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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

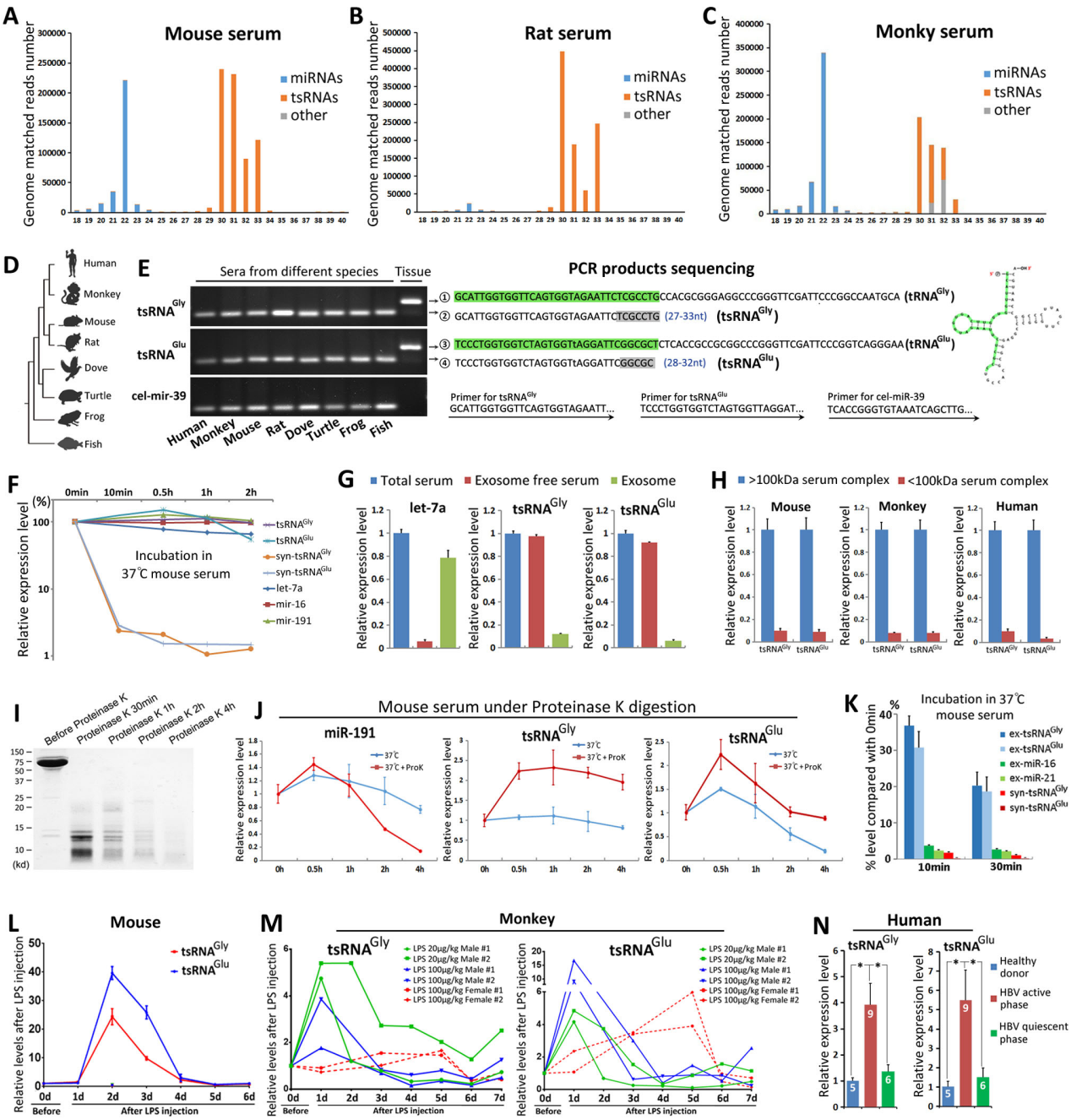

Figure 1 Identification and characterization of serum tsRNAs and their response to body infection. (**A**–**C**) Length distributions of small RNAs and the abundant tsRNAs in the sera of mouse, rat, and monkey. (**D**) Species along the evolution tree for examination of serum tsRNAs. (**E**) RT–PCR analysis 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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