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Differential expression of dicer, miRNAs and inflammatory markers in diabetic Ins2+/– Akita hearts

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

Abstract

Diabetic cardiomyopathy is a leading cause of morbidity and mortality, and Insulin2 mutant ($Ins2^{+/-}$) Akita is a genetic mice model of diabetes relevant to humans. Dicer, miRNAs and inflammatory cytokines are associated with heart failure. However, the differential expression of miRNAs, dicer and inflammatory molecules are not clear in diabetic cardiomyopathy of Akita. We measured the levels of miRNAs, dicer, pro-inflammatory tumor necrosis factor alpha (TNFα), and anti-inflammatory interleukin 10 (IL-10) in C57BL/6J (WT) and Akita hearts. The results revealed increased heart to body weight ratio and robust expression of brain natriuretic peptide (BNP: a hypertrophy marker) suggesting cardiac hypertrophy in Akita. The multiplex RT-PCR, qPCR and immunoblotting showed up regulation of dicer whereas miRNA array elicited spread down regulation of miRNAs in Akita including dramatic down regulation of let-7a, miR-130, miR-142-3p, miR-148, miR-338, miR-345-3p, miR-384-3p, miR-433, miR-450, miR-451, miR-455, miR-494, miR-499, miR-500, miR-542-3p, miR-744, and miR-872. Conversely, miR-295 is induced in Akita. Cardiac TNFα is up regulated at mRNA (RT-PCR and qPCR), protein (immunoblotting), and cellular (immunohistochemistry and confocal microscopy) levels, and is robust in hypertrophic cardiomyocytes suggesting direct association of TNFα with hypertrophy. Contrary to TNFα, cardiac IL-10 is down regulated in Akita. In conclusion, induction of dicer and TNFα, and attenuation of IL-10 and majority of miRNAs contributes to diabetic cardiomyopathy in Akita.

Keywords

BNP; diabetes; heart failure; hypertrophy; IL-10; TNFα

Introduction

Insulin2 mutant (Ins2+/–) Akita mouse is an attractive animal model for diabetes because Insulin2 gene of mouse is an ortholog of human Insulin, and mutation in Insulin gene causes human juvenile diabetes (1). Akita mice become diabetic spontaneously at the age of 3–4

[*]Address of correspondence, Paras Kumar Mishra, pkmish01@louisville.edu; parasbhu2005@gmail.com, Chavali Vishalakshi, v0chav01@louisville.edu, 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.

Conflict of Interest
Authors confirm that there are no conflicts of interest.

weeks and show robust hyperglycemia by 12 weeks (2–7). Therefore, it is a better than the induced diabetes models such as NOD-autoimmune depletion of beta cells (8), OVE 26-the non-specific gene expression of beta cells (9), and Streptozotocin- or Alloxan-chemicals destruction of beta cells (10–12). These mice develop cardiac fibrosis, endothelium-myocyte uncoupling (5;13), impaired contractility of cardiomyocytes and cardiac dysfunction (4;14). However, the role of microRNA (miRNA), dicer and inflammation in diabetic cardiomyopathy in Akita is nebulous.

MiRNAs are newly discovered, 19–23 nucleotides long, non-coding regulatory RNAs that modulate gene expression either by mRNA degradation or translational repression (15;16). The differential expression of miRNAs is associated with heart failure (17–22) and diabetes (23–26). The distinct role of individual miRNA in cardiac dysfunction is corroborated by the fact that alteration in the levels of miR-133 causes cardiac hypertrophy (27;28), arrhythmia (29–31), fibrosis (32;33) and epigenetic modifications (34). Therefore, it is important to assess the differential expression of miRNAs in the heart of diabetic Akita. Dicer is an RNase III endonuclease essential for maturation of all pre-miRNAs (26;35–40). Dicer is indispensable for cardiac development because targeted deletion of dicer causes impaired development of ventricular myocardium and pericardial edema that lead to embryonic lethality at day 12.5 (41). The cardiac–specific knock out of dicer causes progressive dilated cardiomyopathy and ultimately heart failure (42). Even the conditional deletion of dicer in postnatal myocardium induces cardiomyocytes dysfunction and sudden cardiac arrest (43). Therefore, dicer plays crucial role in cardiac development and maintenance of cardiac functions. Ablation of dicer also inhibits global miRNA maturation that causes disarray in miRNA mediated regulatory networks. Interestingly, dicer is also regulated by miRNAs such as let-7 in human lung cancer (44) and miR-103/107 in breast cancer (45). MiRNAs also regulates inflammatory molecules including pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) and anti-inflammatory cytokine interleukin-10 (IL-10) (46–49). There is a dynamic change in the levels of TNFα and IL-10 during pathological myocardial remodeling (50). TNFα is not only produced by immune cells rather synthesized by cardiomyocytes in response to injury or stress (51). The infusion of pathological dose of TNFα promotes left ventricular dysfunction and remodeling in rats (52). TNFα is also associated with diabetes (53) and contributes to heart failure (54;55). On the other hand, IL-10 inhibits TNFα and ameliorates pathological cardiac remodeling (56–58). Since, inflammation exacerbates heart failure (59), it is important to assess the levels of TNFα and IL-10 in Akita hearts. Therefore, we determined the levels of dicer, miRNAs, TNFα and IL-10 in Akita hearts.

Materials and Methods

Animal models

Twelve week old male C57BL/6J and Ins2+/− Akita mice were procured from Jackson Laboratory (Bar harbor, ME, USA). Akita mice have same genetic background as C57BL/6J mice. Therefore, we used C57BL/6J as wild type (WT) control for Akita. Mice were kept in the animal facility of University of Louisville with normal chaw diet and drinking water in *ad libitum*. They were sacrificed following the protocol approved by the Institutional Care

and Use Committee of University of Louisville. The standard protocol and guidelines of National Institute of health (NIH) and *Guide for the care and Use of Laboratory Animals* and the regulation of the Animal Welfare Act was followed.

Experimental Protocols

For measuring the blood glucose level, mice were kept on fasting (without food but with drinking water) for 8 hrs. To collect blood, tail vein was punctured and 1–2 drops of blood was transferred onto glucose strips of glucometer. The diabetics' mice have the blood glucose level ≥250 mg/dL and WT mice have ≤80mg/dL. Mice (both **diabetic and WT**) were anaesthetized with tribromoethanol (240mg/kg body weight, i. p.) and hearts were extracted under deep anesthesia. For RNA and protein extraction, hearts were snap frozen in liquid nitrogen and stored at −80°C. For histological sections, hearts were arrested in diastole by injecting 0.2ml/100g body weight of a 20% KCl (i. v.) and perfused with 0.9% saline (Teknova, CA, USA, Catalogue # S5815). They were fixed in 4% paraformaldehyde and embedded with tissue freezing medium for cryosectioning (5μM). For all the experiments, sample size (n) was at least 5.

RNA extraction and quality assessment

The *miR*Vana RNA isolation kit Ambion (Part Number #AM1560) was used for RNA isolation. The quality and quantity of RNA was analyzed by NanoDrop 1000, where the ratio of 260: 280 and 260: 230 with a value of ~2 was considered as good quality RNA. The detailed protocol is described elsewhere (60).

Semi-quantitative RT-PCR and quantitative qPCR

We used 1μg RNA for reverse transcribed by two step protocol of Promega Kit (Promega, WI, USA, # A3800) as elaborated in our previous publication (60). The PCR program for amplification of dicer, TNFα, IL-10 and 18S was 95°C−7.00 min, (95°C−0.50min, 55°C −1.00 min, 72°C−1.00 min) × 34, 72°C−5.00 min and 4°C for infinity. As a loading control, 18S (Ambion # AM1716) was used. The primers used for dicer, TNFα, IL-10 and BNP are as follows: dicer-Forward: 5′atgcaaaaaggaccgtgttc3′, Reverse: 5′caaggcgacatagcaagtca3′; TNFα-Forward: 5′gccgatttgctatctcatac3′, Reverse: 5′tgggtagagaatggatgaac3′; IL-10-Forward: 5′agaaatcaaggagcatttga3′, Reverse: 5′acactcgctcagagacagat3′; and BNP-Forward: 5′ctgtcccagatgattctgtt3′, Reverse: 5′taagagatatgctgcccaat3′.

For gel electrophoresis of RT-PCR product, 1% agarose with 0.008 % ethidium bromide was used. The bands were observed under UV light and their intensity was analyzed by ChemiDoc device (BioRad, CA, USA).

For quantitative amplification (qPCR) of mRNA, Stratagene Mx3000P Real-time PCR device was employed. The Syber-green method was used with the PCR cycle of 95°C−10 min; (95°C−0.30 min, 55°C−1.00 min, 72°C−0.30 min) × 40; 95°C−1.00, and 55°C−0.30 min. The extended protocol is reported in our previous publication (60).

Multiplex-RT-PCR

To rule out the variation in RNA quantity and quality, and initial quantization errors during PCR, we used multiplex RT-PCR to amplify dicer in WT and Akita hearts. Multiplex RT-PCR amplifies two sets of primer in a single PCR, where one primer set acts as invariant endogenous control (18S), while the other amplifies the target gene (dicer, IL10 TNFa and BNP). Gel electrophoresis show two bands; one for endogenous control and other for the target gene.

MiRNA Assay

The miRNA array and individual assay was performed using Applied Biosystems kit and following their protocols. The Taqman microRNA reverse transcription kit (Part # 4366596), miR-223 (Part #007896) and sno234 (endogenous control) (Part # 1234) were used and the protocol is elaborated elsewhere (60).

Protein extraction and Western Blotting

Proteins were extracted from snap frozen hearts using Radio-Immuno-Precipitation Assay (RIPA, catalogue # BP-115D, Boston BioProducts, MA, USA) lyses buffer. The quantity of proteins was estimated by Bradford method using SoftMax Pro 4.6 program and Molecular Devices plate reader. The routine Western blotting was performed using SDS-PAGE gel electrophoresis. The primary antibodies of TNFα (LS Biosciences, WA, USA; # C18838), IL-10 (LS Biosciences, WA, USA; # B4913), Dicer (Santa Cruz; # sc-30226) and GAPDH (Millipore, CA, USA; # MAB 374) were diluted in the ratio of 1: 1000. The secondary antibodies, anti-mouse IgG-HRP (Santa Cruz Biotech; # sc-2005) and anti-rabbit IgG-HRP (Santa Cruz Biotech; #sc 2054) were diluted in the ratio of 1:3000. We also used secondary antibody for molecular size marker (BioRad Precision Protein Strep Tactin-HRP conjugate, (Bio-Rad; # 161-0381). The membranes were developed and band intensity was analyzed by ChemiDoc software (BioRad, CA, USA).

Multiplex-immunoblotting

To analyze the expression of target (TNFα) and control (GAPDH) proteins simultaneously, we incubated the nitrocellulose membranes with the primary antibodies of both TNFα and GAPDH. Similarly, the secondary antibodies corresponding to both TNFα and GAPDH were used. The membranes were developed as routine Western blotting and band intensity was analyzed for quantification.

Immunohistochemistry (IHC) and confocal microscopy

The IHC and confocal microscopy protocols were same as described earlier (13;61). In brief, histological sections of the heart from WT and Akita mice were washed with Phosphate Buffered Saline (PBS) (Invitrogen, GIBCO, #14190), permeabilized with 0.02% Triton X-100 (Fisher BioReagents, #BP-151) for 20 min, washed with PBS (three time for 5 min), blocked with 1% BSA in PBS, incubated with TNFα primary antibody (LS Biosciences, WA, USA; # C18838) diluted in the ratio of 1:100 for overnight at 4°C, washed with PBS, incubated with anti-rabbit Alexa-fluor 647 (Invitrogen; #A21245) diluted in the ratio of 1: 200 for 2 hr at room temperature, washed with PBS, incubated with DAPI (1: 1000 dilution)

for 30 min, mounted with FluoroGel (GTX; #28214) and observed under confocal microscope (Olympus). The intensity of red color was determined by Image-Pro software to compare the cellular expression of TNFα in WT and Akita hearts.

Statistical analyses

All the statistical values were presented as mean ± SE. Student t-test was performed to calculate the differences in mean between two groups. A p-value of <0.05 was considered as statistically significant difference.

Results

Cardiac hypertrophy in Akita

The geometrical shape of heart in WT is elongated (American football) but in Akita, it is changed to round shape (soccer ball) (Fig.1 A i), which is common in dilated cardiomyopathy (62). To determine cardiac hypertrophy, we measured the heart to body weight ratio (HW/BW) in WT and Akita hearts. The significant increase in HW/BW in Akita (Fig.1 A ii) suggests cardiac hypertrophy. To corroborate the finding at molecular level, we measured the levels of BNP (hypertrophy marker) by qPCR in the heart tissue of both WT and Akita. There is robust up regulation of BNP in Akita hearts (Fig. 1B) extending support to cardiac hypertrophy.

Dicer is induced in Akita hearts

Dicer level is low in end-stage heart failure but elevated in the hearts with left ventricle assist device (LVAD) (42). Therefore, we measured dicer at mRNA and protein levels in WT and Akita hearts. The multiplex-RT-PCR (Fig. 2A (i) and (ii)) and qPCR (Fig. 2B) show increased transcription of dicer in Akita, which is translated into protein (Fig 2 C i and ii). The increased level of dicer is similar to the condition of LVAD, where the heart is in recovery stage (42).

MiRNAs are attenuated in Akita hearts

Since differential expression of miRNAs contributes to pathological cardiac remodeling leading to heart failure (18;26), we measured the levels of miRNAs in WT and Akita hearts using miRNA array. The miRNA array analyses revealed that out of 242 miRNAs, 65 miRNAs are attenuated, where let-7a, miR-345-3p, miR-384-3p, miR-433 and miR-455 are ~ 80% down regulated in Akita (Fig. 3A and B). Conversely, miR-295 is induced (~3 fold up regulated) in Akita (Fig. 3B). We also confirmed the results by individual miRNA assay, which is in line with the miRNA array analyses (Fig. 3C and D). Since miR-223 is anti-inflammatory and cardio-protective (63;64), we also measured the levels of miR-223. The results show significant down regulation of miR-223 in Akita hearts (Fig. 3E).

TNFα is induced and associated with cardiac hypertrophy in Akita

Since TNFα is a pro-inflammatory cytokine that contributes to heart failure (54;55), we measure the levels of TNFα in WT and Akita hearts. The RT-PCR (Fig. 4A i and ii), and qPCR (Fig. 4B) results show significant up regulation of TNFα mRNA in Akita suggesting

its increased transcription. We used two endogenous controls (18s and GAPDH) to verify the induction of TNFα in Akita hearts (Fig. 4A and B). The multiplex –immunoblotting show that transcriptional message is translated at protein level in Akita hearts (Fig. 4C i and ii). We also determined the cellular levels of TNFα in WT and Akita hearts using IHC and confocal microscopy. There is increased expression of TNFα in Akita hearts (Fig. 4Di and ii). In the ventricular sections of Akita hearts, TNFα is mostly localized in the hypertrophic (comparatively bigger in size than the normal cardiomyocytes) cardiomyocytes (indicated by arrow), and almost absent in the normal size cardiomyocytes (indicated by star) (Fig. 4E). The localization of TNFα with hypertrophic cardiomyocytes suggests that TNFα is directly associated with cardiac hypertrophy in diabetic Akita.

IL-10 is attenuated in Akita hearts

The anti-inflammatory IL-10 is measured in WT and Akita hearts at both mRNA and protein levels. The RT-PCR (Fig. 5A i and ii) and qPCR (Fig. 5B) analyses show inhibition of transcription of IL-10 in Akita hearts. The immunoblotting results revealed that mRNA message is translated at protein levels in Akita (Fig. 5C). The mitigation of IL-10 suggests inhibition of anti-inflammatory cytokine in Akita hearts.

Discussion

Despite development in therapy regime, heart failure is a leading cause of morbidity and mortality in the world (65). The incidence of heart failure increases 2–4 folds with diabetes (66), which is rapidly increasing across the globe (67;68). However, the mechanism of heart failure in diabetes is still elusive. The newly discovered non-coding, tiny miRNAs are emerged as novel class of regulatory RNA that plays pivotal role in diabetes and heart failure (18;25;26;69–71). It is documented that differential expression of specific miRNA contributes to heart failure such as miR-1, -133, -150 in hypertrophy, miR-29, -133 in fibrosis, miR-1, -133 in arrhythmia, and miR-126 in angiogenesis (26;31). The miRNA maturation enzyme dicer is also reported to alter in the pathological and reverse cardiac remodeling (42). It is documented that miRNA regulates inflammation (48;64;72;73), which is induced in stress condition and contributes to heart failure (53;59;74;75). The pro-inflammatory TNFα plays key role in myocardial remodeling (54–56) and blocking of TNFα reverses the LV remodeling (55). On contrary, induction of anti-inflammatory IL-10 suppresses TNFα (56), and attenuates LV remodeling after myocardial infarction (MI) (57). The genetic deletion of IL-10 exacerbates but IL-10 treatment ameliorates pressure overload induced pathological cardiac remodeling (58) suggesting that IL-10 is a potent anti-inflammatory cytokine. Therefore, we investigated the cardiac levels of dicer, miRNAs, TNFα and IL-10 in diabetic Akita.

It is reported that the geometry of left ventricle (LV) alters to spherical dilatation in the apex in the failing heart (76). We found that shape of Akita heart has tendency to become spherical in the apical region of LV when compared to the WT heart (Fig 1A i). It extends support to cardiomyopathy of Akita hearts (14;77). The pathological condition of the heart is also evaluated by cardiac hypertrophy, where cardiomyocytes increase in size making the heart bigger than the normal size. The cardiac hypertrophy often is assessed by HW/BW

ratio and at molecular level by BNP expression. In Akita, increase in HW/BW (Fig 1A ii) and significant up regulation of BNP (Fig 1B) suggest cardiac hypertrophy. It supports pathological cardiac remodeling and dysfunction in diabetes (4;5;77). These changes are accompanied by induction of dicer both at mRNA and protein levels (Fig 2Ai–Cii). However, miRNAs are largely attenuated (Fig 3A–E) except miR-295 (Fig3 B). Since targeted deletion of dicer attenuates mature miRNAs (42), up regulation of dicer is anomalous to down regulation of miRNAs in Akita hearts. In the failing heart dicer is significantly down regulated but LVAD improves dicer to a moderate level (42). The induction of dicer in Akita can be explained by simulating the condition of the heart maneuvering to reverse the pathological remodeling as in case of LVAD. The spread down regulation of miRNAs in Akita hearts suggests inhibition of biogenesis of majority of miRNAs in diabetes. The miRNA profiling revealed several candidate miRNAs such as let-7a, miR-130a,-142-3p, -148,-338, -345-3p, -384-3p,-433, -450,-451,-455,-499,-500,-542-3p,-744, and -872, which are down regulated, and miR-295, which is up regulated in diabetic Akita (Fig 3 A–B). It is reported that miR-130a is involved in angiogenesis (78). In Akita, miR-130a is ~70% down regulated that may have implications on angiogenesis. Similarly, inhibition of miR-133a is associated with cardiac hypertrophy (27), fibrosis (33) and matrix remodeling (79). The cardiac hypertrophy (Fig 1Aii and B) and fibrosis (5) in Akita is in line with the above findings. The only miRNA we found up regulated in Akita is miR-295 (Fig 3A). It is documented that miR-295 is a mammalian –specific miRNA that express especially in early embryonic stage (80). In the failing heart, miRNAs undergo fetal reprogramming that is switching of expression profile from adult to embryonic condition (81). Fetal reprogramming is an adaptive mechanism to recover from adverse condition. The ~3 fold increase in the levels of miR-295 suggests pathological condition of Akita hearts. The miR-223 is an anti-inflammatory miRNA (82). The attenuation of miR-223 in Akita hearts suggest increased inflammation. Although inflammation can be cardioprotective in damaged myocardium if triggered ephemerally (83), chronic inflammation is detrimental (84). The robust up regulation of pro-inflammatory TNFα in Akita at mRNA (Fig 4Ai–B), protein (Fig 4Ci–ii) and cellular (Fig 4Di–ii) levels corroborates induction of inflammation in Akita hearts. Since TNFα causes LV remodeling (54;55), and beta-adrenergic receptor–mediated cardiac hypertrophy (85), we localized TNFα at cellular level both in normal and hypertrophic cardiomyocytes. The increased level of TNFα in the hypertrophic cardiomyocytes (shown by arrow head in Fig 4E), and decreased level of TNFα in normal cardiomyocytes (pointed with star in Fig 4E) suggests a direct association of TNFα with cardiac hypertrophy in Akita. The increased inflammation is also attributed to the inhibition of IL-10. It is documented that IL-10 suppresses TNFα induced cardiomyocytes apoptosis (56). Also, IL-10 inhibits inflammation and mitigates pathological LV remodeling (57). We found that IL-10 is down regulated in Akita both at mRNA (Fig 5Ai–B) and protein (Fig 5Ci–ii) levels indicating the inflammation mediated cardiac dysfunction in Akita.

Conclusion

Diabetic cardiomyopathy in Akita is associated with differential expression of dicer, miRNAs, TNFα and IL-10. The localization of TNFα in hypertrophic cardiomyocytes

points to role of TNF in cardiac hypertrophy. Our results revealed several novel putative candidate miRNAs involved in diabetic cardiomyopathy.

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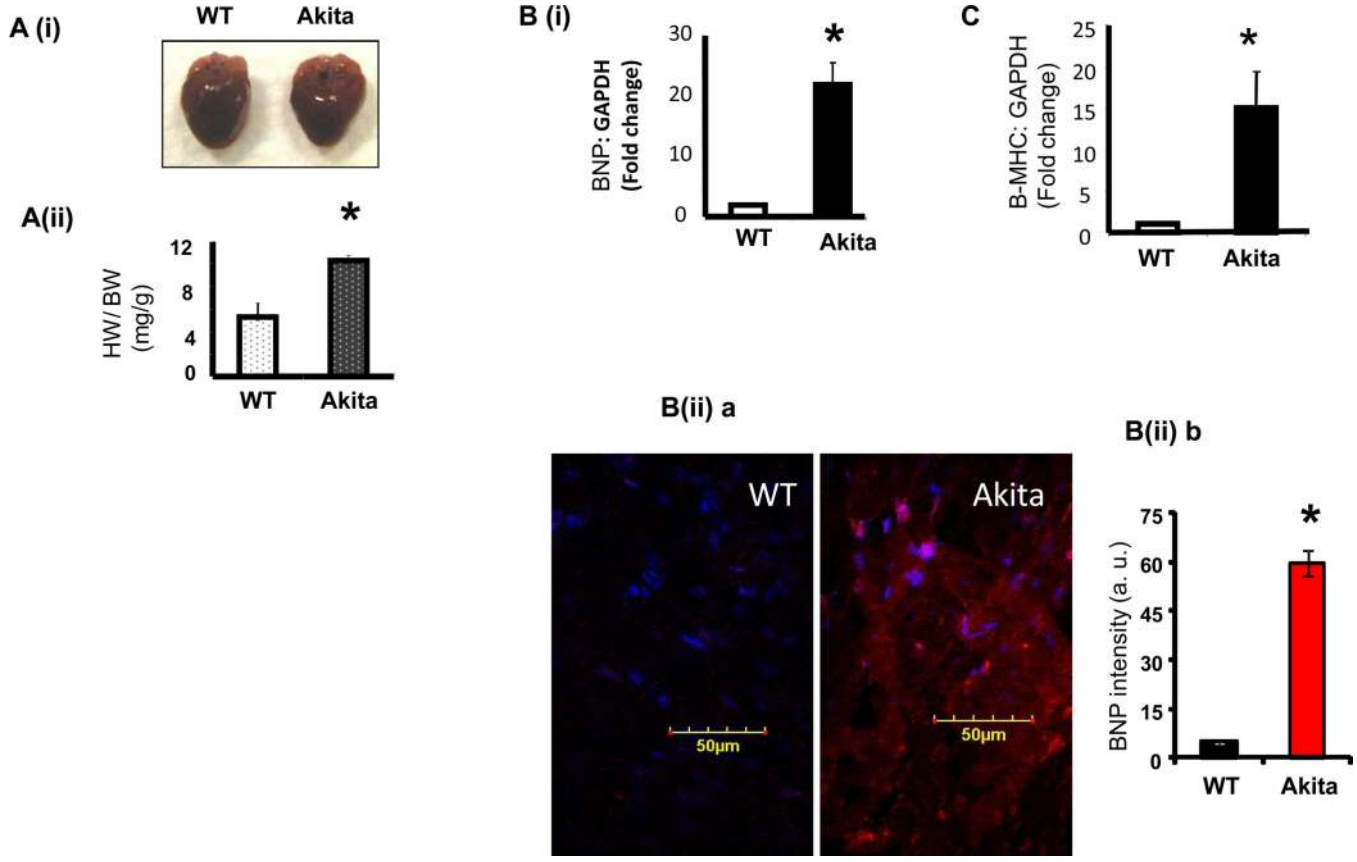


Figure 1. Comparative analysis of cardiac hypertrophy in WT and Ins2+/− Akita mice

A (i). Representative heart morphology of WT and Akita with bar graph showing ratio of heart to body weight (HW/BW). A **(ii)**. Representative longitudinal section of the heart from WT and Akita mice. **B (i)**. The qPCR analysis of brain natriuretic peptide (BNP) in WT and Akita hearts. GAPDH is a loading control. Data are represented as mean ± SE. *, $p<0.05$; n= 4.

B (ii) a. Representative immunohistochemistry figure of BNP (red color) in the WT and Akita hearts. Dapi (blue) color stains nuclei. **B (ii) b**. Bar graph showing the intensity of red color in the heart. *, $p<0.05$; n= 3.

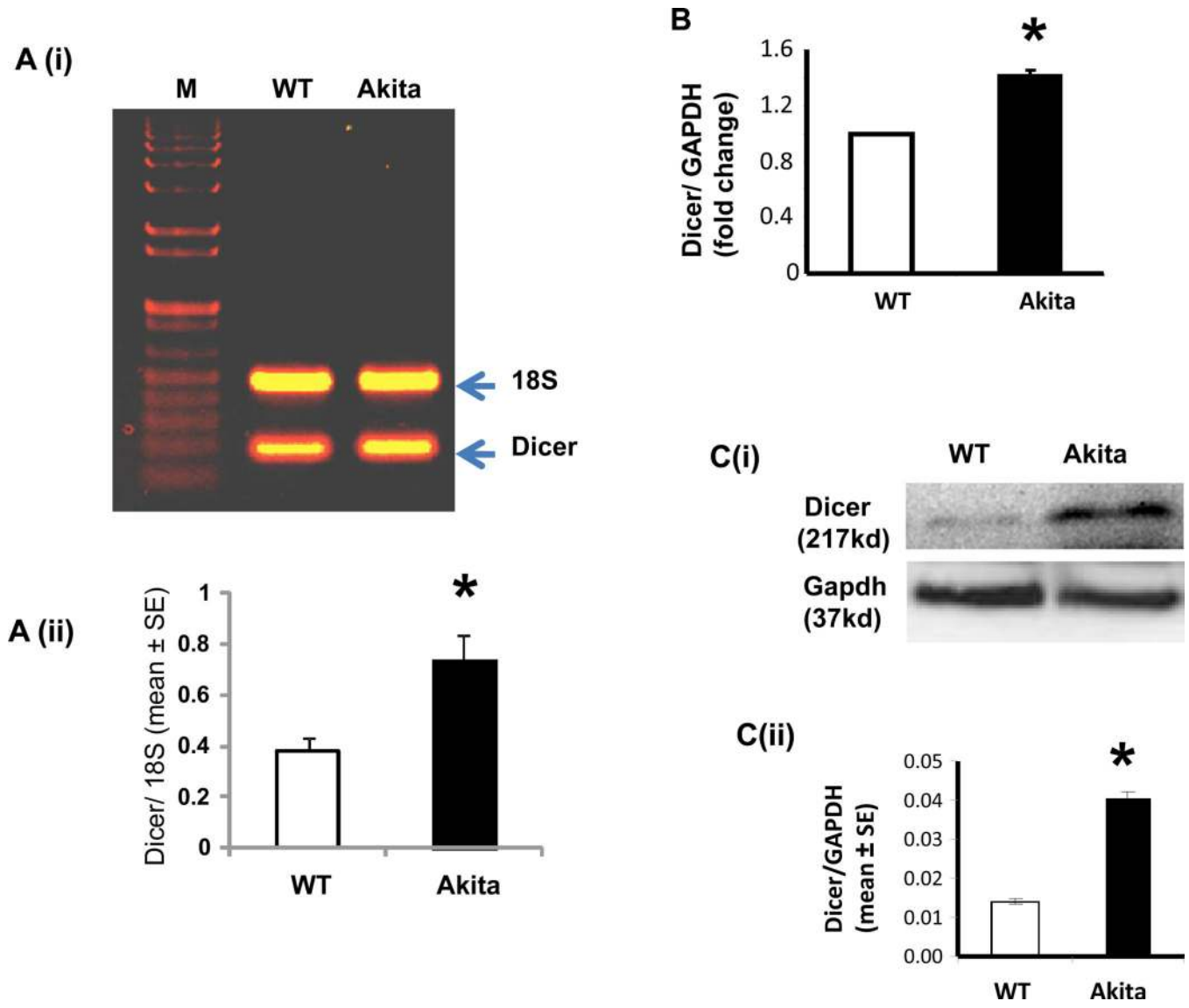
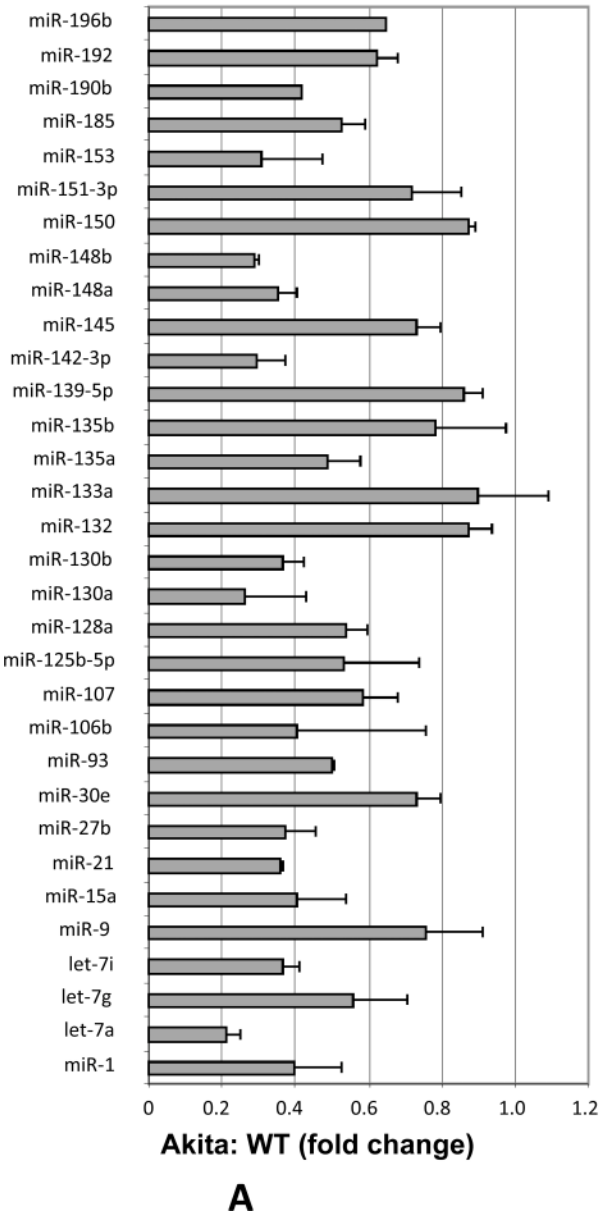


Figure 2. Differential expression of dicer in WT and Ins2+/− Akita hearts

A. **(i)**. Representative multiplex-RT-PCR of dicer and 18S in WT and Akita. The 18S RNA is a loading control. **(ii)**. The bar graph show densitometry analyses of intensity of bands normalized with 18S. The values are mean ± SE. *, $p<0.05$; n= 4. **B**. The qPCR analyses of dicer normalized with GAPDH in WT and Akita. **C**. **(i)**. Representative band of immunoblotting of dicer in WT and Akita hearts. GAPDH is a loading control. **(ii)**. The bar graph represents densitometry analyses of intensity of bands normalized with GAPDH. The values are mean ± SE. *, $p<0.05$; n= 4.



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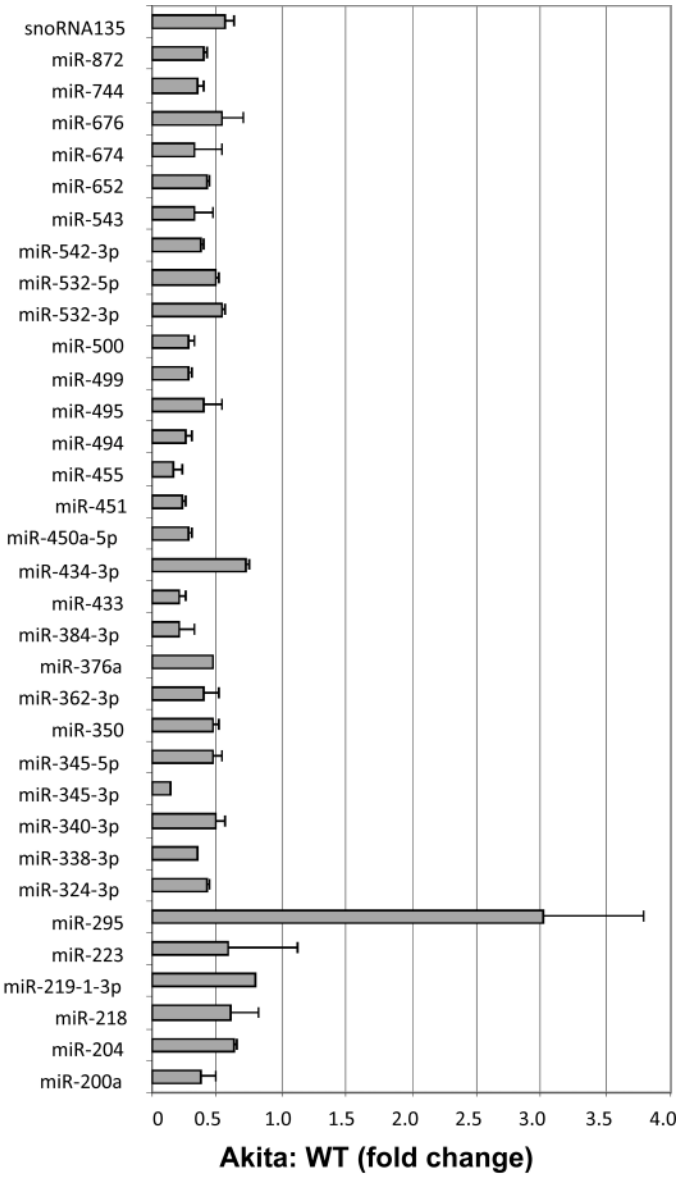

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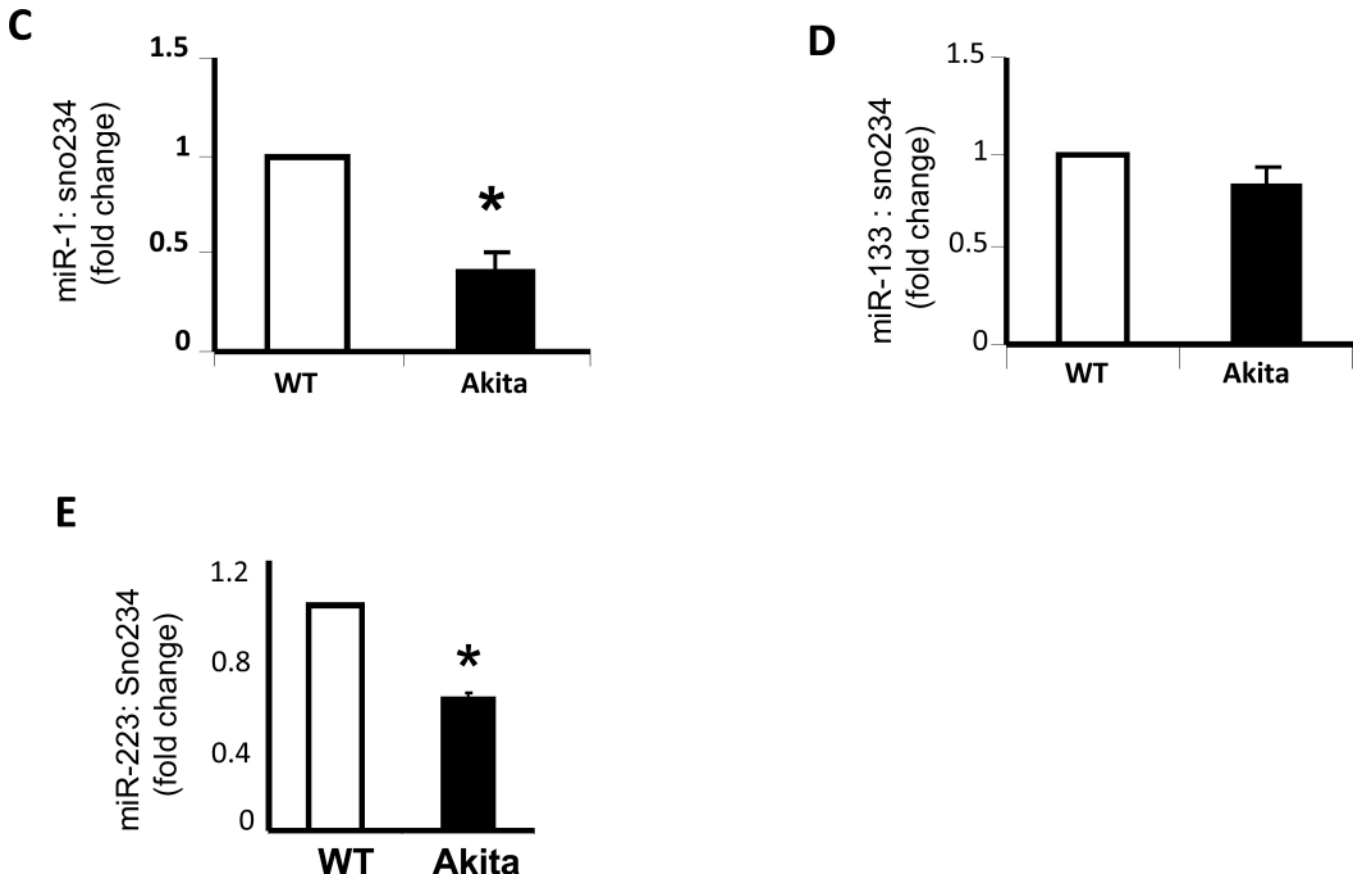

Figure 3. The miRNA profiling of WT and Akita hearts

A–B. The miRNA array shows attenuation of majority of miRNAs but induction of miR-295 in Ins2+/− Akita. The sno234 is used as an endogenous control. **C–E**. Individual miRNA assay shows inhibition of miR-1 (C), miR-133 (D) and miR-223 (E) in Ins2+/− Akita. The values are represented in fold change with standard error. *, $p<0.05$. n=2 (for array) and 4 for (individual assay).

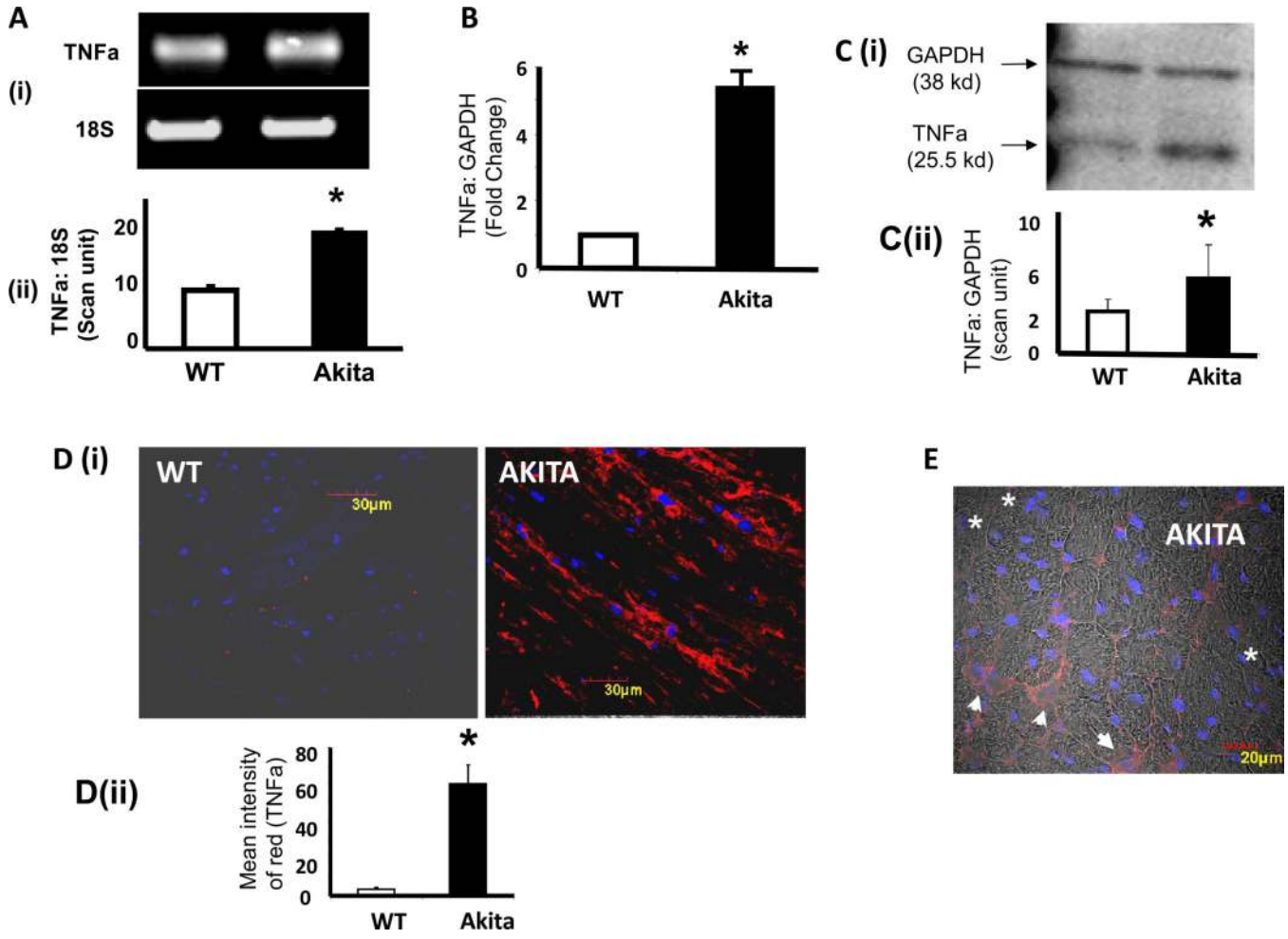

Figure 4. Differential expression of TNFα in Ins2+/− Akita hearts

A. **(i)**. Representative RT-PCR of TNFa in WT and Akita hearts. The 18S RNA is a loading control. **(ii)**. The bar graph represents densitometry analyses of band intensity (mean ± SE) normalized with 18S. *, $p<0.05$, n= 4. **B**. The qPCR analyses of TNFα in WT and Akita hearts. GAPDH is an endogenous control. *, $p<0.05$; n= 4. **C. (i)**. Representative multiplex-immunoblotting of TNFα in WT and Akitas. GAPDH is a loading control. **(ii):** The densitometry analyses of band intensity (mean ± SE) normalized with GAPDH. *, $p<0.05$, n= 4. **D**. **(i)**. Representative immunohistochemistry of TNFα (red color). The 5µM cryosections of the heart are stained with TNFα primary and Texas Red secondary antibodies and observed under confocal microscope. The blue color (Dapi staining) is showing nucleus. **(ii)**. The bar graph represents intensity of red color in WT and Akita. The values are presented as mean ± SE. *, $p<0.05$; n= 3. **E**. The cellular localization of TNFa (red color) in hypertrophic (arrow head) and normal (star) cardiomyocytes of Akita. The blue color (Dapi staining) is showing nucleus.

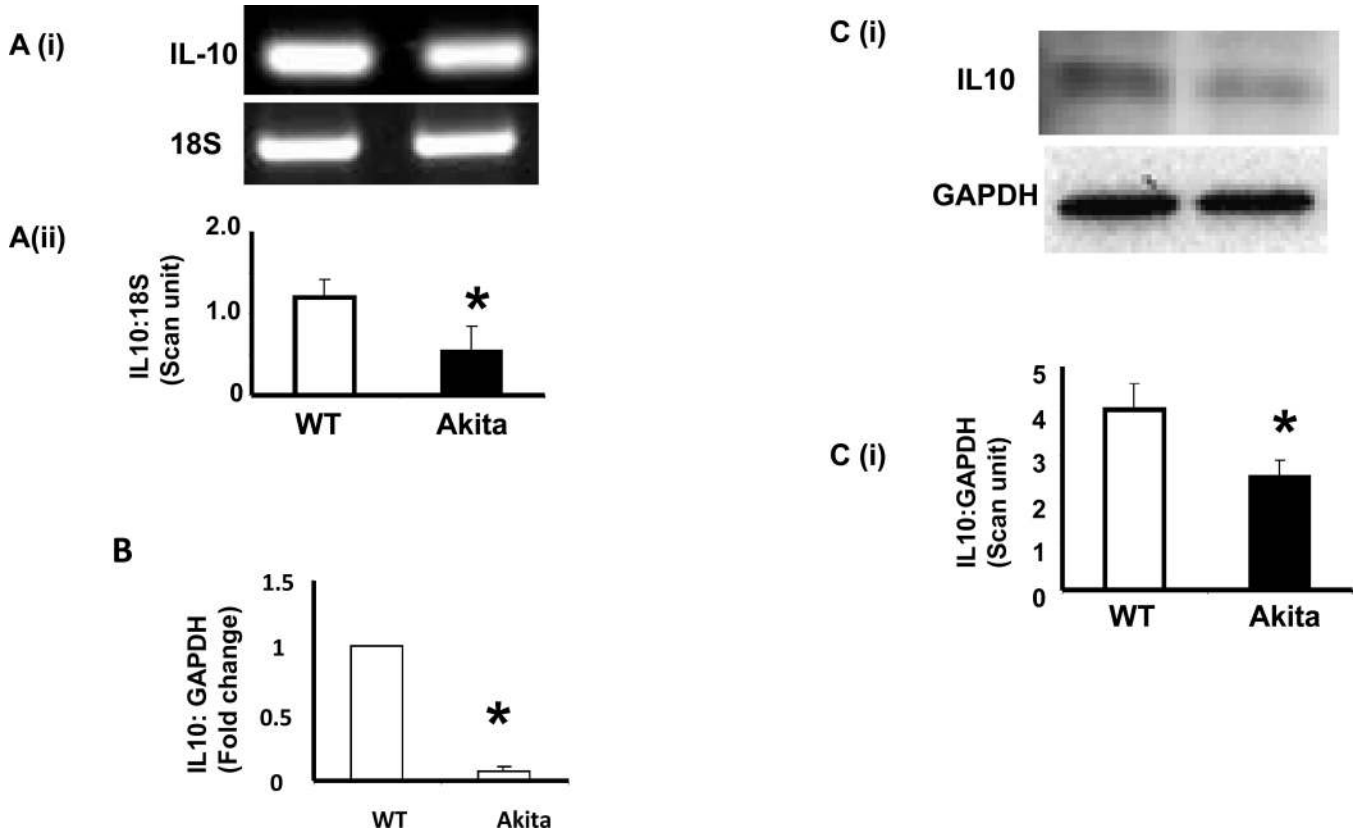

Figure 5. Differential expression of IL-10 in Ins2+/– Akita hearts

A. **(i)**. Representative RT-PCR of IL-10 in WT and Akita. The 18S RNA is a loading control. **(ii)**. The densitometry analyses of band intensity normalized with 18S in WT and Akita. The bar graph represents mean ± SE of band intensity determined by arbitrary scan unit. *, $p<0.05$; n= 4. **B**. The qPCR analyses of IL-10 normalized with GAPDH in WT and Akita. **C. (i)**. Representative immunoblots for IL-10 in WT and Akita. GAPDH is a loading control. **(ii):** The bar graph represents densitometry analyses of band intensity in scan arbitrary unit normalized with GAPDH in WT and Akita. The values are presented as mean ± SE. *, $p<0.05$, n= 4.