

SUPPLEMENTAL METHODS AND RESULTS

SUPPLEMENTAL MATERIALS:

LncRNA:

LncRNA Preparation and sequencing:

The workflow is as follows: Total RNA sample RNA test → rRNA removal → double-stranded cDNA synthesis → repair ends, add “A” overhang and adaptor → cDNA second-strand degradation → fragment selection and PCR amplification → library quality test → Illumina sequencing.

Mapping to reference genome:

Reference genome and gene model annotation files were downloaded from a genome website browser (NCBI/UCSC/Ensembl) and paired-end clean reads were mapped to the reference genome using HISAT2 software.

LncRNA and mRNA identification and Quantification:

Mapped read assembly was performed by StringTie. StringTie, with ‘--library-type’ and other default parameters, was used to assemble the mapped reads of each sample. All the transcripts from all the samples were merged through Cuffmerge. StringTie-eB was used to obtain an estimate of both lncRNAs and mRNA transcripts in each sample. By summing up the transcript fragments per kilo-base of exon per million fragments mapped (FPKMs) in each gene group, gene FPKMs were computed.

Novel gene prediction:

StringTie (genome-guided transcriptome assembly approach) was used to assemble the set of transcripts obtained during the mapping step and Cuffcompare was used to compare StringTie assemblies to reference annotation files and help sort out new genes from known ones. The class codes are as follows: "u" for "Potentially novel gene" and the set of "j" (Potentially novel isoform: at least one splice junction is shared with a reference transcript), "u" (Unknown, intergenic transcript), "i" (A transfrag falling entirely within a reference intron), "o" (Generic exonic overlap with a reference transcript), and "x" (Exonic overlap with reference on the opposite strand) for "Potentially novel isoform".

LncRNA Target prediction:

Potential co-expression between lncRNAs and mRNAs was evaluated using Pearson Correlation. A correlation >0.7 and a p -value <0.05 was considered positive.

Alternative splicing analysis:

rMATS was utilized to perform alternative splicing analysis. Alternative splicing events for all major types of alternative splicing patterns were identified and the P value and FDR were calculated for differential splicing. These include exon skipping (SE), alternative 5' splice sites (A5SS), alternative 3' splice sites (A3SS), mutually exclusive exons (MXE), and retained introns (RI).

Variant Calling:

Picard tools and Samtools were used to sort, mark duplicated reads and reorder the bam alignment results of each sample, followed by HaplotypeCaller tool in GATK software to complete variant discovery, including SNPs and INDELs. Finally, ANNOVAR was used to functionally annotate genetic variants detected from diverse genomes.

CircRNA:

CircRNA Preparation and sequencing:

The workflow is as follows: Total RNA QC → rRNA digestion → linear RNA digestion → dsDNA synthesis → adapter ligation → size selection and cDNA second-strand digestion → PCR library QC and sequencing.

Quality control and mapping to reference genome:

FASTQ format raw data were processed by Novogene in-house scripts. The reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) and Paired-end clean reads were mapped to the reference genome using Spliced Transcripts Alignment to a Reference (STAR) software, which exhibits better alignment precision and sensitivity than other RNA-seq aligners for both experimental and simulated data.

CircRNA identification and distribution:

CIRCexplorer2 was used to detect circRNAs as well as to identify alternative back-splicing and back-spliced circRNA variants. Step-1 includes Step1: Circular RNA fusion junction read alignment and parsing; Step2: De novo assembly for circular RNA transcripts; Step3: Characterization of alternative back-splicing, including two types of alternative back-splicing events (alternative 5' back-splice site and alternative 3' back-splice site). The density of total mapped reads in each chromosome was calculated. Circos was used to analyze the relationship between chromosome length and total reads.

Quantification:

The expression of circRNAs is represented by the fragments that are mapped to the back-spliced exon-exon junction sites. Normalized RNA-seq fragments that are mapped to a specific back-spliced exon-exon junction by total mapped fragments were used along with the raw fragment numbers to quantitate circRNA expression. With FPM (Fragments mapped to back-spliced junction Per Million mapped fragments), circRNAs from different samples with distinct sequencing depths were compared.
$$\text{FPM} = (\text{Fragments mapped to a specific back-spliced junction} \times 10^6) / \text{Total mapped fragments}$$

Small RNA:

Library preparation for Small RNA sequencing:

From total RNA, sequencing libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) and index codes were added to attribute sequences to each sample. Briefly, NEB 3' SR Adaptor was directly and specifically ligated to the 3' end of miRNA, siRNA and piRNA. The SR RT Primer hybridized to the excess of 3' SR Adaptor and transformed the single-stranded DNA adaptor into a double-stranded DNA molecule. The 5' ends adapter was ligated to 5' ends of miRNAs, siRNA and piRNA. The first strand cDNA was synthesized using M-MuLV Reverse Transcriptase (RNase H⁻). PCR amplification was performed, and PCR products were purified on an 8% polyacrylamide gel. DNA fragments corresponding to 140~160 bp (the length of small noncoding RNA plus the 3' and 5' adaptors) were recovered and dissolved in elution buffer. The library quality was assessed on the Agilent Bioanalyzer 2100 system using High Sensitivity Chips.

Quality control:

FASTQ format raw data were processed by Novogene in-house perl and python scripts. Clean data reads were obtained from this step by removing reads with poly-N, 5' adapter contaminants, ploy A/T/G/C, without 3' adapter, the insert tag, and low quality.

Reads mapping to the reference sequence:

Using Bowtie, the small RNA tags were mapped to the reference sequence [1].

Known miRNA alignment:

miRBase20.0 was used as a reference to identify known miRNAs, by using the mapped small RNA tags to the reference. Then, modified mirdeep2 [2] and srna-tools-cli were used to obtain potential miRNA and draw secondary structures. Custom scripts were used to obtain the miR counts and base bias on the first position of identified miRNAs.

Novel miRNA prediction:

miREvo [3] and mirdeep2 [2] were used to predict novel miRNAs through exploration of the RNA secondary structures, the Dicer cleavage sites, and the minimum free energy of the previously unannotated small RNA tags. Simultaneously, custom scripts were used to obtain the miRNA counts and base bias on the first position of all identified miRNA.

Small RNA annotation summary:

To make every unique small RNA mapped to only one annotation, the following priority rule was followed: known miRNA > rRNA > tRNA > snRNA > snoRNA > repeat > gene > NAT-siRNA > gene > novel miRNA > ta-siRNA. The total rRNA proportion was used as a sample quality indicator.

miRNA editing analysis:

Positions 2~8 of mature miRNAs are called seed regions and are highly conserved. The miRNA targets may be different with potential changes in nucleotides in this region. In the analysis pipeline, miRNAs that may have base edits were detected by aligning all the sRNA tags to mature miRNA, allowing one mismatch.

Target gene prediction:

miRanda was used to predict the target genes of miRNAs.

Quantification of miRNA:

miRNA expression levels were estimated by TPM (transcript per million) using the following criteria [4]:

Normalization formula: Normalized expression = mapped read count/Total reads*1000000.

SUPPLEMENTAL RESULTS:

Supplementary Table S1. Quality Control (LncRNA and CircRNA):

	Control	HypoTH	HyperTH	HypoTH + T3
Raw Reads	40414882±2080 208.475	41862489±306 531.005	41492816.333±3 98483.857	54496711±123019 52.995
Clean Reads	40052519.333± 2050072.277	41114499±214 828.350	40750017.333±3 99893.012	53719779±120724 61.82
Raw Data (G)	12.133±0.636	12.566±0.088	12.433±0.12	16.366±3.667
Clean Data (G)	12±0.608	12.366±0.067	12.233±0.12	16.133±3.634
Error (%)	0.026±0.003	0.026±0.003	0.03±0	0.03±0
Q20 (%)	97.813±0.102	97.043±0.742	95.826±0.069	95.4±0.191
Q30 (%)	93.863±0.321	92.176±1.652	89.443±0.15	88.643±0.442
GC (%)	47.39±0.105	47.36±0.202	47.793±0.1	47.35±0.158

Values are mean ± SE. The details of the variables are described below:

- **Raw reads:** Reads count from the raw data, four rows as a unit, with statistics of reads count for every sequencing;
- **Clean reads:** Clean data are read count filtered from raw data. The statistical method is similar to raw reads. All the following analyses are based on clean data;
- **Raw data (Bases; Giga):** Base number of raw data. (Number of raw reads) * (sequence length), converting unit to G;
- **Clean data (Bases; Giga):** Base number of raw data after filtering. (Number of clean reads) * (sequence length), converting the unit to G;
- **Error rate (%):** Base error rate of whole sequencing;
- **Q20 (%):** Phred values greater than 20 base number contain the percentage of total bases. (Base number of Phred value > 20) / (Total base number)*100;
- **Q30 (%):** Phred values greater than 30 base number contain the percentage of total bases. (Base number of Phred value > 30) / (Total base number)*100;
- **GC content (%):** The percentage of G&C base numbers of total bases. (G&C base number) / (Total base number)*100.

Supplementary Table S2. Differentially expressed LncRNAs between HypoTH and Control groups.

Transcript	Gene Name	Gene ID	HypoTH Group Expression	Control Group Expression	Log2 (Fold Change)	p-value (Adjusted/Corrected)
NR_028422.1	C130080 G10Rik	100303644	0.891445	34.444394	-5.271979596	0.027635415
XR_867569.1	Gm40124	105244531	5.430553333	0	Inf	0.027635415
TCONS_00004800	Synpo2	118449	1.056803667	0	Inf	0.027635415
TCONS_00009217	1700030F04Rik	72263	1.307807667	0	Inf	0.027635415
XR_380269.2	Gm38485	102636048	1.170262333	0	Inf	0.027635415
TCONS_00022369	LOC118568312	118568312	375.3674317	0	Inf	0.027635415
TCONS_00022949	LOC118568312	118568312	27.22169733	0	Inf	0.027635415
TCONS_00022951	LOC118568312	118568312	20.39411167	0	Inf	0.027635415
TCONS_00025736	mt-Rnr2	17725	16.98086167	0	Inf	0.027635415
TCONS_00025779	-	XLOC_015265	12.45470933	0	Inf	0.027635415
NR_045640.1	Gm20199	100504377	1.138684333	0.103314	3.462260187	0.031451684
NR_168272.1	Gm42067	105246846	1.114302333	4.448331667	-1.99712364	0.033344077
TCONS_00025777	-	XLOC_015265	26.76265467	3.527620333	2.923454027	0.0355126
XR_004934374.1	Gm26944	102635071	0.987436667	0	Inf	0.048831628

The lncRNAs that are downregulated in HypoTH are given in blue and those that are upregulated are given in red. LncRNAs common within HypoTH vs. Control and HyperTH vs. HypoTH (below) comparisons are highlighted in bold.

Supplementary Table S3. Differentially expressed LncRNAs between HyperTH and HypoTH groups.

Transcript	Gene Name	Gene ID	HyperTH Group Expression	HypoTH Group Expression	Log2 (Fold Change)	p-Value (Adjusted/Corrected)
NR_131913.1	Lincred1	100996933	0.373112333	2.218947333	-2.572193471	0.005399615
XR_380324.2	Gm36827	102640859	2.307737	0.259222667	3.154215038	0.007224164
TCONS_00011627	Chd9	109151	2.979505	0.92774	1.683280218	0.011849041
XR_871656.2	Gm36827	102640859	0	5.332116333	-Inf	0.011849041
NR_166471.1	Foxo6os	402730	7.148917	0.057763	6.95143522	0.011849041
NR_015601.1	Fam120aos	68128	0.710750333	4.032742667	-2.504346573	0.011849041
TCONS_00004800	Synpo2	118449	0	1.056803667	-Inf	0.011849041
TCONS_00025736	mt-Rnr2	17725	0	16.98086167	-Inf	0.011849041
XR_003950992.1	Gm52092	115488289	3.039186667	0.023409333	7.020457629	0.012025606
NR_045170.1	Gm10336	328186	53.12925967	12.389056	2.100440346	0.01313389
XR_880771.2	Gm42208	105247026	0.641111333	2.688013667	-2.067893656	0.01751405
NR_168057.1	D330025C20Rik	665054	0.547646333	2.623150667	-2.259984259	0.024594455
NR_040309.1	2310069G16Rik	69659	3.403704333	0.273044	3.639900361	0.027401014
TCONS_00013019	Gm28979	102633035	2.465509333	0.005822	8.726155159	0.029175858
XR_867957.2	Gm12701	105244634	1.073003	3.434677333	-1.678520463	0.031663793
NR_168272.1	Gm42067	105246846	4.790582333	1.114302333	2.104060319	0.032840751
NR_033503.1	E530011L22Rik	320301	0.085753667	1.51838	-4.146190725	0.033507249
XR_386629.3	Gm35268	102638787	4.213745667	0.927016	2.184437094	0.034294928
XR_001779774.2	Gm36827	102640859	3.463763	0.637568667	2.441687585	0.03699232

NR_166847.1	Gm11967	10263 9588	9.905372	0.943594	3.391973007	0.03950929 5
NR_166742.1	Gm41717	10524 6427	1.20793633 3	3.9691416 67	- 1.716282639	0.04262102 6
NR_045269.2	Sp3os	10050 3849	0.94919766 7	3.1166576 67	- 1.715219239	0.04507131 9
XR_004941109.1	LOC118568566	11856 8566	0.82317366 7	2.6728333 33	- 1.699101144	0.04715887 9

LncRNAs common within HypoTH vs. Control and HyperTH vs. HypoTH comparisons are highlighted in bold. Those that are downregulated in HyperTH are given in blue and upregulated are given in red.

Supplementary Table S4. Differentially expressed LncRNAs between HypoTH+T3 and HypoTH groups.

Transcript	Gene Name	Gene ID	HypoTH+T3 Group Expression	HypoTH Group Expression	Log2 (Fold Change)	p-Value (Adjusted/Corrected)
NR_015601.1	Fam120a os	68128	0.86721266 7	4.03274266 7	- 2.217303615	0.01818002
XR_003950992.1	Gm52092	11548 8289	2.02625333 3	0.02340933 3	6.4355869	0.02698759 2
TCONS_00022791	9630028I 04Rik	31972 0	0.00352633 3	3.753323	- 10.05578388	0.03616698
XR_376121.3	Gm31678	10263 3985	0.59443533 3	2.50116333 3	- 2.073007495	0.04814049 2

LncRNAs common within HypoTH+T3 vs. HypoTH and HyperTH vs. HypoTH comparisons are highlighted in bold. Those that are downregulated in HypoTH+T3 are given in blue and those that are upregulated are given in red.

Supplementary Table S5. Differential expression of circRNAs between HyperTH and Control groups.

Gene	HyperTH group expression	Control Group Expression	log2(fold change)	p-Value (adjusted/corrected)
mmu_circ_4456	60.14525446	1002.420908	-4.058889532	0.004635362
mmu_circ_5616	80.19367261	1122.711417	-3.807351926	0.001404521
mmu_circ_2471	60.14525446	741.7914716	-3.624486861	0.018317977
mmu_circ_3252	60.14525446	621.5009627	-3.369229916	0.044420573
mmu_circ_1539	140.3389271	982.3724895	-2.807353026	0.010540753
mmu_circ_799	160.3873452	942.2756532	-2.554587257	0.015979686
mmu_circ_2045	280.6778541	1323.195598	-2.237038155	0.006376503
mmu_circ_1548	6876.607426	28208.12434	-2.036341746	2.87E-47
mmu_circ_2258	320.7746904	1202.905089	-1.906889743	0.018317977
mmu_circ_960	481.1620357	1423.437689	-1.564783962	0.040139341
mmu_circ_384	521.258872	1503.631361	-1.528378352	0.040139341

CircRNAs that are downregulated in HyperTH are given in blue.

Supplementary Table S6: sRNA statistics.

	Statistics of total reads mapped to ncRNA				Statistics of unique reads mapped to ncRNA			
Types	Control	HypoTH	HyperTH	HypoTH+T3	Control	HypoTH	HyperTH	HypoTH+T3
rRNA	5378602±793107.95	4294684±693529.87	1931337.66±208317.10	2569843.33±403666.28	54321.33±1454.24	44578±7973.23	28013.66±1076.90	28732.66±2516.73
rRNA:+	5378229±793135.09	4294575±693527.08	1931194±208323.52	2569727.66±403668.02	54217.33±1469.08	44527.33±7967.56	27954.66±1083.39	28683.33±2514.63
rRNA:-	373±31.39	109±8.33	143.66±6.77	115.66±21.40	104±15.04	50.66±6.94	59±8.19	49.33±10.33
tRNA	172074±16057.01	57565.33±25871.95	180905.66±61400.73	68582.33±17868.34	5362±173.30	3300±1219.04	2384±151.01	1632.33±214.79
tRNA:+	169937.33±16281.08	56488.33±25405.17	178670.66±61591.30	67864±17786.59	4912.66±179.14	3031.66±1100.99	2178.33±134.94	1521.66±198.04
tRNA:-	2136.66±262.50	1077±480.37	2235±209.69	718.33±102.23	449.33±7.67	268.33±118.51	205.66±27.85	110.66±17.29
snRNA	57910±8162.93	55011±17756.16	16260±586.06	13548.33±2093.37	6649.33±391.67	5823±1582.51	2107.66±45.84	2024±199.85
snRNA:+	57889.66±8161	54989±17750.46	16249.66±587.44	13538.66±2090.88	6634±391.33	5808.66±1577.68	2100±47.03	2018.33±198.33
snRNA:-	20.33±2.03	22±6.08	10.33±1.45	9.66±3.28	15.33±0.33	14.33±4.98	7.66±1.45	5.66±1.67
snoRNA	177770.33±6664.29	77646.66±23296.94	41398±739	41220±8455.35	18908.66±1117.95	14585.33±4259.05	5960.66±196.87	5453±611.58

snoRNA :+	17776 4±666 5.53	77636.6 6±2329 3.46	41390±7 38.27	41216.33± 8455.40	18903.6 6±1118 .47	14576. 66±42 56.21	5956±19 7.05	5450±611. 53
snoRNA :-	6.33±1 .33	10±3.5 1	8±1.15	3.66±0.67	5±1.73	8.66±2 .85	4.66±0.8 8	3±0.58

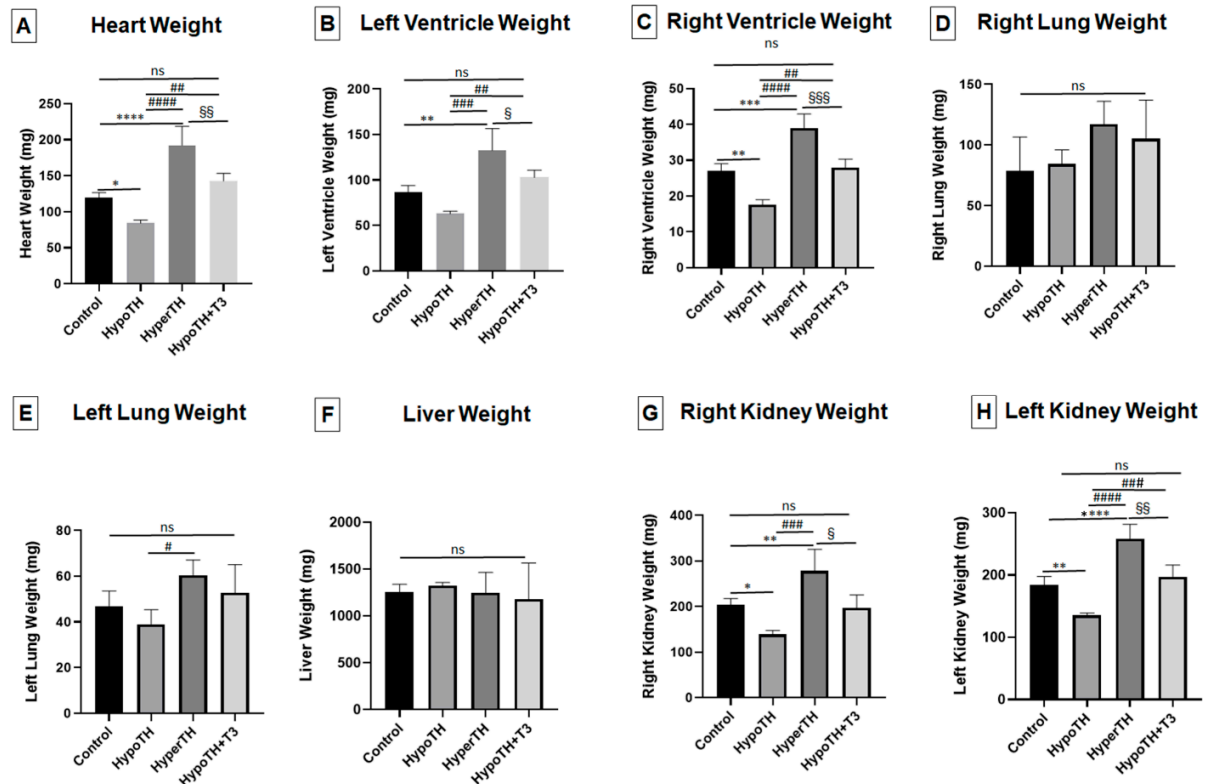
Statistics of total reads and unique reads mapped to ncRNA are presented. The small RNA reads were annotated with sequences from Rfam and matched reads were removed from the different small RNA types; rRNA: ribosomal RNA; tRNA: transfer RNA; snRNAs: Small nuclear RNAs; snoRNAs: Small nucleolar RNAs

Supplementary Table S7. Summary of the small RNA Quality Control.

Sample	Reads	Bases (G)	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
Control	30587688±32455 38.15	1.53±0.16	0±0	99±0	96±0	52±0
HypoTH	24307605±14735 89.89	1.21±0.08	0±0	99±0	97±0	53±0
HyperTH	22084188.33±11 00643.28	1.10±0.05	0±0	99±0	96±0	52±0
HypoTH + T3	23937723.33±20 29995.81	1.19±0.10	0±0	99±0	97±0	52±0

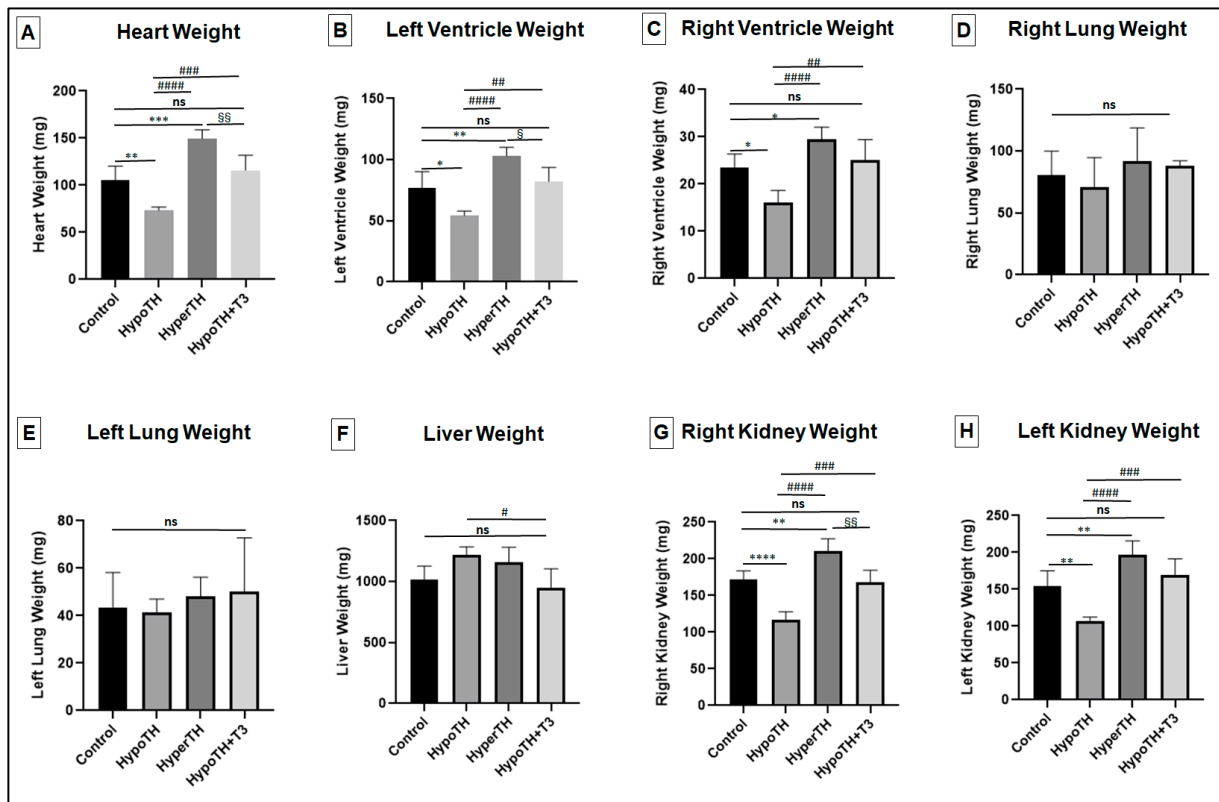
- Reads: Statistics of the original sequence data;
- Bases: Sequence number multiplied by the length of the sequence, expressed in gigabase pairs (Gbp);
- Error rate: Sequencing error rate;
- Q20: Percentage of bases whose Phred values exceed 20;
- Q30: Percentage of bases whose Phred values exceed 30;
- GC content: G and C bases as a percentage of all bases.

Supplementary Figure S1. Morphometrics: Males.



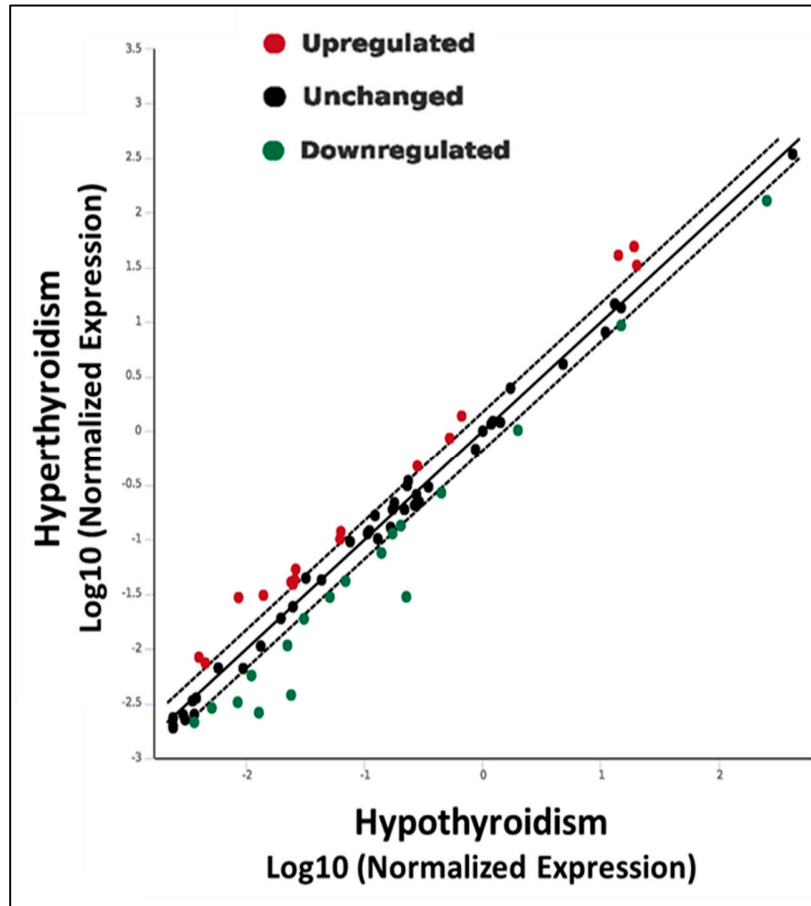
Supplementary Figure S1. Morphometrics (males): Alterations in (A) Heart, (B) Left Ventricle, (C) Right Ventricle, (D) Right Lung, (E) Left Lung, (F) Liver, (G) Right Kidney, and (H) Left Kidney weights when subjected to HypoTH, HyperTH and HypoTH + oral T3 in male mice. All values are mean \pm standard deviation; n=3-5 per group. HyperTH: Hyperthyroidism; HypoTH: Hypothyroidism; T3: Triiodothyronine; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. controls; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs. HypoTH; §p<0.05, §§p<0.01, §§§p<0.001 vs. HyperTH; ns: not significant.

Supplementary Figure S2. Morphometrics - Females:



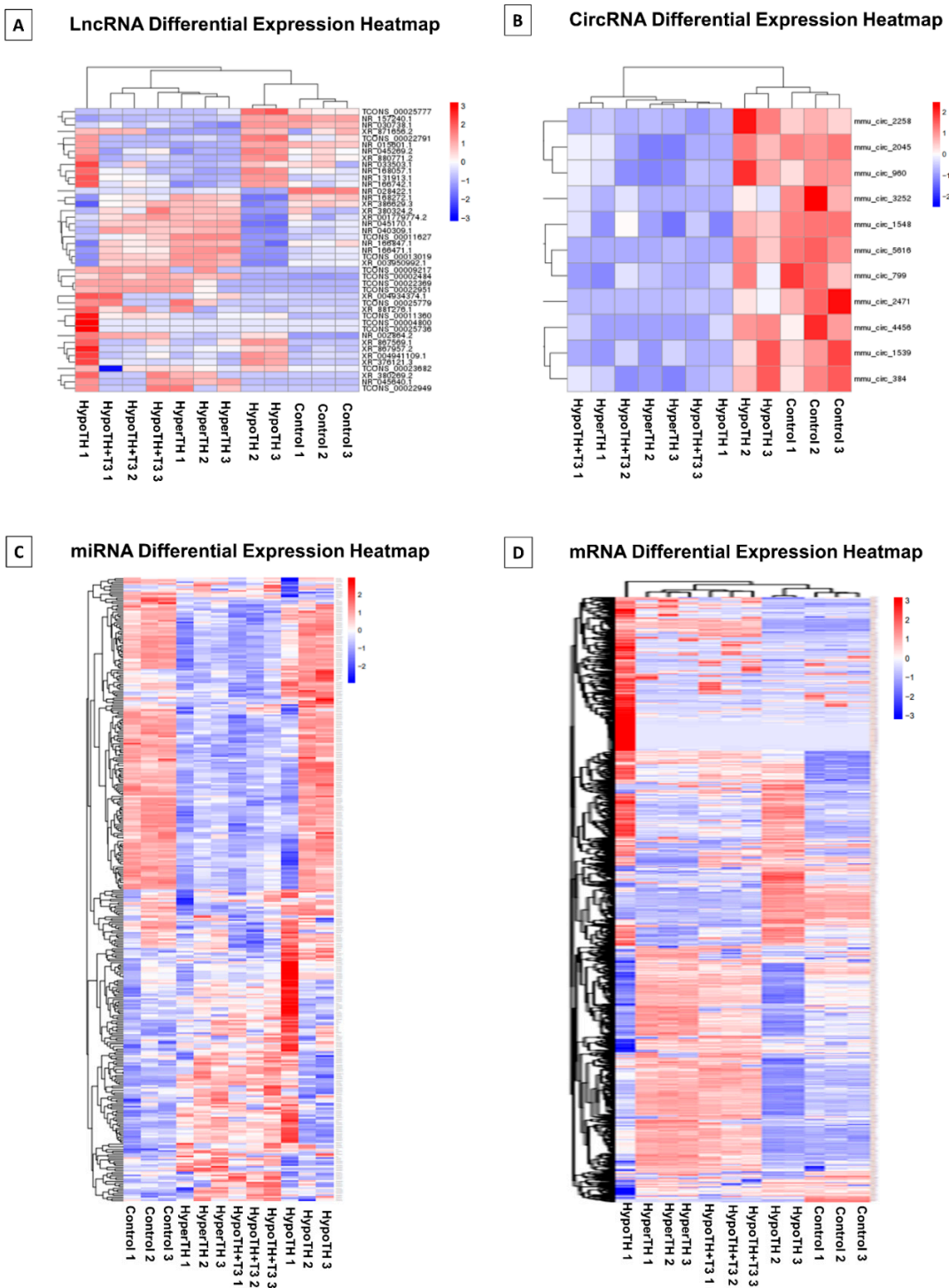
Supplementary Figure S2. Morphometrics (Females): Alterations in (A) Heart, (B) Left Ventricle, (C) Right Ventricle, (D) Right Lung, (E) Left Lung, (F) Liver, (G) Right Kidney, and (H) Left Kidney weights when subjected to HypoTH, HyperTH and HypoTH + oral T3 in female mice. All values are mean \pm standard deviation; n=3-5 per group. HyperTH: Hyperthyroidism; HypoTH: Hypothyroidism; T3: Triiodothyronine; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. controls; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs. HypoTH; §p<0.05, §§p<0.01 vs. HyperTH; ns: not significant.

Supplementary Figure S3. Significant impairment in cardiac inflammatory lncRNA expression under altered TH conditions.

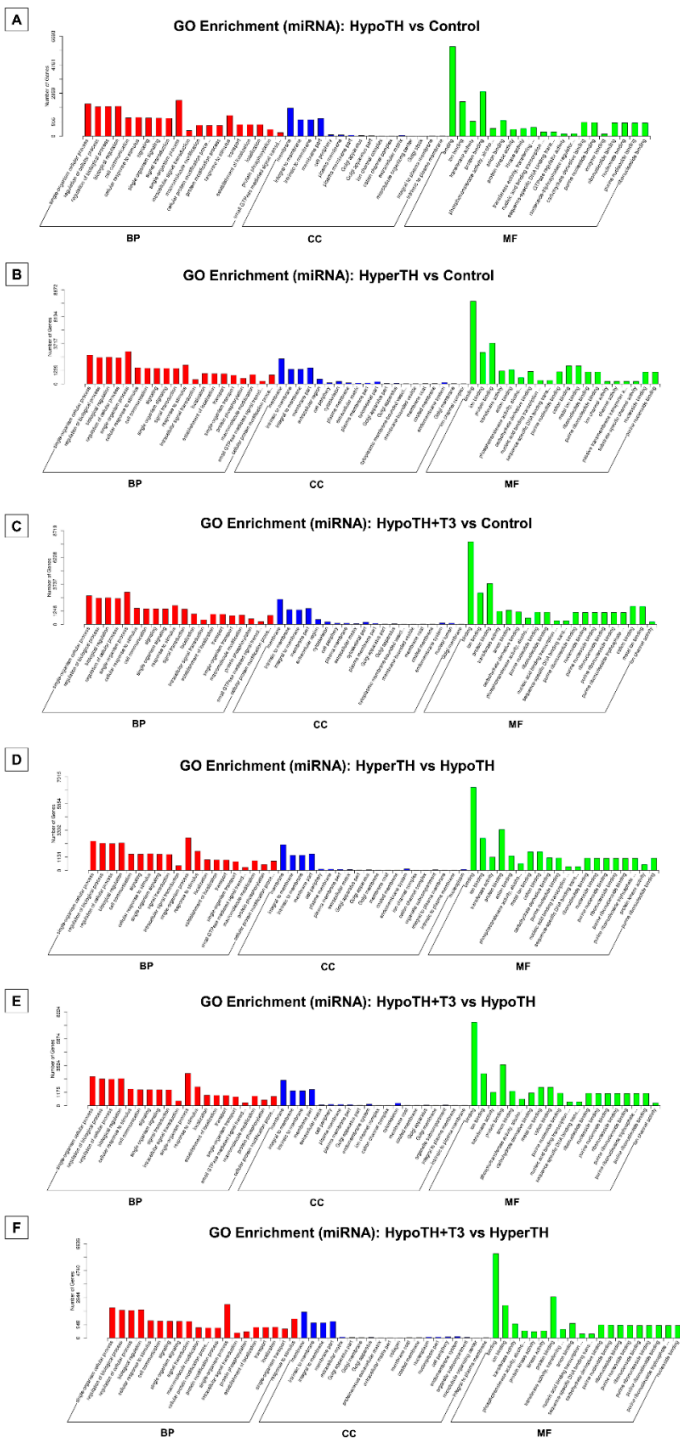


Supplementary Figure S3. Left ventricular lncRNA-specific qPCR arrays showed aberrant real-time expression with altered TH levels; n=4/group; $\geq \pm 1.5$ -fold change; $p < 0.05$

Supplementary Figure S4. Differential expression heatmaps (hierarchical clustering) of (A) lncRNAs, (B) circRNAs, (C) miRNAs, and (D) mRNAs. Rows indicate significantly differentially expressed RNAs and columns represent individual samples from all the four groups.



Supplementary Figure S5. GO Enrichment analysis of miRNA groupwise comparisons.



Supplementary Figure S5. GO Enrichment analysis of miRNA groupwise comparisons. Top 20 GO enrichment (Biological process, BP; Cellular compartment, CC; and Molecular function, MF) hits are plotted for (A) HypoTH vs. Control; (B) HyperTH vs. Control; (C) HypoTH+T3 vs. Control; (D) HyperTH vs. HypoTH; (E) HypoTH+T3 vs. HypoTH; and (F) HypoTH+T3 vs. HyperTH group. All the hits plotted here are statistically significant with $p(\text{adj/corr}) < 0.05$.

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