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Parental noncoding RNA expression dynamics across sperm, oocyte, and zygote

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Abstract

Fertilization ensures the flow of information from the parent to the progeny via fusion of male and female gametes orchestrated by a cascade of events guided by parental noncoding RNA (ncRNA) molecules. These parental ncRNAs play an essential role toward rewiring the germ cell profile to make them compatible for successful fertilization, thereby resulting in the formation of the zygote. They further modulate the zygotic profile to support the postfertilization events. In this work, an in-depth meta-analysis of small and long RNA-seq data corresponding to sperm, oocyte, and one-cell stage zygote of *Mus musculus* has been performed, followed by subsequent wet bench experiments. Our findings provide a comprehensive long noncoding RNA (lncRNA) and microRNA (miRNA) profile contributed by the parental gametes. Further, a set of novel lncRNAs have been identified, which have a potential role toward modulating sperm and oocyte fertility in the pre-fertilization stage. Moreover, a fine-tuned miRNA expression dynamics has been observed between the germ cells and the zygote to increase the competency of the germ cells for fertilization in the pre-fertilization stage. Its subsequent effects have been observed on early embryonic development in the postfertilization stage.

Introduction

Mammalian fertilization is the initial event of our life process characterized by fusion of male and female gametes. Parental noncoding RNA (ncRNA) molecules play a key role toward modulating the cascade of events [1], which leads to the formation of a totipotent zygote [2] that refers to the first stage of a developing mammalian embryo. As the embryo progresses from pre- to postimplantation stage, significant structural and transcriptional changes take place within the embryonic lineage to ensure the success of a series of crucial biological events, such as oocyte activation [3, 4] and maternal-to-zygotic transition coordinated with zygotic genome activation (ZGA) [5, 6], followed by the first cell-fate decision and lineage-specific differentiation, laying the foundation for the subsequent phase of gastrulation.

On the contrary, infertility is a condition of reproductive impairment caused primarily due to duplication and ineffective differentiation of germ cells/parental gametes, both of which are controlled by a set of ncRNAs [7]. ncRNAs play an important role in maintaining cell identity and regulating gene expression in pre- as well as postfertilization stages. Both long ncRNAs (lncRNAs) and small ncRNAs (sncRNAs) like microRNAs (miRNAs) have been found to participate in the regulation of oogenesis and spermatogenesis [8] by interacting with their target mRNAs [9].

lncRNAs are mostly transcribed by RNA polymerase II and are longer than 200 nts in length and can act as molecular bait and scaffolds through molecular guidance [10]. Several studies have reported the contribution of lncRNAs in germ cells at various developmental stages that were identified in mouse testes. A large number of differentially expressed (DE) lncRNAs have also been detected at various developmental stages of germ cells [11–13]. lncRNATsx, for example, was specifically expressed in pachytene spermatocytes and Tsx knockout mice showed decreased testicular weight and spermatocyte apoptosis [10]. Also, recent research on mouse and ovine oocytes revealed that some lncRNAs are expressed particularly in specific stages of oocyte maturation [14, 15]. Ovarian lncRNAs are found to be associated with follicular and luteal phases of the estrous cycle maintaining the fertility of large white sows [16]. Further, dynamic pattern of lncRNA expression has been found to be essential for normal preimplantation embryo development in rabbits [17].

MiRNAs, on the other hand, are small ncRNAs, which control the regulation of mRNAs by targeting them posttranscriptionally. MiRNA family like miR-17–92 is found to regulate

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Table 1. Input dataset and experimental grouping for (A) long RNA sequencing data and (B) small RNA sequencing data

Sample type	Experimental grouping	Accession ID	No. of samples	Sample condition/age/	Strain
A					
Sperm	Test 1	GSE173659	5	Adult male	C57BL/6
		GSE88737	4	3–5 months	C57BL/6
Oocyte	Test 2	GSE53386	4	2 months, Metaphase II Oocyte	CD-1
		GSE86470	3	Full grown oocyte	CBA/CAJxC57Bl6/J F1 hybrid mice
		GSE66582	2	MII Oocyte	C57BL/6N
		E-MTAB-2950	1	12–14-day-old mouse MII oocyte	B6D2F1
Zygote	Control	GSE53386	4	one-cell stage	CD-1
		GSE86470	3	one-cell stage	CBA/CAJxC57Bl6/J F1 hybrid mice
		GSE66582	2	one-cell stage	C57BL/6N x DBA/2N
		E-MTAB-2950	1	one-cell stage	B6D2F1
B					
Sperm	Test 1a	GSE70198 (caput)	2	8 weeks	Swiss
	Test 1b	GSE70198 (corpus)	2	8 weeks	
	Test 1c	GSE70198 (cauda)	2	8 weeks	
Oocyte	Test 2	GSE174504	6	16 weeks	C57BL/6
Zygote	Control	GSE83581	4	one-cell embryo	FVB/N

the self-renewal of spermatogonia stem cells (SSCs) [18]. Current studies also showed that human spermatozoal or seminal plasma miRNAs could act as potential biomarkers of male infertility, e.g. person with asthenospermia (a condition with reduced sperm motility) generally exhibit lower levels of miR-135b and miR-10b [19]. In several species, DE miRNAs between mature and immature oocytes reveals their role during different stages of oocyte development [20–22]. In porcine, it has been observed that miR-10a-5p, miR-10b, and miR-183 are upregulated and miR-27b-3p is downregulated in MII stage when compared to Germinal vesicle (GV) stage of the developing oocyte [23]. In contrast, miR-365-5p, miR-584, and miR-628 are found exclusively in GV oocytes of bovine system [22]. Further, miRNAs are also essential during preimplantation embryo development [24].

All these reveal the importance of lncRNAs and miRNAs in germ cell development that leads to successful fertilization and subsequently toward postfertilization events. But the detailed contribution of the maternal and paternal ncRNA profile toward transferring essential information from pre- to postfertilization state and fine-tuning these events remains to be deciphered. These motivated us to elucidate the general as well as differential parental ncRNA profile within mature fertile sperm and oocyte as well as zygote in mouse system, followed by an in-depth analysis to understand their modes of regulation, which fine-tunes fertilization as well as postfertilization events. An in-depth meta-analysis has been performed to understand the flow of parental information from germ cells to the zygote. Subsequent experiments have been performed to strengthen our findings.

Materials and methods

Data collection for the *in silico* analysis and experimental grouping

All the long and small RNAseq data corresponding to sperm, oocyte, and zygote for *in silico* analysis were obtained from GEO database (https://www.ncbi.nlm.nih.gov/geo/) and are provided in Table 1.

To focus on the gene expression profile that influences mouse pre- and postfertilization events irrespective of strain-specific differences, we have collected the earlier mentioned data from different mouse strains. DE miRNA, DE lncRNA, and DE mRNA profiles in sperm (from caput, corpus, and cauda of the epididymis separately) and oocyte have been obtained with zygote as control group.

Raw data normalization and analysis

Read quality was assessed for each sample (both long and small RNAseq data) using FastQC (http://www.bioinformaticsbabrahamacuk/projects/fastqc), and low-quality reads (quality <35) were removed using Cutadapt 3.4 [25]. The trimmed long RNA reads were mapped to the mouse mm10 reference genome retrieved from Ensembl (https://www.ensembl.org/) using spliced aligner tool Hisat2 2.1.0 [26]. SAMtools 0.1.19 [27] was used to sort and index the BAM files. Transcript counts were generated using FeatureCounts [28], followed by batch correction with ComBat-seq [29] and differential analysis with DESeq2 [30]. DE mRNAs and lncRNAs were screened using a Fold Change (FC) cut off of ≥ 2.0 and ≥ 1.5 [31], respectively, along with false discovery rate (FDR) ≤ 0.05.

For miRNA detection, the trimmed small RNA reads (for both the online and the in-house datasets) were aligned with mature mouse miRNA sequences, retrieved from miRBase Release 22 [32] using Megablast [33]. In-house custom scripts have been used to detect the general profile for each group followed by batch correction with ComBat-seq and differential analysis using DESeq2 (with FC cut off of ≥ 2.0 and FDR < 0.05).

The DE mRNAs were further subjected to Ingenuity Pathway Analysis (IPA) (www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/) for their functional annotation. We subsequently did a rigorous literature survey and consulted the database Panglaodb [34], which hosts single-cell data analysis for different tissues and provides cell type-specific marker information so as to filter the cell type-specific genes.

Table 2. Details of the *in vivo* experiment with Balb/c mice

Group no.	Age (in weeks)	No. of male mice	Weights of male mice (g)	No. of female mice	Weights of female mice (g)
1	4–6 weeks	5	30 ± 5	10	25 ± 5
2		10	25 ± 5	20	20 ± 5
3		5	25 ± 5	10	25 ± 5
4		10	30 ± 5	20	25 ± 10
5		10	25 ± 10	20	20 ± 10
6		5	25 ± 5	10	25 ± 10
7		5	25 ± 10	10	20 ± 10

In each group the male:female mice ratio was restricted to 1:2 ratio. In order to obtain optimum yield of zygotes and oocytes, female mice were kept in two separate cohorts in equal numbers, and one of the cohorts was allowed to mate as per standard mouse breeding guidelines. A part of the sperm, oocyte, and zygote sample obtained from the first two groups of animals (as 2 replicates) were sent for sequencing.

Co-expression analysis of mRNA and lncRNA

Co-expression analysis between mRNA and lncRNA was performed using Pearson's correlation coefficient with a screening criterion of Pearson's correlation coefficient (r) > 0.6 and P ≤ .05 using *rcorr* function of Hmisc (https://cran.r-project.org/web/packages/Hmisc) library in R, to investigate the potential role of lncRNAs in mouse during pre- and postfertilization events.

miRNA target gene prediction

miRNA target genes were comprehensively identified using TargetScan Release 8 [35]. Potential target pairs of 8mer, 7mer-m8, and 7mer-A1 within the 3′ UTR regions [35] of the DE mRNAs were screened. Following miRNA target prediction using TargetScan, we employed ParasoR [36] utilizing a 21-bp sliding window to assess target site accessibility, aiming to strengthen the robustness of the predictions by checking the accessibility of the predicted binding sites. This approach ensured the inclusion of both primary and secondary seed sites of the miRNA, allowing for comprehensive consideration of binding potential while accounting for RNA accessibility influenced by secondary structure.

Animal experiment and ethical declaration

Female and male Balb/c mice, 50 male and 100 female in numbers ∼4–6 weeks old (details provided in Table 2), were obtained from Centre for Translational Animal Research, Bose Institute, Kolkata, India, and were maintained as per the guidelines of the animal ethical committee in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The animal experiments were conducted in accordance with CPCSEA guidelines and all experimental protocols have been approved by the animal ethics committee of Bose Institute (Ref. No. IAEC/BI/008/2021, dated 28th September 2021) registered with the CPCSEA.

To obtain maximum ovulation and enhanced reproductive yield, the female mice in different groups were super ovulated by administering 5 IU pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) obtained from Sigma intraperitoneally in a consecutive manner at an interval of 48 h to attain the maximal yield. This treatment was executed after the females attained an age of 5–6 weeks and weight 20–30 g as per the standard norms for superovulation [37]. Moreover, the time of PMSG and hCG administration was strictly adhered between 1:00 and 2:00 PM for both the consecutive treatments at 48 h intervals for optimum results. Following superovulation, the female mice were kept in two separate cohorts in equal numbers and one of the cohorts was allowed to mate (Supplementary Fig. S1).

After 36–40 h of mating, the male and female mice were sacrificed. The entire epididymis was isolated from the male mice (Fig. 1A). Mouse spermatozoa were collected by directly puncturing cauda epididymis of euthanized mice in sterile 1× Phosphate Buffered Saline (PBS) from Invitrogen. The sample was screened by observing under the microscope. The spermatozoa isolated were visualized under microscope to see if they were healthy, active, and motile. About 90 ± 5% of sperms showed normal morphological features i.e. an intact acrosome with long slender tails that are not coiled or folded. Upon visualization, ∼85 ± 5% of the sperms were found to be motile, showing higher progressive motility. Progressive motility refers to the ability of the sperm to swim in a straight line or large circles, making them more potent to swim up the reproductive tract and fertilize an oocyte and is regarded as a suitable marker for determination of good-quality sperms [38, 39].

To estimate sperm count and assess membrane integrity, samples were stained with 0.2% Trypan Blue [40] and counted using a hemocytometer. To eliminate any potential somatic cell contamination, samples were treated with a somatic cell lysis buffer (composition: 0.1% SDS and 0.5% Triton X-100) for 10 min at 4°C, following established protocols [41, 42]. After lysis, the sperm suspension was washed and re-examined microscopically to confirm the absence of somatic cells (Fig. 1B). These sperm samples were further stored in multiple replicates in QIAzol Lysis Reagent (Cat. 217 004) at −80°C for subsequent RNA isolation and downstream experiments.

Following euthanasia, the oviducts were isolated from the female mice. Mouse oocytes and zygotes were collected by carefully trimming the fat tissue and finely chopping the oviducts in sterile 1× PBS (Fig. 1C). Oocytes (Fig. 1D) and one-cell stage zygotes (Fig. 1E) were carefully differentiated based on their morphological differences and isolated by observing under the microscope. The morphology of oocytes appeared uniformly circular with shiny and smoothened appearance, whereas that of the zygotes appeared somewhat granular and dense. Further, the presence of thick zona pellucida, intact polar body, thin perivitelline space, and healthy cytoplasm indicated the occurrence of healthy oocytes [43]. The zygotes and oocytes were also stored in Qiazol lysis solution in replicates for further experiments.

RNA isolation, library preparation, sequencing, and analysis

RNA isolation and library preparation followed by sequencing were performed by Genotypic Technology Pvt. Ltd., Bangalore, India. Cells were resuspended in 200 µl of TRIzol. Equal volume of 95%–100% ethanol was added and mixed thoroughly. Trizol-ethanol mixture was loaded onto Zymo Spin IC column, and centrifuged at 10 000 × g for 30 seconds. The column was washed with 400 µl of RNA wash buffer. RNA was eluted in 15 µl nuclease-free water (Ambion, Cat#AM9938) and stored at –80°C. For trancriptome, RNA sequencing libraries were prepared with Illumina-

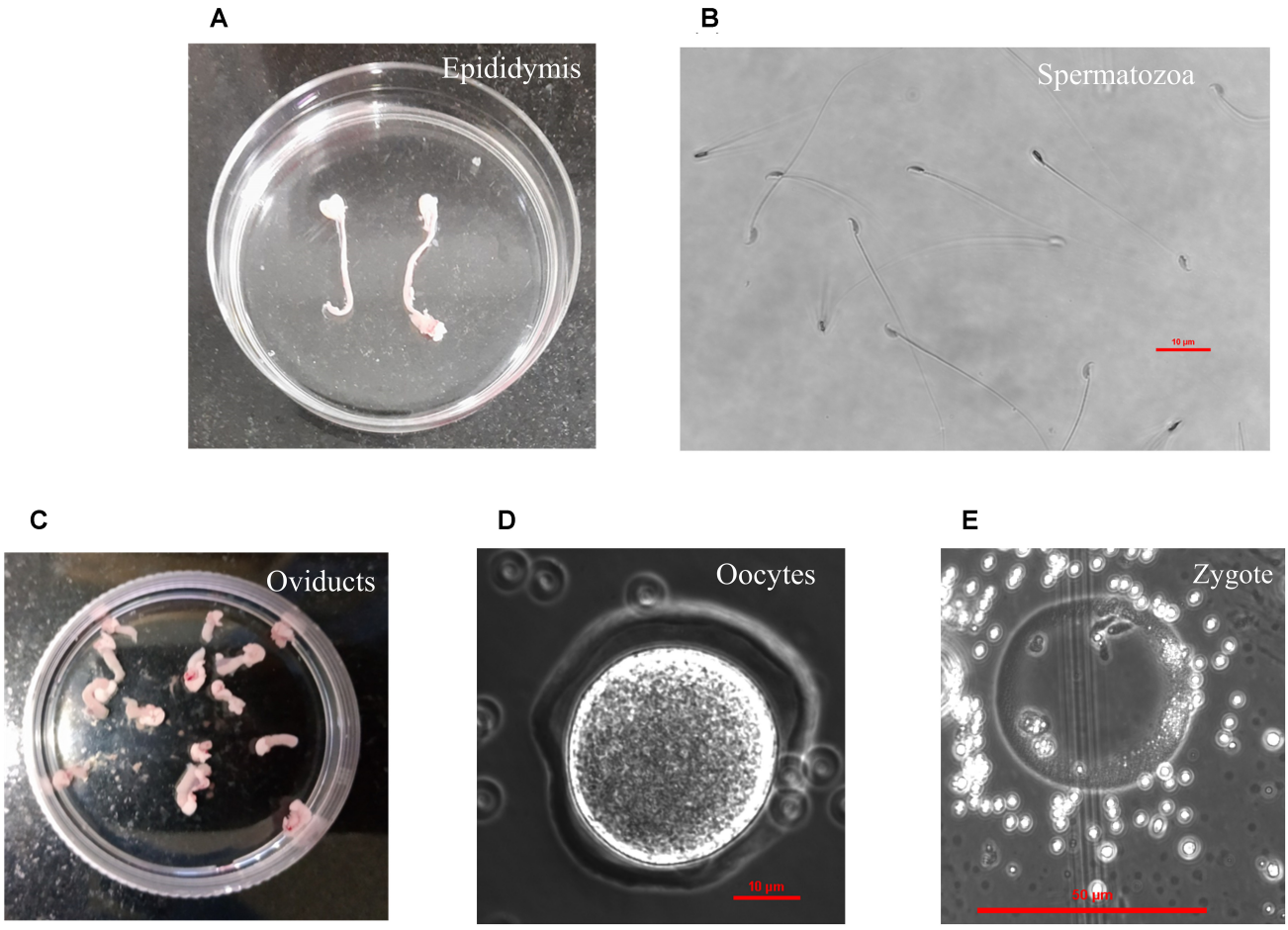

Figure 1. (**A**) Mouse spermatozoa collected by directly puncturing cauda epididymis of euthanized mice. (**B**) Mouse sperm after removal of somatic cells, (**C**) mouse oviducts, (**D**) healthy oocytes, and (**E**) one-cell stage zygote. Mouse oocytes and zygotes were collected by carefully trimming the fat tissue and finely chopping the oviducts. The morphology of oocytes appeared uniformly circular with clearly visible zona pellucida that makes a notable difference between oocytes and zygotes.

compatible NEBNext® UltraTM II Directional RNA Library Prep Kit (New England BioLabs, MA, USA) at Genotypic Technology Pvt. Ltd, Bangalore, India. Purified cDNA was end-repaired, adenylated, and ligated to Illumina multiplex barcode adapters as per NEBNext® UltraTM II Directional RNA Library Prep protocol followed by second-strand excision using USER enzyme at 37°C for 15 min.

Small RNA sequencing library was prepared with NEXTflexTM Small RNA Sample Preparation protocol (Bioo Scientific Corporation, Austin, Texas, USA). Illumina-compatible sequencing libraries were quantified by Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and fragment size distribution analysis was carried out on Agilent 2200 Tapestation and on Agilent 2100 Bioanalyzer for transcriptomic and small RNA data, respectively.

In-house long and small transcriptomic datasets have been processed and analyzed following the same protocol as mentioned in Raw data normalization and analysis section. This data has been deposited in NCBI's Gene Expression Omnibus and is accessible through the accession number GSE269614.

Quantitative real-time PCR validation

RNA isolation for quantitative real-time polymerase chain reaction (qRT-PCR) validation was done using miRNeasy Mini Kit (Qiagen, Cat. 217004) to isolate long and small RNA separately from sperm, oocyte, and zygote.

The reverse transcription reactions for long RNA, were performed using verso cDNA synthesis kit (Thermo Scientific, Cat. AB1453A) according to the manufacturer's protocol. cDNA synthesized from 300 ng of total RNA was used for qRT-PCR reaction. For small RNA, reverse transcription reaction was performed using miRCURY LNA miRNA PCR Starter Kit (Cat. 339320). Subsequently, qRT-PCR reactions were performed to measure the expression levels of DElncRNAs and DEmiRNAs using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Cat. A25742), using three biological replicates.

Gene and lncRNA primers were designed in consultation with PrimerBank (https://pga.mgh.harvard.edu/primerbank/) and NCBI Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). They were further checked using Sigma Aldrich OligoEvaluator™ (http://www.oligoevaluator.com/). The primer list is provided in Supplementary Table S1. Additional requirements include PCR products with a size between 80 and 200 bp and an annealing temperature of roughly 63°C. qRT-PCR reactions were performed using PowerUp SYBR Green master mix (Thermo Fischer Scientific, A25742). miRCURY LNA miRNA PCR Assay primers (Cat.339306 -YP00205695 and YP00206071) were

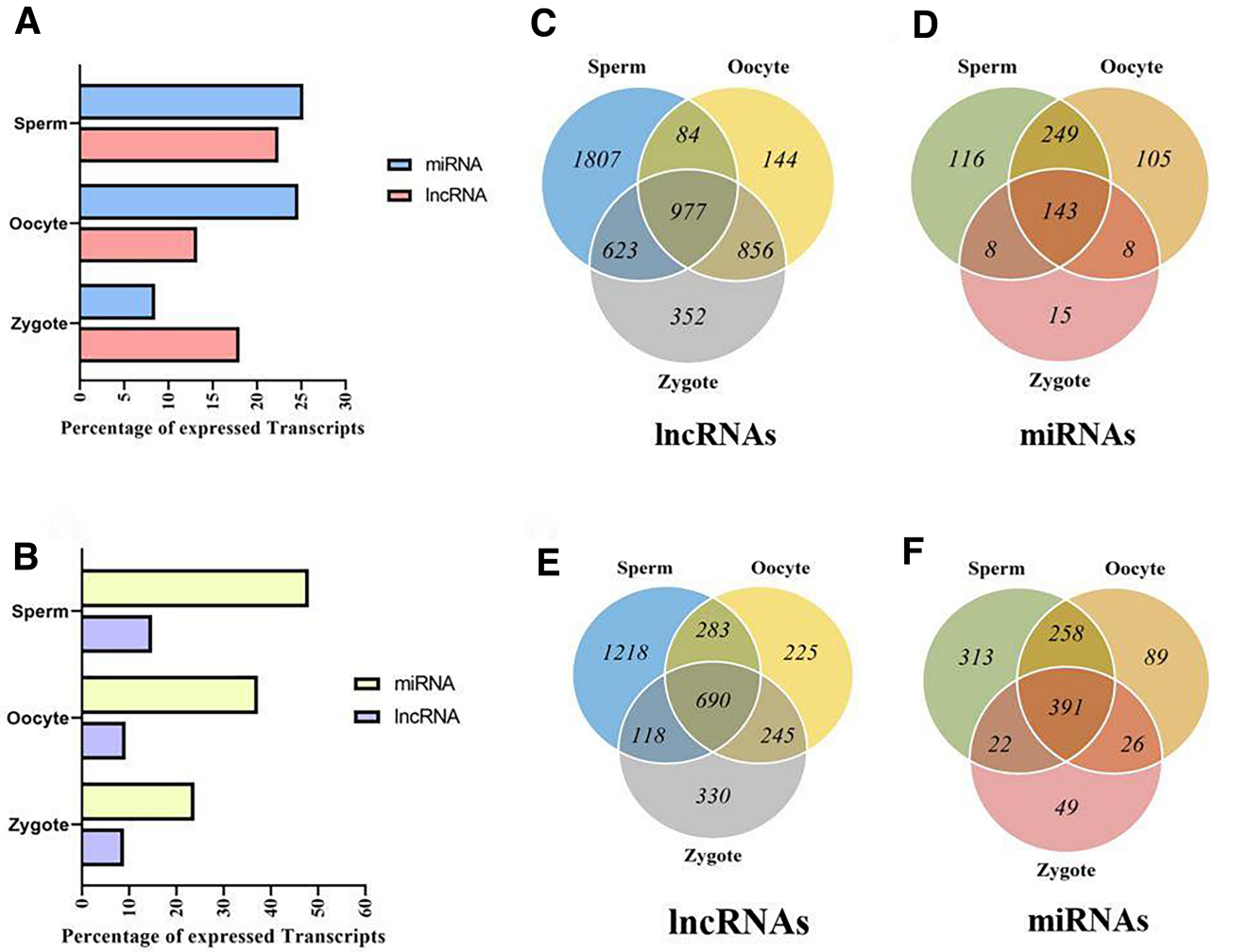

Figure 2. General ncRNA expression profile within sperm, oocyte, and zygote. Bar chart shows percentage of expressed lncRNA and mature miRNA transcripts in sperm, oocyte, and zygote obtained from (**A**) online available data and (**B**) in-house generated data. Venn diagram shows (**C**) 977 lncRNA transcripts and (**D**) 143 mature miRNA transcripts to be commonly expressed in sperm, oocyte, and zygote from online available data analysis and (**E**) 690 lncRNA transcripts and (**F**) 391 mature miRNA transcripts to be commonly expressed in sperm, oocyte, and zygote from in-house generated data analysis.

ordered from Qiagen and used for miRNA quantitative analysis.

Out of the oocyte and zygote samples obtained from 100 female and sperm samples from 50 male mice, samples were pooled to create several biological replicates. For each biological replicate, qRT-PCR for lncRNAs and their correlated mRNA pairs as well as miRNAs and their target mRNAs was performed in triplicate as technical replicates to account for machine variability. The mean Ct value from these technical replicates was used for downstream calculation. FC and statistical analysis were conducted based on the results of three independent biological replicates.

Results

General expression profile of mouse ncRNAs in pre- and postfertilization stages

In order to find out the parental contribution of ncRNAs viz., miRNAs and lncRNAs, in pre- and postfertilization events prior to ZGA, we first looked into the gene expression dataset available online at NCBI (https://www.ncbi.nlm.nih.gov) (input dataset provided in Table 1) and got the general ncRNA expression profile across sperm, oocyte, and one-cell stage zygote in *Mus musculus*.

Global small and long ncRNA transcriptome analysis revealed the general lncRNA and miRNA profile across sperm, oocyte, and zygote (shown in Fig. 2A) as the percentage of expressed transcripts (both lncRNA and miRNA) with respect to the entire set of annotated lncRNA and miRNA genes within the mouse reference genome (mm10). Our observation reveals the presence of highest number of lncRNA and miRNA transcripts in sperm (depicted by the bar diagrams in Fig. 2A). Deeper investigation into the lncRNA and miRNA profile (depicted by the Venn diagrams in Fig. 2C and D) revealed that the commonly shared 977 lncRNA and 143 miRNA transcripts between sperm, oocyte, and zygote are more as compared to their exclusive contribution to the zygote. Overall, the general ncRNA expression profile reveals sperm to be the largest contributor of miRNAs and lncRNAs as compared to oocyte.

In order to check the robustness of our findings from the meta-analysis done on the online available datasets, we cross-checked the pattern (mentioned earlier) within our in-house

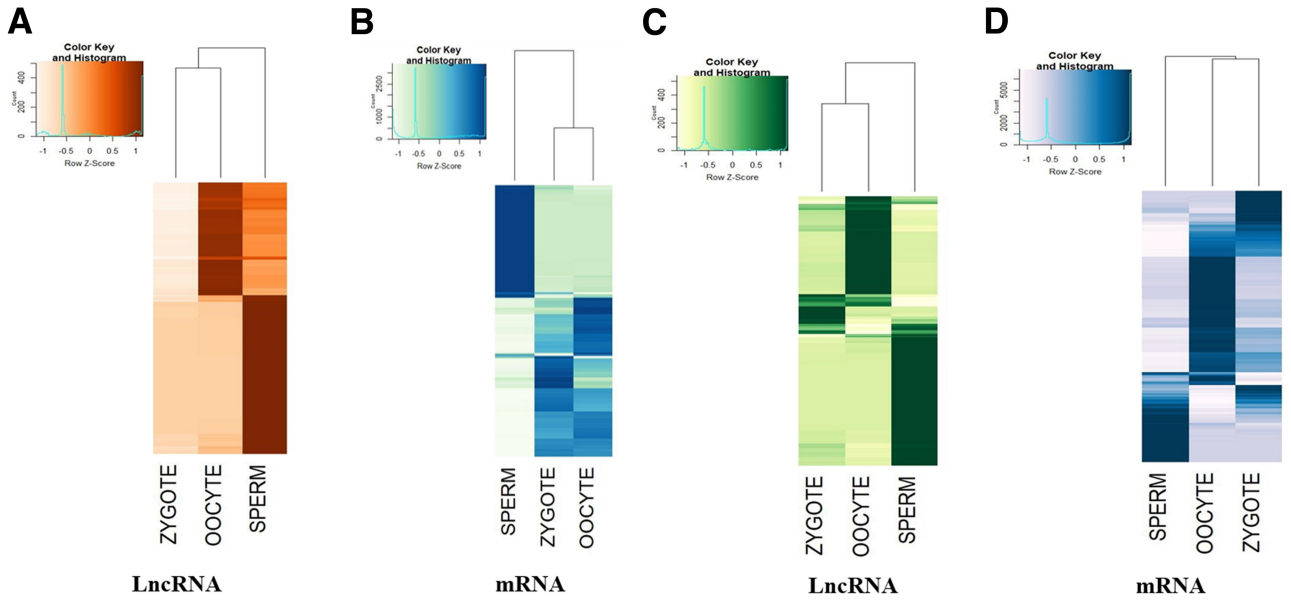

Figure 3. Hierarchical clustering representing DE (**A**) lncRNAs and (**B**) mRNAs corresponding to the online available dataset; (**C**) lncRNAs and (**D**) mRNAs corresponding to the in-house dataset.

generated small and long RNAseq data corresponding to sperm, oocyte, and zygote, obtained from our *in vivo* experiment and observed the same pattern for both lncRNAs and miRNAs (Fig. 2B, E, and F).

Differential expression profile of mouse ncRNAs and mRNAs in sperm and oocyte with respect to zygote

Next, we investigated the set of DE miRNAs, DE lncRNAs, and DE mRNAs within sperm and oocyte with respect to zygote so as to find out their expression dynamics in pre- and postfertilization stages. Detailed differential analysis (with FDR cut off of ≤ 0.05, and FC cut off ≥ 2.0 for mRNAs and ≥ 1.5 for lncRNAs) was performed for long RNAs (lncRNAs and mRNAs) using the dataset in Table 1. Interestingly the corresponding DE profile of lncRNA and mRNA transcripts (as provided in Supplementary File 1) revealed a significant degree of similarity between the oocyte and the zygote, which is also evident from earlier reports [44–46]. Among the entire set of lncRNAs that are commonly found in sperm and zygote (as revealed in the Venn diagram provided in Fig. 2), ~46% of them are differentially regulated between them. On the contrary, only ~0.8% of the lncRNAs that are commonly found in oocyte and zygote are differentially regulated between them. Further the DE profile of lncRNAs as obtained from in-house sequencing data (provided in Supplementary File 2) also reveals that a greater percentage of lncRNAs are differentially regulated between sperm and zygote as compared to that between oocyte and zygote.

Among the mRNAs that are commonly found in sperm and zygote ~35.5% of them are differentially regulated between them whereas, only ~2.4% of the mRNAs that are commonly found in oocyte and zygote are differentially regulated between them.

Both these results reveal that lncRNAs play a dominant sperm-specific role in pre-fertilization stage as the differential profile of it is very different from that of zygote. Further, the oocyte and zygote seem to be similar with respect to the differential profile of lncRNAs and mRNAs (as is evident from a very little number of lncRNAs and mRNAs being DE between oocyte and zygote). Hierarchical clustering based on the DE profile of the lncRNAs and mRNAs corresponding to the online available dataset (Fig. 3A and B) and the in-house dataset (Fig. 3C and D) also showed clustering of the oocyte and zygote together, with the sperm samples clustering separately.

On the other hand, for sperm miRNA data analysis (for the online dataset) the small RNAseq data corresponding to sperm samples from caput, corpus, and cauda of the epididymis have been analyzed separately with that from zygote as control. Since cauda contributes the mature sperm population that fertilizes the oocyte, a set of cauda-specific DEmiRNAs has been finally screened. These miRNAs are also DE in the sperm samples from the caput and corpus (with respect to zygote) as well, although their expression patterns vary widely across the three regions making them interesting candidates to explore further.

The hierarchical clustering of the cauda-specific DE miRNAs from both online and in-house data (depicted in Fig. 4 and the data provided in Supplementary Files 1 and 2) reveal that sperm and oocyte are closer to each other and distant from zygote. This suggests that an almost equal percentage of miRNAs are DE among those that are commonly found in sperm and zygote, and in oocyte and zygote. Such miRNA expression dynamics reveal that a specific set of these miRNAs gets downregulated and another set gets upregulated in zygote. This provides a clue regarding the role of these parental miRNAs in respective germ cells as well as in early embryos, making the germ cells compatible for successful fertilization and subsequently supporting early embryonic development. An in-depth investigation within these miRNAs revealed an exclusive set of sperm-borne miRNAs and oocyte-borne miRNAs along with seven and two miRNAs that have been found to be upregulated and downregulated, respectively, in both sperm and oocytes with zygote as control.

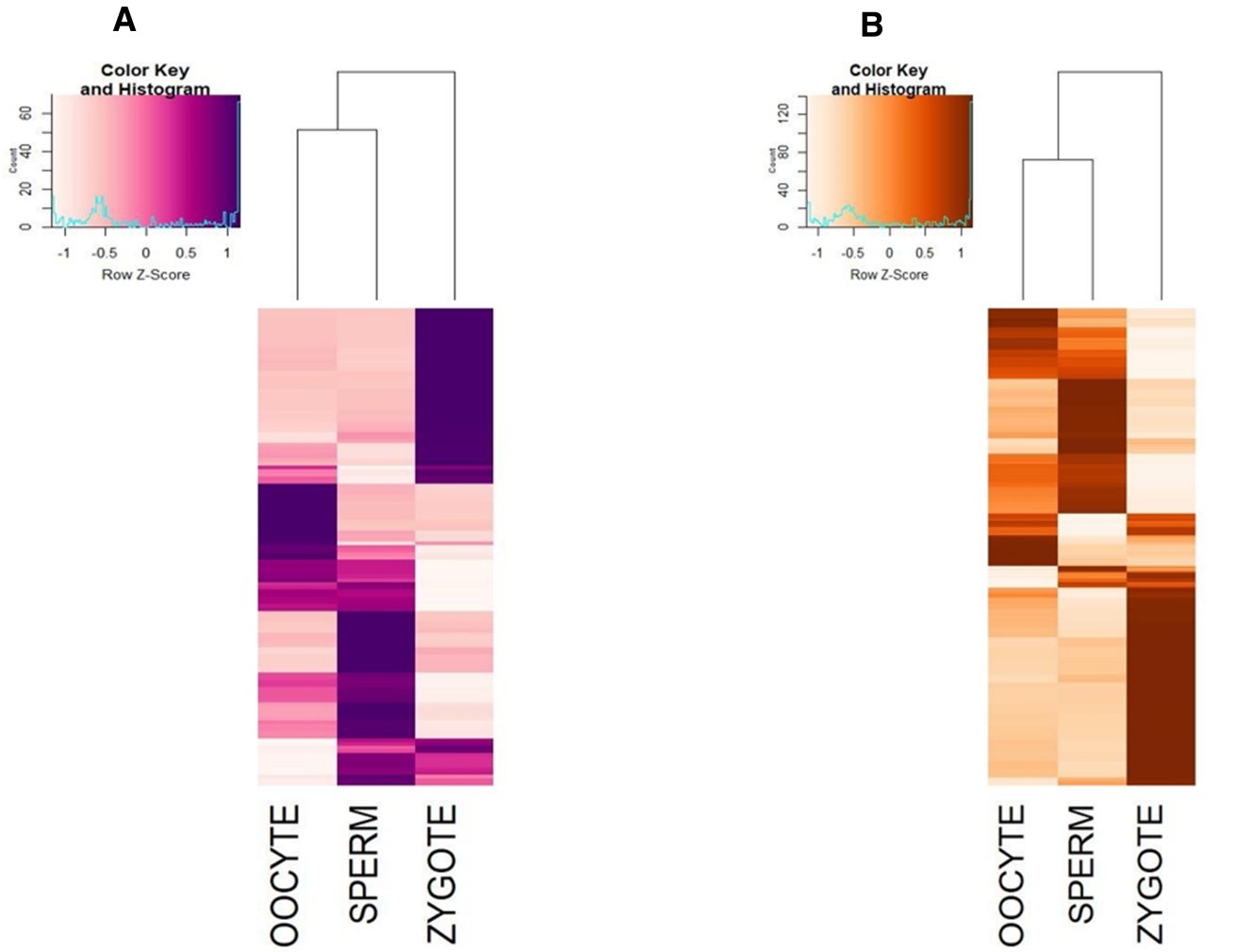

Figure 4. Hierarchical clustering of the DE miRNAs corresponding to (**A**) the online available dataset and (**B**) in-house dataset.

Sperm-borne DE miRNAs: From both the online available and in-house small RNAseq data, mmu-mir-486a-5p, mmu-mir-486b-5p, and mmu-mir-423-5p have been found to be exclusively downregulated in sperm cells as compared to that in zygote. Among these, miR-486-5p has been reported to be associated with sperm motility in cattle [47]. Further, miR-423-5p plays a key role in maintaining sperm motility promoting oxidative stress and has been found to be upregulated in the sperm samples of asthenozoospermic individuals, a condition that affects sperm motility [48]. This miRNA has also been detected as one of the highly expressed miRNAs in developing germ cells of infertile patients [49].

Oocyte-borne DE miRNAs: mmu-let-7i-5p, mmu-let-7g-5p, and mmu-miR-3962 have been found to be exclusively upregulated in oocytes as compared to that in zygote from both the online and the in-house small RNA-seq data. let-7i-5p and let-7g-5p have a reported role in oocyte development as revealed by the work of Wei *et al.* [50].

The function of the target genes corresponding to these common and exclusive sets of miRNAs has been discussed within "miRNA-target mRNA pairs" section.

Screening the important set of DE genes

The DE mRNAs obtained from both the analysis of the online available dataset and our in-house generated data underwent further screening based on their functional annotation provided by IPA (result provided in Supplementary File 3). We further did an exhaustive literature mining using a systematic approach to ensure thoroughness and reproducibility. Studies from the past ten years were prioritized, with particular attention given to highly cited articles and those published in high-impact, peer-reviewed journals. Further, we consulted a well-cited database named Panglaodb [34], which hosts mouse single-cell RNA sequencing studies and provides cell-specific information to filter the final set of DE mRNAs. The enriched functions revealed by the screened set of genes have been represented in Fig. 5A (sperm versus zygote) and B (oocyte versus zygote). The pathways in Fig. 5A are directly related to sperm fertility, spermiogenesis, embryo development, etc., and those in Fig. 5B are related to female reproductive system and embryonic development. Overall, Fig. 5 depicts certain pre- and postfertilization events, which are significantly enriched in our dataset. These enriched pathways and functions depicted in Fig. 5 truly reveal the significance of the filtered genes for pre- and postfertilization events involving the germ cells and the zygote, respectively.

lncRNA–mRNA co-expression analysis

With this filtered set of DE mRNAs and the DE lncRNAs (obtained from both the analysis of the online available dataset

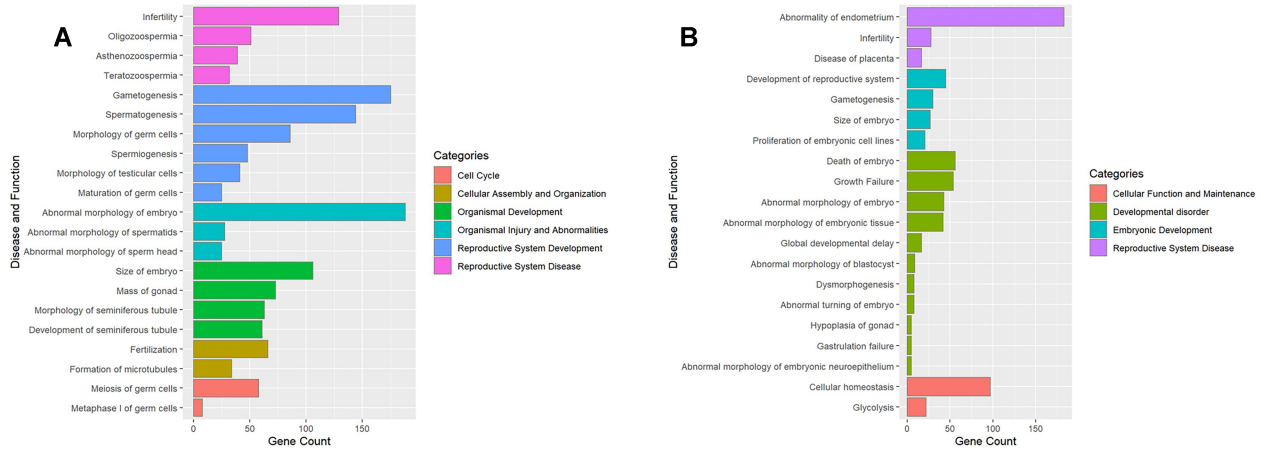

Figure 5. Enriched functions of the DE mRNAs between (**A**) sperm and zygote, and (**B**) oocyte and zygote. Enriched functions have been screened based on, *P*-value cutoff ≤.005.

and our in-house generated data), we did lncRNA–mRNA co-expression analysis and screened those mRNA targets that demonstrated significant positive correlation ($r > 0.6$, $P \leq .05$). For the DE lncRNAs between sperm and zygote, the final significant set of co-expressed lncRNA–mRNA pairs have been organized into co-expression networks. Figure 6 depicts the network featuring prominent lncRNAs (all of which are upregulated in sperm) with one or more connections to their target mRNAs. Notably, the visual elements include oval shapes representing lncRNAs, hexagons symbolizing genes, and varying thickness in the connecting lines reflecting the correlation strength, where a thicker line corresponds to a higher correlation value. On the contrary, for the DE lncRNAs between oocyte and zygote, a significant set of co-expressed lncRNA–mRNA pairs has been obtained only for two lncRNAs. Supplementary Table S2 containing Supplementary Table S2A and B, depicts the function of these lncRNA target genes, which once again reveals that lncRNAs play a significant role in the pre-fertilization stage, mainly related to sperm motility and oocyte maturation. Further it corroborates with the conclusion drawn from the DE profile obtained for the lncRNAs as provided before.

miRNA-target mRNA pairs

To assess the functions of the DE miRNAs, obtained from both the online and our in-house generated data, we did a target prediction analysis between the DE miRNAs and DE genes using TargetScan [35], followed by Parasor (as mentioned in "Materials and methods" section). In our analysis we have found gene *RogdI* to be one of the potential targets of the exclusively sperm-borne downregulated miRNA-mmu-mir-423-5p (Supplementary Table S3). Interestingly, it has been reported that *RogdI* is involved in sperm movement, capacitation, and acrosome reaction, and reduced expression of it in sperm indicates male infertility [51]. Further genetic variation present in the loci of this gene has been associated with sire conception rate corresponding to male fertility as reported in bovine system [52]. On the contrary, none of the miRNA-target genes have been obtained for the exclusive set of oocyte-borne miRNAs.

We next investigated the set of miRNAs commonly upregulated or downregulated in sperm and oocytes with zygote as control. The target genes along with their functions corresponding to the seven and two miRNAs that have been found to be upregulated and downregulated, respectively, in both sperm and oocytes have been provided in Supplementary Table S3. It is important to note that their target genes vary in both sperm and oocytes. This reveals that the same set of DE miRNAs modulate different sets of functions in these germ cells. Among these, our observation reveals two important DE miRNAs viz. mir-30a-5p and miR-200b-3p upregulated in sperm (isolated from epididymis) as well as in oocyte (isolated from the oviduct) with respect to zygote. Earlier reports reveal the importance of these miRNAs in sperm fertility [53, 54] as well as maintaining female fertility [55, 56]. This has led us to screen these two miRNAs for their further validation in sperm, oocyte, and zygote. We have thereafter performed qRT-PCR expression validation for these two miRNAs in sperm and oocyte, with zygote as control.

Validation of lncRNAs and their correlated gene partners in sperm and oocyte with respect to zygote

Comparative qRT-PCR was carried out between sperm and zygote and oocyte and zygote to check the expression of the significant set of lncRNAs and their correlated target genes as provided in Supplementary Table S2. For qRT-PCR-based validation, primer pairs were designed for the upregulated lncRNAs and their correlated mRNA pairs. qRT-PCR result of the lncRNAs and their target genes in sperm and oocyte with zygote as control has been shown in Fig. 7. We successfully validated the statistically significant upregulated expression of six lncRNAs in sperm and two lncRNAs in oocyte (Fig. 7) with zygote as control. We also checked the expression of their correlated mRNAs in the respective systems. In the case of sperm, the upregulated lncRNAs are 1700027A15Rik, Ube4bos3, Malat1, 1700019L13Rik, 1700009J07Rik, and 1810049I09Rik. We detected a total of seven correlated genes with upregulated expression: *Pgk2*, *Prm1*, *Spem1*, *Spz1*, *Tnp2*, *Actl7a*, and *D1Pas1* (Fig. 7A). Notably, all these coding genes are exclusively associated with sperm-specific activities [34]. For oocytes, expression of two lncRNAs viz. Gm10553 and D830035M03Rik, and that of their correlated genes *Rtn4*, *Mdh1*, and *Cnot7*, have been validated (Fig. 7B). These coding genes are associated with functions involved in oocyte development [57–59].

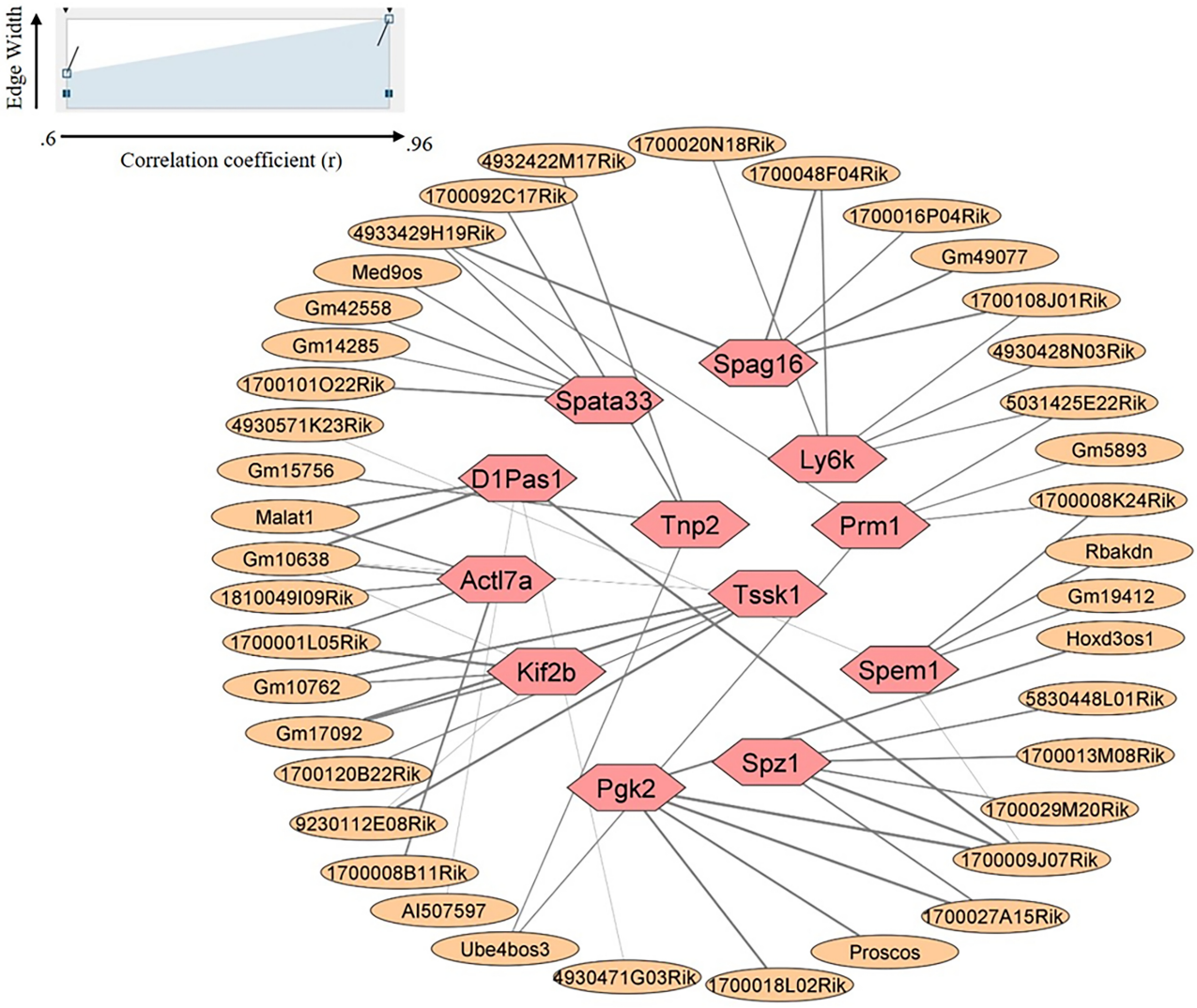

Figure 6. LncRNA–mRNA co-expression network for DE lncRNAs between sperm and zygote. Positively correlated pairs with Pearson's correlation coefficient cut-off of (r) > 0.6 and $P \leq .05$ have been screened.

Validation of the miRNAs and their target gene partners in sperm and oocyte with respect to zygote

As mentioned earlier, miR-200b-3p and miR-30a-5p were screened finally for expression validation along with their target genes in the respective systems compared to zygote. We successfully validated the upregulated expression of two miRNAs, miR-200b-3p and miR-30a-5p in both sperm and oocyte samples compared to zygote (Fig. 8). Additionally, their target genes, *Med28*, *Sugt1*, *Egln1*, and *Arpp19*, were validated to be downregulated in sperm (Fig. 8A), while *Dph3*, *Ndufa4*, and *Eef1e1* were downregulated in oocyte samples (Fig. 8B) with zygote as control in both cases. These findings align with existing literature, which suggests that these target genes play various roles in embryonic development (as is also evident from Supplementary Table S3).

Discussion and conclusion

The fusion of two parental gametes resulting in the formation of a zygote is essential to give rise to a new life, complete with a multitude of specialized tissues and a fully developed organism. ncRNAs play a crucial role in governing the maturation and fertility status of the germ cells, which act as determinants for successful fertilization. Further they function as versatile gene expression modulators at different stages of embryonic development. Figure 9 depicts the detailed mode of interaction of the lncRNAs and miRNAs with their target genes thus contributing toward pre- and postfertilization stages. In this work, we have looked into the contribution of parental miRNAs and lncRNAs in the transfer of information from sperm and oocyte to the one-cell zygote. However, we cannot entirely rule out the effect of early zygotic transcription, which has been reported to begin at minor ZGA at the one-cell stage [60]. We adopted both *in silico* and *in vivo* techniques to obtain long and small RNA profiles of murine sperm, oocyte, and zygote and subsequently identify the significant set of ncRNA modulators and their target genes, playing a significant role in pre- and postfertilization events. Thereafter, we validated the expression of significant set of lncRNAs and their target genes as well as miRNA and their target genes.

Interestingly, certain specific ncRNA gene expression patterns were obtained in pre-and postfertilization stages. The general expression profile revealed that sperm produces the

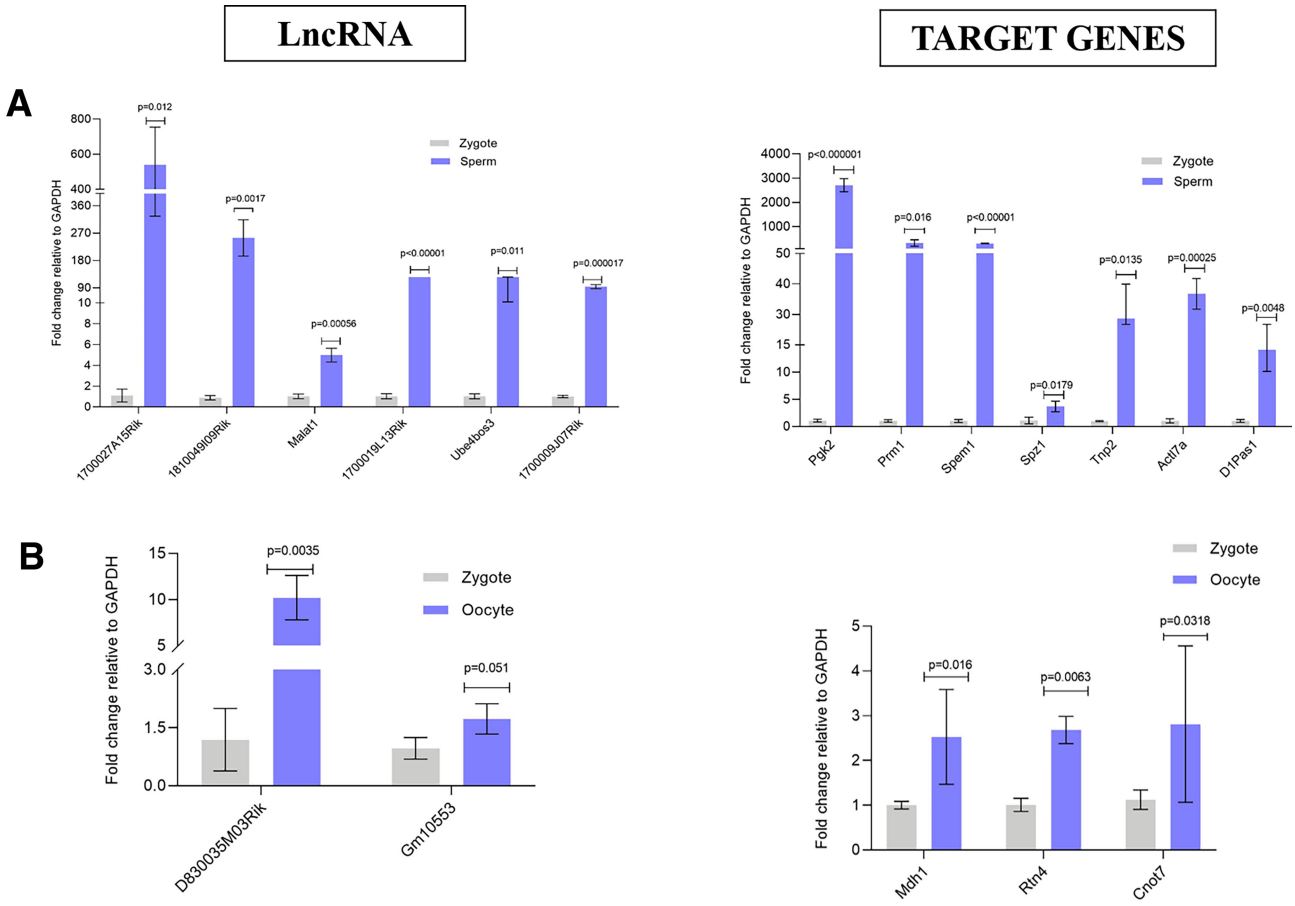

Figure 7. qRT-PCR result of lncRNAs and their target genes in (**A**) sperm and (**B**) oocyte with zygote as control.

highest number of lncRNA and miRNA transcripts as compared to oocyte. Despite that, the common contribution of lncRNA and miRNA transcripts from both sperm and oocyte is more than their exclusive contribution to the zygote. Differential profiles reveal a higher degree of similarity between the oocyte and zygote in terms of the DE lncRNAs and DE mRNAs, whereas that of sperm is very distinct. This unveils the importance of lncRNAs in pre-fertilization stage, especially toward sperm maturation and making them compatible for fertilization. On the contrary, miRNAs play a significant role in both pre- and postfertilization stages.

To elucidate the function of the lncRNAs specifically in pre-fertilization stage, we conducted co-expression analyses between DE lncRNAs and mRNAs and validated their expression. Most of the validated lncRNAs are relatively novel, as their roles in the relevant field have not yet been thoroughly explored.

Among the other validated upregulated lncRNAs, 1700027A15Rik previously identified in the testis [61] plays a regulatory role in spermatogenesis. When they suppressed this lncRNA, it disrupted spermatogenesis, leading to the loss of germ cells. Reducing 1700027A15Rik levels also resulted in fewer round and elongating spermatids in abnormal seminiferous tubules, highlighting its potential importance in these processes [61]. Malat1 in sperm plays a significant role in determining sperm motility. Its expression levels are connected with ROS-related defects in sperm function [62]. LncRNA Gm10553 and D830035M03Rik, have been consistently observed to be upregulated in our *in silico*, *in vivo*, and qPCR experiments within the oocyte with respect to the zygote. Their correlated target genes play a crucial role in oocyte maturation [57, 58] and maturation of oocyte granulosa cells [59].

The miRNA expression dynamics within sperm along its journey via caput to corpus and then to the cauda epididymis after exiting from the testis reveals a very interesting pattern that prepares the sperm further to make it competent for fertilization [63]. Further, oviductosomes, which are nano-sized extracellular vesicles secreted in the oviductal luminal fluid by oviductal epithelial cells bear repertoires of miRNAs throughout the estrous cycle [55]. It is interesting to note that after undergoing modifications within the epididymis, the sperm undergo final maturation i.e. capacitation in the oviductosomes [64]. We validated the expression of two such DE miRNAs viz. mir-30a-5p and miR-200b-3p upregulated in sperm (isolated from epididymis) as well as in oocyte (isolated from the oviduct) with respect to zygote.

In humans, miR-30a has been proposed to be a significant player in female reproductive physiology and embryogenesis [65]. On the other hand, reports reveal the importance of miR-200b for mouse ovulation and its importance for maintaining female fertility [56]. This is one of those miRNAs that is gained upon entry into the epididymis and has an increasing expression during epididymal maturation of the sperm [55]. It has also been reported to be detected in human spermatozoa of healthy individuals of different age groups [66]. Further miRNAs belonging to the miR-30 and miR-200 families play an important role in embryonic development [67]. All

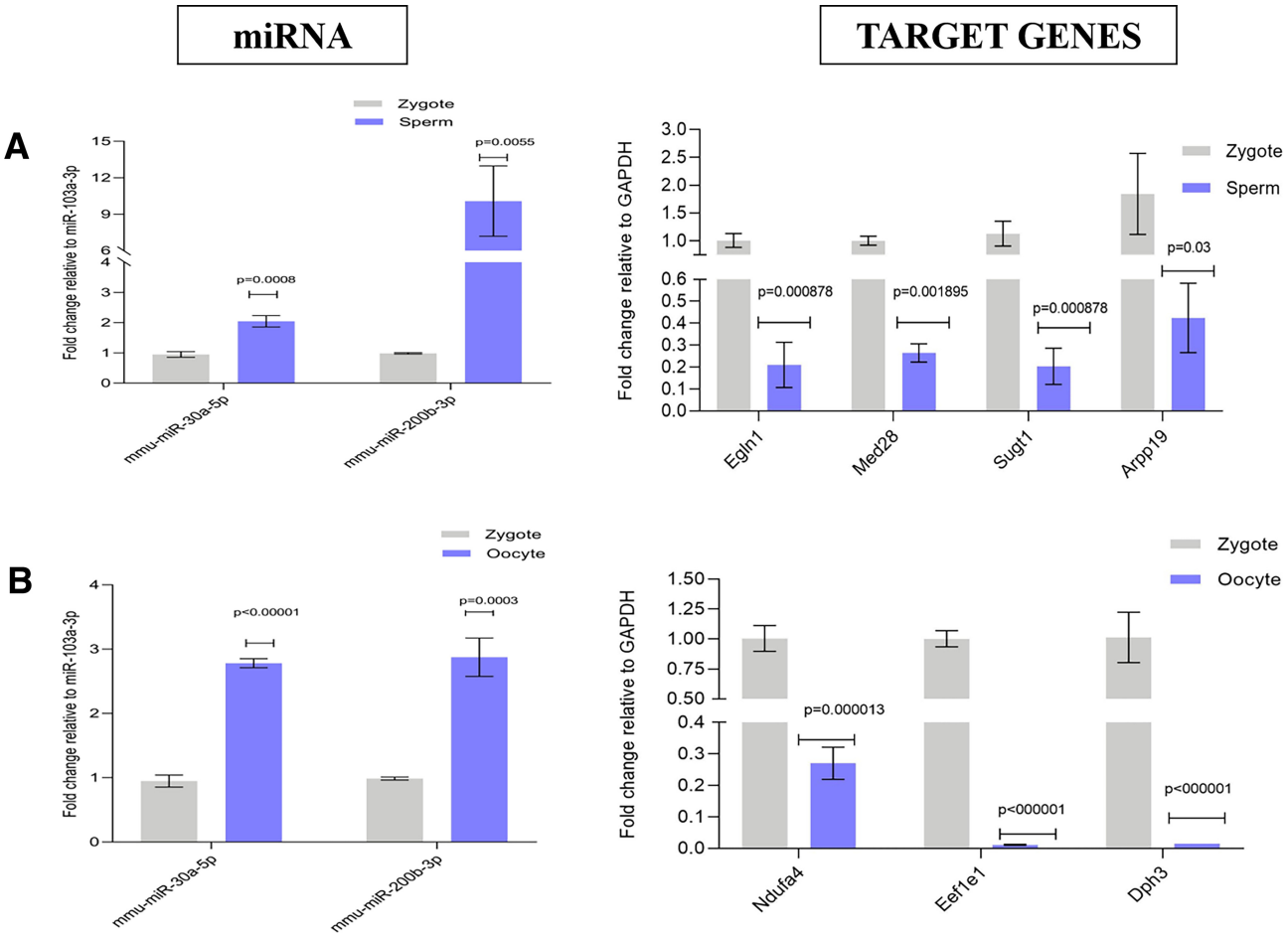

Figure 8. qRT-PCR result of miRNAs and their target genes in (**A**) sperm and (**B**) oocyte with zygote as control.

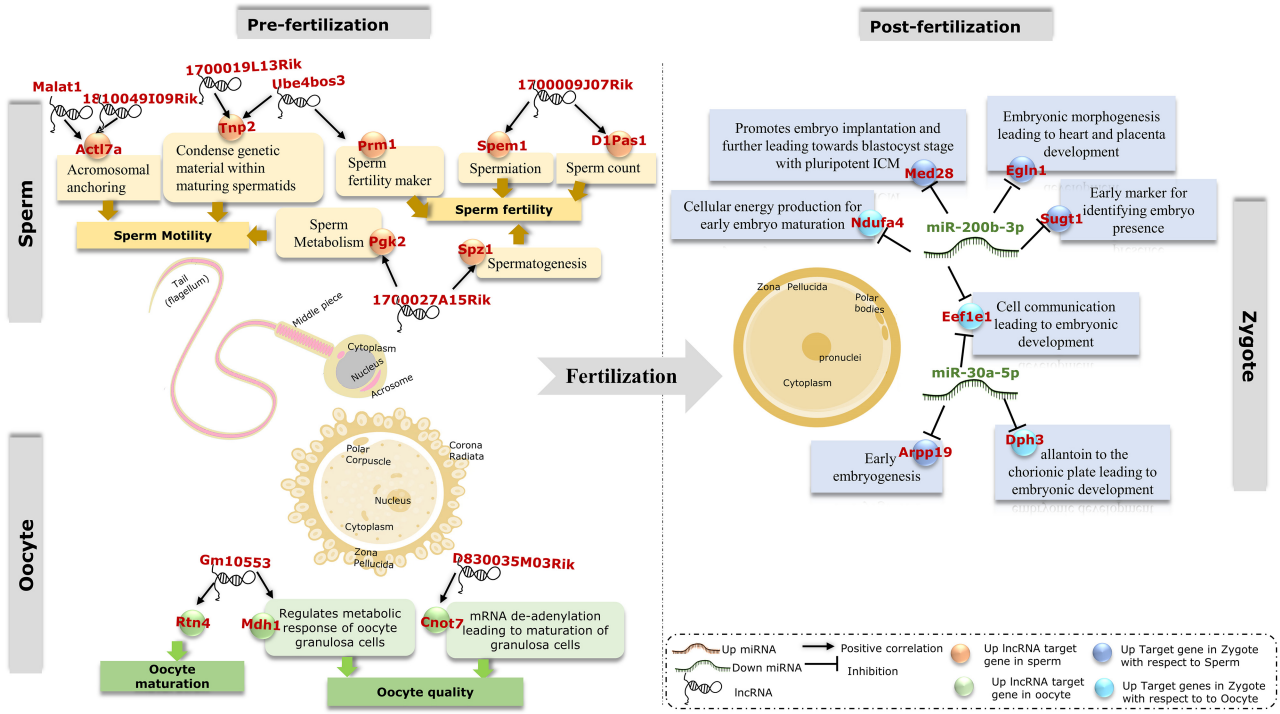

Figure 9. Schematic diagram depicting the detailed mode of action of the lncRNAs, miRNAs, and their target genes in the pre- and postfertilization stages.

these reveal the importance of both of these miRNAs in pre-fertilization stage as well as in postfertilization. Hence, critical levels of these miRNAs are needed to initiate embryonic development just after fertilization. This speculation gets further strengthened as we look into the function of their target genes, which are downregulated in sperm and oocyte. All these target genes (Supplementary Table S3) get upregulated just after fertilization and play a very important role in embryonic development.

The miRNA expression dynamics between the germ cells and the zygote reveal their substantial role toward increasing the competency of sperm and oocytes for fertilization, which could be a fascinating avenue for future research exploration. Further, these findings also open avenues for further investigation into the role of their target genes in early embryonic development.

To summarize, this study underscores the substantial impact of parental gametes on fertilization as well as early embryonic development. Our findings provide a comprehensive lncRNA and miRNA profile contributed by oocytes and sperm prior to fertilization and subsequently their role in post-fertilization. Moreover, the set of novel lncRNAs that have been identified has a potential role during pre-fertilization stage. Further, in-depth investigations exploring their contribution toward maintaining germ cell fertility, will strengthen our conclusion.

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Author's contribution: Z.G. hypothesized, conceptualized, and provided overall supervision of the study. Z.G., A.V., and A.D. initially designed the experiments, which were further fine-tuned by B.G. and T.D. The animal experiments were done by A.V., A.M., P.S., and K.J. Data analysis was done by T.D., B.G., A.V., and A.D. T.D. and B.G. performed the expression validation experiments fine-tuned by A.S. T.D., B.G., A.S., A.D., P.S., and Z.G. drafted the manuscript.

Supplementary data

Supplementary data is available at NAR Genomics & Bioinformatics online.

Conflict of interest

None declared.

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Data availability

The in-house long and small transcriptomic datasets corresponding to mouse sperm, oocyte, and zygote have been deposited in NCBI's Gene Expression Omnibus and are accessible through the accession number GSE269614.

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