Electronic supplementary material (ESM)

ESM methods

1. Breeding protocol and study protocol of eNOS deficient mice

eNOS knockout mice of the C57BL/6J strain and their wild-type (wt) littermate were obtained from Gödecke et al. who described the whole procedure of their generation [1]. These animals were bred in-house according to the procedure described in the ESM Fig.1. Study design and experimental protocols were conducted according to the local institutional guidelines for the care and use of laboratory animals and approved by the animal welfare ethical committee of the state of Berlin.

Male and female offspring were kept for 24 weeks and analysed separately. Body weight, length and abdominal diameter of the F2 generation were measured daily until day 13, thereafter body weight daily until day 40 and weekly hereafter until week 20 of the experiment. Blood pressure was measured using the tail cuff method as previously described in week 24 [2]. Plasma creatinine was measured in week 23. Fasting glucose testing was performed in study week 21 and an intraperitoneal glucose tolerance test (IPGTT) in study week 24. Regarding IPGTT, the animals were fasted overnight, injected intraperitoneally with 2mg Glucose/g body weight, then blood samples were collected through the tail vein at 0, 15 and 60 minutes to measure plasma glucose and insulin as described previously [3-5]. Collecting blood samples after longer duration i.e 90 or 120 minutes was not done accounting for animal welfare and to avoid potential loss of animals.

2. Effects of nitric oxide deficiency on sperm development and epigenetic alterations in the sperm

A total of 30 C57BL/6J male mice were randomized into three groups and treated for a consecutive 12 weeks. The control group was given normal drinking water (n =10). The second group received drinking water containing L-NAME (0.15 mg/ml, approximately 15 mg/kg/day; n = 10; Sigma, St Louis, MO, USA). The third group received drinking water containing L-NAME (2 mg/ml, approximately 200 mg/kg/day; n =10). Daily water consumption was estimated individually for every animal 1 week before the experiment. A regular chow diet was used to feed all experimental subjects.

3. Sperm total DNA methylation
Mature sperm was isolated from cauda epididymis. Sperm total DNA methylation was performed as described before [6]. Briefly, DNA was extracted using a QIAamp DNA Mini Kit from Qiagen (Hilden, Germany). The concentration and quality of the RNA-free DNA solution were determined by a NanoDrop ND-1000 spectrophotometer. DNA hydrolysis was carried out using DNA Degradase Plus from Zymo Research (Freiburg, Germany). DNA methylation was assessed by liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC-ESI/MS/MS) technique as described previously [7].

4. Sperm count and small RNA libraries construction

Mature sperm were isolated from cauda epididymis of male C57BL/6J mice and processed for RNA extraction as previously described [8, 9]. In brief, sperm were released from cauda epididymis into 5ml phosphate buffered saline (PBS) maintained at 37°C for 15 min incubation, then 10 ul semen was taken and placed on a MAKLER sperm counting plate and observed by an optical microscope (Olympus BX 53) at ×400 magnification to assess sperm concentration. After incubation, nylon mesh (pore size: 70 mm) was used to filter the suspension. The sperm were then treated with somatic cell lysis buffer (0.1% SDS, 0.5% Triton X in DEPC H2O) for 40 min on ice to eliminate somatic cell contamination, after which the sperm be pelleted by centrifugation at 600g for 5 minutes. after removal of suspension, the sperm pellet was resuspended and washed twice. The sperm pellet was added with TRIzol reagent, homogenated, followed by RNA extraction. Small RNA libraries were constructed according to Small RNA Sample PreKit (Illumina), the small RNA libraries were prepared followed by library quality validation for sequencing.

Deep sequencing, quality control and Small RNA - seq data analysis

For each RNA library, 10 million reads (raw data) were generated by Illumina HiSeq. After quality control, small RNA tags were mapped to mouse genome to analyze their distribution and expression on the genome and annotated with miRNA, tRNA, rRNA and other small noncoding RNA from miRBase19, Genbank and Rfam databases using blastn. To analyze differential expression of small RNAs between L-NAME treated and normal mice sperm, miRNA reads were normalized by TPM (transcripts
per million reads. Those miRNAs that had P value smaller than 0.05 and had the fold change of at least 2 were considered as significantly changed miRNAs.

5. testicular morphology

Small tissue samples of testicle were obtained, fixed and processed by routine histological techniques. Tissue sections of 5 μm thickness were stained with hematoxylin–eosin (H&E) and observed under a microscope (Olympus BX53). Sections were evaluated according to the modified Johnsen scoring system as previously described [10, 11]. The sloughing rate of maturing sperm cells is also calculated (at least 50 seminiferous tubules per sample were analyzed) as previously described [12].

6. Liver morphology

Hematoxylin and Eosin Staining was done after washing the livers in PBS buffer, fixation in 4% (w/v) paraformaldehyde in PBS, embedding in paraffin and cutting into 3 μm slices using a Microm HM230 Microtom. For liver slices, the hepatic venules and their adjacent portal fields were identified by sinusoidal connection [13]. 10 lobules of every liver were thus identified using a Zeiss (Oberkochen, Germany) Axiovert 100 microscope (200x) and photographed with a Leica EC3 digital camera using LAS EZ software (Leica, Wetzlar, Germany). Linear lobular dimensions were measured from the centre of the hepatic vein to the centre of three related portal vein branches using ImageJ (version 1.410, NIH shareware). The mean radius of lobules was calculated for each animal. The extent of lobular inflammation was graded as described previously [14]: score 0, no inflammatory foci; score 1, fewer than two foci per × 200 field; score 2, two to four foci per × 200 field; and score 3, more than four foci per × 200 field.

Red Oil Staining was done as described elsewhere [15]. 30 pictures were taken per organ using an Olympus (Shinjuku, JP) BH-2 microscope (400x) and a digital camera CFW-1310C (Scion Corporation, Frederick, MD). The lipid content and the number and size of lipid droplets were quantified with the ImageJ program.

Liver immunohistochemistry: Liver sections were deparaffinized, boiled with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) with microwave for 10 min for antigen retrieval, and incubated overnight at 4°C with rabbit polyclonal anti-mouse CD68 antibody (ab125047, Abcam, Cambridge, UK) in 4% BSA/PBS. For
immunostaining, an anti-rabbit staining kit (CTS005, R&D Systems, Minneapolis, MN) and peroxidase anti-peroxidase with the streptavidin-biotin system were used. Immunostaining was followed by hematoxylin for nuclear counterstaining. The number of CD68-positive macrophages in the liver was quantified as described previously [16].

7. Pancreas morphology

Hematoxylin and Eosin-stained pictures of whole tissue slide and of every islet of Langerhans were taken using Zeiss Axiovert 100 microscope (25x/200x) and Leica EC3 digital camera. The islets were counted, and the islet area was measured using ImageJ software to calculate the islet density and the mean islet area per slide.

Pancreas Immunohistochemistry: Beta cell content of islets of Langerhans was measured using immunohistological staining of insulin. We used an antibody against insulin (ab7842, abcam, Cambridge, UK) and a secondary antibody (ab6907, abcam) diluted in antibody diluent (Dako, Glostrup, DK) and for visualisation the ABC staining system (sc2023, Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s instructions. All islets per slide were photographed using an Olympus BH-2 microscope (200x) and CFW-1310C digital camera. Using ImageJ software, the total islet area and the beta cell area was measured (see also Figure 5). Content of β-cells in islets was expressed as the percentage of positively stained area in the total islet area.

8. Liver glycogen content

Glycogen content was determined using the amyloglucosidase method as described before [17]. Briefly, liver was incubated with 1N KOH (95°C, 30 minutes). Glycogen was precipitated using saturated sodium sulfate solution (Na₂SO₄) and 95% (v/v) Ethanol and washed twice in 60% (v/v) Ethanol. Resuspended glycogen was degraded with 0.1% (w/v) Amyloglucosidase (Sigma-Aldrich, St. Louis, MO) in acetate buffer (0.2 M sodiumacetate, 0.46% (v/v) acetic acid, pH 4.8) for 2 hours at 40°C. Glucose concentration was measured colorimetrically using the Glucose (HK) Assay Kit (Sigma-Aldrich). Double measurements were performed, and glycogen content was expressed in relation to liver weight.
9. Quantitative real time PCR

We analysed a list of candidate genes involved in liver fat and carbohydrate metabolism as described recently [18]. We have choosen this list, because we wanted to investigate whether a heterozygous paternal eNOS knockout that is not transmitted to the next generation has the same effect on the offspring’s phenotype – a fatty liver phenotype – as we recently described in the offspring of female heterozygous eNOS mice [6]. RNA extraction from liver tissue, reverse transcription PCR and design of specific primer were done like previously described [19]with the exception that primer were obtained from Sigma-Aldrich, Eurofins (Ebersberg, GER) and Biolegio (Nijmegen, NE).

The PCR was performed on a Mx3000P thermal cycler (Stratagene, La Jolla, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), Sensi Mix or SensiFast low ROX kit (Bioline, London, UK) in accordance with instructions for use. All samples were analysed in triplicate. The PCR reaction efficiency has been proofed by linear regression method and the relative quantity of analysed genes was calculated with the ΔΔCt method as described elsewhere [20]. In short, the Ct values of gene of interest were normalized to the geometric mean of the reference genes HPRT, β-Actin and 18S. These values were normalized against the mean value of the reference group. Sequences of used primers are listed in ESM Table 1.

10. Quantification of gene specific DNA methylation

Quantification of gene specific DNA methylation was achieved with immunoprecipitation of methylated genomic DNA (MeDIP), with minor modifications as described by Weber et al. [21]. Briefly, genomic DNA was extracted from liver tissue by proteinase K treatment, RNAse digestion, phenol-chloroform extraction and precipitation with isopropanol. The DNA was sonicated to obtain random fragments between 300 and 1000 bp. 2 µg of the fragmented DNA was denatured for 10 min at 95°C and precipitated over night at 4°C with 10 µg of monoclonal antibody against 5-methylcytosine (Zymo Research) in IP buffer (10 mM sodium phosphate (pH 7.0), 140 mM NaCl, 0.05% Triton X-100). 20 µl of MagnaChip protein G magnetic beads (Millipore) were added and incubated for 2 h at 4°C to capture the antibodies. Magnetic beads were washed two times with IP buffer and treated with proteinase K for 3 h at 50°C. The methylated DNA was recovered by phenol-chloroform extraction and ethanol precipitation.
For the analysis of GR exon 1A and PGC1α promoter, specific primers were created (ESM Table 1). To calculate the proportion of methylated DNA in specific target sequences the content in enriched methylated DNA and input DNA were compared and normalized against the mean value of the reference group.

11. Statistics

A formal power calculation to plan group size was not possible due to two reasons first the litter size of wildtype offspring cannot be exactly planned a priori. Second, we did not know the resulting phenotype of wildtype offspring from heterozygous eNOS knockout fathers. Thus, there was no clear assumptions about the effect size and variation that we might expect. Given that, we decided that we needed to have a least 10 animals per group and planed our experiments based on this assumption. This approach to plan the study was approved by the responsible animal welfare committee.

Statistical analysis was done using GraphPad Prism 6 software (GraphPad, La Jolla, CA). All values are presented as mean ± SEM. For the statistical analysis of IPGTT glucose and insulin, two-way analysis of variance (2-way ANOVA) test followed by Bonferroni post-hoc test was conducted. The unpaired Student’s t-test and Pearson correlation analysis were applied for normally distributed data, while the Mann-Whitney U test and Spearman correlation analysis were used for non-normally distributed data. The data were non-normally distributed; thus the Mann-Whitney-U test and Spearman’s rank correlation were applied. To correct for multiple testing in the gene expression analysis, a false discovery rate (FDR) cut off was set at 0.05 (observation of no more than 5% false positives) [22, 23]. Statistically significant differences were considered as \( p \leq 0.05 \).
**ESM Table 1:** Primers used for real time PCR and MeDIP

| Target Gene | Primer Pair | Length of amplification product (bp) |
|-------------|-------------|--------------------------------------|
| 18S         | fw 5’CGGCTACCACATCCAAGGAA’ 3  
              rev 5’GCTGGAATTACCGCGGCT’ 3 | 187 |
| Acc1 (Acaca, ENSMUSG00000020532) | fw 5’TTTCACATGAGATCCAGCATG’ 3  
              rev 5’GCCACAGTGAAATCTCGTTG’ 3 | 92 |
| β-Actin (Actb, ENSMUSG00000029580) | fw 5’GATATCGCTTGCCTGTC’ 3  
              rev 5’CATCACACCTGGTGCT’ 3 | 123 |
| Acs13 (ENSMUSG00000032883) | fw 5’CTGTTCCGGAATCATGGA’ 3  
              rev 5’GAAAAACAAAGCGGTCACACA’ 3 | 158 |
| Acs14 (ENSMUSG00000031278) | fw 5’CCAGAAAACCTTGAGCGTTCC’ 3  
              rev 5’TGCCTCTCGGTCTCTAGTCCA’ 3 | 172 |
| AMPK-α2 (Prkaa2, ENSMUSG00000028518) | fw 5’AAAGACATAACGGAACATGAAT’ 3  
              rev 5’CTTCACAGCCTCATCGTCAA’ 3 | 105 |
| Cdkn1a (ENSMUSG00000023067) | fw 5’CAGACCAGCTGTAGATTTTC’ 3  
              rev 5’GCAGGCACGTTATATACAGGA’ 3 | 204 |
| CHREBP (Mlxipl, ENSMUSG00000005373) | fw 5’GAAGATGCTTATGTGGCAATG’ 3  
              rev 5’GGCGGTAATTGGTGAAGAAA’ 3 | 109 |
| CPT1a (ENSMUSG00000024900) | fw 5’CGCACATTACAAGGACATGG’ 3  
              rev 5’TCTGCTCTGCCGTTTGT’ 3 | 158 |
| eNOS (Nos3, ENSMUSG00000028978) | fw 5’GGGAAAGCTGCAGCTTTG’ 3 | 111 |
| Gene        | Forward Primer | Reverse Primer | Transcript Length |
|-------------|----------------|----------------|-------------------|
| FAS (Fasn, ENSMUSG00000025153) | fw 5` GGATTCGGTGTATCCTGCTG '3 | rev 5` TGGGCTTGTCTCGCTCTAAC '3 | 171 |
| Fbp1 (FBPase) (ENSMUSG00000069805) | fw 5` ATCAAAGCCATCTCGTCTGC '3 | rev 5` ATTTGCCCTTTCTCTCAGGT '3 | 217 |
| G6Pase (G6pc, ENSMUSG00000078650) | fw 5` GACTGTGGGCGATCAATCTCC '3 | rev 5` TCACAGGTGACAGGGAACTG '3 | 165 |
| Gck (ENSMUSG00000041798) | fw 5` AAGTCCCACGATGTGTTC '3 | rev 5` CTTCCCTGTAAGGCAAGAAC '3 | 100 |
| GR (Nr3c1, ENSMUSG00000024431) | fw 5` ACACGTCAGCACCCCATAAT '3 | rev 5` AGGCCGCTCAGTGTTTCTA '3 | 160 |
| Gys2 (ENSMUSG00000030244) | fw 5` GAGGAAGCCTGATGTAGTGACTC '3 | rev 5` TCCAGATGACCATAGAAATGACC '3 | 138 |
| Hprt (ENSMUSG00000025630) | fw 5` CAGGCCAGACTTTGTTGGAT '3 | rev 5` TTGCGCTCATCTAGGCTTT '3 | 147 |
| HSL (Lipe, ENSMUSG0000003123) | fw 5` ACCTGCTTTGGTTCAACCTGGA '3 | rev 5` CTGGCACCTCACTCCATAG '3 | 111 |
| IGF-BP 1 (lgfbp1, ENSMUSG00000020429) | fw 5` CAGCATGAAGAGGCAAGG '3 | rev 5` CTATAGGTGCTGATGCGGTTC '3 | 152 |
| Gene Name                  | Accession Number | Forward Primer | Reverse Primer | Length |
|---------------------------|------------------|----------------|----------------|--------|
| IGF-BP 2 (Igfbp2, ENSMUSG00000039323) | fw 5' AGGTCCCTGGAGCGGATCT '3 | rev 5' CATCTTGCACTGCTTAAAGGTTG '3 | 125 |
| IGF-BP 3 (Igfbp3, ENSMUSG00000020427) | fw 5' TGCTCCAGGAAAAACATCAGTG '3 | rev 5' GGAGTGGATGGAACCTTGAA '3 | 110 |
| iNOS (Nos2, ENSMUSG00000020826) | fw 5' TGCTCCAGGAAACATCAGTG '3 | rev 5' CCATGATGTCACATTCCTGC '3 | 152 |
| Nampt (ENSMUSG000000020572) | fw 5' CACCGACTGAGTCAAGGTTACTC '3 | rev 5' TTTACGCGGCAATCAGATTAGG '3 | 83 |
| Nrf1 (ENSMUSG00000058440) | fw 5' TCATCTCGTACCACAGAGCCACC '3 | rev 5' TTTGTTCCACCTCCTCCATCAC '3 | 182 |
| Pdk4 (ENSMUSG000000019577) | fw 5' CTCTTCAAGAATGCCATGAGG '3 | rev 5' TCGGTCAGAAATCCTTGATGTAAG '3 | 120 |
| PEPCK (Pck1, ENSMUSG00000027513) | fw 5' ATACATGGTGCCGGCCTTTC '3 | rev 5' GACAACCTGTTGGCGCTTCTC '3 | 204 |
| PK-L (Pklr, ENSMUSG00000041237) | fw 5' AGTATGGAAGGGCCAGCA '3 | rev 5' AGAGGGTGTCCAGGAAGGAT '3 | 130 |
| PGC1a (Ppargc1a, ENSMUSG00000029167) | fw 5' AGTCACCAATGACCCCAAG '3 | rev 5' GGAGTTGTGGGAGGAGGTAG '3 | 106 |
| PPARγ (Pparg, ENSMUSG00000004040) | fw 5' CAGGCCCTCATGAAGAAGCCTT '3 | rev 5' GGATCCCGCGCAGTTAAGATCA '3 | 176 |
| Gene                  | Description               | Forward Primer (5' to 3') | Reverse Primer (5' to 3') | Length |
|-----------------------|---------------------------|---------------------------|---------------------------|--------|
| PPARα (Ppara, ENSMUSG00000022383) | fw 5' TCTGGAAAGCTTTTGTTTGGC 3' | rev 5' TTCGACACTCGATGTTTCAGG 3' | 176    |
| SREBF1c (Srebf1, ENSMUSG00000020538) | fw 5'CTGTCGGGGGTAGCGTCTG 3' | rev 5'CGGGAAGTCACTGTCTTTGGT 3' | 112    |
| Tfam (ENSMUSG00000003923) | fw ACACCCAGATGCAAAACTTTC 3' | rev CTTTGTATGCTTTCCACTCAGC 3' | 122    |
| PGC1a promoter (5 CpG) | fw 5' TCCGGTTTAGAGTTGGTG CG 3' | rev 5'CCATCCAGCTCCCAGTACGAC 3' | 380    |
| GR exon 1A (11 CpG)    | fw ACGCAAAGGAAAGAACATGCC 3' | rev CCCAGACACTCTAAGCAAGCA 3' | 349    |
ESM Table 2. Differentially expressed miRNAs between low-dose L-NAME (0.15mg/ml DW) group and control group

| miRNA         | logFC     | P Value       |
|---------------|-----------|---------------|
| **Down-regulated miRNA** |           |               |
| mmu-miR-615-3p  | -1.188126408 | 0.019959695 |
| mmu-miR-193a-5p | -1.27414962  | 0.038590132  |
| mmu-miR-199b-5p | -1.275405992 | 0.039922001  |
| mmu-miR-144-3p  | -1.45308533  | 0.01248127   |
| mmu-miR-132-3p  | -1.610251375 | 0.020140258  |
| mmu-miR-8114    | -1.661256834 | 0.006551138  |
| novel_184       | -4.18149069  | 0.048205786  |
| novel_187       | -4.301847207 | 0.046457366  |
| novel_158       | -4.427062846 | 0.04252077   |
| novel_189       | -4.572081065 | 0.044273836  |
| novel_145       | -4.831490776 | 0.041621203  |
| novel_141       | -5.059573644 | 0.043502905  |
| novel_228       | -5.152814224 | 0.048448563  |
| novel_137       | -5.223232961 | 0.047526908  |
| novel_170       | -5.235373352 | 0.042099932  |
| novel_258       | -5.341004355 | 0.046318068  |
| novel_238       | -5.52641261  | 0.04578849   |
| novel_147       | -5.737488916 | 0.015507201  |
| novel_248       | -5.947660942 | 0.018018269  |
| novel_156       | -6.393873119 | 0.002402113  |
| novel_152       | -6.502969632 | 0.02018211   |
| novel_139       | -8.381870524 | 0.034513278  |
| novel_133       | -9.400424176 | 0.033183276  |
| **Up-regulated miRNA** |           |               |
| novel_66        | 4.793368    | 0.028573      |
| novel_58        | 5.434276    | 0.027511      |
| novel_29 | 5.556467 | 0.027116 |
| novel_77 | 5.975698 | 0.037912 |
| novel_31 | 6.866177 | 0.011476 |
ESM Table 3. Differentially expressed miRNAs between high-dose L-NAME (2mg/ml DW) group and control group

| miRNA            | logFC   | P Value     |
|------------------|---------|-------------|
| **Down-regulated miRNAs** |         |             |
| mmu-miR-1843a-5p | -1.022555086 | 0.014345528 |
| mmu-miR-503-5p  | -1.085633053 | 0.009853503 |
| novel_33         | -1.192791837 | 0.002824878 |
| mmu-miR-7230-3p  | -1.515984779 | 0.009723282 |
| novel_28         | -1.955217119 | 0.000764771 |
| mmu-miR-5099     | -2.205040054 | 0.035678986 |
| mmu-miR-497a-3p  | -2.269705466 | 0.006030488 |
| novel_84         | -2.280232855 | 0.027527084 |
| mmu-miR-181d-3p  | -3.226742279 | 0.049292565 |
| mmu-miR-7015-3p  | -3.4442631   | 0.009004629 |
| mmu-miR-802-5p   | -3.477390754 | 0.010386511 |
| mmu-miR-1934-5p  | -3.687785393 | 0.039205574 |
| mmu-miR-128-1-5p | -4.083115839 | 0.030951033 |
| novel_184        | -4.109186325 | 0.049069552 |
| novel_187        | -4.230642111 | 0.047614594 |
| novel_158        | -4.352196753 | 0.043753659 |
| novel_189        | -4.498326021 | 0.046217523 |
| novel_145        | -4.757665544 | 0.043398889 |
| novel_88         | -4.772178861 | 0.043502864 |
| novel_79         | -4.923206711 | 0.04426357  |
| novel_120        | -4.947664744 | 0.049891703 |
| novel_141        | -4.991829808 | 0.044516861 |
| novel_114        | -5.085712365 | 0.042926355 |
| novel_21         | -5.086738329 | 0.015148642 |
| novel_258        | -5.271151919 | 0.049804242 |
| novel_42         | -5.637582224 | 0.044847908 |
| novel_147        | -5.670669564 | 0.015130277 |
| novel_248     | -5.886025363 | 0.019359849 |
| novel_156    | -6.321290758 | 0.002119395 |
| novel_152    | -6.43052072  | 0.022028445 |
| novel_78     | -6.483633127 | 0.002054752 |
| novel_107    | -6.510814481 | 0.022250835 |
| novel_139    | -8.312733009 | 0.036698075 |
| novel_133    | -9.333723567 | 0.037405496 |
| novel_121    | -10.0821985  | 0.036392588 |

**Up-regulated miRNAs**

| mmu-miR-320-3p | 1.000850931 | 0.005684666 |
| mmu-miR-365-3p | 1.072714256 | 0.018210289 |
| mmu-miR-146a-5p| 1.207131152 | 0.010556529 |
| mmu-miR-292a-5p| 1.818042926 | 0.03852769 |
| mmu-miR-219c-5p| 2.686836576 | 0.010766331 |
| mmu-miR-6970-5p| 2.695362472 | 0.043526378 |
| mmu-miR-218-1-3p| 2.787253559 | 0.048315625 |
| mmu-miR-7219-3p| 3.09932527  | 0.033766784 |
| mmu-miR-3060-3p| 3.247223663 | 0.018640336 |
| novel_66     | 4.592328379  | 0.014903218 |
| novel_215    | 5.690671331  | 0.038178473 |
| novel_77     | 6.36285523   | 0.044196678 |
ESM Table 4: Described functions of already described miRNAs identified in mouse sperm after low dose L-NAME treatment mimicking paternal heterozygous deficiency.

| miRNA     | Main findings                                                                                                                                                                                                 | Reference |
|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| mmu-miR-615-3p | microRNA mmumiR-615-3p is reduced under conditions of endoplasmic reticulum (ER) stress, wherein it regulates the expression of C/EBP homologous protein (CHOP) and determines cellular sensitivity to cell death. | [24]      |
|           | CircZNF609 is involved in the pathogenesis of focal segmental glomerulosclerosis by sponging miR-615-5p.                                                                                                       |           |
| mmu-miR-193a-5p | miR-193a alleviates diabetic neuropathic pain in a mouse model through the inhibition of HMGB1 expression.                                                                                                     | [26]      |
|           | miR-193a/b-3p overexpression attenuates liver fibrosis through suppressing the proliferation and activation of HSCs.                                                                                              | [27]      |
|           | mmu-miR-193 influenced embryo implantation by regulating growth factor receptor-bound protein 7 expression.                                                                                                      | [28]      |
| mmu-miR-199b-5p | miR-199b-5p is an important regulator in medullary TEC proliferation through targeting Fzd6 to activate Wnt signaling and cell cycle signaling.                                                                | [29]      |
|           | miR-199b as a regulator of the phenotypic switch during vascular cell differentiation derived from iPS cells by regulating critical signaling angiogenic responses.                                                | [30]      |
|           | miR-199b is a direct calcineurin/NFAT target gene that increases in expression in mouse and human heart failure.                                                                                                 | [31]      |
| mmu-miR-144-3p | miR-144 is involved in extracellular matrix remodeling post MI and its loss leads to increased myocardial fibrosis and impaired functional recovery.                                                               | [32]      |
|           | Downregulation of microRNA-144 inhibits proliferation and promotes the apoptosis of myelodysplastic syndrome cells through the activation of the AKAP12-dependent ERK1/2 signaling pathway.                               | [33]      |
| miR-144 | miR-144 maybe a potential regulator of the development of atherosclerosis via changes in vimentin signaling. | [34] |
| --- | --- | --- |
| Circulating exosomal miR-144-3p inhibits the mobilization of endothelial progenitor cells post myocardial infarction via regulating the MMP9 pathway. |  |
| Circulating exosomal miR-144-3p inhibits the mobilization of endothelial progenitor cells post myocardial infarction via regulating the MMP9 pathway. | [35] |
| mmu-miR-132-3p | miR-132-3p priming enhances the effects of mesenchymal stromal cell-derived exosomes on ameliorating brain ischemic injury | [36] |
| mmu-miR-132-3p | Targeted silencing of miRNA-132-3p expression rescues disuse osteopenia by promoting mesenchymal stem cell osteogenic differentiation and osteogenesis in mice | [37] |
| mmu-miR-132-3p | Brown adipocyte-derived exosomal miR-132-3p suppress hepatic Srebf1 expression and thereby attenuate expression of lipogenic genes | [38] |
| mmu-miR-132-3p | MiR-132 controls pancreatic beta cell proliferation and survival through Pten/Akt/Foxo3 signaling | [39] |
| mmu-miR-8114 | No functional related literature found |  |
**ESM Table 5:** Body weight, organ weights, systolic blood pressure and plasma creatinine

| Variable                              | Both sexes | XY          | XX          |
|---------------------------------------|------------|-------------|-------------|
|                                       | F:WT; M:WT | F:+/-eNOS; M:WT | F:+/-eNOS; M:WT |
|                                       | (n=45-50)  | (n=26-27)   | (n=10)      |
| Final body weight (g)                 | 27.25±0.99 | 27.07±1.11  | 34.18±0.89  | 21.80±0.42  | 23.70±0.83 ** |
| Relative Liver Weight (% of body weight) | 4.31±0.06  | 4.26±0.08   | 4.56±0.07   | 4.44±0.10   | 4.11±0.08   | 4.16±0.11   |
| Relative Kidney weight (% of body weight) | 0.59±0.01  | 0.61±0.01   | 0.61±0.02   | 0.64±0.02   | 0.57±0.01   | 0.59±0.02   |
| Relative Heart weight (% of body weight) | 0.51±0.01  | 0.49±0.01   | 0.50±0.01   | 0.49±0.02   | 0.51±0.01   | 0.49±0.02   |
| Systolic blood pressure (mmHg)        | 102.70±2.43 | 106.10±2.16 | 95.65±3.22  | 99.04±2.14  | 108.30±3.20 | 110.30±2.76 |
| Plasma creatinine (µmol/l)            | 52.16±0.02 | 49.50±0.03  | 48.62±0.02  | 46.85±0.03  | 55.69±0.03  | 50.39±0.04  |

XX: female offspring; XY: male offspring. Data are given as mean±SEM and **: p<0.01 vs. father wt/mother wt
**ESM Table 6: Histological findings in the liver**

| Variable                                  | Both sexes       | XY             | XX              |
|-------------------------------------------|------------------|----------------|-----------------|
|                                            | F:WT; M:WT       | F:+/-eNOS; M:WT| F:+/-eNOS; M:WT |
|                                            | (n=48)           | (n=22)         | (n=9-10)        |
| Liver Lobular Dimension (mm)              | 0.07±0.001       | 0.07±0.001     | 0.07±0.002      | 0.07±0.003 | 0.07±0.002 |
| Liver Connective Tissue Content (% area)  | 0.16±0.02        | 0.17±0.03      | 0.12±0.03       | 0.11±0.04 | 0.18±0.03  | 0.20±0.04 |
| Liver Fat Content (% area)                | 0.73±0.16        | 1.09±0.34      | 0.87±0.32       | 0.20±0.06 | 0.61±0.10  | 1.67±0.51 |
| Liver Lipid Droplet Density (droplets/mm²)| 2701.0±411.2     | 3066.1±689.0   | 2537.7±779.2    | 4980.7±255.5 | 2850.8±352.1 | 4406.6±969.9 |
| Liver Lobular Inflammation (score)        | 0.31±0.13        | 0.33±0.14      | 0.29±0.18       | 0.33±0.21 | 0.33±0.21  | 0.33±0.21 |
| Number of CD68-Positive Immune Cells      | 0.85±0.10        | 0.92±0.08      | 0.86±0.14       | 1.00±0.00 | 0.83±0.17  | 0.83±0.17 |
| (score) in the liver                      |                  |                |                 |            |              |              |

XX: female offspring; XY: male offspring. Data are given as mean±SEM.
| Parameter                        | Liver glycogen | IPGTT plasma glucose (0 min) | IPGTT plasma glucose (15 min) | IPGTT plasma glucose (60 min) | IPGTT plasma glucose (AUC) |
|---------------------------------|----------------|-----------------------------|-------------------------------|-------------------------------|----------------------------|
| **Both sexes**                  |                |                             |                               |                               |                           |
| Liver glycogen                  | 1.0            | 0.128                       | -0.017                        | 0.161                         | 0.087                      |
| IPGTT plasma insulin (0 min)    | 0.305*         | -0.054                      | 0.031                         | 0.054                         | 0.001                      |
| IPGTT plasma insulin (15 min)   | 0.2            | -0.008                      | 0.425**                       | 0.399**                       | 0.414**                    |
| IPGTT plasma insulin (60 min)   | 0.107          | 0.038                       | 0.449**                       | 0.536**                       | 0.545**                    |
| IPGTT plasma insulin (AUC)      | 0.276          | 0.016                       | 0.435**                       | 0.449**                       | 0.426**                    |
| **XX**                          |                |                             |                               |                               |                           |
| Liver glycogen                  | 1.0            | -0.004                      | -0.183                        | 0.143                         | -0.007                     |
| IPGTT plasma insulin (0 min)    | 0.201          | -0.148                      | -0.033                        | 0.330*                        | 0.074                      |
| IPGTT plasma insulin (15 min)   | 0.114          | -0.129                      | 0.508**                       | 0.428**                       | 0.464**                    |
| IPGTT plasma insulin (60 min)   | -0.059         | 0.105                       | 0.354*                        | 0.619**                       | 0.504**                    |
| IPGTT plasma insulin (AUC)      | 0.183          | 0.07                        | 0.396*                        | 0.594**                       | 0.430*                     |
| **XY**                          |                |                             |                               |                               |                           |
| Liver glycogen                  | 1.0            | 0.24                        | 0.22                          | 0.076                         | 0.115                      |
| IPGTT plasma insulin (0 min)    | 0.34           | 0.04                        | 0.135                         | -0.293                        | -0.114                     |
| IPGTT plasma insulin (15 min)   | 0.419*         | 0.124                       | 0.266                         | 0.196                         | 0.214                      |
| IPGTT plasma insulin (60 min)   | 0.337          | -0.069                      | 0.440*                        | 0.254                         | 0.391*                     |
| IPGTT plasma insulin (AUC)      | 0.452*         | -0.035                      | 0.473*                        | 0.208                         | 0.328                      |

XX: female offspring; XY: male offspring. *: p<0.05, **: p<0.01.
**ESM Table 8**: Liver NOS expression, real time PCR quantification

| Variable  | Both sexes | XY | XX |
|-----------|------------|----|----|
|           |            |    |    |
| eNOS (Nos3) |            |    |    |
| F:WT; M:WT (n=20) | 1.00 ± 0.06 | 1.00 ± 0.07 | 1.00 ± 0.11 |
| F: +/- eNOS; M:WT (n=20) | 1.03 ± 0.08 | 1.04 ± 0.07 | 1.02 ± 0.15 |
| iNOS (Nos2) |            |    |    |
| F:WT; M:WT (n=10) | 1.00 ± 0.13 | 1.00 ± 0.19 | 1.00 ± 0.19 |
| F: +/- eNOS; M:WT (n=10) | 0.92 ± 0.12 | 0.84 ± 0.10 | 1.00 ± 0.22 |

XX: female offspring; XY: male offspring. Data are given as mean±SEM.
eNOS knockout mice of the C57BL/6J strain and their wild-type (wt) littermate were used. Female wt mice were cross-bred with homozygous male eNOS knockout mice. The resulting male heterozygous eNOS knockout (eNOS+/−) mice were then again crossed with female wt mice. Only wt offspring of this breeding procedure (F2 generation) entered the study. These mice were compared to wt mice resulting from crossing male wt and female wt mice. Heterozygous animals used for breeding of the F2 generation were all derived from different dams i.e siblings were not used.
ESM Fig.2:

Birth weight, length and abdominal diameter as well as the change in body weight over the time
XX: female offspring; XY: male offspring. F:WT; M:WT : wildtype offspring of wildtype fathers and wildtype mothers and F: +/- eNOS; M:WT : wildtype offspring of eNOS heterozygous fathers and wildtype mothers
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