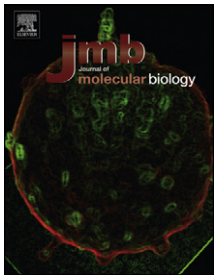

Post-Transcriptional Regulation of Renalase Gene by miR-29 and miR-146 MicroRNAs: Implications for Cardiometabolic Disorders

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Abstract

Renalase, a recently identified oxidoreductase, is emerging as a novel regulator of cardiovascular and metabolic disease states. The mechanism of regulation of renalase gene, especially at the post-transcriptional level, is completely unknown. We set out to investigate the possible role of microRNAs in regulation of renalase gene in this study. Computational predictions using multiple algorithms coupled with systematic functional analysis revealed specific interactions of miR-29a/b/c and miR-146a/b with mouse and human renalase 3′-UTR (*un*translated *r*egion) in cultured cells. Next, we estimated miR-29b and miR-146a, as well as renalase expression, in genetically hypertensive blood pressure high and genetically hypotensive blood pressure low mice. Kidney tissues from blood pressure high mice showed diminished (~1.6- to 1.8-fold) renalase mRNA/protein levels and elevated (~2.2-fold) miR-29b levels as compared to blood pressure low mice. A common single nucleotide polymorphism in human renalase 3′-UTR (C/T; rs10749571) creates a binding site for miR-146a; consistently, miR-146a down-regulated human renalase 3′-UTR/luciferase activity in case of the T allele suggesting its potential role in regulation of renalase in humans. Indeed, genome-wide association studies revealed directionally concordant association of rs10749571 with diastolic blood pressure, glucose and triglyceride levels in large human populations ($n \approx$ 58,000–96,000 subjects). This study provides evidence for post-transcriptional regulation of renalase gene by miR-29 and miR-146 and has implications for inter-individual variations on cardiometabolic traits.

Introduction

Renalase, a recently discovered flavoprotein with oxidoreductase activity, is expressed in several tissues including kidney, heart and nervous system [1,2]. Apart from its enzymatic function, renalase may also act as a signaling molecule to protect against acute kidney injury via interaction with a cell surface receptor, such as the plasma membrane calcium ATPase isoform PMCA4b [3,4]. The renalase gene is positioned on chromosome 19 and 10 in mouse and human, respectively [5,6]. Human renalase (*RNLS*) occurs as 7 isoforms (hRenalase1–hRenalase7 yielding proteins of 342, 315, 232, 200, 181, 163 and 139 amino acid residues, respectively) [3]; among these isoforms, *RNLS* isoform 1 and *RNLS* isoform 2 are prominently expressed [2,7]. On the other hand, mouse renalase (*Rnls*) has been reported to have 2 isoforms (*Rnls* isoform 1 and *Rnls* isoform 2 yielding proteins of 342 and 249 amino acids, respectively) [5,8].

Accumulating evidence in the literature shows that renalase is a major regulator for hypertension, diabetes, stroke and coronary heart disease [9–11]. For example, knockout mouse for renalase exhibited increased plasma catecholamines and high blood

pressure; consistently, injection of purified renalase into rats significantly decreased the mean arterial blood pressure [1,12]. The amount of this protein is profoundly diminished in sub-nephrectomized rats and end-stage renal disease and chronic kidney disease patients [6,11,13]. In addition, three common single nucleotide polymorphisms (SNPs) in human *RNLS* (viz. rs2576178 located in the promoter region, rs2296545 located in exon 2 and rs10887800 located in intron 6) have been associated with cardiometabolic disease states including hypertension, stroke and diabetes in different human populations [9,10,14–17]. Therefore, it is important to understand the regulation of this gene under basal and pathophysiological conditions.

Recent studies by us and others have revealed that several transcription factors (viz. Sp1, STAT3 and ZBP89) play crucial roles to regulate renalase transcription and that nicotine, dopamine and epinephrine activate renalase gene expression in various cell types [11,18,19]. The mouse *Rnls* promoter has also been shown to harbor multiple hypoxia response elements that bind to hypoxia-inducible factor 1α and enhance the renalase expression providing protection against myocardial ischemic reperfusion injury [20]. However, the mechanism of regulation of renalase at the post-transcriptional level remains completely unknown.

Here, we probed for the possible regulatory role of microRNAs (miRNAs) in governing the expression of renalase. Systematic functional analysis by mouse *Rnls* 3′-UTR (*un*translated *r*egion)/luciferase reporter assays and measurement of endogenous renalase protein levels after ectopic expression/inhibition of specific miRNAs revealed interactions of miR-29 and miR-146 miRNAs with mouse/human renalase 3′-UTR. In corroboration, genetically hypertensive blood pressure high (BPH) mice showed diminished protein levels of renalase and elevated levels of miR-29b as compared to genetically hypotensive blood pressure low (BPL) mice. Moreover, a common genetic variation in the 3′-UTR of human *RNLS* gene (C → T; rs10749571) altered interaction with miR-146a and the SNP displayed association with several physiological/biochemical cardiometabolic traits, suggesting potential role of miR-146a in regulating human *RNLS* gene *in vivo*. These observations provide new insights into the pathogenetic mechanisms of cardiometabolic disease states.

Results

Prediction of putative miRNA binding sites in the mouse *renalase* 3′-UTR

To identify miRNAs that may interact with the mouse *Rnls* 3′-UTR, we used 11 computational analysis programs (viz. miRWalk, Microt4, mirBridge, miRmap, miRNAMap, PICTAR2, RNA22, RNAhybrid, TargetScan, miRanda and PITA), resulting in the prediction of 1012 miRNAs. Among those potential miRNAs, four were predicted by at least seven programs (viz. mmu-miR-29b-3p, mmu-miR-146a-5p, mmu-miR-146b-5p and mmu-miR-758-3p; Table 1). Interestingly, the putative binding sites for these miRNAs are closely spaced toward the 3′-end of the UTR between 251 bp and 311 bp (Fig. 1). Further analysis of these miRNAs on their thermodynamic energy values (for interaction with the renalase 3′-UTR) by PITA and RNAhybrid programs showed that mmu-miR-29b, mmu-miR-146a and mmu-miR-146b had a PITA $\Delta\Delta G$ value of less than −10 and also RNAhybrid ΔG value of less than −20 kcal/mol (Table 1). Notably, other members of the miR-29 family (viz., miR-29a and miR-29c) were also predicted by at least six programs (Table 1). Thus, members of miR-29 and miR-146 families appeared as strong candidates for binding with mouse *Rnls* 3′-UTR.

Down-regulation of mouse renalase 3′-UTR/luciferase activity by miR-29 and miR-146 family members

To test interactions of miR-29 family with the mouse *Rnls* 3′-UTR (Fig. 2A), we carried out a series of co-transfection experiments in HEK-293 and N2a cells. First, the wild-type and mutated 3′-UTR/luciferase reporter constructs were co-transfected with increasing concentrations of a miR-29b expression plasmid. While the wild-type 3′-UTR construct showed diminished luciferase activity in HEK-293 cells (one-way ANOVA $F = 14.06$, $p = 0.0014$; up to ~63%, $p < 0.001$), as well as N2a cells (one-way ANOVA $F = 4.754$, $p = 0.0346$; up to ~30%, $p < 0.05$), the mutated 3′-UTR construct failed to show any significant changes (Fig. 2B and C). Next, co-transfection of the wild-type 3′-UTR was carried out with other members of the miR-29 family (viz. miR-29a and miR-29c that share the same seed sequence AGCACCAU). Repression of luciferase activities was observed in HEK-293 cells (one-way ANOVA $F = 20.21$, $p = 0.0004$; up to ~44%, $p < 0.05$ for miR-29a; up to ~70%, $p < 0.001$ for miR-29b; Fig. 2D) and N2a cells (one-way ANOVA $F = 6.323$, $p = 0.0166$; up to ~46%, $p < 0.05$ for miR-29b; up to ~50%, $p < 0.05$ for miR-29c; Fig. 2E). Thus, the effect of miR-29b to down-regulate the mouse *Rnls* 3′-UTR activity was consistent in both these cell types.

Similarly, we tested interactions of miRNAs belonging to miR-146 family with the mouse *Rnls* 3′-UTR (Fig. 3A). Increasing concentrations of a miR-146a expression plasmid caused a dose-dependent down-regulation of 3′-UTR activity in HEK-293 cells (one-way ANOVA $F = 13.04$, $p = 0.0013$; up to ~40%, $p < 0.001$) and N2a cells (one-way ANOVA $F = 4.918$, $p = 0.0318$; up to ~29%, $p < 0.05$); on the