

RESEARCH ARTICLE

Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic development

Shuiqiao Yuan, Andrew Schuster, Chong Tang, Tian Yu, Nicole Ortogero, Jianqiang Bao, Huili Zheng and Wei Yan*

ABSTRACT

Although it is believed that mammalian sperm carry small noncoding RNAs (sncRNAs) into oocytes during fertilization, it remains unknown whether these sperm-borne sncRNAs truly have any function during fertilization and preimplantation embryonic development. Germline-specific *Dicer* and *Drosha* conditional knockout (cKO) mice produce gametes (i.e. sperm and oocytes) partially deficient in miRNAs and/or endo-siRNAs, thus providing a unique opportunity for testing whether normal sperm (paternal) or oocyte (maternal) miRNA and endo-siRNA contents are required for fertilization and preimplantation development. Using the outcome of intracytoplasmic sperm injection (ICSI) as a readout, we found that sperm with altered miRNA and endo-siRNA profiles could fertilize wild-type (WT) eggs, but embryos derived from these partially sncRNA-deficient sperm displayed a significant reduction in developmental potential, which could be rescued by injecting WT sperm-derived total or small RNAs into ICSI embryos. Disrupted maternal transcript turnover and failure in early zygotic gene activation appeared to associate with the aberrant miRNA profiles in *Dicer* and *Drosha* cKO spermatozoa. Overall, our data support a crucial function of paternal miRNAs and/or endo-siRNAs in the control of the transcriptomic homeostasis in fertilized eggs, zygotes and two-cell embryos. Given that supplementation of sperm RNAs enhances both the developmental potential of preimplantation embryos and the live birth rate, it might represent a novel means to improve the success rate of assisted reproductive technologies in fertility clinics.

KEY WORDS: Sperm, Small noncoding RNAs, Male infertility, Embryonic development, Assisted reproductive technologies, Maternal transcripts, *In vitro* fertilization, Epigenetics

INTRODUCTION

Spermatozoa (i.e. sperm), as the male gamete, are responsible for not only delivering the paternal genome, but also for providing essential factors for fertilization and preimplantation embryonic development (Krawetz, 2005; Sone et al., 2005). Recent data also suggest that in addition to the paternal proteins and genome, sperm also contribute the paternal epigenome in the forms of specific paternal DNA methylation, retained histones and sperm-borne RNAs during fertilization and early embryonic development (Gannon et al., 2014; Jenkins and Carrell, 2012). Both coding and noncoding RNAs have been detected in sperm (Krawetz, 2005; Pessot et al., 1989) and small noncoding RNA (sncRNA) species including microRNAs (miRNAs) (Amanai et al., 2006; Yan et al.,

2008), endogenous small interfering RNAs (endo-siRNAs) (Song et al., 2011), piwi-interacting RNAs (piRNAs) (Ghildiyal and Zamore, 2009; Grivna et al., 2006), and tRNA-derived small RNAs (Kawano et al., 2012; Peng et al., 2012) appear to be abundant in sperm. Similarly, oocytes also contain various species of sncRNAs (McGinnis et al., 2015; Sirotkin, 2012). Interestingly, only endo-siRNAs have been shown to be essential for oogenesis and the other types of sncRNAs appeared to be either nonfunctional or dispensable during oogenesis (Dixon et al., 2012; Hong et al., 2008; Leese et al., 2001). Zygotic expression of sncRNAs plays an essential role in early development (Ohnishi et al., 2010; Viswanathan et al., 2009; Yang et al., 2008). However, it is unclear whether sperm-borne (i.e. paternal) or oocyte-borne (i.e. maternal) sncRNAs are required for fertilization and/or preimplantation development.

The biogenesis of miRNAs and endo-siRNAs requires two RNase III enzymes, Drosha and Dicer (also known as Dicer1) (Lee et al., 2006; Suh and Blelloch, 2011; Yang and Lai, 2011). Drosha is responsible for the processing of miRNA primary transcripts into precursor miRNAs (pre-miRNAs) in the nucleus, whereas Dicer cleaves the pre-miRNAs into mature miRNAs in the cytoplasm. The miRNA production requires both RNase III enzymes, whereas endo-siRNA production needs only Dicer (Lee et al., 2006; Suh and Blelloch, 2011; Yang and Lai, 2011). By generating male germline-specific *Dicer* or *Drosha* conditional knockout (cKO) mice, we have demonstrated that these cKO male mice produce a small number of normal-looking sperm despite low sperm counts and reduced motility (Wu et al., 2012). Therefore, we decided to utilize these cKO male mice to investigate whether the Dicer or Drosha cKO spermatozoa are deficient in miRNAs and endo-siRNAs; if so, we will use these miRNA- and endo-siRNA-deficient sperm to perform intracytoplasmic sperm injection (ICSI) to study whether ablation of paternal sncRNAs could lead to defects in fertilization and/or preimplantation development, and whether supplementation of normal sperm-derived sncRNAs and total RNAs could rescue the defects. By generating female germ cell-specific *Drosha* cKO mice, we also obtained miRNA-deficient oocytes (Yuan et al., 2014), with which ICSI was performed using *Dicer* or *Drosha* cKO sperm to examine the relative importance of paternal and maternal sncRNAs. Here, we report that sperm-borne sncRNAs are indeed important for preimplantation embryonic development.

RESULTS

miRNAs and/or endo-siRNAs are partially deficient in *Dicer* and *Drosha* cKO spermatozoa

We previously generated male germ cell-specific *Dicer* (*Stra8-iCre; Dicer^{lox/del}*, herein called *Dicer* cKO) and *Drosha* (*Stra8-iCre; Drosha^{lox/del}*, herein called *Drosha* cKO) conditional KO mice by crossing the *Stra8-iCre* line with *Dicer* or *Drosha loxP* mice

Department of Physiology and Cell Biology, University of Nevada School of Medicine, 1664 North Virginia Street, MS 0575, Reno, NV 89557, USA.

*Author for correspondence (wyang@medicine.nevada.edu)

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(Wu et al., 2012). Both *Dicer* and *Drosha* cKO males were infertile as a result of low sperm counts, low sperm motility and abnormal sperm morphology (Fig. 1A–F), resembling oligoasthenoteratozoospermia (OAT) in humans. We collected cKO epididymal spermatozoa and analyzed *Dicer* and *Drosha* mRNA levels using real-time quantitative polymerase chain reaction (qPCR). Both *Dicer* and *Drosha* mRNA levels were reduced drastically in cKO sperm (Fig. 1G), which is consistent with our previous data showing significantly reduced levels of *Dicer* and *Drosha* in *Dicer* and *Drosha* cKO pachytene spermatocytes and round spermatids, respectively (Wu et al., 2012). Notably, although *Dicer* mRNA levels were not affected in *Drosha* cKO sperm, *Drosha* mRNA levels were significantly reduced in *Dicer* cKO sperm compared with those in WT sperm (Fig. 1G).

To determine whether *Dicer* and *Drosha* cKO sperm are deficient in sncRNAs, we performed sncRNA deep sequencing (sncRNA-Seq) (Fig. 2). A total of 379 miRNAs were identified in the sperm of WT, *Dicer* cKO and *Drosha* cKO mice (Fig. 2A and Tables S1–S8). In *Dicer* cKO sperm, ~47% of miRNAs were dysregulated, including ~15% upregulated and 32% downregulated (Fig. 2A,B

and Tables S1–S3). Among the downregulated miRNAs, 10% were downregulated by 2- to 10-fold (Fig. 2A,B and Table S2), and 22% by >10-fold, which were defined as absent (Fig. 2A,B and Table S3). Interestingly, ~53% were unchanged (Fig. 2B and Table S4). Similarly, in *Drosha* cKO sperm, ~52% of miRNAs were dysregulated, with ~19% upregulated and 33% downregulated, compared with WT controls (Fig. 2A,B and Tables S5–S7). Among the downregulated miRNAs, 14% were downregulated by 2- to 10-fold, whereas 19% were absent (down by >10-fold) (Fig. 2A,B and Tables S6, S7). Approximately 48% of miRNAs were not altered in *Drosha* cKO sperm (Table S8).

Due to a lack of endo-siRNA annotation in murine sperm, we performed a search for novel endo-siRNAs *in silico* using our previous method (Schuster et al., 2015), and identified 711 putative novel endo-siRNAs (Table S9). 65 out of the 711 endo-siRNAs identified were abundantly expressed (>2 copies) in at least one of the sperm samples from WT, *Dicer* cKO and *Drosha* cKO mice. In *Dicer* cKO sperm, 4 of 65 (accounting for ~6%) endo-siRNAs were significantly downregulated (by >10 fold) and only 1 (accounting for ~2%) was upregulated (Fig. 2C,D and Table S10). As expected, in *Drosha* cKO sperm, none of the 65 endo-siRNAs showed any

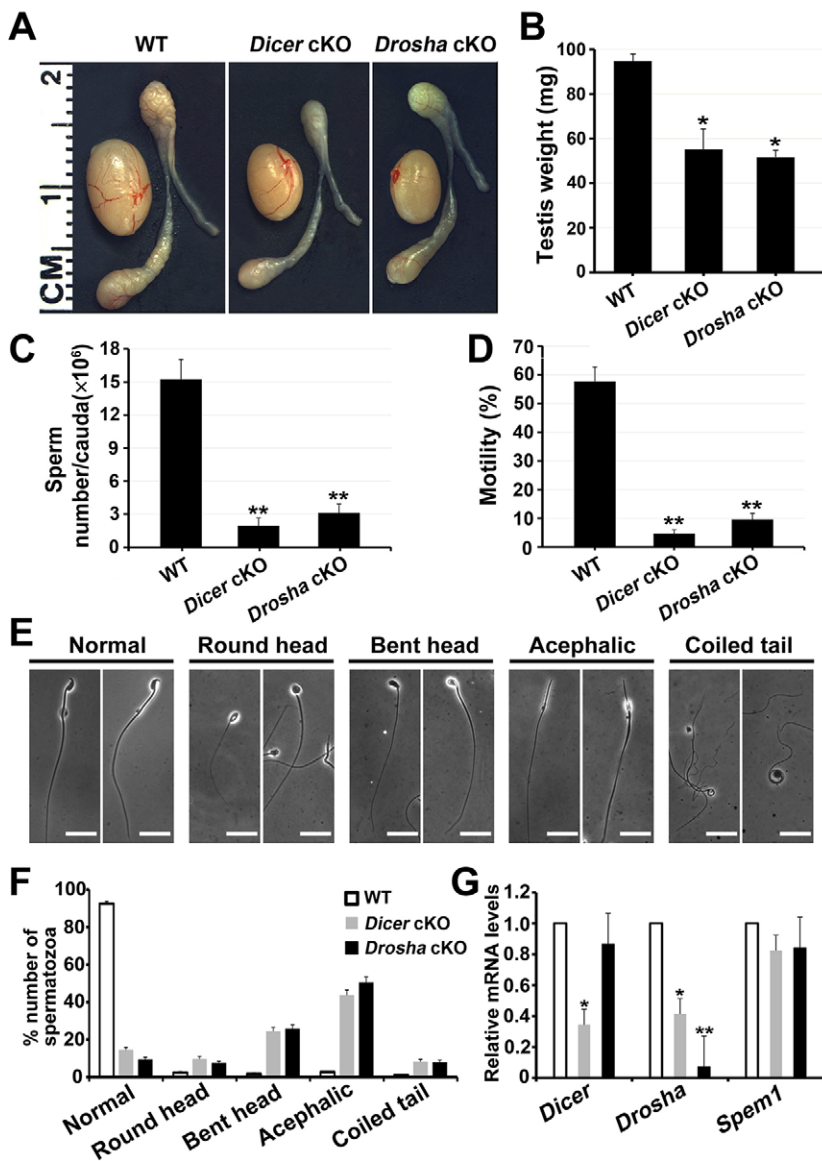


Fig. 1. Inactivation of *Dicer* and *Drosha* in spermatogenic cells leads to oligo asthenoteratozoospermia. (A) Gross morphology of adult WT, *Dicer* cKO (*Stra8-Cre; Dicer^{lox1del}*) and *Drosha* cKO (*Stra8-Cre; Drosha^{lox1del}*) testes and epididymides. (B) Testis weight of WT, *Dicer* cKO and *Drosha* cKO mice. (C) Sperm counts (C) and motility (D) of human tubal fluid (HTF)-activated epididymal spermatozoa collected from WT, *Dicer* cKO and *Drosha* cKO mice. (E) Phase-contrast microscopic images showing epididymal spermatozoa with normal and abnormal morphology in *Dicer* and *Drosha* cKO male mice. Scale bars: 25 μ m. (F) Quantitative analyses of epididymal spermatozoa with normal and abnormal morphology in WT, *Dicer* and *Drosha* cKO male mice. (G) qPCR analyses of *Dicer*, *Drosha* and *Spem1* mRNA levels in WT, *Dicer* cKO and *Drosha* cKO sperm. All data are based on analyses using biological triplicates and are presented as means \pm s.e.m. * P <0.05; ** P <0.01 (vs WT controls).

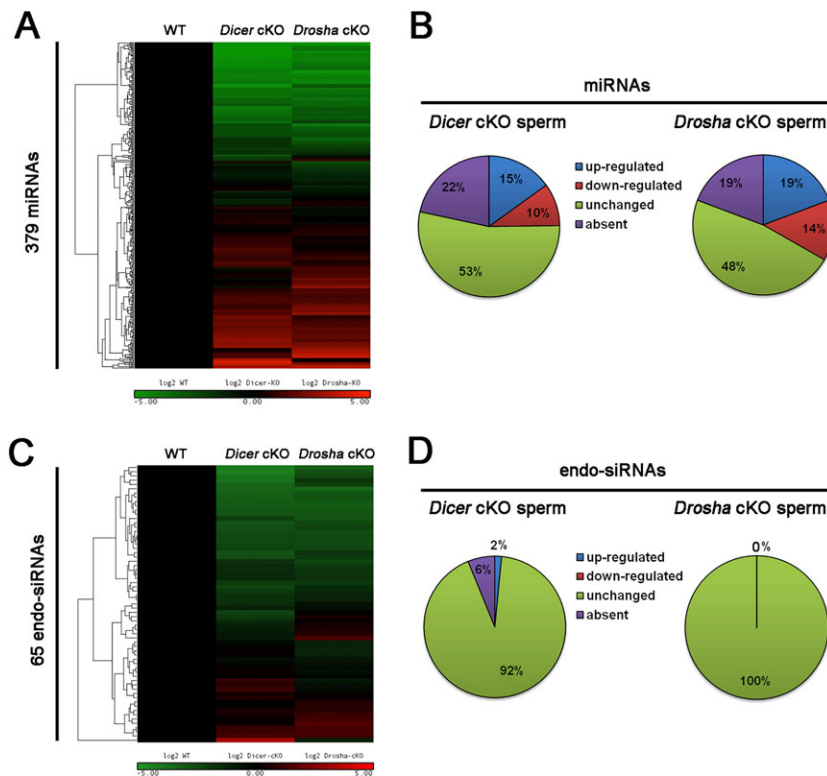


Fig. 2. Dicer and Drosha cKO sperm display altered miRNA and endo-siRNA expression profiles, as revealed by sncRNA-Seq analyses. (A) Heat map showing a total of 379 miRNAs identified in WT, Dicer cKO and Drosha cKO sperm. (B) Pie charts illustrating the proportions of changed (≥ 2 -fold) and unchanged (< 2 -fold) miRNAs in Dicer (left) or Drosha (right) cKO sperm. Adjusted P -values (P_{adj}) < 0.05 were considered to be significantly up- or down-regulated. (C) Heat map showing 65 known endo-siRNAs identified in WT, Dicer cKO and Drosha cKO sperm. (D) Pie charts illustrating proportions of the changed (≥ 2 -fold) and unchanged (< 2 -fold) endo-siRNAs in Dicer (left panel) or Drosha (right panel) cKO sperm. Adjusted P -values (P_{adj}) < 0.05 were considered to be significantly up- or down-regulated. The original sncRNA-Seq data can be found in Tables S1–S8 and S10.

significant changes when compared with the WT controls (Fig. 2C, D and Table S10). miRNA profiles were altered in both Dicer and Drosha cKO sperm, whereas the endo-siRNA profiles were affected only in Dicer cKO sperm, further supporting the current concept that endo-siRNAs are Dicer dependent and Drosha independent (Kim et al., 2009; Wu et al., 2012). Overall, the Dicer or Drosha cKO spermatozoa were not completely devoid of, but rather partially deficient in miRNAs and/or endo-siRNAs.

Eggs fertilized by spermatozoa with aberrant miRNA and endo-siRNA profiles through ICSI display reduced preimplantation developmental potential

A partial depletion of miRNAs and endo-siRNAs in Dicer or Drosha cKO sperm is, in fact, advantageous to our purpose because it allows mature spermatozoa to be produced despite abnormal miRNA and endo-siRNA profiles in these mutant sperm. To test

whether sperm with altered sperm miRNA and endo-siRNA contents could support fertilization and early embryonic development, we injected the control (WT) and cKO sperm into WT oocytes (C57BL/6J), and evaluated the developmental potential by counting the number of embryos that reached each of the five developmental stages, including two pronuclei (2PN), 2-cell, 4-cell, morula and blastocyst. Dicer cKO sperm-derived ICSI embryos displayed significantly reduced developmental potential in all five stages evaluated, compared with WT controls (Table 1 and Fig. 3). By contrast, Drosha cKO sperm-derived ICSI embryos only showed a significant reduction in developmental potential starting at the 4-cell stage and thereafter (Table 2 and Fig. 4). In general, Drosha cKO spermatozoa appeared to be more competent than the Dicer cKO spermatozoa in supporting fertilization and preimplantation embryonic development, based on the fact that close-to-normal fertilization rate and zygote to 2-cell transitions

Table 1. Preimplantation development of embryos derived from ICSI using WT oocytes and Dicer cKO sperm with or without sperm RNA supplementation

| Injected content | Total no. of surviving oocytes (no. of experiments) | Number of embryos at each stage | | | | |
|---|---|---------------------------------|--------------------------|--------------------------|-------------------------|-----------------------------|
| | | 2PN (% of total) | 2-cell (% of 2PN) | 4-cell (% of 2-cell) | Morula (% of 2-cell) | Blastocyst (% of 2-cell) |
| WT sperm | 171 (10) | 150 (87.72) ^a | 121 (80.67) ^a | 109 (90.08) ^a | 97 (80.17) ^a | 61 (50.41) ^a |
| Dicer cKO sperm | 226 (15) | 165 (73.01) ^b | 113 (68.48) ^b | 71 (62.83) ^b | 57 (50.44) ^b | 34 (30.08) ^b |
| Dicer cKO sperm+WT sperm small RNA | 239 (14) | 170 (71.13) ^b | 111 (65.29) ^b | 76 (68.47) ^b | 66 (59.46) ^b | 39 (35.14) ^b |
| Dicer cKO sperm+WT sperm total RNA | 109 (7) | 82 (75.23) ^b | 44 (53.66) ^b | 35 (79.55) ^a | 28 (63.64) ^b | 22 (50.00) ^a |
| Dicer cKO sperm+vehicle | 81 (5) | 60 (74.07) ^b | 32 (53.33) ^b | 16 (50.00) ^c | 9 (28.13) ^c | 7 (21.88) ^b |
| Dicer cKO sperm+WT sperm total RNA+RNase (1:10 molar ratio) | 54 (3) | 39 (72.22) ^b | 25 (64.10) ^b | 11 (44.00) ^c | 9 (36.00) ^c | 4 (16.00) ^b |
| Dicer cKO sperm+WT heart total RNA | 42 (2) | 31 (73.81) ^b | 19 (61.29) ^b | 10 (52.63) ^c | 7 (36.84) ^c | 5 (26.32) ^b |
| None (control for parthenogenesis) | 30 (2) | 0 | 0 | 0 | 0 | 0 |

2PN, 2 pronuclei. Statistical analyses were conducted using χ^2 test; values with different superscripts are significantly different ($P < 0.05$).

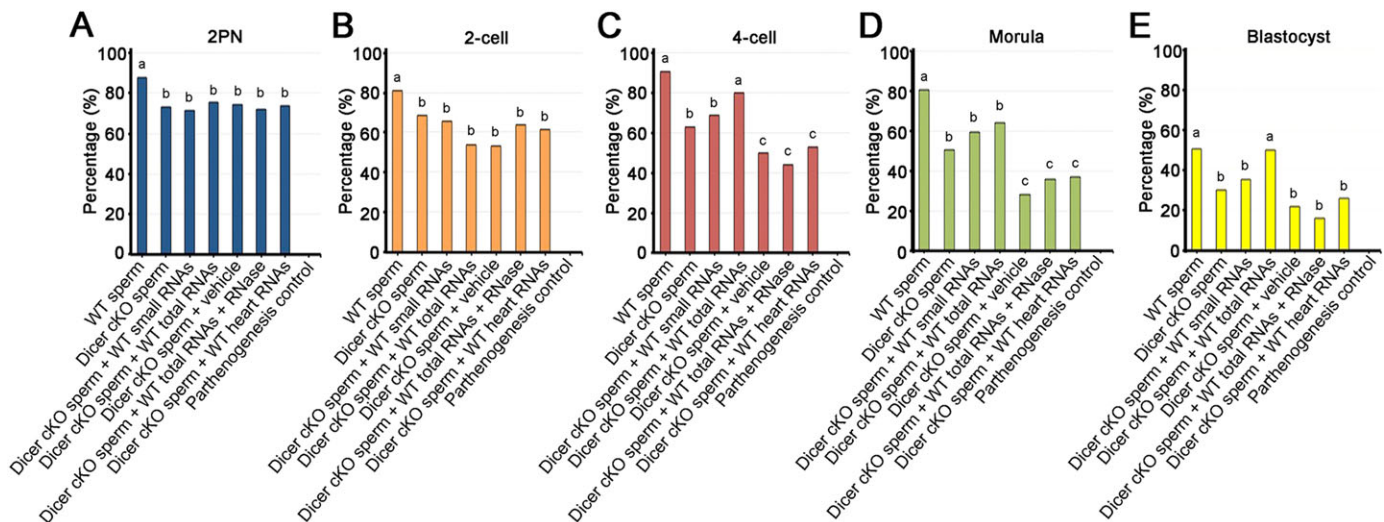


Fig. 3. Histogram showing the rate of development of embryos derived by ICSI using WT oocytes and Dicer cKO sperm with or without sperm RNA supplementation. (A) 2PN, (B) 2-cell, (C) 4-cell, (D) morula and (E) blastocyst embryos. Values labeled with different letters (a, b or c) are significantly different, based on χ^2 test ($P < 0.05$).

were observed in Droscha cKO sperm-fertilized eggs (Fig. 4A,B), but not in the Dicer cKO sperm-derived eggs (Fig. 3A,B).

Supplementation of WT sperm RNA enhances preimplantation development of ICSI embryos derived from spermatozoa with aberrant miRNA or endo-siRNA profiles

The compromised developmental potential of embryos derived from ICSI using Dicer and Droscha cKO sperm might not necessarily reflect the deficiency in sncRNA contents because these spermatozoa were mostly defective as a result of disrupted spermiogenesis. Although we injected only sperm heads with normal-looking morphology, potential structural defects in the injected sperm heads might have also contributed to the reduced developmental potential observed in the ICSI embryos. If the compromised developmental potential were truly caused by the loss of sperm-borne miRNAs/endo-siRNAs, supplementation of normal paternal RNA contents would enhance the developmental potential. Otherwise, the reduced developmental potential might well have been due to other structural or functional defects of the sperm head, which have nothing to do with sperm-borne sncRNAs. Therefore, we injected total, or small RNAs, isolated from WT sperm into eggs fertilized by Dicer and Droscha cKO spermatozoa through ICSI. Injection of the WT sperm small RNA fractions into ICSI eggs

fertilized by Dicer cKO spermatozoa slightly improved the embryonic developmental potential at the 4-cell, morula and blastocyst stages, although the overall developmental potential remained lower than that of the WT controls (Table 1 and Fig. 3). Interestingly, when WT sperm total RNA was injected into the ICSI eggs fertilized by Dicer cKO spermatozoa, ~50% of ICSI-derived 2-cell embryos developed into blastocysts. This rate is similar to that of WT controls, suggesting a full rescue can be achieved when Dicer cKO sperm-fertilized eggs are supplemented with WT sperm-borne total RNA (Fig. 3E).

Using a similar strategy, we studied the effects of injecting WT sperm total RNAs, or small RNAs only, into eggs fertilized by Droscha cKO spermatozoa through ICSI (Table 2 and Fig. 4). Supplementation of WT sperm small RNAs significantly increased the developmental potential from 2-cell to blastocyst stages from ~26% to ~41%, which is close to the developmental rates in WT controls (~50%) (Fig. 4E). Although at a lower efficiency, supplementation of WT sperm total RNAs also enhanced the developmental potential of Droscha cKO sperm-fertilized ICSI embryos from ~26% to ~34% (Table 2).

Four types of controls were included to determine whether the rescue truly resulted from sperm-borne RNAs. The first was a vehicle control that monitored the potential inhibitory or activating

Table 2. Preimplantation development of embryos derived from ICSI using WT oocytes and Droscha cKO sperm with or without sperm RNA supplementation

| Injected content | Total no. of surviving oocytes (no. of experiments) | Number of embryos at each stage | | | | |
|---|---|---------------------------------|----------------------------|-----------------------------|-----------------------------|---------------------------|
| | | 2PN (% of total) | 2-cell (% of 2PN) | 4-cell (% of 2-cell) | Morula (% of 2-cell) | Blastocyst (% of 2-cell) |
| WT sperm | 171 (10) | 150 (87.72) ^a | 121 (80.67) ^a | 109 (90.08) ^a | 97 (80.17) ^a | 61 (50.41) ^a |
| Droscha cKO sperm | 164 (12) | 133 (81.10) ^a | 100 (75.19) ^{a,b} | 64 (64.00) ^b | 52 (52.00) ^b | 26 (26.00) ^b |
| Droscha cKO sperm+WT sperm small RNA | 149 (10) | 119 (79.87) ^{a,b} | 88 (73.95) ^{a,b} | 68 (77.27) ^c | 61 (69.32) ^{a,c} | 36 (40.91) ^a |
| Droscha cKO sperm+WT sperm total RNA | 79 (4) | 66 (83.54) ^a | 44 (66.67) ^{b,c} | 35 (79.55) ^{a,c} | 28 (63.64) ^{b,c} | 15 (34.09) ^{a,b} |
| Droscha cKO sperm+vehicle | 97 (8) | 69 (71.13) ^{b,c} | 45 (65.22) ^{b,c} | 27 (60.00) ^{b,d} | 26 (57.78) ^{b,c} | 11 (24.44) ^{b,c} |
| Droscha cKO sperm+WT sperm total RNA+RNase (1:10 molar ratio) | 64 (3) | 45 (70.31) ^{b,c} | 23 (51.11) ^c | 10 (43.47) ^{b,d} | 9 (39.13) ^{b,d} | 4 (17.39) ^{b,c} |
| Droscha cKO sperm+WT heart total RNA | 41 (2) | 31 (75.61) ^{a,b,c} | 24 (77.42) ^{a,b} | 17 (70.83) ^{b,c,d} | 15 (62.50) ^{b,c,d} | 7 (29.17) ^{b,c} |
| None (control for parthenogenesis) | 30 (2) | 0 | 0 | 0 | 0 | 0 |

2PN, 2 pronuclei. Statistical analyses were conducted using χ^2 test; values with different superscripts are significantly different ($P < 0.05$).

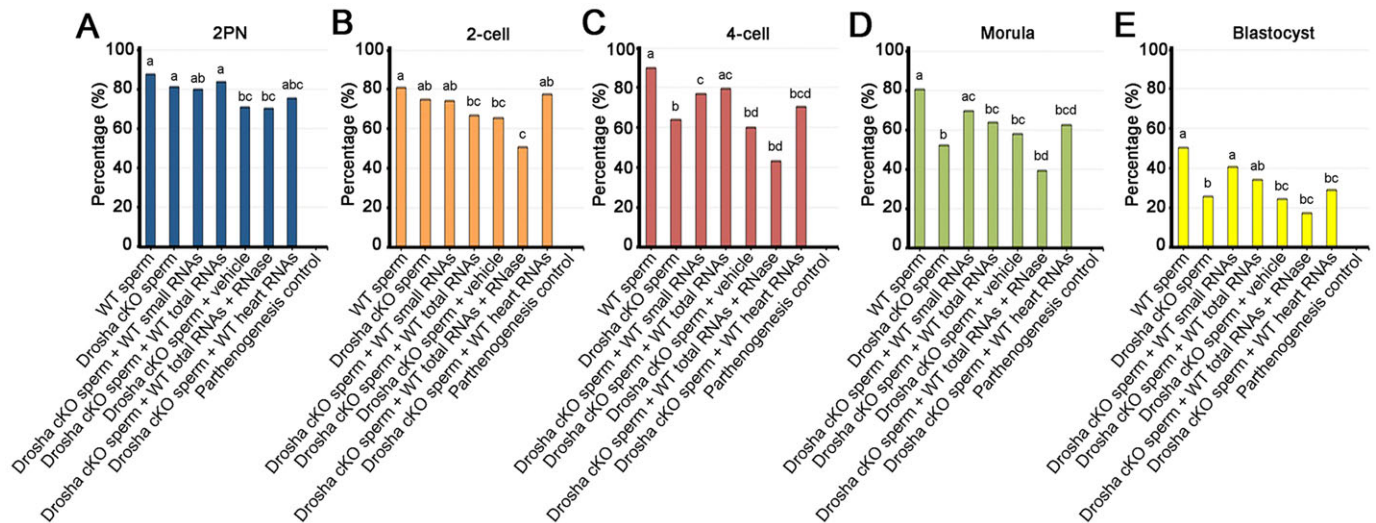


Fig. 4. Histogram showing the rate of development of embryos derived by ICSI using WT oocytes and Drosha cKO sperm with or without sperm RNA supplementation. (A) 2PN, (B) 2-cell, (C) 4-cell, (D) morula and (E) blastocyst embryos. Values labeled with different letters (a-d) are significantly different, based on χ^2 test ($P < 0.05$).

factors contained in the solution used for diluting sperm RNAs. The second control was sperm RNA treated with RNase A prior to injection, which would indicate whether the effects observed were RNA dependent. The third was a somatic cell RNA control, which contained total or small RNAs isolated from the heart. This control would tell whether the effects observed were sperm RNA specific. The fourth was a control for parthenogenesis. The much-reduced developmental potential in the vehicle control suggests potential damage to developing embryos when injected with vehicle only (Tables 1, 2 and Figs 3, 4). A lack of rescuing effects in the second and the third controls (i.e. ‘sperm RNA + RNase’ and somatic cell RNA) indicate that the improved developmental potential observed in supplementation with WT sperm total or small RNAs was both RNA dependent and sperm RNA specific. Overall, a lack of rescue in all of the four types of controls suggests that the rescue effects observed were sperm-borne RNA specific and RNA dependent.

Supplementation of WT sperm RNAs significantly improves the birth rate of ICSI embryos derived from spermatozoa with aberrant miRNA and endo-siRNA profiles

To evaluate the post-implantation development of the embryos derived from partial miRNA- or endo-siRNA-deficient spermatozoa supplemented with WT sperm-borne RNA, we transferred 2-cell embryos into the oviducts of recipient mice, and allowed them to develop to term. In the WT control group, ~28% of transferred 2-cell embryos led to live-born pups, whereas only 4–8% of

transferred 2-cell embryos from ICSI using Dicer cKO or Drosha cKO sperm developed to term with live-born pups (Table 3 and Fig. 5), suggesting a decreased post-implantation developmental potential in embryos derived from ICSI using partial miRNA- or endo-siRNA-deficient spermatozoa. By contrast, the birth rates were almost doubled (from 8.5% to 14.8%) and tripled (from 4.3% to 12.7%), for transferred 2-cell embryos derived from ICSI using Dicer cKO spermatozoa followed by supplementation of WT sperm total RNA and by Drosha cKO spermatozoa with subsequent supplementation of WT sperm small RNAs, respectively (Table 3 and Fig. 5). Such a significant improvement suggests that early supplementation of normal sperm RNA can drastically enhance not only the preimplantation development, but also the post-implantation development and the birth rate of ICSI embryos derived from sperm partially deficient in miRNAs and endo-siRNAs in mice. Both the male and female offspring, derived from ICSI using both Dicer and Drosha cKO spermatozoa with or without supplementation of WT sperm RNAs, developed normally with normal fertility when they reached adulthood.

Maternal miRNAs are dispensable for both fertilization and preimplantation development

It has been demonstrated that miRNA are dispensable for oocyte maturation, and miRNA-deficient oocytes from *Zp3-Cre; Dgcr8^{lox/lox}* or *Zp3; Drosha^{lox/lox}* females are fertile (Ma et al., 2010; Suh et al., 2010; Yuan et al., 2014). However, endo-siRNAs appear to be essential for oocyte maturation because Dicer cKO oocytes display

Table 3. Pups born through transfer of 2-cell embryos derived from ICSI using WT oocytes and WT, Dicer cKO, Drosha cKO sperm with or without supplementation of sperm-borne RNAs

| Injected content | No. of 2-cell embryos transferred (no. of experiments) | No. of recipients | No. of live-born offspring (%) | Pup genotype |
|-------------------------------------|--|-------------------|--------------------------------|------------------------------|
| WT sperm | 65 (3) | 4 | 18 (27.69) ^a | WT |
| Dicer cKO sperm | 47 (3) | 4 | 4 (8.51) ^{b,c} | <i>Dicer</i> ^{+/-} |
| Drosha cKO sperm | 47 (3) | 3 | 2 (4.26) ^c | <i>Drosha</i> ^{+/-} |
| Dicer cKO sperm+WT sperm total RNA | 54 (3) | 4 | 8 (14.81) ^{a,b} | <i>Dicer</i> ^{+/-} |
| Drosha cKO sperm+WT sperm small RNA | 71 (4) | 5 | 9 (12.68) ^b | <i>Drosha</i> ^{+/-} |

Statistical analyses were conducted using χ^2 test; values with different superscripts are significantly different ($P < 0.05$).

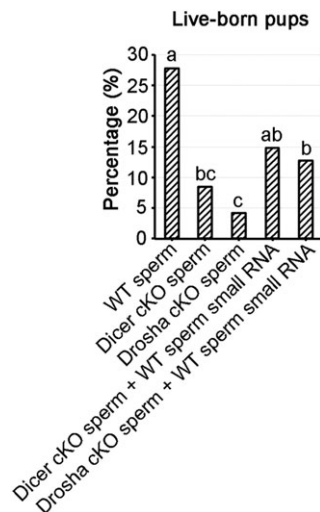


Fig. 5. Histogram showing the rate of live-born pups derived from ICSI using WT oocytes and WT, Dicer cKO, or Drosha cKO sperm with or without supplementation of sperm-borne RNAs. Bars labeled with different letters (a–c) are significantly different, based on χ^2 test ($P < 0.05$).

spindle defects and are infertile, whereas *Dgcr8* cKO or Drosha cKO oocytes are completely normal and fertile (Murchison et al., 2007; Suh et al., 2010; Yuan et al., 2014). To investigate whether a lack of both maternal and paternal miRNAs and/or endo-siRNAs would have additive adverse effects on fertilization and preimplantation development, we injected Dicer and Drosha cKO spermatozoa into Drosha cKO oocytes and evaluated the developmental potential (Table 4 and Fig. 6).

No differences in developmental potential were observed between embryos derived from WT sperm injected into Drosha cKO versus WT oocytes (Tables 1, 2 and 4). However, we observed reductions in the fertilization rate and early developmental potential between embryos derived from ICSI using Dicer cKO sperm injected into WT and Drosha cKO oocytes (Tables 1 and 4). These results suggest that the absence of maternal miRNAs has little or no effect on fertilization and preimplantation development, and a lack of both maternal and paternal miRNAs does not worsen the preimplantation development. However, the embryos derived from ICSI using Drosha cKO spermatozoa and Drosha cKO oocytes displayed reduced developmental potential in all five stages observed. This drastically reduced preimplantation developmental potential might reflect an absolute requirement for the zygotic or embryonic *Drosha* expression during preimplantation development because it has been documented that *Drosha*-null embryos die at ~E6.5 (Yuan et al., 2014). Supplementation of WT sperm RNAs, either total or small, failed to rescue the defective developmental potential (Table S11), supporting the notion that the defects are derived from the oocytes rather than Drosha cKO spermatozoa.

Paternal miRNAs are indeed delivered to the oocytes during fertilization and can persist beyond the 2PN stage

To determine whether sperm-borne miRNAs are indeed delivered into oocyte and persist in early embryos, we performed snRNA-Seq using WT oocytes and 2PN embryos derived from ICSI using WT, Dicer cKO and Drosha cKO spermatozoa (Fig. 7). A total of 82 and 85 miRNAs (>3 reads per million) were identified in WT oocytes and WT 2PN embryos, respectively (Fig. 7 and Table S12A,B); 36 and 86 miRNAs were identified in Dicer cKO and Drosha cKO sperm-derived 2PN embryos (Table S12A,B). Comparative analyses of the miRNAs profiles among WT sperm, WT oocytes and 2PN embryos derived from ICSI using WT, Dicer cKO and Drosha cKO spermatozoa, identified 14 miRNAs that were present in WT 2PN embryos and sperm, but not in WT oocytes (Fig. 7 and Table S12C). Among the 14 miRNAs, 5 were present in the Drosha cKO 2PN embryo and none was found in the Dicer cKO 2PN embryo (Fig. 7 and Table S12C). These data prove that sperm-borne miRNAs can be delivered to the oocytes during fertilization and many can persist beyond the 2PN stage.

Embryos derived from ICSI using Dicer or Drosha cKO spermatozoa display aberrant gene expression profiles during preimplantation development

To further explore the underlying molecular mechanism, we analyzed the expression profiles of 96 genes known to be essential for preimplantation development (Guo et al., 2010; Levy, 2001; Messerschmidt et al., 2014; Zheng and Dean, 2007). Using the latest microfluidics-based, high-throughput qPCR system (Fluidigm BioMark HD), we simultaneously analyzed the expression levels of the 96 genes in single WT oocytes, and embryos at 2PN, 2-cell, 4-cell, morula and blastocyst stages derived from ICSI using WT, Dicer and Drosha cKO spermatozoa. Dysregulation of genes was observed at all stages (Fig. 8 and Table S13). Although levels of the maternal transcripts were reduced from oocytes to the 2PN stage in WT controls, a similar decrease was observed in Drosha but not Dicer cKO sperm-derived 2PN embryos (Fig. 8, genes framed in blue). At the 2-cell stage, five genes (*Nes*, *Nodal*, *Vim*, *Cdkn2d* and *Stat5b*) were activated and highly expressed in WT embryos, whereas these genes failed to be activated in 2-cell embryos derived from either Dicer or Drosha cKO sperm (Fig. 8, framed genes). Although numerous genes were dysregulated (up- or down-regulated) in subsequent stages, this most likely reflects secondary effects of the initial disruptions in 2PN and 2-cell embryos, caused by deficiencies in paternal miRNAs and/or endo-siRNAs. In general, the expression profiles of Drosha cKO sperm-derived embryos appeared to be closer to those of the WT controls than the Dicer cKO sperm-derived embryos. This is consistent with the overall better developmental potential of Drosha cKO sperm-derived embryos, compared with Dicer cKO sperm-derived ones.

Table 4. Preimplantation development of embryos derived from ICSI using Drosha cKO oocytes and WT, Dicer cKO and Drosha cKO sperm

| Sperm genotype | Total no. of surviving oocytes (no. of experiments) | Number of embryos at each stage | | | | |
|----------------|---|---------------------------------|-------------------------|-------------------------|-------------------------|--------------------------|
| | | 2PN (% of total) | 2-cell (% of 2PN) | 4-cell (% of 2-cell) | Morula (% of 2-cell) | Blastocyst (% of 2-cell) |
| WT | 125 (7) | 109 (87.20) ^a | 99 (90.83) ^a | 75 (75.76) ^a | 70 (70.70) ^a | 51 (51.52) ^a |
| Dicer cKO | 140 (6) | 103 (73.57) ^b | 86 (83.50) ^a | 70 (81.40) ^a | 57 (66.28) ^a | 30 (34.88) ^b |
| Drosha cKO | 142 (9) | 100 (70.42) ^b | 72 (72.00) ^b | 24 (33.33) ^b | 16 (22.22) ^b | 9 (12.50) ^c |

2PN, 2 pronuclei; statistical analyses were conducted using χ^2 test; values with different superscripts are significantly different ($P < 0.05$).

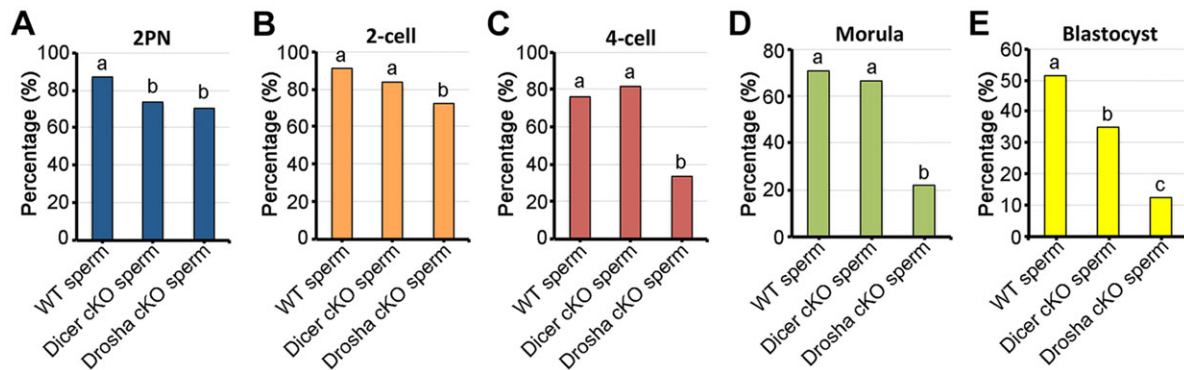


Fig. 6. Histogram showing the rate of development of 2PN embryos derived from ICSI using Drosha cKO oocytes and WT, Dicer cKO and Drosha cKO sperm. (A) 2PN, (B) 2-cell, (C) 4-cell, (D) morula and (E) blastocyst embryos. Values labeled with different letters (a, b, or c) are significantly different, based on χ^2 test ($P < 0.05$).

The significant dysregulation of genes at 2PN and 2-cell stages might represent the primary effects of deficiencies in paternal miRNAs and/or endo-siRNAs. Therefore, we further examined whether those dysregulated genes were targets of those miRNAs or endo-siRNAs that are deficient in Dicer or Drosha cKO spermatozoa. In Dicer cKO sperm-derived 2PN and 2-cell embryos, ~64% (25 out of 39) of dysregulated genes all had their targeting miRNAs significantly (>2-fold) dysregulated in Dicer cKO sperm (Fig. 9A and Table S14). In Drosha cKO sperm-derived 2PN and 2-cell embryos, ~52% (17 of 33) of dysregulated genes could be targeted by miRNAs that were significantly dysregulated in Drosha cKO sperm (Fig. 9B and Table S14). For example, *Neurog3* is drastically upregulated, whereas *Vim* is significantly downregulated in both Dicer and Drosha cKO sperm-derived 2PN and 2-cell embryos; miRNAs targeting *Neurog3* (miR-17/17-5p/20ab/20b-5p/93/106ab/427/518a-3p/519d and miR-124/124ab/506) were also significantly dysregulated in Dicer and Drosha cKO sperm (Fig. 9 and Tables S14, S15). Similarly, miRNAs that target *Vim* (miR-124/124ab/506, miR-138/138ab and miR-320abcd/4429) were also dysregulated in Dicer and Drosha cKO sperm (Fig. 9 and Tables S14, S15). *Neurog3* encodes a protein belonging to a basic helix-loop-helix (bHLH) transcription factor involved in neurogenesis and spermatogenesis (Hong et al., 2008; Stewart and Behringer, 2012). *Vim* encodes a class III intermediate filament protein widely expressed in the developing embryo and in cells of mesenchymal origin in the adult (Graw, 1996). Premature activation of *Neurog3*

and failure in activation of *Vim* reflect the disrupted early developmental program in Dicer and Drosha cKO sperm-derived embryos.

Taken together, these data suggest that the normal paternal miRNA and endo-siRNA profiles might have effects on the proper turnover of maternal transcripts, as well as the timely initiation of the early transcriptional program during fertilization and zygote to 2-cell transition. Moreover, the effects of the paternal miRNAs are most likely mediated through post-transcriptional regulation of the maternal and early zygotic mRNAs, for example, miRNA-mediated mRNA stability control.

Expression patterns of H3K4me2, H3K4me3 and H3K9me3 are normal in 2PN and 2-cell embryos derived from Dicer and Drosha cKO sperm

As an essential epigenetic mechanism, histone modifications regulate gene expression during development, for example, methylation of histone H3 at different lysine residues can either activate (H3K4me, H3K36me, H3K79me) or repress (H3K9, H3K27, H4K20) transcription (Jenuwein and Allis, 2001; Talbert and Henikoff, 2010). Recent reports have shown that noncoding RNAs (ncRNAs) are involved in epigenetic regulation of gene expression either through post-transcriptional regulation of important epigenetic regulators, including DNA methyltransferases, histone methyltransferases and deacetylases (Sato et al., 2011) or by serving as a 'sequence guide' and thus directing chromatin modifying machineries to the correct loci for epigenetic modifications (Maruyama et al., 2012). To explore whether

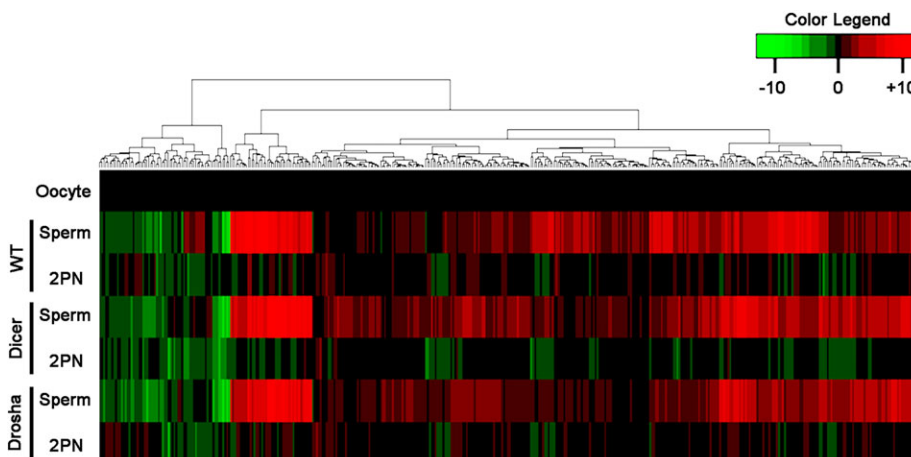


Fig. 7. Heat map showing miRNA profiles in WT oocytes, WT sperm and 2PN embryos derived from ICSI using WT, Dicer cKO and Drosha cKO sperm, as determined by low-input sncRNA-Seq. The expression levels were normalized against those in WT oocytes. Biological duplicates were analyzed for each group. The original sncRNA-Seq data are shown in Table S12.

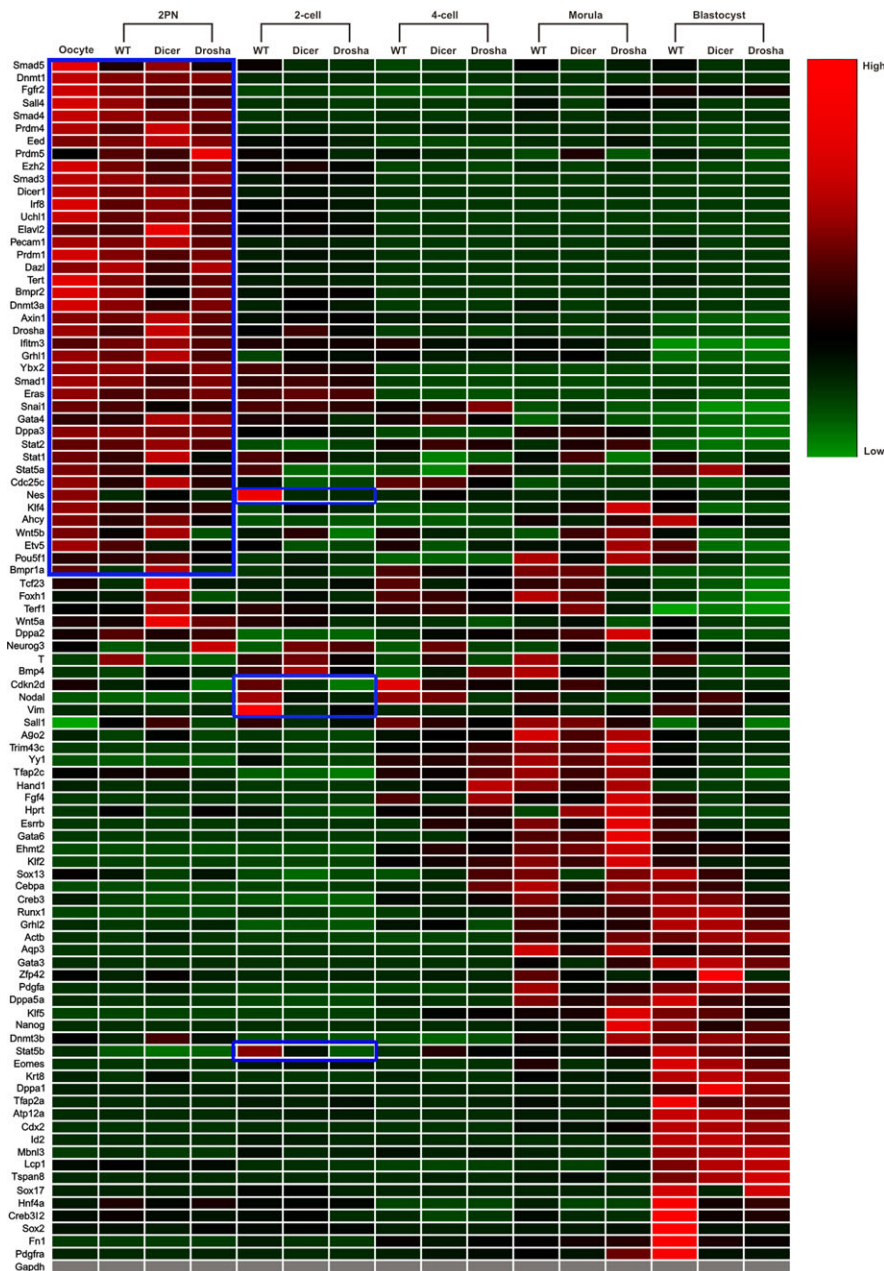


Fig. 8. Heat map showing expression profiles of 96 genes in single oocytes and preimplantation embryos derived from ICSI using WT, Dicer and Drosha cKO sperm at 2PN, 2-cell, 4-cell, morula and blastocyst stages. The high-throughput quantitative real-time PCR (HT qPCR) analyses were performed using the Fluidigm BioMark HD HT qPCR system. *Gapdh* was used as an internal control for data normalization. Biological triplicates were analyzed for each genotype (i.e. WT, Dicer and Drosha cKO). The original HT qPCR data can be found in Table S13.

an initial lack of sperm-borne sncRNAs could affect histone modifications during the 2PN to 2-cell transition, we examined the expression patterns of H3K4me2, H3K4me3 and H3K9me3 in 2PN and 2-cell embryos derived from ICSI using Dicer and Drosha cKO spermatozoa. At the 2PN stage, H3K4me2 was expressed in both male and female pronuclei in all four types of embryos (WT, Dicer cKO sperm derived, Drosha cKO sperm derived and *Drosha* null). Both H3K4me3 and H3K9me3 were detected only in female pronuclei of the 2PN embryos of all four types (Fig. S1). At the 2-cell stage, H3K4me2, H3K4me3 and H3K9me3 were detected in the nuclei of all four types of embryos (Fig. S1). No discernable differences were observed among all four types of embryos. Given the limited resolution of the immunofluorescence method used, one cannot preclude epigenetic changes in specific chromatin regions despite the lack any discernable changes in the global histone methylation patterns. Nevertheless, our data suggest that the altered paternal miRNA/endo-siRNA contents do not have a significant

impact on the three specific types of histone modifications in 2PN and 2-cell embryos.

DISCUSSION

The incomplete depletion of miRNAs and endo-siRNAs in Dicer and Drosha cKO sperm is consistent with the persistent expression of *Dicer* and *Drosha* mRNAs in spermatogenic cells even after Cre-mediated gene deletion (Wu et al., 2012). Several possibilities exist, which might explain why around half of the total sperm-borne miRNAs remain unchanged and a small proportion even get upregulated in cKO sperm. First, although the *Dicer* or *Drosha* gene was inactivated by late pachytene or round spermatid stages, their transcripts might have been synthesized, then stabilized and stored in ribonucleoproteins, a mechanism well documented for numerous transcripts that are needed for the haploid phase of spermatogenesis (Iguchi et al., 2006). The stored transcripts can then be translated into proteins even in the absence of the gene in haploid

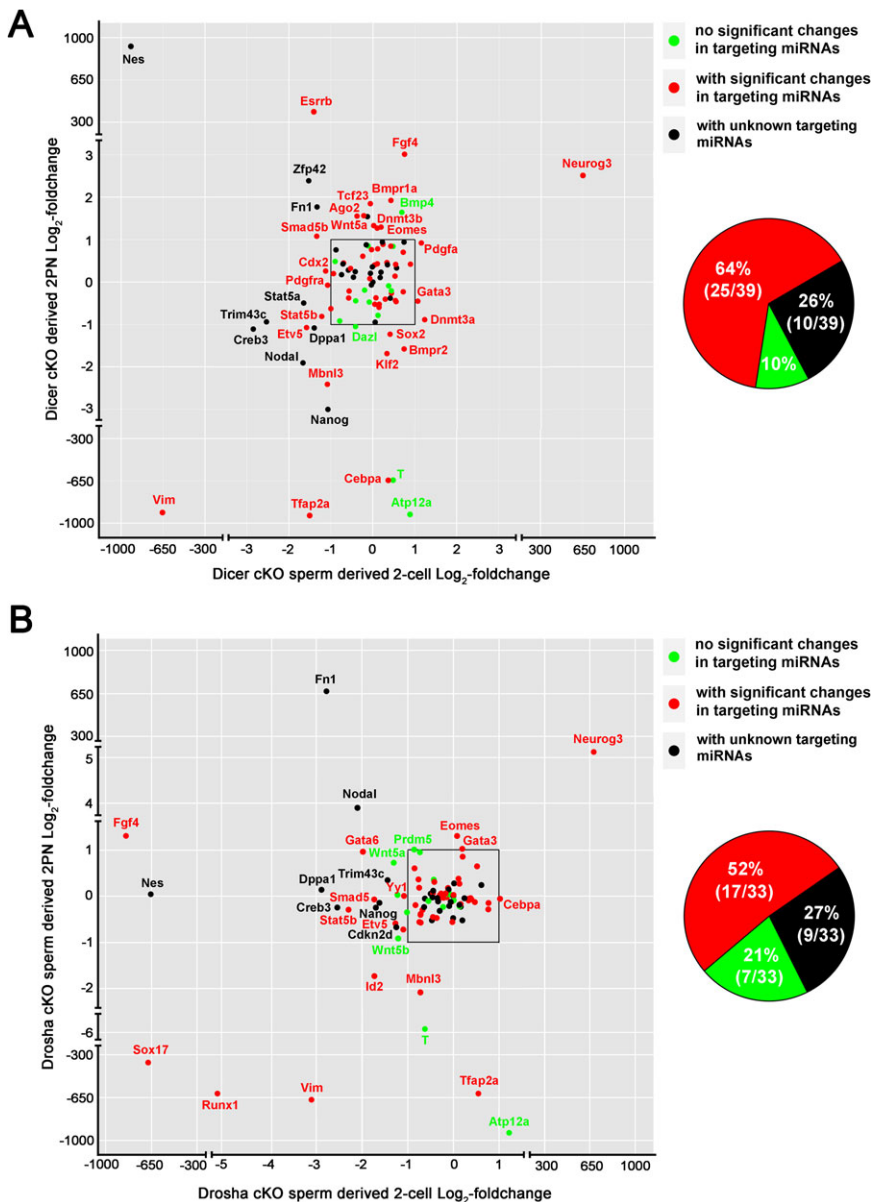


Fig. 9. Fold changes of dysregulated genes with or without significantly altered targeting miRNAs in Dicer and Drosha cKO sperm in 2PN and 2-cell embryos derived from ICSI using Dicer and Drosha cKO sperm. (A) Log₂ fold-changes of 96 genes analyzed in Dicer cKO sperm-derived 2PN and 2-cell embryos. Genes targeted by significantly altered miRNAs (fold changes ≥ 2 , $P_{adj} < 0.05$) in Dicer cKO sperm are in red font, and those without significantly changed targeting miRNAs are in green font. Transcripts without known targeting miRNAs in the databases are in black font. (B) Log₂ fold-changes of 96 genes analyzed in Drosha cKO sperm-derived 2PN and 2-cell embryos. Genes targeted by significantly altered miRNAs (fold changes ≥ 2 , $P_{adj} < 0.05$) in Drosha cKO sperm are in red font, and those without significantly changed targeting miRNAs are in green font. Transcripts without known targeting miRNAs in the databases are in black font. The original data on HT qPCR assays and miRNA target analyses can be found in Tables S14, S15.

germ cells. Similarly, pre-miRNAs may be synthesized and stored in ribonucleoproteins prior to the Cre-mediated gene inactivation in late pachytene spermatocytes and round spermatids. These pre-miRNAs could then be processed in spermatids and eventually packed into sperm. Second, those unchanged or upregulated miRNAs or endo-siRNAs might be more stable and thus would exist for an extended period of time and eventually could be packaged into sperm. The stabilization of miRNAs can be achieved through binding to circular RNAs or mRNAs (Aravin et al., 2007; Peng and Lin, 2013). Third, the cytoplasm of pachytene spermatocytes and developing spermatids are interconnected through intercellular bridges (IBs) (Haglund et al., 2011; Hermo et al., 2010). IBs allow for sharing of the cytoplasmic contents, including organelles and mRNAs (Ventela et al., 2003). Therefore, if the *Dicer* or *Drosha* gene is not deleted in one cell, the transcripts synthesized by this cell would be able to cross the IBs and reach its neighboring cells. Lastly, those miRNAs and endo-siRNAs might be produced through the non-canonical pathway (Pek et al., 2012), which does not require *Dicer* or *Drosha*. Nevertheless, with reduced levels of *Dicer* or *Drosha* transcripts, although some

spermatids manage to complete spermiogenesis, the spermatozoa in the cKO testes or epididymides are low in number, largely deformed and do not display normal motility, resembling human oligo-asthenoteratozoospermia (OAT). On the basis of testicular histology, sperm morphology, sperm counts and sperm motility, the phenotype of our *Dicer* and *Drosha* cKO mice appears to be less severe than that of *Dicer1* and *Dgcr8* cKO mice, as reported previously (Zimmermann et al., 2014). The discrepancy might well result from different Cre deleter lines (our *Stra8-Cre* versus their *Ddx4-Cre*) and different mouse strains used in these two studies. The OAT phenotype implies that the cKO spermatozoa are structurally and functionally compromised, which explains why those cKO males are infertile through natural mating. Because our goal was to show the effects of altered sperm miRNA and endo-siRNA contents on fertilization and early embryonic development, we purposely chose those normal-looking sperm heads for injection for the following reasons: (1) abnormalities in other parts of the sperm (e.g. flagellum) can be ignored; (2) those normal-looking sperm heads should have fewer structural defects, making them closer to the control sperm heads

except for altered miRNA and endo-siRNA profiles. Further supporting this notion, the almost complete rescue by the injection of WT sperm total RNA into Dicer cKO sperm-fertilized eggs implies that the defects largely lie in the RNA contents in Dicer cKO sperm and the rescuing effects are RNA dependent.

Differential effects were observed between supplementation experiments using total versus small sperm RNA fractions, for example, WT sperm total RNA appears to be more efficient than small RNAs in rescuing developmental defects in ICSI embryos derived from Dicer cKO sperm although total RNAs contains small RNAs. This discrepancy can be explained by the fact that total RNAs could contain factors such as large noncoding RNAs and/or mRNAs that are absent in small RNA preparations, which might have contributed the rescuing effects observed. Alternatively, in the total RNAs, small RNAs are diluted and thus, more optimal for the rescue. By contrast, sperm small RNAs seem to be more efficient than sperm total RNAs in rescuing the developmental defects of eggs fertilized with Drosha cKO sperm. Since we injected the same volume of total RNAs or small RNAs at the same concentrations into eggs, the difference in efficacy might result from the dilution of small RNAs in the total RNA samples. Our data suggest that WT sperm total RNAs can rescue the reduced developmental potential in Dicer cKO sperm-derived embryos, and WT sperm small RNAs can enhance the developmental potential of Drosha cKO sperm-derived embryos. These data also suggest that that Dicer cKO sperm have a more profound RNA deficiency (e.g. defective in both large and small RNA species), whereas Drosha cKO sperm are mainly deficient in sncRNAs. This is also supported by the fact that Drosha cKO sperm, in general, perform better than Dicer cKO sperm in supporting fertilization and preimplantation development prior to the 4-cell stage. Overall, our data demonstrate that sperm RNA supplementation enhances both preimplantation development and birth rate when sncRNA-deficient sperm (e.g. Dicer or Drosha cKO sperm) are used for ICSI. It would be interesting to study whether such a strategy is equally efficient in ICSI using sperm derived from other infertile mice with OAT. If this method can universally enhance the ICSI outcome, it might be considered for future application in assisted reproductive technology (ART) clinics to enhance success rates.

If paternal miRNAs or endo-siRNAs are truly functional, they must act mainly prior to, or soon after, zygotic genome activation, which occurs mainly at late zygote and 2-cell stages (Yuan et al., 2015). Although our data suggest a role of bulk paternal sncRNAs in post-fertilization development, it remains unclear how individual miRNAs and endo-siRNAs function at the molecular levels. A previous study reports that a paternal miRNA, miR-34c, is essential for the first cleavage, based on injection of miR-34c inhibitor into zygotes (Liu et al., 2012); however, this claim is not supported by the fact that *Mir34c*-null male mice are completely fertile (Wu et al., 2014). In fact, miR-34c belongs to a family of six miRNAs (miR-34a/b/c and miR-449a/b/c) encoded by three miRNA clusters (*Mir34a*, *Mir34b/c* and *Mir449*), which all contain the same seed sequence for 3'UTR recognition and thus are all functionally redundant. Inactivation of one of the three miRNA clusters does not cause any phenotype during either development or in adulthood (Wu et al., 2014). Nevertheless, our profiling analyses on maternal and early zygotic genes suggest that the paternal- or sperm-borne sncRNAs might have a role in regulating proper maternal mRNA turnover during zygote to 2-cell transition. Disruptions in both maternal transcript turnover and zygotic gene activation would impact, in theory, all early events, consequently leading to an arrest mostly at the 2-cell stage. It remains an interesting future topic to

investigate how paternal transcripts lead to degradation of maternal transcripts and how proper maternal transcript turnover affects zygotic gene activation. The fact that the majority of the dysregulated genes in 2PN and 2-cell embryos derived from ICSI using Dicer or Drosha cKO sperm are direct targets of miRNAs deficient or dysregulated in Dicer or Drosha cKO sperm strongly suggests that the paternal miRNAs act on their target mRNAs, which are mostly maternal transcripts, in the 2PN and 2-cell embryos. Many of the paternal miRNAs are also present in oocytes as the maternal miRNAs (Dixon et al., 2011; Hong et al., 2008). It remains puzzling that paternal miRNAs, rather than the same sets of maternal miRNAs, can affect maternal and early zygotic transcripts. However, this is consistent with the earlier findings, showing that maternal miRNAs appear to be functionally suppressed or non-functional during oocyte maturation and fertilization (Dixon et al., 2012; Hong et al., 2008; Leese et al., 2001). The underlying physiological significance of this phenomenon remains an interesting topic for future investigation.

In summary, we demonstrate that aberrant sperm-borne miRNA and endo-siRNA profiles correlate with reduced preimplantation developmental potential, which can be rescued by supplementation of wild-type sperm total RNAs or small RNAs in mice. Our data suggest that paternal miRNAs and endo-siRNAs are important for initiating the normal developmental program during early preimplantation development, especially from fertilization to the 2PN to 2-cell transition.

MATERIALS AND METHODS

Reagents and media

All reagents used were purchased from Sigma unless otherwise stated. The modified CZB-HEPES medium containing 20 mM HEPES-Na, 5 mM NaHCO₃, and 0.1 mg/ml polyvinyl alcohol (cold water soluble) was used for collecting sperm or oocytes. The CZB medium, supplemented with 5.56 mM D-glucose and 4 mg/ml BSA (Fraction V, Calbiochem), was used for culturing oocytes before ICSI, as previously described (Chatot et al., 1990; Kimura and Yanagimachi, 1995; Yanagimachi et al., 2004). The medium used for culturing fertilized embryos after ICSI was EmbryoMax KSOM medium supplemented with amino acids (KSOM+AA; Millipore, MR-121-D).

Generation of postnatal germ cell-specific Dicer or Drosha cKO mice

The Institutional Animal Care and Use Committee (IACUC) of the University of Nevada, Reno, approved all animal work in this study. The Cre-loxP strategy was used to generate the germline conditional knockout mice. *Stra8-iCre;Dicer^{lox/del}* (called Dicer cKO), *Stra8-iCre;Drosha^{lox/del}* (called Drosha cKO) male mice and *Zp3-iCre;Drosha^{lox/del}* female mice were generated as our previously reported (Wu et al., 2012; Yuan et al., 2014).

Oocyte preparation and ICSI

WT and *Zp3-iCre;Drosha^{lox/del}* female mice at 5–10 weeks of age were superovulated by intraperitoneal injection of 5 IU of pregnant mare's serum gonadotropin (PMSG), followed by intraperitoneal injection of 5 IU of human chorionic gonadotropin (hCG) 48 h later. Mature oocytes (MII stage) were collected from the oviducts 14–16 h after hCG injection, and freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase in HEPES-CZB at 37°C for 2–3 min. The cumulus-free oocytes were washed and kept in the CZB medium for at least 1 h in an incubator at 37°C with air containing 5% CO₂ before ICSI. An extended ICSI protocol can be found in the supplementary Materials and Methods.

Total and small RNA isolation from mouse sperm

The total RNA was isolated using the mirVana miRNA Isolation Kit (Life Technologies) following the manufacturer's instructions with modifications at the lysis stage. In brief, after addition of lysis buffer, the frozen sperm pellets were homogenized at low settings for 90 s, followed by incubation for 5 min at 65°C. Complete lysis of sperm heads was verified by microscopic examination. Once a total lysis of sperm heads was achieved, the samples were then placed on ice and the default protocol was resumed. To determine the quantity and quality, sperm RNA samples were analyzed using the RNA 6000 Nano chips run on an Agilent 2100 Bioanalyzer (Agilent). An RNA integrity number (RIN) of 2–4 indicates good sperm RNA quality.

Injection of sperm total and small RNA into post-ICSI oocytes

The concentration of sperm total or small RNAs was adjusted to 20–100 pg/μl and an aliquot of 1–2 μl of the RNA solution was loaded into a microinjection needle (Eppendorf, 930000043). Zygote microinjection was performed in HEPES-CZB medium ~2 h after ICSI following standard procedures (Nagy et al., 2003). Each zygote was injected with ~1–2 pl small or total RNA solution and a successful injection was verified by a visible, minor expansion of the cytoplasmic membrane. To cause RNA degradation, sperm total RNAs were treated with RNase A (Invitrogen, 12091-021) at a molar ratio of 1:10 (sperm RNA:RNase A) at 37°C for 30 min. The final concentration of RNase A in the sperm total RNA degradation products was ~5–10 pg/μl; given that the injection volume was ~1–2 pl, ~5–10×10⁻⁶ pg RNase A might have been injected into a zygote. Injected zygotes were then transferred into pre-balanced KSOM+AA medium and cultured in an incubator with air containing 5% CO₂ at 37°C.

Evaluation of developmental potential of ICSI-derived preimplantation embryos

ICSI and sperm RNA-supplemented ICSI oocytes were allowed to develop in KSOM+AA medium in an incubator with air containing 5% CO₂ at 37°C. The number of 2PN stage embryos was counted 6–8 h after ICSI or 3–4 h after RNA supplementation. Subsequently, the numbers of embryos at 2-cell, 4-cell, morula and blastocyst stages were examined at 24 h, 48 h, 72 h and 96 h, respectively. To evaluate the potential of post-implantation development, two-cell embryos were transferred into the oviducts of pseudopregnant CD1 females. Cesarean section was performed on day 19 after embryo transfer and live-born pups were counted.

High-throughput single-cell qPCR

MII oocytes were collected from the oviducts 14–16 h after hCG injection and treated with hyaluronidase to remove cumulus cells. Single embryos were collected at 6–8 h (2PN stage), 24 h (2-cell stage), 48 h (4-cell stage), 72 h (morula stage), 96 h (blastocyst) after ICSI using WT, Dicer or Drosha cKO sperm. An extended high-throughput qPCR method can be found in the supplementary Materials and Methods.

Quantitative real-time PCR (qPCR)

WT, Dicer cKO and Drosha cKO sperm total RNA was subjected to DNA removal using a DNA-free DNase (Ambion), followed by cDNA synthesis using the SuperScript III First-strand Kit (Invitrogen) according to the manufacturer's instructions. cDNA concentrations were measured using a NanoDrop 2000 spectrophotometer, and then diluted to a concentration of 25 ng/μl to serve as cDNA templates. SYBR Green-based real-time

quantitative PCR was performed to examine mRNA expression levels. *Gapdh* was used as an internal control for data normalization.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in HEPES-CZB medium for 1 h and washed three times in 0.1 M glycine with 0.3 mg/ml BSA at room temperature (RT). The embryos were permeabilized in 0.2% Triton X-100 in PBS for 15 min and blocked using a blocking solution containing 2% BSA in PBS for 1 h at RT, followed by incubation with the following antibodies diluted in the blocking solution for 1 h at RT: rabbit anti-H3K4me2 polyclonal antibody (Millipore, 07-030, 1:300 dilution), rabbit anti-H3K4me3 polyclonal antibody (Diagenode, pAb-003-050, 1:300) and rabbit anti-H3K9me3 (AbCam, ab8898, 1:500). After washing in the blocking solution, embryos were incubated with fluorescence-conjugated, species-specific secondary antibodies [Alexa Fluor 594 goat anti-rabbit IgG(H+L); Molecular Probes, A11012, 1:2000] for 1 h at RT. Finally, the embryos were counterstained with 4',6-diamidino-2-phenylindole dilacatate (DAPI, Sigma) for indirect immunofluorescent assays using a fluorescence microscope (Zeiss, HAL100).

Small noncoding RNA deep sequencing (sncRNA-Seq) and data analyses

Sperm sncRNA libraries were prepared using the standard protocol of an Ion Total RNA-Seq Kit v2 (Invitrogen) and biological triplicates for each sample type (WT, Dicer cKO, Drosha cKO) were prepared. For low-input sncRNA-Seq, 20–30 WT oocytes or 2PN embryos derived from ICSI using WT, Dicer cKO and Drosha cKO spermatozoa (biological duplicates for each pooled sample) were collected followed by lysis in a buffer containing 1% Salkosyl, 20 mM Tris-HCl pH 8.0, 20 mM KCl, 100 mM DTT. The extended sncRNA-seq and data analysis processing for sperm, oocyte and 2PN embryos can be found in supplementary Materials and methods. Data have been deposited into the Gene Expression Omnibus (accession number GSE73824).

Bioinformatic analysis

miRNA families predicted to target the 96 early genes examined were identified using TargetScan (Berezikov et al., 2005; Lewis et al., 2005). A detailed protocol can be found in the supplementary Materials and methods.

Statistics

Data are presented as means±s.e.m. and statistical differences between datasets were assessed by one-way ANOVA or Student's *t*-test using SPSS16.0 software. *P*≤0.05 was considered significant and *P*≤0.01 was considered highly significant. ICSI data were analyzed using χ^2 tests, compared with the WT group and *P*≤0.05 was regarded as significant.

Competing interests

The authors declare no competing or financial interests.

Author contributions

W.Y. and S.Y. conceived and designed the study; S.Y., A.S., C.T., T.Y., N.O., J.B. and H.Z. performed the experiments; W.Y. and S.Y. wrote the manuscript.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.131755/-/DC1>

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