MECHANISTIC STUDY OF

HIGH FAT DIET-INDUCED CARDIOMYOPATHY

A Dissertation

by

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ABSTRACT

High-fat diet (HFD)-induced obesity is a risk factor contributing to cardiovascular disease. Excessive accumulation of fatty acids in the heart can cause cardiac dysfunction and heart failure, and is referred to as lipotoxic cardiomyopathy. A number of pathological conditions can create this lipotoxic environment in the heart, such as obesity, insulin resistance, and diabetes mellitus. The deleterious effects of hyperlipidemia on the heart are well recognized. Many signaling pathways associated with cardiac lipotoxicity have been suggested. However, there is limited insight into the epigenetic signaling mechanism of the heart in response to excessive fatty acids preceding metabolic and vascular disorders, and the associated epigenetic mechanisms promote lipotoxic cardiomyopathy. Therefore, the main purpose of this study is to delineate the initial epigenetic signaling mechanism in response to lipid overload and define its contribution to lipotoxic cardiomyopathy. In our mouse model, we found that the animals that have been fed with high fat diet (60% kcal) for 2 weeks are predisposed to hemodynamic stress (transverse aortic constriction) with an increase of miR-23a and miR-23b expression levels. Also, these 2 miRs target on a histone lysine 36 methyltransferase called Setd2 and repress its expression, which might cause the cardiomyopathy predisposed phenotype. In vivo, we identified the above hypothesis by using Setd2 cardiac specific haploinsufficiency mice and Setd2 cardiac-specific transgenic mice. In conclusion, we observed that high fat diet caused heart predisposed to hypertrophy through upregulation of miR-23a and miR-23b, and repress Setd2.

DEDICATION

I dedicate this dissertation to my family, my mentor and all of my friends, especially

to my parents for providing a of the top-line education and the best environment in my whole life;

to my husband for the unconditional support and encouragement, and tolerating the long working hours during my Ph.D. training;

to Dr. Jiang Chang for his guidance and support;

to Weijia Luo, Kelsey Andrade and Yuan Dai for their friendship and consistent help;

to all my friends that I have met during the training for all the support, experiences, friendship, and the traveling time that we spent together, may you always have happiness.

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NOMENCLATURE

CVDs	Cardiovascular Diseases
HCM	Hypertrophic Cardiomyopathy
DCM	Diabetic Cardiomyopathy
DAGs	Diacylglycerols
HFD	High Fat Diet
PA	Palmitic Acid
FFA	Free Fatty Acid
TG	Triglyceride
IGT	Impaired Glucose Tolerance
T2DM	Type 2 Diabetes Mellitus
EF	Ejection Fraction
PKC	Protein Kinase C
AMPK	AMP-Activated Protein Kinase
HAT	Histone Acetyltransferases
HDACs	Histone Deacetylases
MI	Myocardial Infarction
WGA	Wheat Germ Agglutinin
ChIP	Chromatin Immunoprecipitation
miR-23b	Micro RNA 23b
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase

H3K36me3	Tri-Methylated Histone 3 Lysine 36
Setd2	Set domain containing protein 2
3’UTR	3 Prime Untranslational Region
MHC-TG	Myosin Heavy Chain Drive Overexpression in Transgenic Mice

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CHAPTER I

INTRODUCTION

High-fat diet (HFD)-induced obesity is a risk factor contributing to cardiovascular disease. Excessive accumulation of fatty acids in the heart can cause cardiac dysfunction and heart failure, and is referred to as lipotoxic cardiomyopathy. A number of pathological conditions can create this lipotoxic environment in the heart, such as obesity, insulin resistance, and diabetes mellitus. The deleterious effects of hyperlipidemia on the heart are well recognized. Many signaling pathways associated with cardiac lipotoxicity have been suggested. There is still limited insight into the epigenetic signaling mechanism of the heart in response to excessive fatty acids preceding metabolic and vascular disorders, and the associated epigenetic mechanisms that promote lipotoxic cardiomyopathy. Therefore, the main purpose of the study is to delineate the initial epigenetic signaling mechanism in response to lipid overload and define its contribution to lipotoxic cardiomyopathy.

About 60-80% of ATP generation depends on β-oxidation of fatty acids in the adult heart. A precise balance of lipid uptake and oxidation is critical for normal cardiac function. There is accumulating evidence from both basic research and clinical observations showing that an excessive amount of fatty acids in the heart is a deleterious factor that impairs cardiac function and structure; this process is called lipotoxic cardiomyopathy. Pathological disorders such as obesity, insulin resistance, and diabetes mellitus can elicit this lipotoxic environment in the heart. Therefore, a better

understanding of the molecular regulation that underlies lipotoxic cardiomyopathy is highly important and significant.

CHAPTER II

HIGH FAT DIET CAUSED MICE HEART PREDISPOSED TO HEMODYNAMIC CHALLENGE DUE TO MIR-23 UPREGULATION

Overview of This Part

High-fat diet (HFD)-induced obesity is a risk factor contributing to cardiovascular disease[1]. Excessive accumulation of fatty acids in the heart can cause cardiac dysfunction and heart failure, and is referred to as lipotoxic cardiomyopathy. A number of pathological conditions can create this lipotoxic environment in the heart, such as obesity, insulin resistance, and diabetes mellitus. The deleterious effects of hyperlipidemia on the heart are well recognized. Many signaling pathways associated with cardiac lipotoxicity have been suggested. However, there is limited insight into the epigenetic signaling mechanism of the heart in response to excessive fatty acids preceding metabolic and vascular disorders, and the associated epigenetic mechanisms promote lipotoxic cardiomyopathy. Therefore, the main purpose of this study is to delineate the initial epigenetic signaling mechanism in response to lipid overload and define its contribution to lipotoxic cardiomyopathy. In this study, we found that the animals fed with high fat diet (60% kcal) for 2 weeks are predisposed to hemodynamic stress (transverse aortic constriction) with an increase of miR-23a and miR-23b expression levels, In vivo, by using adeno-associate virus, miR-23a and miR-23b overexpression in cardiomyocyte are achieved. Mice with miR-23a and/or miR23b

overexpression were predisposed to hemodynamic stress, while blocking these two miRs by antagomiRs reverse the dilated cardiomyopathy even with the presence of high fat diet and TAC. The data strongly suggest that the increase of the two miRs might be responsible for HFD-induced cardiaclipotoxicity.

Literature Review

High Fat Diet and Metabolism Change

Presently, a diet enriched in fat, especially saturated fatty acid, has been largely reported to be associated with arthritis, cancer, cardiovascular disease, diabetes, hypertension, obesity or stroke[1-8]. The mechanism behind the pathology changes are the broken balance in between fatty acid uptake and utilization. Under physiological conditions, lipid taken from diet will be broken down under β-oxidation and release energy in the form of ATP to supply the body needs. While under a high fat condition, the lipid supply exceeds oxidative capacity, which leads to the imbalance between uptake and oxidation of fatty acid, especially during the early exposure of high fat diet when none of the metabolism compensation change developed. All the above changes will lead to the upregulation of triglyceride level and free fatty acid level in circulation and lipid deposition in peripheral organs such as liver, adipose tissue, heart and muscle, which is called ectopic fat deposition. Ectopic fat is associated with insulin resistance and type 2 diabetes mellitus (T2DM). It has been widely accepted for the correlation between fatty acid infusion and insulin resistance since 1963 in animal models as well, which indicated

that fatty acids impaired insulin-mediated glucose uptake[9]. Multiple molecular pathways are involved, such as Diacylglycerol (DAG) and Novel Protein Kinase C (PKC)s[10], Ceramides and Akt2[11],etc.

Lipid Metabolism Balance

Lipid metabolism is the break down or storage of fats for energy, these fat are obtained from consuming food or synthesized by liver. More than 95% of dietary lipids are triglycerides (TGs); the rest are free fatty acids (FFAs), cholesterol, phospholipids, and fat-soluble vitamins. Dietary triglycerides and cholesterol esters are transported by chylomicrons, and in adipose and muscle tissue, apoprotein C-II (apo C-II) on the chylomicron activates endothelial lipoprotein lipase (LPL) to convert 90% of chylomicron triglycerides to fatty acids and glycerol, which are taken for energy use or storage. Due to the hydrophobic nature of lipids, they need to be transported by lipoproteins that are synthesized by the liver to the cytoplasm and hydrolyzed into glycerol and fatty acids. Lipoproteins circulate through the blood continuously until the TGs that they contain are taken up by peripheral tissues or the lipoproteins themselves are cleared by the liver.

Factors that stimulate hepatic lipoprotein synthesis generally lead to elevated plasma cholesterol and TG levels, the same time, diet fat overtake has the same effect. Fatty acid can be oxidized into acetyl-coA and produce ATP when demanded. Carnitine acyl transferase I limits the speed of fatty acid oxidation. While acetyl-coA can used for

synthesize fatty acid and energy storage when the amount built up, this is reason that take in more calories than body needs leads to lipid deposition.

Lipid Accumulation and Lipid Toxicity in Heart

Under physiological conditions, fatty acid can be stored in different types, among which triglyceride is the most harmless intracellular lipid storage that consists of a glycerol backbone bound with three fatty acids, majority of the triglycerides are stored in adipocytes with only minimal lipid accumulation in liver or muscle. While the fatty acid uptake and utilization mismatch leads to acetyl-coA, as the central molecular of protein, carbohydrate and lipid metabolism, came from the breaking down of the macro nutrients, going back toward adipose tissue, cause pathological conditions such as insulin resistance, glucose intolerance, leptin increase, adiponectin decrease, etc. Fatty acid deposited in the non-adipose tissue, such as kidneys, liver, heart and skeletal muscle, will cause the lipotoxicity through the lipid intermediates such as fatty acyl-CoAs, diacylglycerol and ceramides[12].Increased cardiac lipid content has been linked to impaired systolic function and increased left ventricular mass[13]. Recent studies with more advanced imaging methods showed increased intramyocardial lipid content in patients with non-ischemic heart failure[14], which is further exacerbated with obesity[15], diabetes mellitus[14, 15], and metabolic syndrome[16]. The link between cardiac lipid accumulation and metabolic cardiovascular complications, such as diabetes mellitus and metabolic syndrome has been reported more than 50 years ago[17]. The deleterious effects

of hyperlipidemia on the heart have been widely studied and many pathway associated with cardiac lipotoxicity have been suggested[18-22].

Heart Energy Supply under Physiology and Pathological Condition

Different from other organs in the body that use glucose as their priority energy supply, the heart uses fatty acid. Under physiological condition, cardiac metabolism of the adult heart primarily depends on fatty acid utilization for oxidative phosphorylation and ATP generation[18]. The physiologic balance of lipid oxidation and uptake prevents excess lipids accumulation. Due to the tremendous energy demanding of the heart, oxidation of large amounts of fatty acid requires a fully oxygen supply. Nonetheless, under pathological conditions such as most forms of heart failure, obesity, diabetes, ischemia, sepsis that lead to fatty acid oxidation altered and often also accumulation of lipids. Meanwhile the heart switches its major energy supply from lipid toward glucose and reduced gene expression involved in fatty acid metabolism with relative increase in expression of gene involved in glucose metabolism[18]. Also the changes accompanied with reduced myocardial ATP production of due to less ATP molecules generated form glycolysis compared with fatty acid oxidation. Notably, in failing myocardium lipids accumulation in subjects with diabetes and obesity with increased levels of toxic intermediates leading to more severe lipotoxicity that already been mention above.

Diabetes and High Fat Diet

Dietary consumption of fatty acid, exotic fatty acid is the major contributor to breaking the balance of fatty acid uptake and oxidation. Plasma free fatty acid levels can be reduced by insulin. Consequently, elevations of circulating plasma FFA concentration can be prevented if large amounts of insulin can be secreted. If hyperinsulinemia cannot be maintained, plasma FFA concentration will not be suppressed normally, and the resulting increase in plasma FFA concentration will lead to the compensate increase in fatty acid oxidation and acetyl-coA level. Acetyl-coA appears to reduce the activity of pyruvate dehydrogenase and result in gluconeogenesis[23]. Meanwhile these individuals are resistant to insulin-stimulated glucose uptake which leads to hyperglycemia. Consistent high fat diet will lead to insulin resistance and hyperinsulinemia due to the above mechianism[24], as well as other pathological changes including impaired glucose tolerance (IGT), increased plasma triglyceride concentration, and decreased high-density lipoprotein cholesterol concentration, all of which are diagnostic standards of type II diabetes and are associated with increased risk for cardiovascular diseases (CAD).

Diabetic Cardiomyopathy

According to International Diabetes Federation reports in 2015, diabetes might leads to cardiac complications such as cardiac vascular injury, atherosclerotic plaque formation, and even myocardial infarction[25].Among the above complications, myocardial disorder without hypertension or coronary artery disease, named as diabetic cardiomyopathy

(DCM), has received much attention in recent years[26]. DCM has been noticed in both human and animal models with Type 1 or Type 2 diabetes[27]. Diabetic Cardiomyopathy is one of those diseases that occurs to the heart muscles in diabetic patients.

Hyperinsulinemia, insulin resistance and hyperglycemia are independent risk factors for the development of diabetic cardiomyopathy[28]. One of the common symptoms noticed is systolic dysfunction and its role in symptomatic heart failure, left ventricular dilation and atherosclerosis or hypertension. Literature has reported rodent models with chronic diabetes present abnormalities in left ventricle diastolic functions with or without systolic dysfunction. Also, diastolic dysfunction has been described in patients with Type I and Type II diabetes has been recognized as the abnormality of cardiac tissue independent from vascular defeat in diabetes[29].

Insulin resistance and hyperinsulinemia increase systemic metabolic disorders, activate the SNS, activate RAAS, prompt oxidative stress, mitochondrial dysfunction and endoplasmic reticulum stress and impair calcium homeostasis.

Meanwhile the abnormal lipid metabolism accentuates diabetes cardiomyopathy. Increased circulating triacylglycerol levels lead to increased fatty acid delivery to cardiomyocytes and, thus, enhanced fatty acid β-oxidation and impaired insulin metabolic signaling in diabetic hearts[30]. These effects result in cardiac fibrosis,

hypertrophy, cardiomyocyte death, dysfunction of the coronary microcirculation and eventually heart failure.

Hypertrophic Cardiomyopathy

The most common stimuli for cardiac hypertrophy are long-standing hypertension; valvular insufficiency and stenosis; myocardial infarction (MI) or ischemia associated with coronary artery disease (CAD); congenital malformations; myocarditis due to an infectious agent; familial hypertrophic and dilated cardiomyopathies; and diabetic cardiomyopathy[31, 32]. The above stimuli cause the individual cardiomyocytes to grow in length and/or width as means of increasing cardiac pump function and decreasing ventricular wall tension, this process inducing a state of compensated hypertrophy state, while in the long term, myocardial hypertrophy predisposes individuals to arrhythmia, heart failure, and even sudden death[33, 34]. In vivo and in vitro studies have revealed a serial signaling pathway and different molecules that are involved in the development of cardiac hypertrophy, such as G-protein coupled receptor activation induced protein kinase C (PKC) activation[35], internal sensory apparatus such as integrin and Z-disc that transduce biomechanical stress signals through attached signaling molecules and activate downstream small GTPase[36, 37], MAPK signaling[38], calcineurin–NFAT pathway[39], PI3k –AKT-mTOR pathway[40], Class II HDACs[41]. Recent studies reported that cardiomyocyte growth can lead to concentric hypertrophy(HCM) or eccentric hypertrophy(DCM) based on the different tension index(calcium vs time-profile) which the indicating the

Ca^{2+} affility and contractile force of the cardiomyocyte decide the cardiomyocyte growth in width (HCM) or length (DCM)[42-44].

High Fat Diet Regulation on Micro-RNA Level in Heart

The biological and pathological functions of microRNAs have been widely recognized in many organs including the heart. Intensive studies have focused on the essential role of microRNAs in heart development and different cardiac diseases[45-47]. Very few microRNAs have been identified to be directly associated with lipid-induced cardiac toxicity[47-49]. Recently, two excellent studies have observed the upregulation of miR-195 and miR-451 in correlation to lipid overload. The responsive increase in the expression levels of the two miRs promote cardiomyocyte death and cardiac hypertrophy through inhibition of their downstream targets[48, 49]. The two studies either investigate the mechanism in isolated cardiomyocytes or mice that fed with high fat diet for 20 weeks. There are also studies that focus on different types of high fat diet caused micro-RNA profile change[50]. The mechanism underlying early exposure to high fat diet as the cause of cardiac micro-RNA profile change remains unknown. Continual discovery of critical miRs in response to excessive lipid stress is critically needed to uncover the initial signaling that triggers the detrimental cascades into disease and will also close the current knowledge gap in lipotoxic cardiomyopathy studies. The purpose of our study is to fill this gap.

Materials and Methods

Cell Culture and Palmitic Acid Treatment

Human embryonic kidney epithelial cell line 293T (HEK 293T) was obtained from ATCC. Cells were cultured in DMEM with 10% FBS.

Palmitic acid (P0500 Sigma Aldrich) was dissolved in DMSO and added into culture medium at final concentration 1μM. For the control group, the same amount of DMSO were added.

miR-mimic, miR-inhibitor Used in in vitro Assay

Overexpression of miR-23a and miR-23b were achieved by transient transfections of CMV-miR23a or CMV-miR23b expression vectors. The miRIDIAN microRNA Hairpin Inhibitors were used to inhibit micro RNAs in the in vitro assay (GE Healthcare Dharmacon, Lafayette, CO). Antago-miR-control: IN-001005-01-20, antago-miR-23b-3p: IH-310379-07- 0010, antago-miR-23a-3p: IH-300494-05-0010) at the final concentration of 100nM.

Generation of Recombinant Adeno-associate Virus

The recombinant adeno-associated virus used to express miR-23a and miR-23b (Ad-AAV) was generated by using DNA double strain synthesis (IDT), synthesizing 66-base-pair miRNA precursors sequence with primers

5′-AGTCAGATGTACAGTTATAAGCACAAGAGGACCAG-3′ for mmu-miR-23a and 5′-TTATTCAAGATCCCGGGGCTCTTCC-3′ for mmu-miR-23b. The fragment was inserted into vector AAV-U6-GFP.

5′-AGTCAGATGTACAGTTATAAGCACAAGAGGACCAG-3′ was used as a control fragment. For adeno-associated virus production, AAV-U6-GFP, AAV-DJ/8 and pHelper plasmid (Cell Biolabs) were co-transfected into 293T cells using calcium transfection. Virus were harvest after 72 hours through 4 thaw-freeze cycles. Virus title was measured by AAV-quick title kit (VPK-145, Cell Biolabs, San Diego, CA).

Gene Transient Transfection

All gene transient transfections were performed using the NEON transfection system (MPK5000; Life Technologies). 293T cells were suspended by the density of 5*10^7 cells/ml, electroporation parameter was 1100 voltage, 20ms pulse width, 2 pulses, the efficiency of transfection is more than 80% under this condition.

Cells were immediately seeded after the electroporation transfection.

High Fat Diet Feeding

The Institutional Animal Care and Use Committee (IACUC) of Texas A&M University Health Science Center Institute of Biosciences and Technology approved the experiments with animals. Male mice with C57BL/6 background, at the age of 10 weeks

were used, and fed with high fat diet (fat provide 60% Kcal, Research Diet, D12492), the control group was fed with normal chow. After feeding for 2 weeks, echocardiography was performed to record the cardiac function and molecular markers were checked in heart.

Cardiac Function Assessment by Echocardiography

For cardiovascular function measurements, a Vevo770 High-Resolution Micro-Imaging System (VisualSonics, Toronto, ON, Canada) with 30-MHz probe (RMV-707B) was used.

Male mice in each group were anesthetized with 3–4% isoflurane, and then switched to 1–1.5% isoflurane mixed with 100% oxygen. At the level of the papillary muscles, 2-dimensional guided M-mode echocardiography was obtained from anterior (+septum) and posterior walls.

Transverse Aortic Constriction

Mice are divided into different groups. First group, mice have been fed with 2 weeks of high fat diet or normal chow; second group mice been injected with the adeno-associate virus specifically expressing miR-23a or miR-23b in cardiomyocyte; third group mice have been injected with antagomiR and fed with high fat diet for 2 weeks. Those mice are anesthetized with 3% isoflurane with 100% oxygen, the thymus was retracted to expose the transverse aorta. Between the right innominate and left carotid artery, an

aortic constriction was achieved by tying a 6-0 suture against a 3 mm length of 27 gauge needle. After two knots, the 27 gauge needle was promptly removed, which yielded a constriction of ~0.3 mm as the outer diameter of the 27 gauge needle. The procedure imposed a 60–80% aortic constriction on the animal. To minimize the variation of the constriction among the animals, only the mice with a ratio of right-to-left carotid artery flow velocity from 5 : 1 to 10 : 1 were used. The artery flow velocity was measured by a Pulsed Doppler (Indus Instruments, Houston, TX, USA). As a control, a sham operation without occlusion was performed on age-matched littermate mice.

Hematoxylin and Eosin Staining, Immunostaining

Mice hearts were perfused and fixed by Langendorff Perfusion System (Radnoti, 120108). The whole heart paraffin sections were applied to Hematoxylin and Eosin Staining (51275, Fluka; HT110132-1L, Sigma-Aldrich) and images were captured under 1.25X magnification with Leica DM2000 histology microscope.

Paraffin sections of the whole heart were used for hematoxylin & eosin, wheat germ agglutinin (WGA) (W11262, Thermo Fisher Scientific, NY, USA), Pictures were taken under the 40x microscope objective. A total of 20 staining pictures from each group were quantified by Leica Application Suite Imaging Software (Version 4.0, Germany). Nuclei were visualized by DAPI staining. The images were acquired by fluorescence microscopy.

Wheat Germ Agglutinin Staining

Paraffin sections of the whole heart were used for Wheat Germ Agglutinin (WGA) Staining. The tissue sessions were deparaffinized and incubated with Wheat Germ Agglutinin (Vector Laboratories, Burlingame, CA) at approximately 20 µg/ml in PBS to the sections and incubated for 30 minutes at room temperature. The tissue sessions were wash with TPBS (PBS + 0.05% Tween™ 20) for 3 times. Nuclei were visualized by 1 µg/ml DAPI staining for 5 min. Images were captured under the 40x microscope objective with Leica DM2000 histology microscope. One image was from the top of the interventricular septum and four images were from the left ventricle wall. A total of 20 staining pictures from each mouse were quantified by Leica Application Suite Imaging Software (Version 4.0, Germany).

Micro-RNA Sequencing Assay and RNA Sequencing Assay

Total RNA was isolated from the hearts of 10-wk-old mice using miRNeasy kit (QIAGEN, Cat No./ID: 217004), and 3 µg was used for RNA-seq or miR-seq. The assessment was performed using the Qiagen RT2 Profile PCR Array.

Data analyses were performed with R (www.r-project.org), a publicly available statistical tool for data analysis. Signaling pathway analysis was performed with Ingenuity (www.ingenuity.com).

Retro-Orbital Sinus Injection

----[[[[Using a 28-gauge insulin syringe and inserting the needle into the retro-bulbar space, mice were anesthetized with 0.2ml 2.5% avertin during the procedure. A maximal 150ul volume can be injected. Mice were infected with 1×10^{12} Geno Copy of virus by injection into the retro-orbital sinus. For antagomiR treatment experiments, mice started with high fat diet received a shot of antagomiR through retro-orbital sinus of 80mg/kg body weight. A drop of ophthalmic anesthetic (0.5% proparacaine hydrochloride ophthalmic solution, Alcon Laboratories, Inc., Fort Worth, TX) was applied on the eye after receiving the injection.

Adult Mouse Cardiomyocyte Isolation and Culture

Adult mouse cardiomyocytes were isolated using the isolation kit (Cellutron Life Technologies). Mouse cardiomyocytes were isolated from 10- to 15-week-old adult male mice by enzymatic digestion with the Langendorff perfusion system (120108, Radnoti, CA, USA) following the Cellutron protocol. The cells were collected by centrifuging at 1000 rmp for 1 min and cultured onto laminin-coated dishes with AW medium for later experiments.

RNA Isolation and qRT-PCR (Quantitative Polymerase Chain Reaction) Analysis

Isolation of total RNA from mouse heart was performed using TRIzol reagent (Life Technologies). cDNAs were synthesized from 1 μg total RNA with the qScript One-Step RT-qPCR kit (Quanta Biosciences). Quantitative RT-PCR was performed using SYBR

Green PCR Master Mix (Life Technologies) in the StepOnePlus Real-Time PCR System according to the manufacturer's instructions.

Transcripts were quantified by quantitative PCR (qPCR) analysis. Total RNA was prepared by miRNeasy kit (QIAGEN, Cat No./ID: 217004), 0.5ug RNA was used for reverse transcription(QUANTA, 95048).

miR-23a and miR-23b transcription were quantified by quantitative PCR (qPCR) analysis. Total RNA was prepared by miRNeasy kit (QIAGEN, Cat No./ID: 217004); 10 ng RNA was used for reverse transcription by TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thomas Fisher, A28007,Waltham, MA). Probes for target miRs were purchased from Thomas Fisher scientific Waltham, MA (hsa-miR-23a-3p: 478532; hsa-miR-23b-3p: 477991; hsa-miR-191-5p: 477952). miR-191-5p expression level was used for qPCR normalization. Expression levels were determined by the $2^{-\Delta\Delta Ct}$ threshold cycle method.

Statistical Analysis

Data are presented as means ± s.d. Unpaired, two-tailed student's *t* test was used for two group comparisons. One-way ANOVA followed by Student-Newman-Keuls test was used for multiple group comparisons.

All statistical analyses were performed in software SigmaPlot 11.0 (Systat, San Jose, CA, USA). The P value < 0.05 was considered as significant difference.

Results of This Part

Short-Term (2-Week) HFD is a Detrimental Factor that Exacerbates Cardiac Dysfunction and Hypertrophy after Hemodynamic Challenge

In this mouse model after 2 weeks feeding of high fat diet, there was no significant change in cardiac function or morphology in the mouse heart from the high fat diet (HFD) group (Fig.1 Sham) compared with mice fed with normal diet (ND). After the diet treatment followed by 3 weeks transverse aortic constriction (TAC), mice in the HFD group developed dilated cardiomyopathy with significantly enlarged cardiomyocytes and worsened cardiac function compare to the normal diet (ND) group. (Fig.1 TAC)

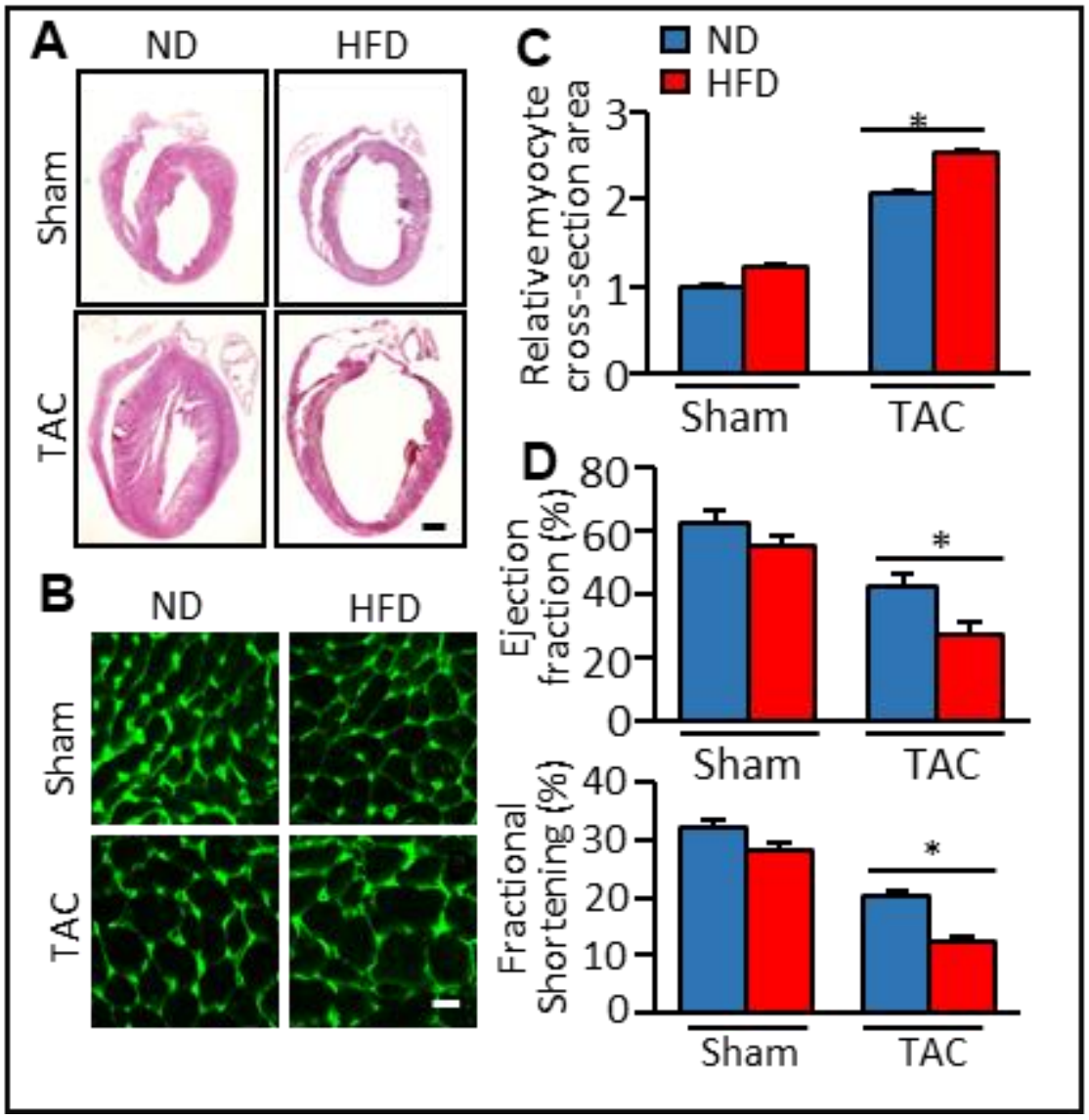

Figure. 1. Short-term (2-week) HFD is a detrimental factor that exacerbates cardiac function and promotes hypertrophy. (A). H&E staining for mouse myocardial section. Scale bar: 1 mm. (B). Wheat germ agglutinin (WGA) staining exhibits significant increase in cardiomyocyte size in HFD heart after 3-week TAC. Scale bar: 10 μm. (C). Quantification of the cardiomyocytes size. (D). Cardiac function assessments show that HFD mice develop heart failure after TAC. TAC: transverse aortic constriction; ND: normal diet; HFD: high fat diet.. * $P<0.05$, n=5.

2-week HFD Induces Profound Increases in miR-23a and miR-23b in Mouse Heart

To rule out the pathways that are involved in the development of the above phenotype, miR-seq was performed on the high fat diet fed 2-week mouse heart. The result indicated that there are over 100 microRNA expression levels are increased in the 2-week HFD mouse heart and, within those miRs, miR-23a and miR-23b are specifically enriched in the heart. Quantitative PCR was used to further confirm this result and found that after feeding with HFD for 2 weeks, miR-23a and miR-23b increased 18.37- and 12.82-fold compared to the normal chow fed mouse heart. To exclude the effect of TAC in the development of the predisposed cardiac hypertrophy phenotype that happens in high fat diet fed heart, miR-23a and miR-23b levels in the TAC heart compare with the heart from Sham group were checked, the result indicated that TAC itself can only induce a slight increase on these 2 miRs, 0.35-fold increase of miR-23a and 0.78-fold increase of miR-23b. (Fig.2)

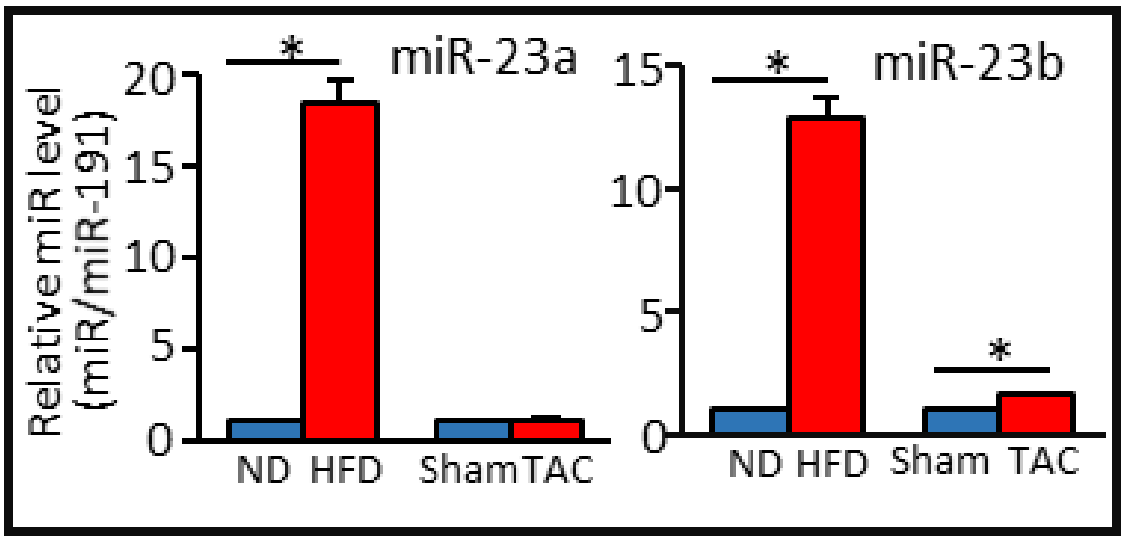

Figure. 2. 2-week HFD induces profound increases in miR-23a and miR-23b in mouse heart. The levels of two miRs are quantified by qPCR. A moderate increase in miR-23b is also detected in 3-week TAC mouse heart. *P<0.05, n=5.

Upregulation of miR-23a and miR-23b are Responsible for HFD Induced Lipotoxic Cardiomyopathy

To further investigate whether the upregulation of these 2 miRs caused predisposition to TAC, forced expression of miR-23a and miR-23b separately or jointly in mouse heart were achieved by the adeno-associate virus system, which successfully boosted the expression level 1.7-fold (Fig.3a) in heart when treated with mice with the AAV DJ/8:miR-23a or AAV DJ/8:miR-23b. Co-expression of these 2 miRs did not cause redundancy. The mice are viable and normal under physiological conditions. Similar to the 2-week high fat diet fed mice, mice injected with AAV DJ/8:miR-23a or AAV DJ/8:miR-23b showed severe cardiac dysfunction compared with the control

mice(Fig.3). The cardiac morphology indicated a thicker left ventricular wall and developed more severe cardiac hypertrophy (Fig.4a) with enlarged cardiomyocyte size (Fig.4 b&c), as compared to the control mice after 3 weeks of TAC.

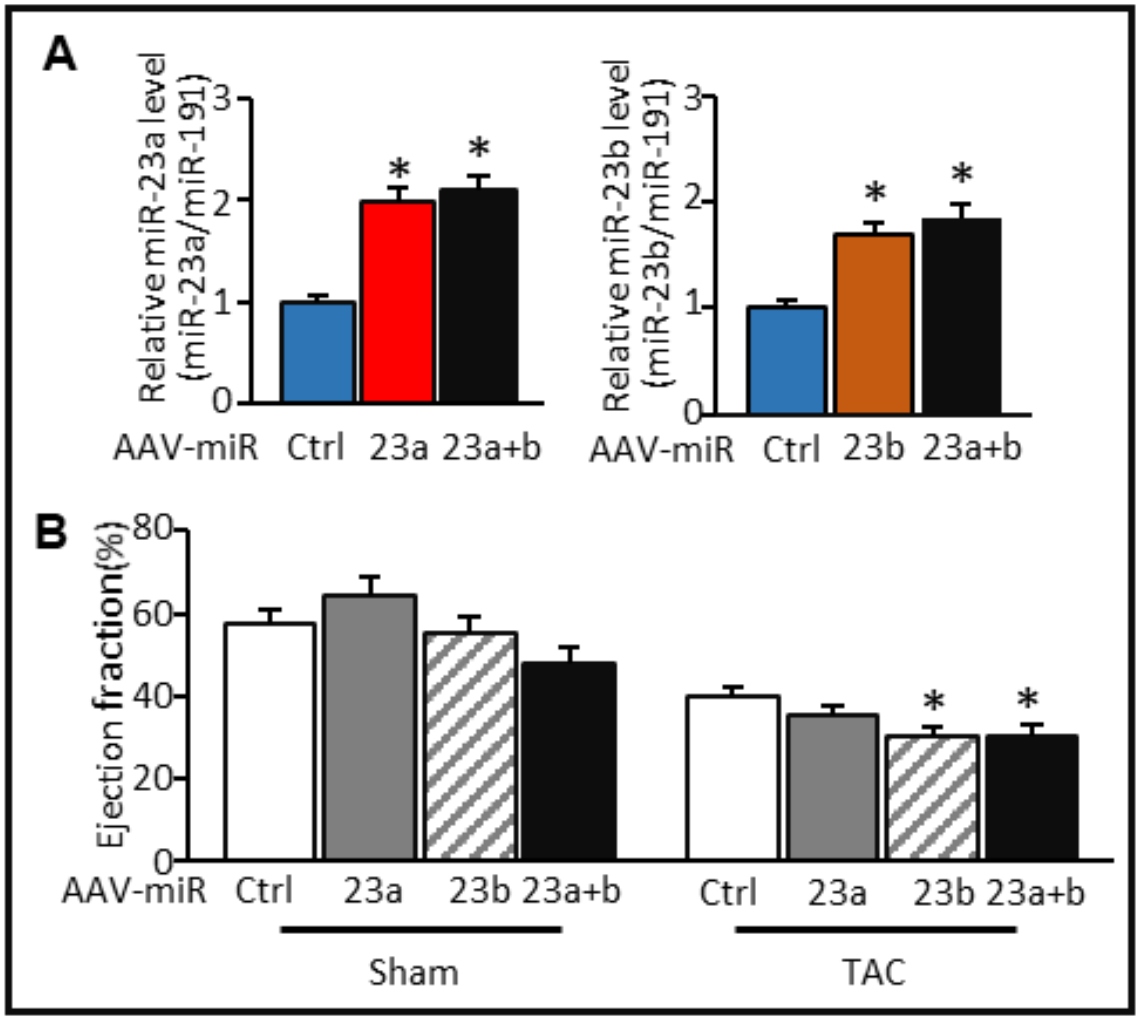

Figure. 3. Overexpression of miR-23 exacerbates cardiac function after TAC. (A).The increase in miR-23 expression in mouse heart is achieved by intravenous delivery of adeno-associate virus (AAV) expressing miR-23. The qPCR analysis was conducted two weeks after the virus injection (B). AAV-miR-23-treated mice showed deleterious cardiac function compared to control mice in response to 3weeks of TAC. * $P<0.05$, $n=5$.

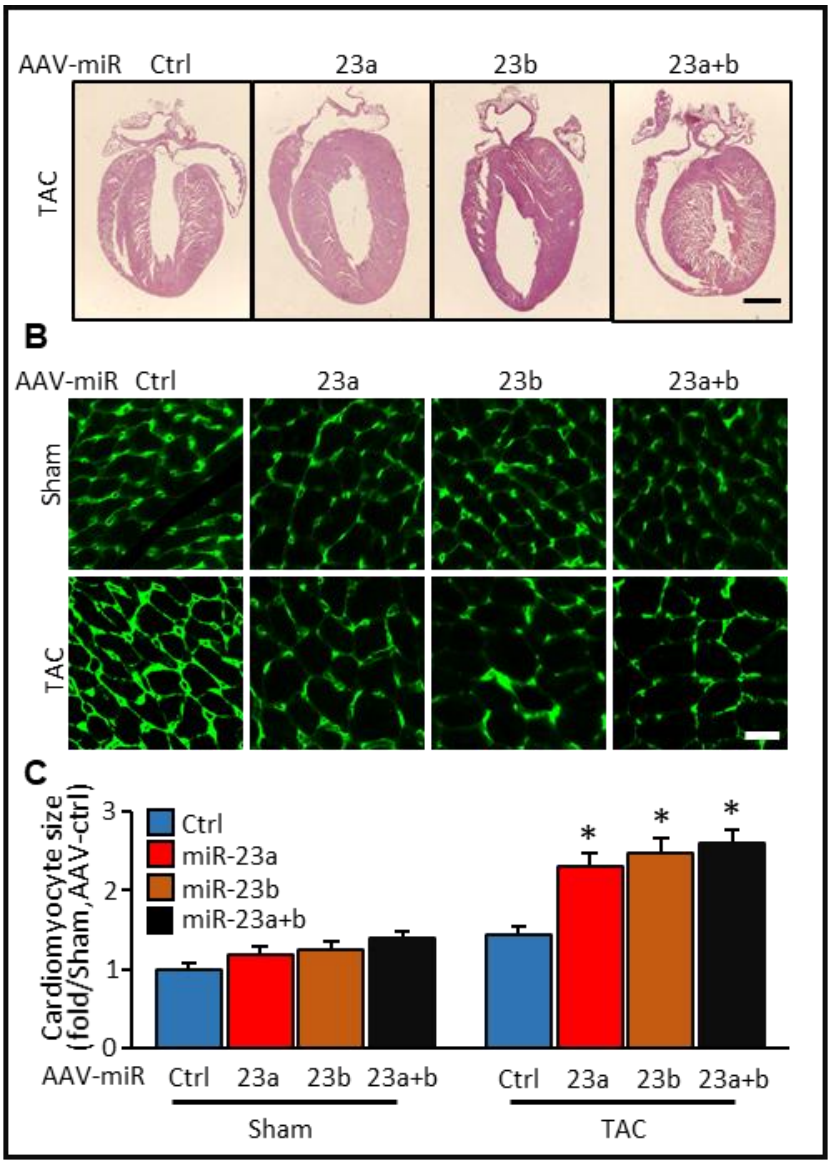

Figure. 4. Overexpression of miR-23 exacerbates cardiac hypertrophy after TAC. (A). H&E staining of mouse heart sections after different adeno-associate virus infection which specifically expresses miR-23a and /or miR-23b in cardiomyocyte and 3-week TAC. Scale bar represents 1mm. (B). Wheat germ agglutinin (WGA) staining exhibits significantly enlarged cardiomyocytes from the AAV-miR-23 treated mouse heart after 3-week TAC compared with the control hearts. Scale bar: 10 μm. (C). Quantification of cardiomyocyte size. * $P<0.05$, n=5.

Blocking miR-23a and /or miR-23b will Rescue Dilated Cardiomyopathy Triggered by High Fat Diet and TAC

Since the gain-of function experiment achieved a similar phenotype as that observed in high fat diet fed mice after 3 weeks of TAC, loss of function experiments were also performed to investigate whether miR-23a and miR-23b is not only necessary but sufficient to cause the cardiomyopathy phenotype. On day 0, C57BL/6 mice were injected with antagomiR (23a, 23b or 23a and b, ctrl) and started being fed with high fat diet; 2 weeks after the injection of antagomiR, significant downregulation of miR-23a and miR-23b in mice hearts were observed in the antagomiR group (mir-23a, miR-23b, miR-23a&b) compared with the control mice (Fig.5a). Then followed by 3 weeks of TAC, the antagomiR group mice have significantly preserved cardiac function (Fig.5 b) and showed less severe cardiac hypertrophy(Fig.6 a-c).

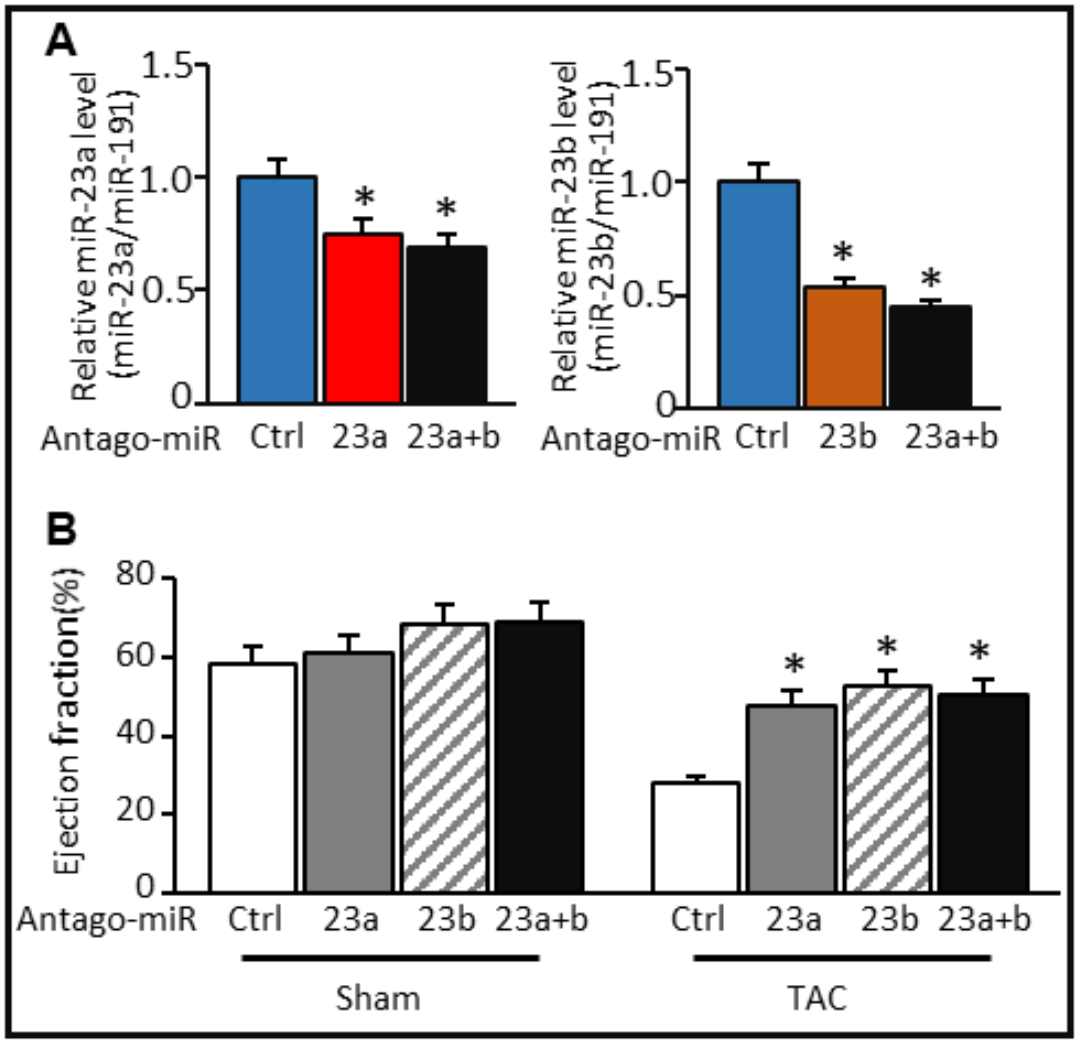

Figure. 5. Repression of miR-23 partially rescue cardiac dysfunction after HFD+TAC. (A). Repression of miR-23 expression in mouse heart is achieved by intravenous delivery of Antago-miR-23. The qPCR analysis is conducted two weeks after the agents injection. (B). Antago-miR-23-treated mice showed preserved cardiac function compared to control mice in response to 3 weeks of TAC. * $P<0.05$, n=5.

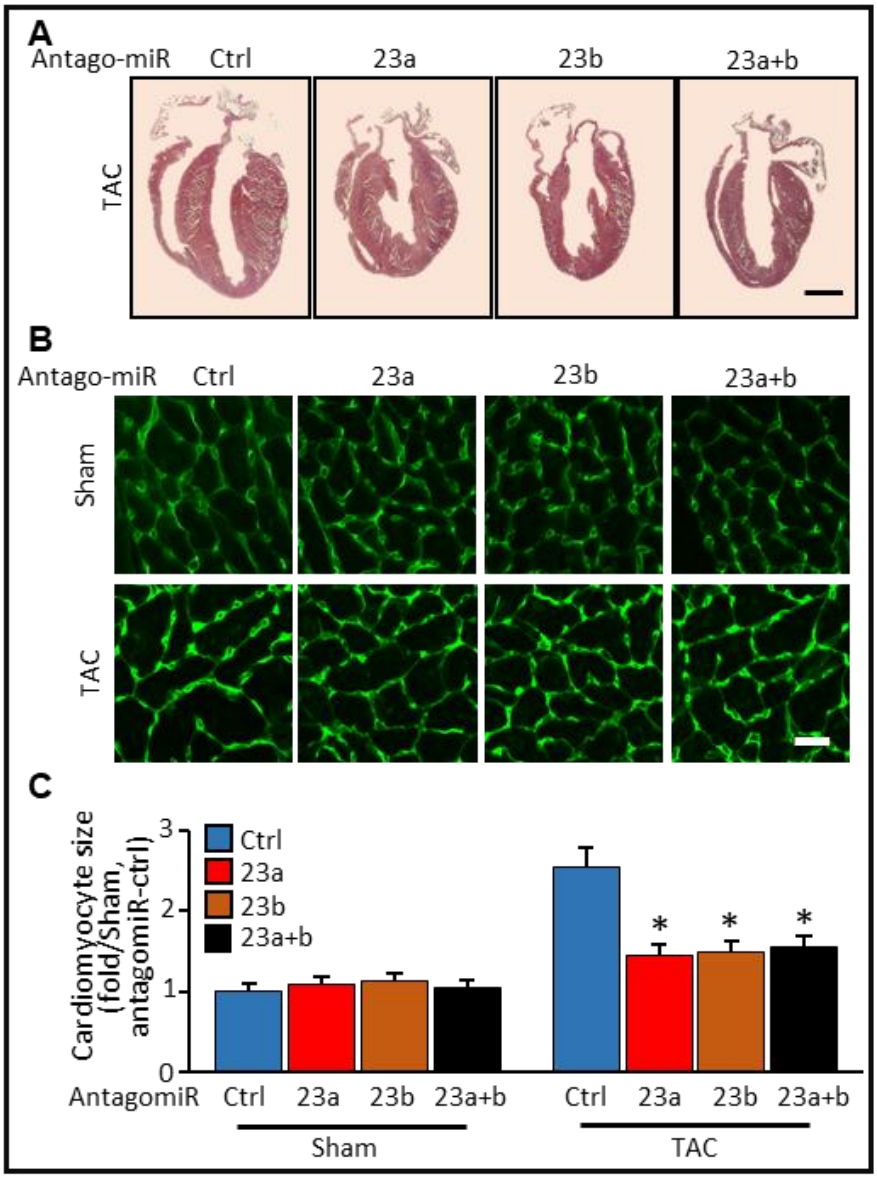

Figure. 6. Repression of miR-23 partially rescued cardiac hypertrophy after HFD+TAC. (A). H&E staining for mouse heart sections after HFD2W+TAC3W. Scale bar: 1mm. (B). Representative image Wheat germ agglutinin (WGA) staining exhibits resistance of cardiomyocytes enlargement from the Antago-miR-23 treated mouse heart after 3-week TAC compared to control heart. Scale bar: 10 μm. (C). Quantification of cardiomyocyte size. * $P<0.05$, n=5.

miR-23a and miR-23b are High Fat Sensors

Based on our observation earlier in this study, miR-23a and miR-23b were significantly upregulated under a short-term high fat diet (HFD) *in vivo* and a 24-hour Palmitic Acid (PA) treatment in vitro. To investigate the dynamic change of miR-23a and miR-23b levels in response to high fat diet/ palmitic acid, 293T cells have been treated with palmitic acid for 24 hours; then upon retracting the treatment, miR-23a and miR-23b levels were monitored at 4 hrs, 8 hrs, 12 hrs, 24hrs, 48 hrs after the retraction. In vivo, c57bl/6 mice at 10-12 weeks were fed with high fat diet for 2 weeks then switched back to normal chow. miR-23a and miR-23b expression levels were monitored after 1 day, 3 day, 7day and 14 days after the diet switch. The dynamic change of miR-23a and miR-23b expression levels were observed both *in vivo* and *in vitro*, in which the expression levels of these two miRs were boosted by high fat diet or palmitic acid treatment, while slowly returning to the baseline after the HFD treatment was retracted. (Fig.7)

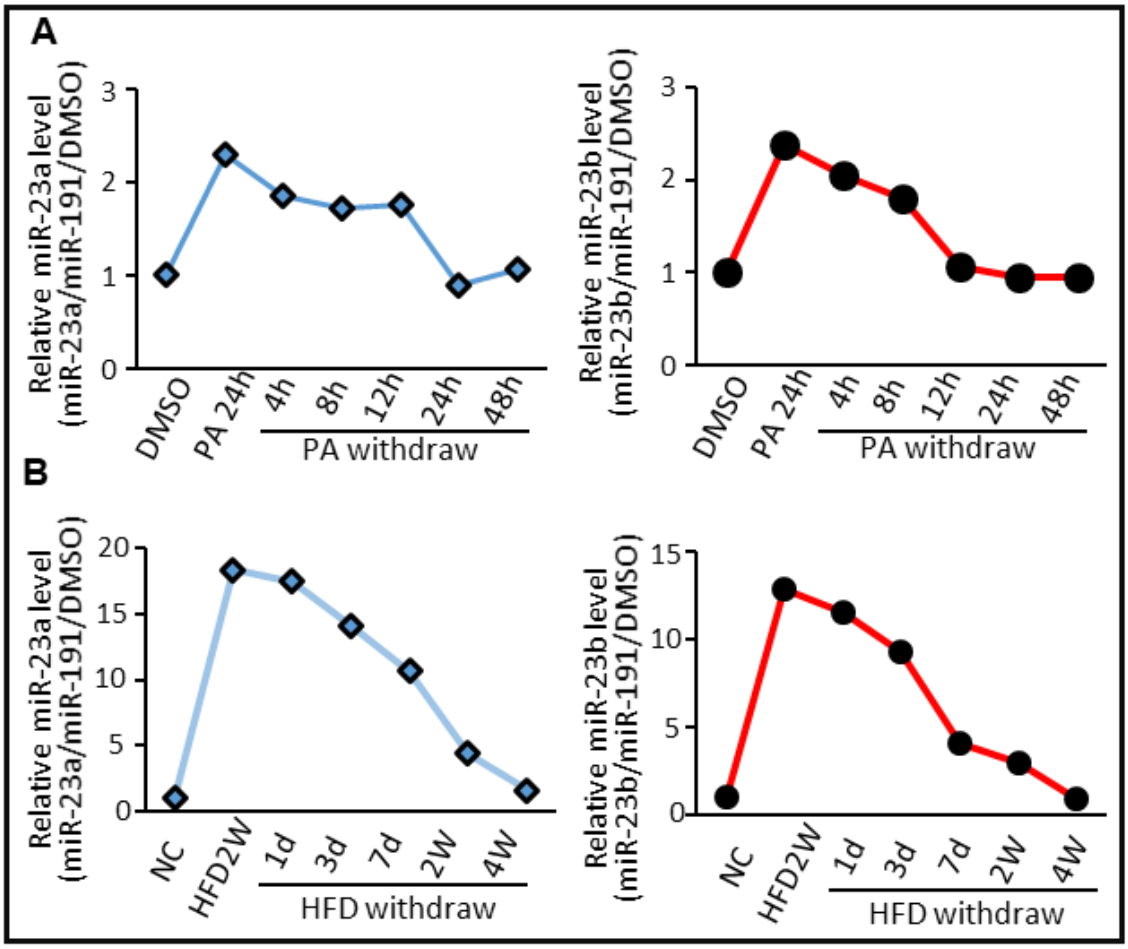

Figure. 7. miR-23a and miR23b are lipid sensors. (A). Excessive fatty acids triggered an upregulation of the miR-23a and miR23b and the increase of the two miRs recede upon fatty acid withdrawal in 48 hrs in 293 T cell. (B). Mice fed with high fat diet for 2 weeks were observed with significant upregulation of miR-23a and miR-23b in hearts, and the increase of the two miRs receded after high fat diet was withdrawn in 4 weeks, , n=2.

Discussion of This Part

High Fat Diet in Cardiovascular Disease

High fat diet is enriched with saturated fatty acid that more easily causes the imbalance of fatty acid intake and oxidation compared to normal diet[51]. Lipid overload-induced cell/organ dysfunction, referred to as lipotoxicity, was first reported in Roger Unger's seminal paper in 1994[52].

An elevated level of plasma free fatty acids was sufficient to cause β-cell dysfunction in the Zucker diabetic fatty rat, which resulted in insulin resistance before non-insulin-dependent diabetes mellitus. Since then, lipotoxicity has been widely recognized in animal models and human diseases including lipotoxic cardiomyopathy[14, 18-20, 53-55].

Lipotoxic cardiomyopathy is closely associated with many long-term chronic metabolic syndromes such as obesity, insulin resistance, and diabetes mellitus[18-20, 54, 56]. Prolonged high levels of circulating fatty acids and triglycerides, which commonly exist in these metabolic syndromes, will affect the cardiac metabolism by changing from using fatty acid into glucose, which further leads to the increase of toxic products such as Diacylglycerols (DAGs), ceramides, medium chain acyl carnitines[57] that cause cardiac dysfunction and injury.

Thus, new therapeutic strategies aimed at reducing cardiac and circulation lipid accumulation will be a primary approach to prevent lipotoxic heart disease.

Because of the complexity of the disease, **the initial molecular signaling that triggers cardiac lipotoxicity *preceding* metabolic syndromes is relatively understudied and remains a challenge in the field.**

Therefore, the majority of the studies on cardiac lipotoxicity have been closely associated with diabetic and obesity cardiomyopathy investigations due to their natural connection and overlap.

Cardiac Lipotoxicity

Given the importance of excessive fatty acid-induced cardiac complication, many signaling pathways have been found to be activated by lipid accumulation and have been linked to this cardiac toxicity[18-22, 55, 56], such as lipid overload-induced endoplasmic reticulum (ER) stress, and ER stress-induced mitochondria dysfunction and cell death; mitochondria dysfunction, and mitochondria-mediated apoptosis; excessive generation of ROS; lipid metabolic intermediates: generation and accumulation of diacylglycerol (DAG), ceramide and acyl carnitines; Altered AMP-activated protein kinase (AMPK) signaling; Altered circulating factors such as adipokines; Autophagy activation; Inflammatory response; Activation of mitogen-activated protein kinases; Altered PPAR

signaling; P53, PKC, PKB, FOXO signaling pathways. All above are the current paradigm of excessive lipid-induced cardiac toxicity.

Epigenetic Regulation in Gene Expression

The term of epigenetic is emerged in the 1990s, which refers to changes in chromosome without alteration in the DNA sequence. Epigenetic modifiers include 1) non-coding RNA such as microRNA (miR) and long non-coding RNA (lnc RNA), 2) histone modification such as acetylation, methylation etc. 3) DNA methylation and 4) chromatin remolding such as chromatin opening and closing. Epigenetic processes are essential for development and differentiation, but they can also arise in mature humans and mice, either by random change or under the influence of the environment[58]. Non-coding RNAs especially small RNAs are well- known as repressors in all known animal and plant genomes[59], which mediates translational blocking and mRNA degradation. Histone acetyl transferase and histone deacetylase were first described in 1996 with gene expression regulator functions[60, 61]; other than acetylation, histone lysine residue methylation status also mediates transcription activities. Histone lysine methylation can either be activating such as H3K4 methylation or repressive such as H3K9 tri-methylation. DNA methylation is initiated by DNA methyltransferases DNMT3A and DNMT3B, and are maintained by DNMT1[62], which will further recruit the methyl-CpG –binding proteins (MeCP2) and histone deacetylase complex and lead to histone deacetylation and gene silencing[63, 64]. This provides the first molecular evidence linking DNA methylation and histone deacetylation with transcription repression. Meanwhile,

Tamaru et.al also found that the methylation status of Histone 3 controls DNA methylation which further controls downstream gene expression[65], and indicating that the above 4 categories of epigenetic regulations are not functional parallel while they may form a network and regulate each other in different layers.

Above all the epigenetic modifiers, our group was first sequencing the micro-RNA in mouse heart that has been fed with high fat diet for 2 weeks for the purpose to investigate whether the changes in Micro RNAs affected by high fat diet will contribute to the development of cardiac lipotoxicity. Micro RNA are single-strand noncoding RNAs that function by binding to miRNA-inducing silencing complex (RISC) to generate the mature miRNA, which represses the expression of fully or partially complementary target mRNAs[66]. With micro-RNA sequencing indicating that miR-23a and miR-23b significantly upregulated and further verified by quantitative-PCR, and further investigation indicating that the 2 miRs are function as lipid sensor and later manipulate gene expression as epigenetic regulators.

Micro-RNA in High Fat Induced Cardiac Pathological Disorders

In recent years, microRNAs and epigenetics have emerged as important mechanistic factors that regulate many pathological disorders. A numbers of studies have shown that high fat diet in the long run is associated with cardiac contractile dysfunction and remolding due to the increase of toxic lipid intermediates[67-71]. **The significance of**

epigenetic factors in lipid accumulation-induced cardiomyopathy is undervalued; there is limited study that connects exceeding fat accumulation predisposes the heart towards cardiac hypertrophy with either micro RNA or histone modifier. Recently, microRNA has been broadly reported to be related to multiple disease development such as cancer[72-74], cardiovascular disease[75-79], nervous system disorder[80, 81], digestion disorder[82, 83], obesity[84, 85], etc. **Very few microRNAs have been identified that directly associate with lipid-induced cardiac toxicity[47-49]**, and the limited studies either used isolated cardiomyocyte or applied high fat diet to mice for 20 weeks. Therefore, in heart, the miRs early signature profiles in response to lipid accumulation *preceding* metabolic disorders remains largely unknown. Continual discovery of miRs in response to lipid stress is critically needed to fill the current knowledge gap.

miR-23 in Cardiac Hypertrophy and Heart Failure

Presently, very limited knowledge of the biological functions of miR-23a and miR-23b in the heart [86-88]. In humans, a lower myocardial expression level of miR-23a in heart failure patients was suggested to correlate with a better recovery of left ventricular function[89]. The elevated circulating miR-23a was detected in acute myocardial infarction as well as unstable angina pectoris patients[90], and its plasma levels were correlated with the degree of coronary stenosis[91].

In animals, mechanical stretch by TAC and activation of calcineurin can increase miR-23a and miR-23b expressions in the mouse heart[87, 88]. Our group confirmed the observation and showed that TAC led to a moderate (<1-fold) increase in the two miRs. We detected a 17- and 12-fold increase in miR-23a and miR-23b, respectively, in HFD mouse hearts, suggesting that HFD is a strong inducer of the expression of the two miRs. Furthermore, we found that overexpression of miR-23a and /or miR-23b in mice sensitized the animal heart to hemodynamic challenge, and the mice developed hypertrophy and heart failure, which indicates that the responsive upregulation of the two miRs is a detrimental factor causing exacerbated cardiac lipotoxicity in animals. Regarding miR-23 downstream targets, two factors were identified by others: muscle specific ring finger protein 1 (MuRF1, also called TRIM63) and forkhead box O3 (Foxo3)[86, 92]. The studies reported that the upregulation of miR-23a inhibited the expression of MuRF1 and Foxo3, leading to cardiac hypertrophy[86, 92]. Although, we did not observe any significant decreases of MuRF1 and Foxo3 expression in our HFD mouse model, indicating a possible different mechanism and target by miR-23 under lipid overload conditions.

Manipulation of microRNAs has been demonstrated as a promising and attractive therapeutic strategy[93, 94]. Since there is **no study about the biological functions of miR-23a and miR-23b in cardiac lipotoxicity**, our discovery is novel and has translational significance for the diagnosis and treatment of cardiac lipotoxicity. Both miR-23a and miR-23b can be unique and specific targets for microRNA-mediated therapy for cardiac

lipotoxicity. This concept is in line with one recent report showing a responsive elevation of miR-23b in the mouse liver after HFD[95].Meanwhile, the two miRs are highly sensitive to hyperlipidemia, and can serve as biomarkers for dyslipidemia-mediated organ dysfunction.

CHAPTER III

DECREASED EXPRESSION LEVEL OF SETD2 IS RESPONSIBLE FOR MIR-23A AND/OR MIR-23B-MEDIATED LIPOTOXIC CARDIOMYOPATHY

Introduction and Literature Review

Epigenetic Regulation in Cardiac Hypertrophy

Epigenetics indicates of a DNA sequence-independent genome functions regulated pattern. In recent years, many epigenetic markers have been discovered on nuclear histones which function as fine tuners for DNA exposure and read-out, among them including acetylation, methylation and phosphorylation[96]. Epigenetic mechanisms control chromatin structure through histone modifications and DNA methylation. The major modifications are acetylation and deacetylation on specific lysine residues of histone, which are operated by Histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation opens and activates chromatin, while deacetylation of histones and DNA methylation compact chromatin making it transcriptionally silent. Both can activate or repress gene transcription. New evidences shows that HDACs function in the regulation of lipid and other metabolic pathways will open new perspectives to the pathophysiology of metabolic disorders[97]. Environmental influence on Epigenetic modifications have produced a special interest in finding the cardiac hypertrophy origin. Within multiple epigenetic modifications, methylation of lysine residues on the histoneH3 and H4 N- terminal tails are responsible for controlling

gene expression[98-100]. Therefore, the balance of histone lysine residues methylation/demethylation is important for genomic integration and gene expression[101, 102]. Both the localization and the degree of methylation (mono-, di-, or tri-methylation) of the histone lysine residues affect differential gene expression[102-104]. Evidence suggests that loss of regulation of these lysine methylation properties could lead to promoting congenital heart diseases and cardiac hypertrophy in adulthood[105, 106]. Different from the established role of histone modifiers and HDACs in heart failure transcriptional regulation[107, 108], relatively little is known about DNA methylation in cardiac hypertrophy. Recent data suggested DNA methylation can respond to external stimuli and correlates with the cardiomyocyte functional state in cardiovascular disease[109, 110]. Stenzig et al. show beneficial effects of non-nucleosidic DNMT inhibitors on cardiomyocyte contractile function[111]. Thus, in contrast to the above-described epigenetic mechanisms that modulate gene expression at the transcription level, miRNAs control gene expression at the post-transcriptional level. Each miRNA might regulate the expression of hundreds of target mRNAs. miRNA genes are organized within the genome in the host (protein-coding or non-coding) genes introns of or in host exons, as well as intergenic regions as independent genes[112]. miRNAs interact with RNA-induced silencing complex (RISC). RISCs bind to the 3′- UTR (untranslated region) of specific mRNA targets. Either translation is blocked or the mRNAs are degraded[113]. A shift toward a fetal miRNA transcription pattern is responsible for part of the cardiac proteome modifications during hypertrophy and failure[113]. Genome-wide association study has shed light on the miRs expression pattern

change in hypertrophy heart, while the mechanisms under those miRs changes are still largely unknown.

Study of Micro-RNA in Heart

The biological and pathological functions of microRNAs have been widely recognized in many organs including the heart. Intensive studies have focused on the essential role of microRNAs in heart development and different cardiac diseases[45-47, 114]. A number of studies have indicated miRNA expression signatures of human hearts[115-117] and mouse hearts[87, 88, 118-120] and have demonstrated miRNA arrays that are mis-expressed, either positively and/or negatively, in cardiac disease. Meanwhile, the researchers have found that a shift toward a fetal miRNA transcriptome is responsible for at least part of the cardiac proteome modifications occurring with cardiac hypertrophy and heart failure. Genome-wide miRNA expression profiling study provided evidence that miRNAs signatures of three human heart pathologies— dilated cardiomyopathy, ischemic cardiomyopathy and aortic stenosis— are distinct; 43 out of 87 examined miRNAs are differentially expressed in at least one of these disease groups[115]. A following study also demonstrated commonalities and differences in 33 miRNAs expression in ischemic cardiomyopathy and agnogenic dilated cardiomyopathy[117].

The factors that affect cardiac miRNA transcription are still incompletely understood, but it could be conjectured that different miRNA expressions could be clinically important for many cardiovascular pathologies if used for diagnostic and/or prognostic

purposes[115]. Applied with combination of mRNA profiling, miRNA transcriptome was indicated to be a delicately perceptive biomarker of severe heart failure[121].

Except for pathogenesis and etiology, the progression of disease is another significant aspect of cardiovascular disease; more than half of patients with cardiomyopathy progress unyieldingly towards heart failure and death. While the causes of such progression are not well investigated, the miRNA transcriptomes study could uncover major features of this process. For sure, studies using mouse models are providing evidence that the miRNA is dynamically regulated, not only in the process of ageing[120] but also in the progression of myocardial infarction, hypertrophy and heart failure[88, 118-120, 122]. Differences between the miRNA transcriptome in hypertrophy with that in heart failure might have been partly documented in patient samples[115].

During disease progression, the miRNA transcriptome alterations could be reversible at certain points: Matkovitch et.al have provided evidence that upon biomechanical support treatment, miRNA transcription level in heart-failure samples goes back to normal, even if this was not reflected by mRNA profile normalization[121]. Therefore, it seems that miRNAs are more sensitive to acute alterations in pathophysiological status than mRNA [121]. Some miRNAs have been found more commonly dysregulated than others in heart disease patients, including miR-133, miR-1, miR-30 and miR-150 are often downregulated;—and miR-23a, miR-125, miR-195, miR-21, miR-199 and miR-214 are often upregulated. Although grouping data from different diseases, models, stages and

analysis methods should be performed cautiously, it might be speculated that some miRNAs represent 'core mediator' miRNAs that have central causative roles in the pathogenesis of heart disorders. Some of these miRNAs have been tested in model systems to see whether or not their misexpression is sufficient to produce an effect on phenotype (usually heart size). For example, miR-23a, miR-23b, miR-24, miR-195 and miR-214 (found upregulated with disease) provoked increased cell size when transfected in neonatal cardiac myocytes, and miR-195 was sufficient to provoke pathological cardiac growth and dilated cardiomyopathy when overexpressed in transgenic mice[87]. Similarly, inhibition of miR-133, an miRNA found downregulated in human hypertrophic cardiomyopathy, was sufficient to induce hypertrophy in wild-type mice[123]. Databases are being developed to provide comprehensive lists of the dysregulated miRNAs found in various diseases and models, including those of the heart [123].

miR-133, together with miR-1, is particularly interesting as a 'key' miRNA for hypertrophy, whose expression level has been found reduced in animal models of hypertrophy induced either by pathologic (surgically-induced pressure overload) or physiologic (Akt overexpression or exercise) stresses[124]. Although it might not be surprising that both miR-1 and miR-133, as they are central to cardiac biology, are found altered with hypertrophy regardless of its underlying cause, it does raise question of whether and how miRNA dysregulation differs between heart growth induced by pathological and physiological means. It might be speculated that miRNAs that are mis-expressed differentially upon pathologic hypertrophy, when compared with physiologic

hypertrophy, have a causative role in the cardiac dysfunction associated with unfavorable cardiac remodeling. Future studies should focus on testing such hypotheses. Meanwhile, investigating the miRNA profiles differences between inherited cardiac diseases and the acquired forms with similarities might also reveal specific miRNAs that have a causative role in disease development, which might be used as diagnostic or therapeutic agents in pre-clinic medicine.

The target of miRNAs involved in the development of different diseases, such as the two muscle-specific miRNAs, miR-1 and miR-133, target at least two transcription factors: Hand2 and SRF during cardiogenesis[125]. Hand2 and SRF further cooperate in the re-induction of an embryonic pattern of protein expression[126], that provoke heart growth through hypertrophy. As well, miR-1 target at RASA1, Cdk9, and Rheb, which involved in cell growth, and fibronectin, which is an extracellular matrix protein[88]. Also, miR-1 has been reported to target at calmodulin and Mef2a that are Ca^{2+}-signaling pathway components; when miR-1be repressed with disease, calmodulin and Mef2a were boosted and resulted in stimulation of prohypertrophic genes[127].miR-133a-1/2 double knockout mice show thin-walled hearts and septal defects, and mice surviving to adulthood presented dilated cardiomyopathy[128]. miR-133 has been reported as targeting small GTPase RhoA and Cdc42, also WHSC2, which are involve in cardiac genesis and induces fetal gene program in cardiomyocytes[124]. The above 2 studies documented that miR-133 has a key role in myocardial development, hypertrophy and cardiac function,

while the complexity of miR-133 regulatory mechanism is still underlined, which needs to be further elucidated.

The miR-29 family of miRNAs, in particular, has been linked to fibrosis[122], which is the major feature of abnormal myocardial. A large number of extracellular-matrix-related genes were found to be targets of miR-29, including ELN, FBN1, COL1A1, COL1A2 and COL3A1. miR-29 is downregulated by TGF-β in myofibroblasts, which indicate that miR-29 is a negative regulator of matrix-related genes, and its repression is involved in the increased collagen deposition during pathological remodeling and progression to heart failure. miR-30c and miR-133 which are highly expressed in the heart have also been reported as being implicated in cardiac fibrosis[129] and target at CTGF, which becomes upregulated with heart disease and contributes to myocardial remodeling through increased collagen deposition. Another study documented that miR-21is upregulated in heart failure cardiac fibroblast and increasing MAPK signaling by repressing SPRY1, which might be responsible for fibroblast survival that is independent from cardiomyocyte loss[130]. miR-21 has also been reported as being implicated in cardiomyocyte death whose expression is upregulated in cardiac myocytes in response to H_2O_2 treatment, and protecting against H_2O_2-mediated cell death though targeting at PDCD4[131]. Together with miR-1 and miR-24, miR-21 is upregulated after ischemic preconditioning, functioning as cardiac a protector by upregulating eNOS, HSP70, and HSF1[132]. Other than being involved in the hypertrophic progress, miR-1and miR-133 have been reported to be involved in the regulation of cell death[133]. Overexpression

of miR-1 provokes apoptotic cell death in H2c9 cells, whereas miR-133 protects against H_2O_2-induced apoptosis[133] through targets HSP60 and HSP70 for miR-1, and CASP9 for miR-133. Meanwhile, miR-1 is boosted in ischemic cardiomyopathy and myocardium with coronary artery disease, which might contribute to the cardiac apoptosis under these conditions.

Under pathological conditions, increased myocardial mass might not be paired with appropriate angiogenesis that is associated with normal growth patterns, as pointed out two decades ago[134]. miR-130a, miR-17-92 cluster and miR-378 target MEOX2 and/or HOXA5, THBS1 and/or CTGF, and Sufu[135, 136] have been identified as proangiogenic miRNAs. Meanwhile, miR-221 and miR-222 by targeting *c-kit* and regulating eNOS indirectly inhibit endothelial cell migration, proliferation and angiogenesis[137]. Also numbers of miRNAs with predicted angiogenic related targets are upregulated in rat myocardial endothelial cells with type 2 diabetes and lead to impaired angiogenesis such as miR-320 target at Flk1, VEGFc, IGF-1, IGF-1R and FGF, documented as important angiogenic miR; miR-126, has been shown to negatively regulate VEGF in endothelial cells after myocardial infarction through SPRED1 and PIK3R2[138, 139].

One of the well-studied miRNAs linked to cardiac pathology is miR-208, which is encoded within an α-MHC gene intron and controls the β-MHC expression in response to cardiac stress[140]. This miRNA has been found upregulated with hypertrophy and heart

failure. miR-208-null mice that underwent pressure overload or pathological remodeling did not experience that hypertrophic or fibrotic processes that respond to stress. THRAP1, co-regulator of the thyroid hormone receptor was identified as miR-208 target, which is upregulated in the absence of miR-208, results in increased protein actions on the hormone response elements which will consequently repressed β-MHC promoter. Thus, miR-208 postnatally regulates α-MHC expression as an on–off switch[116, 119].

Although, the early signature profiles of miRs in response to lipid accumulation in the heart preceding metabolic disorders or cardiac remodeling (hypertrophy) remains largely unknown. Continual discovery of critical miRs in response to excessive lipid stress is crucially needed to uncover the initial signaling that triggers the detrimental cascades into disease; and will also close the current knowledge gap in lipotoxic cardiomyopathy studies. This study identified that miR-23a and miR-23b are highly sensitive to hyperlipidemia, and both are excellent candidates as initiating factors, which might fill a knowledge gap within the field.

Histone Modifier in Response to High Fat Treatment

Histone modifiers include histone methyltransferase, demethylases, acetyltransferases, deacetylases etc. The combination of those modifiers change the lysine residue on histone and affect gene transcription activity[141]. Limited studies have reported a close and systematic relationship between high fat diet and histone modifications in multiple

organs. Currently, the relationship between histone modification and lipotoxic cardiomyopathy is largely unknown. Recent study by Sun et.al has indicated that high fat diet causes HDAC3 downregulation in hepatocytes that strongly induce expression of lipid-sequestering LDs-coating protein Perilipin 2 and leads to insulin hypersensitive and impaired glucose production, further causing severe hepatosteatosis and, notably increased insulin sensitivity without changes in body weight or insulin signaling [142]. Another group has reported that 6-month-old male offspring from maternal high fat diet has a significant suppressed expression of Sirt-1 and HDAC1 in Hypothalamic Arcuate Nucleus that may contribute to hyperphagia and obesity in HF male offspring[143]. The third group found that excision of *Hdac3* in heart and muscle later in development caused a severe hypertrophic cardiomyopathy and heart failure when adding on high fat diet for a few weeks, due to down-regulation of myocardial mitochondrial bioenergetic genes, specifically those involved in lipid metabolism[144]. In one recent cell culture study, palmitate treatment induced significant changes in H3 methylation in β cells[145]. Whether these histone modifications caused β cell dysfunction or the consequences of the β cell death resulted in epigenetic changes remains unknown[145].

None of the above 3 studies elucidate that how histone modifiers expression changes due to response to high fat treatment or their downstream targets that involved in the molecular mechanism of disease development in metabolism related organs or even cardiovascular system. It is of great interest and importance to understand the biology of histone modifier changes under high fat diet regulation and how histone modifiers

function as mediators in cardiovascular disease development in response to environmental stimuli.

Materials and Methods

Cell Culture and Palmitic Acid Treatment

Human embryonic kidney epithelial cell line 293T (HEK 293T) was obtained from ATCC. Cells were cultured in DMEM with 10% FBS.

Palmitic acid (P0500 Sigma Aldrich) was dissolved in DMSO and added into culture medium at final concentration 1μM. For the control group, the same amount of DMSO were added.

Gene Transient Transfection

All gene transient transfections were performed using the NEON transfection system (MPK5000; Life Technologies). 293T cells were suspended by the density of 5*10^7 cells/ml, electroporation parameter was 1100 voltage, 20ms pulse width, 2 pulses, the efficiency of transfection is more than 80% under this condition.

Cells were immediately seeded after the electroporation transfection.

High fat diet feeding and Palmitic Acid Treatment

The Institutional Animal Care and Use Committee (IACUC) of Texas A&M University Health Science Center Institute of Biosciences and Technology approved the experiments with animals.

Male mice with C57BL/6 background, at the age of 10 weeks, are fed with high fat diet (fat provide 60% Kcal, Research Diet, D12492). After feeding for 2 weeks, echocardiography was performed to record the cardiac function.

Cardiac Function Assessment by Echocardiography

For cardiovascular measurements, a Vevo770 High-Resolution Micro-Imaging System (Visual Sonics, Toronto, ON, Canada) with 30-MHz probe (RMV-707B) was used.

Male mice in each group, were anesthetized with 3–4% isoflurane, and then switched to 1–1.5% isoflurane mixed with 100% oxygen. At the level of the papillary muscles, 2-dimensional guided M-mode echocardiography was obtained from anterior (+septum) and posterior walls.

Generation of Cardiac Muscle-Specific Setd2 Knockout Mice and Setd2 Overexpression Transgenic Mouse Lines

To avoid the constitutive Setd2 knockout result in early embryonic lethality, $Setd2^{flox/flox}$ mice were bred with α-MHC-Cre mice to generate $Setd2^{f/f,\ \alpha\text{-MHC-Cre}}$ mice, respectively.

Human c-terminal Setd2 cDNA was subcloned into the α-myosin heavy chain (α-MHC) promoter expression vector for pronuclear microinjection with a C57/B6 background.

Genomic DNA isolated from the tails of the mice was used for genotyping purposes. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Texas A&M University Health Science Center-Houston.

Chromatin Immunoprecipitation (ChIP) Assay

To define the interaction of Setd2 with its histone dependent targets in vivo in the development of cardiac hypertrophy, a ChIP assay was performed.

Hearts from 10- week-old $Setd2^{f/f,\ \alpha\text{-MHC-Cre}}$ and $Setd2^{f/f}$ mice were digested into single cardiomyocyte suspension and fixed with formaldehyde and lysed by sonication.

The specific Histone3 lysine36 tri-methylation antibody anti-H3K36me3(Abcam, ab-9050, Cambridge, MA) was used to pull down-DNA fragment.

Transverse Aortic Constriction

Mice that have been fed with 2 weeks of high fat diet (60% KCal) or normal chow are anesthetized with 3% isoflurane with 100% oxygen. The thymus was retracted to expose the transverse aorta. Between the right innominate and left carotid artery, an aortic constriction was achieved by tying a 6-0 suture against a 3 mm length of 27 gauge

needle. After two knots, the 27 gauge needle was promptly removed, which yielded a constriction of ~0.3 mm as the outer diameter of the 27 gauge needle. The procedure imposed a 60–80% aortic constriction on the animal. To minimize the variation of the constriction among the animals, only the mice with a ratio of right-to-left carotid artery flow velocity from 5 : 1 to 10 : 1 were used. The artery flow velocity was measured by a Pulsed Doppler (Indus Instruments, Houston, TX, USA). As a control, a sham operation without occlusion was performed on age-matched littermate mice.

Hematoxylin and Eosin Staining, Immunostaining and Immunoblotting

Mouse hearts were perfused and fixed by Langendorff Perfusion System (Radnoti, 120108). The whole heart paraffin sections were applied to Hematoxylin and Eosin Staining (51275, Fluka; HT110132-1L, Sigma-Aldrich) and images were captured under 1.25X magnify with Leica DM2000 histology microscope.

Protein samples for Western blot analysis were extracted and separated as described previously[146], and the immunoblotting densitometry was quantified by Gel Logic 6000 PRO Imaging System (Carestream Health, Inc.). Antibodies were from the following sources: anti-setd2(Cell Signaling Technology,#23486, Danvers, MA), anti-H3K36me3(Abcam, ab-9050, Cambridge, MA), anti-GAPDH (Santa Cruz, sc-20357, Dallas, TX), anti-β-actin(Santa Cruz, sc-8432, Dallas, TX), anti-lamin B1(Santa Cruz, sc-20682, Dallas ,TX).Equal protein loading was verified by the intensity of the GAPDH blot (Santa Cruz, sc-20357, TX, USA).

Paraffin sections of the whole heart were stained for wheat germ agglutinin (WGA) (Vector Laboratories, Burlingame, CA), pictures were taken under the 40x microscope objective. A total of 20 staining pictures from each group were quantified by Leica Application Suite Imaging Software (Version 4.0, Germany). Nuclei were visualized by DAPI staining. The images were acquired by fluorescence microscopy.

Wheat Germ Agglutinin Staining and Quantification

Paraffin sections of the whole heart were used for Wheat Germ Agglutinin (WGA) Staining. The tissue sessions were deparaffinized and incubated with Wheat Germ Agglutinin (Vector Laboratories, Burlingame, CA) at approximately 20 µg/ml in PBS to the sections and incubated for 30 minutes at room temperature. Washed with TPBS (PBS + 0.05% Tween™ 20) 3 times. Nuclei were visualized by 1 µg/ml DAPI staining for 5 min. Images were captured under the 40x microscope objective with Leica DM2000 histology microscope. One image was from the top of the interventricular septum and four images were from the left ventricle wall. A total of 20 staining pictures from each mouse were quantified by Leica Application Suite Imaging Software (Version 4.0, Germany), single cardiomyocyte size was calculated based on a cross-section area marked by the staining.

RNA Sequencing Assay

Total RNA was isolated from the hearts of 10-week-old mice using miRNeasy kit (QIAGEN, Cat No./ID: 217004), and 3μg was used for RNA-seq. The assessment was performed using the Qiagen RT2 Profile PCR Array.

Data analyses were performed with R (www.r-project.org), a publicly available statistical tool for data analysis. Signaling pathway analysis was performed with Ingenuity (www.ingenuity.com).

Dual-Luciferase Activity Assay for Measurement of Setd2 3'-UTR Activity

Dual-luciferase assay was performed as described in our earlier study[147], the 1kb region of Setd2 3'UTR was subcloned into a pGL3-basic vector expressing the luciferase reporter gene (Promega, Madison, WI, USA). A similar vector with the miR-23a and miR-23b binding site mutant was constructed as a mutant reporter. 293-T cells were seeded in triplicates in a 12-well plate after transit transfection using Neon system. The cells in each group were then transfected with 1 ug of Setd2 3'-UTR firefly reporter plasmid and 20 ng of Renilla reporter plasmid together with 1ug of miR-23 expression vector or control vector. The cells were harvested 24 hour later. Dual-luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). The Renilla luciferase activity and the protein concentration were used for the normalization purpose.

RNA Isolation and qRT-PCR (Quantitative Polymerase Chain Reaction) Analysis

Isolation of total RNA from mouse heart was performed using TRIzol reagent (Life Technologies). cDNAs were synthesized from 1 μg total RNA with the qScript One-Step RT-qPCR kit (Quanta Biosciences). Quantitative RT-PCR was performed using SYBR Green PCR Master Mix (Life Technologies) in the StepOnePlus Real-Time PCR System according to the manufacturer's instructions.

Transcripts were quantified by quantitative PCR (qPCR) analysis. Total RNA was prepared by miRNeasy kit (QIAGEN, Cat No./ID: 217004); 0.5ug RNA was used for reverse transcription(QUANTA, 95048).

The forward and reverse PCR primers (5′ to 3′) were as follows:

Setd2 (mouse): TACTTGGGAAAGCCCAGGAG/ GCTTTTCTTTGCCAGCTCAC,

Setd2 (human): AACTGGTTCCTTCAGGCTCA/ CTTCATTTTCCCTCCTGCTG,

GAPDH (mouse): GAGTCAACGGATTTGGTCGT/TTGATTTTGGAGGGATCTCG,

GAPDH(human): CATGGCCTTCCGTGTTCCTA/CCTGCTTCACCACCTTCTTGAT,

GAPDH expression levels were used for qPCR normalization.

Expression levels were determined by the $2^{-\Delta\Delta Ct}$ threshold cycle method.

Statistical analysis

Data are presented as means ± s.d. Unpaired, two-tailed student's *t* test was used for two group comparisons.

One-way ANOVA followed by Student-Newman-Keuls test was used for multiple group comparisons.

All statistical analyses were performed in software SigmaPlot 11.0 (Systat, San Jose, CA, USA). The P value < 0.05 was considered as significant difference.

Results of This Part

Setd2 is Downregulated in High Fat Diet Fed Two Weeks Mouse Heart

Non-bias RNA–sequencing data provide preliminary evidence that after 2-week high fat diet, over 100 gene expression change in mouse heart. With bioinformatic analysis to narrow down targets, in those genes, Setd2, which encoded histone methyltransferase that specific targets at tri-methylate histone lysine 36, was the only histone modifier that significantly downregulated. Further verification by quantitative-PCR and immunoblotting, indicated that Setd2 in hearts of mice that fed with high fat diet for 2 weeks decreased to 40% compared with mice that fed with normal diet (Fig.8).

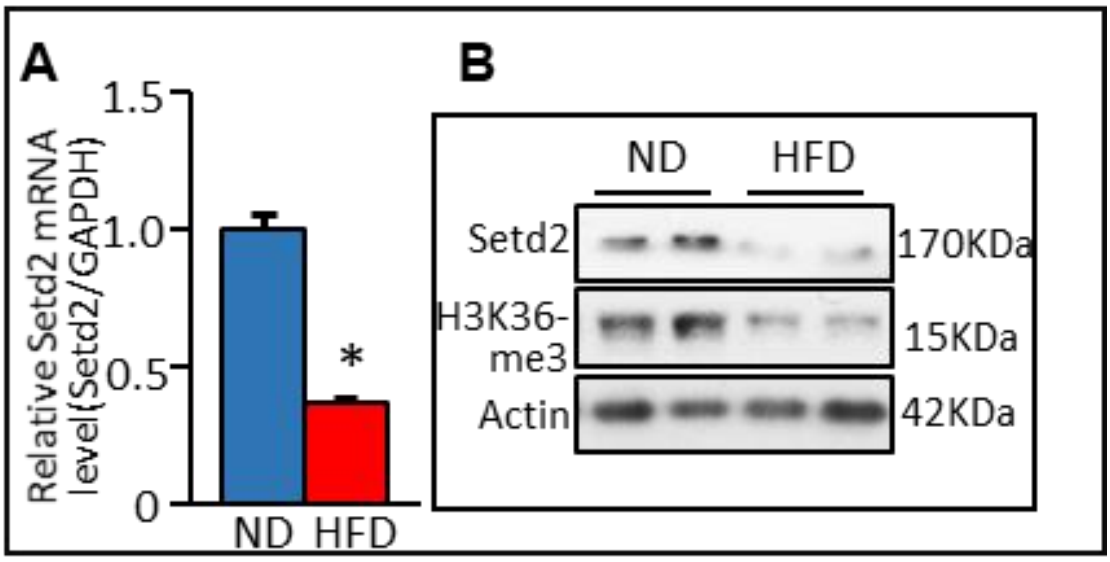

Figure. 8. Short-term (2-week) HFD is a detrimental factor that exacerbates cardiac dysfunction and hypertrophy by downregulation of Setd2. (A). QPCR shows a significant decrease in Set2 transcript in 2-week HFD heart which further verified RNA-Sequencing data. (B). An attenuated Set2 and H3K36me3 level is detected in HFD fed 2 weeks mouse heart by Western blot. * $P<0.05$, n=5.

miR-23a and miR-23b Negatively Regulate Setd2

With bioinformatic analysis, miR-23a and miR-23b have both been found to target at the 3'-UTR of SET domain containing 2 (Setd2), a Histone 3 lysine 36 tri-methyltransferase with the most significant change in RNA-sequencing result. And the binding site for these 2 miRs on Setd2 3'UTR is highly conserved across species (Fig.9a). Luciferase activity assay shows that forced expression of miR-23a and miR-23b can repress the luciferase activity of vector that containing Setd2 3'UTR, while when the binding site is mutated, the repression effect caused by miR-23a and miR-23b is blunted (Fig.9b).

To determine whether miR-23a and miR-23b could silence Setd2 expression, forced expression of miR-23a, miR-23b or both of the miRs in 293T cell were achieved. Setd2 mRNA was repressed 89% by miR-23a and 85% by miR-23b, 92% by miR-23a and 23b together (Fig.10a). Western blot from the cohort cell lysis shows the same trend. While antagomiR can reverse the trend as shown (Fig.10b), which further verified that Setd2 is directly negatively regulated by miR-23a and miR-23b.

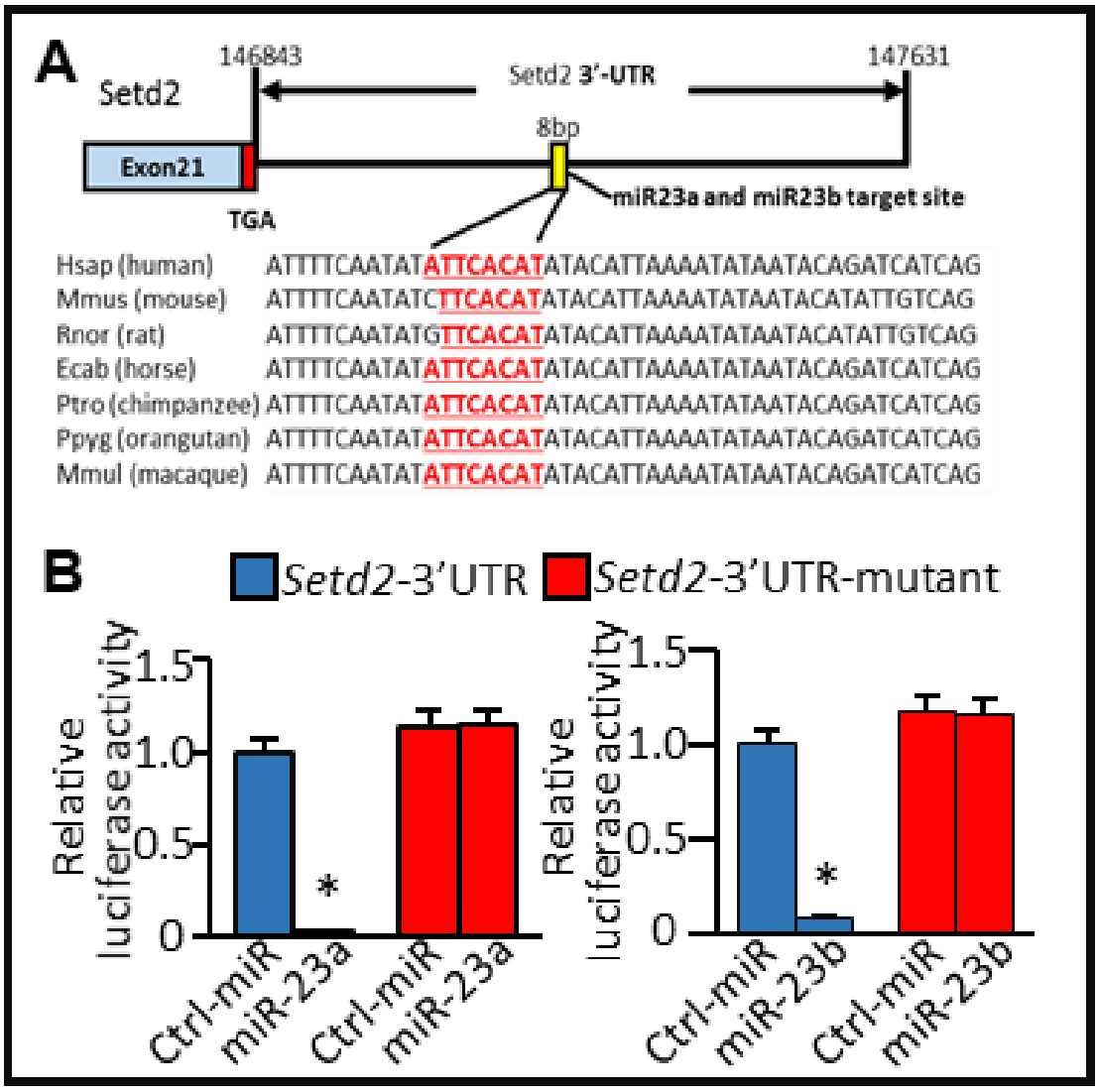

Figure. 9. miR-23 bind Setd2 3'UTR and repress its expression. (A). Prediction of one putative targeting site (red) of miR-23 in the 3'-UTR of Setd2, and the comparison of targeting site sequence among different species. (B). miR-23a and miR-23b represses Setd2 3'-UTR reporter gene luciferase activity. Mutation of miR-23 putative target site blocks the repressive effect of miR-23 on the target. * $P<0.05$.

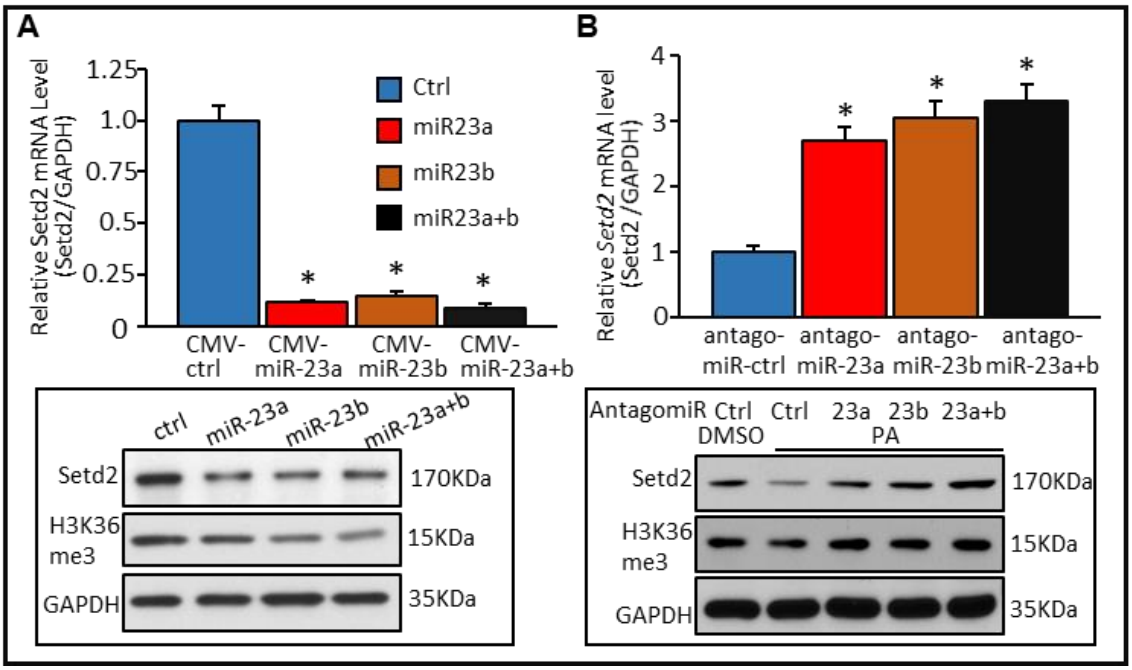

Figure. 10. miR-23 repress Setd2 expression in vitro. (A). Forced expression of miR-23 in 293 T cell by transit transfection represses Setd2 in both mRNA level and protein level, and its downstream target H3K36me3, as well. (B). Blunt miR-23 is achieved by antagomiR, and re-boost Setd2 expression and H3K36me3 separately and synergistically. * P<0.05, n=5.

Setd2 Downregulation is Responsible for the Predisposed Cardiac Hypertrophy Induced by TAC in High Fat Diet Fed Mice

Based on the in vitro results, our group tried to investigate whether high fat induced cardiac lipotoxicity by repressing Setd2 through miR-23a and miR-23b upregulation in mouse model. In the cohort heart that been injected with AAV DJ/8:miR-23a or AAV DJ/8:miR-23b, or both, Setd2 mRNA level was found repressed in high fat diet fed 2

weeks mice heart (Fig 11a). Setd2 and its downstream target H3K36me3 protein level were significantly downregulated after 2 weeks of injection (Fig 11b). In contrast, antagomiR partially blunted miR-23's repression on Setd2 and its downstream target H3K36me3 in mouse heart after 2 weeks of injection. (Fig.11 c&d).

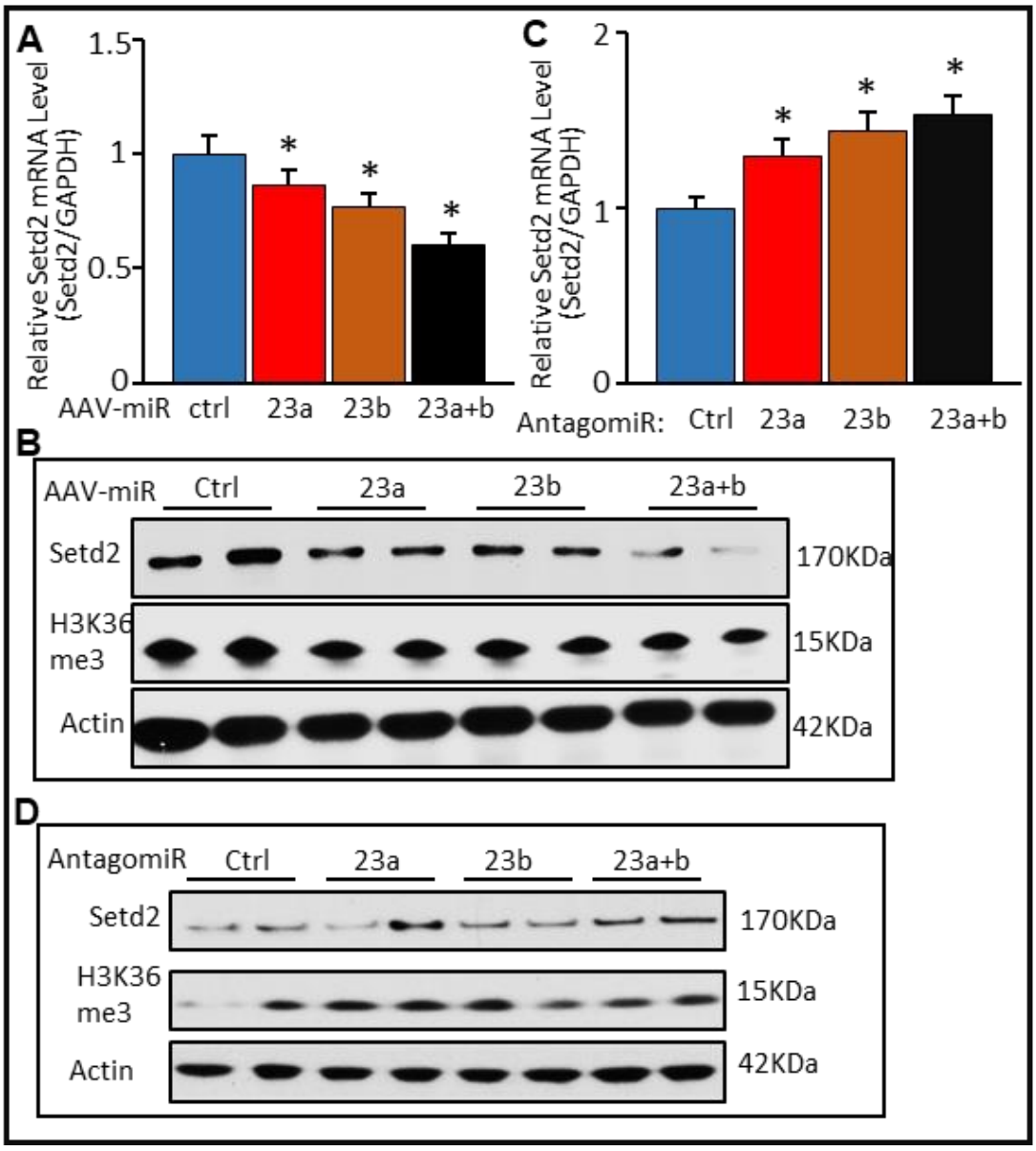

Figure. 11. miR-23 represses Setd2 expression in vivo (A). Overexpression of miR-23 in mouse cardiomyocyte archived by adeno-associate virus repress Setd2 expression level. (B). Attenuated Set2 and H3K36me3 expression by forced expression of miR-23 separately or synergistically in cardiomyocyte. (C&D). AntagomiR treatment for reboost Setd2 mRNA and protein level and H3K36me3 in mouse heart. * $P<0.05$, n=5

Setd2 Knockout Indicate Multiple Cardiac Diseases Related Genes Expression Alteration

Further correlation between loss of Setd2 and cardiac hypertrophy was indicated by a deep sequencing result performed on Setd2 cardiac specific knockout (MHC-Setd2$^{-/-}$) mouse heart. RNA-seq shows multiple sets of genes with significant expression changes are related to cardiac hypertrophy, such as growth factor activity and binding; anion channel activity, heart contraction, hypoxia adaption, etc.. Deep sequencing data prove the concept that loss of Setd2 is strongly related to cardiac hypertrophy and provides further study direction (Fig.12.).

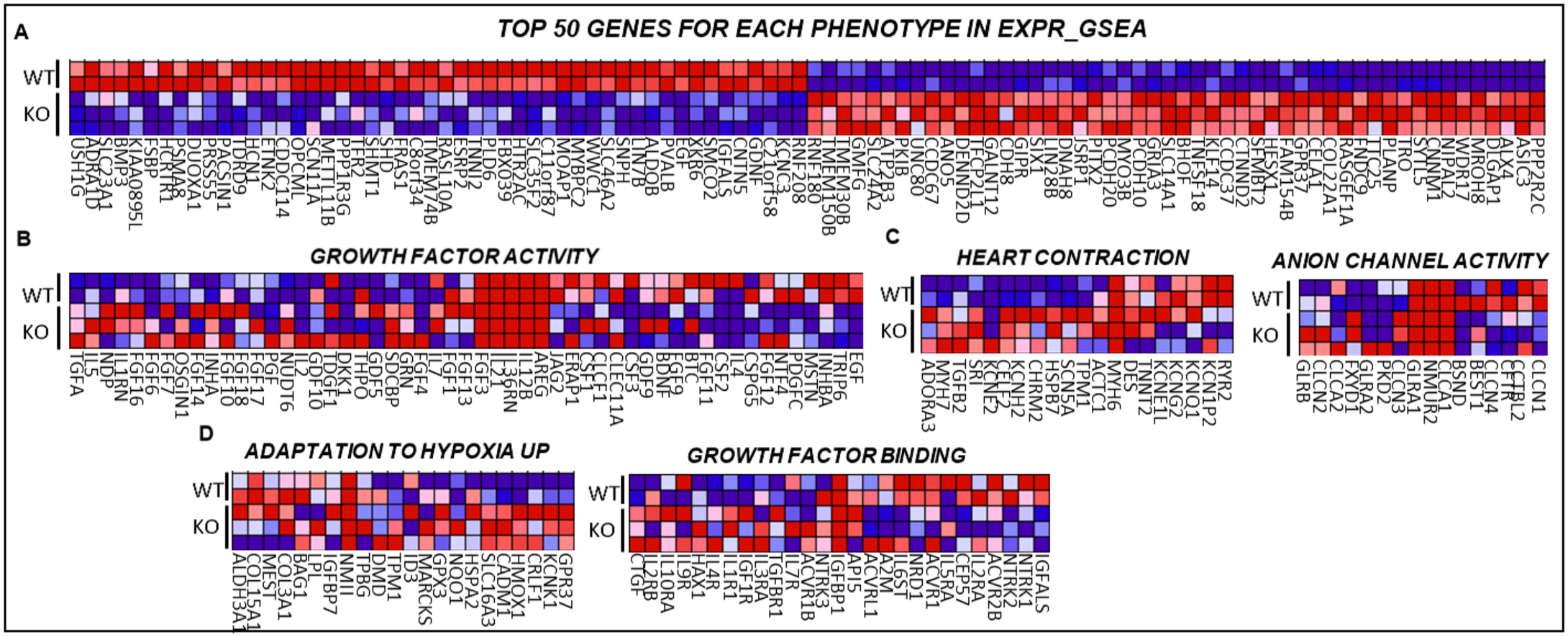

Figure. 12. Cardiac disease-related gene expression profile in Setd2 knockout heart. (A). Top 50 genes with statically significant changes. (B). Genes related to growth factor activity. (C). Genes related to heart contraction and anion channel activity. (D). Genes related to hypoxia adaption and growth factor binding.

Setd2$^{F/+}$, α-MHC-Cre Mice are Predisposed to TAC; Setd2 Cardiac Specific Transgenic Mice Partially Rescue the Cardiac Hypertrophy in Response to High Fat Diet and TAC

To explore the biological significance of the downregulation of Setd2 by miR-23a and miR-23b and the consequence of decreased H3K36 tri-methylation in the high fat diet fed heart, genetic-loss and –gain of Setd2 mouse lines were generated, the cardiac-specific Setd2-haploinsufficient (MHC-Setd2$^{+/-}$) mice (Fig.13), and the cardiac-specific Setd2 overexpression transgenic(Tg-MHC-Setd2) mice (Fig.14). By taking advantage of these genetic Setd2 mouse models, cardiac-specific Setd2-haploinsufficient (MHC-Setd2$^{+/-}$) mice that mimic the Setd2 downregulation in mouse heart after fed with high fat diet for 2 weeks, 3 weeks of TAC can induce severe cardiac dysfunction and hypertrophy in MHC-Setd2+/- mice compared with wild type littlemates (Fig.13) . While the cardiac-specific Setd2 overexpression can partially rescue the phenotype, shown as preserved cardiac function and less severe cardiac hypertrophy (Fig.14).

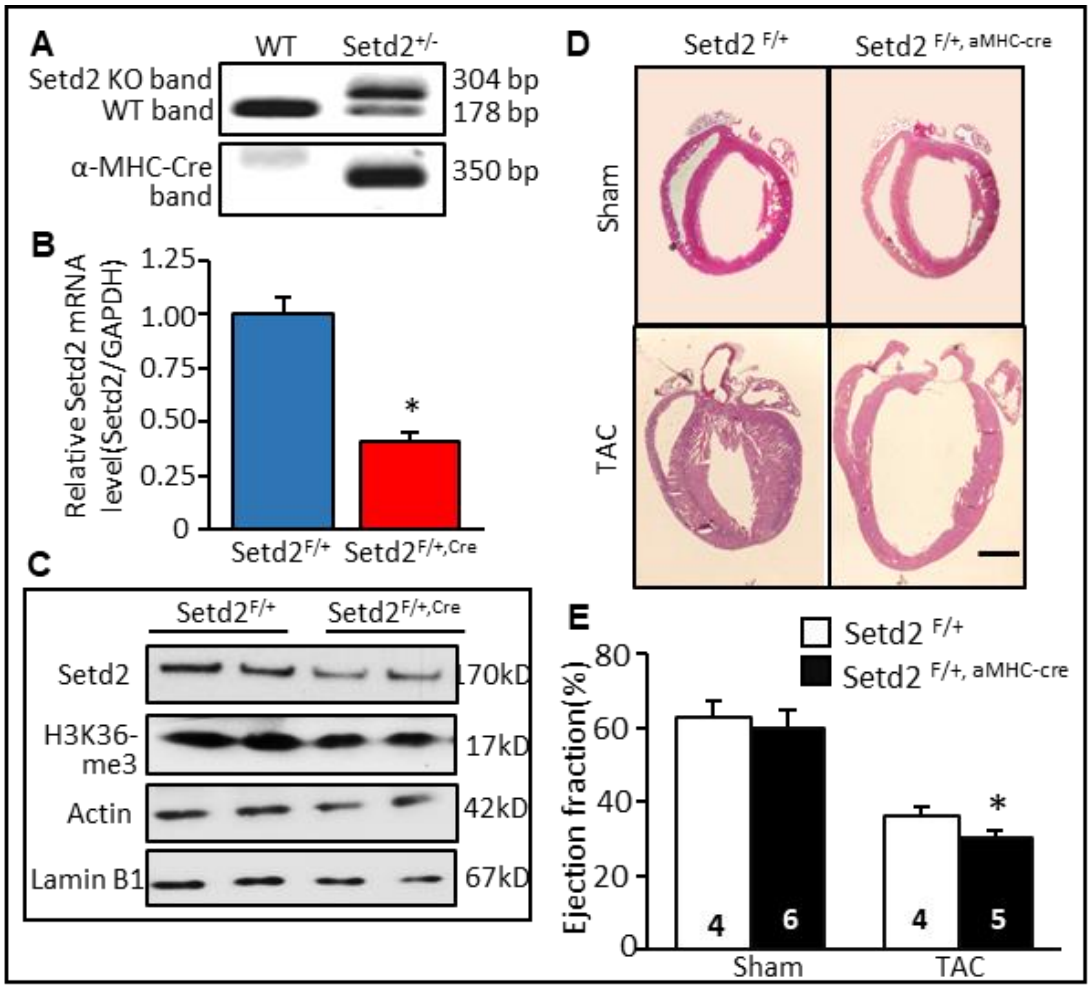

Figure. 13. Setd2$^{F/+,}$ a-MHC-cre mice are predisposed to TAC. (A). Generation of cardiac-specific Setd2 knockdown mice. (B). Setd2 mRNA level was quantified by qPCR, normalized with GAPDH. (C). Setd2 protein level was verified. The decreased H3K36me3 level is detected in the Setd2 haploinsufficient heart by immunoblot. (D). Setd2$^{F/+,}$ a-MHC-cre mice are predisposed to TAC and developed dilated cardiomyopathy. (G). Echocardiography showing Setd2$^{F/+,}$ a-MHC-cre mice has a larger decrease in cardiac function than its control, and developed heart failure by the end of 3 weeks of TAC.. * $P<0.05$. n=5

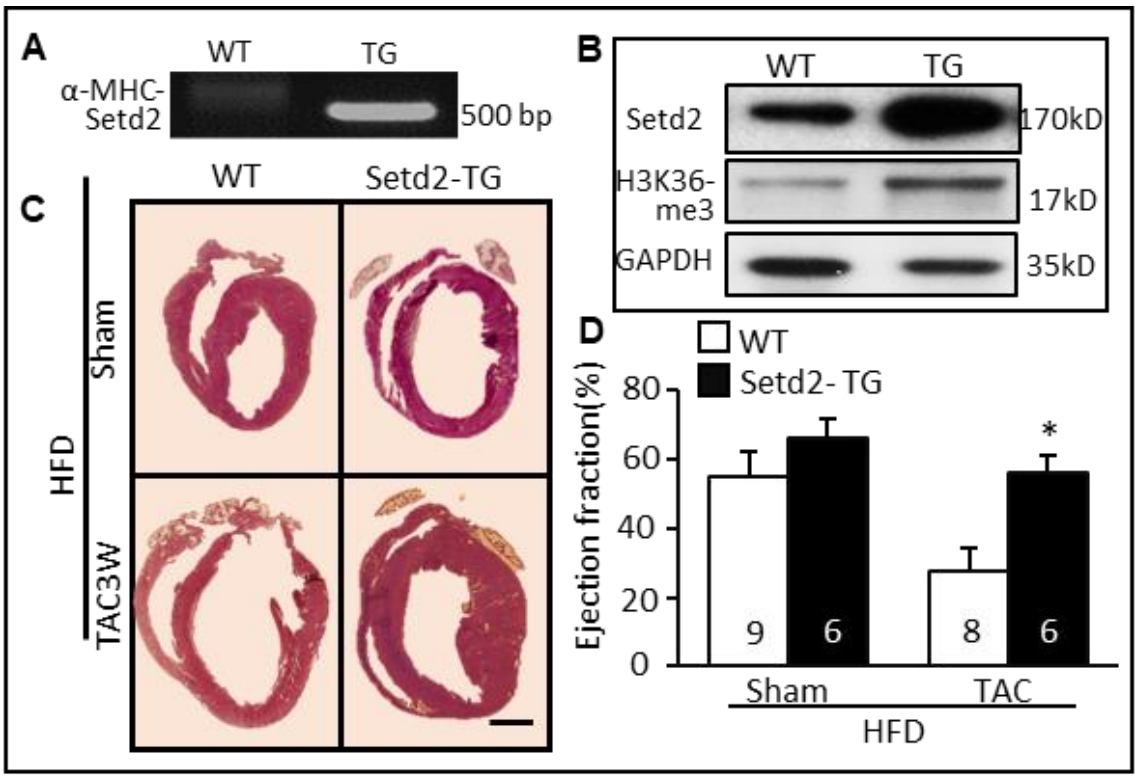

Figure. 14. Setd2 cardiac-specific transgenic mice (Setd2-TG) partially rescue cardiac hypertrophy and heart failure in response to high fat diet and TAC (A). Generation of Setd2 cardiac-specific overexpression mice; the inserted sequence was detected by PCR. (B). Setd2 overexpression was verified by immunoblot, and its downstream target H3K36me3 as well. Setd2-TG mice are predisposed to TAC and developed dilated cardiomyopathy. (C). Forced Setd2 overexpression in cardiomyocyte can partially rescue the development of dilated cardiomyopathy after high fat diet and 3 week of TAC. (D). Setd2-TG mice showed preserved cardiac function when challenged with high fat diet followed by TAC. * $P<0.05$.

Discussion of This Part

Histone Modification in Lipotoxic Cardiomyopathy

Histone modification is an essential part of epigenetic regulation. Methylation and acetylation of lysine residues in the amino termini of histones 3 and 4 (H3 and H4, respectively) are typical examples of histone modifications, by which genetic accessibility can be altered in response to environmental stimuli.

Currently, **the relationship between histone modification and lipotoxic cardiomyopathy is largely unknown**. In one recent cell culture study, palmitate treatment induced significant changes in H3 methylation in β cells[145]. However, whether these histone modifications caused β cells dysfunction or the consequences of the β cell death resulted in the epigenetic changes remains unknown[145].

Furthermore, a number of important questions remain unanswered: what is the signature pattern of HFD-induced histone modifications; and whether the changes of the histone modifications contribute to myocardial lipotoxicity.

Our preliminary data showed a 64% decrease in Setd2 transcripts in the 2-week HFD mouse hearts compared to normal controls. The tri-methylation level of histone 3 lysine 36 (H3K36me3) was reduced significantly. Further evidence has linked the responsive upregulation of miR-23a and miR-23b in early exposure to high fat diet with repressed

Setd2 expression both in vivo and in vitro. The above preliminary study drives the hypothesis short-term(2 weeks) administration of high fat diet in mouse will not cause any metabolism change, while predisposed miR-23a and miR-23b elevation in heart, which leads to predisposed to cardiac hypertrophy even heart failure after hemodynamic stress due to the loss of a histone methyltransferase Setd2.

From the data of RNA-Seq and miR-Seq results, among hundreds of upregulated genes and miRNAs, with the help of bioinformatic analysis to match miRNAs and their potential targets, two miRNAs and a histone methyltransferase stand out. It is well known that miRNAs function as gene repressors by binding to the 3'UTR of a target gene and block its translation, or directly degrade the mRNA. By applying the bioinformatic system of Target Scan, it has been revealed that on the 3'-UTR of Setd2 exist a common binding site of miR-23a-3p and miR-23b-3p. It is well known that microRNA has multiple targets and a specific gene can be regulated by different microRNAs. To further investigate the relationship between the 2 miRs and Setd2, a luciferase vector contaiing Setd2 3'UTR was built to indicate that miR-23a and miR-23b can bind to Setd2 3'UTR and repress the luciferase activity and mutation of the binding site that can fully blunt the repression of miR-23a-3p and miR-23b-3p. Even this project has provide solid evidence that miR-23a and miR-23b negatively regulate Setd2 through its 3'UTR during early exposure to high fat diet, the RNA-Seq and miR-Seq data can provided further subjects for future studies.

We propose a working model in this study and provide evidence indicating that miR-23a and miR-23b are "lipid sensors". High fat diet induced the upregulation on these 2 micro RNA in the heart, and function as antagonists to histone methyltransferase Setd2 through binding to Setd2 3'UTR and repress the transcription and translation of this histone modifier, Setd2 downregulation caused the cardiac hypertrophy signal cascade, further leads to heart predispose to hemodynamic stress. This is the first study in which miR-23a and miR-23b functions as upstream sensors that conduct epigenetic regulation of the development of lipotoxic cardiomyopathy in prediabetes. The process is dynamically regulated, down-regulation of Setd2 reduces the tri-methylation of Histone 3 lysine 36, the latter promotes a cascade of signaling pathways changes which are highly involved in cardiovascular diseases.

Role of Setd2 in Cardiac Hypertrophy

The pathological functions of Setd2 have been largely associated with clear renal cell carcinoma as a tumor suppressor[148, 149]. Genetic global silencing of *Setd2* in mice was embryonically lethal with compromised vascular development[150]. Recent emerging evidence indicates that Setd2 also participates in inhibiting liver cancer stem cell proliferation[151] in which Setd2 function is consistent with our results through different mechanisms. We recently reported that Setd2 was essential for skeletal muscle cell proliferation and differentiation. Genetic silencing of *Setd2* abolished myotube formation and inhibited muscle cell growth by repressing myogenein transcriptional activity and activating cyclin-dependent kinase inhibitor

p21[152]. The Setd2 deficiency-medicated proliferative effect is in line with our finding in cardiomyocyte. It is important to note that the basic knowledge of Setd2 in a normal heart and in a lipid-induced pathological situation remains unknown,

To explore the biological significance of the downregulation of Setd2 and the consequence of decreased H3K36 tri-methylation in the heart, we generated genetic loss- and -gain-of-function mouse lines: the cardiac-specific Setd2-haploinsufficient (MHC-Setd2$^{+/-}$) and the cardiac-specific Setd2 overexpression transgenic mice (Tg-MHC-Setd2). The Setd2 heterozygous mice, which mimic high fat diet induced Setd2 repression in the heart, exhibited normal cardiac contractile function under physiological conditions. While they are predisposed hemodynamic challenge with dilated cardiomyopathy, demonstrate that the decreased level of H3K36 tri-methylation is detrimental for normal cardiac function, which triggers a cascade of harmful gene activation, leading to HFD-induced cardiac toxicity.

The RNA-seq has provided multiple pathways that can be involved in heart contraction, anion channel activity growth factor activity and binding; all might contribute to the dilated cardiac hypertrophy caused by high fat diet plus hemodynamic stress. The chromatin immunoprecipitation (ChIP) sequencing will provide evidence of the genes that have been regulated by histone 3 lysine 36 methylation in Setd2 cardiac specific

knockout mice, which further indicated that cardiac hypertrophy related genes are positively regulated with early exposure to high fat diet due to loss of Setd2.

As noted above, 2-week of high fat diet contributes to predispose to hemodynamic challenge in mouse heart; it is difficult to separate the effects of pure high fat from glucose increase in the present investigation. A link between the high circulation glucose and cardiac hypertrophy was suggested in the 1980s and 1990s[153, 154], while the relationship between early high fat diet and cardiovascular susceptibility to stress has not been addressed in previous studies. Our group also did a parallel experiment by feeding the mice fructose water for 2 weeks, then performed TAC on them. Three week after TAC, cardiac function was significantly dropped with Setd2 repression, while there was no significant change of miR-23a and miR-23b. This pilot experiment indicated that loss of Setd2 leads to higher susceptibility to stress and cardiac hypertrophy, even heart failure. Future study should be focus on investigating Setd2 downregulation mediated cardiac hypertrophy, as well as miR-23a and miR-23b function as lipid sensors mediated on other metabolism-related signaling pathways.

It is important to note that this study does have limit due to the study model and the cell treatment that has been applied. Animals are under high fat diet for only 2 weeks which extended the individual variation in response to high fat diet and later the hemodynamic stress (TAC), this limit can be compensated for by expansion of

the sample size. In vitro, this study used palmitic acid to mimic the high fat diet condition in cell culture, which is a saturated fatty acid. There are other studies that apply linoleic acid or other unsaturated fatty acid, that or a mixture of saturated and unsaturated fatty acid to cardiomyocyte. The unsaturated fatty acids were reported to have an unignorably cardiac protective function in high glycose treated cardiomyocyte[155]. In addition to the current study, the responsive upregulation of miR-23a and miR-23b to other fatty acid treatment should be investigated as well. Also, it is still unknown that how a high fat diet environment triggers the upregulation of these 2 miRs, which could lead to further investigations.

In summary, this study demonstrates an essential role of epigenetic regulation in the development of lipotoxic cardiomyopathy during early high fat diet exposure. Upregulation of miR-23a and miR-23b predisposes the animal hearts to transition to dilated cardiomyopathy and heart failure through repressed Setd2 expression. Blocking of Setd2 function is partially responsible for this maladaptive transition. Inhibition of miR23a and miR23b is sufficient to rescue the Setd2-mediated cardiac hypertrophy in the high fat diet fed animals even after TAC. Genetically forced overexpression of Setd2 in cardiomyocyte or blocking miR-23a and miR-23b by applying antagomiR will rescue the dilated cardiomyopathy phenotype. The finding of miR-23a and miR-23b effects on Setd2, and Setd2 inhibition caused cardiac hypertrophy shed light on a new concept of epigenetic regulation in the

development of cardiac lipotoxicity in prediabetes caused by high fat diet. Given the novel “lipid sensor” role of miR-23a and miR2-3b and their inhibition on Setd2-caused cardiomyopathy, our findings provide a potential alternative target for pharmacological manipulation in lipotoxic cardiomyopathy prevention.

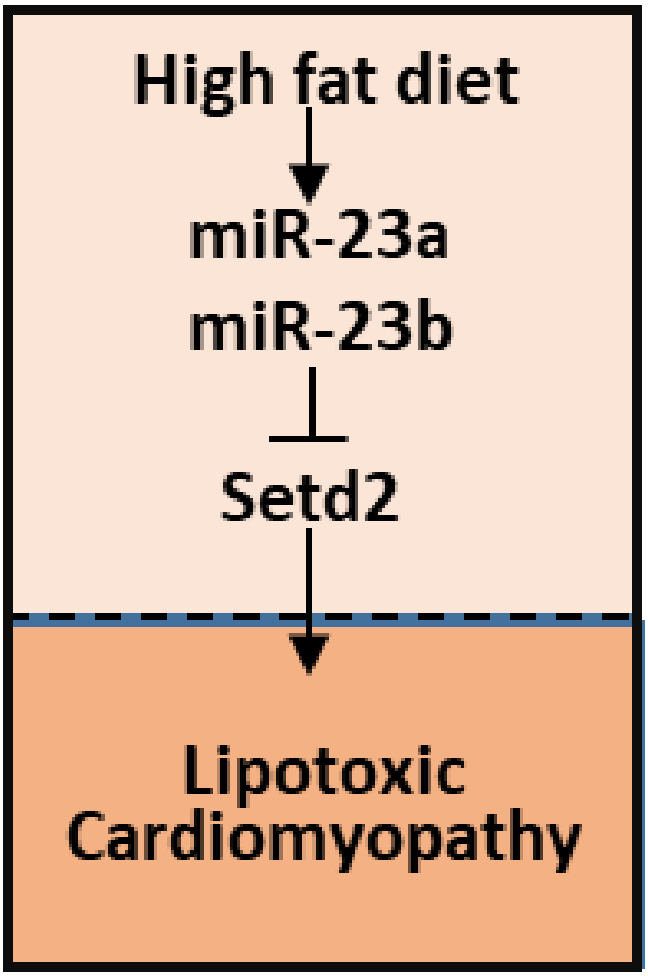

Figure. 15. Molecular mechanism of lipotoxic cardiomyopathy. Short-term excessive fatty acid exposure upregulates miR-23a and miR-23b expression, which in turn represses Setd2 expression. The downregulation of Setd2 leads to lipotoxic cardiomyopathy.

CHAPTER IV

CONCLUSION

The epigenetic signaling mechanism leading to excessive lipid-induced cardiac complication before obesity, coronary atherosclerosis or obesity-associated metabolic syndromes is yet to be accomplished. While in this study, I found one of the epigenetic regulatory pathway that involved in lipid-overload induced cardiomyopathy in the early exposure. And this is the first time to show that histone methyltransferase *setd2* can be repressed by microRNA-23a and 23b and further leads to cardiomyopathy.

The innovation of this study can be concluded as:

1) miR-23a and miR-23b are identified as lipid overload sensors;
2) Upregulation of miR-23a and/or miR-23b is linked to lipotoxic cardiomyopathy;
3) Discovery of Setd2 as a downstream target of miR-23a and miR-23b;
4) Setd2 is obligatory for normal cardiac function;
5) Downregulation of Setd2 is responsible for miR-23a and miR-23b-mediated lipotoxic cardiomyopathy.

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