miRNAs, target genes expression and morphological analysis on the heart in gestational protein-restricted offspring

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Abstract

Gestational protein restriction was associated with low birth weight, hypertension and higher prevalence of cardiac disorders in adults. Several mechanisms, including epigenetics, could be related with the cardiovascular phenotype on protein-restricted offspring. Thus, we investigated the morphological cardiac effects of gestational protein restriction and left ventricle miRNAs and target genes expression pattern in both 12-day and 16-week old gestational protein-restricted male offspring. Pregnant Wistar rats were allocated into two groups, according to protein supply during pregnancy: NP (normal protein diet- 17%) or LP (low protein diet—6%). Dams on the gestational protein-restricted diet had lower body weight gain and higher food intake. Gestational protein-restricted offspring had low birth weight, followed by rapidly body weight recovery, hypertension, and increased myocytes cross-sectional area and collagen fraction at 16-week old age. At 12-days old, miR-184, miR-192, miR-376c, miR-380-3p, miR-380-5p, miR-451, and miR-582-3p had increased expression, and miR-547 and miR-743a had decreased expression in the gestational protein-restricted left ventricle. At 16-week old, let-7b, miR-125a-3p, miR-142-3p, miR-182 and miR-188-5p had increased expression and let-7g, miR-107, miR-127, miR-181a, miR-181c, miR-184, miR-324-5p, miR-383, miR-423-5p and miR-484 had decreased expression in gestational protein-restricted left ventricle. Target predicted gene expression analysis showed higher expression of Dnmt3a, Oxct1, Rictor and Trps1 and lower expression of Bbs1 and Calml3 in 12-day old protein-restricted offspring. 16-week old protein-restricted offspring had higher expression of Adrbk1, Bbs1, Dnmt3a, Gpr22, Inppl1, and Oxct1 genes. In conclusion, gestational protein restriction was related to offspring low birth weight, increased systolic blood pressure and morphological heart alterations that could be related to early heart miRNA expression changes that perpetuate into adulthood and which are associated with the regulation of essential genes involved in cardiovascular development, heart morphology, function, and metabolism.

Introduction

Several epidemiological and experimental studies have shown associations between gestational protein restriction, low birth weight and a higher prevalence of cardiovascular disease in

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adulthood [1,2]. Initially, it was thought that the mechanisms causing cardiovascular changes in protein-restricted offspring might be secondary to the development of arterial hypertension [3] and endocrine changes, such as insulin and leptin resistance [4,5]. Alternatively, taking into account the evidence, studies have shown that primary insults in heart development itself might predispose to cardiovascular dysfunction later in life. Thus, protein restriction in the intrauterine environment results in permanent changes in cardiac structure and function [6,7]. Several authors have shown maternal protein restriction to lead to impairment in offspring cardiomyocyte proliferation and differentiation [8,9], reduction of cardiomyocyte number [10,11], fibrosis [7,13] and, ultrastructural changes, such as increased β/α- myosin heavy chain ratio in left ventricle (LV) and increased N2B/ N2BA titin isoforms in LV sarcomeres, that may lead to impaired cardiac function later in life [11,12]. However, information regarding the molecular mechanisms of the etiopathogenesis of these cardiac changes is still scarce.

MicroRNAs (miRNAs) are genomic-encoded small noncoding RNAs of approximately 22 nucleotides in length that play an essential role in post-transcriptional regulation of target gene expression [14,15]. miRNAs control gene expression post-transcriptionally by regulating mRNA translation or stability in the cytoplasm [16]. Although only recently discovered [17], it has become clear that miRNAs are critical components of diverse regulatory networks in animals [14].

Functional studies indicate that miRNAs are involved in critical biological processes during development and in cell physiology [15,18], and changes in their expression are observed in several pathologies [18,19]. Currently, it is known that miRNAs are not only involved in cardiovascular development and physiology [20,21] but also in several cardiovascular diseases [22,23]. Therefore, the expressional study of miRNAs and target genes that undergo miRNA-mediated regulation in the heart may help the understanding of the mechanisms underlying the cardiovascular phenotype on protein-restricted offspring.

This study aimed to evaluate the miRNAs and predicted gene expression pattern on rat LV in both 12-day and 16-week old gestational protein-restricted male offspring to elucidate the possible molecular mechanisms involved with the etiology of the cardiac phenotype observed in gestational protein-restricted offspring. Furthermore, we wished to evaluate the effects of maternal protein restriction on food consumption and body weight of both pregnant dams and offspring, systolic blood pressure in 16-wk old offspring and on cardiac morphometric parameters in both 12-d and 16-wk old offspring.

Material and methods

Animals and diets

The Institutional Ethics Committee on the Use of Animals (CEUA/UNICAMP) approved the experimental protocol (protocol number #315) and the general research guidelines on animal care established by the Brazilian College of Animal Experimentation (COBEA) and by NIH Guide for the Care and Use of Laboratory Animals were followed throughout the investigation. The experiments were conducted as described in detail previously [7,24] on age-matched rats of 12-week-old sibling-mated *Wistar HanUnib* rats (250–300 g). The local colonies originated from a breeding stock supplied by the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science, UNICAMP, Brazil. Male and female weanling *Wistar HanUnib* rats were housed and maintained under a 12-hour day/night cycle (lights on 06.00–19.00 h) at constant temperature (22±2˚C), with standard chow (Nuvital, Curitiba, PR, Brazil) and water available *ad libitum*. From 12 to 14 weeks of age, the animals were mated. Pregnant dams were singly-caged and randomly assigned either the regular protein diet (NP, 17% casein) or isocaloric low protein diet (LP, 6% casein) throughout the entire pregnancy. Body

weight and food intake were evaluated weekly in pregnant dams (NP: n = 21; LP: n = 31). Protein intake in each week was calculated considering the total food intake and the protein content of each diet. Birth weight and anogenital distance were measured in male offspring (NP: n = 103; LP: n = 107). Litter size was adjusted on the birth's day to eight pups per litter to ensure equal access to breastfeeding. At 12 days after birth, half of the male offspring of each dam was euthanized (NP-12d and LP-12d groups). At 21 days after birth, the remaining male offspring were weaned and caged separately. Body weight was evaluated weekly from birth to 16 weeks after birth, and food intake was assessed daily from weaning to 16 weeks after birth when they were euthanized (NP-16w and LP-16w groups).

Blood pressure measurement

The systolic blood pressure was weekly measured in conscious male offspring from 6 to 16 weeks age (NP-16w: n = 9; LP = 16w: n = 18), employing an indirect tail plethysmography method. Briefly, an indirect tail-cuff method using an electro-sphygmomanometer combined with a pneumatic pulse transducer/amplifier was used (IITC Life Science—BpMonWin Monitor Version 1.33). Measurements were conducted at the same time during the day. This indirect approach allowed repeated measurements with close correlation (correlation coefficient = 0.975) compared with direct intra-arterial recording. The mean of three consecutive readings was taken as the blood pressure.

LV weight measurement

At 12 days and 16 weeks of age, some male offspring from different litters were deeply anesthetized with a mixture of ketamine (50 mg/kg body weight, *i.p.*) and xylazine (1 mg/kg body weight, *i.p.*) and were decapitated using sharp guillotine. Heart LV was dissected (NP-12d: n = 6; LP-12d: n = 6; NP-16w: n = 8; LP-16w: n = 13), weighed and stored at -80˚C. The tibia length was also measured (NP-12d: n = 6; LP-12d: n = 3; NP-16w: n = 5; LP-16w: n = 11).

Histological analysis

At 12 days and 16 weeks of age, some male offspring from different litters were anesthetized and had the heart LV perfused with a heparinized saline solution (1%) and with a 4% (w/v) paraformaldehyde solution in 0.1M phosphate buffer (pH 7.4). After perfusion, the LV was dissected, fixed for 24 hours in the paraformaldehyde solution, and then embedded in paraplast (Sigma-Aldrich, USA). Five-micrometer-thick sections were stained with hematoxylin and eosin (HE) or picrosirius red. The measurements were performed from digital images that were collected by a video camera attached on an Olympus microscope (x40 magnification lens), and the images were analyzed by Image J software. The cross-sectional area (CSA) was measured with a digital pad and the selected cells were transversely cut so that the nucleus was in the center of the myocyte and determined as an average of at least 30 myocytes per animal (NP-12d: n = 10; LP-12d: n = 11; NP-16w: n = 10; LP-16w: n = 13). The heart interstitial collagen volume fraction, marked by picrosirius, was calculated as the ratio between the connective tissue area and connective tissue plus myocyte areas, from 30 microscope fields of digitalized images of each animal (NP-12d: n = 9; LP-12d: n = 11; NP-16w: n = 10; LP-16w: n = 13). Perivascular collagen was excluded from analysis.

Heart LV miRNA expression

Four male offspring from different litters were used in each group for the miRNA expression analysis. Total ribonucleic acid (RNA) was extracted from LV samples using Trizol reagent (Life Technologies, USA) [25]. Total RNA was quantified (Take3 micro-volume plate—Epoch

spectrophotometer; BioTek, USA). The RNA integrity was evaluated by electrophoresis on a denaturing agarose gel stained with GelRed Nucleic Acid Gel Stain (Uniscience, USA) and the RNA purity was assessed by the ratio of absorbance at 260 and 280 nm. Briefly, 450 ng RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit and Megaplex RT Primers Rodent Pool A (Life Technologies, USA), according to the manufacturer's guidelines. Complementary DNA (cDNA) was amplified using a TaqMan Rodent MicroRNA Array A v2.0 with TaqMan Universal PCR Master Mix on QuantStudio 12K Flex System (Life Technologies, USA), according to the manufacturer's instructions. Data analysis was performed using relative gene expression evaluated using the comparative quantification method [26]. The U87 gene was used as a reference gene. Mean relative quantity was calculated and miRNAs differentially expressed between groups (LP-12d versus NP-12d and LP-16w versus NP-16w) were evaluated. miRNA data have been generated following the MIQE guidelines [27].

Target prediction

In silico target, the prediction was performed for differentially expressed miRNAs using the combined analysis of three algorithms based on conservation criteria TargetScan [28], microRNA.org [29] and PicTar [30]. Results were taken from each search analysis and cross-referenced across all the three research results. To exclude the hypertension effect on gene expression, only targets predicted in both 12-day and 16-week old animals were considered. Furthermore, only targets genes expressed in cardiac tissue were used for the analysis. To offer experimental support to *in silico* predicted targets, we evaluated the gene expression by RT-qPCR and quantified the protein levels by western blot analysis.

Heart LV predicted gene expression

Total RNA was extracted from LV of eight offspring in each group using the Trizol method [25]. The total RNA quantity, purity and integrity was assessed as previously described for miRNAs expression analysis. For the cDNA synthesis, High Capacity cDNA reverse transcription kit (Life Technologies, USA) was used. For real-time PCR, 2 μl cDNA (40 ng/ μl) was added to a master mix comprising 10 μl TaqMan Fast Advanced Master Mix (Life Technologies, EUA), 1 μl primer mix and 7 μl water for reaction. Water was used in place of cDNA as a non-template control. The cycling conditions were: 50˚C for 2 minutes, 95˚C for 20 seconds, 50 cycles of 95˚C for 1 second and 60˚C for 20 seconds. Amplification and detection were performed using the StepOne Plus (Life Technologies, EUA) and data acquired using the StepOne Software v2.1 (Life Technologies, EUA). Ct values were converted to relative expression values using the ΔΔCt method with offspring heart data normalized to GAPDH as a reference gene. IDT Integrated DNA Technologies provided primers for mRNA RT-qPCR.

Western blot analysis

Fifteen animals in each group were used to perform the protein level analysis by western blot. LV was homogenized in solubilization buffer (100mM Tris-hydroxymethil-aminomethane pH7.4, 10mM sodium pyrophosphate, 100mM sodium fluoride, 10mM ethylenediaminetetra-acetic acid, 10mM sodium vanadate, 2mM phenylmethylsulfonyl fluoride and 0,1mg/ml aprotinin) using a polytron PTA 20S generator (model PT 10/35) Brinkmann Instruments, Westbury, N.Y., USA) at maximum speed. The tissue extracted were incubated with 10% volume Triton-X 100 and then centrifuged at 11.000 rpm at 4˚C for 40 minutes. Supernatant proteins were quantified using Biuret method. The samples were mixture with Laemmli buffer containing 100mM dithiothreitol, heating at 95˚C for 5 minutes. Each sample (120ug of protein) were subjected to gel electrophesis in Bio-Rad minigel apparatus (Mini-Protean

SDS-Page, Bio-Rad Laboratories, Hercules, C.A., USA). Electrotransfer of proteins from the gel to the nitrocellulose membranes was performed for 90 minutes at 120V. Non-specific protein binding was reduced by incubating the membrane for 1 hour at ambient temperature in blocking buffer (5% bovine serum albumin (BSA), 10mM Tris, 150mM NaCl and 0,02% Tween 20). Primary antibodies were diluted in 3% BSA, 10mM Tris, 150mM NaCl and 0,02% Tween 20. Secondary antibodies were diluted in 1% BSA, 10mM Tris, 150mM NaCl and 0,02% Tween 20. Antibodies used were: Bbs1 (sc-134455, Santa Cruz Biotechnology, Santa Cruz, CA, rabbit polyclonal, 1:500); Calml3 (ab155130, Abcam, Cambridge, MA, rabbit polyclonal, 1:500); Dnmt3a (ab23565, Abcam, rabbit polyclonal, 1:500); Oxct1 (ab105320, Abcam, rabbit polyclonal, 1:500); Rictor (sc-99004, Santa Cruz, rabbit polyclonal, 1:500); Trps1 (sc-26974, Santa Cruz, goat polyclonal, 1:500); Alpha-tubulin (#2144, Cell Signalling Technology, Dancers, MA, rabbit polyclonal, 1:1000); Goat-anti-rabbit (31460, Pierce Biotechnology, Waltham, MA 1:5000) and Rabbit-anti-goat (31402, Pierce Biotechnology, 1:5000). Immunoreactive bands were detected using the chemiluminescence method (SuperSignal West Pico Chemilluminescent Substrate, Thermo Scientific, USA). Images of the developed radiographs were scanned (HP Deskjet Ink Advantage 4625) and the bands intensities were quantified by optical densitometry using the Scion Image software.

Statistical analysis

Data are expressed as the mean ± standard deviation or as the median with interquartile range [lower quartile—upper quartile] and was previously tested for normality and equality of variance. Comparisons between two groups were performed using Student's t-test when data were normally distributed and the Mann-Whitney test when distributions were non-normal. Comparisons between two groups through the weeks were performed using 2-way ANOVA for repeated measurements test, in which the first factor was the protein content in pregnant dam's diet and the second factor was time. When an interaction was found to be significant, the mean values were compared using Tukey´s post hoc analysis. Significant differences in miRNA expression were detected using a moderated t-test. Data analysis was performed with Sigma Plot v12.0 (SPSS Inc., Chicago, IL, USA). The significance level was 5%.

Results

Effect of protein restriction on body weight and food intake of pregnant rats

Dams on LP diet during pregnancy were lighter on second and third weeks of pregnancy compared with dams on NP diet, despite an equal body weight in the first week of pregnancy ($p_{diet\ x\ time} < 0.001$; $p_{diet} = 0.007$; $p_{time} < 0.001$; Fig 1A). Thus, considering the entire pregnancy weeks, dams in the LP group had lower weight gain than those in the NP group (NP (n = 21): 109.86 ± 20.04 g; LP (n = 31): 87.00 ± 14.01 g; p<0.001).

The weekly food intake was higher in LP dams in the first two weeks of pregnancy compared to NP dams ($p_{diet\ x\ time} = 0.018$; $p_{diet} < 0.001$; $p_{time} < 0.118$; Fig 1B). However, in the last week of pregnancy, there was no difference in food intake between the groups. Despite the higher food intake by LP dams during pregnancy, the assessment of weekly protein intake showed that dams from the LP group were exposed to severe protein restriction during the entire pregnancy ($p_{diet\ x\ time} = 0.018$; $p_{diet} < 0.001$; $p_{time} = 0.069$; Fig 1C).

Effect of gestational protein restriction on offspring phenotype

Male offspring from LP dams had lower birth weight (p<0.001; Fig 2A) and higher anogenital distance (p = 0.018; Fig 2B) compared with offspring from NP dams. At 12 days after birth,
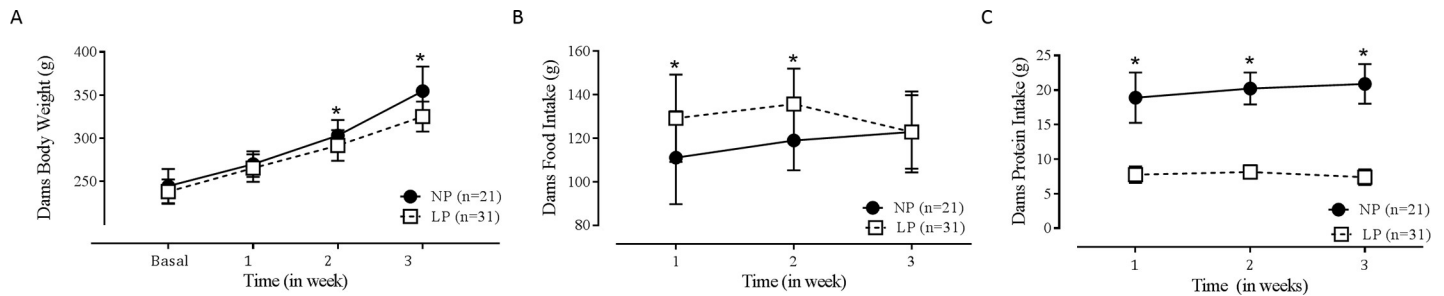
Fig 1. Body weight, food and protein intake of pregnant dams during gestation. (A) Weekly body weight; $p_{interaction} < 0.001$; $p_{diet} = 0.007$; $p_{time} < 0.001$. (B) Weekly food intake; $p_{interaction} = 0.018$; $p_{diet} < 0.001$; $p_{time} = 0.118$. (C) Weekly protein intake; $p_{interaction} = 0.018$; $p_{diet} < 0.001$; $p_{time} = 0.069$. Data were expressed as the mean ± SD. NP (n = 21): normal protein diet group; LP (n = 31): low protein diet group. *Significant difference between week-matched NP x LP groups ($p \leq 0.05$).

offspring from NP-12d and LP-12d groups showed no significant difference on body weight (p = 0.126; Fig 2C).

Measurements on offspring in the 16-week groups showed no interaction between the factors and no significant difference related to protein content in pregnant dam's diet were observed for weekly body weight ($p_{diet \, x \, time} = 0.223$; $p_{diet} = 0.173$; $p_{time} < 0.001$; Fig 3A). Analyzing the weekly food intake, although there was no interaction effect, the protein content in pregnant dam's diet influenced this variable and, in general, LP offspring had lower food intake than NP offspring ($p_{diet \, x \, time} = 0.275$; $p_{diet} < 0.001$; $p_{time} < 0.001$; Fig 3B).

An interaction between the factors protein content in pregnant dam's diet and time was found analyzing the systolic blood pressure from 6 to 16 weeks after birth. Animals from the LP-16w group had higher systolic blood pressure during 9 to 16 weeks of age compared to age-matched NP-16w group ($p_{diet \, x \, time} < 0.001$; $p_{diet} < 0.001$; $p_{time} < 0.001$; Fig 3C).

The morphometric analysis of the heart showed no differences were found for the LV weight comparing NP-12d versus LP-12d groups for both normalization to body weight (p = 0.453) and tibia length (p = 0.337) and then comparing NP-16w versus LP-16w groups for both normalization to body weight (p = 0.796) and tibia length (p = 0.259) (Table 1). Otherwise, the histologic analysis of the heart showed that the LP-16w group had higher myocyte CSA (p<0.001) and higher interstitial collagen volume fraction (p<0.001) compared to the NP-16w group (Table 1; Fig 4).
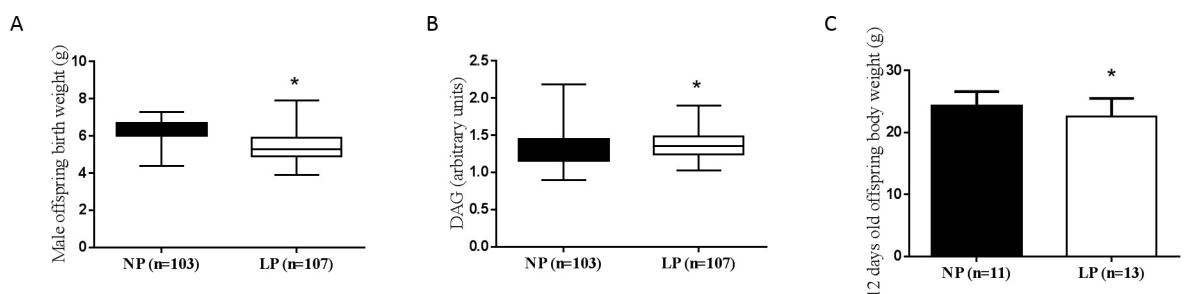
Fig 2. Male offspring birth weight, anogenital distance and male offspring 12 days old body weight. (A) Male offspring birth weight; p<0.001. (B) Male offspring anogenital distance; p = 0.018. (C) 12-days male offspring body weight; p = 0.126. Data were expressed as the median [lower quartile—upper quartile]. NP: normal protein diet group; LP: low protein diet group. *Significant difference between NP x LP groups ($p \leq 0.05$).
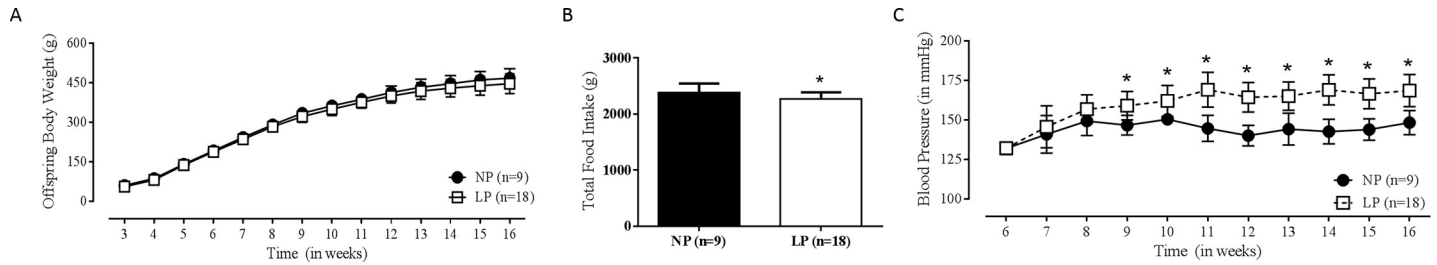
Fig 3. Body weight, food and systolic blood pressure of the 16-week old groups. (A) Weekly body weight. $p_{interaction}$ = 0.223; p_{diet} = 0.173; p_{time}<0.001. (B) Weekly food intake. $p_{interaction}$ = 0.275; p_{diet}<0.001; p_{time}<0.001. (C) Systolic blood pressure. $p_{interaction}$<0.001; p_{diet}<0.001; p_{time}<0.001. Data were expressed as the mean ± SD. NP-16w (n = 9): normal protein diet group followed until 16 weeks old; LP-16w (n = 18): low protein diet group followed until 16 weeks old. *Significant difference between week-matched NP-16w x LP-16w groups (p ≤ 0.05).

Effect of gestational protein restriction on offspring miRNA expression in early-life and adulthood

Regarding heart, left ventricle miRNA expression, protein-restricted diet during pregnancy was significantly associated with male offspring altered miRNAs expression in both early life and adulthood. LP-12d versus NP-12d miRNAs fold-change depicted by volcano-plot showed a significant change in miRNA expression in early life (S1A Fig). LP-12d group was associated with significant up-regulation of mir-184 (p = 0,007), mir-192 (p = 0,019), mir-376c (p = 0,029) mir-380-3p (p = 0,029), mir-380-5p (p = 0,028), mir-451 (p = 0,013) and mir-582-3p (p = 0,029) and significant down-regulation of mir-547 (p = 0,022) and mir-743a (p = 0,004) compared to NP-12d group (Fig 5A). Volcano plot data from LP-16w versus NP-16w depicted in S1B Fig shows a significant change in miRNA expression in adulthood. The LP-16w group had significant up-regulation of let-7b (p = 0.017), mir-125a-3p (p<0.001), mir-142-3p (p = 0.035), mir-182 (p = 0.025) and mir-188-5p (p = 0.029) and significant down-regulation of let-7g (p = 0.045), mir-107 (p = 0.021), mir-127 (p = 0.029), mir-181a (p = 0.045), mir-181c (p = 0.029), mir-184 (p = 0.029), mir-324-5p (p = 0.006), mir-383 (p = 0.002), mir-423-5p (p = 0.006) and mir-484 (p = 0,034) when compared to NP-16w group (Fig 5B).

Target prediction analysis

To test for potential mRNA targets of differentially expressed miRNAs, computational mRNA target prediction was performed. Many target mRNAs were identified for each of the miRNAs, although the number of targets varied per miRNA. By exploring the targets of the miRNAs by computational prediction, we had 165 possible mRNA targets for differentially expressed miRNA in the LP-12d group, and 281 mRNA targets for differentially expressed miRNA in the LP-16w group. Confronting the predicted mRNAs for both 12-day and 16-week old groups,

Table 1. Cardiac left ventricle weight, myocyte cross-sectional area and interstitial collagen volume fraction.

	NP-12d¤	LP-12d¤	p¤	NP-16w¤	LP-16w¤	p¤
LV weight/ Body weight (kg/g)¤	3.11±0.42 (n = 6)	2.97±0.15 (n = 6)	0.453	1.80±0.10 (n = 8)	1.81±0.12 (n = 13)	0.796
LV weight/ Tibial length (kg/mm)¤	6.36±0.63 (n = 6)	5.88±0.75 (n = 3)	0.337	20.48±1.72 (n = 5)	19.38±1.73 (n = 11)	0.259
CSA (µm²)¤	27.4±2.7 (n = 10)	27.6±0.9 (n = 11)	0.777	139.0±6.2 (n = 10)	212.5±14.5 (n = 13)*	<0.001
Interstitial collagen fraction (%)¤	0.94±0.17 (n = 9)	1.00±0.22 (n = 11)	0.553	1.24±0.26 (n = 10)	2.26±0.55 (n = 13)*	<0.001

LV: left ventricle; CSA: myocyte cross-sectional area. Data are expressed as the mean±SD. NP-12d: normal protein group followed until 12 days old; LP-12d: low protein group followed until 12 days old. NP-16w: normal protein group followed until 16 weeks old; LP-16w: low protein group followed until 16 weeks old.
*Significant difference between age-matched groups (p ≤ 0.05).
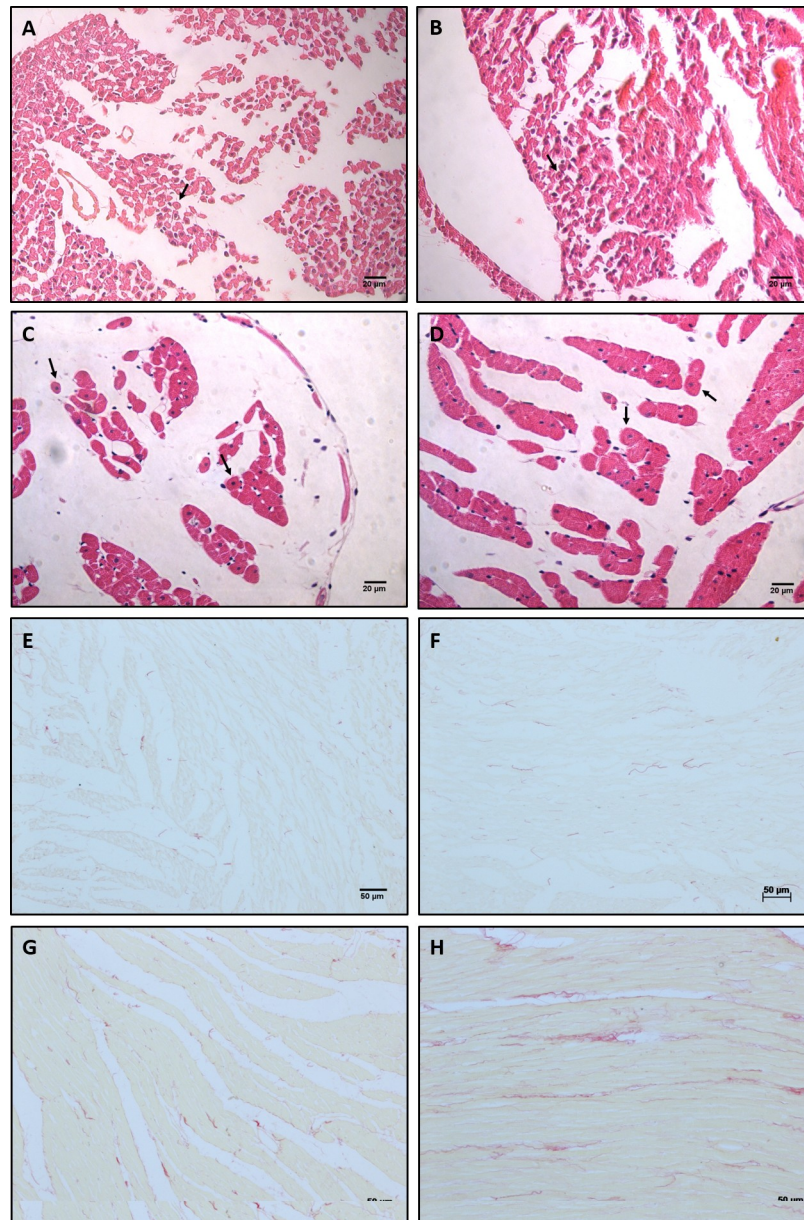
Fig 4. Histological representative images of 12 days and 16 weeks old animals. Representative myocyte cross-sectional area in (A) NP-12d; (B) LP-12d; (C) NP-16w; (D) LP-16w; Representative interstitial collagen fraction in (E) NP-12d; (F) LP-12d; (G) NP-16w; (H) LP-16w.

https://doi.org/10.1371/journal.pone.0210454.g004

we had 54 possible targets predicted simultaneously for both groups. Furthermore, selecting only the mRNA targets expressed in heart, we had 24 possible mRNA targets. Table 2 shows all the 24 mRNA targets considered for the analysis and the respective regulatory miRNA.

Experimental support for predicted regulatory targets: Target's mRNA RT-qPCR analysis

miRNAs can regulate post-transcriptional gene expression by targeting mRNAs for degradation. To explore the potential extent of miRNA-directed regulation of mRNA levels, RT-qPCR

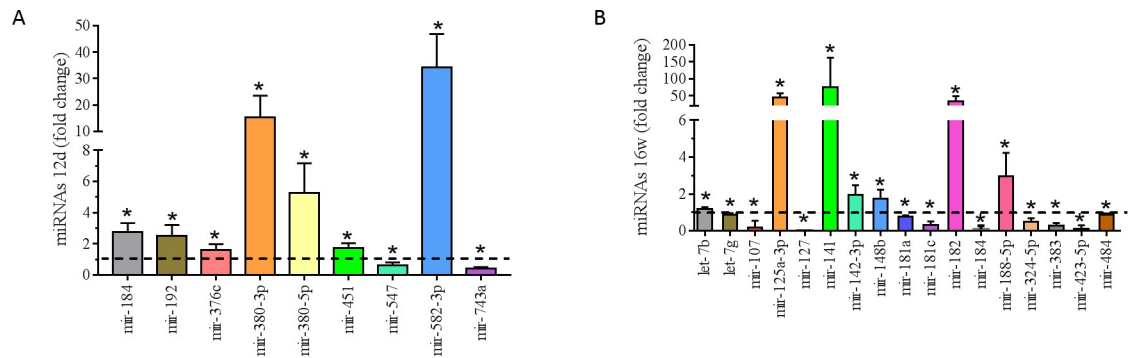**Fig 5. Differentially expressed miRNA of 12 days and 16 weeks old animals.** (A) Fold-change and miRNA expression values in LP-12d (n = 4) versus NP-12d (n = 4); (B) Fold-change and miRNA expression values in LP-16w (n = 4) versus NP-16w (n = 4). *Significant difference between week-matched groups (p ≤ 0.05).

was used to measure mRNAs predicted to be targeted by the differentially expressed miRNA. The sequences of the primers used are shown in S1 Table. The results of the RT-qPCR analysis showed that the expression of Bbs1 and Calml3 genes were downregulated and that the expression of Dnmt3a, Oxct1, Rictor and Trps1 genes were upregulated in LP-12d versus NP-12d animals (Fig 6A). Furthermore, the expression of Adrbk1, Bbs1, Dnmt3a, Gpr22, Inppl1, and Oxct1 genes were upregulated in LP-16w versus NP-16w animals (Fig 6B). The expression of the other analyzed genes did not differ between groups.

Experimental support for predicted regulatory targets using western blot analysis

We quantified encoded proteins of genes whose expression was changed in the 12d groups thereby to exclude the possible effect of hypertension in the modulation of gene expression. The results of western blot analysis showed that LP-12d animals had lower levels of Bbs1 (NP-12d: 100.0±1.3; LP-12d: 94.7±1.8; p = 0.027) and Calml3 (NP-12d: 100.0±4.33; LP-12d: 84.3 ±4.1; p = 0.019) proteins in LV than NP-12d animals. Dnmt3a (NP-12d: 100.0±4.6; LP-12d: 141.3±11.5; p = 0.017) and Oxct1 (NP-12d: 100.0±0.9; LP-12d: 112.3±3.0; p = 0.037) proteins levels in LV were higher in LP-12d than NP-12d animals. No significant difference between these groups was found for Rictor (NP-12d: 100.0±2.6; LP-12d: 106.4±3.9; p = 0.176). The LP-16w animals had higher levels of Bbs1 (NP-16w: 100.3±3.4; LP-16w: 111.6±3.1; p = 0.002) and Oxct1 (NP-16w: 100.0±3.1; LP-16w: 133.3±12.7; p = 0.037) proteins in LV compared to NP-16s animals. No significant difference between these groups was found for Calml3 (NP-16w: 100.0±3.0; LP-16w: 102.3±4.2; p = 0.232), Dnmt3a (NP-16w: 100.0±5.2; LP-16w: 108.3±7.5; p = 0.424) and Rictor (NP-16w: 100.0±5.1; LP-16w: 102.6±5.8; p = 0.749) (Fig 5). Trps1 protein was not detected (Fig 7).

Discussion

In the present study, protein-restricted offspring showed altered expression of a large number of heart LV miRNAs and predicted target gene expression was observed in both early life and adulthood LP offspring. Additionally, LP offspring had low birth weight, higher systolic blood pressure and changes in cardiac LV morphological parameters in adulthood compared with age-matched NP rats. Furthermore, this study showed that protein-restricted dams had a lower body mass gain and higher food consumption during pregnancy compared to NP rats. These results are supported by previous studies that showed the orexigenic stimulus and

Table 2. Predicted mRNAs for the differentially expressed miRNAs in NP-12d versus LP-12d and NP-16w versus LP-16w groups.

Gene symbol	Gene Name	Differentially expressed miRNA that could regulate the gene	
		NP-12d/ LP-12d	NP-16w/ LP-16w
Adrbk1	*Adrenergic receptor kinase, beta 1*		mir-423-5p
Akap12	*A kinase (PRKA) anchor protein (gravin) 12*	mir-184	mir-184
Amotl1	*Angiomotin-like 1*	mir-184	mir-184
Bbs1	*Bardet-Biedl syndrome 1 (human)*	mir-184	mir-184
Calml3	*Calmodulin-like 3*	mir-743a	
Dab2	*Disabled 2, mitogen-responsive phosphoprotein*	mir-743a	
Dnmt3a	*DNA methyltransferase 3A*	mir-582-3p	mir-423-5p mir-484
Gpr22	*G protein-coupled receptor 22*	mir-192	mir-182
Hbegf	*Heparin-binding EGF-like growth factor*	mir-376c	mir-127
Hic2	*Hypermethylated in cancer 2*	mir-547	mir-127 mir-125a-3p mir-484
Inppl1	*Inositol polyphosphate phosphatase-like 1*	mir-184	mir-184
Insr	*Insulin receptor*	mir-743a mir-582-3p	
Jcad	*RIKEN cDNA 9430020K01 gene*	mir-743a	mir-423-5p
Mcf2l	*Mcf.2 transforming sequence-like*	mir-184	mir-184
Mmp8	*Matrix metallopeptidase 8*	mir-184	mir-184
Nfat5	*Nuclear factor of activated T cells 5*	mir-380-5p	mir-324-5p
Odc1	*Ornithine decarboxylase, structural 1*	mir-743a	mir-423-5p
Oxct1	*3-oxoacid CoA transferase 1*	mir-743a	mir-324-5p
Ppp2ca	*Protein phosphatase 2, catalytic subunit*	mir-547	mir-141
Rictor	*RPTOR independent companion of MTOR, complex 2*	mir-192	let7g mir-142-3p mir-188-5p
Sirt1	*Sirtuin 1*		mir-141
Tgfbr1	*Transforming growth factor, beta receptor I*		mir-125a-3p
Trps1	*Trichorhinophalangeal syndrome I*	mir-547	mir-484
Ubn1	*Ubinuclein 1*	mir-184	mir-184

NP-12d: normal protein group followed until 12 days old; LP-12d: low protein group followed until 12 days old. NP-16w: normal protein group followed until 16 weeks old; LP-16w: low protein group followed until 16 weeks old.

reduced mass gain on protein-restricted rats compared to isocaloric normal protein rats [31,32,33].

In protein-restricted chow, the carbohydrate content is approximately 15% higher than in normoproteic standard rodent chow. In this way, previous reports have shown that gastric emptying in LP animals is faster than in NP animals and consequently, the orexigenic signaling for food intake is rapidly triggered [32,33]. However, despite the increased food consumption, the experimental protein-restricted model proposed in the present study was kept.

In rats, the anogenital distance can be influenced by the embryo position in the womb, as well as by the sex of surrounding embryos due to the action of released steroid hormones [34]. Furthermore, anogenital distance is a sensitive marker of hormonal changes in rodents, especially from high and persistent steroid serum levels [35]. The placental enzyme 11beta-hydroxysteroid dehydrogenase (11β-HSD) type 2 catalyses the interconversion of maternal active corticosterone with inert 11-dehydrocorticosterone. In gestational protein restricted models,
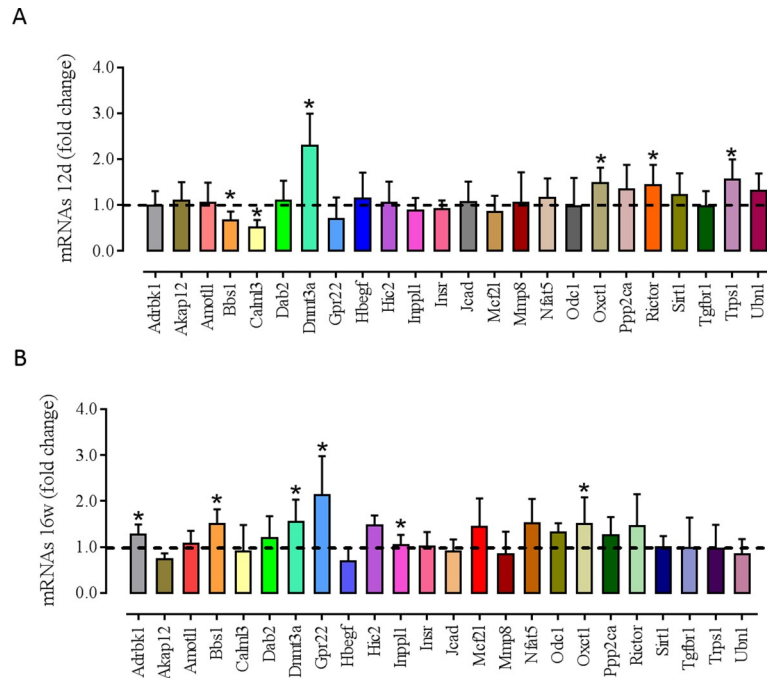
A

B

Fig 6. Targets mRNA expression of 12 days and 16 weeks old animals. (A) Fold-change of mRNA expression in LP-12d (n = 8) versus NP-12d (n = 8); (B) Fold-change of mRNA expression in LP-16w (n = 8) versus NP-16w (n = 8). *Significant difference between week-matched groups (p ≤ 0.05).

the lower concentration and decreased the activity of 11β-HSD2 enzyme is one of one of the mechanisms involved in the high exposure of the fetus to maternal glucocorticoids [36]. Thus, early fetal exposure to higher maternal glucocorticoids levels in gestational protein-restricted offspring may be responsible, at least in part, for the increased anogenital distance observed in protein-restricted offspring.

Gestational protein restriction is also associated with decreased intrauterine growth and low birth weight [10,31]. In the current study, we confirm these results, and we show that male offspring birth weight from protein-restricted dams was lower when compared to offspring from dams fed with a standard protein chow. The body mass was assessed from birth up to 16 weeks of life, and beyond the second week of age, we did not find a significant difference between LP and age-matched NP offspring. The recovery of offspring body mass after delivery in protein-restricted dams, known as "catch-up" is associated with a higher growth rate compared to the normal growth curve [37]. Furthermore, studies have demonstrated that fast mass gain after birth, in maternal LP offspring, is itself a risk factor for the development of hypertension [38], reduced peripheral insulin sensitivity and disorder in insulin secretion [39], increased predisposition to obesity [40], metabolic syndrome [41] and increased cardiovascular risk [42]. Despite the occurrence of "catch-up" growth in 16-wk old LP offspring in the present study, this change was not related to increasing postnatal food intake when compared to age-matched NP animals.

16-wk old LP offspring showed an increased systolic blood pressure from the 9th week onwards when compared to age-matched NP animals. Several mechanisms may influence the development of hypertension in adults submitted to protein restriction during the intrauterine period. Clinical and experimental studies show that low birth weight due to both intrauterine growth restriction (IUGR) and maternal protein-restricted diet, are related to the reduction in
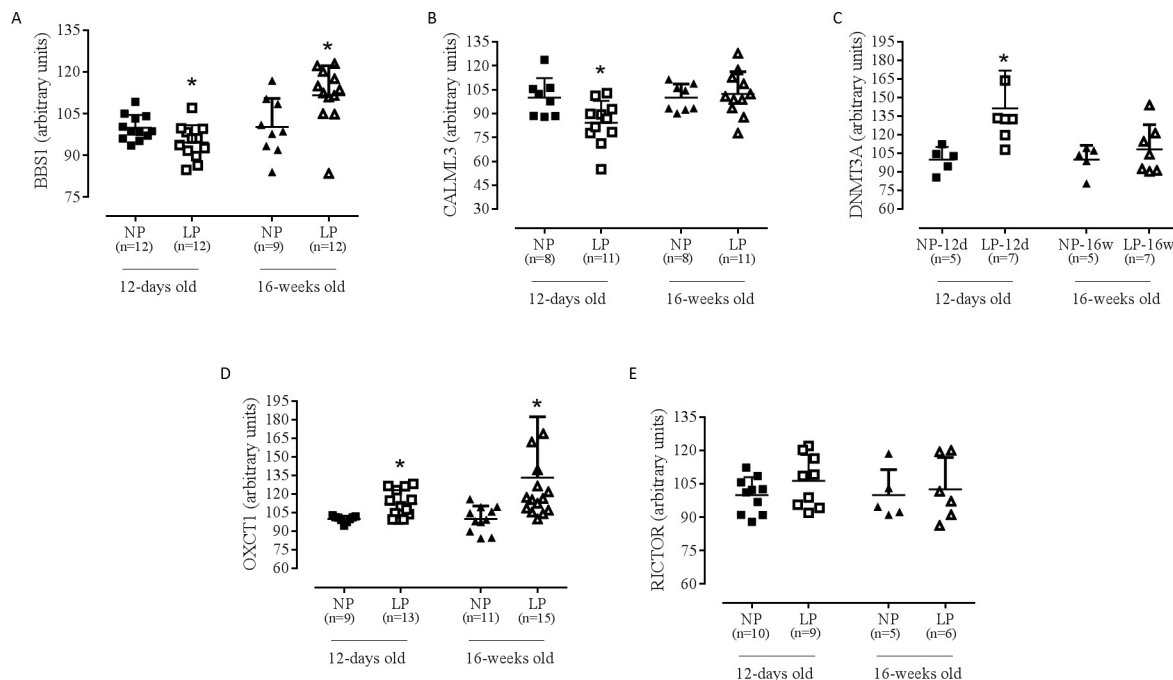
Fig 7. Western blot analysis of BBS1, Calml3, Dnmt3a, Oxct1 and Rictor of 12 days and 16 weeks old animals. (A) BBS1 in LP-12d (n = 12) versus NP-12d (n = 12) and LP-16w (n = 12) versus NP-16w (n = 9); (B) Calml3 in LP-12d (n = 11) versus NP-12d (n = 8) and LP-16w (n = 11) versus NP-16w (n = 8); (C) Dnmt3a in LP-12d (n = 7) versus NP-12d (n = 5) and LP-16w (n = 7) versus NP-16w (n = 5); (D) Oxct1 in LP-12d (n = 13) versus NP-12d (n = 9) and LP-16w (n = 15) versus NP-16w (n = 11); (E) Rictor in LP-12d (n = 9) versus NP-12d (n = 10) and LP-16w (n = 6) versus NP-16w (n = 5). Data were expressed as the mean ± SD *Significant difference between week-matched groups (p ≤ 0.05).

the number of nephrons [24,43]. This kidney change, in turn, may alter glomerular hyperflow/hyperfiltration. Renal hyperperfusion/hyperfiltration accelerates glomerulosclerosis that naturally occurs with aging. The early loss of functional kidney units feeds a vicious cycle that perpetuates itself and determines the progressive increased renal retention of sodium and water and, consequently, enhanced arterial blood pressure [12,44]. However, the mechanisms related to hypertension development due to maternal nutritional impairment are complex and multifactorial. Although the impairment of nephrogenesis was associated with the hypertensive framework, fetal overexposure to glucocorticoids is a crucial component of this process [45]. Furthermore, endothelial dysfunction and loss of modulatory function performed by the vascular endothelium appear to be another critical element to the etiology of hypertension [46].

Regarding the evaluation of heart LV morphological findings, the current study has not shown any change in a whole organ or LV weight in both 12-day and 16-wk old LP offspring. However, the cardiac histological analysis in 16-wk old gestational protein-restricted rats showed a striking increase in the myocyte cross-sectional area associated with interstitial collagen expression in the LV. The literature is controversial about the heart weight of gestational protein-restricted offspring. In the rodent model, lower heart weight is often reported [10,11]. Otherwise, both higher [47] and equal heart weight [13,31] have also been reported in rats. These discrepant results may be related to several factors such as different strains used, protein-restricted intake levels, a period of hypoprotein diet restriction as well as differences in postnatal growth and arterial pressure values of LP offspring [37].

Additionally, the present study confirms previous studies showing the higher collagen content in heart LV in gestational protein-restricted rats compared with age-matched NP

offspring [7,13]. The higher accumulation of collagen in the LV may compromise the myocardium elasticity and could be associated with functional cardiac disorders in adulthood [48]. Several authors have suggested that fibrosis occurs by hemodynamic overload imposed by arterial hypertension development. Furthermore, higher apoptosis [49] and reduced cardiomyocyte number [10] may explain the cardiomyocyte hypertrophy accompanied by increased collagen deposition in the left ventricle in LP offspring.

Regarding the miRNAs expression analysis, this work has identified nine miRNAs differentially expressed in 12-day old LP compared to age-matched NP offspring (upregulated miRNAs: mir-184, mir-192, mir-376, mir-380-3p, mir-380-5p, mir-451, mir-582-3p and, downregulated miRNAs: mir-547, mir-743a) and fifteen differentially expressed miRNAs in 16-wk old LP (upregulated miRNAs: let-7b, mir-125a-3p, mir-182, mir-188-5p and, downregulated miRNAs: let-7g, mir-107, mir-127, mir-181a, mir-181c, mir-184, mir-324-5p, mir-383, mir-423-5p, mir-484) compared to age-matched NP rats. Identification and validation of miRNA targets are of fundamental importance to gain a comprehensive understanding of miRNA function on modulation of cardiac phenotype in the present animal model of gestational protein restriction.

Analyzing the 12-day LP group, we observed the translation modulation of the following mRNAs encoding proteins Bbs1, Calml3, Dnmt3a, Oxct1, Rictor and Trps1. Furthermore, analyzing the 16-wk old LP group, we observed the translation modulation of the following mRNAs encoding proteins Adrbk1, Bbs1, Dnmt3a, Gpr22, Inppl1, and Oxct1. Then, to exclude the possible bias due to hypertension in the LP-16w group, we evaluated the levels of proteins encoded by the genes that had altered expression in an LP-12d group versus NP-12d group. Thus, we performed western blot analysis to quantitate the level of Bbs1, Calml3, Dnmt3a, Oxct1, Rictor and Trps1 proteins. Trps1 protein level was not detected. The LP-12d group had lower Bbs1 and Calml3 protein levels and higher Dnmt3a and Oxct1 protein levels compared to the NP-12d group. Rictor protein level was similar in both LP-12d and NP-12d groups. The lp-16w group had higher Bbs1 and Oxct1 protein levels compared to the NP-16w group. Calml3, Dnmt3a and Rictor proteins levels did not differ between LP-16w and NP-16w animals.

Thus, it is evident that for some mRNAs targets the result of expression and protein level analysis was different from that expected by the respective miRNA analysis. Despite the lower levels of miR-743a, the expression and the protein level of the predicted target Calml3 gene was surprisingly lower in LP-12d versus NP-12d group. Similarly, the higher expression and protein level of Dnmt3a in LP-12d versus NP-12d groups was contrary to the expected higher expression of miR-582-3p. Also, the higher expression of miR-192 in LP-12d group disagreed with the higher expression and with the unchanged Rictor protein level in LP-12d versus NP-12d group. Furthermore, the higher expression of miR-182, the higher expression of the predicted target Gpr22 gene was surprisingly higher in LP-16w versus NP-16w group.

Several factors may explain this discrepancy between the expected and the obtained results after the analysis of miRNAs and their predicted targets expression. First, although not widely applicable, studies have suggested that miRNAs could also act as positive regulators of transcription [50,51]. Furthermore, it is evident that even the best available algorithms fail to identify a significant number of miRNA-gene interactions [52]. miRNA target prediction currently manages to detect 60% of all available targets and to provide one valid target in approximately every three predicted targets [53]. Finally, miRNAs integrate a high complexity network of gene expression regulation, and they have the potential to regulate a large part of the transcriptome [54]. Thus, each miRNA could regulate the expression of several mRNAs' targets, and the expression of each mRNA target could be potentially regulated by several miRNAs [55]. However, despite these discrepancies, in both 12-day and 16-week old animals, gestational

protein restriction induces differential miRNAs expression seems to have a modulatory function on the expression of specific genes that has been associated to cardiac morphology, metabolism, and function.

The Adrbk1 protein, also known as Grk2, under normal conditions, acts together with β-arrestin to promote the desensitization, internalization and reduce expression of β-adrenergic receptors after catecholamine stimulus [56]. However, hypertension [57] and heart failure [58] are associated with increased expression and activity of Grk2, which are initially linked to the prevention of excessive β-adrenergic stimulation. However, with chronic stimulation, a vicious cycle begins, and increasingly high levels of Grk2 contribute to heart failure progression [56]. Furthermore, the Grk2 expression is related to insulin resistance and increased mitochondrial stress [59, 60]. In fact, LP offspring have reduced β-adrenergic responsiveness and attenuated adrenergic and insulin signalling [61]. Thus, higher expression of this gene in 16-week old protein restricted animals could be related to higher systolic blood pressure, evidenced since the ninth week of life.

The Inppl1 gene encodes a Ship2 protein that acts as a negative regulator of the insulin signaling pathway, decreasing the insulin sensitivity due to inhibition of Glut4 translocation [62]. Also, the Ship2 function is related to the inactivation of the PI3K-Akt signaling pathway [63]. Furthermore, Ship2 acts directly as docking protein to cytoskeletal proteins, focal adhesion proteins, and receptors associated with phosphatase and tyrosine kinase proteins [64,65]. In fact, several authors have been shown that gestational protein restriction is related to impaired glucose homeostasis, hyperinsulinemia and insulin resistance in adulthood [66,67,68].

Bbs1 protein is a structural component of cilia basal body and features a well-characterized role in the ciliary formation, stability, and function [69]. The bbs1 expression is related to reduced expression of insulin and leptin receptor plasmatic [70,71]. Furthermore, Bbs1 gene mutation is associated with higher susceptibility to congenital cardiac defects [72], heart valves and atrioventricular canal defects, dextrocardia and dilated cardiomyopathy [73].

Dnmt3a protein is one component of the DNA methylation epigenetic mechanism and, together with Dnmt3b and Dnmt3l are responsible for the methylation pattern establishing genomic DNA during the initial embryogenesis [74]. DNA methylation dynamics are essential during cardiovascular development as well as in the progression of cardiovascular disease. Heart failure in mice was associated with altered DNA methylation pattern that resembles the newborn pattern [75]. In an infarction rat model, Dnmt3a expression is increased due to the lower expression of mir-29a and mir-30c, and these changes correlate with post-ischemic tissue remodeling [76]. Furthermore, increased Dnmt3a expression is associated with lower RASSF1A expression in cardiac fibroblasts inducing, thereby, cardiac fibrosis [77]. Thus, the higher collagen accumulation in 16-week old protein restricted animals could be, at least in part, due to Dnmt3a action.

Oxct1 protein is a critical component of the ketone body's metabolism [78]. Although fatty acids are the primary energy substrate for myocardium [79], ketone body metabolism is physiologically crucial during the neonatal period [80]. In new-born rodents, ketogenesis is related to reduced white adipose tissue and altered availability of substrates after birth, since, during lactation, the availability of lipids is greater than carbohydrates while in the intrauterine period, the opposite availability occurs [78,80]. Furthermore, the heart energy demand increases after birth [78]. Ketone body metabolism is also essential in heart failure [78], and the change in energy substrate after cardiac injury seems to be a protective role against cardiac injury and ventricular remodeling [81]. However, the understanding of the pathophysiology of this metabolic switch as well as the context in which these changes are adaptive or maladaptive is limited [82]. Hepatic ketogenesis is stimulated, and plasma levels of ketone bodies increase in a heart failure model proportional to increase in blood pressure, leading to a reduction in

fatty acids oxidation and increase in ketone body oxidation during the progression of cardio-myopathy [82].

Thus, we conclude that all morphological heart alterations that were observed here in the protein-restricted offspring could be, at least in part, due to changes in cardiac miRNA expression. Some of the miRNAs differentially expressed in gestational protein restriction modulate the expression of several genes whose function is associated with cardiac morphogenesis and morphology by regulating cell polarity, the cytoskeletal dynamics and intracellular trafficking, cell proliferation, and growth, extracellular matrix deposition, and apoptosis. Furthermore, some miRNAs differentially expressed in this experimental model modulate the expression of genes whose function is associated with cardiac metabolism and function in the cardiovascular system. Although several studies have determined a close relationship between abnormal miRNA expression and human cardiac functional disorders, as far as we know, our study is the first description of changes in miRNA expression caused by gestational protein restriction that may modulate heart structure in early life and cause disease onset in later life.

Supporting information

S1 Fig. Volcano plot analysis of 12 days and 16 weeks old animals. (A) MiRNAs volcano plot in LP-12d versus LP-12d groups; (B) MiRNAs volcano plot in LP-16w versus LP-16w groups. Balls above the red dashed line indicates the miRNAs differentially expressed between groups.
(DOCX)

S2 Fig. Western blot gels. (A) BBS1 blots of 12 days old offspring; (B) BBS1 blots of 16 weeks old offspring; (C) Calml3 blots of 12 days old offspring; (D) Calml3 blots of 16 weeks old offspring; (E) Dnmt3a blots of 12 days old offspring; (F) Dnmt3a blots of 16 weeks old offspring; (G) Oxct1 blots of 12 days old offspring; (H) Oxct1 blots of 16 weeks old offspring; (I) Rictor blots of 12 days old offspring; (J) Rictor blots of 16 weeks old offspring.
(DOCX)

S1 Table. Primers sequences.
(DOCX)

S2 Table. Targets mRNA expression values of NP-12d, LP-12d, NP-16w and LP-16w groups.
(DOCX)

S1 File. Dataset.
(XLSX)

Acknowledgments

Author Contributions

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Writing – review & editing: José Antonio Rocha Gontijo, Patrícia Aline Boer.

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