Dynamic expression of long noncoding RNAs reveals their potential roles in spermatogenesis and fertility†

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Abstract

Mammalian reproduction requires that males and females produce functional haploid germ cells through complex cellular differentiation processes known as spermatogenesis and oogenesis, respectively. While numerous studies have functionally characterized protein-coding genes and small noncoding RNAs (microRNAs and piRNAs) that are essential for gametogenesis, the roles of regulatory long noncoding RNAs (lncRNAs) are yet to be fully characterized. Previously, we and others have demonstrated that intergenic regions of the mammalian genome encode thousands of long noncoding RNAs, and many studies have now demonstrated their critical roles in key biological processes. Thus, we postulated that some lncRNAs may also impact mammalian spermatogenesis and fertility. In this study, we identified a dynamic expression pattern of lncRNAs during murine spermatogenesis. Importantly, we identified a subset of lncRNAs and very few mRNAs that appear to escape meiotic sex chromosome inactivation, an epigenetic process that leads to the silencing of the X- and Y-chromosomes at the pachytene stage of meiosis. Further, some of these lncRNAs and mRNAs show a strong testis expression pattern suggesting that they may play key roles in spermatogenesis. Lastly, we generated a mouse knockout of one X-linked lncRNA, Tslrn1 (testis-specific long noncoding RNA 1), and found that males carrying a Tslrn1 deletion displayed normal fertility but a significant reduction in spermatozoa. Our findings demonstrate that dysregulation of specific mammalian lncRNAs is a novel mechanism of low sperm count or infertility, thus potentially providing new biomarkers and therapeutic strategies.

Summary Sentence

Long non-coding RNAs are critical for mammalian spermatogenesis.

Key words: meiosis, spermatogenesis, epigenetics, lncRNAs, CRISPR/Cas9.
Introduction

Advances in transcriptomic technologies in the past decade have led to the identification of thousands of novel mRNA-like transcripts that have no significant protein encoding capacity [1–4]. These transcripts are referred to collectively as long noncoding RNAs (lncRNAs), and many lncRNAs show strong tissue-specific expression patterns, with the brain and testis showing the highest number of expressed lncRNAs [5]. To date, only a small percentage of all annotated lncRNAs have been functionally and/or mechanistically characterized; nonetheless, these studies have demonstrated in vitro and in vivo that lncRNAs are essential for development and viability [6–8]. Given the numerous lncRNAs that are expressed in human and mouse testis, we postulated that a subset of these lncRNAs may play critical roles in spermatogenesis and fertility. Currently many causes of human infertility are yet to be identified despite extensive studies of protein-coding genes raising the possibility that some of these cases could be driven by dysregulation of regulatory noncoding RNAs such as lncRNAs. Indeed, a recent study in Drosophila utilizing CRISPR/Cas9 has demonstrated that many lncRNAs are required for spermatogenesis and fertility [9]. Furthermore, studies in mammalian species have identified regulatory elements (e.g., enhancers) and regulatory noncoding RNAs that are spermatocyte- or spermatid-specific, further supporting the hypothesis that lncRNAs may impact mammalian spermatogenesis and fertility [10–13].

During mammalian spermatogenesis, differentiating type B spermatagonia divide mitotically to give rise to preleptotene spermatocytes, which commit to undergoing meiosis. Primary spermatocytes propagating through prophase I of meiosis can be divided into at least five distinct stages known as: leptotene, zygotene, pachytene, diplotene, and diakinesis before entering the first meiotic division [14,15]. The second meiotic division is required to give rise to haploid round spermatids that undergo spermiogenesis to produce fully differentiated testicular spermatocytes. A number of studies have demonstrated that the expression of mRNAs is dynamically regulated during the progression of spermatogenesis, which led to the identification of proteins that are essential for spermatogenesis and fertility [16–23]. Also, recent studies in model organisms such as Schizosaccharomyces pombe suggest that not only proteins but also regulatory noncoding RNAs may orchestrate key processes in meiosis such as meiotic recombination [24], and possibly other genetic and epigenetic events.

During prophase I, homologous autosomes align and synapse prior to the first meiotic division. By contrast, the X- and Y-chromosomes, which share limited sequence homology, pair only at the homologous pseudoautosomal regions to form the XY body at the pachytene stage [25]. Previous studies have shown that prior to or at the time of XY body formation at pachytene, X- and Y-linked protein-coding genes become transcriptionally silenced in a process known as meiotic sex chromosome inactivation (MSCI) [26,27]. To date, no mRNA genes are known to escape MSCI [28], and this is in sharp contrast to X inactivation (Xi) in female somatic cells where ~20% of protein-coding genes escape Xi [29]. However, two studies identified a subset of microRNAs that escape MSCI [28,30]; raising the possibility that newly identified regulatory lncRNAs may also escape MSCI and/or contribute to spermatogenesis and fertility.

A previous study examined lncRNA expression in male germ cells; however, the authors did not purify specific stages of the male germ line but isolated mixed population of cells at different time points of development [31]. Thus, in this study, we purified key stages of male spermatogenesis by STA-PUT to fully characterize the transcriptome of lncRNAs at specific stages of spermatogene-
sis. Bioinformatic analyses demonstrated dynamic changes in gene expression during the progression of spermatogenesis of lncRNAs. Importantly, we also identified a subset of X- and Y-linked lncRNAs and very few mRNAs that appear to escape MSCI. Finally, we generated a knockout mouse model of a candidate X-linked lncRNA that is highly expressed during the pachytene stage and observed normal fertility in mice but a significant reduction in sperm count. Our findings demonstrate that lncRNAs represent a new frontier in our understanding of mammalian spermatogenesis and potentially unknown causes of low sperm count and infertility.

Materials and methods

Mice studies

The Institutional Animal Care and Use Committee of Case Western Reserve University and the University of Texas San Antonio approved all animal protocols that were required to complete the studies.

STA-PUT unit gravity sedimentation

We utilized the STA-PUT method to isolate enriched populations of specific spermatogenic cell types from the mouse testis as previously described [19]. These cell types included combined type A and type B spermatogonia, preleptotene, combined leptotene/zygotene, pachytene spermatocytes, and round spermatids. Each cell type is approximately 85%–95% pure. Total RNA was isolated from each cell type and subjected to next generation RNA-sequencing (RNA-seq) as we previously described [32,33].

RNA-sequencing and analysis

Total RNA (1 μg) from each cell type was used to generate next generation RNA-seq libraries using Illumina TruSeq stranded library preparation kit per manufacturer’s protocol. RNA-seq reads were aligned to mouse genome (mm10) and gene expression was quantified using Fragments Per Kilobase of exon per Million mapped reads (FPKM) values. Using CuffDiff, differentially expressed mRNAs and lncRNAs were identified using the following parameters: ≥2-fold change, P-values ≤ 0.01, and q-values ≤ 0.05. All RNA-seq raw data are deposited in GEO (accession number: GSE100964).

Quantitative real-time Polymerase chain reaction (PCR)

RNA was converted to cDNA using RNA to cDNA EcoDry™ Premix Random Hexamers (Clontech). Primer pairs were designed using primer3 software with most spanning exon–exon boundaries. Maxima SyBr Green/ROX qPCR Master Mix (Thermo Scientific) was used for qRT-PCR. A comparative Ct quantification was performed with a hold stage of 50°C for 2 min and 95°C for 10 min followed by 40× cycles of 95°C for 15 s and 60°C for 1 min, and finally a melt curve at 95°C for 15 s, 60°C for 1 min, and a ramp to 95°C at 0.3°C increments. Analysis was done using the 2ΔΔCt method with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene [34].

Production of Tslrn1 knockout mouse

To produce Tslrn1 knockout mice using CRISPR/Cas9, guide RNAs were selected surrounding the 16 kb transcribed region of Tslrn1. Each guide was tested in vitro using Guide-it sgRNA in vitro transcription and screening kit (Clontech) for the ability to guide Cas9 nuclease activity to the given DNA sequence. One gRNA on each side of the Tslrn1 gene, gRNA1 and gRNA2,
were produced and injected (50 ng/μl) along with Cas9 protein (50 ng/μl) (PNABio) in the pronucleus of C57Bl/6 one-cell embryos. Embryos were then placed in pseudo-pregnant females to develop. The 25 resulting founder mice were genotyped for the presence and/or absence of Tslrn1 using PCR with the following primers (P1-TGTGCAAACCTGAGCCCTGTC, P2-TGGGAGTCTTTGGCATAAACC, P3-TGGACTAATGGCCAGGAGTT, and P4-CCAGGGGCTACTGAGTACA). The resulting bands were verified through Sanger sequencing. Quantitative RT-PCR described above was also utilized for Tslrn1 expression in the testes from Tslrn1 knockout and wildtype mice.

Characterization of Tslrn1 knockout male mice
Males aged 12–16 weeks of age were sacrificed, and sperm count, motility and morphology were assessed. Sperm count was assessed by dissecting out epididymis from each animal, placing it in 1 ml of medium supplemented with 3 mg/ml bovine serum albumin. Each epididymis was cut 10–12 times to allow sperm to exude for 15 min and counted on a hemocytometer. Sperm motility was performed with 10 μl of sperm suspension on a glass slide with a coverslip. A total of 200 spermatozoa were observed in 10 random fields for progressive and nonprogressive motility expressed as percent motile sperm. Sperm morphology was evaluated using a thin sperm suspension air-dried on a glass slide and fixed in 75% ethanol for 2 min, briefly dipped in water, stained with hematoxylin for 1 min and washed again in water for 15 s. The spermatozoa were categorized into normal head, head without hook, amorphous head, vacuolated head, and banana-shaped head. The number of spermatozoa with head abnormalities was expressed as abnormal sperm as a percentage. For histology, testes were removed, fixed in 10% formalin for 48 h, paraffin-embedded, and 5 μm thick sections were cut, placed on glass slides and stained with hematoxylin and eosin. For fertility, males aged 8–10 weeks of age were bred with wildtype females aged 6 weeks of age. At least 5 males of each genotype were mated individually with a female for at least 4 months. Number of litters and litter size were recorded.

Results
Numerous long noncoding RNAs are differentially expressed during the progression of spermatogenesis
We initially purified enriched populations of premeiotic spermatogonia (combined type A and B), meiotic pachytene spermatocytes and postmeiotic round spermatids using the STA-PUT method as we previously described [19]. We isolated total RNA from three biological replicates of each cell type and subjected the RNA to strand-specific paired-end RNA-seq and subsequent bioinformatics analysis using a pipeline we recently described [32,33]. We identified mRNAs and lncRNAs that are differentially expressed between spermatogonia and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNAs and pachytene spermatocytes, and between pachytene spermatocytes and spermatids (Figure 1). Specifically, we found 11 772 mRNas were detected at high levels in pachytene spermatocytes despite the coincident occurrence of MSCI; however, only a small number of these transcripts appear to escape MSCI (see below); and (iv) ~47 X-linked lncRNAs are expressed/reactivated postmeiotically in round spermatids (Supplemental File S4). These findings prompted us to further examine the expression of several X- and Y-linked lncRNAs and mRNAs.

Very few X-linked mRNAs are highly expressed at pachytene despite meiotic sex chromosome inactivation
RNA-seq analysis revealed a list of mRNAs that are expressed at the pachytene stage despite MSCI. However, a detailed analysis of these X- and Y-linked mRNAs demonstrated that they are also expressed ~5–10-folds higher in round spermatids. Since the STA-PUT method results in ~95% purity of pachytene cells with ~5% contamination of round spermatids, it is very critical to take this into consideration. Thus, we concluded that many of these mRNAs are unlikely to escape MSCI and their expression level at pachytene is likely due to the contamination with round spermatids. However, very few mRNAs showed higher expression levels in pachytene than spermatogonia and round spermatids raising the possibility that these very few mRNAs may escape MSCI. To that end, we decided to validate the RNA-seq observations using qRT-PCR to further analyze each spermatogenic cell type. First, we examined the expression of testis expressed gene 11 (Tex11) mRNA, which is known to be expressed in spermatogonia, and then to be subject to MSCI in spermatocytes before it is reactivated in round spermatids, as a positive control. Consistent with previous findings [26], Tex11 showed the expected expression pattern in our qRT-PCR analysis (Figure 2). Next, we examined the expression of other mRNAs that appears to escape MSCI and found that three mRNAs show higher expression levels in pachytene spermatocytes than all other stages examined. Specifically, DMRT-like family C1b (Dmrtc1b), sosondowah ankyrin repeat domain family member D (Sowad), and mcf.2 transforming sequence (Mcf2) mRNAs are expressed at higher levels in pachytene spermatocytes than other spermatogenic cell types examined (Figure 2, shown in red). These findings support the idea that few mRNAs may escape MSCI [23], and are consistent with our previous observation that specific regions of the XY sex body retain activin chromatin marks [35].

A subset of X- and Y-linked long noncoding RNAs is highly expressed at pachytene and appears to escape meiotic sex chromosome inactivation
Expression analysis of X- and Y-linked lncRNAs during spermatogenesis demonstrated that some lncRNAs are expressed in pachytene...
Figure 1. Numerous X- and Y-linked mRNAs and IncRNAs are differentially expressed during spermatogenesis. Heatmaps of differentially expressed X- and Y-encoded mRNAs and IncRNAs are shown. Differential expression of genes was calculated between spermatogonia versus pachytene and pachytene versus round spermatids using RNA-seq FPKM values. All experiments were performed in triplicates and genes were considered differentially expressed if they show equal or greater than two-fold change in expression and $P < 0.05$, $q < 0.05$. Blue color indicates low expression and red color indicates high expression.
spermatocytes at much higher levels than in either spermatogonia or round spermatids, suggesting that some of these lncRNAs may also escape MSCI. This was further tested by qRT-PCR using RNA isolated from purified populations of multiple spermatogenic cell types including spermatogonia (A and B combined), preleptotene, leptotene/zygotene, pachytene spermatocytes, and round spermatids. This analysis confirmed that these lncRNAs show higher expression in pachytene spermatocytes than in any of the other spermatogenic cell types (Figure 2, shown in green).

To further test if these lncRNAs are germ cell specific or enriched, we also examined the expression of top lncRNAs identified in our analysis in a panel that included both sertoli and mouse embryonic fibroblasts (MEFs) in addition to spermatogonia, pachytene, and round spermatids. X-linked Tsx, which is known to be highly expressed in germ cells and low in sertoli cells, was used as a positive control. As expected, Tsx expression was low in both sertoli and MEFs, and highest in pachytene spermatocytes (Supplemental Figure S1). Both the X-linked lncRNAs 1700019B21Rik (Tslrn1) and
Several novel lncRNAs show strong testis-specific expression. We examined the expression of several mRNAs and lncRNAs that show high expression at pachytene in several mouse tissues and identified a few mRNAs and lncRNAs that show very specific or enriched expression in the testis. Trpc5os also showed very low expression in sertoli and MEFs as compared to pachytene (Supplemental Figure S1). By contrast, the mRNA Trpc5 and the X-linked lncRNA Firre, which do not appear to escape MSCI, are both expressed at comparable levels in sertoli and pachytene spermatocytes (Supplemental Figure S1). Also, the Y-linked lncRNAs RP24-290B7.4 and RP24-382F19.1, which also appear to escape MSCI, are highly expressed at pachytene as compared to both sertoli and MEFs (Supplemental Figure S2). Taken together, these results suggest that several lncRNAs are possibly escaping MSCI, and that these results are not due to artifact caused by contamination of the pachytene spermatocyte population with round spermatids.

Testis-specific expression of long noncoding RNAs and mRNAs

We examined the expression of lncRNAs and mRNAs that appears to escape MSCI across multiple mouse tissues to determine if any of these transcripts are also testis-specific or testis-enriched, which would suggest a spermatogenesis-specific function. We first examined the expression of Dmrtc1b, Sowahd, and Mcf2 mRNAs in a panel of mouse tissues and found that both Dmrtc1b and Sowahd show strong testis-enriched expression, whereas Mcf2 does not (Figure 3, shown in red). Next, we examined the expression of several X- and Y-linked lncRNAs in the same panel and identified 1700019B21Rik, which we refer to as testis-specific lncRNA.
Figure 4. A few IncRNAs and mRNAs are highly expressed in male but not in female germ cells. The expression of key IncRNAs and mRNAs was examined in several mouse tissues including female ovaries at 18dpc when pachytene oocytes are enriched as compared to adult ovaries. We found two IncRNAs (Tslrn1 and Trppc5os) and two mRNAs (Sowahd and Dmrtc1b) to be highly expressed in male testis but in neither 18dpc nor adult ovaries suggesting potential male-specific functions.

1 (Tslrn1), and Trpc5os as testis-enriched transcripts, but expression of the three other IncRNAs did not appear to be testis-enriched (Figure 3, shown in green). To confirm that Trpc5os is indeed an independent antisense IncRNA from the coding Trpc5 mRNAs, we also examined the expression of Trpc5 mRNA in the same panel, and confirmed that Trpc5 mRNA shows an expression pattern that is distinct from that of Trpc5os IncRNA (Figure 3, compare Trpc5os to Trpc5 expression). In addition, we observed no expression of Tslrn1, Trpos5, Dmrtc1b, or Sowahd in ovaries from day 18 embryos, when pachytene cells are most prevalent, suggesting that these RNAs may serve a male-specific role in meiosis (Figure 4).

Lastly, we examined the expression of both SOWAHD and DMRTC1B in a panel of human tissues, and found that both genes are highly expressed in human testis; both are also expressed in several other human tissues revealing key differences between humans and mice in this respect (Supplemental Figure S3). In summary, these results demonstrate that some X-linked mRNAs and IncRNAs, which we identified as potential escapees from MSCI, are testis-enriched transcripts, suggesting potentially important functional roles for these transcripts in spermatogenesis and male fertility.

Tslrn1 knockout males are fertile but display significant reduction in sperm counts

To examine a possible role of one of the key IncRNAs identified in our gene expression analyses during spermatogenesis, we deleted the entire 16 kb transcribed region of Tslrn1 using the CRISPR/Cas9 genome editing system (Figure 5A). Out of 25 founders, we observed 5 mice (20%) with complete deletion of Tslrn1. We established two founder lines from these mice, designated as DEL1 and DEL2, and confirmed the absence of the Tslrn1 transcribed region through DNA sequencing and gene expression analyses (Figure 5B–E). Next, we investigated the effect of Tslrn1 absence on male fertility. We observed no difference in litter sizes produced by wildtype mice and mice from the DEL1 and DEL2 lines (Table 1). In addition, there were no significant differences in body weight, body length, or testis size among these groups. Testes histology revealed no differences between wildtype and Tslrn1 knockout lines (Figure 6A and B). Interestingly, however, while sperm motility and morphology were not different from wildtype mice (Figure 6C and D), sperm counts from both Tslrn1 knockout lines were reduced by ~20% compared to wildtype littermates (Table 1). The sperm counts were performed using 12–16-week-old mice and this reduction was consistent. These data demonstrate that loss of Tslrn1 IncRNA in males results in reduced sperm count without a reduction in litter size or major defects in testis histology.

Discussion

Previous studies have shown that the process of spermatogenesis is a complex cellular differentiation process that requires a dynamic gene
Figure 5. Creation and verification of a Tslrn1 knockout mouse. (A) Schematic of the deletion strategy of Tslrn1 using CRISPR/Cas9. Guide RNAs were selected within 600 bp of the first and last exon of Tslrn1 (gRNA1 and gRNA2, respectively) to completely delete the Tslrn1 transcribed region. Primers (P1–P4) were designed to detect the presence or absence of Tslrn1 sequence. (B) PCR primers were designed to amplify an approximately 650 bp product (P1 and P2) when Tslrn1 was absent and a 273 bp product when Tslrn1 is present (P3 and P4). (C) Two Tslrn1 deleted founder lines were identified and their sequence is shown compared to wildtype sequence. Guide RNA1 sequence (red capitalized bases) and gRNA2 sequence (green capitalized bases) as well as their respective PAM sequence (3 colored lowercase bases) are displayed for context of each deletion line (DEL1 and DEL2). (D) Sanger sequencing result of DEL1 line is shown. (E) Quantitative RT-PCR of Tslrn1 expression from testes of wildtype and knockout males from DEL1 and DEL2 founder lines. Relative quantities to wildtype 1 (WT1) testes are shown.
expression pattern of both coding and noncoding genes (mRNAs, microRNAs, and piRNAs) [36]. Based on our RNA-seq analysis of enriched populations of specific spermatogenic cell types, we have now demonstrated that lncRNAs are also dynamically expressed at specific stages of spermatogenesis. Although the functions of the vast majority of lncRNAs remain unknown, we anticipate that some of these lncRNAs play key roles in spermatogenesis and fertility. Indeed, a recent study in Drosophila has shown that some lncRNAs are required for spermatogenesis and/or fertility [9], suggesting that a subset of mammalian lncRNAs could also be essential for spermatogenesis.

In this study, we investigated the potential role of a testis-enriched lncRNA, Tslrn1, that also shows high expression in pachytene spermatocytes, by creating a knockout mouse. We found that male mice lacking Tslrn1 displayed a significant reduction in the amount of spermatozoa. This reduction had no apparent effect on fertility, as all Tslrn1 knockout mice were capable of siring litters and without reduction in litter size as compared to wildtype. One possibility to explain these observations is that other lncRNAs with redundant functions to Tslrn1 may compensate for Tslrn1 loss. Our findings are not completely surprising as previous studies have shown that the knock out (KO) of protein-coding genes with well-characterized functions in the testis can be dispensable for male fertility due to potential redundancy [37]. Thus, male germ cells may have compensatory mechanisms of some critical mRNAs and lncRNAs to progress through spermatogenesis. Lastly, the increased expression of Tslrn1 at pachytene during male meiosis and its absence at similar stages in female meiosis could indicate a male-specific function, which should be further evaluated. In addition, the function and mechanisms of Tslrn1 during spermatogenesis requires further characterization in future studies to fully understand how the KO of this lncRNA results in sperm reduction.

Meiotic sex chromosome inactivation is a key epigenetic process that occurs during prophase I of meiosis and results in repression of both X- and Y-chromosomes [27]. It was previously shown that some X-linked microRNAs escape MSCI [28,30]. Although the functions of these microRNA escapees are yet to be determined, these findings suggest that some microRNAs may regulate key genes required for the meiotic or postmeiotic progression of spermatogenesis. In this study, we determined that a subset of lncRNAs and

### Table 1. Body parameters and reproductive data of Tslrn1<sup>−/−</sup> male mice.

<table>
<thead>
<tr>
<th>Genotype (n)</th>
<th>Body&lt;sup&gt;a&lt;/sup&gt; weight (g)</th>
<th>Body&lt;sup&gt;a&lt;/sup&gt; length (cm)</th>
<th>Testis&lt;sup&gt;a&lt;/sup&gt; weight (mg)</th>
<th>Sperm&lt;sup&gt;a&lt;/sup&gt; count (millions)</th>
<th>Litter&lt;sup&gt;b&lt;/sup&gt; size</th>
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<tr>
<td>Tslrn1&lt;sup&gt;+/+&lt;/sup&gt; (8)</td>
<td>28.9 ± 1.9</td>
<td>9.23 ± 0.5</td>
<td>100.5 ± 18.7</td>
<td>22.5 ± 3.7</td>
<td>6.49 ± 1.8</td>
</tr>
<tr>
<td>Tslrn1&lt;sup&gt;−/−&lt;/sup&gt;-DEL1 (6)</td>
<td>27.7 ± 2.2</td>
<td>9.39 ± 0.5</td>
<td>113.8 ± 26.7</td>
<td>16.9 ± 1.5&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>6.20 ± 1.5</td>
</tr>
<tr>
<td>Tslrn1&lt;sup&gt;−/−&lt;/sup&gt;-DEL2 (6)</td>
<td>29.6 ± 1.8</td>
<td>9.51 ± 0.3</td>
<td>109.2 ± 5.9</td>
<td>17.6 ± 3.8&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>6.45 ± 2.1</td>
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<sup>a</sup>Values are means ± SD of mice aged 12–16-week-old.

<sup>b</sup>Values are means ± SD of at least 10 litters per genotype.

<sup>∗</sup>P ≤ 0.03 compared to Tslrn1<sup>+/+</sup>.

![Figure 6. Testis histology, sperm motility, and morphology of Tslrn1 knockout mice.](https://example.com/figure6)

**Figure 6.** Testis histology, sperm motility, and morphology of Tslrn1 knockout mice. Histology of the testis from (A) wildtype and (B) Tslrn1 knockout mice post hematoxylin and eosin staining (X200). (C) Sperm motility expressed as percent of sperms with progressive movement and (D) abnormal sperm morphology is calculated as percentage of total sperms examined from wildtype and two Tslrn1 knockout lines (DEL1 and DEL2).
a few mRNAs may also escape MSCI, suggesting a role of these genes during mammalian spermatogenesis. These findings should be confirmed in future studies using RNA Fluorescence In Situ Hybridization (RNA FISH). Although little is currently known about the functions of lncRNAs in mammalian meiosis, identification of such lncRNAs provides a shortlist of key candidates for functional studies.

In conclusion, our data support the need for further studies of lncRNAs that we and others have identified that show testis-specific and/or enrichment at specific stages during spermatogenesis and oogenesis. These studies may reveal new mechanisms governing gametogenesis and fertility in mammals, paving the way toward novel insights of fertility.

**Supplementary data**

Supplementary data are available at **BIOLR** online.

Supplemental information contains Supplemental Experimental Procedures, three figures, and one table that are available online. Supplemental Figure S1. lncRNAs that appear to escape MSCI show low expression in sertoli cells. qRT-PCR analysis of Tslr1 and Trpc5os in a panel that includes both sertoli and MEFs as well as spermatagonia, pachytene, and round spermatids demonstrate that both of these lncRNAs show low expression in sertoli cells and MEFs. By contrast, Firre lncRNA and Trpc5 mRNA are both expressed in sertoli cells.

Supplemental Figure S2. Two Y-chromosome encoded lncRNAs are highly expressed at pachytene with low expression in sertoli and MEFs. Quantitative RT-PCR of two Y-encoded lncRNAs, which appear to escape MSCI, shows very low expression in sertoli and MEFs.

Supplemental Figure S3. Sowahd mRNA is highly expressed in human testis. The expression of Sowahd and Dmrtc1b, which show high expression in mouse testis, is examined in a panel of human tissues. While both genes show very strong expression in mouse testis as compared to other tissues, they both are expressed in several human tissues including the testis suggesting key differences in regulation between human and mouse.

Supplemental Files S1–S4. These files contain RNA-seq analysis of mRNAs and lncRNAs expression in spermatogonia, pachytene, and round spermatids.

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Next generation RNA Sequencing (RNA-seq) was performed at the CWRU Genomics Core Facility. Tslr1 knockout mice were generated by the transgenic core facility at CWRU using the CRISPR-Cas9 technology.

**Conflict of Interest:** All authors declare that there is no conflict of interest to report.

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