

## RESEARCH ARTICLE

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

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## 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<sup>lox/del</sup>*, herein called *Dicer* cKO) and *Drosha* (*Stra8-iCre; Drosha<sup>lox/del</sup>*, herein called *Drosha* cKO) conditional KO mice by crossing the *Stra8-iCre* line with *Dicer* or *Drosha loxP* mice

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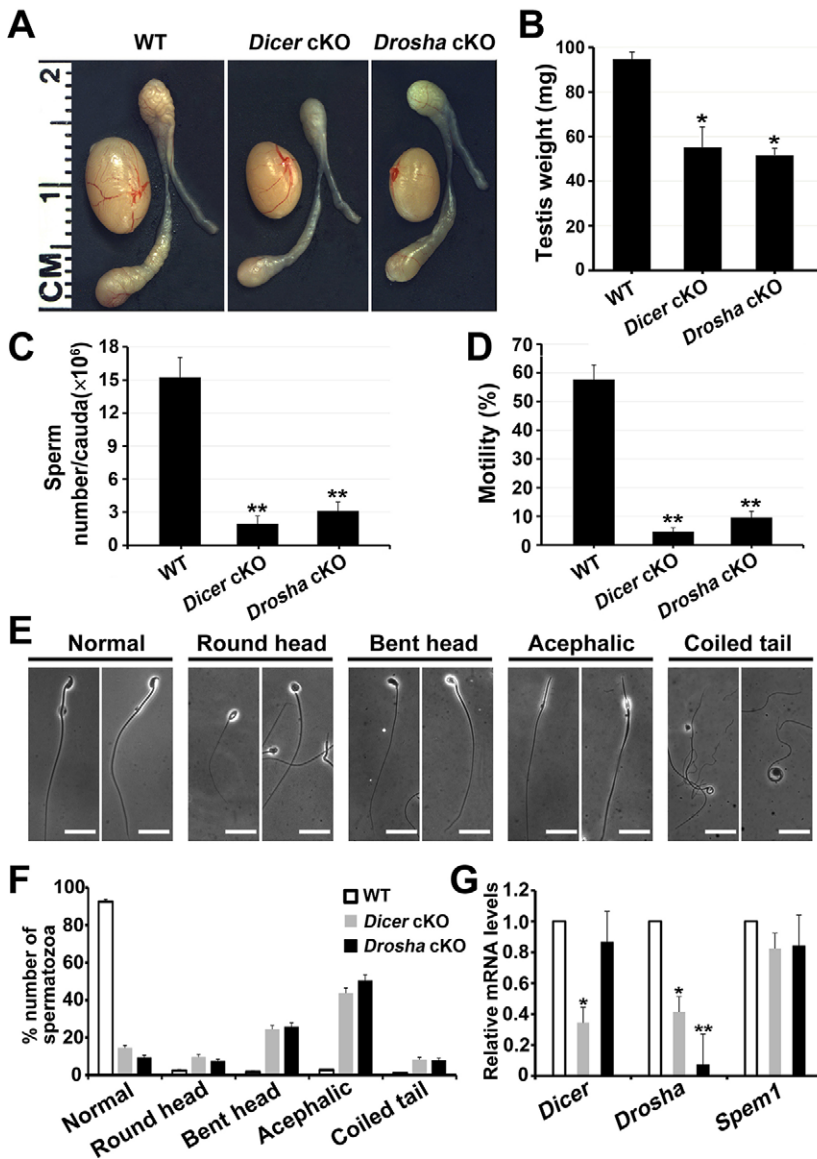
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(Wu et al., 2012). Both *Dicer* and *Drosha* cKO males were infertile as a result of low sperm counts, low sperm motility and abnormal sperm morphology (Fig. 1A–F), resembling oligoasthenoteratozoospermia (OAT) in humans. We collected cKO epididymal 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).

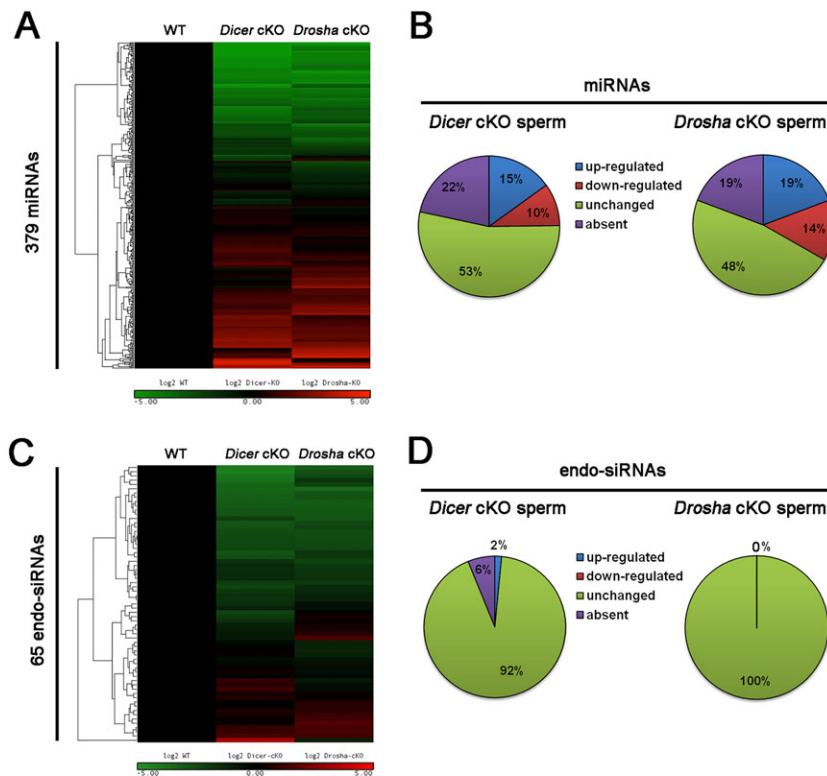
To determine whether *Dicer* and *Drosha* cKO sperm are deficient in sncRNAs, we performed sncRNA deep sequencing (sncRNA-Seq) (Fig. 2). A total of 379 miRNAs were identified in the sperm of WT, *Dicer* cKO and *Drosha* cKO mice (Fig. 2A and Tables S1–S8). In *Dicer* cKO sperm, ~47% of miRNAs were dysregulated, 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 (*Stras8-Cre; Dicer<sup>lox/del</sup>*) and *Drosha* cKO (*Stras8-Cre; Drosha<sup>lox/del</sup>*) 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  $\mu$ 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 ( $\geq 2$ -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 ( $\geq 2$ -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

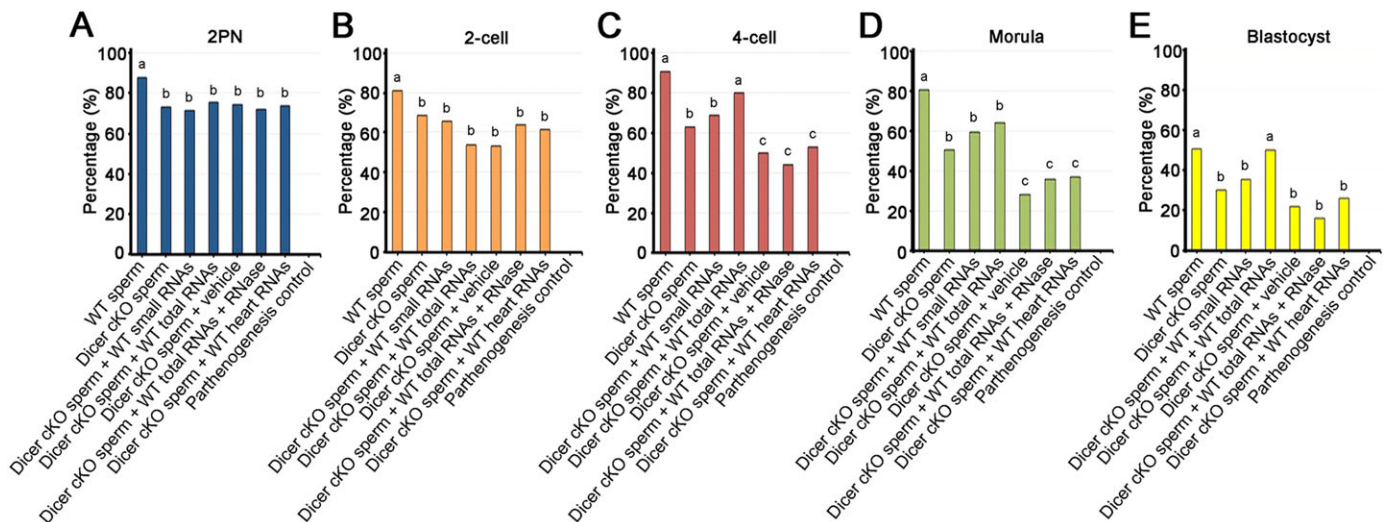
A partial depletion of miRNAs and endo-siRNAs in Dicer or Drosha cKO sperm is, in fact, advantageous to our purpose because it allows mature spermatozoa to be produced despite abnormal miRNA and endo-siRNA profiles in these mutant sperm. To test

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) <sup>a</sup>	121 (80.67) <sup>a</sup>	109 (90.08) <sup>a</sup>	97 (80.17) <sup>a</sup>	61 (50.41) <sup>a</sup>
Dicer cKO sperm	226 (15)	165 (73.01) <sup>b</sup>	113 (68.48) <sup>b</sup>	71 (62.83) <sup>b</sup>	57 (50.44) <sup>b</sup>	34 (30.08) <sup>b</sup>
Dicer cKO sperm+WT sperm small RNA	239 (14)	170 (71.13) <sup>b</sup>	111 (65.29) <sup>b</sup>	76 (68.47) <sup>b</sup>	66 (59.46) <sup>b</sup>	39 (35.14) <sup>b</sup>
Dicer cKO sperm+WT sperm total RNA	109 (7)	82 (75.23) <sup>b</sup>	44 (53.66) <sup>b</sup>	35 (79.55) <sup>a</sup>	28 (63.64) <sup>b</sup>	22 (50.00) <sup>a</sup>
Dicer cKO sperm+vehicle	81 (5)	60 (74.07) <sup>b</sup>	32 (53.33) <sup>b</sup>	16 (50.00) <sup>c</sup>	9 (28.13) <sup>c</sup>	7 (21.88) <sup>b</sup>
Dicer cKO sperm+WT sperm total RNA+RNase (1:10 molar ratio)	54 (3)	39 (72.22) <sup>b</sup>	25 (64.10) <sup>b</sup>	11 (44.00) <sup>c</sup>	9 (36.00) <sup>c</sup>	4 (16.00) <sup>b</sup>
Dicer cKO sperm+WT heart total RNA	42 (2)	31 (73.81) <sup>b</sup>	19 (61.29) <sup>b</sup>	10 (52.63) <sup>c</sup>	7 (36.84) <sup>c</sup>	5 (26.32) <sup>b</sup>
None (control for parthenogenesis)	30 (2)	0	0	0	0	0

2PN, 2 pronuclei. Statistical analyses were conducted using  $\chi^2$  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  $\chi^2$  test ( $P < 0.05$ ).**

were observed in Drosha 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 Drosha 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 Drosha 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).

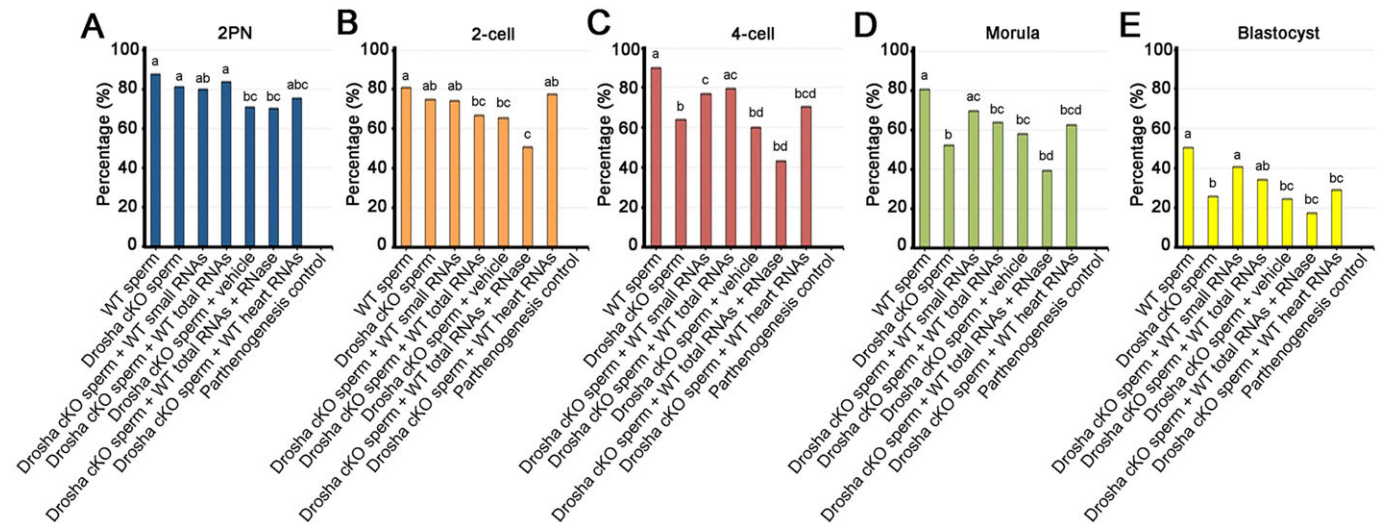
Using a similar strategy, we studied the effects of injecting WT sperm total RNAs, or small RNAs only, into eggs fertilized by Drosha cKO spermatozoa through ICSI (Table 2 and Fig. 4). Supplementation of WT sperm small RNAs significantly increased the developmental potential from 2-cell to blastocyst 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 Drosha 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 Drosha 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) <sup>a</sup>	121 (80.67) <sup>a</sup>	109 (90.08) <sup>a</sup>	97 (80.17) <sup>a</sup>	61 (50.41) <sup>a</sup>
Drosha cKO sperm	164 (12)	133 (81.10) <sup>a</sup>	100 (75.19) <sup>a,b</sup>	64 (64.00) <sup>b</sup>	52 (52.00) <sup>b</sup>	26 (26.00) <sup>b</sup>
Drosha cKO sperm+WT sperm small RNA	149 (10)	119 (79.87) <sup>a,b</sup>	88 (73.95) <sup>a,b</sup>	68 (77.27) <sup>c</sup>	61 (69.32) <sup>a,c</sup>	36 (40.91) <sup>a</sup>
Drosha cKO sperm+WT sperm total RNA	79 (4)	66 (83.54) <sup>a</sup>	44 (66.67) <sup>b,c</sup>	35 (79.55) <sup>a,c</sup>	28 (63.64) <sup>b,c</sup>	15 (34.09) <sup>a,b</sup>
Drosha cKO sperm+vehicle	97 (8)	69 (71.13) <sup>b,c</sup>	45 (65.22) <sup>b,c</sup>	27 (60.00) <sup>b,d</sup>	26 (57.78) <sup>b,c</sup>	11 (24.44) <sup>b,c</sup>
Drosha cKO sperm+WT sperm total RNA+RNase (1:10 molar ratio)	64 (3)	45 (70.31) <sup>b,c</sup>	23 (51.11) <sup>c</sup>	10 (43.47) <sup>b,d</sup>	9 (39.13) <sup>b,d</sup>	4 (17.39) <sup>b,c</sup>
Drosha cKO sperm+WT heart total RNA	41 (2)	31 (75.61) <sup>a,b,c</sup>	24 (77.42) <sup>a,b</sup>	17 (70.83) <sup>b,c,d</sup>	15 (62.50) <sup>b,c,d</sup>	7 (29.17) <sup>b,c</sup>
None (control for parthenogenesis)	30 (2)	0	0	0	0	0

2PN, 2 pronuclei. Statistical analyses were conducted using  $\chi^2$  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  $\chi^2$  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.

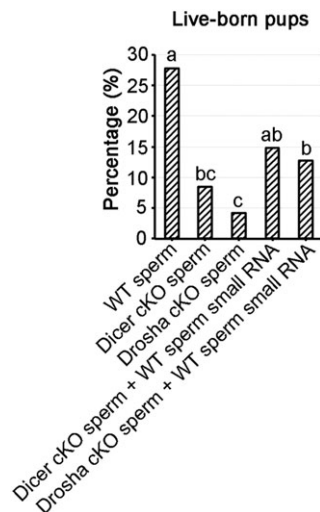
#### Maternal miRNAs are dispensable for both fertilization and preimplantation development

It has been demonstrated that miRNA are dispensable for oocyte maturation, and miRNA-deficient oocytes from *Zp3-Cre; Dgcr8<sup>lox/lox</sup>* or *Zp3; Drosha<sup>lox/lox</sup>* 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) <sup>a</sup>	WT
Dicer cKO sperm	47 (3)	4	4 (8.51) <sup>b,c</sup>	<i>Dicer</i> <sup>+/-</sup>
Drosha cKO sperm	47 (3)	3	2 (4.26) <sup>c</sup>	<i>Drosha</i> <sup>+/-</sup>
Dicer cKO sperm+WT sperm total RNA	54 (3)	4	8 (14.81) <sup>a,b</sup>	<i>Dicer</i> <sup>+/-</sup>
Drosha cKO sperm+WT sperm small RNA	71 (4)	5	9 (12.68) <sup>b</sup>	<i>Drosha</i> <sup>+/-</sup>

Statistical analyses were conducted using  $\chi^2$  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  $\chi^2$  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.

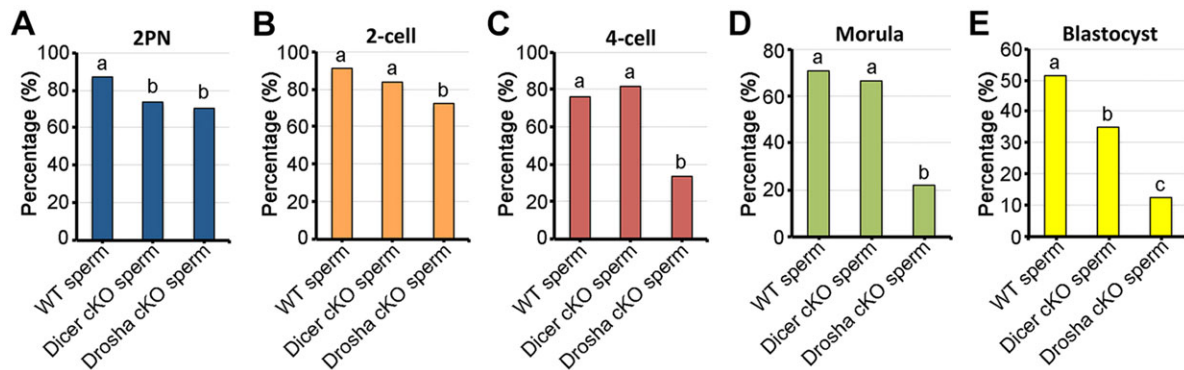
### Embryos derived from ICSI using Dicer or Drosha cKO spermatozoa display aberrant gene expression profiles during preimplantation development

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) <sup>a</sup>	99 (90.83) <sup>a</sup>	75 (75.76) <sup>a</sup>	70 (70.70) <sup>a</sup>	51 (51.52) <sup>a</sup>
Dicer cKO	140 (6)	103 (73.57) <sup>b</sup>	86 (83.50) <sup>a</sup>	70 (81.40) <sup>a</sup>	57 (66.28) <sup>a</sup>	30 (34.88) <sup>b</sup>
Drosha cKO	142 (9)	100 (70.42) <sup>b</sup>	72 (72.00) <sup>b</sup>	24 (33.33) <sup>b</sup>	16 (22.22) <sup>b</sup>	9 (12.50) <sup>c</sup>

2PN, 2 pronuclei; statistical analyses were conducted using  $\chi^2$  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  $\chi^2$  test ( $P < 0.05$ ).

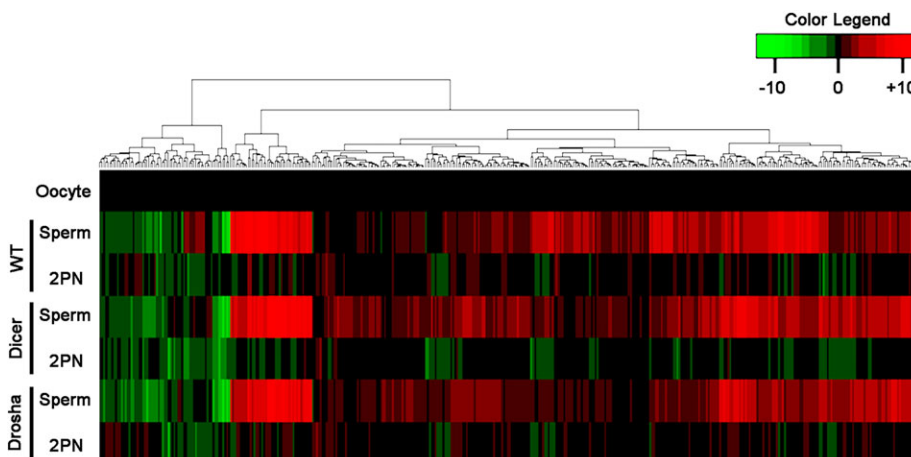
The significant dysregulation of genes at 2PN and 2-cell stages might represent the primary effects of deficiencies in paternal miRNAs and/or endo-siRNAs. Therefore, we further examined whether those dysregulated genes were targets of those miRNAs or endo-siRNAs that are deficient in Dicer or Drosha 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.

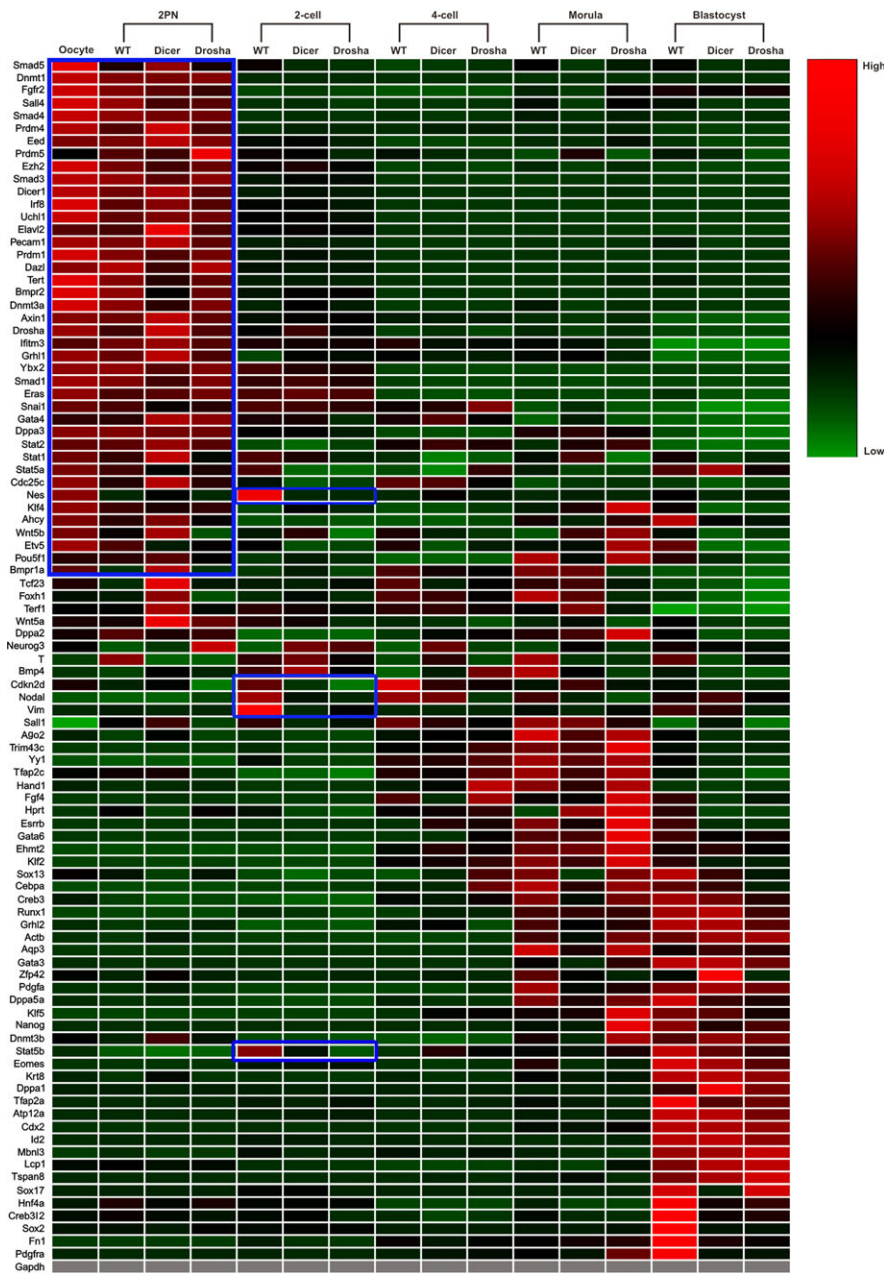
Taken together, these data suggest that the normal paternal miRNA and endo-siRNA profiles might have effects on the proper turnover of maternal transcripts, as well as the timely initiation of the early transcriptional program during fertilization and zygote to 2-cell transition. Moreover, the effects 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 snRNA-Seq.** The expression levels were normalized against those in WT oocytes. Biological duplicates were analyzed for each group. The original snRNA-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.

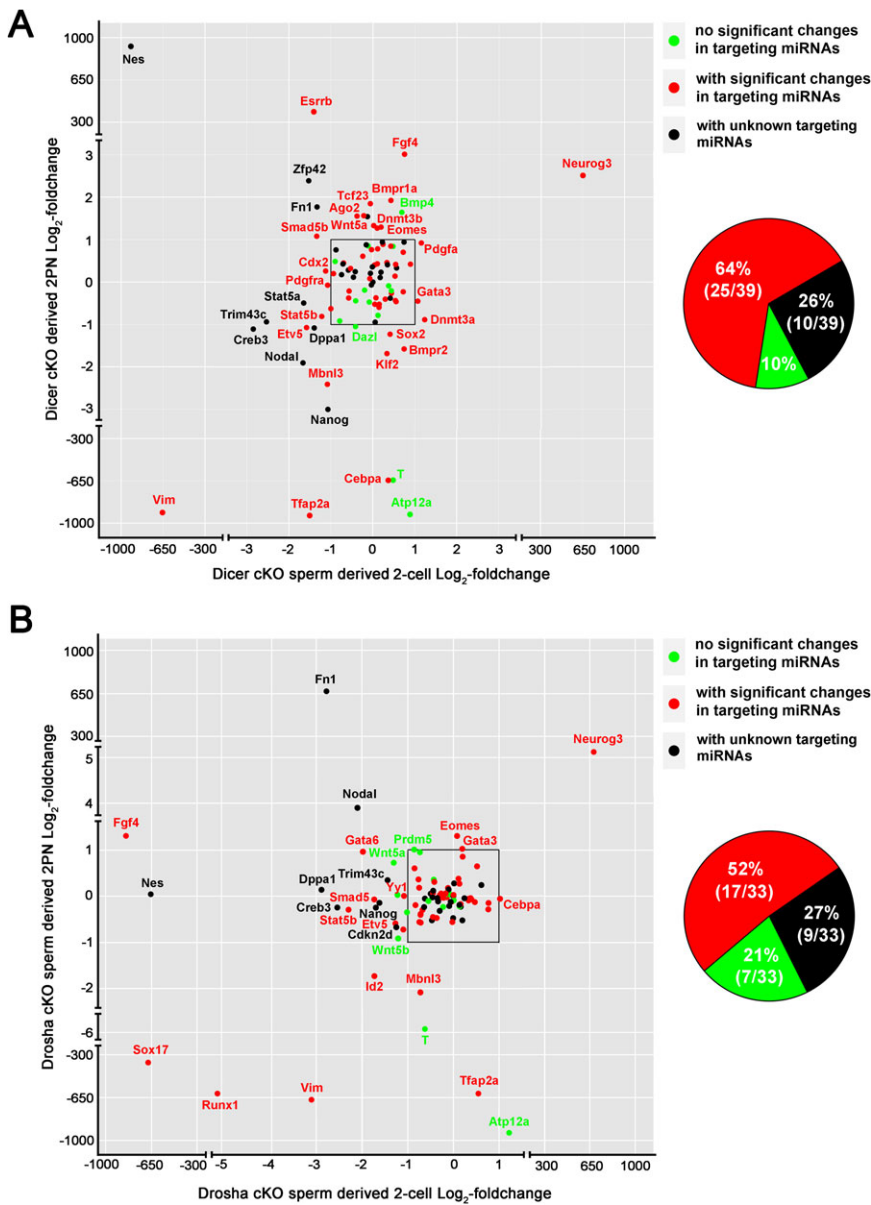
an initial lack of sperm-borne sncRNAs could affect histone modifications during the 2PN to 2-cell transition, we examined the expression patterns of H3K4me2, H3K4me3 and H3K9me3 in 2PN and 2-cell embryos derived from ICSI using Dicer and Drosha cKO spermatozoa. At the 2PN stage, H3K4me2 was expressed 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 discernible 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 discernible 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)  $\text{Log}_2$  fold-changes of 96 genes analyzed in *Dicer* cKO sperm-derived 2PN and 2-cell embryos. Genes targeted by significantly altered miRNAs (fold changes  $\geq 2$ ,  $P_{\text{adj}} < 0.05$ ) 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)  $\text{Log}_2$  fold-changes of 96 genes analyzed in *Drosha* cKO sperm-derived 2PN and 2-cell embryos. Genes targeted by significantly altered miRNAs (fold changes  $\geq 2$ ,  $P_{\text{adj}} < 0.05$ ) 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 Droscha 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 Droscha 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 Droscha cKO sperm are mainly deficient in sncRNAs. This is also supported by the fact that Droscha 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 Droscha 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 Droscha cKO sperm are direct targets of miRNAs deficient or dysregulated in Dicer or Droscha 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 NaHCO<sub>3</sub>, 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 Droscha 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<sup>lox/del</sup>* (called Dicer cKO), *Stra8-iCre;Droscha<sup>lox/del</sup>* (called Droscha cKO) male mice and *Zp3-iCre;Droscha<sup>lox/del</sup>* female mice were generated as our previously reported (Wu et al., 2012; Yuan et al., 2014).

### Oocyte preparation and ICSI

WT and *Zp3-iCre;Droscha<sup>lox/del</sup>* 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% CO<sub>2</sub> 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<sup>-6</sup> 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% CO<sub>2</sub> 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% CO<sub>2</sub> 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 Droscha 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 Droscha 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, Droscha cKO) were prepared. For low-input sncRNA-Seq, 20-30 WT oocytes or 2PN embryos derived from ICSI using WT, Dicer cKO and Droscha cKO spermatozoa (biological duplicates for each pooled sample) were collected followed by lysis in a buffer containing 1% Sarkosyl, 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.  $P \leq 0.05$  was considered significant and  $P \leq 0.01$  was considered highly significant. ICSI data were analyzed using  $\chi^2$  tests, compared with the WT group and  $P \leq 0.05$  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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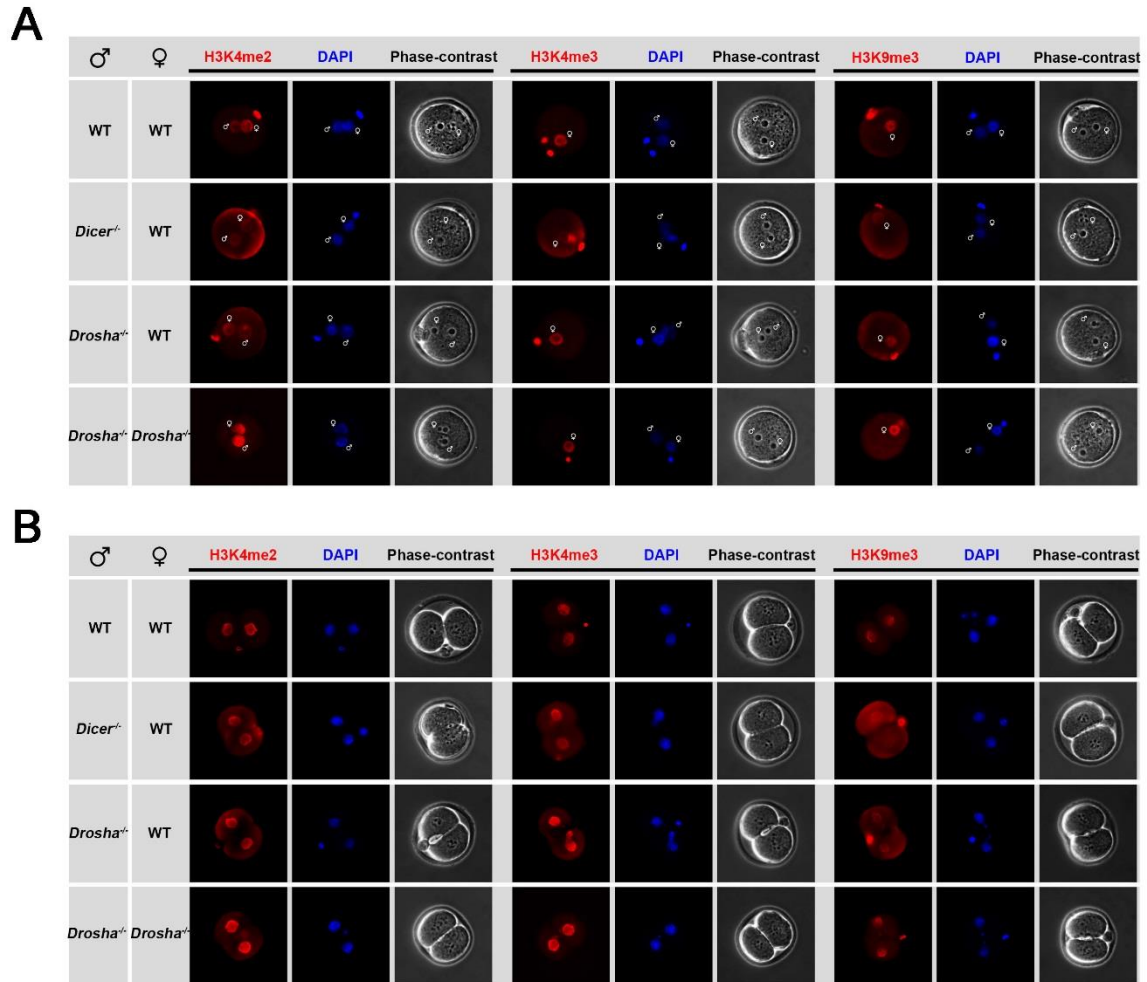
### Supplementary information

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

### References

- Amanai, M., Brahmajosyula, M. and Perry, A. C. F. (2006). A restricted role for sperm-borne microRNAs in mammalian fertilization. *Biol. Reprod.* **75**, 877-884.
- Aravin, A. A., Sachidanandam, R., Girard, A., Fejes-Toth, K. and Hannon, G. J. (2007). Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science* **316**, 744-747.
- Berezikov, E., Guryev, V., van de Belt, J., Wienholds, E., Plasterk, R. H. A. and Cuppen, E. (2005). Phylogenetic shadowing and computational identification of human microRNA genes. *Cell* **120**, 21-24.
- Chatot, C. L., Lewis, J. L., Torres, I. and Ziomek, C. A. (1990). Development of 1-cell embryos from different strains of mice in CZB medium. *Biol. Reprod.* **42**, 432-440.
- Dixon, R. E., Britton, F. C., Baker, S. A., Hennig, G. W., Rollings, C. M., Sanders, K. M. and Ward, S. M. (2011). Electrical slow waves in the mouse oviduct are dependent on extracellular and intracellular calcium sources. *Am. J. Physiol. Cell Physiol.* **301**, C1458-C1469.
- Dixon, R. E., Hennig, G. W., Baker, S. A., Britton, F. C., Harfe, B. D., Rock, J. R., Sanders, K. M. and Ward, S. M. (2012). Electrical slow waves in the mouse oviduct are dependent upon a calcium activated chloride conductance encoded by Tmem16a. *Biol. Reprod.* **86**, 1-7.
- Gannon, J. R., Emery, B. R., Jenkins, T. G. and Carrell, D. T. (2014). The sperm epigenome: implications for the embryo. *Adv. Exp. Med. Biol.* **791**, 53-66.
- Ghildiyal, M. and Zamore, P. D. (2009). Small silencing RNAs: an expanding universe. *Nat. Rev. Genet.* **10**, 94-108.
- Grav, J. (1996). Genetic aspects of embryonic eye development in vertebrates. *Dev. Genet.* **18**, 181-197.
- Grivna, S. T., Beyret, E., Wang, Z. and Lin, H. (2006). A novel class of small RNAs in mouse spermatogenic cells. *Genes Dev.* **20**, 1709-1714.
- Guo, G., Huss, M., Tong, G. Q., Wang, C., Li Sun, L., Clarke, N. D. and Robson, P. (2010). Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst. *Dev. Cell* **18**, 675-685.
- Haglund, K., Nezis, I. P. and Stenmark, H. (2011). Structure and functions of stable intercellular bridges formed by incomplete cytokinesis during development. *Commun. Integr. Biol.* **4**, 1-9.
- Hermo, L., Pelletier, R.-M., Cyr, D. G. and Smith, C. E. (2010). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 4: intercellular bridges, mitochondria, nuclear envelope, apoptosis, ubiquitination, membrane/voltage-gated channels, methylation/acetylation, and transcription factors. *Microsc. Res. Tech.* **73**, 364-408.
- Hong, X., Luense, L. J., McGinnis, L. K., Nothnick, W. B. and Christenson, L. K. (2008). Dicer1 is essential for female fertility and normal development of the female reproductive system. *Endocrinology* **149**, 6207-6212.
- Iguchi, N., Tobias, J. W. and Hecht, N. B. (2006). Expression profiling reveals meiotic male germ cell mRNAs that are translationally up- and down-regulated. *Proc. Natl. Acad. Sci. USA* **103**, 7712-7717.
- Jenkins, T. G. and Carrell, D. T. (2012). The sperm epigenome and potential implications for the developing embryo. *Reproduction* **143**, 727-734.
- Jenuwein, T. and Allis, C. D. (2001). Translating the histone code. *Science* **293**, 1074-1080.
- Kawano, M., Kawaji, H., Grandjean, V., Kiani, J. and Rassoulzadegan, M. (2012). Novel small noncoding RNAs in mouse spermatzoa, zygotes and early embryos. *PLoS ONE* **7**, e44542.
- Kim, V. N., Han, J. and Siomi, M. C. (2009). Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.* **10**, 126-139.
- Kimura, Y. and Yanagimachi, R. (1995). Intracytoplasmic sperm injection in the mouse. *Biol. Reprod.* **52**, 709-720.
- Krawetz, S. A. (2005). Paternal contribution: new insights and future challenges. *Nat. Rev. Genet.* **6**, 633-642.
- Lee, Y., Han, J., Yeom, K.-H., Jin, H. and Kim, V. N. (2006). Drosha in primary microRNA processing. *Cold Spring Harb. Symp. Quant. Biol.* **71**, 51-57.
- Leese, H. J., Tay, J. I., Reischl, J. and Downing, S. J. (2001). Formation of Fallopian tubal fluid: role of a neglected epithelium. *Reproduction* **121**, 339-346.
- Levy, R. (2001). Genetic regulation of preimplantation embryo survival. *Int. Rev. Cytol.* **210**, 1-37.
- Lewis, B. P., Burge, C. B. and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**, 15-20.
- Liu, W.-M., Pang, R. T. K., Chiu, P. C. N., Wong, B. P. C., Lao, K., Lee, K.-F. and Yeung, W. S. B. (2012). Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proc. Natl. Acad. Sci. USA* **109**, 490-494.
- Ma, J., Flehr, M., Stein, P., Berninger, P., Malik, R., Zavan, M., Svoboda, P. and Schultz, R. M. (2010). MicroRNA activity is suppressed in mouse oocytes. *Curr. Biol.* **20**, 265-270.
- Maruyama, R., Suzuki, H., Yamamoto, E., Imai, K. and Shinomura, Y. (2012). Emerging links between epigenetic alterations and dysregulation of noncoding RNAs in cancer. *Tumour Biol.* **33**, 277-285.
- McGinnis, L. K., Luense, L. J. and Christenson, L. K. (2015). MicroRNA in ovarian biology and disease. *Cold Spring Harb. Perspect. Med.* **5**, a022962.
- Messerschmidt, D. M., Knowles, B. B. and Solter, D. (2014). DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. *Genes Dev.* **28**, 812-828.
- Murchison, E. P., Stein, P., Xuan, Z., Pan, H., Zhang, M. Q., Schultz, R. M. and Hannon, G. J. (2007). Critical roles for Dicer in the female germline. *Genes Dev.* **21**, 682-693.
- Nagy, A., Gertsenstein, M., Vintersten, K. and Behringer, R. (2003). *Manipulating the Mouse Embryo*, 3rd edn. NY: Cold Spring Harbor Laboratory Press.
- Ohnishi, Y., Totoki, Y., Toyoda, A., Watanabe, T., Yamamoto, Y., Tokunaga, K., Sakaki, Y., Sasaki, H. and Hohjoh, H. (2010). Small RNA class transition from siRNA/piRNA to miRNA during pre-implantation mouse development. *Nucleic Acids Res.* **38**, 5141-5151.
- Pek, J. W., Anand, A. and Kai, T. (2012). Tudor domain proteins in development. *Development* **139**, 2255-2266.
- Peng, J. C. and Lin, H. (2013). Beyond transposons: the epigenetic and somatic functions of the Piwi-piRNA mechanism. *Curr. Opin. Cell Biol.* **25**, 190-194.
- Peng, H., Shi, J., Zhang, Y., Zhang, H., Liao, S., Li, W., Lei, L., Han, C., Ning, L., Cao, Y. et al. (2012). A novel class of tRNA-derived small RNAs extremely enriched in mature mouse sperm. *Cell Res.* **22**, 1609-1612.
- Pessot, C. A., Brito, M., Figueroa, J., Concha, I. I., Yanez, A. and Burzio, L. O. (1989). Presence of RNA in the sperm nucleus. *Biochem. Biophys. Res. Commun.* **158**, 272-278.
- Sato, F., Tsuchiya, S., Meltzer, S. J. and Shimizu, K. (2011). MicroRNAs and epigenetics. *FEBS J.* **278**, 1598-1609.
- Schuster, A., Henning, G. W., Ortogero, N., Luong, D. and Yan, W. (2015). In silico identification of novel endo-siRNA. *Methods Mol. Biol.* **1218**, 341-351.
- Sirotkin, A. V. (2012). Application of RNA interference for the control of female reproductive functions. *Curr. Pharm. Des.* **18**, 325-336.
- Sone, Y., Ito, M., Shirakawa, H., Shikano, T., Takeuchi, H., Kinoshita, K. and Miyazaki, S. (2005). Nuclear translocation of phospholipase C-zeta, an egg-activating factor, during early embryonic development. *Biochem. Biophys. Res. Commun.* **330**, 690-694.
- Song, R., Hennig, G. W., Wu, Q., Jose, C., Zheng, H. and Yan, W. (2011). Male germ cells express abundant endogenous siRNAs. *Proc. Natl. Acad. Sci. USA* **108**, 13159-13164.
- Stewart, C. A. and Behringer, R. R. (2012). Mouse oviduct development. *Results Problems Cell Differ.* **55**, 247-262.
- Suh, N. and Belloch, R. (2011). Small RNAs in early mammalian development: from gametes to gastrulation. *Development* **138**, 1653-1661.
- Suh, N., Baehner, L., Moltzahn, F., Melton, C., Shenoy, A., Chen, J. and Belloch, R. (2010). MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Curr. Biol.* **20**, 271-277.
- Talbert, P. B. and Henikoff, S. (2010). Histone variants—ancient wrap artists of the epigenome. *Nat. Rev. Mol. Cell Biol.* **11**, 264-275.
- Ventela, S., Toppari, J. and Parvinen, M. (2003). Intercellular organelle traffic through cytoplasmic bridges in early spermatids of the rat: mechanisms of haploid gene product sharing. *Mol. Biol. Cell* **14**, 2768-2780.
- Viswanathan, S. R., Mermel, C. H., Lu, J., Lu, C.-W., Golub, T. R. and Daley, G. Q. (2009). microRNA expression during trophoblast specification. *PLoS ONE* **4**, e6143.
- Wu, Q., Song, R., Ortogero, N., Zheng, H., Evanoff, R., Small, C. L., Griswold, M. D., Namekawa, S. H., Royo, H., Turner, J. M. et al. (2012). The RNase III enzyme DROSHA is essential for microRNA production and spermatogenesis. *J. Biol. Chem.* **287**, 25173-25190.
- Wu, J., Bao, J., Kim, M., Yuan, S., Tang, C., Zheng, H., Mastick, G. S., Xu, C. and Yan, W. (2014). Two miRNA clusters, miR-34b/c and miR-449, are essential for normal brain development, motile ciliogenesis, and spermatogenesis. *Proc. Natl. Acad. Sci. USA* **111**, E2851-E2857.
- Yan, W., Morozumi, K., Zhang, J., Ro, S., Park, C. and Yanagimachi, R. (2008). Birth of mice after intracytoplasmic injection of single purified sperm nuclei and detection of messenger RNAs and MicroRNAs in the sperm nuclei. *Biol. Reprod.* **78**, 896-902.
- Yanagimachi, R., Wakayama, T., Kishikawa, H., Fimia, G. M., Monaco, L. and Sassone-Corsi, P. (2004). Production of fertile offspring from genetically infertile male mice. *Proc. Natl. Acad. Sci. USA* **101**, 1691-1695.
- Yang, J.-S. and Lai, E. C. (2011). Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. *Mol. Cell* **43**, 892-903.
- Yang, Y., Bai, W., Zhang, L., Yin, G., Wang, X., Wang, J., Zhao, H., Han, Y. and Yao, Y.-Q. (2008). Determination of microRNAs in mouse preimplantation embryos by microarray. *Dev. Dyn.* **237**, 2315-2327.
- Yuan, S., Ortogero, N., Wu, Q., Zheng, H. and Yan, W. (2014). Murine Follicular Development Requires Oocyte DICER, but Not DROSHA. *Biol. Reprod.* **91**, 39.

- Yuan, S., Tang, C., Zhang, Y., Wu, J., Bao, J., Zheng, H., Xu, C. and Yan, W.** (2015). mir-34b/c and mir-449a/b/c are required for spermatogenesis, but not for the first cleavage division in mice. *Biol. Open* **4**, 212-223.
- Zheng, P. and Dean, J.** (2007). Oocyte-specific genes affect folliculogenesis, fertilization, and early development. *Semin. Reprod. Med.* **25**, 243-251.
- Zimmermann, C., Romero, Y., Warnefors, M., Bilican, A., Borel, C., Smith, L. B., Kotaja, N., Kaessmann, H. and Nef, S.** (2014). Germ cell-specific targeting of DICER or DGCR8 reveals a novel role for endo-siRNAs in the progression of mammalian spermatogenesis and male fertility. *PLoS ONE* **9**, e107023.



**Fig. S1.** Immunohistochemical analyses of H3K4me2, H3K4me3, and H3K9me3 in 2PN and 2-cell embryos derived from ICSI using WT, *Dicer*, and *Drosha* cKO sperm. (A) H3K4me2, H3K4me3 and H3K9me3 immunostaining patterns in 2PN embryos. (B) Immunostaining of H3K4me2, H3K4me3 and H3K9me3 in 2-cell embryos. Four types of ICSI were analyzed, including WT sperm + WT oocyte, *Dicer* cKO sperm + WT oocyte, *Drosha* cKO sperm + WT oocyte, and *Drosha* cKO sperm + *Drosha* cKO oocyte.

**Table S1. miRNAs that are up-regulated in Dicer cKO sperm, as determined by sncRNA-Seq.** miRNAs with a  $\log_2$  expression (relative to the WT control) value above 1 and adjusted p-values ( $p_{\text{adj}}$ )  $<0.05$  were considered to be significantly up-regulated. Both the  $\log_2$  expression and  $p_{\text{adj}}$  values were determined by DESeq2.

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**Table S2. miRNAs that are down-regulated in Dicer cKO sperm, as determined by sncRNA-Seq.** miRNAs with a  $\log_2$  expression (relative to the WT control) value between -1 and -3.322 (i.e. a fold change of 10), and adjusted p-values ( $p_{\text{adj}}$ )  $<0.05$  were considered to be significantly down-regulated. Both the  $\log_2$  expression and  $p_{\text{adj}}$  values were determined by DESeq2.

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**Table S3. miRNAs that are absent in Dicer cKO sperm, as determined by sncRNA-Seq.** Significantly down-regulated miRNAs with a fold change greater than 10 ( $\log_2$  value  $< -3.322$ ) were considered as absent miRNAs. Significantly down-regulated miRNA are defined as miRNA with a  $\log_2$  expression (relative to the WT control) below -1 and adjusted p-values ( $p_{\text{adj}}$ ) less than 0.05 (both determined by DESeq2).

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**Table S4. miRNA with no significant changes in Dicer cKO sperm, as determined by sncRNA-Seq.** miRNAs that did not have both a  $\log_2$  expression (relative to the WT control) value above 1 or below -1, and adjusted p-values ( $p_{\text{adj}}$ )  $< 0.05$  were not considered to be significantly changed in the Dicer-cKO sperm. Both the  $\log_2$  expression and  $p_{\text{adj}}$  values were determined by DESeq2.

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**Table S5. miRNAs that are up-regulated in Drosha cKO sperm, as determined by sncRNA-Seq.** miRNAs with a  $\log_2$  expression (relative to the WT control) value above 1 and adjusted p-values ( $p_{\text{adj}}$ )  $< 0.05$  were considered to be significantly up-regulated. Both the  $\log_2$  expression and  $p_{\text{adj}}$  values were determined by DESeq2.

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**Table S6. miRNAs that are down-regulated in Drosha cKO sperm, as determined by sncRNA-Seq.** miRNAs with a  $\log_2$  expression (relative to the WT control) value between -1 and -3.322 (i.e., a fold change of 10), and adjusted p-values ( $p_{\text{adj}}$ )  $< 0.05$  were considered to be significantly down-regulated. Both the  $\log_2$  expression and  $p_{\text{adj}}$  values were determined by DESeq2.

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**Table S7. miRNA that are absent in Drosha cKO sperm, as determined by sncRNA-Seq.**

Significantly down-regulated miRNAs with a fold change greater than 10 ( $\log_2$  value  $< -3.322$ ) were considered as absent miRNAs. Significantly down-regulated miRNAs are defined as miRNA with a  $\log_2$  expression (relative to the WT control) below -1 and adjusted p-values ( $p_{\text{adj}}$ )  $< 0.05$  (both determined by DESeq2).

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**Table S8. miRNA with no significant changes in Drosha cKO sperm, as determined by sncRNA-Seq.**

miRNAs that did not have both a  $\log_2$  expression (relative to the WT control) value above 1 or below -1, and adjusted p-values ( $p_{\text{adj}}$ )  $< 0.05$  were not considered to be significantly changed in the Drosha-cKO sperm. Both the  $\log_2$  expression and  $p_{\text{adj}}$  values were determined by DESeq2.

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**Table S9. Novel sperm-borne endo-siRNAs and their putative targets.** A. The nucleotide sequences of novel endo-siRNAs identified from sncRNA-Seq analyses. B. Putative targets for each novel sperm endo-siRNA. The list of transcripts used in this study was comprised of cDNA from ENSEMBL (release 75; downloaded July 18<sup>th</sup>, 2014), refMrna from UCSC Genome Browser (mm10; downloaded July 18<sup>th</sup>, 2014), and repetitive elements from Repbase (v19.06; downloaded July 18<sup>th</sup>, 2014).

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**Table S10. endo-siRNA expression in Dicer and Drosha cKO sperm, as determined by sncRNA-Seq.** endo-siRNA expression levels (relative to the WT control) are provided as  $\log_2$  values. endo-siRNA that had  $\log_2$  values above 1 or below -1, and adjusted p-values ( $p_{\text{adj}}$ ) < 0.05, were considered significantly up or down-regulated, respectively. Significantly down-regulated endo-siRNA with a fold change greater than 10 ( $\log_2$  value < -3.322) were considered as absent endo-siRNA. Absent and up-regulated endo-siRNA are highlighted in yellow and red, respectively. Both the  $\log_2$  and  $p_{\text{adj}}$  values were determined using DESeq2. endo-siRNAs with less than 1 normalized read in at least one sample type were removed prior to DESeq2 analysis, and are not shown above.

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**Table S11. Preimplantation development of embryos derived from ICSI using Drosha cKO oocytes and Drosha cKO spermatozoa with or without sperm RNA supplementation.**

Injected content	Total No. of surviving oocytes (no. of experiments)	No. of oocytes with 2PN (% of $\frac{2PN}{\text{Total}}$ )	No. of 2-cell embryos (% of $\frac{2\text{-cell}}{2PN}$ )	No. of 4-cell embryos (% of $\frac{4\text{-cell}}{2\text{-cell}}$ )	No. of morula (% of $\frac{\text{morula}}{2\text{-cell}}$ )	No. of Blastocyst (% of $\frac{\text{Blastocyst}}{2\text{-cell}}$ )
Drosha cKO	142 (9)	100 (70.42)	72 (72.00)	24 (33.33)	16 (22.22)	9 (12.50)
Drosha cKO + Small RNAs	106 (6)	81 (76.42)	48 (59.25)	26 (54.17)*	19 (39.58)*	9 (18.75)
Drosha cKO + Total RNAs	111 (6)	91 (81.98)*	59 (64.84)	28 (47.45)	16 (28.81)	9 (15.25)

Note: exp., experiment; Statistic analyses were conducted using  $\chi^2$  test, \*  $p < 0.05$ , \*\*  $p < 0.01$ , as compared to Drosha cKO sperm.

**Table S12. sncRNA-Seq data on miRNA expression in WT oocytes and 2PN embryos derived from ICSI using WT, Dicer cKO and Drosha cKO sperm.**

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**Table S13. High throughput real-time quantitative PCR (HT-qPCR) data on expression levels of 96 genes in single oocytes and preimplantation embryos at 2PN, 2-cell, 4-cell, morula and blastocyst stages, which were derived from ICSI using WT, Dicer and Drosha cKO sperm.**

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**Table S14. Fold changes of dysregulated genes (highlighted in red, green or grey, 39 out of 96) in Dicer cKO sperm-derived 2PN and 2-cell embryos and fold changes of miRNAs that target these dysregulated genes in Dicer cKO sperm.**

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**Table S15. Fold changes of dysregulated genes (highlighted in red, green or grey, 33 out of 96) in Droscha cKO sperm-derived 2PN and 2-cell embryos and fold changes of miRNAs that target these dysregulated genes in Droscha cKO sperm.**

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**Table S16. Sequences of primers used for high throughput qPCR analyses.**

Gene symbol	Forward (5' → 3')	Reverse (5' → 3')
<i>Actb</i>	GACGGCCAGGTCATCACTAT	ATGCCACAGGATTCCATACC
<i>Ahcy</i>	CCCTACAAAGTCGCGGACATC	GAGGCTGAGTACATCTCCCG
<i>Aqp3</i>	GCTTTTGGCTTCGCTGTCAC	TAGATGGGCAGCTTGATCCAG
<i>Atp12a</i>	ATGCGCCGGAAAACAGAAATC	CCTCCTCCTGACTCTTGTTGG
<i>Axin1</i>	CTCCAAGCAGAGGACAAAATCA	GGATGGGTTCCCCACAGAAATA
<i>Bmp4</i>	AGGAGGAGGAGGAAGAGCAG	CCTGGGATGTTCTCCAGATG
<i>Bmpr1a</i>	CTGTACATAAGGCATTGACAGG	TAATGGTAAACTTCATAAGGCACC
<i>Bmpr2</i>	CTGCTTTAACCTCCTGTCAGC	GGAGACCACTTTGGATAAGCAC
<i>Cdc25c</i>	CCCTGAATCTCCGAAAGACA	CGAAAGGTCAAGGCAACATT
<i>Cdkn2d</i>	CTTCTTCACCGGGAGCTG	TGCTGGACTTCCAAACATCA
<i>Cdx2</i>	TCACCATCAGGAGGAAAAGTG	CTGCGGTTCTGAAACCAAT
<i>Cebpa</i>	CAAGAACAGCAACGAGTACCG	GTCCTGGTCAACTCCAGCAC
<i>Creb3</i>	AAGGCTCCGCTGGACTTAGA	TGTGGAAGGGAGTAGTTGTGA
<i>Creb3l2</i>	CATGTACCACACGCACTTCTC	CCACCTCCATTGACTCGCTC

<i>Dazl</i>	ATACCTCCGGCTTATACAAGTGT	GACTTCTTTTGC GGCCATTT
<i>Dicer1</i>	GAGCTACAGGATCGCCAAGTC	GTGGAGCTGTGGTTCTGGTC
<i>Dnmt1</i>	GCCAGTTGTGTGACTTGGAA	AGTGTGTGTTCCGTTCTCCA
<i>Dnmt3a</i>	GACTCGCGTGCAATAACCTTAG	GGTCACTTTCCCTCACTCTGG
<i>Dnmt3b</i>	CTCGCAAGGTGTGGGCTTTTGTAAC	CTGGGCATCTGTCATCTTTGCACC
<i>Dppa1</i>	ATGATGTCCCTTCAAGTCCTCA	TGTGTTGGGATCACTTCAGTGT
<i>Dppa2</i>	TCAACGAGAACCAATCTGAGGA	GCGTAGCGTAGTCTGTGTTTG
<i>Dppa3</i>	AGGCTCGAAGGAAATGAGTTTG	TCCTAATTCTTCCCATTTCG
<i>Dppa5a</i>	AAGGAGTGCTGAAGCTGGAGG	CAGCTTAACCTGCATCCAGGTC
<i>Drosha</i>	GGACCATCACGAAGGACACT	CACGGGTCTCTTGTTTTGT
<i>Eed</i>	AAGGGCACATAGAGCATTAGAC	CGAGCAGGAAGACAGTACAAAG
<i>Ehmt2</i>	CTTCTTCAGCTCCAGGGACATC	GAATGCTTGCACTTCTCAGAGC
<i>Ago2</i>	TGCTAGGTCTCCACAGTGTCTC	CAATCTCTCGGACTTGCTACTG
<i>Elavl2</i>	ACACAGCCAATGGTCCAACC	TTCCCGGAGTCAACTGGTGA
<i>Eomes</i>	CCCTATGGCTCAAATTCCAC	TGGGGTTGAGTCCGTTTATG
<i>Eras</i>	GTAGCTGTGGCTGCTCTGTAG	GATGTCTGTGGTAACTTGGTCCG
<i>Esrrb</i>	GGCGTTCTTCAAGAGAACCA	CCCCTTTGAGGCATTTTTCAT
<i>Etv5</i>	TCAGTCTGATAACTTGGTGCTTC	GGCTTCCTATCGTAGGCACAA
<i>Ezh2</i>	TCAGGAACCTTGAGTACTGTGG	CTTTGCAGCTGGTGAGAAGGC
<i>Fgf4</i>	CGAGGGACAGTCTTCTGGAG	TCGGTAAAGAAAGGCACACC
<i>Fgfr2</i>	AATCTCCCAACCAGAAGCGTA	CTCCCAATAAGCACTGTCTT
<i>Fn1</i>	GGAGGGAAAACAGAGCTTGA	GGGTCTACTCCACCGAACAA
<i>Foxh1</i>	GACCTGCTCTGTGATCTAGAC	ATGCTGTACCAGGAAAGGAGC

<i>Gapdh</i>	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT
<i>Gata3</i>	CTTATCAAGCCCAAGCGAAG	CATTAGCGTTCCTCCTCCAG
<i>Gata4</i>	CTACCTGTGCAATGCCTGTG	GGTGGTGGTAGTCTGGCAGT
<i>Gata6</i>	TTGCTCCGTAACAGCAGTG	GTGGTCGCTTGTGTAGAAGGA
<i>Grhl1</i>	CAGTTCAGCTCTGGCACTCA	GAGGGGAAGAAAACCTCCTG
<i>Grhl2</i>	AAGCCCAGTGCAACAACCTCC	TGTCCGGTCCTCTGTAGGTTT
<i>Hand1</i>	TCAAAAAGACGGATGGTGGT	GCGCCCTTTAATCCTCTTCT
<i>Hnf4a</i>	CACGCGGAGGTCAAGCTAC	CCCAGAGATGGGAGAGGTGAT
<i>Hprt</i>	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
<i>Id2</i>	CCAGAGACCTGGACAGAACC	ATTCAGATGCCTGCAAGGAC
<i>Ifitm3</i>	TGGTCCTCAGCATCCTGATGG	AGGGTGAAGCACTTCAGGACC
<i>Irf8</i>	CATGCAAACAGAATCCTTGAG	GAACAGGTTGTCCTGGCTTCG
<i>Klf2</i>	CTCAGCGAGCCTATCTTGCC	CACGTTGTTTAGGTCCTCATCC
<i>Klf4</i>	GCGAGTCTGACATGGCTGT	AGAGAGTTCCTCACGCCAAC
<i>Klf5</i>	CTCCGGAGACGATCTGAAAC	TCTTGTCTGGACCAGCTCCT
<i>Krt8</i>	GCACTCAGGAGAAGGAGCAG	ATGTTGCTCCTCGACGTCTT
<i>Lcp1</i>	TCCGTGTCTGACGAAGAAATG	GCGGCCTTGAACAAGTCAT
<i>Mbnl3</i>	GTCCGGTTCTGATTTCTGGA	CTGAAATTCACGGCAAACCT
<i>Ybx2</i>	CTTCTATCGAAGGCGGTTTG	GCTGTTGATCACCTCCAAT
<i>Nanog</i>	ATGCCTGCAGTTTTTCATCC	GAGGCAGGTCTTCAGAGGAA
<i>Nes</i>	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA
<i>Neurog3</i>	AGTGCTCAGTTCCAATCCAC	CGGCTTCTTCGCTTTTTGCTG
<i>Nodal</i>	AGCCACTGTCCAGTTCTCCAG	GTGTCTGCCAAGCATAACATCTC

<i>Pdgfa</i>	TGTGCCCATTCGCAGGAAG	GAGGTATCTCGTAAATGACCGTC
<i>Pdgfra</i>	TCCATGCTAGACTCAGAAGTCA	TCCCGGTGGACACAATTTTTC
<i>Pecam1</i>	ACGCTGGTGCTCTATGCAAG	TCAGTTGCTGCCCATTCATCA
<i>Pou5f1</i>	AGAGGATCACCTTGGGGTACA	CGAAGCGACAGATGGTGGTC
<i>Prdm1</i>	AGCATGACCTGACATTGACACC	CTCAAACTCTCATGTAAGAGGC
<i>Prdm4</i>	ACTCCCTGTGAGCAGGCACAC	CAGCAGAGTCCTCTACTCAAGCC
<i>Prdm5</i>	GGCAGAGTGCCATTTCTGCCATAAG	CACATGGTGTGGTTCCTTGCCG
<i>Runx1</i>	GCCATGAAGAACCAGGTAGC	GACGGTGATGGTCAGAGTGA
<i>Sall1</i>	CTCAACATTTCCAATCCGACCC	GGCATCCTTGCTCTTAGTGGG
<i>Sall4</i>	AGTGTCACCTGCCAATAGCC	GGCTGTGCTCGGATAAATGT
<i>Smad1</i>	CTTCTGTTTCGCAAATCAACTGG	AATGAACAGAGTTACCAGGTTTGG
<i>Smad3</i>	GTGGACTCTTGTTTAGGAGGC	GGACACGGCTCTTTAACAATGG
<i>Smad4</i>	GTATCTTAGGGCAAGACTGCAGAC	GTCTGTGGTACAGTCAATGTGTC
<i>Smad5</i>	TACAGAGCGAGTGCTTGTGTTC	CATACACAGAAGGAGGCAGACC
<i>Snai1</i>	CACACGCTGCCTTGTGTCT	GGTCAGCAAAGCACGGTT
<i>Sox13</i>	CTCATCAGCCTGGACTCCTC	TCTTGATGTGGCTGCTGTTC
<i>Sox17</i>	CTTTATGGTGTGGGCCAAAG	GCTTCTCTGCCAAGGTCAAC
<i>Sox2</i>	GCGGAGTGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
<i>Stat1</i>	ACCTTACACCCCGAGTGCAG	CTTGGTCATTTCAATTTGGCATGG
<i>Stat2</i>	ATGGCACTGCTTCTTTCTGCTC	ACGAAGGACTTCAGGACAACAG
<i>Stat5a</i>	GGTGAAGGCGACCATCATCAG	GTGGTACTCCATGACGCAACAG
<i>Stat5b</i>	GAAGTGGCTATCTCCTACAACG	GTGGCTGCCTCAATTTATACAC
<i>T</i>	ATCAGAGTCCTTTGCTAGGTAG	GTTACAATCTTCTGGCTATGC

<i>Tcf23</i>	GAGTCGCATCAACAGGACAAG	GTCTTCACCCGAGTCCGTT
<i>Tfap2a</i>	GAAGACTGCGAGCACGTAGAA	GTCCTTGTTAATAGGGATGGCG
<i>Tfap2c</i>	CAGAGGGCTCGAAGGATTC	AGAGATGTTGTCCCGGAGAG
<i>Terf1</i>	GAGACTGGAGGCTGGATG	CTCTTTCTCTCCCCCTCCTC
<i>Tert</i>	GGAGATGAAAAAGGACATCGAG	CTGCTCCAGTAGGAAAATCCTG
<i>Trim43c</i>	AGGCTCAAATCCAGCAGAA	CTTCACTGAGGGGTGGACAT
<i>Tspan8</i>	TCTGGGTATGTGGTACTGAT	AGGGGTTCGTGCTAGAGTCTC
<i>Uchl1</i>	TCACGGCCAGCATGAAAA	ACAGGAGTTTCCGATGGTCTG
<i>Vim</i>	CGGCTGCGAGAGAAATTGC	CCACTTTCCGTTCAAGGTCAAG
<i>Wnt5a</i>	TATCCATTCATGGGCTTGGTAG	TTGCAATTCATTGTAGCTGAGG
<i>Wnt5b</i>	CCTGATAACTGGGATGGTAGCC	GGTTGGAGTGACTTGAGACCAC
<i>Yy1</i>	GCCAGATGCTGATGTTCAAGT	TTGTTGCCCTTTCTGTTACACG
<i>Zfp42</i>	TCCATGGCATAGTTCCAACAG	TAACTGATTTTCTGCCGTATGC



## Supplementary Materials and Methods

### Intracytoplasmic sperm injection (ICSI)

ICSI was carried out as described (Kimura and Yanagimachi, 1995; Stein and Schultz, 2010), with some medications. Briefly, WT and cKO sperm were suspended in 500 $\mu$ l HEPES-CZB medium followed by centrifugation at 700g for 5min. The sperm pellet was resuspended with 200 $\mu$ l NIM/PVA medium (Stein and Schultz, 2010), and the suspension was sonicated four times for 15 second each. After sonication, ~50% of the sperm heads were separate from the tails; an aliquot of 1-2 $\mu$ l sperm suspension was diluted with 50 $\mu$ l NIM/PVA medium. Single sperm head was picked up from the sperm NIM/PVA suspension, and injected it into WT or *Zp3-Cre; Drosha<sup>lox/del</sup>* oocytes using a Piezo drill under the control of an electric micromanipulator (TransferMan NK2, Eppendorf) at room temperature. Injection of ~15 oocytes was completed within 5 min. Injected oocytes were then transferred to KSOM+AA medium covered by mineral oil followed by culture at 37°C in an incubator with humidified atmosphere and 5% CO<sub>2</sub>. Each ICSI process was completed within 2h after oocyte collection.

### Single cell high-throughput qPCR (HT qPCR)

Single oocytes and embryos were harvested individually into tubes with one oocyte or one embryo in each. 5 $\mu$ l CellDirect 2X Reaction Mix lysis buffer (Invitrogen, One-Step qRT-PCR Kit, Grand Island, NY) was added into each tube containing one single oocyte or embryo before snap freezing in liquid nitrogen followed by storage at -80°C. Single cell high-throughput qPCR (HT qPCR) was performed in two steps. The first included reverse transcription and specific target amplification (STA). In brief, total RNA from single MII oocytes and single embryos was reverse-transcribed to cDNAs, and the cDNAs were then pre-amplified for 18~20 cycles using a mixture of 96 pairs of primers designed to amplify 96 genes of interest (Table S16). The pre-amplified amplicons were cleaned up using ExoSAP-IT (Affymetrix) to eliminate unincorporated primers. The second step was the HT qPCR, which was performed in the 96 X 96 chips using the Biomark™ HD system (Fluidigm, South San Francisco, CA), according to the protocols provided by the manufacturer. The final data were analyzed using the Biomark real-time PCR analysis software (Fluidigm, Version 3.1.2). *Gapdh* was used as an internal control for data normalization. A heat-map was generated based on normalized values and the original HT qPCR dataset can be found in Table S13.

### Small noncoding RNA deep sequencing (sncRNA-Seq)

The lysates were processed using the Ion Total RNA-Seq Kit v2 with modifications. In brief, 100% alcohol was used in the purification step to minimize the sample loss, and 30 amplification cycles were used to ensure the capture of low-copy small RNAs. The pre-amplified libraries were then subjected to small RNA enrichment and removal of amplification noise by size selection using a Pippin Prep™ Kit 3010. Barcodes were added using the Ion Xpress RNA-Seq Barcode 1-16 kit (Invitrogen). The final sncRNA cDNA libraries were sequenced on the Ion Proton sequencer (Life Technologies) using the P1 chips in Nevada Genomics Center. In the sperm sncRNA-Seq experiments, one replicate from the WT group and one from the Dicer cKO group were not annotated

due to poor sequencing quality ( $R^2$  values between other replicates  $> 0.1$ ), leaving duplicates for WT and Dicer cKO, and triplicate sequencing data for Drosha cKO samples for annotation.

### **SncRNA-Seq data analysis processing**

The oocyte and 2PN embryo sncRNA-Seq data was annotated as follows: Reads shorter than 15nt were discarded. The remaining reads were matched to known murine sncRNA, consisting of miRNA (miRBase, release 21), tRNA (Genomic tRNA Database, mm10), piRNA (piRNABank), rRNA (ENSEMBL, release 76), snoRNA (ENSEMBL, release 76), snRNA (ENSEMBL, release 76), and mitochondrial RNA (ENSEMBL, release 76) using Sequery (up to 2 mismatches allowed). Unmatched reads were matched to mouse testis (Song et al., 2011) and putative sperm endo-siRNAs with Sequery (0 mismatches allowed). The remaining unmatched reads were aligned to the mouse genome (mm10) via Bowtie (settings `-n 2 -k 3 --best -S --al -q`) (Langmead et al., 2009). Aligned reads were matched to the genomic coordinates of known murine miRNA, tRNA, rRNA, snoRNA, snRNA, and mitochondrial RNA (same databases used previously). Read counts were obtained by in-house Python scripts. Raw reads were normalized to the number of total aligned reads (via Bowtie,  $n = 2$ ) and are provided as reads per million aligned reads (RPM). Genes with fewer than 3 RPM in every oocyte and 2PN sample were not included in subsequent analyses. To compare the miRNA present in the sperm, oocyte, and 2PN data, miRNA that had at least 3RPM were defined as being 'present' in the sample. After applying this cutoff, we determined which miRNA were 'present' in WT 2PN and sperm, but not WT oocyte. The list of WT 2PN and sperm specific miRNA was then compared to the miRNA that were 'present' in the Drosha cKO and Dicer cKO sperm data.

For sperm sncRNA-Seq data, prior to novel endo-siRNA identification, known sncRNAs and novel miRNAs were removed using a previously described method, with slight modifications (Ortogerro N, 2015). Known murine sncRNA populations were acquired from miRBase, the genomic tRNA Data Base, piRNABank, ENSEMBL (rRNA, snoRNA, snRNA, mito-tRNA, mito-rRNA) using the most up-to-date versions at the time of download (6/24/2014) (Sai Lakshmi and Agrawal, 2008; Chan and Lowe, 2009; Flicek et al., 2014; Kozomara and Griffiths-Jones, 2014). Endo-siRNAs from a previous study in testis were also included (Song et al., 2011). Novel miRNA identification was performed using miRDeep\* (An et al., 2013). Novel endo-siRNA analysis was performed using a previously described method (Schuster A, 2015). The murine transcriptome used consisted of sequences from ENSEMBL (cDNA sequences), UCSC (refMrna), and Repbase (repetitive sequences, e.g. retrotransposons), using the most up-to-date versions at the time of download (7/18/2014) (Jurka et al., 2005; Flicek et al., 2014; Karolchik et al., 2014). Endo-siRNA and miRNA expression levels were determined via Sequery (v1.0) using the newly identified sperm endo-siRNA and mature miRNA (miRBase, release 20) sequences as the reference, respectively (Ortogerro et al., 2013; Kozomara and Griffiths-Jones, 2014). Up to 2 mismatches were allowed for miRNA matching; 0 mismatches were allowed for endo-siRNA matching. Raw reads were normalized to account for sequencing depth by dividing each sncRNA read count by the respective library's total number of aligned reads (aligned with Bowtie,  $k = 1$ ,  $n = 2$ ) and multiplying by a factor of a million resulting in reads per million (RPM) values (Langmead et al., 2009). Differential expression was measured by DESeq2 under default parameters, using raw read counts lacking all sncRNA species that had fewer than 3 normalized reads in all sample types (Drosha-cKO, Dicer-cKO, and WT). Differential expression analysis for miRNA and endo-siRNA was performed separately. Significant dysregulation was defined as having an adjusted p-value  $\geq 0.05$  and a  $\log_2$  fold-change  $\geq 1$  (up-regulated) or  $\leq -1$  (down-regulated) (Michael I Love, 2014).

## RNA-Seq raw data analysis

Multiple programs (i.e., Bowtie and Sequery) were used to process our sncRNA-Seq data in order to account for confounding factors unique to sncRNA annotation. Many sncRNA databases (e.g., miRBase) use consensus sequences and oftentimes, slight variations are present in the 5' and 3' boundaries of sncRNA genes. To ensure that we did not identify any previously known sncRNAs as endo-siRNAs in our subsequent analysis, it was critical that we annotated our sncRNA-Seq data as thoroughly as possible. The programs we selected allowed us to use very up-to-date sncRNA references and to incorporate lesser-known sncRNAs (e.g., small nucleolar RNA, snoRNA). The first program we used in our annotation pipeline, Sequery, matches the individual sequences (reads) in the sncRNA-Seq data against the consensus sequences of known mouse sncRNA (reference), supplied by the user. The number of mismatches between the reads and reference sequences that was allowed was two for all the sncRNAs except endo-siRNAs; because endo-siRNAs bind to their targets with complete complementarity, their sequences are not as variable as other sncRNAs (e.g., miRNAs), so zero mismatches were allowed for endo-siRNA-to-read matching. Next, the reads which did not match to any sncRNA sequences in the reference were aligned to the mouse genome using Bowtie. Our decision to use Bowtie instead the newer version, Bowtie2, was based on the former being faster and more sensitive when aligning short reads (>50nt). The genomic coordinates (e.g., Chr1: 1000-1020) for each aligned read were matched against the coordinates of known mouse sncRNA genes, using Python scripts we developed in-house. Therefore, using coordinates allowed us to account for aforementioned boundary variations that are sometimes an issue for sncRNA genes (e.g., with the criteria we used, a read aligned at Chr1:1000-1020 would be matched to a gene at Chr1:1003-1023). By running our sncRNA-Seq data through the two programs, we accounted for sncRNA variations and ensured that the remaining reads did not represent currently known sncRNA.

## Bioinformatic analysis

TargetScan was used for predicting the identified miRNA families to target the 96 genes. Fold changes of the identified miRNAs in Dicer and Drosha cKO sperm were then determined based on our sncRNA-Seq data (Tables S1-S8). For a particular dysregulated mRNA, the absolute values of fold changes of all its targeting miRNAs were summed, and the total fold changes  $\geq 2$  were regarded as miRNAs with significant effects on their target mRNAs.  $\log_2$  fold changes of dysregulated mRNAs at 2PN and 2-cell stages were plotted, and the dysregulated mRNAs with significant changes in their targeting miRNAs in cKO sperm (total fold changes  $\geq 2$ ) were indicated by red fonts. Those dysregulated mRNAs without significant changes in their targeting miRNAs in cKO sperm (total fold changes  $< 2$ ) were indicated by green fonts. The transcripts without known targeting miRNAs in the databases were marked with black font.

## References

- An, J., Lai, J., Lehman, M. L. and Nelson, C. C. (2013) 'miRDeep\*: an integrated application tool for miRNA identification from RNA sequencing data', *Nucleic Acids Res* 41(2): 727-37.
- Chan, P. P. and Lowe, T. M. (2009) 'GtRNAdb: a database of transfer RNA genes detected in genomic sequence', *Nucleic Acids Res* 37(Database issue): D93-7.
- Flicek, P., Amode, M. R., Barrell, D., Beal, K., Billis, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fitzgerald, S. et al. (2014) 'Ensembl 2014', *Nucleic Acids Res* 42(Database issue): D749-55.
- Jurka, J., Kapitonov, V. V., Pavlicek, A., Klonowski, P., Kohany, O. and Walichiewicz, J. (2005) 'Repbase Update, a database of eukaryotic repetitive elements', *Cytogenet Genome Res* 110(1-4): 462-7.
- Karolchik, D., Barber, G. P., Casper, J., Clawson, H., Cline, M. S., Diekhans, M., Dreszer, T. R., Fujita, P. A., Guruvadoo, L., Haeussler, M. et al. (2014) 'The UCSC Genome Browser database: 2014 update', *Nucleic Acids Res* 42(Database issue): D764-70.
- Kimura, Y. and Yanagimachi, R. (1995) 'Intracytoplasmic sperm injection in the mouse', *Biol Reprod* 52(4): 709-20.
- Kozomara, A. and Griffiths-Jones, S. (2014) 'miRBase: annotating high confidence microRNAs using deep sequencing data', *Nucleic Acids Res* 42(D1): D68-D73.
- Langmead, B., Trapnell, C., Pop, M. and Salzberg, S. L. (2009) 'Ultrafast and memory-efficient alignment of short DNA sequences to the human genome', *Genome Biol* 10(3): R25.
- Michael I Love, W. H., Simon Anders (2014) 'Moderated estimation of fold change and dispersion for RNA-Seq data with DESeq2', *BioRxiv*: 1-50.
- Ortoger, N., Hennig, G. W., Langille, C., Ro, S., McCarrey, J. R. and Yan, W. (2013) 'Computer-assisted annotation of murine Sertoli cell small RNA transcriptome', *Biol Reprod* 88(1): 3.
- Ortoger N, H. G., Luong D, Yan W (2015) Computer-assisted annotation of small RNA transcriptomes. in M. Sioud (ed.) *RNA Interference*, vol. 1218: Humana Press.
- Sai Lakshmi, S. and Agrawal, S. (2008) 'piRNABank: a web resource on classified and clustered Piwi-interacting RNAs', *Nucleic Acids Res* 36(Database issue): D173-7.
- Schuster A, H. G., Ortoger N, Luong D, Yan W (2015) In silico identification of novel endo-siRNA. in M. Sioud (ed.) *RNA Interference*, vol. 1218.
- Song, R., Hennig, G. W., Wu, Q., Jose, C., Zheng, H. and Yan, W. (2011) 'Male germ cells express abundant endogenous siRNAs', *Proc Natl Acad Sci U S A* 108(32): 13159-64.
- Stein, P. and Schultz, R. M. (2010) 'ICSI in the mouse', *Methods Enzymol* 476: 251-62.