Supporting Information

Wang et al. 10.1073/pnas.0813371106

SI Text

Materials and Methods. *Animals and the animal model.* Six-month-old male Balb/C mice were fasted for 24 h before a single i.p. injection of either varying concentrations of acetaminophen in PBS (treatment group) or PBS (control group). Mice were euthanized at different time points after exposure; plasma (with EDTA as anticoagulant) and liver samples were collected for pathological examination and biochemical analyses.

To assess the tissue damage induced by acetaminophen overdose, the sections of liver tissues from treated and control animals were examined by a pathologist, and the ALT level was also determined in plasma obtained from the same animal by using a standard end-point colormetric assay kit (TECO Diagnostics).

RNA isolation from plasma. Total RNA, including miRNA from plasma, was isolated by using the miRNeasy kit (Qiagen) with minor modifications (S. Zhang and K. Wang, unpublished work). In brief, 700 μL of QIAzol reagent was added to 200 μL of plasma sample. The sample was mixed in a tube, followed by the addition of 3 μL of miSPIKE, spiked-in miRNA, at a concentration of 0.1 μM (IDT) and 140 μL of chloroform. After mixing vigorously for 15 s, the sample was then centrifuged at 12,000 $\times$ *g* for 15 min. The upper aqueous phase was carefully transferred to a new collection tube, and 1.5 vol of ethanol were added. The sample was then applied directly to a silica membrane containing column and the RNA was bound and cleaned by using buffers provided by the manufacturer to remove impurities. The immobilized RNA was then collected from the membrane with a low salt elution buffer. The quality and quantity of the RNA was evaluated by 260/280 ratio and Agilent 2100 Bioanalyzer (Agilent Technologies). The efficiency of small RNA isolation is monitored by the amount of spiked-in miRNA recovered by using PCR with sequence specific primers (IDT).

Array hybridization and data analyses. To assess the level and composition of miRNA, miRNA arrays from Agilent Technologies were used. The RNA samples were labeled and processed according to manufacturer's recommended protocols. In brief, $\approx$100 ng of total RNA was dephosphorylated with calf intestinal alkaline phosphatase, followed by denaturing with heat in the presence of dimethyl sulfoxide (DMSO). A cyanine dye, cyanine 3-cytidine bisphosphate (pCp), was then joined to the dephosphorylated single-stranded RNA (including miRNA) by T4 RNA ligase. MicroBioSpin 6 columns (Bio-Rad) were used to remove any unincorporated cyanine dye from the samples. The purified labeled miRNA probes were hybridized to 8 $\times$ 15 K mouse miRNA microarrays in a rotating hybridization oven at 10 rpm for 20 h at 55 °C. After hybridization, the arrays were washed in Agilent GE Wash Buffer 1 with Triton X-102, followed by Agilent GE Wash Buffer 2 with Triton X-102. After washing, all slides were immediately scanned at 5-μm resolution by using a PerkinElmer ScanArray Express array scanner. The resulting images were quantified by using Agilent's Feature Extraction software. The differentially expressed miRNAs were identified by using a standard protocol developed for mRNA gene arrays. To increase the reliability of the data and the ease of detection, miRNA species with hybridization intensities $<$1.5 times the average hybridization intensity (mean) were excluded from analysis. The miRNA clustering analysis was performed with the Hierarcical clustering algorithm provided in MultiExperiment Viewer, MeV4.0 software package (www.tm4.org/mev.html).

Real-time quantitative RT-PCR analysis. The expression levels of miRNA were confirmed with a SYBR-based quantitative PCR (QPCR) using individual miRNA-specific primers (Qiagen). The first-strand miRNA-cDNA PCR template was generated from $\approx$50 ng of total RNA according to the manufacturer's instructions. Approximately 2.5 ng of cDNA was then used in the PCR. The PCR product that corresponded to the specific miRNA species was first confirmed on a 6% polyacrylamide gel. The level of specific miRNA based on SYBR green intensity was then monitored with the 7900HT real-time PCR system from Applied Biosystems. The QPCR results were analyzed by SDS 2.2.2.

Results. *Significant elevation of plasma RNA quantity in acetaminophen-overdosed mice.* The amount of total RNA isolated from control and 24 h after acetaminophen-overdosed liver samples were similar, ranging from 150 μg to 300 μg from $\approx$100 mg of tissue. However, the amount of RNA isolated from plasma was quite different between control and treated samples. In the control samples, the amount of total RNA obtained ranged from 0.2 to 0.5 μg from 200 μL of plasma. The plasma from 24 h after acetaminophen-overdosed animals provided 2–18 μg of RNA from 200 μL of plasma, a 10- to30-fold increase of the RNA in the plasma.

Direct correlation between plasma RNA levels with tissue injury. In addition to proteins, it has been reported that plasma contains significant levels of nucleic acids including DNA and RNA. It has long been believed that DNA and RNA as well as some of the intracellular proteins such as lactate dehydrogenase (LDH), ALT, and AST appear in the plasma as a result of cell death through normal physiological renewal as well as necrotic or apoptotic processes occurring in various pathological conditions. The level of circulating DNA and liver-specific RNA have been suggested as good indicators for drug-induced tissue injury. In our study, we also observed a gradual increase of RNA in plasma with the progression of tissue injuries induced by acetaminophen. At 24 h after acetaminophen exposure, the amount of plasma RNA showed an increase as high as 70-fold (from $\approx$0.2 to 0.3 μg from 200 μL of control plasma samples to as high as 18 μg in drug-overdosed plasma samples). At the same time point, the ALT level, a well-established indicator for hepatocyte injury, was increased by 360-fold, which suggests that the loss of cellular integrity makes a significant contribution to the increase of plasma RNA levels (Fig. S3). The circulating RNA concentration by itself may be a good indicator for drug-induced tissue injury, but lack of specificity makes it unlikely to be as informative as changes in specific miRNA levels in the plasma.

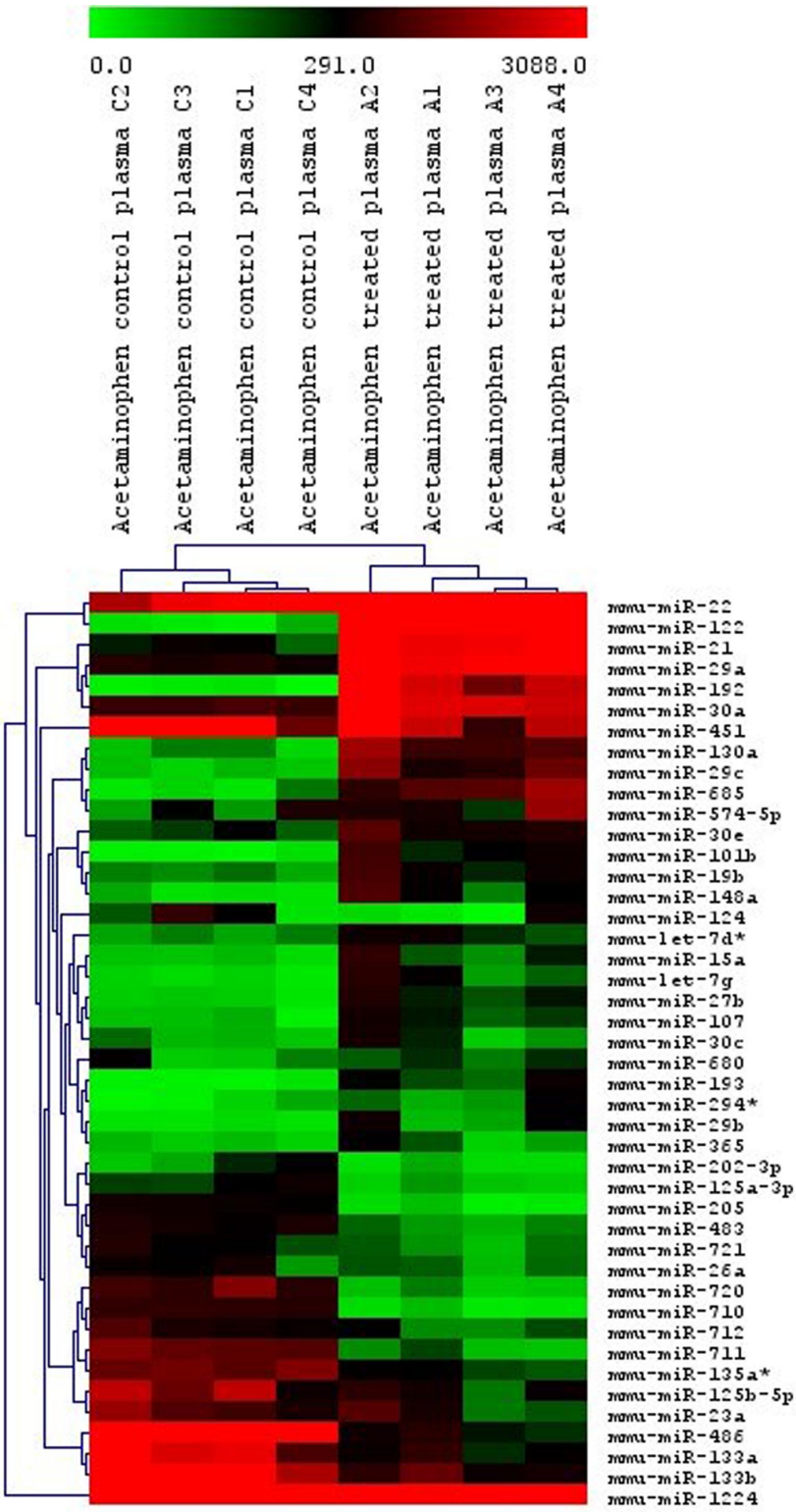

Fig. S1. Cluster analysis of plasma miRNAs. Cluster analysis of 44 miRNAs that showed a >2-fold change between control and acetaminophen-overdosed plasma samples. The progressively brighter shades of red indicate a gradual increase of the miRNA concentration, whereas the shades of green indicate a gradual decrease of miRNA expression levels. The identities of miRNAs are listed on the right, whereas the sample names are on the top.

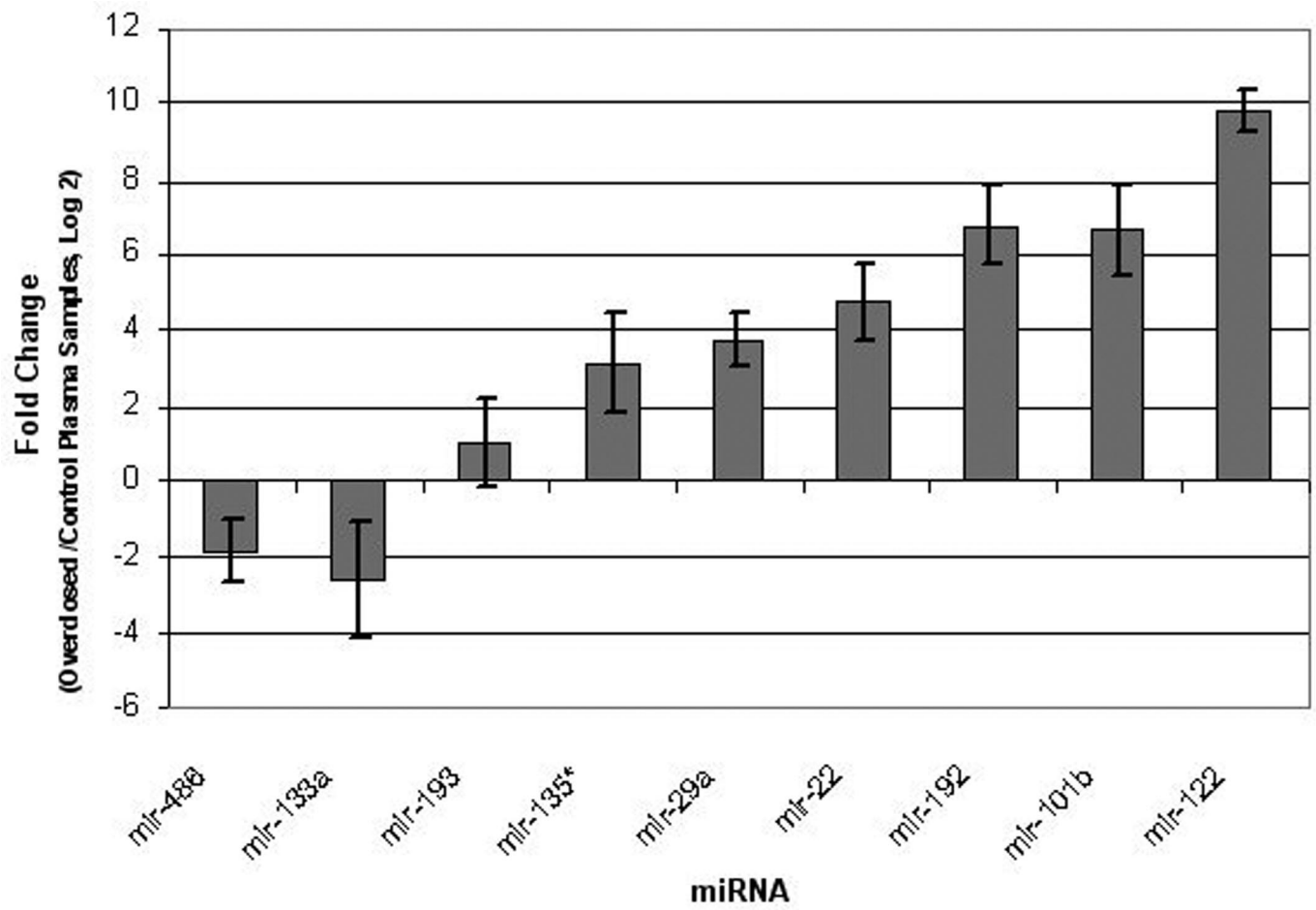

Fig. S2. Confirmation of differentially expressed miRNA species in plasma by QPCR. The identities of miRNA species used in QPCR are indicated. Results are shown as log2 of the ratio of the mean of 4 independent overdosed plasma samples and the mean of 4 independent control plasmas. The standard derivations are shown as error bars.

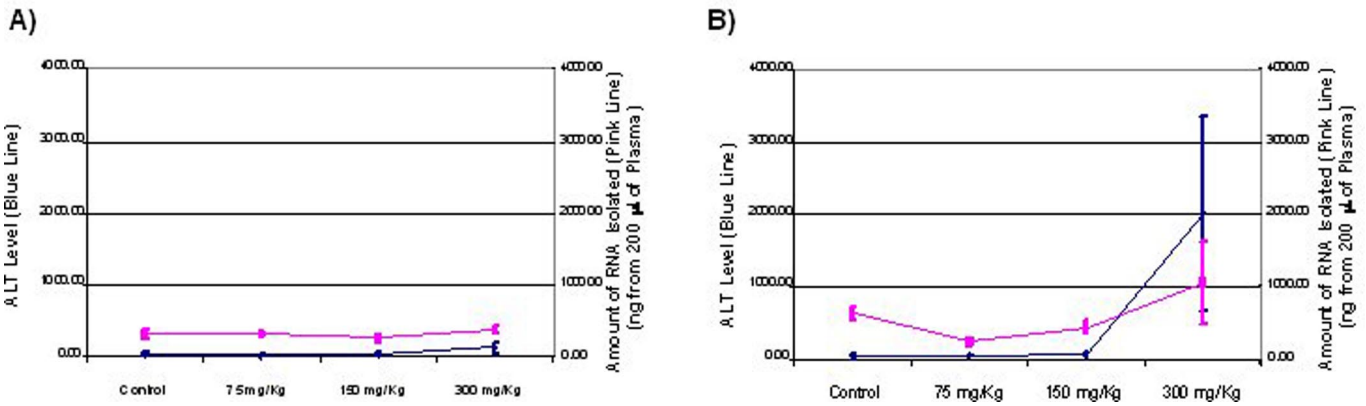

Fig. S3. Amount of RNA isolated from plasma correlates well with the ALT levels. The trends between the amount of RNA isolated from 200 μL of plasma (pink line) and the ALT (blue line) levels from samples collected from mice 1 (*A*) and 3 (*B*) h after exposed to different doses of acetaminophen (indicated on *x* axis) are similar.

Table S1. Detectable miRNAs in the liver and plasma by microarray

Liver tissues		Plasma samples	
Control	Treated	Control	Treated
mmu-let-7b	mghv-miR-M1–2	**mmu-let-7b**	**mmu-let-7b**
mmu-let-7c	mmu-let-7d*	**mmu-let-7c**	**mmu-let-7c**
mmu-miR-101b	**mmu-miR-122**	**mmu-miR-1224**	mmu-let-7d*
mmu-miR-122	**mmu-miR-1224**	**mmu-miR-124**	mmu-let-7 g
mmu-miR-1224	mmu-miR-188-5p	mmu-miR-125a-3p	mmu-miR-1
mmu-miR-130a	mmu-miR-197	**mmu-miR-125b-5p**	mmu-miR-101b
mmu-miR-15a	mmu-miR-207	**mmu-miR-133a**	mmu-miR-106b
mmu-miR-192	**mmu-miR-21**	**mmu-miR-133b**	mmu-miR-107
mmu-miR-193	**mmu-miR-22**	**mmu-miR-135a***	mmu-miR-122
mmu-miR-194	mmu-miR-297a	**mmu-miR-140***	**mmu-miR-1224**
mmu-miR-19b	mmu-miR-297b-3p	**mmu-miR-16**	**mmu-miR-124**
mmu-miR-21	**mmu-miR-29a**	**mmu-miR-188-5p**	**mmu-miR-125b-5p**
mmu-miR-212	**mmu-miR-30a**	mmu-miR-199b	mmu-miR-130a
mmu-miR-22	mmu-miR-328	mmu-miR-202-3p	**mmu-miR-133a**
mmu-miR-26a	mmu-miR-466c-5p	mmu-miR-205	**mmu-miR-133b**
mmu-miR-29a	mmu-miR-466d-3p	**mmu-miR-21**	**mmu-miR-135a***
mmu-miR-29b	mmu-miR-466f-3p	**mmu-miR-22**	**mmu-miR-140***
mmu-miR-29c	mmu-miR-466 g	**mmu-miR-223**	mmu-miR-148a
mmu-miR-30a	mmu-miR-466 h	**mmu-miR-23a**	mmu-miR-15a
mmu-miR-30e	mmu-miR-467a*	**mmu-miR-24**	**mmu-miR-16**
mmu-miR-487b	mmu-miR-467b*	**mmu-miR-25**	**mmu-miR-188-5p**
mmu-miR-494	mmu-miR-467e*	mmu-miR-26a	mmu-miR-192
mmu-miR-720	mmu-miR-468	**mmu-miR-29a**	mmu-miR-193
	mmu-miR-483	**mmu-miR-30a**	mmu-miR-19b
	mmu-miR-483*	**mmu-miR-30d**	**mmu-miR-21**
	mmu-miR-485*	mmu-miR-328	**mmu-miR-22**
	mmu-miR-494	**mmu-miR-378**	**mmu-miR-223**
	mmu-miR-574-3p	**mmu-miR-451**	**mmu-miR-23a**
	mmu-miR-574-5p	mmu-miR-483	**mmu-miR-24**
	mmu-miR-669a	**mmu-miR-486**	**mmu-miR-25**
	mmu-miR-669c	**mmu-miR-574-5p**	mmu-miR-27a
	mmu-miR-671-5p	**mmu-miR-671-5p**	mmu-miR-27b
	mmu-miR-672	mmu-miR-680	mmu-miR-294*
	mmu-miR-689	**mmu-miR-689**	**mmu-miR-29a**
	mmu-miR-709	**mmu-miR-709**	mmu-miR-29b
	mmu-miR-710	mmu-miR-710	mmu-miR-29c
	mmu-miR-711	mmu-miR-711	**mmu-miR-30a**
	mmu-miR-720	**mmu-miR-712**	mmu-miR-30b
	mmu-miR-721	mmu-miR-720	mmu-miR-30c
	mmu-miR-877*	mmu-miR-721	**mmu-miR-30d**
		mmu-miR-762	mmu-miR-30e
		mmu-miR-92a	mmu-miR-365
		mmu-miR-93	**mmu-miR-378**
			mmu-miR-423-5p
			mmu-miR-451
			mmu-miR-486
			mmu-miR-574-5p
			mmu-miR-671-5p
			mmu-miR-685
			mmu-miR-689
			mmu-miR-709
			mmu-miR-712
			mmu-miR-92a

The common miRNA species between treated (24 h after acetaminophen exposure) and control are listed in bold characters.

Table S2. Detectable miRNAs by microarray among the 6 organs surveyed

miRNA	Predominately expressed tissue
mmu-let-7a	Brain
mmu-let-7b	Lung
mmu-let-7c	Brain
mmu-let-7d	Brain
mmu-let-7e	Brain
mmu-let-7f	Brain
mmu-let-7 g	Brain
mmu-let-7i	Lung
mmu-miR-1	Kidney
mmu-miR-100	Brain
mmu-miR-101a	Brain
mmu-miR-101b	Liver
mmu-miR-103	Brain
mmu-miR-107	Brain
mmu-miR-10a	Lung
mmu-miR-122	Liver
mmu-miR-1224	Spleen
mmu-miR-124	Brain
mmu-miR-125b-5p	Brain
mmu-miR-126-3p	Lung
mmu-miR-126-3p	Lung
mmu-miR-127	Brain
mmu-miR-128	Brain
mmu-miR-129-3p	Brain
mmu-miR-130a	Lung
mmu-miR-132	Brain
mmu-miR-133a	Heart
mmu-miR-133a*	Heart
mmu-miR-133b	Heart
mmu-miR-136	Brain
mmu-miR-137	Brain
mmu-miR-139-5p	Brain
mmu-miR-141	Lung
mmu-miR-142-3p	Spleen
mmu-miR-142-5p	Spleen
mmu-miR-143	Lung
mmu-miR-144	Spleen
mmu-miR-146a	Spleen
mmu-miR-148a	Liver
mmu-miR-150	Spleen
mmu-miR-151-5p	Lung
mmu-miR-153	Brain
mmu-miR-15a	Liver
mmu-miR-15b	Spleen
mmu-miR-16	Spleen
mmu-miR-181a	Brain
mmu-miR-192	Liver
mmu-miR-193	Liver
mmu-miR-194	Liver
mmu-miR-195	Lung
mmu-miR-199b	Lung
mmu-miR-199b*	Lung
mmu-miR-19b	Spleen
mmu-miR-200a	Lung
mmu-miR-200b	Lung
mmu-miR-200c	Lung
mmu-miR-204	Brain
mmu-miR-208a	Heart
mmu-miR-21	Liver
mmu-miR-218	Brain
mmu-miR-219	Brain
mmu-miR-22	Heart
mmu-miR-223	Spleen
mmu-miR-23a	Lung
mmu-miR-23b	Lung
mmu-miR-24	Lung
mmu-miR-26a	Brain
mmu-miR-26b	Lung
mmu-miR-27a	Lung
mmu-miR-27b	Lung
mmu-miR-29a	Brain
mmu-miR-29b	Brain
mmu-miR-29c	Brain
mmu-miR-300	Brain
mmu-miR-30a	Lung
mmu-miR-30b	Lung
mmu-miR-30c	Lung
mmu-miR-30d	Heart
mmu-miR-30e	Heart
mmu-miR-338-3p	Brain
mmu-miR-34a	Brain
mmu-miR-34b-5p	Lung
mmu-miR-34c	Lung
mmu-miR-376a	Brain
mmu-miR-378	Heart
mmu-miR-434-3p	Brain
mmu-miR-451	Spleen
mmu-miR-486	Heart
mmu-miR-494	Spleen
mmu-miR-497	Lung
mmu-miR-499	Kidney
mmu-miR-652	Lung
mmu-miR-689	Heart
mmu-miR-709	Brain
mmu-miR-720	Liver
mmu-miR-7a	Brain
mmu-miR-9	Brain
mmu-miR-9*	Brain
mmu-miR-92a	Spleen
mmu-miR-99a	Heart

The observed miRNAs are listed on the left and the predominant tissue expressing the miRNA is listed on the right.

Table S3. Possible tissue origin of circulating miRNAs

Tissue	Brain	Heart	Kidney	Liver	Lung	Spleen	Unknown	Total
Control plasma samples	6	7	0	2	5	5	18	43
Acetaminophen-overdosed plasma samples	9	8	1	7	9	6	13	53

Table S4. The relative changes of selected miRNA species in plasma after acetaminophen exposure

Acetaminophen dose ($n = 4$)	Time after exposure, h	mir-486	mir-133a	mir-135*	mir-101b	mir-192	mir-22	mir-193	mir-122
Control	1	0	0	0	0	0	0	0	0
75 mg/kg	1	−0.74 ± 0.72	0.51 ± 1.18	−0.18 ± 1.02	0.47 ± 0.82	0.47 ± 0.95	1.10 ± 1.91	0.27 ± 1.01	−0.04 ± 1.97
150 mg/kg	1	−0.58 ± 0.72	0.48 ± 1.92	0.87 ± 1.24	0.90 ± 1.08	2.46 ± 1.37	1.42 ± 1.52	1.07 ± 1.36	4.50 ± 1.96
300 mg/kg	1	−0.08 ± 0.76	−0.27 ± 1.22	−0.10 ± 1.18	0.39 ± 1.00	1.16 ± 1.26	1.84 ± 1.75	0.00 ± 1.19	2.15 ± 2.07
Control	3	0	0	0	0	0	0	0	0
75 mg/kg	3	−0.40 ± 0.78	−0.60 ± 1.69	1.22 ± 1.17	0.98 ± 1.21	1.38 ± 0.73	−0.11 ± 2.83	0.75 ± 1.25	1.55 ± 1.08
150 mg/kg	3	0.14 ± 1.01	0.15 ± 1.57	0.49 ± 1.28	0.21 ± 1.41	3.86 ± 1.87	−0.33 ± 2.92	0.98 ± 0.94	6.37 ± 2.28
300 mg/kg	3	−0.64 ± 0.78	−1.62 ± 1.63	3.03 ± 1.84	3.22 ± 2.20	5.75 ± 2.55	0.25 ± 2.68	0.97 ± 1.16	8.40 ± 2.71

For each treatment condition, time, and dose, plasma samples collected from 4 independent animals were compared. The relative changes based on QPCR results, reported on a log2 scale, were determined by comparing the means between acetaminophen-exposed samples with its corresponding time point controls. The standard derivation of the sample sets are shown.