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MicroRNA-133a regulates DNA methylation in diabetic cardiomyocytes

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ABSTRACT

We tested the hypothesis that miR-133a regulates DNA methylation by inhibiting Dnmt-1 (maintenance) and Dnmt-3a and -3b (de novo) methyl transferases in diabetic hearts by using Ins2$^{+/-}$ Akita (diabetic) and C57BL/6J (WT), mice and HL1 cardiomyocytes. The specific role of miR-133a in DNA methylation in diabetes was assessed by two treatment groups (1) scrambled, miR-133a mimic, anti-miR-133a, and (2) 5 mM glucose (CT), 25 mM glucose (HG) and HG + miR-133a mimic. The levels of miR-133a, Dnmt-1, -3a and -3b were measured by multiplex RT-PCR, qPCR and Western blotting. The results revealed that miR-133a is inhibited but Dnmt-1 and -3b are induced in Akita suggesting that attenuation of miR-133a induces both maintenance (Dnmt-1) – and de novo – methylation (Dnmt-3b) in diabetes. The up regulation of Dnmt-3a in Akita hearts elicits intricate and antagonizing interaction between Dnmt-3a and -3b. In cardiomyocytes, over expression of miR-133a inhibits but silencing of miR-133a induces Dnmt-1, -3a and -3b elucidating the involvement of miR-133a in regulation of DNA methylation. The HG treatment up regulates only Dnmt-1 and not Dnmt-3a and -3b suggesting that acute hyperglycemia triggers only maintenance methylation. The over expression of miR-133a mitigates glucose mediated induction of Dnmt-1 illustrating the role of miR-133a in regulation of DNA methylation in diabetes.

1. Introduction

Epigenetic modification is the alteration in gene expression without changing the underlying genetic sequences [12]. DNA methylation is the principle form of epigenetic regulation, where addition of a methyl group from *S*-adenosyl-L-methionine (Sam) to 5′cytosine of CpG dinucleotide restricts the accessibility of transcription machinery to the promoter region causing gene silencing and chromosome inactivation [28,33]. DNA methylation is catalyzed by a group of enzymes known as DNA methyl transferase (DNMT in human and Dnmt in mouse): Dnmt-1, -3a and -3b. Dnmt-1 serves as a maintenance methyl transferase that maintains the pre-existing methylation pattern to daughter strands after DNA replication [32]. It is the most abundant methyl transferase in somatic cells and has preference for hemi methylated CpG sites [6]. Dnmt-3a and -3b are de novo methyl transferases required for methylation during embryogenesis that establishes the somatic methylation pattern [24]. However, the regulation of Dnmt-1, -3a and -3b is not completely understood.

MicroRNAs are tiny (~22 nt), conserved, non-coding RNAs that modulate gene expression both at mRNA and protein levels by either mRNA degradation (if seed sequence match perfectly with mRNA) or translational inhibition (if seed sequence have incomplete complimentarity) [20,34]. In the heart, attenuation of miR-133a causes hypertrophy [4], fibrosis [18] and arrhythmia [31], making it a crucial miRNA involved in the heart failure. Interestingly, differential expression of miRNAs is affected by DNA methylation [13]. On the other hand, specific miRNA called epi-miRNA regulates the expression of epigenetic machinery through feed back mechanisms [13]. For example, miR-29 targets Dnmt-1 by regulating transcription factor Sp1 and Dnmt-3a and -3b in lungs cancer [7]. Interestingly, the initiation of epigenetic silencing of genes by DNA methylation depends on the ratio of miRNAs and its target RNA [15] that points to the involvement of miRNAs in modulating the methylation patterns. The attenuation of miR-133a [8] and induction of DNA methylation [23] in diabetic hearts led us to investigate the role of miR-133a in DNA methylation in diabetic cardiomyocytes.

Diabetes is rampant and accounts for a major death toll across the globe [16]. Diabetes enhances the incidents of heart failure 2- to 4-folds as compared to non-diabetic patients [17,25]. Due to differential expression of miRNAs, diabetes is recognized as miRNA-associated disease [11]. Also, the hyperglycemia mediated DNA hyper-methylation suggests that epigenetic regulation plays crucial role in diabetic cardiomyopathy [3,14,23]. The alarming rate of diabetics across the world and its tendency to become a menace warrants a dire need to understand the mechanisms associated with diabetic cardiomyopathy. To this end, the regulation of DNA methylation by miR-133a becomes important as both of them play pivotal regulatory roles in diabetic hearts.

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2. Methods

2.1. Animal models

$Ins2^{+/-}$ Akita mice were used as a genetic model for diabetes. These mice become hyperglycemic at the age of 3–4 week and show robust hyperglycemia and cardiomyopathy at 12 week [1,19]. Therefore, we used 12 week old mice. To avoid gender mediated differences, we used only male mice. We used age and gender matched C57BL/6J as wild type (WT) because they have same genetic background as Akita. Both mice were procured from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal care facility of University of Louisville under controlled temperature (22–24 °C) and light–dark (12 h) cycle with standard chaw and drinking water *ad libitum*. We followed Animal Care and Use Program guidelines of National Institute of Health and Institutional Animal Care and Use Committee of University of Louisville, for sacrificing the animals.

2.2. In vitro assay

We used AT-1 mouse atrial derived HL1 cardiomyocyte cell line because they retain all the properties such as morphological, biochemical, electrophysiological and contractility of cardiomyocytes [5,30]. The HL1 cardiomyocytes are kindly provided by Dr. W.C. Claycomb and maintained in Claycomb complete medium (JRH Biosciences, cat #51800C, Lenexa, KS, USA), which is supplemented with 10% fetal bovine serum (Sigma–Aldrich, cat # F2442, Saint Louis, MO, USA), 1% L-glutamine (Life Technologies, cat # 25030-081, Foster City, CA, USA), 1% nor-epinephrine (Sigma, cat # A-0937, Saint Louis, MO, USA), and 1% Penicillin–Streptomycin (Life Technologies, cat # 15140-122). For treatment, we used serum free plain medium. The protocol is described in details elsewhere [21].

2.3. Transfection and treatment of HL1 cardiomyocytes

To investigate the role of miR-133a in regulation of Dnmt-1, -3a and -3b, HL1 cardiomyocytes were transfected with miR-133a (Genecopoeia, Rockville, MD, USA, cat # MmiR3445-MR03), anti-miR-133a (Anti-miR™ miRNA Inhibitors, Ambion, USA, cat # AM10413) and scrambled (Genecopoeia, Rockville, MD, USA, cat # CmiR0001-MR03) using Lipofectamine 2000 (Invitorgen, cat # 11668-019) transfecting agent and following their protocol. To understand the specific role of miR-133a in mitigation of DNA methylation in diabetes, cardiomyocytes were treated with 5 mM (physiological level) and 25 mM (HG) of glucose (Sigma, cat # G8270) and HG + miR-133a mimic in serum free medium. The treated cells were incubated at 37 °C with 5% carbon dioxide for 24 h. They were processed for RNA and protein extraction following our previously reported protocol [21].

2.4. Multiplex RT-PCR

High quality (260/280–2.0 and 260/230–2.0) RNA was used for multiplex reverse transcription polymerase chain reaction (RT-PCR), where both endogenous control (18S) and target genes (Dnmt-1, -3a and -3b) were amplified together to rule out the variation in the RNA quantity and quality as well as initial quantification errors during PCR. The detailed protocol is elaborated in our earlier report [21]. The primer sequences are shown in Table 1.

2.5. Individual miRNA assay

The miR-133a mimic was tagged with GFP marker. The successful transfection of miR-133a mimic was validated at two levels: (i) green fluorescence due to GFP marker, and (ii) up regulation of miR-133a in transfected cardiomyocytes as compared to the scrambled by miR-133a assay. Similarly, successful transfection of anti-miR-133a was confirmed by miR-133a assay following the earlier described protocol [21]. Sno234 (Applied Biosystems, PN # 001637) was used as endogenous control.

Table 1
The forward and reverse primer sequences for DNA methyl transferase (Dnmt)-3a, and -3b, and GAPDH used for multiplex RT-PCR.

Gene	Forward sequences (5′–3′)	Reverse sequences (5′–3′)
Dnmt-3a	GGGAGAGAGGGAAAATTCTA	GGTTTTCTTCAAGGTTTCCT
Dnmt-3b	GACTGCCTGGAGTTCAGTAG	ACAGGCAAAGTAGTCCTTCA
GAPDH	TAAATTTAGCCGTGTGACCT	AGGGGAAAGACTGAGAAAAC

2.6. Western blotting

RIPA buffer (Boston BioProducts, Worcester, MA, USA) supplemented with protease inhibitor cocktail (Sigma, cat #P8340, Saint Louis, MO, USA) was used for protein extraction from hearts of WT and Akita, and HL1 cardiomyocytes. The standard Western blotting protocol was followed after protein estimation by Bradford method [21]. The primary antibodies for Dnmt-1 (Abcam, cat # AB13537) and GAPDH (Millipore, cat # MAB374) were diluted in the ratio of 1:1000 with overnight incubation at 4 °C. The anti-mouse secondary antibody (Santa Cruz Biotechnology, cat # sc-2005) was diluted at 1:3000 and incubated at room temperature for 2 h. The blots were developed using Pierce ECL substrate (Thermo scientific, cat # 32209) using Molecular Imager Chemi-Doc XRS with Image lab software, version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA). The band intensity was measured using the Bio-Rad Image lab software.

2.7. Statistical analyses

The statistical values were expressed as mean ± standard error (SE). To test the significant differences, we used paired *t*-test and the $p < 0.05$ value was considered statistically significant.

3. Results

3.1. MiR-133a is down regulated in $Ins2^{+/-}$ Akita hearts

To determine whether the expression of cardiac miR-133a altered in diabetic condition, we extracted whole hearts from both WT and Akita mice. The high quality RNA from WT and Akita hearts are used for amplification of both miR-133a and sno234 (endogenous control). The results show significant down regulation of miR-133a in Akita hearts (Fig. 1A).

3.2. Dnmt-1, and -3b are induced but Dnmt-3a is attenuated in $Ins2^{+/-}$ Akita hearts

To determine the levels of de novo and maintenance methylation in diabetic hearts, we measured the expression of Dnmt-1, -3a and -3b by multiplex-RT-PCR, qPCR and Western blotting. Both Dnmt-1 and -3b are up regulated but Dnmt-3a is down regulated in Akita (Fig. 1B–F). Increased levels of Dnmt-1 and -3b suggest that both de novo and maintenance methylation are induced in diabetic hearts. However, attenuation of Dnmt-3a in Akita suggests an intricate cross-talk among Dnmt-1, -3a and -3b, where either Dnmt-1 or -3b or both antagonizes Dnmt-3a in Akita hearts.