ was sufficient to mediate the excess RA repression on early differentiation of C2C12 cells.

**Inhibition of miR-27b-3p partly abrogates early differentiation damage of C2C12 induced by excess RA**
To investigate whether the RA induced inhibition of C2C12 early differentiation was miR-27b-3p dependent, cells were transfected with miR-27b-3p inhibitor when treated with 10 μM RA. The mRNA levels of CaMKIIδ and Myogenin were significantly increased by miR-27b-3p inhibitor with or without RA (Fig. 11), but the MyoD mRNA increase was not significant in presence of RA (Fig. 11), implying that Myogenin was the main target of miR-27b-3p inhibitor when abrogated the disruption of excess RA. Immunofluorescence staining results of myosin further confirm the rescue by miR-27b-3p inhibitor (Fig. 12). Therefore, miR-27b-3p inhibitor could partly rescued C2C12 early differentiation caused by RA especially through regulating Myogenin expression.

Discussion

miRNAs are small non-coding RNAs which regulate gene expression at post-transcription level. miRNAs have been proved involved in skeletal myogenesis by interacting with MRFs and miRNA dysregulation are closely associated with muscular pathology. miR-27b has been shown to inhibit adipocyte differentiation by repressing PPARγ and CREB expression, while TNF-α could stimulate miR-27 expression via NF-kB pathway. Zhang and his colleagues proved that during the porcine myoblast differentiation, miR-27a expression was rebounded back after a transient decrease at early differentiation stage. Furthermore, they found that miR-27a inhibited the Myogenin expression as well as the Akt/FoxO1 pathway in this process. In our latest study, we found that when CaMKIIδ was up-regulated by miR-27b-3p inhibitor in cells treated with 10 μM RA, the Myogenin expression was enhanced more than MyoD, which implied that the rescue results of miR-27b-3p targeted CaMKIIδ in RA induced early myogenic differentiation damage was mainly through Myogenin. Thus, if there is a direct interaction between CaMKIIδ and Myogenin need our further detection.

Ubiquitously expressed and multifunctional CaMKII is the main CaMK in skeletal muscle and its four isoforms (-α,-β,-γ, and -δ) distribute in a cell-type specificity manner. Increased sarcoplasmic CaMKII content affected the skeletal muscle phenotype in vivo. Recently studies showed that the expression of CaMKIIδ was regulated by certain non-coding RNAs in pathologic processes. For example, miR-145 targeted CaMKIIδ directly to regulate reactive oxygen species (ROS)-induced cardiomyocytes apoptosis. CaMKIIδ plays key roles in vascular smooth muscle (VSM) by regulating proliferation, migration and injury-induced vascular neointima formation, and it was reported that miR-30 family members could inhibit neointimal hyperplasia by directly regulating CaMKIIδ expression after vascular injury. In this study, based on the bioinformatics analysis we speculated that CaMKIIδ might be a target of miR-27b-3p in myoblasts. As expected, gain- and loss-of function experiments validated miR-27b-3p negatively regulated CaMKIIδ expression and luciferase reporter assay demonstrated that CaMKIIδ was a target of miR-27b-3p.

CaMKII plays critical role in the Wnt/Ca<sup>2+</sup> pathway, we previous found that Wnt/CaMKIIδ pathway was involved in excess RA-induced abnormal tongue development of embryonic mice. To explore the molecular mechanism for this, we subsequently did miRNA microarray and identified the dysregulated miRNAs in RA-induced abnormal tongue and studies in this paper, we found that CaMKIIδ was a downstream target of miR-27b-3p. Furthermore, our latest studies also indicated that miR-27b-3p and miR-31-5p could target DTNA and CaMKIIδ respectively at late myogenic differentiation stages, implying that miR-27b-3p controls different targets and CaMKIIδ could be regulated by numbers of miRNAs at different stages during myogenic differentiation process. These were consistent with the characters and complex regulatory network of miRNAs. More importantly, miR-27b-3p could target CaMKIIδ and DTNA simultaneously although at different stages, which suggested a potential interaction between CaMKIIδ and DTNA in myogenic differentiation of skeletal muscle.
myoblasts and thus further investigations are needed to confirm.

In conclusion, the downregulation of CaMKIIδ in early myogenic differentiation stage demonstrated the involvement of this molecule in the progression of RA induced abnormal myogenic differentiation. The results of luciferase reporter assay indicated that miR-27b-3p could directly regulate CaMKIIδ expression to exert its antagonism for excess RA in myogenic differentiation. All these indicated that miR-27b-3p and CaMKIIδ may act as biomarkers and potential therapeutic targets for RA induced muscle deformation.

Acknowledgement
The work of this study was supported by National Natural Science Foundation of China (No: 81507962) and the National Natural Science Foundation of China for Youth Scientists (No: 81500827).

Competing Interests
The authors declare there are no COI regarding the publication of this paper.

References
1. Duprey P and Lesens C. Control of skeletal muscle- specific transcription: involvement of paired homeodomain and MADS domain transcription factors. Int J Dev Biol 38: 591-604, 1994
2. Lee GS, Kochhar DM and Collins MD. Retinoid-induced limb malformations. Curr Pharm Des 10: 2657-2699, 2004
3. Xiao J, Cong W, Wang R, Wang B, Wang F, Zhu E-x, Hu H, Katase N and Nagatsuka H. The study of palatal cell proliferation and apoptosis in retinoic acid induced mouse cleft palate varied with different developmental stage. J Hard Tissue Biol 6:125-130, 2007
4. Fabian MR, Sonenberg N and Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 79: 351-379, 2010
5. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W and Tuschl T. Identification of tissue- specific microRNAs from mouse. Curr Biol 12: 735-739, 2002
6. McCarthy JJ. MicroRNA-206: the skeletal muscle-specific myomiR. Biochim Biophys Acta 1779: 682-691, 2008
7. Zhao Y and Srivastava D. A developmental view of microRNA function. Trends Biochem Sci 32: 189-197, 2007
8. Marzi MJ, Puggioni EM, Dall'Olio V, Bucci G, Bernard L, Bianchi F, Crescenzi M, Di Fiore PP and Nicassio F. Differentiation-associated micro-RNAs antagonize the Rb-E2F pathway to restrict proliferation. J Cell Biol 199: 77-95, 2012
9. Lee JJ, Drakaki A, Iliopoulos D and Struhl K. MiR-27b targets PPARγ to inhibit growth, tumor progression, and the inflammatory response in neuroblastoma cells. Oncogene 31: 3818-3825, 2012
10. Crist CG, Montarras D, Pallafacchina G, Rocancourt D, Cumano A, Conway SJ and Buckingham M. Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. Proc Natl Acad Sci U S A 106: 13383-13387, 2009
11. Allen DL and Loh AS. Posttranscriptional mechanisms involving microRNA-27a and b contribute to fast-specific and glucocorticoid-mediated myostatin expression in skeletal muscle. Am J Physiol Cell Physiol 300: C124-C137, 2011
12. Minetti GC, Feige JN, Bombard F, Heier A, Morvan F, Nürnberg B, Leiss V, Birnbaumer L, Glass DJ and Fornaro M. Gu2I signaling is required for skeletal muscle growth, regeneration, and satellite cell proliferation and differentiation. Mol Cell Biol 34: 619-630, 2014
13. Cong W, Liu B, Liu S, Sun M, Liu H, Yang Y, Wang R and Xiao J. Implications of the Wnt5a-CaMKII pathway in retinoic acid-induced myogenic tongue abnormalities of developing mice. Sci Rep 4: 6082, 2014
14. Liu B, Liu C, Cong W, Li N, Zhou N, Tang Y, Wei C, Bai H, Zhang Y and Xiao J. Retinoid acid-induced microRNA-31-5p suppresses myogenic proliferation and differentiation by targeting CamkIIδ. Skelet Muscle 7: 8, 2017
15. Li N, Tang Y, Liu B, Cong W, Liu C and Xiao J. Retinoid acid-induced microRNA-27b-3p impairs C2C12 myoblast proliferation and differentiation by suppressing δ-dystrobrevin. Exp Cell Res 350: 301-311, 2017
16. Goljanek-Whysall K, Sweetman D and Münsterberg AE. microRNAs in skeletal muscle differentiation and disease. Clin Sci (Lond) 123: 611-625, 2012
17. Zyu H, Zhang X, Ding X, Wang H, Chen X, Zhao H, Jia Y, Liu S and Liu Y. miR-27 inhibits adipocyte differentiation via suppressing CREB expression. Acta Biochim Biophys Sin (Shanghai) 46: 590-596, 2014
18. Zhang S, Chen X, Huang Z, Chen D, Yu B, He J, Zheng P, Yu J, Luo J, Luo Y and Chen H. Effects of microRNA-27a on myogenin expression and Akt/FoxO1 signal pathway during porcine myoblast differentiation. Anim Biotechnol 11: 1-7, 2017
19. Takemoto-Kimura S, Suzuki K, Horigane SI, Kamijo S, Inoue M, Sakamoto M, Fujii H and Bito H. Calmodulin kinases: essential regulators in health and disease. J Neurochem 141: 808-818, 2017
20. Eilers W, Jaspers RT, de Haan A, Ferrière C, Valdivieso P and Flück M. CaMKII content affects contractile, but not mitochondrial, characteristics in regenerating skeletal muscle. BMC Physiol 14: 7, 2014
21. Cha MJ, Jang JK, Ham O, Song BW, Lee SY, Lee CY, Park JH, Lee J, Seo HH, Choi E, Jeon WM, Hwang HJ, Shin HT, Choi E and Hwang KC. MicroRNA-145 suppresses ROS-induced Ca2+ overload of cardiomyocytes by targeting CaMKIIδ. Biochem Biophys Res Commun 435: 720-726, 2013
22. Liu YF, Spinelli A, Sun LY, Jiang M, Singer DV, Ginnan R, Saddouk FZ, Van Riper D and Singer HA. MicroRNA-30 inhibits neonatal hyperplasia by targeting Ca(2+)/calmodulin-dependent protein kinase IIδ (CaMKIIδ). Sci Rep 6: 26166, 2016