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MicroRNA-133a regulates DNA methylation in diabetic cardiomyocytes

Vishalakshi Chavali, **Suresh C. Tyagi**, and **Paras K. Mishra**
Department of Physiology & Biophysics, School of Medicine, University of Louisville, Kentucky-40202, USA

Abstract

We tested the hypothesis that miR-133a regulates DNA methylation by inhibiting Dnmt-1 (maintenance) and Dnmt-3a and -3b (de novo) methyl transferase 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) 5mM glucose (CT), 25mM 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 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 elucidating the role of miR-133a in regulation of DNA methylation in diabetes.

Keywords

Dnmt-1; Dnmt-3; Epigenetic modification; epi-miRNA; HL1 cardiomyocytes

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

Corresponding author: Paras Kumar Mishra Department of Physiology & Biophysics School of Medicine, University of Louisville 500 South Preston Street, HSC-A, Room-1216, Louisville, KY 40202, USA Phone: 502-852-3627 Fax: 502-852-6239 pkmish01@louisville.edu.

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 post-transcriptional levels by either degrading mRNA (if seed sequence match perfectly with mRNA) or inhibiting translation (if seed sequence have incomplete complimentarity [19;20]. 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 heart failure.

Diabetes is rampant and accounts for a major death toll across the globe [16]. Diabetes enhances the incidents of heart failure 2-4 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.

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 three-four week and show robust hyperglycemia and diabetic cardiomyopathy at twelve week [1]. Therefore, we used twelve 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 hrs) 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 were kindly provided by Dr. W. C. Claycomb and is maintained in Claycomb complete medium (JRH Biosciences, catalog #51800C, Lenexa, KS,USA), which is supplemented with 10% fetal bovine serum (Sigma-Aldrich, catalog # F2442, Saint Louis, MO, USA), 1% L-glutamine (Life Technologies, catalog # 25030-081, Foster City, CA, USA), 1% norepinephrine (Sigma, catalog # A-0937, Saint Louis, MO, USA), and 1% Penicillin-Streptomycin (Life Technologies, catalog # 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 5mM (physiological level) and 25mM (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 24hrs. 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, 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 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 confirmed 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 also confirmed by miR-133a assay following the earlier described protocol [21]. Sno234 (Applied Biosystems, PN # 001637) was used as endogenous control.

2.6. Western blotting

RIPA buffer (Boston BioProducts, Worcester, MA, USA) supplemented with protease inhibitor cocktail (Sigma, catalog #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 hr. The blots were developed using Pierce ECL substrate (Thermo scientific, Catalog # 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. 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 suggests an intricate cross-talk among Dnmt-1, -3a and -3b, where either Dnmt-1 or -3b or both antagonizes Dnmt-3a in diabetic hearts.

3.3. MiR-133a regulates Dnmt-1, -3a and -3b in cardiomyocytes

To investigate the specific role of miR-133a in regulation of de novo and maintenance methylation in cardiomyocytes, we used HL1 cardiomyocytes for *in vitro* assay. To over express and inhibit miR-133a, we used miR-133a mimic and anti-miR-133a, respectively. The miR-133a mimic and anti-miR-133a are transfected into HL1 cardiomyocytes. Since the miR-133a mimic is tagged with green fluorescence protein (GFP) marker (Figure 2A), the successful transfection is validated by the green color (Fig. 2C) as well as expression of miR-133a (Fig. 2D). The individual miR-133a assay with sno234 as endogenous control revealed that transfection of miR-133a mimic up regulates miR-133a by nearly 5 folds, whereas anti-miR-133a significantly down regulates miR-133a (Fig. 2D).

After validation of miR-133a, we determined the levels of Dnmt-1,-3a and -3b in the three groups: scrambled, miR-133a mimic and anti-miR-133a. The results revealed that miR-133a attenuates whereas anti-miR-133a induces Dnmt-1, -3a and -3b, respectively (Fig. 3A-C).

The differential expression of Dnmt-1, -3a and -3b due to induction and inhibition of miR-133a suggests that miR-133a is involved in regulation of de novo (Dnmt-3a, -3b) and maintenance (Dnmt-1) methylation in the heart.

3.4. MiR-133a mitigates Dnmt-1 in diabetic cardiomyocytes

To investigate the effect of miR-133a on Dnmt-1,-3a and -3b in diabetic cardiomyocytes, we treated HL1 cardiomyocytes with 1) physiological (CT: 5mM) and 2) high dose (HG: 25mM) of D-glucose, and 3) HG pre-treated with miR-133a mimic (miR+HG) for 24 hrs. The levels of Dnmt-1, 3a and -3b are determined in the above three groups. Dnmt-1 was robust (Fig. 4C) but Dnmt-3a and -3b did not show any significant difference between CT and HG groups (Fig. 4A-B). It suggests that acute hyperglycemia triggers mainly maintenance methylation and does not have much effect on de novo methylation. Interestingly, miR-133a mimic mitigates the glucose mediated induction of Dnmt-1 (Fig. 4C) indicating the role of miR-133a in regulation of Dnmt-1. Similarly, miR-133a ameliorates glucose mediated induction of Dnmt-3a and -3b (Fig. 4A-B). These results suggest that miR-133a inhibits DNA methylation in diabetic cardiomyocytes.

4. Discussion

The epigenetic modifications contribute to diabetic complications (21) and are regulated in a feedback manner (11). The epi-miRNAs such as miR-29, -152 and -290 play pivotal role in regulation of epigenetic modifications through DNA methylation [2;22;26;27;29]. The miR-133a plays crucial role in regulation of cardiac hypertrophy [4] and fibrosis [18], and is attenuated in diabetic hearts [8] (Fig. 1A). However, the role of miR-133a in epigenetic modifications is unclear. Therefore, we investigated the effect of miR-133a on epigenetic modifications in diabetic hearts. We selected Insulin2 mutant (Ins2+/−) Akita as genetic model for diabetes because Insuline2 of mice are homologous to human Insulin and mutation in Insulin causes juvenile diabetes in humans [9;10].

We determined the levels of cardiac miR-133a, Dnmt-1(maintenance), - 3a and -3b (de novo) - methyl transferase in diabetic Akita. The cardiac levels of miR-133a was attenuated (Fig. 1A) in Akita. Interestingly, Dnmt-1 and -3b are robust but Dnmt-3a is inhibited in Akita hearts (Fig. 1B-F). Since, both Dnmt-3a and -3b are involved in de novo methylation and Dnmt-3a is inhibited whereas Dnmt-3b is induced in diabetic hearts, it is suggested that there is an intricate regulatory network among the three Dnmt's in diabetic Akita. The down regulation of Dnmt-3a may be either due to inhibitory effect of high glucose or Dnmt-1 and / or Dnmt-3b. The role of miR-133a on regulation of Dnmt-1, -3a and -3b is assessed by over expressing and silencing miR-133a in HL1 cardiomyocyte. The inhibition of miR-133a up regulates Dnmt-1, -3a and -3b, whereas the over expression of miR-133a down regulates Dnmt-1, -3a and -3b (Fig. 3A-C) suggesting the role of miR-133a in regulation of these methyl transferases. The diabetic condition is simulated by treating the HL1 cardiomyocytes with high dose of glucose (HG). Interestingly, the HG treatment for 24hr induces only Dnmt-1 and not Dnmt-3a and -3b (Fig. 4A-C) eliciting that acute hyperglycemia induces only maintenance methylation in the cardiomyocytes and not the de novo methylation. Interestingly, miR-133a mitigates Dnmt-3a and -3b below the levels of CT (Fig. 4A-B)

suggesting the robust inhibitory effect of miR-133a on Dnmt-3a and -3b. The down regulation of both Dnmt-1, 3a and -3b in Akita hearts (Fig.1B-F) indicates that in chronic diabetic condition both de novo and maintenance methyl transferases are activated. Considering the acute and chronic effect of miR-133a in hyperglycemic cardiomyocytes and diabetic Akita hearts, it is concluded that miR-133a plays key role in the regulation of Dnmt-1, -3a and -3b and acts as an epi-miRNA. Since miR-133a is attenuated in Akita hearts (Fig. 1A), we infer that inhibition of miR-133a up regulates Dnmt-1, -3a and -3b that induces maintenance and de novo methylation, respectively in diabetic hearts. However, over expression of miR-133a mitigates attenuation of Dnmt-1, -3a and -3b in diabetic hearts. Future studies on regulation of Dnmt-1, -3a and -3b will unravel the complex cross-talk among Dnmt-1, -3a and -3b in diabetic hearts.

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P.K.M. conceived the idea, performed experiments, analyzed the data and wrote the manuscript. C.V. executed experiments, analyzed the data and contributed to discussion. S.C.T. contributed to the discussion.

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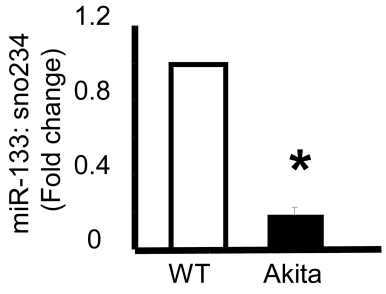

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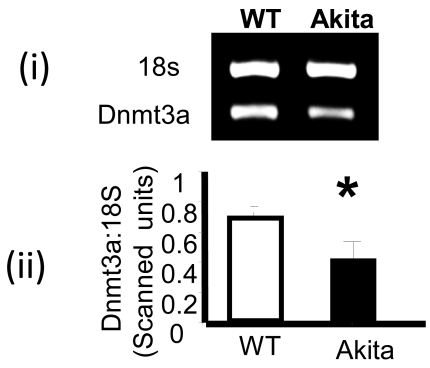

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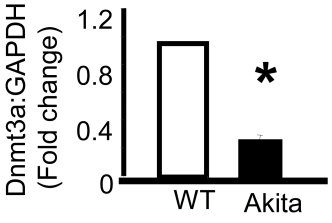

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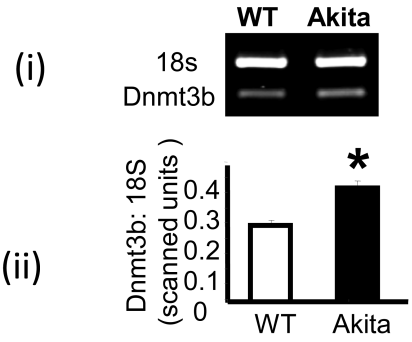

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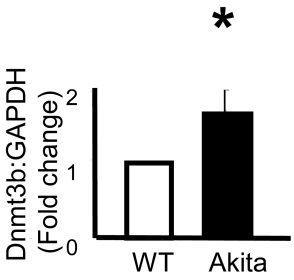

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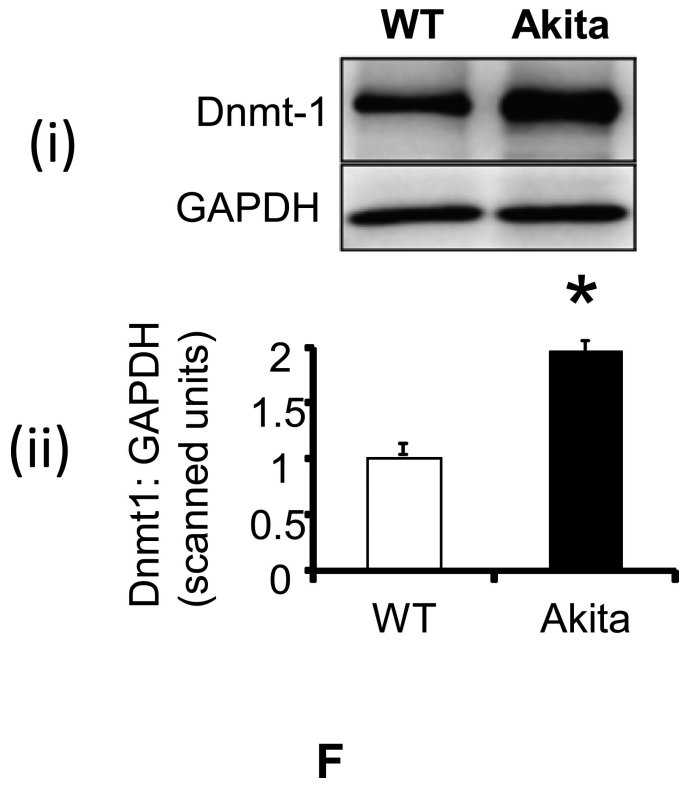

F

Fig. 1.

A. Individual miR-133a assay in WT and Akita hearts. MiR-133a specific reverse transcription and qPCR primers are used for amplification of miR-133a in WT and Akita hearts. The sno234 is used as endogenous control. The bar graph represents fold change with mean ± SE. *, $p<0.05$; n= 4. MiR-133 is down regulated in Akita hearts.

Fig.1. B-C. Quantitative - and semi- quantitative multiplex RT-PCR of DNA methyl transferases (Dnmt- 3a) in WT and Akita hearts. The 18S RNA is a loading control. *; $p<0.05$, n=3.

Fig. 1. D-E. Quantitative - and semi- quantitative multiplex RT-PCR of DNA methyl transferases (Dnmt- 3b) in WT and Akita hearts. The 18S RNA is a loading control. *; $p<0.05$, n=3.

Fig. 1. F. Representative Western blots of DNA methyl transferase (Dnmt-1) in WT and Akita hearts. The GAPDH is a loading control. n= 4; *, $P<0.05$.

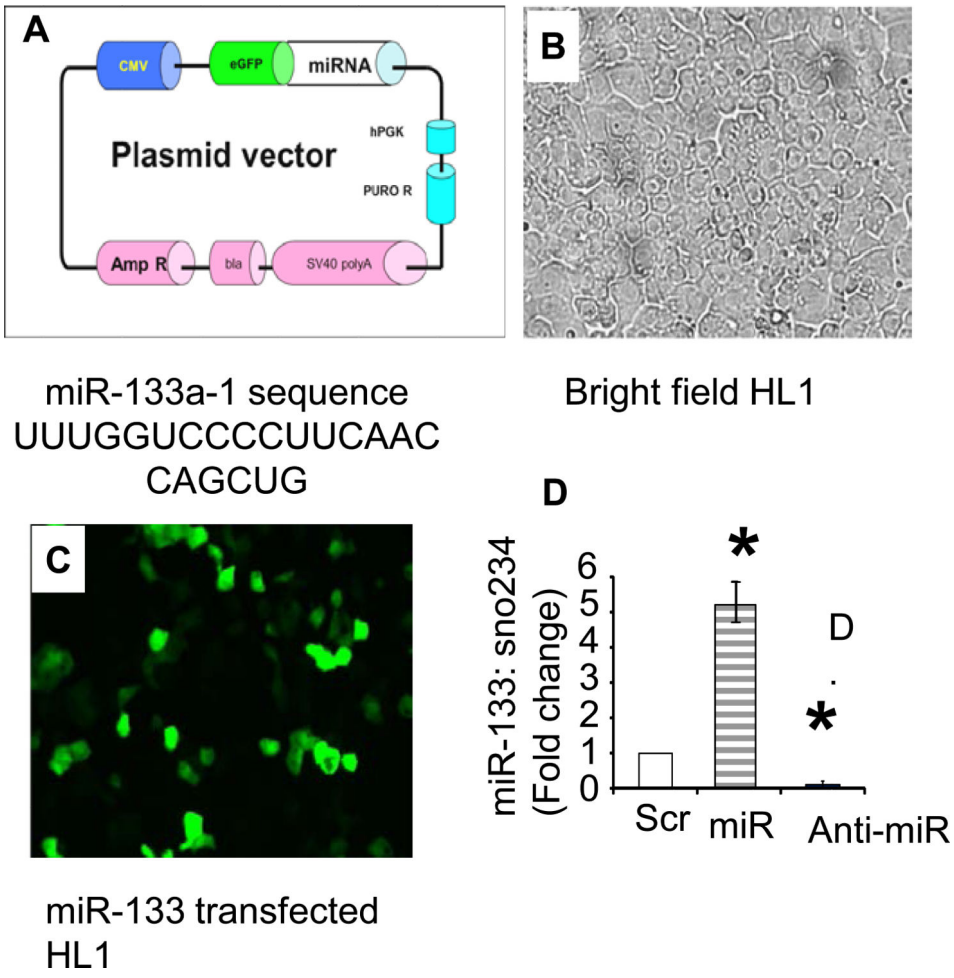

Fig.2.

A.Plasmid construct of miR-133a with GFP marker. **B**. A bright field microscopic view of HL1 cardiomyocytes. **C.** The HL1 cells transfected with miR-133a (green). **D**. QPCR analyses of miR-133a in HL1 cardiomyocytes transfected with scrambled (scr), miR-133a (miR-133) and AntimiR-133. Sno234 is an endogenous control. *; $p<0.05$, n=3.

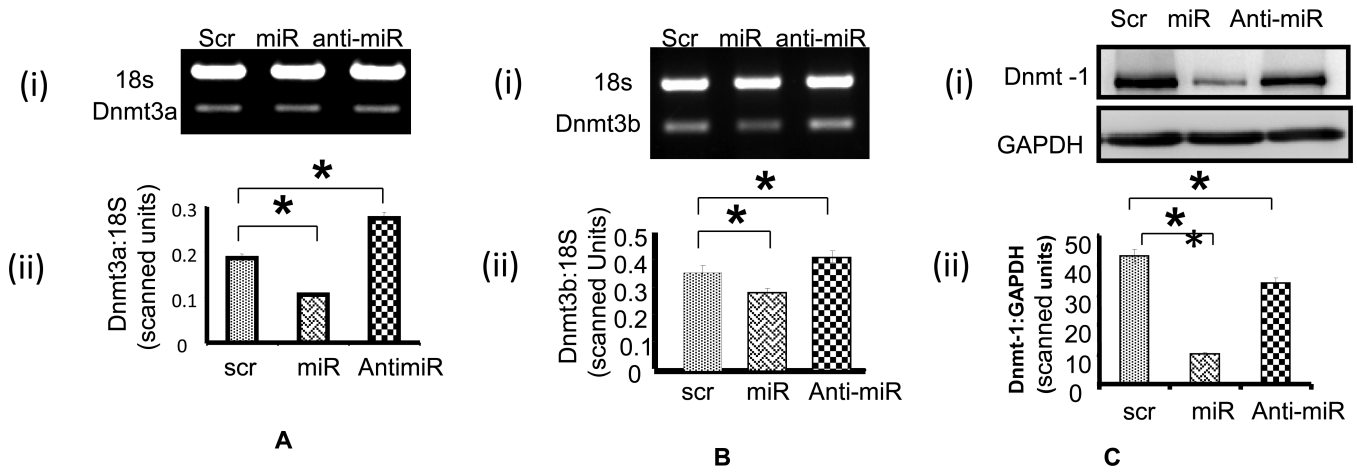

Fig.3.

A-B. Multiplex RT-PCR of DNA methyl transferases (Dnmt-3a and -3b) in scrambled, miR-133a and AntimiR-133a transfected HL1 cardiomyocytes. The 18S RNA is a loading control. **C.** Western blot analyses of DNA methyl transferase (Dnmt- 1) in scrambled, MiR-133 and AntimiR-133 transfected HL1 cardiomyocytes The GAPDH is a loading control. *; $p<0.05$, n=3.

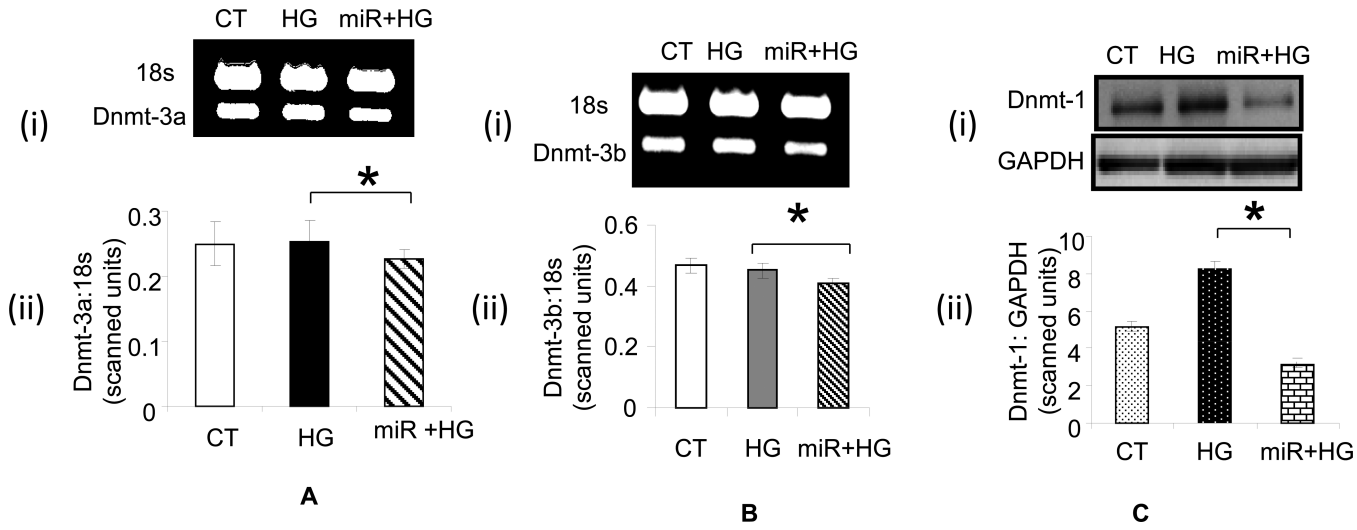

Fig. 4.

A-B. Multiplex RT-PCR and qPCR of DNA methyl transferases (Dnmt- 3a and - 3b) in CT, HG and MiR-133+HG HL1 cardiomyocytes. The 18S RNA is a loading control. **C.** Western blot analyses of DNA methyl transferases (Dnmt-1) in CT, HG and MiR-133+HG HL1 cardiomyocytes The GAPDH is a loading control. *; $p<0.05$, n=3.