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Brief Report

Identification of a cartilage specific novel miRNA which directly targets PRMT3 in rats

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ABSTRACT

Objective: Through experiments to testify a candidate novel miRNA previously discovered by us is a real miRNA and involved in cartilage development. Design: The *miR-novel* and the newly hairpin miRNA transcribed sequence (*pre-miR-novel*) was verified as a genuinely existing miRNA by northern blotting. The predicted secondary structure, sequence alignment and targets of *pre-miR-novel* were performed by "RNAstructure 5.3" program, LASTN2.8.0+/miRbase22 program and RNA hybird program, respective. GO/KEGG pathway analysis also were performed. The *miR-novel* expression in cartilage tissue during development was detected by RT-qPCR and dot blotting. The chondrocyte differentiation model was established to examine whether *miR-novel* is involved in cartilage development. The regulation of PRMT3 expression by novel miRNA was determined with the luciferase reporter gene assay and Western blotting after novel miRNA mimic or inhibitor transfection. Results: It's potential role in specifically regulating rodent cartilage development and associated cellular processes. Furthermore, the expression of protein arginine N-methyltransferase 3 (PRMT3), as a predicted target of the novel miRNA, was found consistently downregulated at rat cartilage during developmental stages and RCJ3.1C5.18 (C5.18) cells during the proliferating and hypertrophic phases of the cartilage development, where the *miR-novel* expression was significantly up-regulated. Both the dual-luciferase reporter gene assay and the up- or down-regulation of *miR-novel* suggest that the later can specifically bind with the *Prmt3 3′-UTR*. Conclusion: Overall, this study provides the first comprehensive evidence that a genuine cartilage-specific novel miRNA directly targets PRMT3 and may regulate multitudinous cellular processes and signal transduction during cartilage development.

1. Introduction

MicroRNAs (miRNAs) have been reported to play important roles in chondrocytesproliferation and differentiation, and in maintaining cartilage homeostasis [1,2]. The modern research focuses on the discovery of novel miRNAs, their target verification and functional annotations, large-scale expression profiling, as well as their roles in buffering biological processes [3]. The differentially expressed repertoire of miRNAs corresponding to different stages of cartilage development may provide useful information and possibility of identifying novel molecular players in cartilage synthesis, repair and other osteochondropathies.

Our previous deep sequencing study has reported a complex repertoire of miRNAs in rat cartilage development [4]. The candidate novel miRNAs are identified [5], after getting rid of the non-coding sequences with free energy more than −20 kcal/mol, subsequently selecting the loci that are embedded within typical stem-loop structures, with

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characteristic of DICER excised sites [6]. Focusing on the miRNA gene clusters expressed during the process of cartilage development in rats, we identified a novel transcript, located on chr20 of rat genome (Table S1). Our current data verifies authenticity of the novel miRNA (*miR-novel)* and exposes the spatio-temporal expression of this miRNA targetting PRMT3 in rat cartilage.

2. Methods

The methods are including in: predicting the secondary structure of the newly hairpin miRNA transcribed sequence (*pre-miR-novel*) by "RNA structure 5.3" program, performing sequence alignment with LASTN2.8.0+ and miRbase22 program, predicting miRNA targets via "RNA hybird programs", feeding the inbred Dark Agouti (DA) rats, culturing the pro-chondrocyte cell line RCJ3.1C5.18 (C5.18), isolating the total RNA, Northern blotting, Dot blotting assay, Western blotting, Immunohistochemical, Luciferase reporter assay. Detailed experimental procedures are described in the supplementary methods.

3. Results and discussion

3.1. A newly transcribed sequence is identified as a genuine cartilage-specific miR-novel, which expresses during cartilage development

The predicted secondary structure of newly hairpin miRNA transcribed sequence (*pre-miR-novel*) had a classic stabilized stem-loop structure by "RNA structure 5.3" program (Energy = −22.3) [Fig. 1(A)]. Both 71 nt *pre-miR-novel* and 22 nt miR-*novel* were detected by northern blotting in rat calvaria-derived pre-chondrocytes (C5.18), harvested at 7th day of in vitro induced differentiation. The cells monolayer showed elevated expression of collagen II (COL2) and Aggrecan (ACAN) in, demonstrating a chondrocyte-specific gene expression, highly sensitive to ECM regulation [7]. Thus, the differentiation induced C5.18 cell line is proved to be a tissue-specific experimental system of cartilage development. Accordingly, the northern blotting is acknowledged as the most efficient and classical method to validate a novel RNA molecule [Fig. 1(B)]. These findings establish that the *miR-novel* is a genuinely existing miRNA in rat chondrocytes.

The femoral head cartilages from rats at D0, D21 and D42 represent newborn/baby (0y), ablactation/child (5y) and juvenile (11y) of the human developmental stages, respectively [4,8]. At D0, only a cartilage tissue exists and no bone structure is present; D21 represents the existence of epiphyseal plate and the primary ossification center, while D42 characterizes the presence of secondary ossification center and a mature articular bone [4,8]. The expression of *miR-novel* was significantly increased at D21 and D42, as determined by RT-qPCR [P=0.0401, P=0.0317, Fig. 1(C) upper part] and Dot blotting [Fig. 1(C) lower part]. On the other hand, it was not detected in kidney, lung, spleen, liver and heart at any stage of the developing tissues in our RT-qPCR system (date not shown). The spatio-temporal expression of miRNA was more important for low abundance of the target mRNA, whose 1–2 copies could have resulted in its overexpression [3]. This suggests that the miR-novel is a cartilage-specific miRNA in rats that may regulate the tissue development.

Analyzing the sequence alignment statistics of transcribed novel hairpin miRNAs showed that conservativeness was fair to middling i.e. 69 nt were 91% accurately matching with the *pre-miR-novel* in total of 30 RNA transcripts [Fig. 1(D) and Table S5]. The rat *pre-miR-novel* sequence was highly conserved comparing to mice, particularly the PRMT3 [Fig. 1(E) and Table S6]. However, the seed sequence mutated from AGAAGGGAG to AGAAGGTAG in human transcriptome [Fig. 1(E) and Table S6]. These results further suggest that this miRNA may specifically regulate rodent cartilage development. Studying species-specific (non-conserved) miRNAs involved in regulating tissue and stage specific processes can be an effective strategy to expose certain regulatory mechanisms, and also because these miRNAs may lose their target mRNAs during evolution [9,10]. In rodents, the most important characteristic is the inability of epiphyseal growth plate to fuse, which leads to lifetime endochondral ossification.

Gene ontology (GO) and KEGG pathway analyses were performed to identify biological function of the *miR-novel* and pathways associated with its target mRNAs assessed through biological processes, cellular components and molecular functions [3,9]. GO analysis showed that *miR-novel* was significantly enriched in cellular processes,

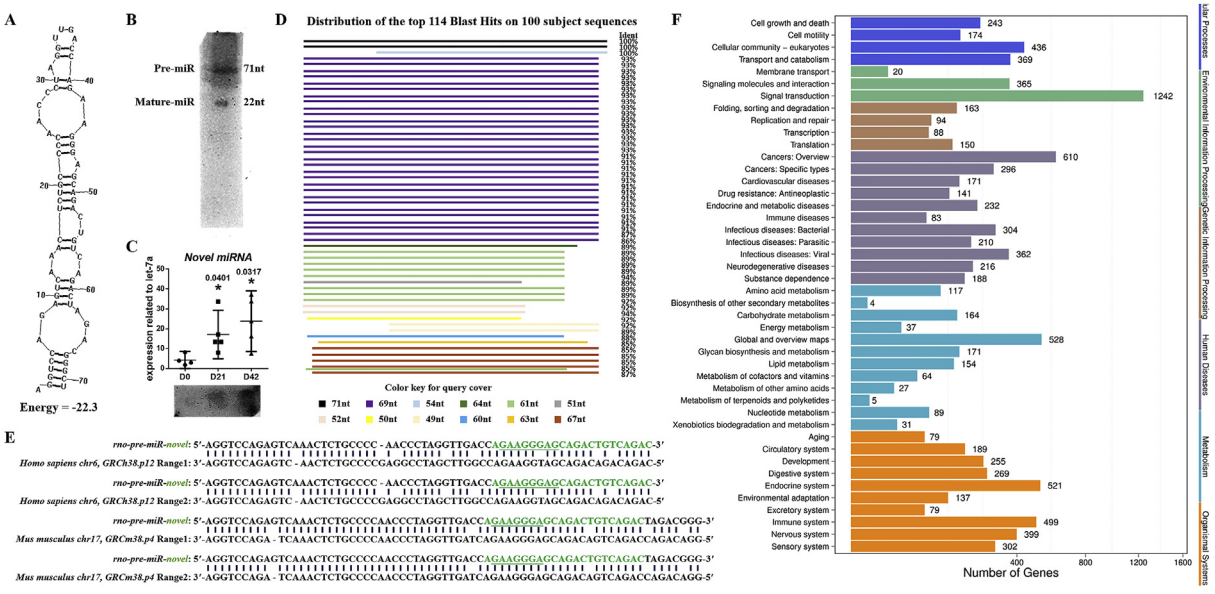

Fig. 1. The newly transcribed sequence is identified a genuine cartilage-specific *miR-novel,* expressing during cartilage development. (A) The secondary structure of the *pre-miR-novel* prediction by "RNA structure 5.3" program. (B) Results of northern blotting identification of *miR-novel* expression, in RCJ3.1C5.18 cells undergoing 7d induced differentiation. (C) The expression of *miR-novel* in left femoral head cartilages collected from DA rats at postnatal days 0 (D0), 21(D21) and 42 (D42) (n = 4, 5, 5). (D) Results of newly hairpin miRNAs transcribed sequence alignment statistics for distribution of the top 114 Blast Hits on 100 subject sequences matching to in BLASTN2.8.0+. (E) Results of hairpin miRNAs transcribed sequence match to *Homo sapiens* and *Mus musculus* in BLASTN2.8.0+. (F) KEGG pathway analysis of the miRNA-novel. The data were expressed as 95% confidence intervals, * stand for $P < 0.05$ with D0 groups.

single-organism process, cell, cell part, biological regulation, metabolic processes, regulation of biological processes, and so on [Fig. S1]. Likewise, KEGG pathway analysis showed enriched signal transduction associated with the *miR-novel* target mRNAs [Fig. 1(F)].

3.2. PRMT3 is a direct target of miR-novel in chondrocytes

Among hundreds of target genes predicted by the RNA hybird programs (Supplementary files), protein arginine N-methyltransferase 3 (PRMT3) was selected for further investigations [Table S4 and Fig. 2(A)]. Not only the GO and KEGG pathways analysis showed enriched cellular processes and signal transduction consistent with PRMT3 [11], but also PRMT3 expression was detected for the first time in cartilage tissue [Fig. 2(B)]. PRMT3 is a member of type I PRMTs family, which functions as a co-factor of methyl-donor S-adenosyl-L-methionine (SAM) for protein arginine labeling. The diverse roles of PRMT3 in the cellular settings for broad localization patterns have been implicated [12–14].

During rats' cartilage development, *miR-novel* showed remarkable up-regulation at D21 and D42 than that of D0 [P=0.0401 and P=0.0317 respectively, Fig. 1(C)]. Contrarily, PRMT3 expression was found significantly decreased during cartilage development i.e. at D21 and D42 [$P < 0.0001, P < 0.0001, P = 0.075$, Fig. 1(B, C) and Fig. S2]. It seems to be in line with our bioinformatics prediction that PRMT3 expression decreases with *miR-novel* up-regulation in differentiating chondrocytes.

Subsequently, dual-luciferase reporter gene analysis showed reduced Rluc/Fluc activity of *Prmt3* WT group (>60%), as compared to control group, suggesting that *miR-novel* could bind with *Prmt3-3′-UTR*. On the other hand, relative activity of Rluc/Fluc from *Prmt3* mutant group was increased up to150% comparing with that of WT group. However, no significant difference between the mutant group and control group could be observed, suggesting that *miR-novel* could not bind with *Prmt3-3′-UTR* because of mutation in the predicted binding sites UCCCUUC to ACACGUA [Fig. 2(A)] and [$P < 0.0001$, Fig. 2(D and E)].

Next, we used mimic and inhibitor sequences of *miR-novel* to up- or down-regulate its expression in differentiating C5.18 cells. The *inhibitor-miR-novel* could up-regulate PRMT3 protein level significantly while

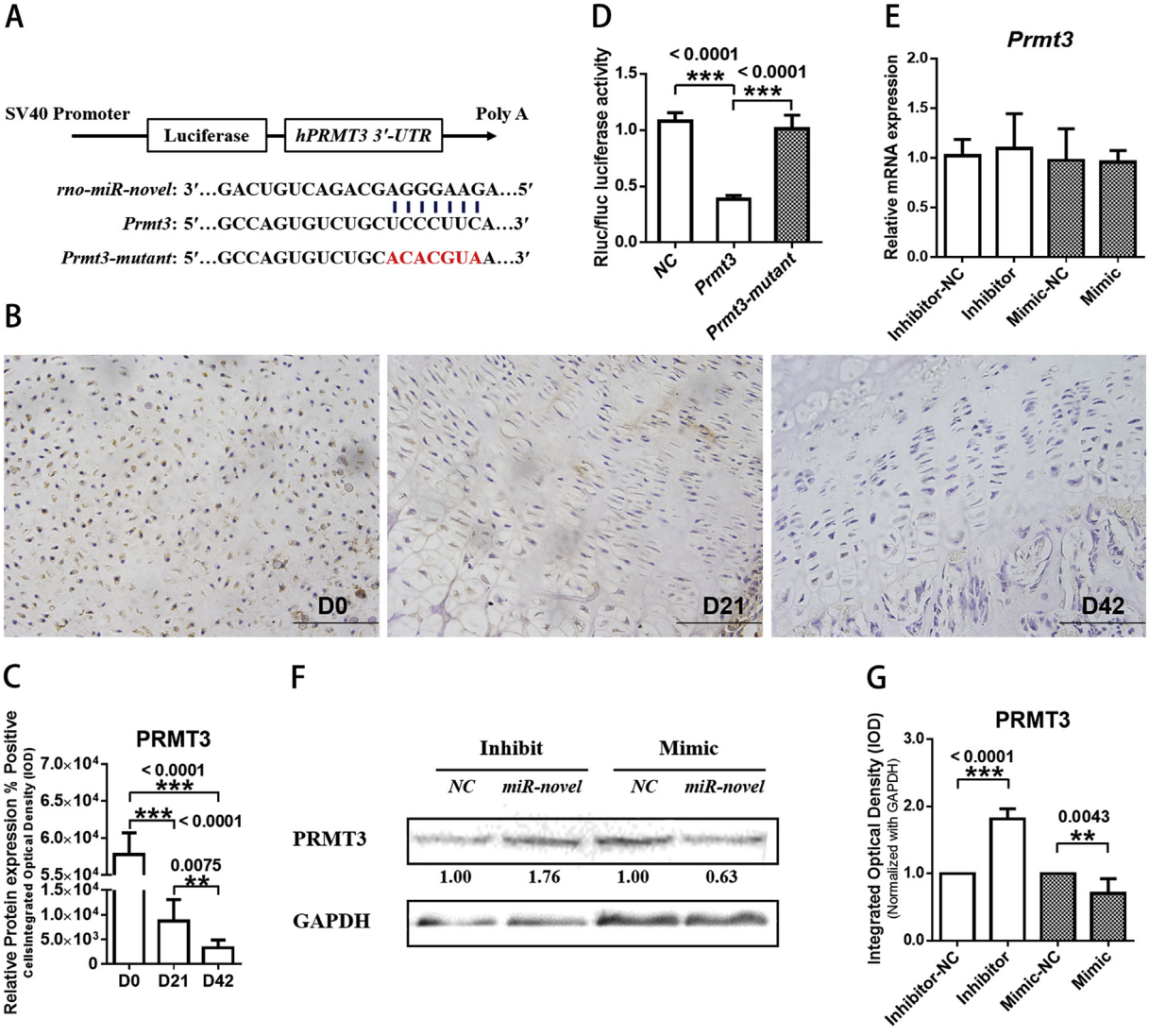

Fig. 2. PRMT3 is a direct target of *miR-novel* in chondrocytes. (A) Schematic diagram of *miR-novel* binding site with *Prmt3 3′-UTR*. (B) The representative IHC images of PRMT5 expression in right femoral heads collected from DA rats at postnatal days 0 (D0), 21(D21) and 42 (D42). (C) The statistical analysis of the Prmt5 protein % positive cells (Integrated Optical Density (IOD)) (n = 5, 5, 5). (D) Results of dual-luciferase reporter gene analysis with wild or mutant *Prmt3 3′-UTR*. (E) mRNA expression of *Prmt3* in RCJ3.1C5.18 cell line transfected with mimic or inhibitor of *miR-novel*. (F) The represent active protein expression of PRMT3 by WB in RCJ3.1C5.18 cell line transfected with mimic or inhibitor of *miR-novel*. (G) Statistical analysis of the Integrated Optical Density (IOD) of PRMT3 Protein expression (Normalized with GAPDH) (n = 3, 3, 3). The data were expressed as 95% confidence intervals, *, ** and *** stand for $P < 0.05$, 0.01 and 0.001 respectively.

mimic-miR-novel down-regulated it [Fig. 2(F, G) and Fig. S3], regardless of *Prmt3* mRNA levels that remained unchanged in all groups [Fig. 2(E)]. These results strongly suggest that *miR-novel* binds specificallywith *Prmt3 3′-UTR*.

PRMT3 forms a stable complex with 40S ribosomal protein S2 (RPS2) and PDCD2L [12,13], and promotes TOP3B topoisomerase activity via arginine methylation of the C-terminus RGG motif [14]. We may say that the specific down-regulation of PRMT3 by the *miR-novel* may suppress ribosome biogenesis by affecting ribosomal RNA precursor (pre-rRNA) processing during chondrocytes differentiation.

4. Conclusion

Overall, this study provides the first comprehensive evidence that a genuinely existing cartilage-specific novel miRNA directly targets PRMT3 and may regulate multitudinous cellular processes and signal transduction pathways during cartilage development.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgments

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Credit author statement

J. S. conceived, designed and supervised the study. J. S., Q. W. and Y. Y. performed most of the experiments, analyzed and interpreted the data. J. S. wrote the original manuscript. S. H., Y. Z., Y. G., M. S., H. H., X. H., F. Z., Q. N., Y. H., P. X. helped the experiments. J. S. and S. L. obtained the funding and critically revised the article for important intellectual content, and take responsibility for the integrity of the work as a whole.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ocarto.2021.100161.

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