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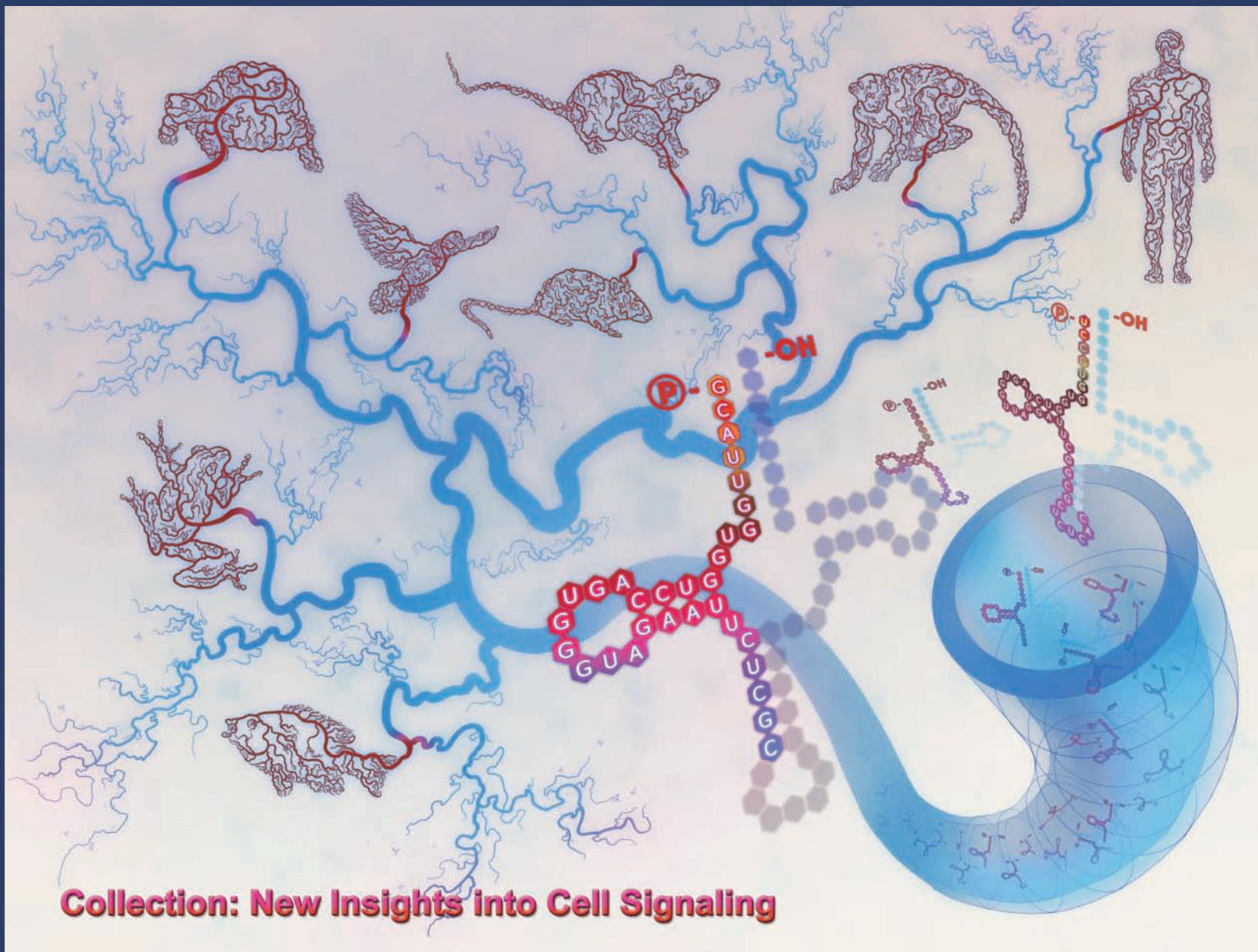

Collection: New Insights into Cell Signaling

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Cover: An ancient class of tRNA-derived small RNAs (tsRNAs) abundantly and conservatively exists in the sera of a wide range of vertebrate species along the evolution tree. The serum tsRNAs show sensitive response to active infection in mouse, monkey, and human being. See pages 172–174 by Zhang et al. for details.

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Letter to the Editor

Identification and characterization of an ancient class of small RNAs enriched in serum associating with active infection

Dear Editor,

The identification of novel serum biomarkers holds great value for diagnosing and monitoring disease conditions due to its convenient and non-invasive nature. Recently, great interests have been shed on serum microRNAs (miRNAs), which emerge as promising biomarkers for a variety of diseases including cancer and metabolic disorders (Cortez et al., 2011). Despite the concentrated attention on serum miRNAs, the reports on the existence and diagnostic value of other serum small RNAs remain surprisingly few. In present study, we identify and characterize an ancient class of tRNA-derived small RNAs (tsRNAs) that abundantly and conservatively exist across a wide range of vertebrate species (from fish to human) and demonstrate their sensitive response to body infection in mouse, monkey, and human being.

We recently identified a novel class of tsRNAs that were derived from the 5′ half of tRNAs (29–34 nt) and highly concentrated in mature mouse sperm under physiological condition and termed them as mature-sperm-enriched tsRNAs (mse-tsRNAs) (Peng et al., 2012). Following this clue, we screened multiple mouse organs by small RNA deep-sequencing (18–40 nt) to reveal potential existence of tsRNAs in other tissues. Under physiological condition, the levels of tsRNAs in most examined tissues (except for bone marrow) were very low (<5%) compared with the well-characterized miRNA population (Supplementary Figure S1). However, the mouse serum showed a surprisingly high percentage of tsRNAs (~70%), exceeding miRNA reads in sum (Figure 1A). High percentage of tsRNAs was also detected in rat serum (~95%) and monkey serum (~43%) (Figure 1B and C), suggesting the existence of abundant serum tsRNAs in more different species. More detailed sequence analysis of serum tsRNAs from mouse, rat, and monkey revealed the preference for some tsRNA species, e.g. $tsRNA^{Gly}$, $tsRNA^{Glu}$, $tsRNA^{Val}$, and $tsRNA^{His}$ (Supplementary Table S1). Then, the sera of a wider range of vertebrate species along the evolution tree, including fish, amphibian, reptile, avian, murine, non-human primate, and human being, were further examined by RT–PCR for $tsRNA^{Gly}$ and $tsRNA^{Glu}$ (Figure 1D and E). The sizes coupled with sequencing results of PCR products (Figure 1E) demonstrated that these serum tsRNAs (at least for $tsRNA^{Gly}$ and $tsRNA^{Glu}$) are an ancient class of small RNAs with highly conserved sequences across all examined vertebrate species.

How could these serum tsRNAs stably exist within an RNase-rich blood environment? Chemically synthesized tsRNAs added into the serum environment (37°C) were rapidly degraded within 10 min, while serum tsRNAs stably existed in the same environment for extended time periods, similar to serum miRNAs (Figure 1F). To data, serum miRNAs are mostly reported to be protected from rapid degradation though either encapsulated in the serum microvesicles (exosomes) or binding with serum proteins (Chen et al., 2012). We therefore tested whether these mechanisms were applicable to the newly discovered serum tsRNAs. By isolating exosomes from the serum (Supplementary Figure S2), we found that, unlike the exosome-enriched let-7a, the tsRNAs were not concentrated in exosomes but remained in serum supernatant (Figure 1G). We next separated serum contents by centrifugal filters with different molecular weight cut-offs (Supplementary Figure S3), and found that the tsRNAs were highly concentrated in serum complex with molecular weight >100 kDa (Figure 1H), suggesting their co-existence with serum protein complexes. Since it has been reported that protein complexes could protect circulating miRNAs from plasma RNases (Arroyo et al., 2011), we applied Protease K digestion on mouse serum, monitoring serum protein contents by silver staining (Figure 1I) and tsRNA levels by quantitative PCR (Figure 1J) at different time points. Interestingly, the tsRNA levels showed an apparent increase at 30 min after Protease K digestion (Figure 1J), accompanied with an overall decrease in the size of detected proteins (Figure 1I). A reasonable explanation for this phenomenon could be that a substantial number of tsRNAs are initially tightly embedded/bound within a large-size serum protein complex, which was resistant to RNA extraction (by Triozl) but more susceptible to Protease K treatment, thus causing more tsRNAs released and extracted after Protease K digestion. During prolonged Protease K treatment, serum tsRNAs did not show significant increase in the degradation rate as observed in miR-191, a serum miRNA whose stabilization depends on protein-binding (Arroyo et al., 2011) (Figure 1J), suggesting other mechanisms contributing to decreased degradation of serum tsRNAs. The tRNAs are known to possess nucleotide modifications at multiple sites, and the cytosine-C5 methylation has shown important roles for the stabilization of tRNAs (Tuorto et al., 2012). To test the possibility that the stability of tsRNAs

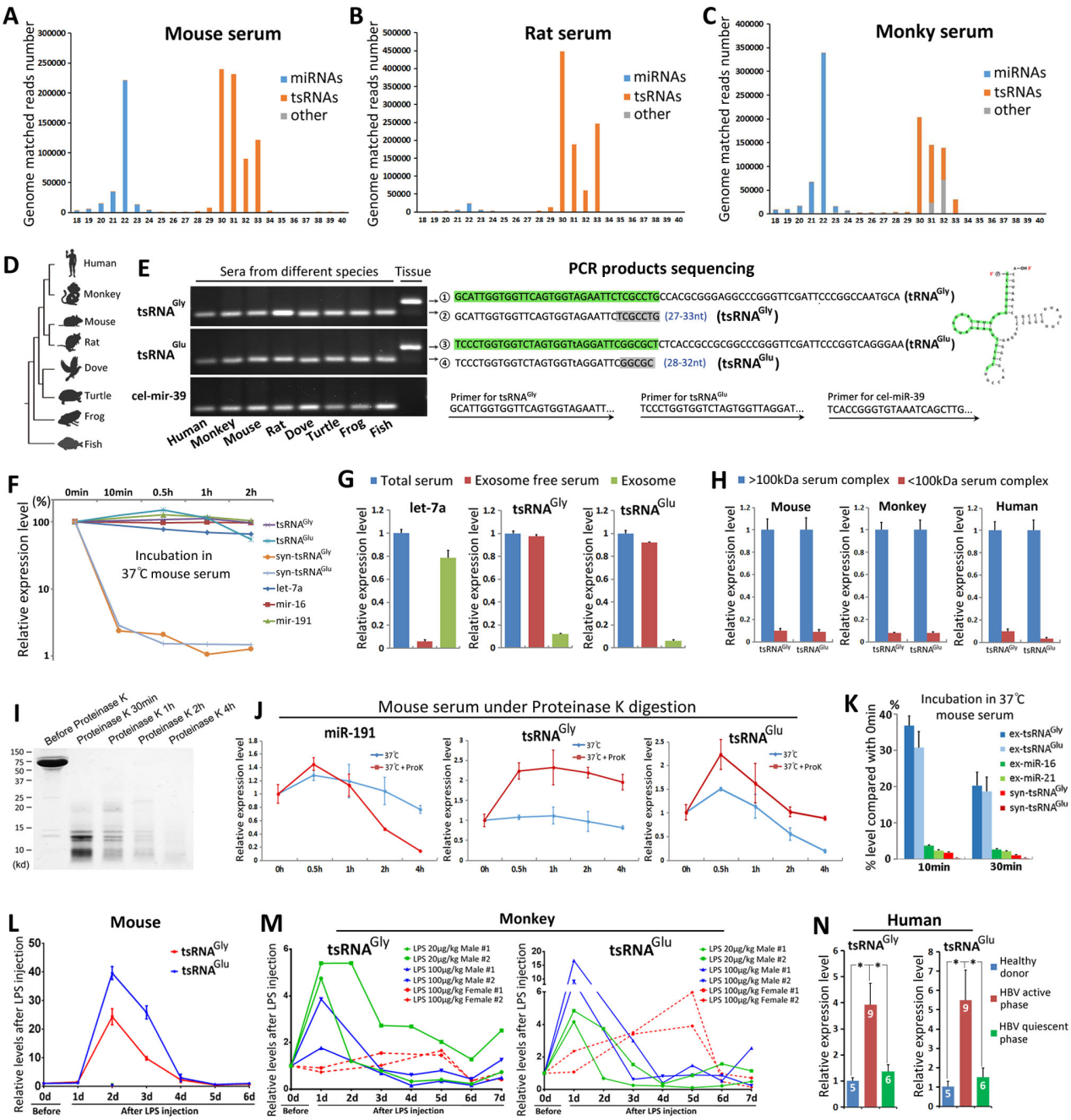

Figure 1 Identification and characterization of serum tsRNAs and their response to body infection. (**A**–**C**) Length distributions of small RNAs and the abundant tsRNAs in the sera of mouse, rat, and monkey. (**D**) Species along the evolution tree for examination of serum tsRNAs. (**E**) RT–PCR analysis of tsRNA^{Gly} and tsRNA^{Glu} in various species, followed by PCR products sequencing. Cel-miR-39 was added in the serum as internal loading control. The variable nucleotides are marked by shade. (**F**) Stability of serum tsRNAs, miRNAs, and chemically synthesized tsRNAs in 37°C mouse serum. (**G**) Examination of let7a, tsRNA^{Gly}, and tsRNA^{Glu} in serum, supernatant, and isolated exosomes by quantitative PCR. Data are normalized and shown graphically as mean ± SEM of three independent samples. (**H**) Examination of tsRNA^{Gly} and tsRNA^{Glu} in different serum components separated by a molecular weight cut-off 100 kDa. Data are normalized and shown graphically as mean ± SEM of three independent samples. (**I**) Silver staining for serum protein contents after Proteinase K digestion. (**J**) Examination of serum miR-191, tsRNA^{Gly}, and tsRNA^{Glu} after Proteinase K digestion by quantitative PCR. Data are expressed as mean ± SEM of three independent experiments for each time point. (**K**) Stability of chemically synthesized tsRNAs and extracted serum tsRNAs and miRNAs in 37°C mouse serum. Data are expressed as mean ± SEM of 2–3 independent experiments. (**L** and **M**) Examination of serum tsRNA^{Gly} and tsRNA^{Glu} in LPS-induced inflammation models in mice (**L**) and monkeys (**M**) by quantitative PCR. For **L**, data are expressed as mean ± SEM of 3–4 mice for each time point. (**N**) Examination of serum tsRNA^{Gly} and tsRNA^{Glu} in healthy human donors and patients under HBV infection (either HBV active phase or quiescent phase) by quantitative PCR. Data are expressed as mean ± SEM of indicated numbers of samples for each group (*$P < 0.05$, *t*-test).

benefits from the nucleotide modifications inheriting from their precursor tRNAs, we compared the stability of chemically synthesized tsRNAs (no nucleotide modification) with tsRNAs/miRNAs extracted from the serum (without protection by binding proteins) by re-adding them into a 37°C serum environment (Supplementary Figure S4). The results clearly showed that tsRNAs, but not miRNAs, extracted from serum were much more stable than chemically synthesized tsRNAs (Figure 1K), supporting the hypothesis that besides protein-binding, nucleotide modifications also increase the stability of serum tsRNAs.

Previous studies at cellular level have demonstrated that tsRNAs could be upregulated under various stresses (e.g. physical, chemical, and virus infection) (Thompson and Parker, 2009; Wang et al., 2013). The existence of abundant serum tsRNAs inspired us to explore whether they are closely linked with pathological conditions. In animal models of LPS-induced acute inflammation, serum tsRNAs showed a rapid increase during first days after LPS injection in both mice (Figure 1L) and monkeys (Figure 1M), followed by a decrease within 6 days, suggesting an active involvement in the acute phase of body inflammation. As shown in Figure 1N, our initial screening of human samples from patients under active hepatitis B virus (HBV) infection (virus replication phase) also revealed a significant upregulation of serum tsRNAs when compared with healthy donors or patients during HBV quiescent phase (in which the virus is inactive). By far, the exact source and mechanism of the surge upregulation of serum tsRNAs during body infection remain unclear. It is possible that serum tsRNAs are derived from bone marrow or immune cells, as they have been found in these compartments (Supplementary Figure S1) (Nolte-'t Hoen et al., 2012). Other cell types might also release tsRNAs into the serum by angiogenin-mediated tRNA cleavage under body stresses (Thompson and Parker, 2009; Ivanov et al., 2011). Since tsRNAs have been reported to play active roles in translational inhibition (Ivanov et al., 2011) or virus replication (Wang et al., 2013) at cellular level, these functions might also exist in mediating infection-induced defensive responses by the whole organism.

In summary, the discovery of abundant serum tsRNAs and their sensitive response to body infection unveils a hidden layer of serum small RNAs closely linking with disease condition. Recently, Dhahbi et al. (2013) reported similar findings on the existence of tsRNAs (named as '5′tRNA halves') in mammalian serum. The biological significance and diagnostic value of serum tsRNAs are intriguing, which warrants further in-depth study and possibly opens a new round of research focus on serum small RNAs.

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Supplementary Material include:

1. Supplementary Methods
2. Supplementary Table S1
3. Supplementary Figures S1-4

Supplementary Methods

Tissue/serum sample collection and RNA extraction

All examined tissue samples (for RNA-Seq) were collected from mice. Serum samples were collected from human, monkey, rat, mouse, dove, turtle, frog and fish, followed by RNAs extraction using the TRizol LS Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA extraction of mouse tissues was performed with standard protocols of TRIzol (Invitrogen, Carlsbad, CA).

Small RNA library construction, data processing and analysis

Small RNA library preparation, sequencing and processing were described previously (Peng et al 2012). The small RNA clean reads were mapped with mouse (mm10), rat (rn5) and monkey (rheMac3) genome by SOAP (Short Oligonucleotide Analysis Package, developed by Beijing Genomics Institute (BGI, Shenzhen, China)). Reads that perfect match to genome were used for further analysis and each kind of RNAs was quantified using the reads per million (RPM) method. Small RNA annotation was performed using Rfam (http://rfam.sanger.ac.uk/) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/). tRNA sequences and their secondary structures were obtained from Genomic tRNA Database (http://gtrnadb.ucsc.edu/). The evolution tree was derived from NCBI Taxonomy (http://www.ncbi.nlm.nih.gov/taxonomy).

RT-PCR and Quantitative PCR of small RNAs

The Reverse Transcription and quantitative assays of the small RNAs were conducted as previously described (Peng et al 2012).Cel-miR-39 was added into serum as an internal loading control during RNA extraction. The amplified PCR products were subcloned into pGEM-T easy vector (Promega) for sequencing.

Isolation of serum exosomes

Exosomes were collected from serum by standard procedures via ExoQuick-TC (System Biosciences). 0.5mL

serum was centrifuged at 12,000 × g for 10 min to eliminate cellular debris. The supernatant was then mixed with 120uL ExoQuick-TC and refrigerated 30 minutes .The mixture was centrifuged at 1,500 × g for 30 min, supernatants were removed to a new eppendorf tube, and the pellet was centrifuged for additional 5 min to remove all fluid .Then the exosome pellet was re-suspended in 100μL buffer. The supernatants and exosomes were stored at -80 °C before use. The protocols were illustrated in Supplementary Figure S2.

Serum components separation with molecular cut-offs

Samples of 0.5mL serum mixed with 0.5mL PBS were subjected to ultrafiltration through Vivaspin® 2 Centrifugal Concentrator with 100kDa MW cut-offs (Sartorius-stedim biotech). The procedures were illustrated in Supplementary Figure S3. Total RNAs were extracted from filtrate and concentrate fractions.

Protease K digestion of serum and silver staining

Mouse serum with or without protease K (1mg/ml) were incubated in 37°C. Samples were collected at different time points (0h, 0.5h, 1h, 2h, 4h) for RNA extraction or protein PAGE-SDS Gel silver staining using ProteoSilver™ Silver Stain Kit (sigma).

Synthetic /endogenous small RNA degradation experiments

The stability of chemically synthesized tsRNAs (by Takara), extracted serum tsRNAs and miRNAs were examined by re-adding them into 37℃ mouse serum as illustrated in Supplementary Figure S4. RNAs isolated from these serum samples were measured by quantitative PCR.

LPS-induced acute inflammation in mice and monkeys

CD1 Mice (female, 7-8 weeks) were purchased from Vital River Laboratories, Beijing, China. Adult monkeys (Macaca fascicularis) were provided by Kunming Biomed International (KBI, Kunming, China), weighing 3.2-9.5 kg and the ages ranging from 5-7 years old. Lipopolysaccharide (LPS) from Escherichia coli serotype 055:B5 was purchased from Sigma Chemical Company. Mice were I.P. injected with 0.5mg/kg of LPS resolved in saline solution. The control group was injected with saline alone. Blood samples were obtained from Day1 to Day6 after LPS injection. Serum was isolated and processed for RNA extraction. For Monkey experiments, 3mL blood was obtained from each monkey before the LPS/saline injection. LPS (20ug/kg or 100ug/kg) or saline were injected intravenously after anesthetization by intramuscular ketamine. For each monkey after

LPS/saline injection, the blood samples (3mL each time) were drawn every 24h for 7 days, the blood were processed for serum isolation and RNA extraction. The curves for tsRNAs changes after LPS injection were obtained after normalizing with the saline group.

Human serum samples

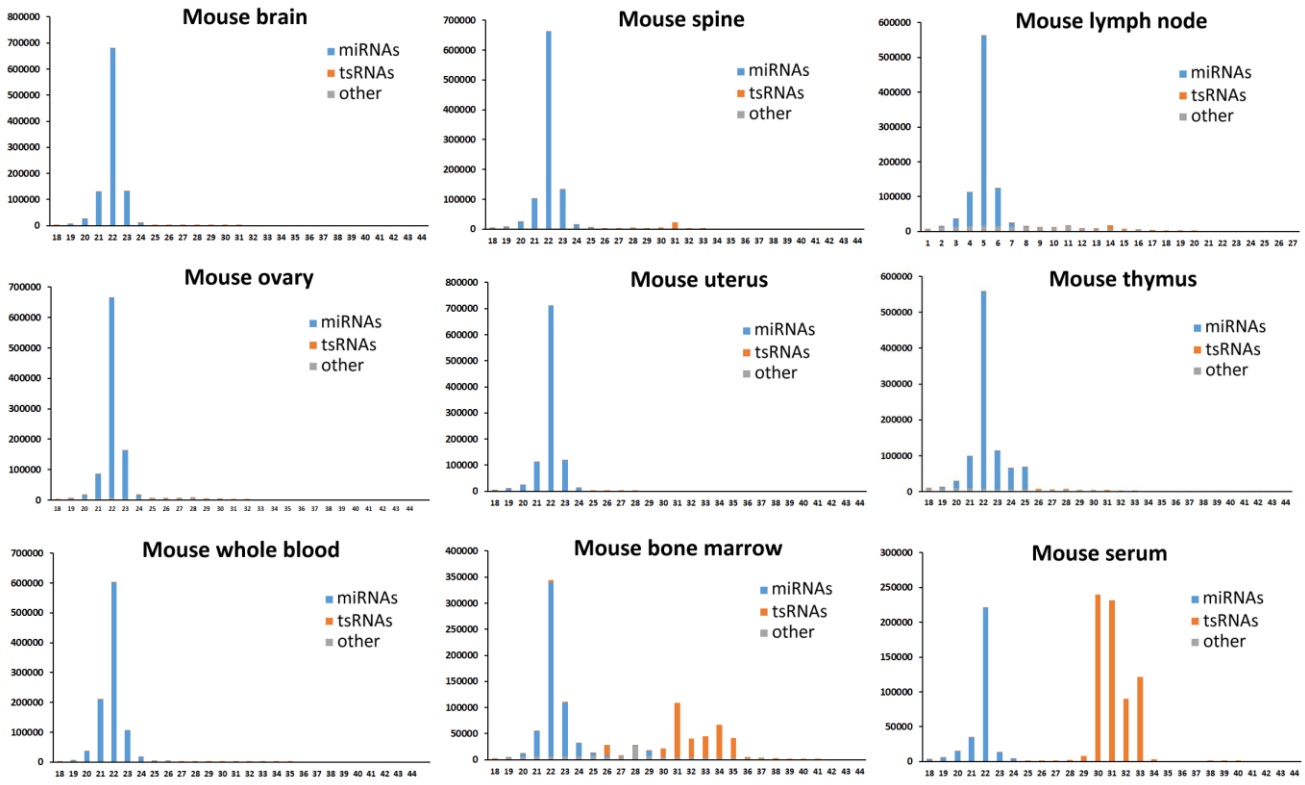
Human serum samples from healthy donors or patients under Hepatitis B virus infection were obtained with all the donors or their guardians providing written consent and ethics permission for the use of blood samples. Patients (ages range from 17-45) under virus infection (HBV replication phase or quiescent phase) were from Shandong Provincial Hospital. For patients under HBV replication phase, the quantities of serum viral load were ranged from 2 million to 70 million (IU/mL). While for patients under HBV quiescent phase, the quantities of serum viral load were < 500 (IU/mL). Healthy donors (ages range from 25-35) were from Chinese Academy of Sciences, Beijing. Blood from healthy donors and patients was collected in Vacuum blood collection tubes and incubated overnight at 4°C for serum obtaining, followed by RNA extraction.

Supplementary References

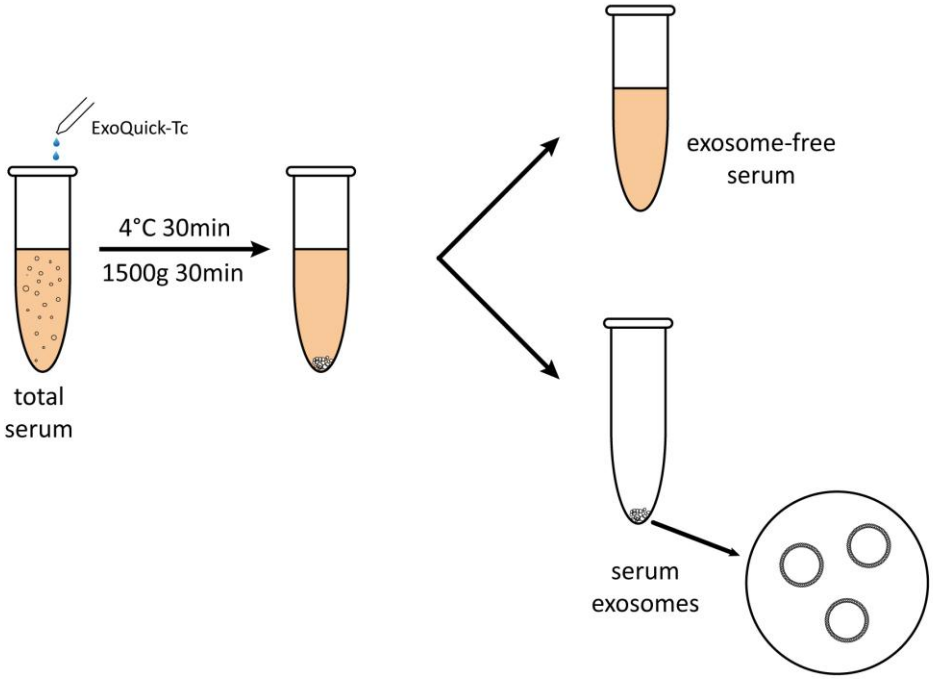
Peng, H., Shi, J., Zhang, Y., et al. (2012). A novel class of tRNA-derived small RNAs extremely enriched in mature mouse sperm. Cell Res. *22*, 1609-1612.

Supplementary Table S1 Predominant tsRNA species from sera of mouse, rat and Monkey.

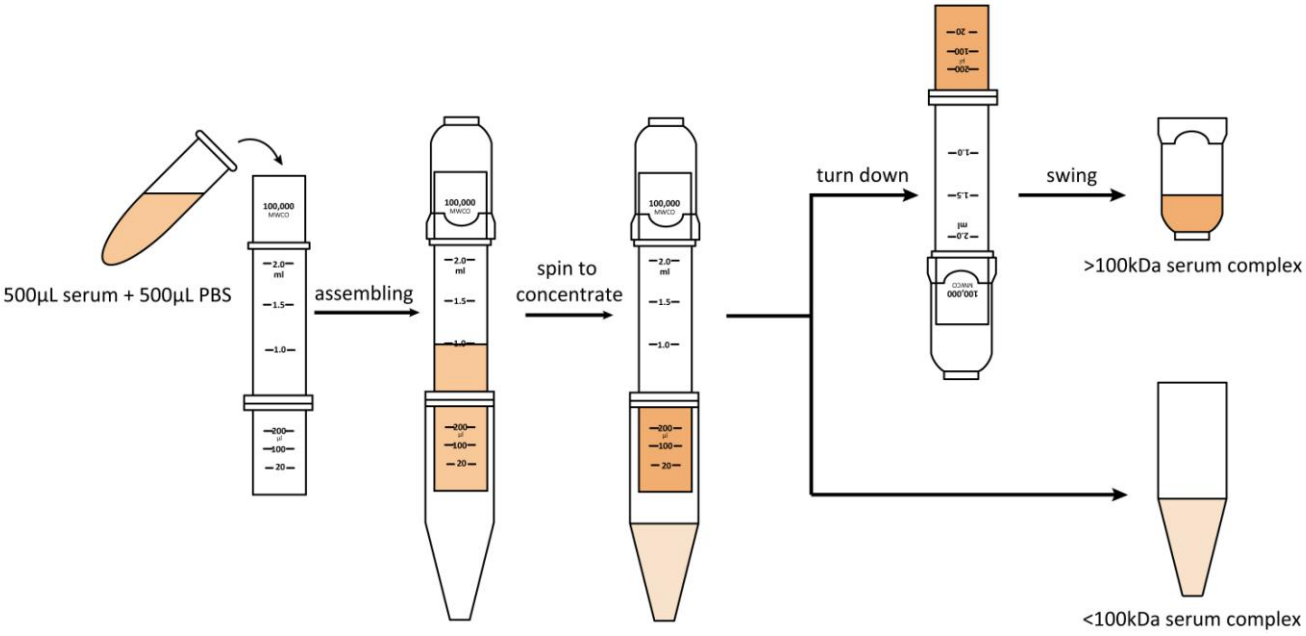
tsRNA species	Sequence (Predomiant)	Mouse *(mus musculus)*		Rat *(Rattus norvegicus)*		Monkey *(Macaca fascicularis)*	
		RPM	%	RPM	%	RPM	%
tsRNA-Gly(GCC)	GCATTGGTGGTTCAGTGGTAGAATTCTCGC	604880.46	60.49%	586958.42	58.70%	877648.68	87.76%
tsRNA-Gly(CCC)	GCATTGGTAGTTCAATGGTAGAATTCTCGCC	31941.68	3.19%	3844.22	0.38%	421.06	0.04%
tsRNA-Glu(CTC)	TCCCTGGTGGTCTAGTGGTTAGGATTCGGCG	39145.53	3.91%	3335.78	0.33%	7637.42	0.76%
tsRNA-Glu(TTC)	TCCCACATGGTCTAGCGGTTAGGATTCCTGGT	1388.62	0.14%	93.34	0.01%	969.35	0.10%
tsRNA-Val(AAC)	GTTTCCGTAGTGTAGTGGTTATCACGTTCGCCT	177326.51	17.73%	290990.24	29.10%	85109.38	8.51%
tsRNA-His(GTG)	GCCGTGATCGTATAGTGGTTAGTACTCTGC	65257.27	6.53%	96265.45	9.63%	6044.68	0.60%
tsRNA-Lys(CTT)	GCCCGGCTAGCTCAGTCGGTAGAGCATGAGAC	24691.23	2.47%	7186.63	0.72%	2780.00	0.28%
tsRNA-Lys(TTT)	GCCCGGATAGCTCAGTCGGTAGAGCATCAGAC	2412.09	0.24%	2352.08	0.24%	1548.82	0.15%
tsRNA-Arg(CCG)	GACCCAGTGGCCTAATGGATAAGGCATCAGCCT	15168.68	1.52%	883.86	0.09%	230.64	0.02%
tsRNA-Arg(TCT)	GGCTCCGTGGCGCAATGGATAGCGCATTGGAC	219.73	0.02%	26.13	0.00%	960.73	0.10%
tsRNA-Met(CAT)	AGCAGAGTGGCGCAGCGGAAGCGTGCTGGGC	9978.64	1.00%	1342.11	0.13%	3588.07	0.36%
tsRNA-Cys(GCA)	GGGGGTATAGCTCAGTGGTAGAGCATTTGACT	4556.96	0.46%	481.89	0.05%	265.52	0.03%
tsRNA-Pro(AGG)	GGCTCGTTGGTCTAGGGGTATGATTCTCGCT	1558.06	0.16%	187.23	0.02%	1371.94	0.14%
tsRNA-Asp(GTC)	TCCTCGTTAGTATAGTGGTTAGTATCCCCGCCT	1215.86	0.12%	42.59	0.00%	42.27	0.00%
tsRNA-Leu(CAG)	GTCAGGATGGCCGAGCGGTCTAAGGC	1173.98	0.12%	17.98	0.00%	76.33	0.01%
tsRNA-Gln(TTG)	GGTCCCATGGTGTAATGGTTAGCACTCTGGAC	798.31	0.08%	37.20	0.00%	54.58	0.01%
tsRNA-Ile(AAT)	ACGCCAAGGTCGCGGGTTCGATCCCCGTACGGGCC	4.73	0.00%	3.87	0.00%	294.66	0.03%
other		18281.65	1.83%	5951.00	0.60%	10955.85	1.10%
RPM = reads per million tsRNA reads							



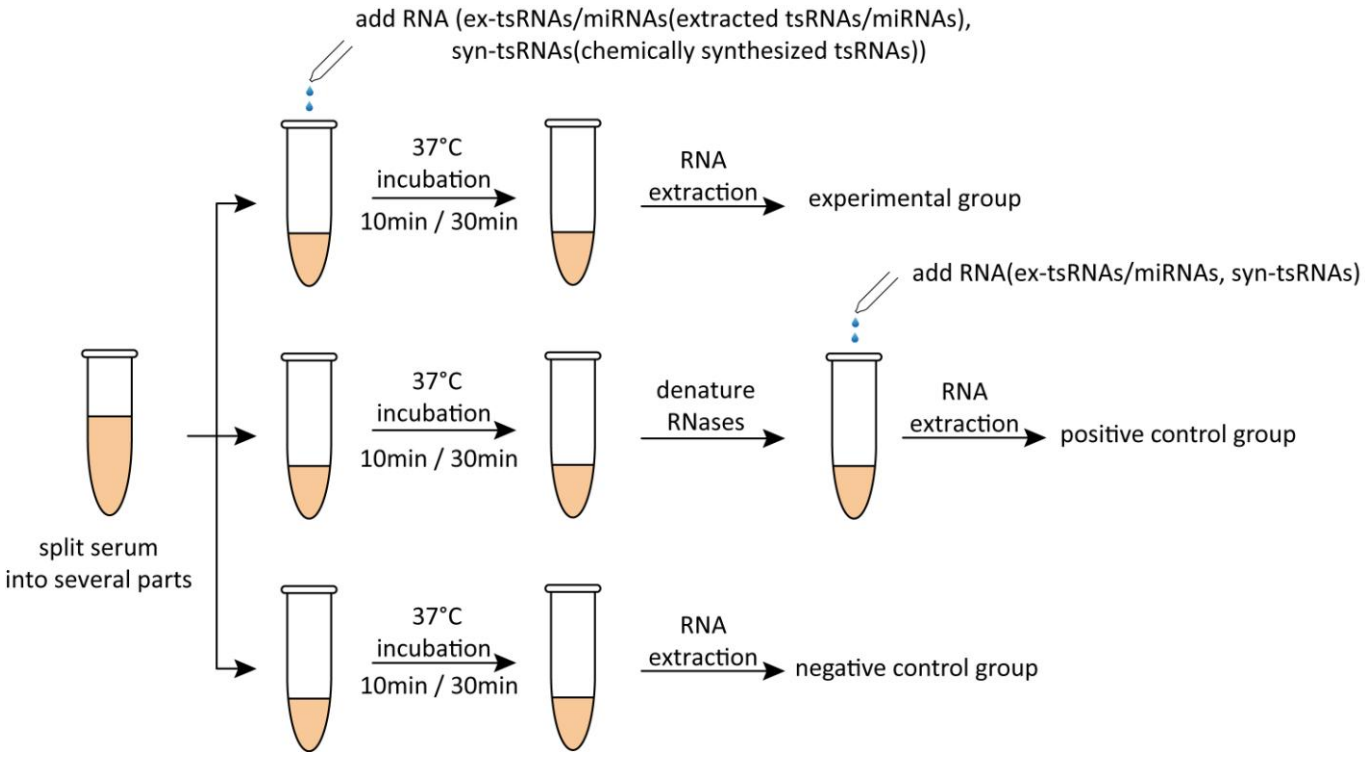
Supplementary Figure S1 Small RNA length distribution and annotations of miRNAs, tsRNAs in mouse tissues and serum.



Supplementary Figure S2 Illustrations of protocol for isolation of serum exosomes.



Supplementary Figure S3 Illustrations of protocol for separating serum components with a molecular weight cut-off 100 kDa.



Supplementary Figure S4 Illustrations of protocol for examination of the stability of chemically synthesized tsRNAs, and tsRNAs/miRNAs extracted from the serum by re-adding them into the serum environment.