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Original Paper

Set7 Deletion Prevents Glucose Intolerance and Improves the Recovery of Cardiac Function After Ischemia and Reperfusion in Obese Female Mice

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Key Words
Obesogenic diet • Set7 • Female • Obesity • Ischemia and reperfusion • Glucose intolerance

Abstract
Background/Aims: An obesogenic diet (high fat and sugar, low fiber) is associated with an increased risk for metabolic and cardiovascular disorders. Previous studies have demonstrated that epigenetic changes can modify gene transcription and protein function, playing a key role in the development of several diseases. The methyltransferase Set7 methylates histone and non-histone proteins, influencing diverse biological and pathological processes. However, the functional role of Set7 in obesity-associated metabolic and cardiovascular complications is unknown. ***Methods:*** Wild type and Set7 knockout female mice were fed a normal diet or an obesogenic diet for 12 weeks. Body weight gain and glucose tolerance were measured. The 3T3-L1 cells were used to determine the role of Set7 in white adipogenic differentiation. Cardiac morphology and function were evaluated by histology and echocardiography. An *ex vivo* Langendorff perfusion system was used to model cardiac ischemia/reperfusion (I/R). ***Results:*** Here, we report that Set7 protein levels were enhanced in the heart and perigonadal adipose tissue (PAT) of female mice fed an obesogenic diet. Significantly, loss of Set7 prevented

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obesogenic diet-induced glucose intolerance in female mice although it did not affect the obesogenic diet-induced increase in body weight gain and adiposity in these animals, nor did Set7 inhibition change white adipogenic differentiation *in vitro*. In addition, loss of Set7 prevented the compromised cardiac functional recovery following ischemia and reperfusion (I/R) injury in obesogenic diet-fed female mice; however, deletion of Set7 did not influence obesogenic diet-induced cardiac hypertrophy nor the hemodynamic and echocardiographic parameters. ***Conclusion:*** These data indicate that Set7 plays a key role in obesogenic diet-induced glucose intolerance and compromised myocardial functional recovery after I/R in obese female mice.

Introduction

Obesity increases the risk of several diseases, such as cancer, hepatic steatosis, type 2 diabetes mellitus, and cardiovascular disorders [1]. Diverse studies have demonstrated that men develop cardiovascular diseases at an earlier age than women [2]. However, obese and insulin resistant women have a higher risk of cardiovascular disease than men [3–5].

The major cause of obesity results from an energy imbalance between calories consumed and calories expended. Mice fed an obesogenic diet, which contains high levels of carbohydrate and fat, is an effective method to model diet-induced obesity in rodents since it recapitulates many obesity-related disorders found in overweight and obese individuals [6]. Despite the increase in obesity levels worldwide, most of the studies related to obesity has been performed in men and male rodents. Therefore, understanding the biological mechanisms involved in obesity-related cardiometabolic disorders in both sexes is necessary to develop new strategies for prevention and treatment.

Studies have demonstrated the role of epigenetic mechanisms, such as DNA methylation, microRNAs, and histone modifications in obesity-related cardiovascular and metabolic disorders [7–10]. The histone-lysine N-methyltransferase Set7 (also known as Setd7, Set9, Set7/9, or Kmt7) was originally described as a histone H3-lysine 4 (H3K4)-specific methyltransferase [11]. The methylation of H3K4 by Set7 prevents chromatin condensation and increases transcription [12]. Set7-dependent methylation of H3K4 has been associated with transcription of genes related to muscle differentiation [13], oxidative stress, inflammation [14], insulin secretion [15], and extracellular matrix proteins [16]. Over the past few years, researchers have determined that Set7 can also methylate non-histone substrates involved in diverse physiological and pathological processes, such as the DNA damage response, cell cycle regulation, chromatin modulation, gene transcription, metabolism, and cell differentiation [17]. Through methylation of proteins, Set7 can influence their function, intracellular localization, and degradation [17]. Several proteins can be methylated by Set7, including estrogen receptor alpha [18], P53 [19], Foxo [20], Akap6 [21], β-catenin [22], Pdx1 [23], Pgc-1α [24], Sirt1 [25], and Nfκb [26]. Functional studies have reported that Set7 affects glucose homeostasis [23, 27] and the response to renal ischemic injury [28]. In addition, streptozotocin-induced type 1 diabetic rats have enhanced Set7 protein levels in the heart, suggesting that Set7 may play a role in diabetic cardiomyopathy [29]. However, the impact of Set7 in obesity-related metabolic and cardiovascular disorders remains unclear.

In this study, we explored the role of Set7 in obesogenic diet-induced metabolic and cardiovascular complications. We found that obese female mice fed an obesogenic diet exhibited higher Set7 protein levels in the heart and perigonadal adipose tissue (PAT). Deletion of Set7 did not affect body weight gain, adiposity, and cardiac hypertrophy in response to an obesogenic diet in female mice. However, loss of Set7 prevented obesogenic diet-induced glucose intolerance and the impaired recovery of cardiac function following I/R injury.

Materials and Methods

Mice and diets

All animal experiments were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Ethic Committee for Animal Research at the Institute of Biomedical Sciences of the University of Sao Paulo (ICB/USP) (CEUA/3979120418) and were performed in the Department of Anatomy at the ICB/USP. The Set7 global knockout (Set7KO) and wild type (WT) mice used in this study were characterized previously [30]. Five-week-old Set7KO and WT female mice were obtained from heterozygous breeding and fed a normal diet (Nd; 10% kcal from fat, 20% kcal from protein and 70% kcal from carbohydrate; PragSolucoes) or an obesogenic diet containing a high-fat diet (HFD; 60% kcal from fat, 20% kcal from protein and 20% kcal from carbohydrate; Research Diets) and sweetened condensed milk (Nestlé) supplemented with mineral and vitamin mix (AIN 93G) for 12 weeks [31]. Five-week-old WT male mice were fed a normal diet or a HFD for 12 weeks. The mice were housed in a temperature- and light- controlled room (22 °C; 12h light-dark cycle) and had water and food *ad libitum*. Body weight gain was monitored weekly. After euthanasia using a CO_2 chamber, heart, PAT, subcutaneous adipose tissue (SAT), retroperitoneal adipose tissue (RAT), and liver were collected, weighted, and stored at -80 °C for further analysis. The weight of the tissues was normalized by tibia length.

Nuclear magnetic resonance

Nuclear magnetic resonance was used to evaluate the body composition (percentages of fat mass and lean mass) of the mice at the last week of dietary feeding (Bruker's minispec LF50 Body Composition Analyzer).

Glucose homeostasis

Intraperitoneal glucose tolerance test (iGTT) and insulin tolerance test (ITT) were performed to assess glucose homeostasis during the last week of the dietary regimen. Female mice were fasted for 6 h. For iGTT, blood glucose levels were measured at baseline (before glucose injection) and after an intraperitoneal injection of glucose (2 g/kg body weight) using a glucometer (Accu-Chek Active, Roche Diagnostics). After 72 hours, ITT was performed in mice fasted for 6 h. Blood glucose levels were assessed before and after insulin injection (0.5 U insulin/kg body weight). The blood glucose disappearance rate (KITT) was calculated to determine insulin sensitivity [32].

Hemodynamic parameters

Systolic blood pressure (SBP) and heart rate (HR) were measured during the last week of the dietary regimen using tail cuff plethysmography (BP-2000 Blood Pressure Analysis System™ of Visitech Systems©). Female mice were conditioned to tail cuff for 7 days before data acquisition. Ten measurements of SBP and HR were obtained per mouse during the morning.

Echocardiography assessment

Echocardiography was performed to evaluate cardiac function and morphology during the last week of the dietary feeding. Female mice were anesthetized with 1.5% isoflurane and echocardiographic analyses were performed using VEVO 2100 system (Visual Sonics) and a 13-24-MHz MicroScan transducer (model MS-550D). The measurements obtained were end-diastolic interventricular septum (IVS;d), end-systolic interventricular septum (IVS;s), LV end-diastolic posterior wall thickness (LVPW;d), LV end-systolic posterior wall thickness (LVPW;s), ejection fraction (EF), fractional shortening (FS), isovolumetric relaxation time (IVRT), isovolumetric contraction time (IVCT), E/A ratio, and E/E' ratio.

Cardiac ischemia-reperfusion model

An *ex vivo* Langendorff perfusion system was used to model cardiac ischemia/reperfusion (I/R), as described previously [33, 34]. After euthanasia, hearts from female mice were removed and cannulated on a non-recirculating Langendorff system (ADInstruments, Castle Hill, NSW, Australia). The hearts were perfused via the aorta with a modified Krebs–Henseleit buffer [NaCl (118 mM), KCl (4.7 mM), $CaCl_2$ (1.75 mM), $MgSO_4$ (1.66 mM), $NaHCO_3$ (24.88 mM), KH_2PO_4 (1.18 mM), dextrose 2 g/l and bidistilled water with a pH of 7.4]

under a constant flow rate (3 ml/min) at 37 ± 1 °C and constant oxygenation (5% CO_2 and 95% O_2). The fresh buffer was prepared and filtered (47 mm Swinnex®, membrane pore 0.22 mM) immediately before heart infusion. The hearts were perfused for 30 min (stabilization period) to establish a baseline recording and then submitted to a global ischemia (zero flow) for 20 min. The flow was restarted and the hearts were reperfused for 45 min (reperfusion period). Ventricular function was determined by a pressure transducer inserted into the left ventricle. Left ventricular developed pressure (LVDP), positive first derivative of left ventricular pressure (+dP/dT), and negative first derivative of left ventricular pressure (−dP/dT) were constantly monitored (PowerLab Chart 7-Lab, AD Instruments, Australia). Functional recovery values are presented for the stabilization period and after 45 min of reperfusion.

Western blotting

Total protein from cell cultures, heart, and PAT was isolated using Ripa Buffer (Tris-HCl 50 mM, NaCl 150 mM, Sodium deoxycholate 0.5%, and Triton 1%) with protease inhibitors. Protein concentration of the samples was quantified using the Bradford method. Fifty μg of each sample was resolved by electrophoresis on polyacrylamide-SDS gels and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was stained with ponceau to evaluate protein transfer efficiency, followed by incubation with the primary antibody overnight at 4ºC and with the secondary antibody at room temperature for 1 h. The membrane was washed three times with TBST (NaCl 150 mM, Tris-base 50 mM, Tween 20 0.1%) between antibodies incubation. The antibodies used were Set7 (#2813, Cell Signaling), α-actinin (sc-15335, Santa Cruz Biotechnology), Gapdh (sc-32233, Santa Cruz Biotechnology), me2-Rpl29 (#19495S, Cell Signaling), β-tubulin (sc-23949), Pparγ (sc-7273, Santa Cruz Biotechnology), Fabp4 (sc-271529, Santa Cruz Biotechnology), Cebpα (sc-365318, Santa Cruz Biotechnology), Serca2 (#9580, Cell Signaling), α-tubulin (sc-5286, Santa Cruz Biotechnology), Casp1 (sc-398715, Santa Cruz Biotechnology), Bcl2 (sc-7382, Santa Cruz Biotechnology), and phospho-Stat3 (#9145, Cell Signalling). The protein bands were visualized using the UVITEC Cambridge (Aliance 9.7) system using a chemiluminescent reagent (Luminata™ Forte) and quantified by densitometry. The results are expressed as relative levels.

Histology

Transverse heart sections were fixed in paraformaldehyde 4% for 24 h and stored in 70% ethanol. The heart samples were dehydrated, embedded in paraffin and sectioned into 5 μm-thick slices. Transverse heart sections were stained with wheat germ agglutinin for quantification of cardiomyocyte area at the papillary muscle level (n=30-50 cardiomyocytes were evaluated per animal). Cardiac fibrosis was evaluated using picrosirius red staining and measured as a relative area in relation to the total cardiac area. Images were analyzed using a light microscope (Nikon®) and quantified using ImageJ software.

White adipocyte culture

White adipocyte cultures were prepared as previously described [35]. The 3T3-L1 cells were differentiated into white adipocytes in medium containing 1 μM dexamethasone, 0.125 mM indomethacin, 1 μM insulin, and 0.5 mM isobutylmethylxanthine for 7 days. To evaluate the role of Set7 in white adipogenesis, 3T3-L1 cells were induced to differentiate into white adipocytes in medium supplemented with (R)-PFI-2 (5 μM), which is a specific Set7 inhibitor [36]. Oil Red O staining was used to assess the intracellular lipid content during adipocyte differentiation.

Quantitative Real Time RT-PCR (qPCR)

Total RNA from heart was isolated with TRIzol (Life Technologies) following the manufacture's recommendations. cDNA was made using SuperScript II RNase H Transcriptase (Invitrogen). The cDNA was amplified by qPCR using SYBR Green Master Mix (Applied Biosystems) and specific primers (Exxtend). The primers used were: *18S*: 5'-GCC ACT TGT CCC TCT AAG-3' and 5'-GTG CAT CGT TCT TAG TTG-3'; for *Myh6*: 5'-TGA CGT CAC CCT CCA ACA TGG-3' and 5'-CAA CTC CCC GTT CTC TGT C-3'; for *Acta1*: 5'-GCT CGG TGA GGA TTT TCA TCA G-3' and 5'-CCT GCC ACA CGC CAT CAT-3'. 18S was used as an internal control since its expression levels were similar between the experimental groups. The relative gene expression levels were calculated using the formula ($2^{-\Delta\Delta Ct}$).

Statistical analyses

The results are presented as mean ± SD. Statistical significance was calculated using two-way ANOVA followed by Bonferroni *post hoc* test and Student's t-test using GraphPad® Prism software. Statistical significance was set at p≤0.05.

Results

Set7 levels are increased in the heart and PAT of obese female mice

Initially, we asked whether Set7 protein levels would be changed in the heart and white adipose tissue of obese male and female mice. As expected, male mice fed a high-fat diet (HFD) exhibited increased body weight compared to their respective controls fed a normal diet (Supplementary Fig. 1A – for all supplementary material see www.cellphysiolbiochem.com). Western blotting analysis revealed that Set7 protein levels were unchanged in the heart (Supplementary Fig. 1B, 1C) of male mice fed a HFD compared to those found in male mice fed a normal diet. However, Set7 protein levels were increased in the perigonadal adipose tissue (PAT) of male mice in response to a HFD (Supplementary Fig. 1B, 1D).

The female mice fed an obesogenic diet displayed increased body weight compared to their respective controls (Fig. 1A), indicating the development of obesity. Interestingly, female mice fed an obesogenic diet exhibited higher Set7 protein levels in the heart (Fig. 1B, 1C) and PAT (Fig. 1B, 1D) compared to those fed a normal diet. Together, these results suggest that obese female mice display increased Set7 protein levels in the heart and PAT.

Pparγ may regulate the transcription of diverse proteins containing SET domain [37]. Western blotting analysis revealed that Pparγ protein levels were increased in the heart (Fig. 1B, 1E) and PAT (Fig. 1B, 1F) of female mice fed an obesogenic diet.

Deletion of Set7 does not affect obesogenic diet-induced obesity in female mice, but prevents glucose intolerance

Since Set7 protein levels were increased in the heart and PAT of obese female mice, we examined the role of Set7 in obesogenic diet-induced metabolic and cardiovascular complications by using a global Set7KO mouse model. Western blotting analysis revealed

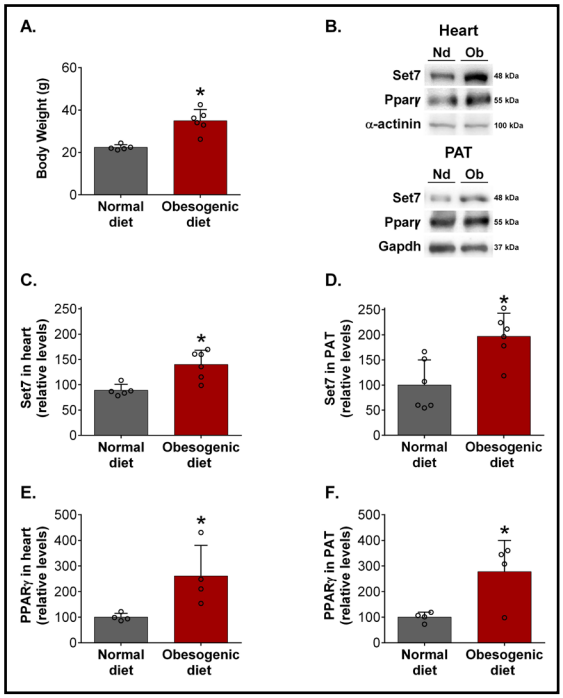

Fig. 1. Set7 protein levels are increased in the heart and perigonadal adipose tissue of obese female mice. (A) Body weight of WT female mice fed a normal diet (Nd) and obesogenic diet (Ob) for 12 weeks (n=5-6). (B) Representative images of western blotting for Set7, Pparγ, α-actinin, and Gapdh. Set7 protein levels in the heart (C) and PAT (D) of WT female mice fed a normal diet (Nd) and obesogenic diet (Ob) evaluated by western blot (n=6). Pparγ protein levels in the heart (E) and PAT (F) of WT female mice fed a normal diet (Nd) and obesogenic diet (Ob) evaluated by western blotting (n=5-6). * vs Nd (p<0.05).

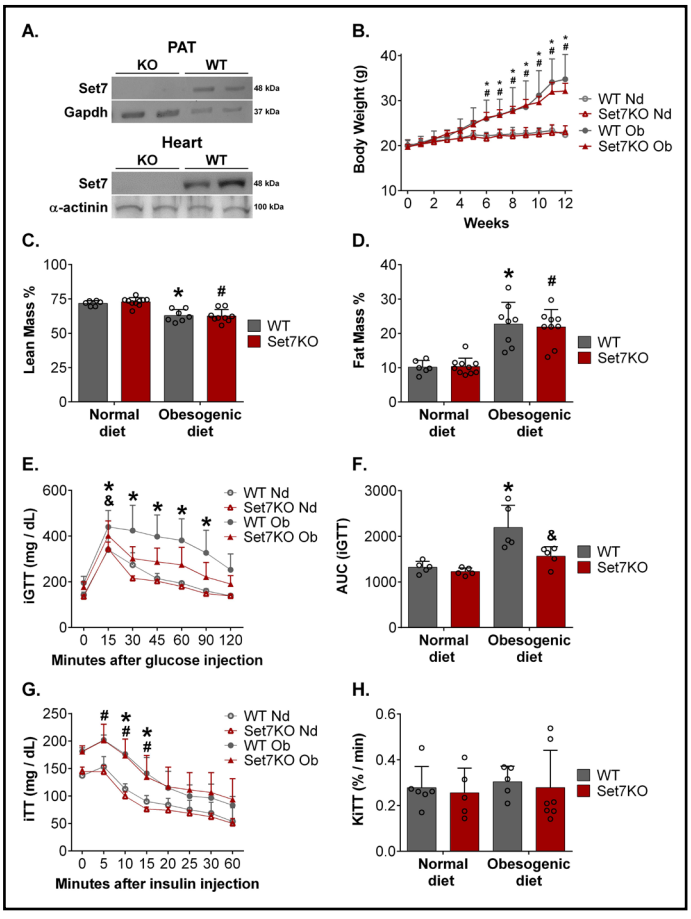

Fig. 2. Deletion of Set7 does not change obesogenic diet-induced obesity, but prevents glucose intolerance in female mice. (A) Validation of Set7 deletion in the PAT and heart of Set7 KO female mice by western blotting. (B) Body weight gain in WT and Set7 KO female mice fed a normal diet (Nd) and obesogenic diet (Ob) (n=6-11). (C) Lean mass and (D) fat mass percentage of WT and Set7 KO female mice fed a normal diet (Nd) and obesogenic diet (Ob) evaluated by nuclear magnetic resonance (n=6-10). (E) Intraperitoneal glucose tolerance test (iGTT) and (F) area under the curve (AUC) of WT and Set7 KO female mice fed a normal diet (Nd) and obesogenic diet (Ob) (n=6-7). (G) Intraperitoneal insulin tolerance test (ITT) and (H) blood glucose disappearance rate (KITT) of WT and Set7 KO female mice fed a normal diet (Nd) and obesogenic diet (Ob) (n=5-7). * WT Ob vs WT Nd; # Set7KO Ob vs Set7KO Nd; & Set7KO Ob vs WT Ob ($p<0.05$).

that Set7KO female mice exhibited undetectable Set7 protein levels in the PAT and heart compared to those found in WT mice (Fig. 2A).

Next, we evaluated the impact of Set7 in obesogenic diet-induced metabolic dysfunctions. As expected, the obesogenic diet regimen increased body weight gain in WT female mice (Fig. 2B). Nonetheless, deletion of Set7 did not affect body weight gain in response to an obesogenic diet. No differences were observed between WT and Set7KO female mice fed a normal diet (Fig. 2B).

Table 1. Deletion of Set7 does not affect obesogenic diet-induced fat mass gain in female mice. The weight of the tissues was normalized by tibia length and the results are presented as mg/mm. Wild type (WT), Set7 knockout (Set7KO), normal diet (Nd), and obesogenic diet (Ob). The results are presented as mean ± standard deviation. Statistical analysis was performed using two-way ANOVA and Bonferroni post-hoc test. *vs WT Nd ($p<0.05$), #vs Set7KO Nd ($p<0.05$)

Parameter	WT Nd (n=7)	Set7KO Nd (n=7)	WT Ob (n=6)	Set7KO Ob (n=6)
Perigonadal adipose tissue	67.2 ± 10.6	54.9 ± 5.7	83.5* ± 14.4	76.9# ± 6.1
Subcutaneous adipose tissue	17.9 ± 8.6	21.9 ± 4.3	118.3* ± 43.9	108.6# ± 34.2
Retroperitoneal adipose tissue	6.0 ± 3.9	8.5 ± 3.1	54.1* ± 21.1	47.9# ± 11.5
Liver	67.1 ± 8.7	54.7 ± 4.4	82.8* ± 13.6	76.8# ± 7.1

We therefore verified whether Set7 deletion could affect fat mass expansion in response to an obesogenic diet. Nuclear magnetic resonance showed that both WT and Set7KO female mice fed an obesogenic diet exhibited reduced relative lean mass (Fig. 2C) and enhanced fat mass (Fig. 2D) in comparison to their respective controls. In line with this finding, analysis of white adipose tissue depots revealed that the obesogenic diet increased the weight of PAT, SAT, and RAT (Table 1) both in WT and Set7KO female mice compared to those fed a normal diet; however, this increase was unaffected by deletion of Set7. Liver weight was increased both in WT and Set7KO female mice fed an obesogenic diet in relation to their respective controls (Table 1).

Given that obese male and female mice had increased Set7 protein levels in the PAT, but Set7 deletion did not change the white adipose tissue gain in response to an obesogenic diet in female mice, we therefore evaluated whether Set7 could affect white adipogenic differentiation. First, we examined the expression of Set7 upon white adipocyte differentiation *in vitro*. Western blotting analysis demonstrated that Set7 protein levels were reduced upon induction of adipocyte differentiation (day 7) compared to 3T3-L1 cells (day 0) (Supplementary Fig. 2A). Next, to characterize the impact of Set7 in white adipocyte differentiation, (R)-PFI-2, which is a selective inhibitor of Set7 [36], was added during the course of white adipogenic induction in 3T3-L1 cells. Western blotting analysis revealed that protein levels of me2-Rpl29, which is the major substrate of the Set7 [38], were reduced by (R)-PFI-2 treatment in white adipocytes (Supplementary Fig. 2B, 2F). Inhibition of Set7 did not affect lipid droplet content in white adipocytes, as assessed by Oil Red O staining (Supplementary Fig. 2B). In addition, protein levels of Pparγ (Supplementary Fig. 2C, 2F), Fabp4 (Supplementary Fig. 2D, 2F), and Cebpα (Supplementary Fig. 2E, 2F), which are factors involved in differentiation and maturation of adipocytes, were unchanged by (R)-PFI-2, suggesting that Set7 does not affect white adipogenesis *in vitro*.

Next, we investigated whether Set7 deletion and obesogenic diet could affect glucose homeostasis. WT female mice fed an obesogenic diet exhibited elevated blood glucose levels during iGTT (Fig. 2E), resulting in increased AUC (Fig. 2F) compared to their respective controls. These results indicate that obesogenic diet induced glucose intolerance. However, Set7KO female mice fed an obesogenic diet did not display increased AUC of the iGTT (Fig. 2F) compared to their respective controls, suggesting that deletion of Set7 prevented obesogenic diet-induced glucose intolerance. Insulin tolerance test (ITT) was also performed to evaluate glucose homeostasis (Fig. 2G). WT and Set7KO female mice fed both diets exhibited similar blood glucose disappearance rate (KITT) (Fig. 2H), indicating that neither obesogenic diet nor Set7 deletion affected insulin sensitivity. Together, these results suggest that Set7 does not contribute to obesogenic diet-induced obesity in female mice, but Set7 deletion protects against obesogenic diet-induced glucose intolerance.

Set7 deletion does not affect obesogenic-diet induced cardiac hypertrophy in female mice

Considering that Set7 protein levels were increased in the heart of obese female mice and that this enzyme can methylate diverse proteins involved in cardiac hypertrophy [20–22, 39–41], we investigated the role of Set7 in obesogenic diet-induced cardiac hypertrophy. Both WT and Set7KO female mice fed an obesogenic diet exhibited cardiac hypertrophy, as assessed by higher HW/TL ratio (Fig. 3A). Consistent with these findings, WGA staining of transverse heart sections demonstrated that obesogenic diet increased cardiomyocyte area both in WT and Set7KO female mice compared to their respective controls (Fig. 3B, 3C). Analysis of qPCR revealed that cardiac *Acta1* mRNA levels were increased in the heart of WT and Set7KO female mice fed an obesogenic diet (Fig. 3D). On the other hand, cardiac *Myh6* mRNA levels were reduced in obesogenic diet-fed WT female mice compared to their respective controls (Fig. 3D). Together, these results indicate that deletion of Set7 does not influence obesogenic diet-induced cardiac hypertrophy in female mice.

Next, we examined the effect of obesogenic diet and Set7 deletion in myocardial fibrosis. No significant changes were detected in cardiac fibrosis among the groups, as assessed by picrosirius red staining (Fig. 3E, 3F), indicating that neither obesogenic diet or Set7 deletion altered myocardial collagen deposition in female mice.

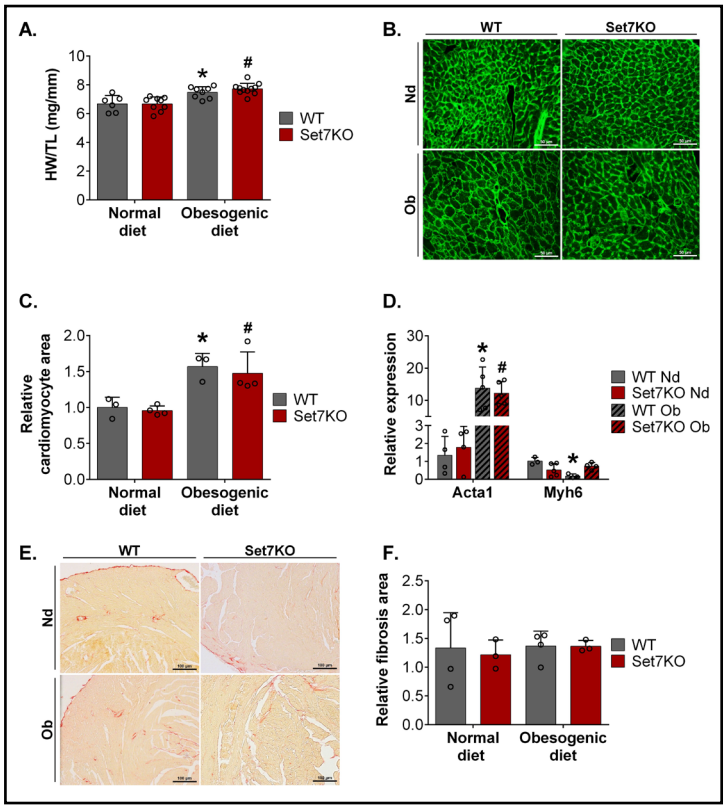

Fig. 3. Deletion of Set7 does not affect obesogenic diet-induced cardiac hypertrophy in female mice. (A) Heart weight normalized to tibia length (HW/TL) in WT and Set7 KO female mice fed a normal diet (Nd) and obesogenic diet (Ob) (n=6-11). (B) Wheat germ agglutinin staining of transverse heart sections and (C) quantification of cardiomyocyte area at the papillary muscle level (n=3). (D) Analysis of hypertrophic markers in the hearts of WT and Set7 KO female mice fed a normal diet (Nd) and obesogenic diet (Ob) evaluated by qRT-PCR (n=3-6). (E) Picrosirius red staining of transverse heart sections and (F) quantification of cardiac fibrosis area (n=3-4). * WT Ob vs WT Nd; # Set7KO Ob vs Set-7KO Nd ($p<0.05$).

Deletion of Set7 does not affect hemodynamic and echocardiographic profile in female mice

We verified whether obesogenic diet and Set7 deletion could modulate hemodynamic and echocardiographic parameters in female mice. SBP and HR (Table 2) were not affected either by obesogenic diet or by deletion of Set7, as assessed by tail cuff plethysmography. Echocardiography revealed that the IVS;d was enhanced in Set7KO female mice fed an obesogenic diet compared to their respective controls (Table 2). The LVPW;d, LVID;d, EF, FS, IVRT, IVCT, E/A ratio, and E/E' ratio were similar between WT and Set7KO female mice fed both diets (Table 2). These findings suggest that hemodynamic parameters and cardiac performance were unchanged by obesogenic diet or by deletion of Set7.

Table 2. Deletion of Set7 does not affect hemodynamic and echocardiographic profile in female mice. The results are presented as mean ± standard deviation. Wild type (WT), Set7 knockout (Set7KO), normal diet (Nd), obesogenic diet (Od), systolic blood pressure (SBP), heart rate (HR), left ventricle (LV), intraventricular septum thickness (IVS), systole (s), diastole (d), left ventricular posterior wall thickness (LVPW), left ventricular interior dimension (LVID), ejection fraction (EF), fractional shortening (FS), isovolumetric relaxation time (IVRT), and isovolumetric contraction time (IVCT). Statistical analysis was performed using two-way ANOVA and Bonferroni post hoc test. *vs WT Nd ($p<0.05$), #vs Set7KO Nd ($p<0.05$)

Parameter	WT Nd (n=3)	Set7KO Nd (n=4)	WT Ob (n=6)	Set7KO Ob (n=6)
SBP (mmHg)	79.3 ± 8.9	76.9 ± 16.2	68.1 ± 13.2	75.7 ± 26.7
HR (bpm)	543.8 ± 186.6	393.4 ± 31.0	371.9* ± 55.3	387.4 ± 20.7
IVS;d (mm)	0.65 ± 0.11	0.63 ± 0.07	0.71 ± 0.10	0.81# ± 0.12
IVS;s (mm)	0.92 ± 0.13	0.94 ± 0.18	0.96 ± 0.10	1.10 ± 0.16
LVPW;d (mm)	0.69 ± 0.08	0.65 ± 0.09	0.72 ± 0.08	0.76 ± 0.11
LVPW;s (mm)	0.90 ± 0.08	0.97 ± 0.07	0.92 ± 0.11	0.96 ± 0.17
LVID;d (mm)	4.27 ± 0.12	4.23 ± 0.10	4.46 ± 0.06	4.36 ± 0.29
LVID;s (mm)	3.15 ± 0.25	3.13 ± 0.39	3.43 ± 0.14	3.41 ± 0.21
EF (%)	51.4 ± 8.4	56.1 ± 4.9	46.5 ± 4.0	48.4 ± 9.2
FS (%)	26.2 ± 5.1	29.1 ± 3.2	23.2 ± 2.4	24.5 ± 5.7
IVRT (ms)	24.9 ± 0.7	18.3* ± 3.6	18.1* ± 2.5	19.3 ± 0.9
IVCT (ms)	22.9 ± 4.2	22.4 ± 4.5	21.3 ± 2.6	19.6 ± 2.1
E/A ratio	1.92 ± 0.28	1.92 ± 0.21	1.64 ± 0.48	1.72 ± 0.29
E/E' ratio	31.2 ± 2.7	22.3 ± 4.9	28.6 ± 4.3	30.2 ± 9.5

Deletion of Set7 prevents cardiac functional deterioration after I/R injury in obese female mice

Given that obesogenic diet increased Set7 protein levels in the heart of female mice, and that Set7 contributes to ischemia/hypoxia injury [27, 28, 42, 43], we investigated whether Set7 could affect cardiac function following I/R injury in obese female mice. First, baseline cardiac function was evaluated at the stabilization period using the *ex vivo* Langendorff perfused heart model. WT and Set7KO female mice fed an obesogenic diet displayed reduced LVDP (Fig. 4A) and −dP/dT (Fig. 4B) at the stabilization period in relation to their respective controls. The +dP/dT was reduced in WT female mice fed an obesogenic diet compared to those fed a normal diet (Fig. 4C), while no difference was observed between Set7KO female mice fed both diets.

After I/R, obese WT female mice displayed reduced LVDP (Fig. 4D), −dP/dT (Fig. 4E), and +dP/dT (Fig. 4F) compared to their respective controls, suggesting that obesogenic diet impaired cardiac functional recovery following I/R injury. In contrast, Set7KO female mice fed an obesogenic diet exhibited a LVDP (Fig. 4D), −dP/dT (Fig. 4E), and +dP/dT (Fig. 4F) similar to those found in their respective controls, suggesting that deletion of Set7 prevents the impaired recovery of cardiac function in response to an obesogenic diet after I/R injury.

Considering that Set7 suppression modulates calcium handling in adult cardiomyocytes [44], we evaluated the protein levels of Serca2 in the hearts after I/R injury. Western blotting analysis showed that Serca2 protein levels were similar among the groups (Fig. 5A, 5B).

Next, we investigated the levels of proteins related to apoptosis, such as Casp1 (proapoptotic protein) and Bcl2 (anti-apoptotic protein) in the heart after I/R injury. The Casp1 protein levels (Fig. 5A, 5C) were similar in the hearts of WT and Set7KO female mice fed both diets after I/R. WT female mice fed an obesogenic diet displayed reduced levels of Bcl2 in the heart compared to their respective controls (Fig. 5A, 5D); however, Bcl2 levels were unchanged in the heart of Set7KO female mice fed an obesogenic diet compared to their respective controls. Together, these findings suggest that Set7 deletion prevents the decrease of cardiac Bcl2 levels induced by obesogenic diet in response to I/R.

A previous study showed that Stat3, a transcriptional factor with cardioprotective effect [45], is phosphorylated, methylated, and inhibited by Set7 [46]. To determine whether

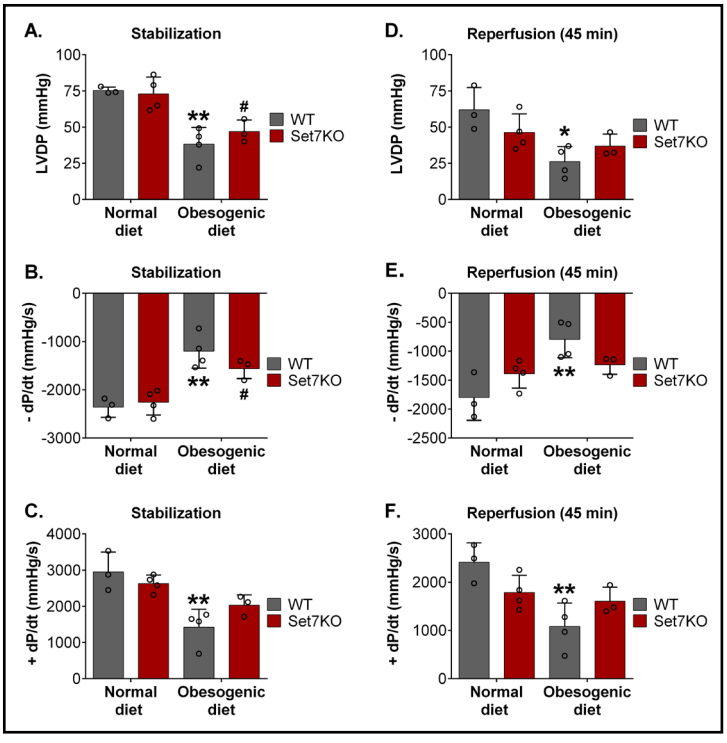

Fig. 4. Deletion of Set7 prevents cardiac functional deterioration after I/R injury in obese female mice. (A) Left ventricular developed pressure (LVDP), (B) positive first derivative of left ventricular pressure (+dP/dT) and (C) negative first derivative of left ventricular pressure (−dP/dT) before the I/R injury (stabilization). (D) LVDP, (E) +dP/dT and (F) −dP/dT after 45 min of cardiac reperfusion of WT and Set7 KO female mice fed a normal diet (Nd) and obesogenic diet (Ob) (n=4-6). * WT Ob vs WT Nd ($p<0.05$); ** WT Ob vs WT Nd ($p<0.01$); # Set7KO Ob vs Set7KO Nd ($p<0.05$).

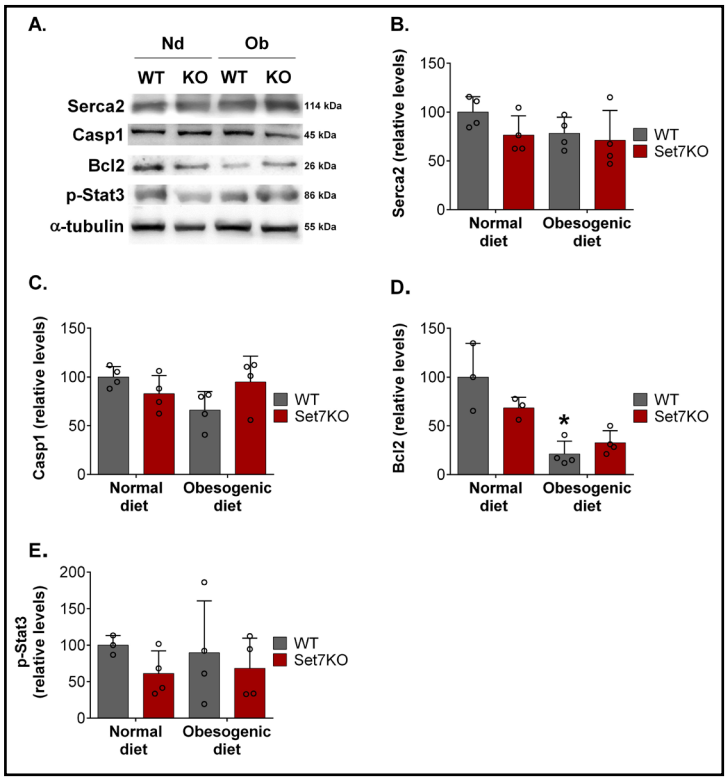

Fig. 5. Set7 deletion prevents the decrease in cardiac Bcl2 levels induced by obesogenic diet after I/R injury. (A) Representative images of western blotting for Serca2, Casp1, Bcl2, phospho-Stat3 (p-Stat3), and α-tubulin. Protein levels of Serca2 (B), Casp1 (C), Bcl2 (D), and p-Stat3 (E) in the heart after I/R injury of WT and Set7 KO female mice fed a normal diet (Nd) and obesogenic diet (Ob) evaluated by western blotting (n=3-4). The protein levels were normalized by α-tubulin and are presented as relative levels. * WT Ob vs WT Nd ($p<0.05$).

Stat3 might be involved in the beneficial effects of Set7 deletion in obese female mice after I/R injury, we evaluated the phospho-Stat3 levels. Western blotting analysis revealed that phospho-Stat3 levels were similar among the groups (Fig. 5A, 5E), suggesting that Stat3 might not be involved in the beneficial effects of Set7 deletion in obese female mice after I/R injury.

Discussion

In this study, we identified that Set7 protein levels were enhanced in the heart and PAT of obese female mice.

Given that Set7 protein levels were increased in the heart and PAT of obese female mice, we explored the functional role of Set7 in obesogenic diet-induced metabolic and cardiovascular disorders by using a global Set7KO mouse model.

As expected, an obesogenic diet regimen increased body weight gain and adiposity in WT female mice. However, deletion of Set7 did not affect body weight gain and the increased adiposity in response to an obesogenic diet, suggesting that this enzyme is not required for obesogenic diet-induced obesity in female mice. The obesogenic diet regimen did not affect insulin sensitivity in WT female mice. This finding is in agreement with previous studies, which demonstrated that female mice are more resistant to develop insulin resistance in response to an obesogenic diet [47–50]. Nonetheless, the obesogenic diet regimen promoted glucose intolerance in WT female mice. This finding is in line with previous studies, which showed that obesogenic diet, containing high levels of carbohydrate and fat, is sufficient to induce glucose intolerance in female rodents [31, 48, 51]. Interestingly, our results revealed that Set7KO female mice did not develop glucose intolerance in response to an obesogenic diet. Consistent with our findings, a previous report showed that the knockdown of Set7 in RCC4 cells increased glucose uptake *in vitro* [27]. Nevertheless, mice with Set7 conditionally deleted in the β cells exhibited glucose intolerance and impaired glucose-stimulated insulin

secretion [23]. Therefore, differences in the model employed to evaluate the impact of Set7 (such as global knockout mouse, tissue-specific knockout mouse, or knockdown *in vitro*) may be involved in the divergent results related to the role of this enzyme on glucose homeostasis. In addition, *Set7* mRNA levels are changed in a temporal manner under HFD in isolated islets of mice, suggesting that this enzyme is an integral component of the β-cell compensatory response [52]. Moreover, the downregulation of Set7 in INS-1 cells increased cell proliferation [52]. Then, differences in the time of the analysis, sex differences, and type of metabolic stress may also explain at least in part the different findings regarding the role of Set7 on glucose homeostasis.

Previously, it was reported that the expression of the histone methyltransferase Setd8 is increased in the white adipose tissue in response to a HFD and during 3T3-L1 adipocyte differentiation [37]. In addition, knockdown of Setd8 attenuated adipocyte differentiation *in vitro*, suggesting that Setd8 is an important regulator of adipogenesis [37]. Here, we found that Set7 protein levels were increased in the PAT of WT female mice fed an obesogenic diet and in the PAT of male mice fed a HFD. Additionally, Pparγ levels were increased in the heart and PAT of WT obese female mice. Pparγ has been shown to regulate the transcription of diverse proteins containing SET domain during adipocyte differentiation, such as Setd5, Setd8, and Setdb1 [37]. Therefore, the higher Pparγ levels in the heart and PAT of obese female mice might be involved in the increase of the Set7 levels.

Moreover, we found that loss of Set7 did not affect obesogenic diet-induced increase in adiposity, suggesting that this enzyme is not required for white adipose tissue expansion in female mice. In addition, we observed that Set7 protein levels were reduced upon white adipocyte differentiation *in vitro*. This finding is consistent with a previous report, which demonstrated that Set7 expression is reduced during brown adipocyte differentiation *in vitro* [53]. Consistent with our *in vivo* findings, the treatment with a Set7 inhibitor did not affect white adipocyte differentiation *in vitro*, indicating that Set7 does not play a key role in white adipogenesis. This result contrasts with a previous study in brown adipocytes, which revealed that knockdown of Set7 reduced the expression of thermogenic genes during brown adipogenic differentiation [54]. Thus, differences in the type of preadipocyte (white vs brown) or in the strategy used to evaluate the impact of Set7 (pharmacological inhibitor vs knockdown) may explain in part the different effects of Set7 on adipogenesis. However, our *in vivo* and *in vitro* results suggest that Set7 does not play a functional role in white adipogenesis and white adipose tissue expansion in response to an obesogenic diet.

In this study, we found that Set7 protein levels were increased in the heart of obese female mice. A previous report demonstrated higher Set7 protein levels in the heart of streptozotocin-induced type 1 diabetic Wistar rats [29]. Studies have shown that Set7 can methylate proteins involved in cardiac homeostasis and hypertrophy, including Akap [21, 40], β-catenin [22, 39], and Foxo [20, 41]. Our results revealed that WT female mice fed an obesogenic diet exhibited cardiac hypertrophy. However, deletion of Set7 did not affect obesogenic diet-induced cardiac hypertrophy, suggesting that this enzyme is not associated with obesity-related myocardial hypertrophy. Here, we also observed that the obesogenic diet did not change myocardial collagen content in female mice. This finding is consistent with our previous study, which showed that obesogenic diet-induced cardiac hypertrophy in female mice is not associated with myocardial fibrosis [31]. Furthermore, we found that Set7 deletion did not alter cardiac collagen content in female mice. Previous researchers have demonstrated that Set7 inhibition or knockdown attenuates fibrosis in different organs [55–57], since this enzyme controls the expression of diverse extracellular matrix proteins, including Col1a1, Ctgf, and Serpine1 [16]. It would be interesting to investigate whether a long-term exposure to an obesogenic diet might affect myocardial fibrosis.

Our findings revealed that Set7 deletion did not alter the echocardiographic or hemodynamic profile in female mice, suggesting that this enzyme does not play a significant role for maintaining cardiovascular homeostasis in the basal condition. Nonetheless, both WT and Set7KO female mice fed an obesogenic diet exhibited lower LVDP and -dP/dT at the stabilization period in an *ex vivo* Langendorff model, suggesting that obesogenic diet

reduced myocardial function. Additionally, obese WT female mice had reduced LVDP, -dP/dT, and +dP/dT after I/R, indicating that obesogenic diet impaired the recovery of cardiac function following I/R injury. These results are consistent with previous reports from our group and others, which demonstrated that obesogenic diet increases the susceptibility of the heart to I/R injury [34, 58–60]. Interestingly, Set7KO female mice fed an obesogenic diet did not display a reduction in LVDP, -dP/dT, and +dP/dT after I/R, suggesting that Set7 deletion protects against the impaired cardiac functional recovery following I/R injury. Our results are in line with previous studies that reported the involvement of Set7 in different models of ischemia. The treatment with Set7 inhibitor improved renal function both in non-diabetic and diabetic rats subjected to ischemic renal injury, indicating that Set7 plays a key role in response to renal ischemia [28]. Likewise, knockdown or inhibition of Set7 in fibroblasts subjected to hypoxia increased the expression of hypoxia-inducible factor target genes, glucose uptake and intracellular adenosine triphosphate levels [27]. Moreover, Set7 inhibition in H9C2 myoblasts submitted to hypoxia decreased ATG16L1 methylation, which activated cell autophagy and prevented cell apoptosis [42]. Therefore, it would be interesting to evaluate whether the inhibition of Set7 might play beneficial effects against cardiac ischemic injury in obese subjects.

Given that Set7 deletion improved cardiac functional recovery after I/R injury in obese female mice, we investigated whether this effect might be associated to changes in the expression of proteins related to calcium handling and apoptotic signaling. A previous study demonstrated that Set7 deletion changes adult cardiomyocyte contraction rhythmicity [44]. Considering that Serca2 is critical in the regulation of cardiac contractility and its overexpression is able to prevent the cardiac function impairment induced by I/R injury [61], we investigated the protein levels of Serca2 in the heart after I/R. Our data revealed that Serca2 levels were not modulated by Set7 deletion or obesogenic diet, suggesting that Serca2 might not be involved in the beneficial effects of Set7 deletion associated with the improved cardiac functional recovery after I/R injury in obese female mice.

Here, we also found that the protein levels of Casp1 were not modulated in the heart by obesogenic diet or Set7 deletion, suggesting that the cardiac functional recovery after I/R in the Set7KO obese female mice might not be associated with pyroptosis [62]. Bcl2 is a protein that decreases apoptosis by inhibiting Bax/Bak signaling [63]. In our study, we found reduced levels of Bcl2 in the heart of WT female mice fed an obesogenic diet. Our findings are in agreement with a previous report, which showed that Bcl2 levels are reduced in the heart of Wistar rats fed an obesogenic diet [64]. Nonetheless, the Set7KO female mice fed an obesogenic diet did not display reduced levels of Bcl2 in the heart, suggesting that Set7 deletion avoids the decrease in Bcl2 levels after I/R. Considering that the increase in Bcl2 expression is able to improve the cardiac recovery after I/R injury [65], it is possible that the lack of reduction in Bcl2 levels in the heart of Set7KO obese female mice may be associated with the lack of impaired cardiac functional recovery following I/R. However, future functional studies are needed to evaluate whether Bcl2 mediates, at least in part, the beneficial effects of Set7 deletion in myocardial functional recovery after I/R in obese female mice.

A previous study showed that Stat3, a transcription factor with cardioprotective effects [45], is phosphorylated, methylated and inhibited by Set7 [46]. Interestingly, activation of Stat3 leads to overexpression of Bcl2 [66]. Here, we found that Stat3 phosphorylation levels were unchanged by the obesogenic diet or Set7 deletion, suggesting that Stat3 might not be involved in the beneficial effects of Set7 deletion in obese female mice after I/R injury.

Study limitations

It is important to mention that in the present study we were not able to assess the influence of Set7 in obesity-induced insulin resistance, since the obesogenic diet regimen did not affect insulin sensitivity in WT female mice. Therefore, further studies are needed to better characterize the functional role of Set7 on glucose homeostasis in response to an

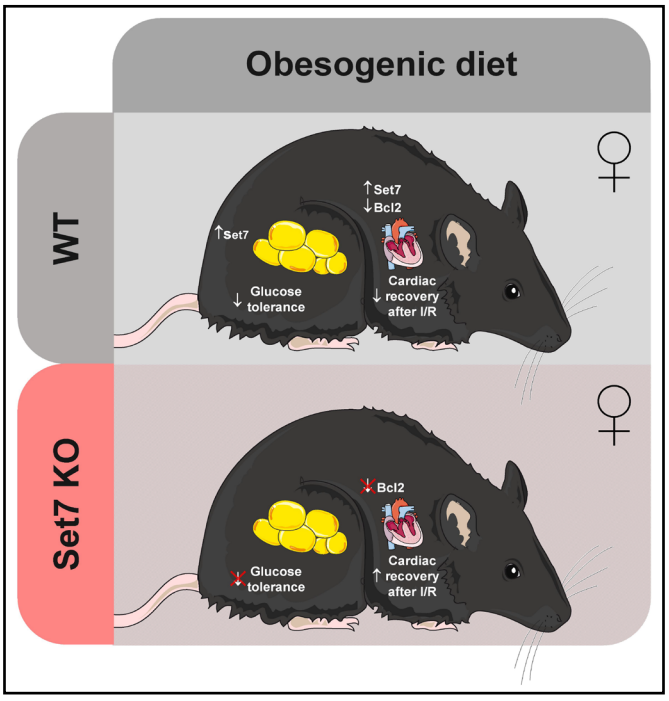

Fig. 6. Proposed model of Set7 in obesogenic diet-induced metabolic and cardiovascular disorders in female mice. Set7 protein levels are increased in the heart and PAT of female mice fed an obesogenic diet. Loss of Set7 prevents obesogenic diet-induced glucose intolerance. Set7 deletion prevents the decrease in the cardiac Bcl2 levels and the compromised cardiac functional recovery induced by obesogenic diet after I/R injury in female mice.

obesogenic diet. In addition, the mechanisms by which Set7 deletion prevented the reduction of cardiac Bcl2 levels in response to I/R in obese mice are unknown. In this sense, further studies are required to identify the mechanisms involved in the beneficial effects of Set7 deletion in obese female mice after I/R injury.

Conclusion

In summary, this study showed that Set7 protein levels are increased in the heart and PAT of female mice fed an obesogenic diet (Fig. 6). In addition, we found that Set7 does not contribute to obesogenic diet-induced obesity and cardiac hypertrophy. In contrast, deletion of Set7 prevents obesogenic diet-induced glucose intolerance and improves cardiac functional recovery after I/R injury in female mice, suggesting that Set7 plays a role in obesity-related cardiometabolic disorders.

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The data of this study are available upon reasonable request to the corresponding author.

Author Contributions

Juliane Miranda: Performed experiments, data analysis and writing - draft. Guilherme Lunardon: Performed experiments, data analysis and writing - review & editing. Vanessa Lima: Performed experiments, data analysis and writing - review & editing. Tábatha de Oliveira Silva: Performed experiments, data acquisition and writing - review & editing. Caroline A. Lino: Performed experiments, data acquisition and writing - review & editing. Leonardo Jensen: Performed experiments and data acquisition. Maria Cláudia Irigoyen: Methodology and writing - review & editing. Ivson Bezerra da Silva: methodology, data acquisition and writing - review & editing. Yao Wei Lu: Resources, methodology and writing - review & editing. Jianming Liu: Resources, methodology and writing - review & editing.

Jose Donato Júnior: Resources, methodology and writing - review & editing. Maria Luiza Barreto-Chaves: Resources, methodology and writing - review & editing. Da-Zhi Wang: Resources, methodology and writing - review & editing. Gabriela Diniz: conceptualization, resources, project administration, supervision, writing - review & editing, and funding acquisition.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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