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Original Article

Regulation of PP2Cm expression by miRNA-204/211 and miRNA-22 in mouse and human cells

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Aim: The mitochondrial targeted 2C-type serine/threonine protein phosphatase (PP2Cm) is encoded by the gene *PPM1K* and is highly conserved among vertebrates. PP2Cm plays a critical role in branched-chain amino acid catabolism and regulates cell survival. Its expression is dynamically regulated by the nutrient environment and pathological stresses. However, little is known about the molecular mechanism underlying the regulation of *PPM1K* gene expression. In this study, we aimed to reveal how *PPM1K* expression is affected by miRNA-mediated post-transcriptional regulation.

Methods: Computational analysis based on conserved miRNA binding motifs was applied to predict the candidate miRNAs that potentially affect *PPM1K* expression. Dual-luciferase reporter assay was performed to verify the miRNAs' binding sites in the *PPM1K* gene and their influence on *PPM1K* 3′UTR activity. We further over-expressed the mimics of these miRNAs in human and mouse cells to examine whether miRNAs affected the mRNA level of *PPM1K*.

Results: Computational analysis identified numerous miRNAs potentially targeting *PPM1K*. Luciferase reporter assays demonstrated that the 3′UTR of *PPM1K* gene contained the recognition sites of miR-204 and miR-211. Overexpression of these miRNAs in human and mouse cells diminished the 3′UTR activity and the endogenous mRNA level of *PPM1K*. However, the miR-22 binding site was found only in human and not mouse *PPM1K* 3′UTR. Accordingly, *PPM1K* 3′UTR activity was suppressed by miR-22 overexpression in human but not mouse cells.

Conclusion: These data suggest that different miRNAs contribute to the regulation of PP2Cm expression in a species-specific manner. miR-204 and miR-211 are efficient in both mouse and human cells, while miR-22 regulates PP2Cm expression only in human cells.

Keywords: PP2Cm; *PPM1K*; miR-204; miR-211; miR-22; species-specific

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Introduction

Protein phosphatase 2C in mitochondria (PP2Cm) is a mitochondrial matrix-targeted serine/threonine protein phosphatase encoded by the gene *PPM1K*[1, 2]. It is a member of the protein phosphatase 2C family and is highly conserved among vertebrates. PP2Cm expression is enriched in brain, heart, and liver[3]. PP2Cm protein is exclusively located in the mitochondrial matrix soluble fraction and modulates mitochondrial function[4]. In our previous work, we have shown that PP2Cm regulates mitochondrial membrane permeability transition pore (MPTP) opening and is critical in cell death regulation. The loss of PP2Cm leads to apoptosis and abnormal cardiac development in zebrafish[1].

PP2Cm is a key regulator of the catabolism of branched-chain amino acids (leucine, isoleucine, and valine, collectively referred as BCAA). The first step of the BCAA catabolic pathway is the conversion of BCAA into branched-chain α-keto acids (BCKA) by a branched-chain amino-transferase (BCAT). Then, BCKA is oxidized by branched-chain α-keto acid dehydrogenase (BCKD) complex, the rate-limiting enzyme in BCAA catabolism. BCKD's expression and activity control BCAA homeostasis[5, 6], and its defect causes Maple Syrup Urine Disease. BCKD activity is negatively regulated by Ser293 phosphorylation of the E1α subunit. Previously, we demonstrated that PP2Cm binds to BCKD complex and dephosphorylates Ser293 of the E1α subunit to activate BCKD[3, 7]. *Ppm1k*-deficient mice develop impaired BCAA

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catabolism and elevated plasma concentrations of BCAA and BCKA[7]. Recently, disrupted BCAA homeostasis has been linked to metabolic, neurological, and cardiovascular diseases[8–10], although the role of PP2Cm in the pathogenesis of these diseases remains to be established.

MicroRNAs (miRNAs) are small noncoding RNAs generated by the nuclear genome and contribute to targeted gene regulation at post-transcriptional or translational level by pairing to the target transcript's 3′-untranslated region (3′UTR)[11–13]. The pairing between the target sequence and the seed sequence (6–8 nt) of the miRNA is regarded as the most important feature for target recognition by miRNAs in mammals[14]. The targeting sites can be highly conserved where they match the miRNA seed sequence perfectly, or less conserved with one or two mismatches. Links have been found between miRNAs and numerous developmental and pathological processes[13, 15]. In particular, miR-204 and miR-211 belong to the same miRNA sub-family and share the same seed sequence: miR-204 is embedded in intron 6 of the *TRPM3* gene (transient receptor potential melastatin 3) and has been shown to be a key regulator in metabolism, cell death, and cardiovascular disease[16–20], and miR-211 is encoded by intron 6 of the *TRPM1* gene[17, 21] and also been shown to play important roles in cell survival and cancer[22–24]. miR-204 is completely conserved between human and mouse. However, mouse miR-211 has a similar mature sequence to its human counterpart with only one nucleotide difference in the non-seed region (www.mirbase.org). Finally, miR-22 is fully conserved between human and mouse and has been reported to be involved in cardiovascular diseases and metabolic regulation[25–27].

Although PP2Cm performs crucial roles in cells, little is known about the regulatory mechanism of *PPM1K* gene expression. In a previous study, we observed the nutrient-dependent transcriptional regulation of PP2Cm in response to BCAA availability[3]. In this study, we revealed a contribution of miRNAs to PP2Cm regulation at the post-transcriptional level in cells from different species.

Materials and methods

Cell culture

HepG2, NIH 3T3, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, USA), penicillin (100 IU/mL) and streptomycin (100 μg/mL) in a humidified 5% CO_2/95% air incubator at 37°C.

Plasmids, miRNA mimics and transfection

The 3′UTR of mouse *Ppm1k* (4216 bp, chr6:57,456,496-57,460,711) and human *PPM1K* (2214 bp, chr4:89,181,531-89,183,744) were identified using the UCSC Genome Browser (http://genome.ucsc.edu/) and cloned into the psiCHECK_2 plasmid (Promega) with *Xho* I and *Not* I sites, respectively. The 3′UTR fragment was located in the 3′ flanking region of the synthetic Renilla luciferase gene. Thus, Renilla luciferase expression can be regulated by the downstream 3′UTR activity[28]. The *PPM1K* 3′UTR of miR-204, miR-211 and miR-22 binding site deletion mutants were generated by PCR-mediated mutagenesis and selected by *Dpn* I (TOYOBO) digestion. Deletion was confirmed by DNA sequencing.

The miRNA sequences were identified from the miRBase database, and all the miRNA mimics were purchased from GenePharma (Shanghai, China) with the following sequences: negative control (miR-NC): 5′-UUCUCCGAACGUGUCACGUTT-3′, miR-204: 5′-UUCCCUUUGUCAUCCUAUGCCU-3′, miR-211: 5′-UUCCCUUUGUCAUCCUUUGCCU-3′, and miR-22: 5′-AAGCUGCCAGUUGAAGAACUGU-3′.

Plasmids and miRNA mimics were transfected using lipofectimine2000 (Thermo Fisher) according to the manufacturer's protocol. For miRNA mimics transfected into mammalian cells, Life Technologies Reverse Transfection Protocol was performed.

Identification of candidate miRNAs targeting PPM1K

Two computational target prediction programs (MiRanda, TargetScan) were applied to analyze miRNAs that potentially targeted the *PPM1K* gene[29]. TargetScan was used to predict the conserved binding region, which is perfectly complementary to bases 2-8 of the miRNA (from 5′ to 3′)[30]. The human and mouse 3′UTR sequences of *PPM1K* were analyzed.

Luciferase reporter assays

Luciferase reporter assays were performed by co-transfecting miRNA mimics and psiCHECK_2 constructs containing wild-type and mutated 3′UTRs into HeLa cells. The transfected cells were rinsed once with cold PBS and lysed with passive lysis buffer (Promega Beijing, Beijing, China) after transfection for 24 h. Luciferase activity was measured using the GloMax-Multi Detection System (Promega) and the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase expression was normalized to the expression of firefly luciferase. Data are represented as mean±SEM from three biological replicates representing three independent experiments.

RNA extraction, reverse transcription and real-time PCR analysis

Total RNA was extracted using TRIzol Reagent according to the manufacturer's protocol. Then, total RNA was reverse transcribed into first-strand cDNA using M-MLV Reverse Transcriptase (Promega). The cDNA transcripts were quantified on a Step-One Plus Real-Time PCR System (Thermo Fisher) using SYBR Green (Thermo Fisher) with the following primer sequences: human *PPM1K*_F: 5′-CACAGATTGGCAAACGGAAA-3′, human *PPM1K*_R: 5′-GCAGGTCCACCGTGTCCAT-3′, mouse *Ppm1k*_F: 5′-TCTCATTGGCAAACGGAAAG-3′, and mouse *Ppm1k*_R: 5′-CAGACAGGTGGGCATAACTCG-3′.

For reverse transcription and real-time PCR of miR-204, miR-211 and miR-22, a miRcute miRNA first-strand cDNA synthesis kit and a miRcute miRNA qPCR detection kit (TIANGEN, Shanghai, China) were used according to the manufacturer's protocol. The miRNA-specific forward primers were purchased from TIANGEN, and the reverse primer

was complementary to the poly A adapter, provided with the miRcute miRNA qPCR detection kit. The results were normalized to U6 small nuclear RNA.

Statistical analysis

Data were expressed as mean±SEM. The two-tailed Student's *t*-test was employed to evaluate the difference between two groups, and $P<0.05$ was considered as significant.

Results

miRNAs regulate the expression of *PPM1K* genes from different species

We used TargetScan (online database, http://www.targetscan.org/) to find the binding sites of miRNAs potentially targeting the *PPM1K* gene via its 3′UTR sequence. The human (2214 base pairs in length) and mouse (4216 base pairs in length) 3′UTR sequences of *PPM1K* were analyzed. Among the miRNA candidates, miR-204, miR-211 and miR-22 are of particular interest. In addition to a less conserved binding site in mouse, miR-204 and miR-211 have conserved binding sites in the 3′UTR region of both the human and mouse *PPM1K* genes (Figure 1A). The miR-22 conserved binding site was only detected in the 3′UTR region of the human but not the mouse *PPM1K* gene (Figure 1A).

Then, we investigated whether miR-204 and miR-211 regulate PP2Cm expression in cells from different species. The mimics of these miRNAs were transfected into human and mouse cell lines, and their abundance was assessed by quantitative RT-PCR. The abundance of miR-204 and miR-211 increased more than 10-fold upon transfection with the corresponding mimic (Figure 1B and 1C, miR-211 data not shown). In both human hepatocellular liver carcinoma cell line HepG2 and mouse embryonic fibroblast cell line NIH 3T3, the endogenous *PPM1K* mRNA level was significantly decreased by the expression of miR-204 or miR-211 mimics but not by the non-specific miRNA control (miR-NC) (Figure 1B and 1C). Therefore, miR-204 and miR-211 expression regulates PP2Cm mRNA expression in both human and mouse cells.

miR-204 and miR-211 directly repress *PPM1K* expression via 3′UTR

To investigate whether *PPM1K* 3′UTR is a direct target of miR-204 and miR-211, we cloned mouse *Ppm1k* 3′UTR (4216 base pairs) and human *PPM1K* 3′UTR (2214 base pairs) fragments into the reporter vector psiCHECK_2, where Renilla luciferase expression can be regulated by a downstream 3′UTR[31], and generated Luc-mouse 3′UTR and Luc-human 3′UTR reporters, respectively. After co-transfecting these reporter vectors with miRNA mimics into cells, the luciferase activity was measured. The results showed that miR-204 and miR-211 significantly inhibited the expression of the luciferase fused with either human or mouse *PPM1K* 3′UTR but did not affect the

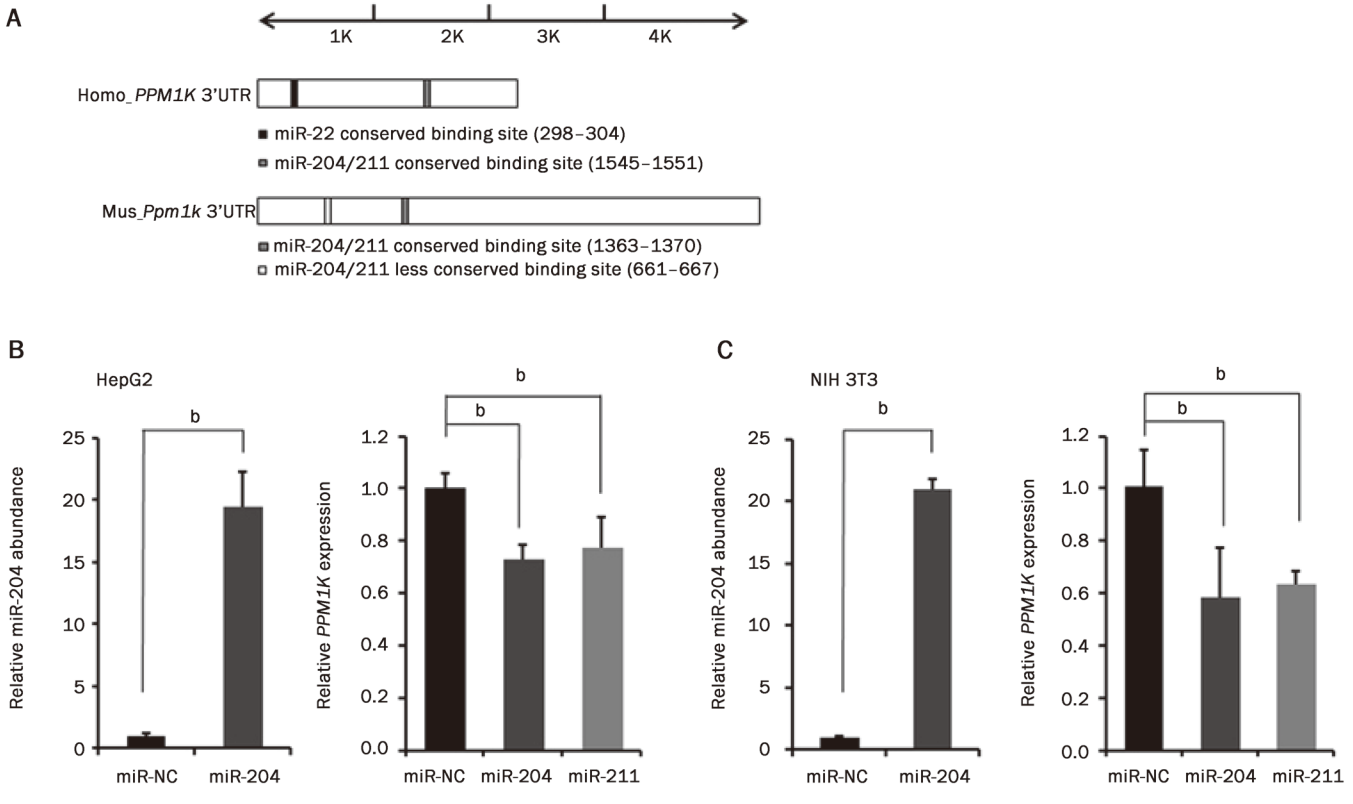

Figure 1. Candidate miRNAs regulate expression of *PPM1K* gene in human and mouse cell lines. (A) Summary of putative miRNAs targeting human and mouse *PPM1K* 3′UTR. (B and C) miR-204 abundance and PP2Cm mRNA level were assessed in HepG2 (B) and NIH 3T3 (C) cells after transfection with miRNA mimics. Data are mean±SEM for three individual samples ($^bP<0.05$).

activity of luciferase without any 3′UTR (named Luc-empty) (Figure 2). These data suggest that miR-204 and miR-211 target the 3′UTR to suppress human and mouse *PPM1K* expression.

Validation of miR-204 and miR-211 recognition motifs

TargetScan predicted numerous putative recognition motifs for miR-204 and miR-211 in the human and mouse *PPM1K* 3′UTRs. One conserved 8-mer seed match motif was identified in genes from both species, and one less-conserved 7-mer-m8 or 7-mer-1A seed match motif was identified in the mouse gene (Figure 1A). To validate the function of these putative miRNA recognition motifs, we generated mutant reporter constructs with targeted deletion of these motifs. For human *PPM1K* 3′UTR (Figure 3A), the deletion of the conserved recognition motifs (1545–1551 base pairs) completely abolished the inhibitory effect of miR-204 and miR-211 (Figure 3B). For mouse *Ppm1k* 3′UTR (Figure 3C), deletion of either the conserved motif (1363–1370 base pairs) or the less-conserved motif (661–667 base pairs) partially abolished the inhibitory effect of miR-204 and miR-211. However, the simultaneous deletion of both sites completely eliminated the effect of miR-204 and miR-211 on the *Ppm1k* 3′UTR (Figure 3D). These data suggest that the putative recognition motifs within the *PPM1K* 3′UTR are functional binding sites for miR-204/211 and are required for their inhibitory effect on PP2Cm expression.

Species-specific regulation of PP2Cm expression by miR-22

The regulatory function of miR-204/211 on the *PPM1K* gene was observed in both human and mouse. TargetScan also predicted a putative miR-22 binding motif in human but not mouse *PPM1K* 3′UTR. Using the 3′UTR luciferase reporter assay, we found that miR-22 inhibited the human *PPM1K* 3′UTR reporter activity but failed to affect the mouse *Ppm1k* 3′UTR reporter (Figure 4A). Deletion of the conserved miR-22 binding site completely abolished miR-22 mediated inhibition of the human *PPM1K* 3′UTR activity (Figure 4B). Furthermore, miR-22 mimic transfection increased the miRNA-22 abundance while decreasing the endogenous PP2Cm mRNA level in human HepG2 cells but not in mouse NIH 3T3 cells (Figure 4C). These data demonstrated that miRNA-22 regulates PP2Cm expression in human but not in mouse.

Discussion

Our data demonstrated that PP2Cm expression can be regulated by multiple miRNAs via 3′UTR targeting at the transcriptional level. miR-204 and miR-211 play conserved roles across human and mouse, while miR-22 may contribute to PP2Cm regulation only in human but not mouse cells. These results suggest that PP2Cm regulation involves different miRNA networks in different species.

PP2Cm is a key regulator of BCAA catabolism[7]. In intact animals, BCAA catabolic activity is accomplished through a coordinated process among different organs. In rodents, the transamination step occurs primarily in skeletal muscle, and the subsequent BCKD-mediated catabolic process predominantly occurs in liver. PP2Cm expression is highly enriched in the liver but low in skeletal muscle, consistently with the relative BCAA catabolic activities in different tissues in mice[3]. The miRNA's regulatory function has been a critical factor in tissue-specific gene expression. Further investigations are warranted to elucidate whether the tissue-specific expression of miR-22/204/211 contributes to the tissue-specific expression of PP2Cm.

The tissue-specific distribution pattern of BCAA catabolic activities is also regulated in a species-dependent manner. Comparing to rodents, where BCAA catabolic activity is primarily restricted to the liver, heart and brain, primate skeletal muscle also consumes a large portion of BCKA[32]. Considering that PP2Cm is a key regulator of BCAA/BCKA catabolic flux, it is possible that different miRNA networks from different species may contribute to the species-specific patterns of catabolic activities. The human *PPM1K* gene has a shorter 3′UTR than its mouse counterpart. However, there is no conserved miR-22 binding site detected in the mouse *Ppm1k* 3′UTR. This difference may represent a case where genetic variation resulted in gaining a new or losing an existing miRNA binding site, leading to different patterns of gene regulation by miRNAs[33]. Thus, it should be further investigated whether species-specific miRNAs involved in PP2Cm

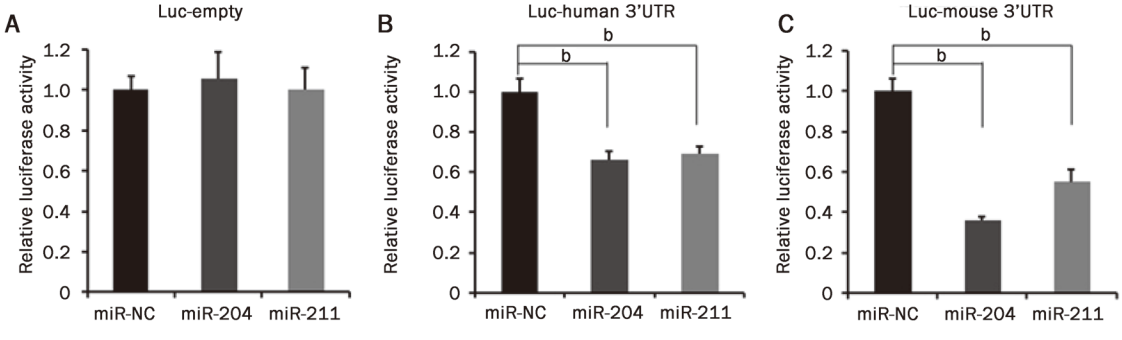

Figure 2. miR-204 and miR-211 suppress *PPM1K* 3′UTR activity. Luciferase assay results from HeLa cells co-transfected with miRNA mimics and luciferase reporter vectors. (A) Luc-empty is the reporter construct without any 3′UTR; (B) Luc-human 3′UTR is the reporter vector containing human *PPM1K* 3′UTR; (C) Luc-mouse 3′UTR is the reporter vector containing mouse *Ppm1k* 3′UTR. Data are mean±SEM for three individual samples ($^bP<0.05$).

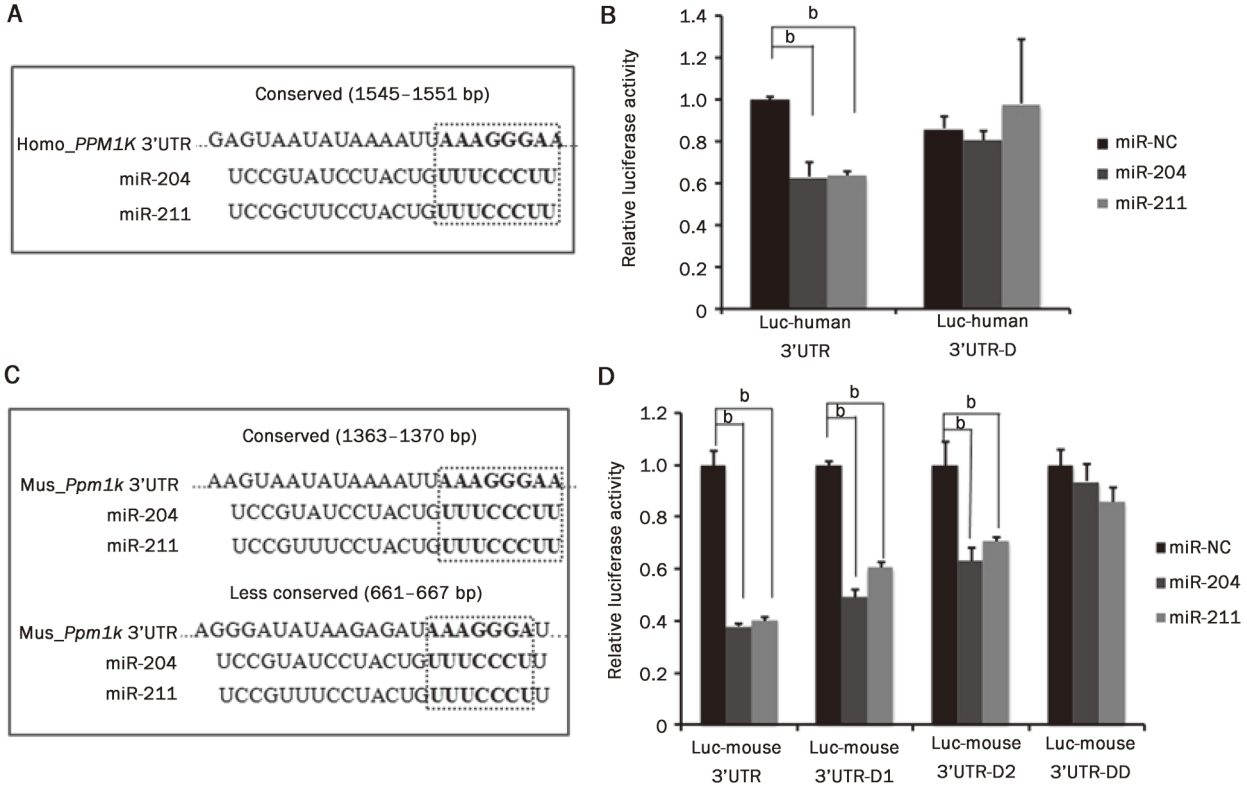

Figure 3. miR-204 and miR-211 directly target recognition motifs in *PPM1K* 3′UTR. (A) Schematic diagram showing the seed sequence of miR-204 and miR-211 and putative conserved recognition motif in human *PPM1K* 3′UTR. (B) Luciferase assay results from HeLa cells co-transfected with miRNA mimics and luciferase reporter vectors. Luc-human 3′UTR-D is the mutated reporter vector where the conserved recognition motif was deleted in human_*PPM1K* 3′UTR. (C) Schematic diagram showing the seed sequences of miR-204 and miR-211, with putative matched conserved and poorly conserved sequences in mouse *Ppm1k* 3′UTR. (D) Luciferase assay results from HeLa cells. Luc-mouse 3′UTR-D1 is the mouse *Ppm1k* 3′UTR reporter vector with conserved site deleted. Luc-mouse 3′UTR-D2 is the mouse *Ppm1k* 3′UTR reporter vector with less-conserved site deleted. Luc-mouse 3′UTR-DD is the mouse *Ppm1k* 3′UTR reporter vector with both sites deleted. Data are mean±SEM for three individual samples ($^bP<0.05$).

regulation contribute to the species-specific catabolic pattern of BCAA.

Cell death occurs during heart failure and may play a causal role in heart failure[34-36]. Meanwhile, mitochondrial dysfunction has been implicated in cardiomyocyte hypertrophy, ischemia/reperfusion injury and heart failure[37-39]. It has been previously reported that PP2Cm plays an essential role in mitochondrial regulation and cell survival[1]. The down-regulation of PP2Cm has been linked to cardiac diseases[1, 3]. However, the molecular mechanism of PP2Cm down-regulation in the stressed heart is not known. The current study suggests a mechanism by which miRNA may contribute to stress-induced PP2Cm down-regulation. Further investigations are warranted to elucidate how and whether these different miRNA networks contribute to PP2Cm down-regulation in the diseased heart.

In conclusion, our data showed that miR-204 and miR-211 both negatively regulate human and mouse PP2Cm expression. However, miR-22 repressed only human but not mouse *PPM1K* 3′UTR activity. The species-specific regulation of PP2Cm via different miRNA networks suggests the complexity of gene regulatory mechanisms involved in BCAA homeostasis and cell survival among different species. On the other hand, miRNA-based therapeutic approaches have been extensively explored in recent years[40]. Meanwhile, BCAA metabolic defects have been linked with metabolic syndrome, cancer, cardiovascular and hepatic diseases, and cell death has also been implicated in numerous diseases[41-43]. Considering the critical role of PP2Cm in BCAA catabolic and cell death regulation, the identification of miRNAs targeting PP2Cm strongly indicates the potential of developing novel therapeutic strategies to treat common disorders.

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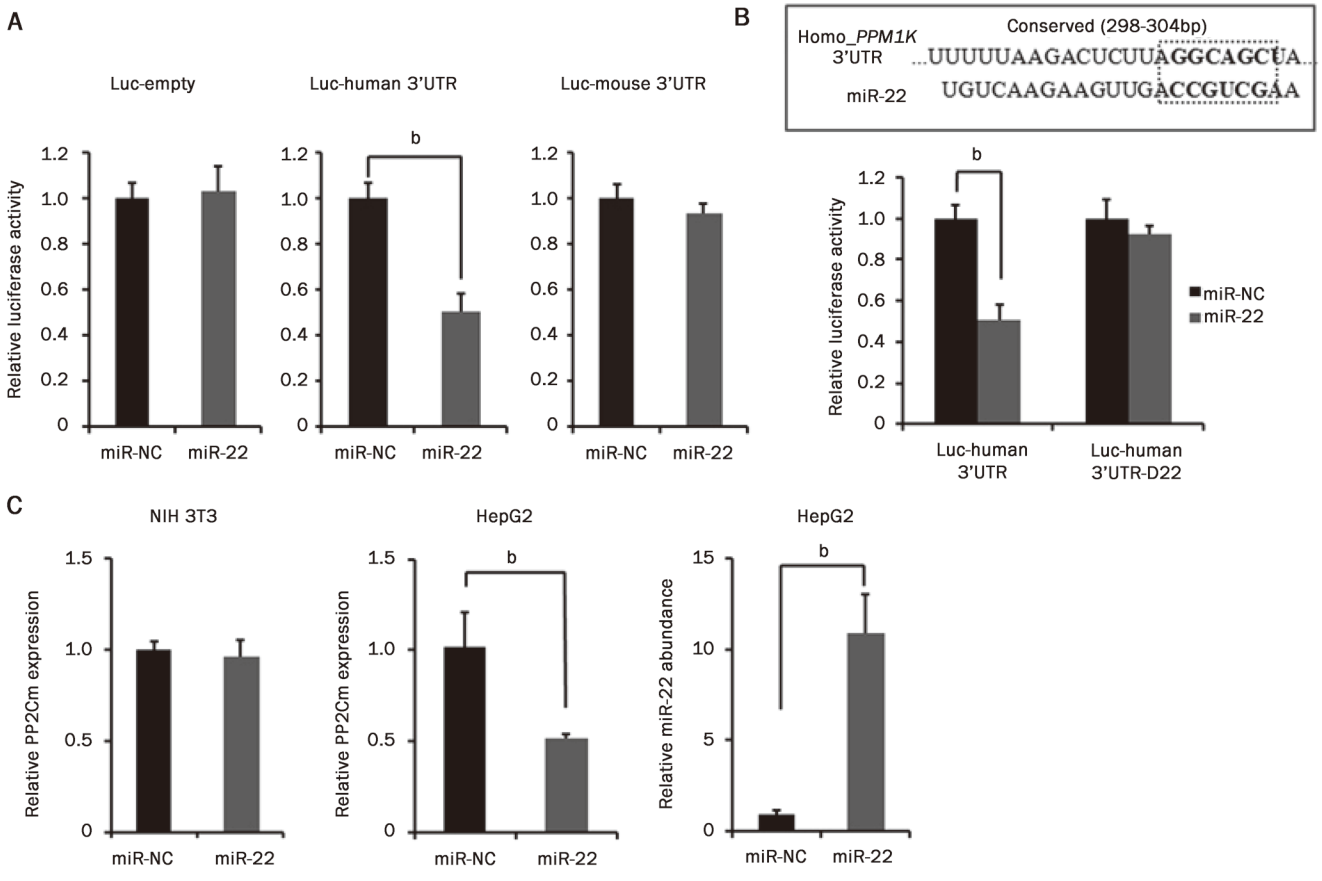

Figure 4. miR-22 regulates human but not mouse PPM1K expression. (A) Luciferase assay results from HeLa cells co-transfected with miRNA mimics and luciferase reporter vectors. Luc-empty is the reporter plasmid psiCHECK_2 without any 3′UTR. Luc-human 3′UTR is the reporter vector containing human PPM1K 3′UTR. Luc-mouse 3′UTR is the reporter vector containing mouse Ppm1k 3′UTR. (B) Top, a schematic diagram shows the seed sequence of miR-22 and putative matched conserved sequence in human PPM1K 3′UTR. Bottom, luciferase assay was performed with the reporter vector containing human PPM1K 3′UTR. Luc-human 3′UTR-D22 is the mutated reporter vector with the miR-22 binding site deleted. (C) miR-22 abundance and human PP2Cm mRNA level in HepG2 and mouse PP2Cm mRNA level in NIH 3T3 cells were assessed after transfection with miRNA mimics. Data are mean±SEM for three individual samples ($^bP<0.05$).

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Author contribution

Bang-fen PAN, Mei-yi ZHOU, and Hai-peng SUN designed the research; Bang-fen PAN, Chen GAO, Shu-xun REN, and Mei-yi ZHOU performed the research; Bang-fen PAN, Mei-yi ZHOU, and Hai-peng SUN analyzed the data; Yi-bin WANG helped to design the overall study and analyzed the data; and all authors contributed to the manuscript preparation.

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