Supplemental Materials

Lnc-ANRIL Protects Against Myocardial Ischemia-Reperfusion Injury by Suppressing Ferroptosis via the miR-7238-3p/GPX4 Axis

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2.5 Real-time quantitative PCR (qRT-PCR) — MIQE-compliant revision

2.5.1 Experimental design

• Objective: Quantify mRNA levels of GPX4, ACSL4, lnc-ANRIL and miR-7238-3p in HL-1 cardiomyocytes and mouse left-ventricular tissue subjected to hypoxia-reoxygenation (H/R) or MI/R.

• Groups: Control (normoxia or sham-surgery), I/R, I/R+lnc-ANRIL-OE, I/R+siANRIL, I/R+miR-7238-3p-mimic, I/R+miR-7238-3p-inhibitor, and respective scrambled controls.

• Biological replicates: n = 6 independent mice or cell cultures per group.

• Technical replicates: triplicate wells per sample; two independent RNA extractions per biological sample (nested design).

2.5.2 Sample

• HL-1 cardiomyocytes: 1×10^6 cells per 60 mm dish; harvested 48 h post-transfection.

• Mouse heart: left-ventricular free wall (30 ± 2 mg) immediately snap-frozen in liquid N_2, stored at −80 °C ≤ 4 weeks.

• Homogenisation: TissueLyser II (Qiagen, 30 Hz, 2 × 30 s, 4 °C) in 1 mL AG RNAex Pro (#AG21101, Accurate Biology).

2.5.3 Nucleic acid extraction

• Kit: AG RNAex Pro reagent with on-column DNase I (RNase-Free DNase Set, Qiagen #79254).

• QC:

– Purity: NanoDrop A260/280 = 2.08 ± 0.03; A260/230 ≥ 1.9.

– Integrity: Bioanalyzer RIN ≥ 8.0 (tissue) or 28S/18S ≥ 1.8 (gel).

• Storage: RNA aliquots (20 μL) at −80 °C, single-thaw use.

2.5.4 Reverse transcription (RT)

• RNA input: 500 ng per reaction.

• Kit: HiScript III RT SuperMix (#R323, Vazyme) with both oligo-dT and random hexamers.

• RT conditions: 50 °C 15 min → 85 °C 5 s → 4 °C hold.

• Controls: minus-RT (no enzyme) for every RNA sample to confirm absence of gDNA.
• RT efficiency: 5-point 1:4 serial dilution, slope −3.24 ± 0.05, $R^2 \geq 0.996$, efficiency 103–105 %.

2.5.5 qPCR target information

• Primers (Table 1) designed with Primer-BLAST (NCBI) and synthesised by Sangon (HPLC purified).
• Amplicon size: 87–338 bp; spans ≥ 1 exon–exon junction for mRNAs.
• Specificity: single melt-peak (±0.2 °C) and single band on 3 % agarose.
• Accession numbers and sequences provided in Table 1.

Table 1. Primers for qRT-PCR

Gene	NCBI Reference Sequence	nucleotide numbers	Sequences (5' - 3')	
GPX4	NM_001037741.4	100	Forward	GAGAGACCTGAACGGAACGCTTTC
			Reverse	TGGGTTTGGTGGTTGCTGGTTG
ACSL4	NM_207625.2	130	Forward	CACCCACTCCCATCTCCCGAAG
			Reverse	TGGCATCTCCCTGGTCCCTTAAC
GAPDH	NM_001289726.2	87	Forward	TGCACCACCAACTGCTTAGC
			Reverse	GGCATGGACTGTGGTCATGAG
Lnc ANRIL	NR_132431.1	338	Forward	TTAGGCAGCAGCAGAACGAAGAAG
			Reverse	AATAGATGGACGACAGTGTTTGGTGAG
miR-669c-3p	-	-	Forward	AACAGTGCGCGTACACACACA
			Reverse	ATCCAGTGCAGGGTCCGAGG
			RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTTTACT
miR-149-5p	NR_029559.1	-	Forward	AAGTCGCTCTGGCTCCGTGT
			Reverse	ATCCAGTGCAGGGTCCGAGG
			RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACGGGAGT
miR-574-5p	NR_029559.1	-	Forward	CTGAGTGTGTGTGTGTGAGTGTGT
			Reverse	ATCCAGTGCAGGGTCCGAGG
			RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACACACACTC
miR-466d-5p	LM380493.1	-	Forward	AACACGCTGTGTGTGCGTACA
			Reverse	ATCCAGTGCAGGGTCCGAGG
			RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACCATGTA
miR-135a-2-3p	LM381920.1	-	Forward	AAGCGCCTTGTAGGGATGGAAG
			Reverse	ATCCAGTGCAGGGTCCGAGG
			RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTTCATG

miR-7238-3p	NR_106097.1	-	Forward	CCTGCTGGCTGTCCTTTGTGT
			Reverse	ATCCAGTGCAGGGTCCGAGG
			RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACGAACAC
U6	X06980.1	99	Forward	GCTCGCTTCGGCAGCACAT
			Reverse	ATGGAACGCTTCACGAAT

2.5.6 qPCR reaction

• Master mix: SYBR qPCR Master Mix (#Q711-02, Vazyme) with ROX reference dye.

• Final reaction: 10 μL total volume containing 2 μL cDNA (1:10 dilution) and 0.4 μM each primer.

• Instrument: qTOWER³ Real-Time PCR Thermal Cycler (Analytik Jena, Germany) in 96-well plates (Axygen PCR-96-FS-C).

• Cycling: 95 °C 30 s; 40 cycles (95 °C 5 s, 60 °C 30 s); melt-curve 65–95 °C (0.5 °C increment).

2.5.7 Controls and calibrators

• NTC (no-template control) and NRT (minus-RT) on every plate.

• Inter-run calibrator: pooled reference cDNA (CV < 5 % across plates).

• Reference genes: GAPDH (mRNA) and U6 (miRNA) validated by geNorm (M-value < 0.5, stability confirmed under H/R).

2.5.8 Data analysis

• Cq determination: automated baseline (cycles 3–15) and threshold set at 0.1 ΔRn.

• Relative quantification: $2^{-\Delta\Delta Cq}$ with efficiency correction (Pfaffl method).

• Exclusion criteria: Cq > 35 or individual Cq SD > 0.3 among triplicates.

• Statistics: GraphPad Prism 10, unpaired t-test or one-way ANOVA with Sidak post-hoc; significance $p < 0.05$.

2.5.9 Reagents and lot traceability

• All reagent lot numbers, and instrument service dates are provided in Supplementary Table S2 to ensure full traceability.

Table 2 All reagent lot numbers, and instrument service dates

Reagent	Company	Lot number (or service dates
TissueLyser II	Qiagen, USA	2022.9
AG RNAex Pro	Accurate Biology, China	#AG21101
AG RNAex Pro reagent with on-column DNase I	Qiagen, USA	#79254
HiScript III RT SuperMix	Vazyme, China	#R323
SYBR qPCR Master Mix	Vazyme, China	#Q711-02
qTOWER³ Real-Time PCR Thermal Cycler	Analytik Jena, Germany	2022.9-2023.12

table 3 Antibodies for western blot

Antibodies	Source	ID
GPX4	Signalway Antibody，America	#21612
ACSL4	Abcam，England	ab15323
GAPDH	Signalway Antibody，America	#48763
SLC7A11	Signalway Antibody，America	#29069
Goat anti-rabbit	Signalway Antibody，America	#L3016
Gaot anti-Mouse	Signalway Antibody，America	#L3032

Figure 1 Schematic diagram of the potential binding site of miR-7238-3p in the promoter region of ACSL4