Early cleavage of preimplantation embryos is regulated by tRNA\textsuperscript{Gln-TTG}–derived small RNAs present in mature spermatozoa

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tRNA-derived small RNAs (tsRNAs) from spermatozoa could act as acquired epigenetic factors and contribute to offspring phenotypes. However, the roles of specific tsRNAs in early embryo development remain to be elucidated. Here, using pigs as a research model, we probed the tsRNA dynamics during spermatogenesis and sperm maturation and demonstrated the delivery of tsRNAs from semen-derived exosomes to spermatozoa. By microinjection of antisense sequences into in vitro fertilized oocytes and subsequent single-cell RNA-seq of embryos, we identified a specific functional tsRNA group (termed here Gln-TTGs) that participate in the early cleavage of porcine pre-implantation embryos, probably by regulating cell cycle–associated genes and retrotransposons. We conclude that specific tsRNAs present in mature spermatozoa play significant roles in preimplantation embryo development.

Couples suffering from male factor infertility are often treated by testicular sperm extraction followed by oocyte fertilization using intracytoplasmic sperm injection (ICSI). However, this treatment completely bypasses the final stage of sperm maturation that takes place in the epididymis and involves uptake of small RNA molecules that are carried over to the zygote through fertilization (1). The nature and function of these small RNAs during fertilization and early embryo development have not yet been sufficiently investigated.

One of the most well-known groups of RNA molecules are transfer RNAs (tRNAs), which have a well-defined role in transferring amino acids during translation (2). However, much less known, tRNAs can be processed to form tRNA-derived small RNAs (tsRNAs). Emerging evidence has demonstrated that these tsRNAs are present in various cell types and able to regulate RNA stability, translation, stress response, and cell proliferation (3).

Spermatogenesis is a complex process involving proliferation and differentiation of spermatogonia, the meiotic divisions of spermatocytes, and spermiogenesis. During the spermiogenesis stage, the haploid round spermatids undergo dramatic morphological and cellular changes to form the highly specialized elongated spermatids and finally form spermatozoa (4, 5). Although they can be used to fertilize oocytes via ICSI, these testicular spermatozoa normally first leave the testis to the epididymis to further mature into fully functional spermatozoa (5). In mice, sperm tsRNAs are scarce in the testis but dramatically enriched in the epididymis (6, 7). These tsRNAs have been reported to be derived from small vesicles that are released from the epididymal epithelium, i.e. epididymosomes, and have recently been confirmed to be involved in sperm maturation (6–9). In addition, tsRNAs have been identified in porcine and human seminal plasma (10, 11). However, little information is available on the correlation between tsRNAs found in seminal plasma and in ejaculated spermatozoa.

Sperm tsRNAs carry paternal epigenetic information and could act as acquired epigenetic factors that influence offspring phenotypes in response to environmental stress (9, 12, 13). Recently, several groups of tsRNAs were identified as potential biomarkers of sperm quality for \textit{in vitro} fertilization (IVF) (14), suggesting that these sperm tsRNAs may be implicated in early embryo development. As a family of transposable elements, mouse endogenous retrovirus type L (MERVL) drives the expression of transcripts specific to embryo genomic activation (EGA) and totipotency (15). Previous studies have shown that depletion of sperm tsRNAs could derepress MERVL-linked genes (6) and that endogenous retroviruses (ERVs) are the targets strongly inhibited by tsRNAs (16). Nevertheless, the
underlying mechanisms for tsRNA functions in fertilization and early embryo development remain to be probed.

Although previous studies have all been using mouse models for functional analysis of sperm tsRNAs (6, 9, 13), development of germ cells and preimplantation embryos differs significantly between rodents and nonrodents including humans. Pigs (Sus scrofa) are increasingly utilized as a large-animal model in biomedical research (17). Moreover, it is relatively easy to collect rodent and porcine epididymis and ejaculated spermatozoa. We therefore used pigs as model animals to study the tsRNA dynamics during spermatogenesis, sperm maturation, and embryo development. We found that tRNA\textsuperscript{Gln-TTG}–derived small RNAs (Gln-TTGs) were highly enriched in ejaculated spermatozoa and that Gln-TTGs played a role during the early cleavage stages of embryo development. Hence, our findings demonstrate that a spermatozoon confers specific tsRNAs to the zygote that play significant roles during preimplantation embryo development.

Results

tRNA dynamics in porcine male germ cells

To uncover the expression profiles of tsRNAs in porcine spermatogenic cells and ejaculated spermatozoa, we first analyzed the small RNA-seq data generated from porcine spermatogonia (SG), pachytene spermatocytes (PS), round spermatids (RS), and ejaculated spermatozoa (SP) in our previous study (18). Of these, the tsRNA sizes range from 25 to 35 nt (Fig. 1, A–D, and Table S1), with 2.1, 2.8, 4.4, and 7.2% falling into the category of tsRNAs in SG, PS, RS, and SP, respectively (Fig. 2A). We then analyzed the derivation of tsRNAs. The majority of these tsRNAs (85–88%) were derived from the 5′ end of tRNAs (Fig. 2B), and tRNAs with the uniform 5′ sequence could produce seven tsRNA families with the uniform 5′ sequence but marginally differing at the 3′ end of the sequence (Fig. 2C).

It has been reported that tRNA\textsuperscript{Glu-CTC} and tRNA\textsuperscript{Gly-GCC}–derived small RNAs (Glu-CTCs and Gly-GCCs) are scarce in mouse testicular germ cells but enriched in epididymal spermatozoa (7, 19). In contrast, our analysis revealed that Glu-CTCs and Gly-GCCs were moderately present in all porcine testicular germ cells and ejaculated spermatozoa (Fig. 3A). Additionally, we specifically identified that one tsRNA family, tRNA\textsuperscript{Gln-TTG}–derived small RNAs (Gln-TTGs), were only moderately expressed in testicular germ cells but highly enriched in ejaculated spermatozoa (Fig. 3A). Gln-TTGs were derived from two subfamilies of tRNA\textsuperscript{Gln-TTG} , i.e. tRNA\textsuperscript{TGl-TTG} and tRNA\textsuperscript{GGl-TTG} , which only differ in one nucleotide at the ninth position (Fig. 3B). Analysis of the cleavage sites showed that most cleavages occurred at positions 32–34 flanking the anticodon loop to generate the 32–34-nt tsRNAs (Fig. 3B and Table S2), indicating that these tsRNAs are cleaved by specific enzymes rather than random tRNA degradation.

tsRNA dynamics during porcine sperm maturation

The enrichment of tsRNAs in ejaculated spermatozoa prompted us to investigate the dynamics of tsRNAs during sperm maturation using the caput, cauda epididymis, and ejaculated spermatozoa from the same individuals (Fig. 4A).

We found that the percentage of tsRNAs among all small RNAs decreased from 19.5 to 16.1% during the transit from caput to cauda epididymis (not statistically significant, Fig. 4B). Then the percentage of tsRNAs among all small RNAs further decreased to 10% in the ejaculated spermatozoa (p < 0.01), significantly different from that in caput spermatozoa (Fig. 4B, p < 0.05). Because 5′ end tsRNAs were mainly present in spermatogenic cells and spermatozoa (Fig. 2B), we turned to analyze their dynamics. Intriguingly, the analysis revealed that 5′ end tsRNAs had a significant drop (~13%, p < 0.05) during maturation in epididymis, followed by a significant increase (~7%, p < 0.05) after ejaculation (Fig. 4C), suggesting the gain of 5′ end tsRNAs in the ejaculated spermatozoa. We also observed that Gln-TTGs were significantly enriched in the ejaculated spermatozoa (Fig. 4D), which was further confirmed by a qRT-PCR analysis (Fig. 4E, p < 0.05).

Next, we isolated exosomes from the caput, corpus, cauda epididymis, and semen (Fig. 5A), and characterized their small RNA payload by an RNA-seq analysis. This revealed that miRNA abundance experienced an increase from caput to cauda exosomes and reached 31% in semen exosomes. By contrast, tsRNA abundance increased from caput (7.5%) to cauda (59.6%) but decreased in semen exosomes (9.9%, Fig. 5B). Notably, 5′ end tsRNAs consistently dominated the tsRNA population (over 90%) in the exosomes from epididymis and declined in semen exosomes (67.8%, Fig. 5C). Nonetheless, tsRNA and miRNA expression profiles in spermatozoa were significantly correlated with those in the corresponding exosomes, as shown by the Spearman’s correlation analysis (r = 0.58–0.77; p < 2.2e-16, Fig. 5D). Hence, semen exosomes, in addition to exosomes from the epididymis, may also deliver tsRNAs to spermatozoa.

To pinpoint this, we incubated the cauda spermatozoa with semen exosomes or without semen exosome incubation. Later, we detected the abundance of tsRNAs in caput, cauda, and ejaculated spermatozoa. Like Gln-TTGs (Fig. 4D), Glu-CTCs and Gly-GCCs were enriched in the ejaculated spermatozoa (Fig. 6, A and B), and all three tsRNA categories could be efficiently shaped by semen exosome incubation (Fig. 6C). Overall, our data demonstrate that semen exosomes can deliver tsRNAs to spermatozoa.

Sperm Gln-TTGs regulate the first cleavage of porcine IVF oocytes

Subsequently, we explored the roles of sperm tsRNAs in fertilized oocytes. We first determined the abundance of tsRNAs in IVF oocytes during oocyte maturation and early embryo development. The results revealed that Glu-CTCs and Gly-GCCs were moderately up-regulated from MII to the two-cell stage (Fig. 7A). Interestingly, Glu-CTCs and Gly-GCCs were increased from the two-cell stage to the four-cell stage (Fig. 7A). However, Gln-TTGs displayed distinct dynamic changes, i.e. they were considerably down-regulated from MII to the one-cell stage and then remained stable (Fig. 7A).

Next, we microinjected 20 μM of the synthesized Glu-CTCs, Gly-GCCs, and Gln-TTGs (overexpression [OE] group); the antisense oligonucleotides of Glu-CTCs, Gly-GCCs, and Gln-TTGs (Anti group); or the scrambled sequence (NC group)
Figure 1. Profiling of tsRNAs during porcine spermatogenesis. A–D, percentage and length distribution of tsRNAs in SG (A), PS (B), RS (C), and SP (D).
into porcine IVF oocytes. We found that the rates of two-cell, four-cell, and blastocyst embryos decreased in the Anti Gln-TTG group (Fig. 7B), whereas depletion of Glu-CTCs or Gly-GCCs had no significant impact on embryo development (Fig. 7C). The reduced rates of embryos at the four-cell and blastocyst stages in the Anti-Gln-TTG group might be attributed to the aberrant first cleavage and therefore the affected two-cell embryos.

To exclude the possibility of the artifact caused by excessive exogenous RNAs, we set 10 μM (half of the initial concentration) and 2 μM (amounting to the total RNAs from ~10 spermatocytes) of RNAs for microinjection (13). Consistently, the reduced concentrations of the antisense sequence of Gln-TTGs led to a similar decrease in the two-cell rate (Fig. 7D). To substantiate the specificity of the observed phenotype, we later conducted a rescue experiment for Gln-TTGs and found that either microinjection of the antisense oligonucleotides (Anti group) followed by the synthesized Gln-TTGs (OE group) or injection of the synthesized Gln-TTGs (OE group) followed by the antisense oligonucleotides (Anti group) could mitigate the effects induced by these RNAs alone (Fig. 7E). In addition, the cleavage rate did not significantly change when the antisense sequence annealed with the sense sequence was injected (Fig. 7E). The overall data thus demonstrate that sperm Gln-TTGs regulate the first cleavage of porcine IVF oocytes.

**Sperm Gln-TTGs regulate cell cycle–associated genes and retrotransposable elements (REs) in porcine IVF oocytes**

To probe the underlying mechanisms for the reduced cleavage rate in the Anti Gln-TTG-IVF oocytes, we performed a single-cell RNA-seq analysis for the Anti Gln-TTG group versus the NC group in two-cell IVF oocytes. This analysis identified 717 differentially expressed genes (DEGs), of which 335 were up-regulated and 382 were down-regulated in the Anti-IVF group (Fig. 8A and Table S3). Gene ontology (GO)–biological process annotation analysis indicated that DEGs in the Anti-IVF group were predominantly linked to cell cycle–associated processes including organelle fission, nuclear division, mitotic cell cycle process, and organelle organization (Fig. 8B), lending further support to the notion that depletion of Gln-TTGs in IVF oocytes causes aberrant cell cycle progress.

Retrotransposon expression is a conventional event in preimplantation embryos (20). It has been reported that microinjection of the antisense oligonucleotides of Gly-GCCs into zygotes activated ERVs, a family of transposons (6). We therefore analyzed the expression of transposons upon Gln-TTG depletion. Overall, the global transcriptomic composition of transposons in the Anti group resembled that in the NC group (Fig. 8B). Further analysis revealed 16 differentially expressed transposons in the Anti-IVF group (Fig. 8C). Of the four REs (LTR4D_SS, LTR6_SS, MLT1I-
Figure 3. The dynamics of tsRNA families during porcine spermatogenesis. A, the number of reads per million (RPM) of tRNA\textsuperscript{Glu-CTC}, tRNA\textsuperscript{Gly-GCC}, and tRNA\textsuperscript{Gln-TTG}\textsuperscript{-}derived small RNAs (Glu-CTCs, Gly-GCCs, and T/Gln-TTGs) during porcine spermatogenesis. B, diagrams of the secondary cloverleaf structure of tRNA\textsuperscript{Gln-TTG}\textsuperscript{-} in two sperm samples. The top three cleavage sites are marked by arrowheads, and the single-nucleotide differences are marked by black circles.
Sperm Gln-TTGs in early embryo development

A

B

Caput spermatozoa

Cauda spermatozoa

Ejaculated spermatozoa

- miRNA
- tRNA
- other

Percentage

25%
20%
15%
10%
5%
0%

Caput spermatozoa

Cauda spermatozoa

Ejaculated spermatozoa

C

Caput spermatozoa

Cauda spermatozoa

Ejaculated spermatozoa

5' end tRNA

other

D

RPM

Caput spermatozoa 1

Caput spermatozoa 2

Caput spermatozoa 3

Cauda spermatozoa 1

Cauda spermatozoa 2

Cauda spermatozoa 3

Ejaculated spermatozoa 1

Ejaculated spermatozoa 2

Ejaculated spermatozoa 3

Length
int, and L1M3b) differentially expressed in the Anti-IVF group (Fig. 8D), LTR4D_SS and L1M3b were predicted to be potential targets of Gln-TTGs by miRanda (Fig. 8E), suggesting potential regulatory roles of Gln-TTGs in retrotransposons (21).

**Sperm Gln-TTGs interact with RNA- and DNA-binding proteins**

A previous study reported that tRNA\textsuperscript{Glu}-derived small RNAs could bind to YBX1, an RNA-binding protein, to mediate mRNA stability in the metastatic progression (22). To gain insights into the potential interaction between Gln-TTGs and
Figure 6. Exosomes transmit tsRNA to cauda spermatozoa. A and B, the RPM of Glu-CTCs (A) and Gly-GCCs (B) in caput, cauda, and ejaculated spermatozoa. C, abundance of tRNA^{Glu-CTC}, tRNA^{Gly-GCC}, and tRNA^{Gln-TTG}-derived small RNAs (Glu-CTCs, Gly-GCCs, and Gln-TTGs) in cauda spermatozoa with incubation of seminal plasma derived exosome (EXO) or without incubation of seminal plasma derived exosome incubation (f-EXO). Square, circle, and triangle symbols represent the expression levels of tsRNAs in each replicate.
**Sperm Gln-TTGs in early embryo development**

A

![Graphs](A) showing the relative expression level of Glu-CTC, Gly-GCC, and Gln-TTG in different developmental stages (GV, MII, 1-cell, 2-cell, 4-cell).

B

- **IVF Oocytes**
- **Microinjection**
- **2-Cell**

![Images](B) illustrating the stages of fertilization and cleavage.

C

- **2-cell Rate**
- **4-cell Rate**
- **Blastoyst Rate**

Comparative study of OE, OE-Glu-CTC, OE-Gly-GCC, OE-Glu-CTC, and Ant-Gly-GCC at a concentration of 20μM.

C

- **2-cell Rate**
- **4-cell Rate**
- **Blastoyst Rate**

Comparative study of OE, OE-Glu-CTC, OE-Gly-GCC, OE-Glu-CTC, and Ant-Gly-GCC at a concentration of 20μM.

D

- **2-cell Rate**
- **Concentration 10μM**
- **Concentration 2μM**

Comparative study of NC, OE, and Ant at different concentrations.

E

- **2-cell Rate**
- **Concentration 10μM**
- **Concentration 2μM**

Comparative study of NC, NC-OE, Ant, NC-Ant, OE-Ant, and Ant-OE at different concentrations.
proteins in embryos, we conducted a Gln-TTG RNA-protein pulldown followed by LC–MS/MS. Because an RNA-protein pulldown requires a large number of embryos, the experiment was instead performed with mature porcine oocytes. Of the proteins pulled down by the synthesized Gln-TTGs, RNA- (e.g. HNRNPA2B1, IFG2BP3, and F1A1) and DNA-binding proteins (e.g. YBX2) were clearly identified (Table S4). Because mature oocytes already contain most maternal proteins (23, 24), it is plausible that, after fertilization, sperm-derived Gln-TTGs interact with maternal RNA- and DNA-binding proteins that have already been in the mature oocytes.

Discussion

Current knowledge of the small RNA payload in spermatogenic cells was gained mainly from the mouse model (6–8, 19). Piwi-interacting RNAs dominate the small RNA repertoire of the mouse spermatocytes, round spermatids, and testicular spermatooza (7). However, tsRNAs become the dominant small RNAs after sperm transition from the testis to epididymis (6, 7). In the present study, we observed significant differences between porcine and mouse models in terms of sperm tsRNA dynamics. In contrast to the highly accumulated tsRNAs in mouse epididymal spermatooza (6, 7, 19), only a small portion (10–20%) of small RNAs in porcine epididymal spermatooza are tsRNAs, similar to a previous report on the human sperm tsRNAs (~20%) (25). Also different from that in mice, we found that the 5’ end-derived porcine tsRNAs dominated the tsRNA population and displayed a downward trend during maturation in epididymis but an upward trend in ejaculated spermatooza, which could be ascribed to the degradation/loss of other small RNAs (6, 7). In addition, the expression patterns of some tsRNAs, such as Glu-CTCs and Gly-GCCs, are distinct between porcine and mouse germ cells (6, 7). The most pronounced feature of the porcine germ cell tsRNAs that we identified in this study is the abundance of Gln-TTGs in porcine epididymal and ejaculated spermatooza.

It is generally accepted that because of the absence of nuclear gene transcription and protein translation, spermatooza remodel their small RNAs through communication with extracellular components (1, 26). The exosomes from epididymis have been proposed as a carrier of tsRNAs (27), enabling efficient shaping of the sperm tsRNA payload (6). Here, we for the first time demonstrate that semen-derived exosomes indeed deliver tsRNAs to spermatooza. This is not surprising, given that RNA molecules in seminal plasma exhibit remarkable stability and resistance to degradation because of the enclosure of RNAs in exosomes (28). Notably, tsRNAs have been reported to be abundant in human semen-derived exosomes (11). Considering the difficulty in isolating human caput and cauda spermatooza, our data lay the ground work for future studies on how human semen-derived exosomes shape the sperm RNA payload and affect sperm function (11).

Spermatozoa obtain small RNAs during maturation in the epididymis (1). ICSI using caput spermatooza led to defective postimplantation embryos, and the phenotypes could be rescued by the specific small RNAs from the cauda epididymis (8). Inconsistently, another group reported the full developmental potential of ICSI embryos using caput spermatooza (29). Although the conflicting results might be attributed to the differences in methods and mouse strains employed (30), the magnitude of sperm-derived small RNAs cannot be overlooked. It has been reported that the 30–40-nt sperm RNA fractions, including tsRNA and rsRNA, contribute to intergenerational inheritance (6, 9, 13). Despite this, specific tsRNAs, and their roles in early embryo development, have not been sufficiently studied. A previous report showed that depletion of Gly-GCCs by antisense sequences changed the MERVL gene expression (6). Here, by microinjecting the antisense oligonucleotides of specific tsRNAs into porcine IVF oocytes, we clearly observed that depletion of Gln-TTGs but not Gly-GCCs induced the aberrant cleavage. To exclude the artificial effects, we further conducted the off-target detection and the RNA rescue experiment, eventually providing the proof of concept that Gln-TTGs function in early embryo development.

Once fertilization is finished, zygotes undergo a rapid cell cycle, switching from the S to the M phase without pausing in a gap phase before EGA (31). It has been reported that most genes at the one-cell to four-cell stage in humans and at the one-cell to two-cell stage in mice were related to cell cycle, protein transport, and GTPase signaling (32). Here, depletion of Gln-TTGs perturbed cell cycle–associated genes in pigs, supporting our presumption that Gln-TTGs influence the early embryo cleavage by modulating the developmental genes.

We found that depletion of Gln-TTGs also altered the expression of REs. Three types of REs, i.e. long interspersed nucleic elements (LINEs), short interspersed nucleic elements, and long terminal repeat (LTR) retrotransposons (also known as ERVs) (33), are found expressed during EGA (34). In the present study, depletion of Gln-TTGs in porcine IVF oocytes led to down-regulation of L1M3b, a family member of LINE-1. This is of significance because the mouse LINE-1 is intensively transcribed at the two-cell stage (35) and modulates chromatin remodeling (36). Interfering with LINE-1 results in half of the embryos arrested at the four- or eight-cell stage (36). In addition, two porcine ERV elements, LTR6_SS and LTR4D_SS, were also down-regulated by Gln-TTG depletion, in line with a recent report describing the ERV expression variation mediated by 5’ end tsRNAs in mice (6). Likewise, 3’ end tsRNAs...
have been reported to be able to post-transcriptionally silence coding-competent ERVs and specifically interfere with reverse transcription and retrotransposon mobility (16). Together, our data complement previous findings that tsRNAs are involved in transcriptional regulation of retrotransposons.

Spermatozoa from different species use diverse repertoires of sperm-specific signaling molecules, and even closely related protein isoforms hold differential properties and function distinctly (37). Use of porcine spermatozoa has certain advantages in physiological and genetic research (38). For instance, pigs produce a high volume of ejaculate (up to 500 ml), which could facilitate studies that require a large number of spermatozoa. In addition, porcine, but not murine spermatozoa, resemble their human counterparts in terms of morphology (e.g. the head morphology, the mitochondrial sheath length and the total sperm length), as well as preimplantation embryo development (e.g. timing of EGA) (39, 40). In this sense, our findings and the knowledge acquired from pigs would greatly fill in the gap caused by using murine and human models only.

To sum up, we provide the proof of concept that Gln-TTGs influence the early embryo cleavage, probably by orchestrating cell cycle–associated genes and retrotransposons via the mechanism illustrated in Fig. 9. Hence, tRNA-derived small RNA molecules in spermatozoa play significant roles during preimplantation embryo development. Many couples suffering from male factor infertility are treated by testicular sperm extraction–ICSI, a procedure that completely bypasses the final stages of sperm maturation. Future studies are thus recommended to elaborate the magnitude of these paternally derived factors, with the long-term goal to facilitate safe reproduction and development in humans.

**Experimental procedures**

**Porcine samples**

Porcine testis and epididymis samples were collected from adult (6-month-old) Landrace boars (S. scrofa) in the Hongxing farm, Heze, Shandong, China. Ovaries were obtained from adult Landrace sows (S. scrofa) in the slaughter house of Besun farm, Yangling, Shaanxi, China. All porcine samples were immediately transported to the laboratory in cold PBS. All experimental procedures involving animals were approved by the institutional animal care and use committees of Northwest A&F University and China Agricultural University.

**Porcine sperm collection and small RNA-seq**

The freshly ejaculated spermatozoa of three Landrace individuals were collected and incubated in prewarmed Modena dilutions to let spermatozoa swim up. The upper fraction of the swim-up spermatozoa was collected followed by centrifugation at 600 × g for 5 min. The sperm pellets were resuspended in lysis buffer (0.1% SDS and 0.5% Triton X-100) and incubated on ice to eliminate somatic cell contamination. After removing
the supernatant, the spermatozoa were resuspended in TRIzol (Takara).

Because the pigs were slaughtered, the epididymis was collected to isolate the caput and cauda spermatozoa as previously described (6, 13). Briefly, the epididymis was carefully dissected into three anatomical segments (the caput, corpus, and cauda). Spermatozoa were released from the caput or cauda by making small incisions on the surface of the tissue. The released spermatozoa were collected by centrifugation at 600 × g for 5 min, followed by a 1× PBS wash. The sperm pellets were resuspended in lysis buffer (0.1% SDS and 0.5% Triton X-100) and incubated on ice to eliminate somatic cell contamination. The purity of sperm samples was confirmed by microscopy. After washing with 1× PBS, the sperm pellets were resuspended in TRIzol (Takara). Total RNAs were extracted according to the manufacturer’s protocol and used for small RNA-seq as previously reported (18).

**Isolation, characterization, and small RNA-seq of porcine caput, corpus, cauda, and semen exosomes**

The tissue was placed in 20 ml of 1× PBS, and multiple incisions were made on the surface of the tissue. The tissue was then subjected to mild agitation, and the luminal fluid was diluted in 1× PBS. Subsequently, the fluid was centrifuged at 2,000 × g for 5 min to remove spermatozoa and then centrifuged at 10,000 × g for 30 min to remove cell debris. The resulting supernatants were further centrifuged at 12,000 × g for 1 h, followed by filtering through a 0.22-µm cell strainer. Later, the acquired fluid was subjected to an ultracentrifugation (the type 70ti tube, Beckman) at 120,000 × g at 4 °C for 2 h. After aspirating the supernatant, the pellets were resuspended in 1× PBS and subjected to a second ultracentrifugation at 120,000 × g at 4 °C for 2 h. The eventually acquired pellets were resuspended in 200 µl of PBS and used for further studies. To isolate exosomes from Landrace semen, 300 ml of semen supernatants were centrifuged at 16,000 × g for 1 h at 4 °C to remove cell debris, followed by filtering through a 0.22-µm filter. The acquired fluid was subjected to an ultracentrifugation at 120,000 × g at 4 °C for 2 h. After removing the supernatant, the pellets were resuspended in 3 ml of 1× PBS and stored at −80 °C for further use.

The morphology and size of purified exosomes were evaluated by a transmission electron microscope (TEM, Hitachi) as previously reported (41, 42). In brief, 50 µl of suspended exosomes were transferred to a carbon-coated grid. After incubation for 10 min at room temperature, the grid was transferred to 50 µl of phosphotungstic acid and incubated for 1 min. The excessive fluid was removed, and the grid was washed with fluid water. After air-drying the grid, the morphology and size were visualized by TEM. Later, the concentration and mean size of particles from caput, corpus, cauda, and semen exosomes were analyzed on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

Western blotting analysis was performed as previously described (43) to detect the exosome biomarker CD63. Briefly, the exosome pellets were lysed and electrophoresed on 10% SDS-PAGE gel. After blocking, the membrane was incubated with the rabbit anti-CD63 (1:600, catalog no. 25682-1-AP, Proteintech) primary antibody at 4 °C overnight. After washing with Tris–HCl–buffered solution with Tween 20 (TBST), the membrane was incubated with the goat anti-rabbit IgG secondary antibody (1:1000) for 1 h at room temperature. The images were captured with the Bio-Rad ChemiDoc XRS Imaging system.

For exosome RNA extraction, samples were suspended in TRIzol (Takara) and then processed following the protocol provided by the manufacturer. The isolated RNAs were used for small RNA-seq as previously reported (18).

**Porcine sperm incubation with exosomes**

The cauda spermatozoa were incubated with exosomes for 2 h at 37 °C, followed by evaluation of sperm motor patterns using a computer-assisted sperm analysis system. Then the spermatozoa were collected, washed with Dulbecco’s PBS three times, and then resuspended in TRIzol for further analysis.

**Microinjection of porcine IVF oocytes**

Ovaries were acquired from the Landrace sows (S. scrofa) in the slaughter house of Besun farm (Yangling, Shaanxi, China). Cumulus–oocyte complexes (COCs) were obtained from follicles with a 2–8-mm diameter using a 10-ml syringe and a 12-gauge needle. The collected COCs were pooled in a sterile centrifuge tube and then dispersed in 100-mm cell culture dishes. After washing by manipulation medium (44), the COCs with multiple layers of cumulus cells were transferred to the maturation medium consisting of M199 (Gibco) supplemented with 10% fetal bovine serum, human menopausal gonadotrophins (FSH:1 LH = 1:1) (0.1 IU/ml), estradiol (1.0 µg/ml), epidermal growth factor (50 ng/ml), uracil (50 µg/ml), and insulin–transferrin–selenium. The cells were incubated at 38.5 °C under a humidified atmosphere of 5% CO₂. After 42–44 h of incubation in the maturation medium, every 10 mature oocytes were pooled in lysis buffer (5 mM DTT, 20 units/ml RNase inhibitor, 1% Nonidet P-40, Thermo) according to a previous study (45). Upon release of RNAs, the samples were treated with RNase-free DNase I (Fermentas) to remove genomic DNA, following the manufacturer’s protocol.

The purchased frozen spermatozoa (Baijunda Science and Technology Development Co., Ltd., Beijing, China) were thawed at 50°C water for 16 s. The thawed spermatozoa were resuspended in 4 ml of TL-HEPES and then washed twice with mTBM (113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, and 8 mg/ml BSA). The motility of spermatozoa was analyzed, and only those above 0.8 were used for IVF. After the oocyte maturation, cumulus cells were removed by pipetting in TL-HEPES containing 0.1% hyaluronidase. Subsequently, the mature oocytes were washed twice in TL-HEPES and then pooled into groups of 10–15 oocytes/45–µl droplet of the fertilization medium (modified Tris-buffered medium containing 1 mM caffeine sodium benzoate and 0.1% w/v BSA). Next, 5 µl of spermatozoa were added into the 45-µl oocyte-containing droplets, followed by co-incubation at 39 °C and 5% CO₂. After 6 h of fertilization, the excessive spermatozoa around oocytes were removed by pipetting in
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NCSU23 medium supplemented with 0.4% w/v BSA (IVC medium). Following fertilization, RNA injection was conducted by using a Femtojet (Eppendorf) microinjector at 400-hPa pressure for 0.1 s with 90-hPa supplementary pressure. The sequences of synthesized RNAs used for microinjection represented the main cleavage products of tRNAs in spermatozoa and were listed in Table S5. The second microinjection of RNAs was conducted 30 min after the first microinjection. Embryos were maintained at 37 °C, 5% CO₂, and 100% humidity until the blastocyst stage. Then germinal vesicle stage oocytes, metaphase II (MII) stage vesicles, one-cell, two-cell, and four-cell embryos (5 samples/group, respectively) were collected for qRT-PCR analysis, and two-cell embryos were stored in lysis buffer for single-cell RNA-seq.

Single-cell RNA-seq

Every five embryo were pooled as previously reported (13). Single-cell RNA-seq was performed by Annoroad Gene Technology Co. Ltd. (Beijing, China). Briefly, the embryos were lysed to release all RNAs, and External RNA Control Consortium agent was added into the released RNAs. Then the RNA was amplified and reversely transcribed into the first-strand cDNA by using the Smart-Seq2 method (46). The single-cell library was constructed from 20 ng of amplified cDNA, and then the library was sequenced on an Illumina Hiseq 2500 platform to generate the 125-bp paired-end reads. The total reads acquired was constructed from 20 ng of amplified cDNA, and then the obtained clean data were mapped to the Ensembl reference genome (Sscrofa10.2.89) with TopHat v2.0.12. The reads were calculated as FPKM to quantify the differential expression of genes by using R package (DESeq).

For single-cell RNA-seq analysis, we first removed the low-quality sequencing output, and then the obtained clean data were mapped to the Ensembl reference genome (Sscrofa10.2.89) with TopHat v2.0.12. The reads were calculated as FPKM to quantify the differential expression of genes by using R package (DESeq). The enrichment of GO terms was calculated by hypergeometric test.

To analyze repeat sequences, positions of reads uniquely mapped to the porcine genome were cross-compared with the positions of repeats extracted from UCSCsusScr11.2 bit database, and the repeat family was annotated to the reads overlapping a repeat sequence. To obtain reliable read counts for the repeat family, RepBase22.12 repeat sequences for pigs were mapped to reads that were not mapped or multimapped to the porcine genome. The mapped reads were then annotated with the repeat family name. The final read counts were calculated by adding the read counts of the two steps.

RNA pulldown

6000 mature porcine oocytes were pooled and lysed to release proteins by using Pierce IP lysis buffer. RNAs (scrambled RNAs, Gln-TTGs, and antisense RNAs) were synthesized by the GenePharma Biotech Company. The 3’ end of RNAs were labeled with desthiobiotin by using a Pierce™ RNA 3’ end desthiobiotinylation kit according to the manufacturer’s guidance. Briefly, a single biotinylated nucleotide was attached to the 3’ end of 50 pmol of nonlabeled RNAs by T4 ligase. The reactions were incubated at 16 °C for 2 h, and then the labeled-RNAs were precipitated and dissolved in nuclease-free water. Next, RNA pulldown was conducted by using the Pierce™ magnetic RNA–protein pulldown kit. In brief, streptavidin–agarose beads were washed twice with 20 mM Tris (pH 7.5). Biotinylated RNAs (50 pmol) were added to streptavidin beads and incubated for 30 min at room temperature, with rotation in 50 μl of RNA capture buffer. After incubation, the immobilized biotinylated RNA–streptavidin complexes were washed by 20 mM Tris (pH 7.5) followed by protein–RNA-binding buffer. After washing, the immobilized biotinylated RNA–streptavidin complexes were incubated with a mixture composed of protein lysates, 50% glycerol, and protein–RNA-binding buffer for 1 h at 4 °C with rotation. After washing with the wash buffer three times, 50 μl of elusion buffer was added to the beads and incubated for 30 min at 37 °C with agitation to acquire the RNA-binding proteins.

LC–MS/MS

The binding proteins were identified by LC–MS/MS at the APT Biotech. Specifically, proteins of each sample were mixed with loading buffer and boiled for 5 min. The proteins were separated on 12.5% SDS-PAGE gel and then visualized by Coomassie Blue R-250 staining. Gel pieces were destained and dried in a vacuum centrifuge. Then the in-gel proteins were reduced with 100 mM DTT, 100 mM NH₄HCO₃ for 30 min at 56 °C. Later, proteins were alkylated with 200 mM iodoacetamide, 100 mM NH₄HCO₃ in the dark for 30 min at room temperature. Gel pieces were rinsed with 100 mM NH₄HCO₃ and Acetonitrile. Gel pieces were digested with 12.5 ng/μl trypsin in 25 mM NH₄HCO₃ overnight. Finally, the peptides were extracted three times with 60% cerium (IV) Ammonium nitrate, 0.1% TFA and were further pooled and dried completely by vacuum centrifuge.

LC–MS/MS analysis was conducted on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Thermo Scientific) for 120 min. The mass spectrometer was operated in positive ion mode. MS data were acquired using a data-dependent top 10 method, dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Automatic gain control target was set to 3e⁶, and maximum inject time was set to 10 ms. Dynamic exclusion duration was 40.0 s. Survey scans were acquired at a resolution of 70,000 at m/z 200, resolution for HCD spectra was set to 17,500 at m/z 200, and isolation width was 2 m/z. Normalized collision energy was 30 eV, and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with the enabled peptide recognition mode.
MS/MS spectra were searched using MASCOT engine (version 2.2), embedded into Proteome Discoverer 1.4 against Uniprot Pig database (50,040 total entries, downloaded May 23, 2018) and the decoy database. For protein identification, the following options were used: peptide mass tolerance = 20 ppm, MS/MS tolerance = 0.1 Da, enzyme = trypsin, missed cleavage = 2; fixed modification: carbamidomethyl (C); variable modification: oxidation (M), FDR ≤ 0.01. The RNA pulldown proteins in the tsRNA or in the Anti group were picked up by subtracting the proteins in the NC group.

**qRT-PCR**

Like a previous study (48), the stem-loop primers for tsRNAs were used for reverse transcription following the manufacturer’s protocol (Roche), and qRT-PCR analysis was performed with FastStart Essential DNA Green Master (Roche) using an IQ-5 (Bio-Rad). The relative expression of tsRNAs in oocytes and embryos was normalized to 5 s of RNA and then calculated using the comparative Ct method \( 2^{-\Delta\Delta Ct} \). qRT-PCR analysis was also performed with FastStart Essential DNA Green Master (Roche) using an IQ-5 (Bio-Rad). The primer information is depicted in Table S5.

**Statistical information**

All experiments were repeated at least three times. Statistical analyses were performed with Student’s t test or one-way analysis of variance followed by Turkey’s test using SPSS version 23.0 statistical software (IBM). The data are presented as the mean ± S.E. of the mean, and the differences were considered statistically significant at \( p \leq 0.05 \) (*) and highly significant at \( p \leq 0.01 \) (**).

**Data availability**

The data sets generated in the current study (RNA-seq) are available in the GEO repository under the accession number GSE124099. The LC–MS/MS data are available via Proteome-Xchange with the identifier PXD019054.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: tsRNA, tRNA-derived small RNA; ICSI, intracellular sperm injection; IVF, in vitro fertilization; MERV, mouse endogenous retrovirus type L; EGA, embryonic genome activation; ERV, endogenous retrovirus; Sg, spermatogonia; Ps, pachytene spermatocytes; Rs, round spermatids; Sp, ejaculated spermatooza; nt, nucleotide(s); RNA-seq, RNA sequencing; OE, overexpression; Anti, antisense oligonucleotide; NC, scrambled sequence; RE, retrotransposable element; DEG, differentially expressed gene; GO, gene ontology; LINE, long interspersed nuclear element; LTR, long interspersed nuclear element; TEM, transmission electron microscope; COC, cumulus–oocyte complex; MII, metaphase II; qRT-PCR, quantitative RT-PCR; TL, Tyrode’s Lactate.

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