Microinjection of antisense oligonucleotides into living mouse testis enables IncRNA function study

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Abstract

Background: Long non-coding RNAs (lncRNAs) have been the focus of ongoing research in a diversity of cellular processes. LncRNAs are abundant in mammalian testis, but their biological function remains poorly known.

Results: Here, we established an antisense oligonucleotides (ASOs)-based targeting approach that can efficiently knock down lncRNA in living mouse testis. We cloned the full-length transcript of lncRNA Tsx (testis-specific X-linked) and defined its testicular localization pattern. Microinjection of ASOs through seminiferous tubules in vivo significantly lowered the Tsx levels in both nucleus and cytoplasm. This effect lasted no less than 10 days, conducive to the generation and maintenance of phenotype. Importantly, ASOs performed better in depleting the nuclear Tsx and sustained longer effect than small interfering RNAs (siRNAs). In addition to the observation of an elevated number of apoptotic germ cells upon ASOs injection, which recapitulates the documented description of Tsx knockout, we also found a specific loss of meiotic spermatocytes despite overall no impact on meiosis and male fertility.

Conclusions: Our study detailed the characterization of Tsx and illustrates ASOs as an advantageous tool to functionally interrogate lncRNAs in spermatogenesis.

Keywords: IncRNA (long non-coding RNA), Tsx, ASO (antisense oligonucleotide), Knock down, Testis injection

Background

Of general curiosity, nearly 98% of the human genome does not have coding functions [1, 2]. Long non-coding RNAs (lncRNAs) are non-protein coding RNAs longer-than-200 nt which may evolve a regulatory function from ‘junk’ transcripts [3–5]. With the rapid identification of lncRNAs through deep sequencing comes the urgent demand for the characterization of their biological functions [6–8]. In mammals, transcription of lncRNAs is shown more widespread and dynamic in testis than other organs [9, 10]. Numerous novel lncRNA genes were discovered in spermatogenic cells, rapidly outpacing the rate of characterizing them [11–14]. Reasons behind this gap include the infrequent emergence of loss-of-function phenotype of lncRNAs as well as the lack of an ideal culturable germline affordable to large-scale lncRNA functional screening [15–17].

Employment of various methods such as CRISPR/Cas9-mediated genome editing, RNA interference (RNAi) or antisense oligonucleotides (ASOs) have been reported in lncRNA loss-of-function studies [18–21]. Although the CRISPR/Cas9 system removes transcripts thoroughly compared with the knockdown, it’s more...
laborious, costly and time-consuming. Also, CRISPR/Cas9 is not applicable in some cases. Unlike mRNA, lncRNA function is not prone to be influenced by small deletions or insertions [18, 22]. Moreover, genetic manipulation of lncRNAs is likely to perturb the overlapping or adjacent genes. To circumvent such problems, in vivo local knockdown by means of short-hairpin RNAs (shRNAs), small interfering RNAs (siRNAs) or ASOs may be a good alternative. In recent reports, testis transduction of shRNAs in adeno-associated viral (AAV) or lentiviral vectors are demonstrated successful in knockdown of either mRNAs [23, 24] or lncRNAs [14, 25]. siRNA microinjection to achieve transient knockdown effect in young testis has proved a simplified method to examine the miogenic function of protein-coding genes [26]. piRNAs can be targeted by ASOs with the aid of electrical transduction in testis [23]. Meanwhile, both siRNAs [27, 28] and ASOs [29–31] have been used for lncRNA knockdown in vivo other than in mouse testis.

ASOs, differing from siRNAs, are synthesized oligonucleotides with a length of 15–20 nt that can hybridize complementarily with an endogenous RNA to form DNA/RNA heteroduplex and then induce recruitment of the nucleus-enriched RNase H followed by target RNA cleavage [18, 32]. Pharmacokinetic studies in mice showed that ASOs have long half-lives that can last several weeks [33–35]. Interestingly, nucleus-localized lncRNAs are more easily targeted by ASOs. In knockdown assays on lncRNAs with different subcellular localization, the knockdown efficiency of ASOs for lncRNAs that primarily located in nucleus is higher than that of siRNAs [36]. Moreover, siRNAs-mediated knockdown affects lncRNAs expression solely at post-transcriptional level, while ASOs can also target upon nascent transcripts and block transcription [37]. In some cases, the transcription or splicing process from the transcribed locus rather than the mature lncRNA products confers the lncRNA functionality [7, 38, 39]. Degradation of transcripts carried out by ASOs, rather than siRNAs, can clarify the lncRNA function in its nascent or on-transcription form [37, 40]. As attractive as the ASO tool is for envisioning its being equivalently applicable in lncRNA knockdown in mammalian testis, a methodological establishment awaits a pioneering attempt.

Considering this technically practical idea a driver of many future investigations to understand lncRNA biological roles in spermatogenesis, we aimed to set up a framework initiated from validating the knockdown effectiveness of testis injection by ASOs through completing a battery of standard phenotypic examinations. To explore ASO application conditions, we first selected a widely expressed, highly nucleus-enriched lncRNA Malat1 [41]. For extending to function study, we knocked down a testis-specific lncRNA Tsx, which represents one rarely documented lncRNA yielding observable phenotype when knocked out in vivo [42, 43]. We defined localization of Tsx and characterized its potential function in sustaining the pool of meiotic germ cells. Comparative assessment of ASOs and siRNAs targeting Tsx highly recommends the former as a better tool for lncRNA function study in testis.

**Results**

**ASOs-mediated lncRNA knockdown is dose-dependent**

To obtain the optimal ASO injection conditions, we started to inject testis with Malat1 (Metastasis associated lung adenocarcinoma transcript 1) which is highly expressed in GC-2 cells and mouse testis [41]. We first examined the knockdown efficiency of two designed ASOs targeting Malat1 transcripts in GC-2 cells by performing fluorescence in situ hybridization (FISH) two days after injection. FISH results showed that the number of Malat1-positive cells was significantly reduced in GC-2 cells transfected with ASO-Malat1-1 (Additional file 1: Fig. S1). Subsequently, we injected ASO-Malat1-1 into testes of 3-week-old mice and expression of Malat1 was measured by RT-PCR at post-injection 2 and 7 days, respectively. The dilutions of ASOs were directly injected from the ductuli efferentes into seminiferous tubules (Fig. 1A). ASOs with Trypan blue as an indicator filled nearly two third of seminiferous tubules after injection (Fig. 1B). Results of RT-PCR showed that nearly 80% knockdown efficiency was tested after 2 days of injection at the dosage of 0.08 nmol (Fig. 1C). However, after 7 days, the expression of Malat1 returned to normal levels (Fig. 1C). To maintain the knockdown effect for a longer period of time, we increased the ASO dosage of initial injection. A maximum volume (4 µL) of higher amounts of ASOs (0.2–1 nmol) were injected into the testis. More than 1 nmol ASOs would become too viscous to be injected fluently. We collected mouse testis 7 days later for RT-PCR test and determined the range of 0.4–1 nmol ASOs that maintained the knockdown efficacy, with no use for cholesterol modification on ASOs (Fig. 1C).

Next, we turned to test another lncRNA Tsx (testis-specific X-linked). We started with cloning the full-length Tsx by 5’-end and 3’-end rapid amplification of cDNA ends (RACE) and identified a 818-nt transcript in mouse testis (Additional file 1: Fig. S2). Three different Tsx-targeting ASOs were injected, among which ASO-Tsx-2 was adopted for its highest knockdown efficiency (Fig. 1D). To confirm the best amount for ASO injection, we compared two different amounts (0.5 nmol and 1 nmol) and assessed separately their 10-day knockdown efficiency by FISH. Nearly half tubules of testes injected at 0.5 nmol showed no reduction of Tsx, while over 80% of the
tubules showed dramatic reduction at 1 nmol (Fig. 1E).

No obvious artificial effect was observed between control and Tsx-knockdown testis sections. Therefore, we selected 1 nmol ASO-Tsx-2 for subsequent experiments.

Tsx is distributed in both nucleus and cytoplasm

Before further evaluating knockdown effect on Tsx, we characterized its expression and localization in postnatal testes. RT-PCR showed that Tsx began to express stably at high levels from 2nd week (2 W) after birth through the 8-week-old (8 W) adulthood. (Additional file 1: Fig. S3A).

We then performed co-localization assays by Tsx FISH in combination with SOX9 immunofluorescence (IF) on seminiferous tubule sections. The results showed that the punctate signals of IncRNA Tsx were partially within the SOX9-positive nucleus of Sertoli cells (Fig. 2A). The rest of Tsx signals spread over from the basal lamina toward lumen, possibly along the cytoplasmic area of Sertoli cells which spaces between germ cells. This speculation was confirmed by co-staining of the Sertoli cytoplasmic marker Vimentin (Fig. 2B). Clearly, Tsx were also shown as individual dots within the SYCP3-marked nucleus of
spermatocytes in 2-week-old testis (Additional file 1: Fig. S3B, indicated by arrows). Moreover, only a few Tsx signals overlapped with the germ cell marker DDX4 (Additional file 1: Fig. S3C, indicated by arrows), consolidating that the majority of Tsx molecules located in the cytoplasmic territory of Sertoli cells but not that of germ cells. Together, Tsx is a nucleocytoplasmic IncRNA in testis.

**ASOs, but not siRNAs, persistently inhibit Tsx expression in vivo**

Next, we designed three pieces of siRNAs sequences, and selected one with highest knockdown efficiency for a comparison with ASOs (Fig. 3A). Two days after injection into 3-week-old testes, preparation of subcellular fractions followed by RT-PCR analysis showed better performance of ASOs in nucleus, while both ASOs and siRNAs were able to significantly reduce the cytoplasmic Tsx levels (Fig. 3B). Co-localization of Tsx and Vimentin confirmed the presence of considerable amounts of Tsx signals in the nucleus of siRNAs instead of ASOs (Fig. 3C, indicated by arrows). For the purpose of achieving sufficient generation of phenotype, we imposed a possibly greatest impact of loss of Tsx on germ cell differentiation by prolonging post-injection treatment of testis up to 10 days. As a result, Tsx levels were suppressed around 60% by ASOs, in contrast with no significance by siRNAs (Fig. 3D). Parallel examination by FISH gave rise
Fig. 3  Comparative evaluation of ASOs and siRNAs in testis. A The RNA level of Tsx detected by RT-PCR in control and knockdown testis treated with three different siRNAs. **p < 0.01, ***p < 0.001, n = 3. B RT-PCR analysis of Tsx expression in cytoplasm and nucleus 2 days after injection of si-Tsx-2 or ASO-Tsx-2. *p < 0.05, **p < 0.01, n = 3. C FISH of Tsx RNA (green) with IF of Vimentin (red). Testes were collected 2 days after injection of siRNAs or ASOs. Nuclei were stained with DAPI. Nuclei of Sertoli cells were indicated by white arrows. Scale bar, 20 μm. D RT-PCR results of Tsx RNA level 10 days after injection of si-Tsx-2 or ASO-Tsx-2. ****p < 0.0001, n = 3. E FISH images of Tsx RNA in testes 10 days after injection of si-Tsx-2 or ASO-Tsx-2. Nuclei were stained with DAPI. Scale bar, 50 μm. A, B, D Values are expressed as mean ± SD. Statistical significance was determined using t tests.
to consistent results (Fig. 3E), suggested that ASOs may have more sustainable knockdown effect in vivo than siRNAs.

**Tsx inhibition by ASOs raises germ cell defects**

To obtain the stable outcome of *Tsx* loss-of-function during spermatogenesis, we uniformed a workable time window from executing ASOs injection at postnatal day-21, when meiotic spermatocytes are relatively enriched, to ceasing ASO treatment after 10 days, which represents developmental length of the prophase I of meiosis [44]. TUNEL assays showed a statistically significant rise of apoptotic cells in ASO- (Fig. 4A), but not in siRNAs-injected testis (Fig. 4B). Our ASO results coincide with the previous description of *Tsx*-knockout mice [43], supporting ASOs a better tool for *Tsx* knockdown than siRNAs. To investigate whether *Tsx* plays a role in meiotic progression, we counted the average numbers of four types of spermatocytes during meiotic prophase I in each seminiferous tubule. Each cell type can be identified based on its correspondence to the specific stages of seminiferous cycle by co-staining of SYCP3, a marker for core composition of synaptonemal complex, and PNA, a marker for acrosome (Fig. 4C, D). Compared with

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**Fig. 4** Physiological outcomes of *Tsx* ASOs in testis. **A, B** TUNEL staining and quantification of TUNEL+ cells in tubules from *Tsx*-knockdown mice at age of 3 weeks injected with ASOs (A) or siRNAs (B). Nuclei were stained with DAPI. Scale bar, 50 μm. Relative number of TUNEL+ cells is determined by counting the number of TUNEL+ cells per field and dividing by the number of tubules in each field. 37 fields including 150 tubules from ASOs-mediated *Tsx*-knockdown testes, 31 fields including 125 tubules from ASO-control testes, 21 fields including 100 tubules from siRNAs-mediated *Tsx*-knockdown testes and 30 fields including 111 tubules from siRNA-control were counted. Each contains three independent samples. *p < 0.05. **C** Quantification of four cell populations between control and *Tsx*-knockdown testes. Number of each cell population per tubule is shown in dot. 81 tubules from 3 tests injected with ASO-*Tsx* and 83 tubules from 3 tests injected with control ASO were analyzed. *p < 0.05. **D** Immunostaining of SYCP3 (green) and PNA (red) on testis sections from control and *Tsx*-knockdown mice. Seminiferous tubule sections were staged by morphology of chromosome and acrosome. Scale bar, 20 μm. **A–C** Values are expressed as mean ± SD. Statistical significance was determined using t-tests.
control, ASO injection resulted in a statistically significant reduction of zygotene and diploteu spermatocytes (Fig. 4C), albeit no stage exhibited evident morphological abnormality (Fig. 4D). Further, we asked whether loss of Tsx affected meiotic chromosome synopsis and recombination. Various stages of spermatocytes were identified through chromosome spread analysis via co-staining of SYCP1 and SYCP3. No significant defects were observed in the synopsis of homologous chromosomes (Additional file 1: Fig. S4A). Staining of MLH1 which marks the site of crossover at late pachytene also showed no difference (Additional file 1: Fig. S4B, C), neither was the change indicated by the number of SOX9-positive Sertoli cells where most Tsx molecules reside (Additional file 1: Fig. S5A, B). Thus, Tsx seems not required for the normal progression of spermatogenesis, whereas it plays a certain role in the balanced maintenance of germ cells among different types as well as their viability.

To further examine the functional consequence of spermatogenesis from mild defects of germ cells, we evaluated the potential effects on litter size resulting from ASOs-mediated Tsx knockdown. Prior to mating test, we prepared another cohort of mice microinjected either with ASO-control or ASO-Tsx at 6-week-old. After 10 days of the post-surgery recovery, we performed mating test by either crossing control males or Tsx-knockdown males with adult wild-type females. Male fertility can be tested when mice reach 7-8 weeks old when germ cells differentiate into mature sperm, explaining the fertility of these animals. Moreover, we added two stringent control groups (surgical group and non-surgical group) to exclude the potential side-effects of surgical procedures on the litter size, although this problem might be minimized through the post-surgery recovery. RT-PCR results demonstrated that the transcript levels of IncRNA Tsx was significantly reduced in males microinjected with ASO-Tsx at 6-week-old, compared to that of control group (Additional file 1: Fig. S6A). Further analysis of IncRNA Tsx by FISH confirmed a decrease in the expression of IncRNA Tsx (Additional file 1: Fig. S6B). Moreover, TUNEL assay revealed an increase in germ cells undergoing apoptosis (Additional file 1: Fig. S6C, D). As expected from the previous genetic IncRNA Tsx knockouts [43], males with Tsx knockdown through ASO microinjection were able to father litters and displayed normal mating behavior with no effect on the litter size or sex ratios (Additional file 1: Fig. S6E, F). These experiments demonstrated that mice with ASOs-mediated Tsx knockdown exhibit defects in germ cell, which is phenotypically similar to the IncRNA Tsx knockouts, although either ASOs-mediated Tsx knockdown or Tsx knockout has no apparent effects on litter size. It’s because the phenotype observed in Tsx knockout is mild, evidenced by the abundant sperm present in Tsx-deficient mice. It is understandable that the remaining sperm in mice either with Tsx knockdown or Tsx knockout is sufficient to produce litters. Nonetheless, our ASOs-based in vivo knockdown approach technically enables a knockout-comparable, complete function study of IncRNA in mouse testis.

Discussion
It remains mysterious how many IncRNAs act usefully in spermatogenesis. In Drosophila, large-scale knock-out screening has yielded dozens of IncRNAs with visual spermatogenic defects [45]. In mice, however, such strategy is neither practicable nor worthy, setting aside that not all types of IncRNAs can be specifically or completely knocked out [22, 46]. Few reports have identified functional IncRNAs via creation of individual knockout mouse models [43] or using shRNA-mediated knockdown [14]. Unlike shRNA, ASO injection avoids intricate procedures of virus packaging. Common administration ways for ASOs such as intravenous fusion and subcutaneous injection allow for repeated administration [35], which is impractical for seminiferous injection in testis. While our understanding of the in vivo IncRNA physiology stays at its infancy, here we have developed a protocol for microinjecting testis with ASOs to interrogate the function of any individual IncRNAs of interest.

Taking Tsx as an example, ASOs were demonstrated to execute high knockdown efficiency in 3-week-old testis so that testicular cells were largely depleted of its signals and exhibited an induction of apoptosis resembling the described Tsx knockout [43]. Analysis of Tsx levels in subcellular fractions showed that ASOs have better performance than siRNAs in knocking down the nucleus-localized IncRNAs, due possibly to the higher accessibility of ASOs to the nucleus [36], and its unique capability of targeting IncRNAs being transcribed [37]. Compared to siRNAs, moreover, ASOs sustained its effect till post-injection 10 days, a duration long enough to ensure adequate phenotypic analysis. These comparative evaluations support ASOs a better choice for making a knockdown model of IncRNA in living testis. In our study, this Tsx-deficiency testis model was used to view the alteration of Tsx levels in specific cell types and in different subcellular compartments by FISH and IF double staining. Tsx was previously reported to be distributed at the periphery of the tubule where Sertoli cells usually locate by In situ hybridization (ISH) [42]. We found that Tsx is a nucleocytoplasmic IncRNA mainly expressed in Sertoli cells and minorly in germ cells. This model was also used to investigate the meiotic defect by employing standard immunofluorescence analyses of either seminiferous sections or chromosome spreads.
Our data revealed the involvement of Tsx in maintaining a balanced pool of spermatocytes. Although mating test results did not reflect the impact of Tsx on the differentiating germ cells, the establishment of knockdown effectiveness in 6-week-old testis as well as exclusion of possible side-effect on mating behavior posed by surgical operation itself has broadened the range of functional assessment of IncRNA to cover male fertility assay. In sum, ASOs-based IncRNA-targeting method, in junction with various follow-up experiments, will facilitate a cascade of investigations to understand the IncRNAs’ roles in mammalian male reproduction.

In recent years, RNA therapeutics based on siRNAs, ASOs and CRISPRi (CRISPR interference) that target transcripts of the genes have emerged. CRISPRi is developed from CRISPR system that has been successfully used in preclinical animal tests and ex vivo modification of iPSCs for transplantation [47–49]. Compared to the infancy of clinical application of CRISPRi, drugs based on siRNAs and ASOs have emerged and been reported to be used in phase III trials [50–52]. Specifically, unlike siRNAs which need a delivery agent, ASOs can be taken up by cells without any delivery agents and has lower biological toxicity. These merits make ASOs more applicable in clinical trials [53–55]. Besides, ASOs can act in the nucleus and intervene in transcription, which makes ASOs widely used in pre-clinical studies of single-gene disorders [56]. The method we established for ASOs-mediated testis knockdown in vivo also offers possibility for pre-clinical application of ASOs in humans with regard to male contraception or improvement of reproductive health [57–59].

Conclusions

Here we have established an ASO microinjection-initiated procedure that is capable of creating a testis-specific knockdown model for IncRNA. Using this model, along with other experiments, we show that: (1) ASOs efficiently knocked down both the IncRNAs Malat1 and Tsx; (2) Tsx is a nucleocytoplasmic IncRNA in testicular cells; (3) Tsx can be efficiently and persistently acted upon by ASOs rather than siRNAs; (4) Tsx deficiency led to a disturbance to the maintaining of meiotic germ cell pool.

Materials and methods

Mice, ASOs, siRNAs and antibodies

C57BL/6 mice were obtained from the Model Animal Research Center of Nanjing University. All mice were housed with 12/12 h light/dark cycles, at 22 °C and allowed free access to water and food.

ASOs and siRNAs were synthesized by Ribobio (Guangzhou, China). Specific sequences are provided in Additional file 2: Table S1. ASOs used in Fig. 1C were diluted with nuclease-free water to different concentrations within 20-500 μM, and injected into the testes of 3-week-old mice with the maximum injection volume (4 μL, with total amount of 1 nmol ASO). Injection volume was increased to 6 μL with total amount of 2.5 nmol ASO when we operated on 6-week-old mice. siRNAs we used in this article were chemically modified with 2′-OM (2-methoxyethyl) and cholesterol to improve the stability and biodistribution [53].

Primary antibodies used in this article are listed as follows: rabbit anti-Vimentin (ab92547, Abcam, USA), rabbit anti-SOX9 (AB5535, Merck Millipore, German), rabbit anti-DDX4 (ab13840, Abcam, USA) and rabbit anti-SYCP3 (ab15093, Abcam, USA), rabbit anti-SYCP1 (ab15090, Abcam, USA), rabbit anti-MLH1 (550,838, BD, USA).

In vivo knockdown through microinjection

Microinjection of ASOs and siRNAs were operated as previously described [60]. Mice of 3-week-old were anesthetized by tri-bromoethanol and one testis was exteriorized through incisions on abdomen. A mixture of 3 µL (5 µL for 6-week-old mice) of ASO dilutions or siRNA dilutions and 1 µL tracer Trypan blue was injected into seminiferous tubule through the microinjection apparatus (FemtoJet 4i, Eppendorf) under a stereoscopic microscope. The testis was placed back to the abdominal cavity after injection. After both sides of testes were finished with injection, the incisions were closed with sutures. Each injected mouse was kept warm by putting hot water bag in cage until they wake up.

RNA extraction and RT-PCR

Testes were collected 48 h or 10 days after injection to measure knockdown efficiency. Total RNA was extracted using TRIZol reagent (Thermo Fisher Scientific, USA) following the manufacturer’s instructions. 1 μg RNA was reversed transcribed into cDNA with PrimeScript RT Master Mix (RR036A, TaKaRa, Tokyo, Japan) according to the manufacturer’s instructions. Real time PCR was performed using TB Green Premix Ex Taq II (RR820A, TaKaRa, Tokyo, Japan) on Applied Biosystems StepOne-Plus Real-Time PCR System. Relative expression of target RNA was determined after normalization to 36B4 gene. The sequences of all primers used in this experiment are provided in Additional file 2: Table S1.

Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from C57BL/6 mouse testis. 5′ RACE and 3′ RACE were performed using the SMARTer RACE 5′/3′ Kit (Clontech, Mountain View, CA) according to the manufacturer’s instructions. The following gene-specific primers (GSP) are used for PCR:
5′-TGGCAAGCAAAACACCCCTAGTTGGC-3′ (5′ RACE GSP);
3′-CTTGGGTATCGCTCCAAACAAGGT-3′ (3′ RACE GSP).

Fluorescence in situ hybridization (FISH) and immunofluorescence
Testes were fixed in 4% PFA overnight at 4 °C, dehydrated by graded ethanol (70%, 95%, 100%) and embedded in paraffin. Embedded testes were sectioned into 5 µm. FISH was performed with RNAscope® multiplex fluorescent reagent kit (Advanced Cell Diagnostics, USA) as manufacturer’s instructions [61]. Target probes were obtained from RNAscope®. The last procedure of FISH, sections were treated in 1×PBS for 5 min and then blocked in blocker (10% FBS, 1% BSA, 1% Triton X-100, 0.05% Tween-20) for 1 h at room temperature (RT). Testis sections were incubated with the following antibodies at 4 °C overnight: rabbit anti-Vimentin (1:250), rabbit anti-SOX9 (1:400), rabbit anti-MLH1 (1:100), rabbit anti-SYCP3 (1:100). Texas red or FITC-conjugated secondary antibodies (Vector Laboratories, USA) were incubated at 37 °C for 1 h and sections were washed 3 times in PBST (1×PBS containing 0.2% Tween-20). Samples were mounted in microslide shield with DAPI. Fluorescent signals were detected on a confocal microscope (LSM800, Carl Zeiss, German).

Isolation of nuclear and cytoplasmic fractions
Subcellular extracts were prepared as described [60]. 30-40 mg testis tissue was homogenized in 400 µL Cytoplasmic Extraction Buffer [CT Buffer, 250 mM sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 1 mM EGTA, 1×protease inhibitor cocktail III, 0.4 µL RNasin (N251B, Promega, USA)] with 100 strokes. The lysis was centrifuged at 300×g for 5 min, and the supernatant was collected as cytoplasmic fraction. Add 1 mL TRizol reagent per 200 µL supernatant and stored at –80 °C. The pellet seen as nuclei fraction was washed three times in 500 µL CT Buffer and centrifuged at 300×g for 5 min. Add 1 mL TRizol homogenize the pellet by 1 mL syringe with 0.4 mm needle. RNA was extracted as above.

TUNEL assays, chromosome spread and immunofluorescence
TUNEL was performed on the testis sections with TUNEL BrightGreen Apoptosis Detection Kit (A112-01, Vazyme, China) according to the manufacturer’s instructions.

Chromosome spread of prophase I spermatocytes were performed as previous described [62]. The following primary antibodies were used in morphology analysis of chromosome: rabbit anti-SYCP1 (1:100), rabbit anti-SYCP3 (1:100), rabbit anti-MLH1 (1:100). Slides were washed in 1×PBS for 5 min, and then blocked with 10% goat serum in PBST (1×PBS containing 0.1% Tween 20) for 1 h at RT. After incubated with primary antibodies overnight at 4 °C, slides were treated with PBST for 3 times. Secondary antibodies were incubated at 37 °C for 1 h. Wash slides in PBST for 3 times. All the slides were mounted in microslide shield with DAPI. Immunofluorescence for all samples was examined under laser scanning confocal microscope (LSM800, Carl Zeiss, German).

Mating test
Twenty-one mating cages were divided into three mating groups: Wild-type (WT) males with WT females, ASO-control males with WT females, and ASO-Tsx males with WT females. Surgical operation was performed on males at 6-week-old. ASOs-injected males were housed with two WT females after 10 days of post-surgery recovery.

Statistical analysis
All values were presented as mean ± SD. Statistical analysis was performed with Student’s t test (*p<0.05; **p<0.01; ***p<0.001) using Prism 7.0 (GraphPad Software, La Jolla, CA, USA). NS means not significant.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13578-021-00717-y.

Additional file 1: Fig. S1. FISH images of Malat1 on GC-2 cells. Fig. S2. 5′ and 3′ RACE results. Fig. S3. Tsx expression in germ cells. Fig. S4. Analysis of impact of Tsx deficiency on meiosis. Fig. S5. Analysis of impact of Tsx deficiency on Sertoli cells. Fig. S6. Functional impact of Tsx knockdown on male fertility.

Additional file 2: Table S1. Sequences of oligonucleotides used in this study.

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Authors’ contributions
KZ, YL and C-YH conceived and supervised the project. Z-HC, LL, X-LS, WL, H-CZ, LY and KZ designed and performed most experiments. Z-LK, B-JZ, J-QZ, S-NY, HW, L-XT and JN contributed to some experiments. KZ and Z-HC wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional information files.
Declarations

Ethics approval and consent to participate
All experiments involving mice were conducted according to the guidelines of the Institutional Animal Care and Use Committee of Nanjing Medical University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, et al. Landscape of transcription in human cells. Nature. 2012;489(7414):101–8.
2. Pennisi E. Genomics. ENCODE project writes eulogy for junk DNA. Science. 2012;337(6099):1159.
3. Palazzo AF, Koomin EV. Functional Long Non-coding RNAs Evolve from Junk Transcripts. Cell. 2020;183(5):1151–61.
4. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell. 2009;136(4):629–41.
5. Yao RW, Wang Y, Chen LL. Cellular functions of long noncoding RNAs. Nat Rev Cancer. 2019;19(5):542–51.
6. Iyer MK, Niknafs YS, Malik R, Singhal U, Sahu A, Hosono Y, et al. The landscape of long noncoding RNAs in the human transcriptome. Nat Genet. 2015;47(3):199–208.
7. Kopp F, Mendell JT. Functional classification and experimental dissection of long noncoding RNAs. Cell. 2018;172(3):399–407.
8. Qian X, Zhao J, Yeung PY, Zhang QC, Kwok CR. Revealing IncRNA Structures and Interactions by Sequencing-Based Approaches. Trends Biochem Sci. 2019;44(1):33–52.
9. Sounimlon M, Becuwe A, Weier M, Brawand D, Zhang X, Gu H, et al. Cellular source and mechanisms of high transcriptome complexity in the mammalian testis. Cell Rep. 2013;6(3):2179–90.
10. Watanabe T, Cheng EC, Zhong M, Lin H. Retrotransposons and pseudogenes regulate miRNAs and IncRNAs via the piRNA pathway in the germline. Genome Res. 2015;25(3):368–80.
11. Trovero MF, Rodriguez-Casuriaga R, Romeo C, Santinaque FF, Francois M, Folle GA, et al. Revealing stage-specific expression patterns of long noncoding RNAs in the mouse testis. RNA Biol. 2020;17(3):350–65.
12. Bao J, Wu J, Schuster AS, Hennig GW, Yan W. Expression profiling reveals developmentally regulated IncRNA repertoire in the male mouse germline. Biol Reprod. 2013;88(3):107.
13. Hong SH, Kwon JT, Kim J, Jeong J, Kim J, Lee S, et al. Profiling of testis-specific long noncoding RNAs in mice. BMC Genomics. 2018;19(1):539.
14. Li K, Xiu B, Luo Y, Zou D, Han R, Zhong S, et al. Panoramic transcriptome analysis and functional screening of long noncoding RNAs in mouse spermatogenesis. Genome research. 2021;31(1):13–26.
15. George MR, Duan Q, Nagle A, Kathinya IS, Huang Y, Rao K, et al. Minimal in vivo requirements for developmentally regulated cardiac long intergenic non-coding RNAs. Development. 2019;146(3).
16. Goudarzi M, Berg K, Pieper LM, Schier AF. Individual long non-coding RNAs have no overt functions in zebrafish embryogenesis, viability and fertility. Elife. 2019;8:9.
17. Richer G, Baert Y, Goossens E. In-vitro spermatogenesis through testis modelling: Toward the generation of testicular organoids. Andrology. 2020;8(4):870–91.
18. Liu SJ, Lim DA. Modulating the expression of long non-coding RNAs for functional studies. EMBO Rep. 2018;19:12.
19. Stojic L, Lun ATL, Mangei J, Mascalchi P, Quarto V, Barr AR, et al. Specificity of RNAi, LNA and CRISPRs as loss-of-function methods in transcriptional analysis. Nucleic Acids Res. 2018;46(12):S950–66.
20. Lucre M, O’Malley MWR, Diermeier SD. Functional Screening Techniques to Identify Long Non-Coding RNAs as Therapeutic Targets in Cancer. Cancers (Basel). 2020;12:12.
21. Stojic L, Lun ATL, Mascalchi P, Ernst C, Redmond AM, Mangei J, et al. A high-content RNA screen reveals multiple roles for long noncoding RNAs in cell division. Nat Commun. 2020;11(1):1851.
22. Li L, Chang HY. Physiological roles of long noncoding RNAs: insight from knockout mice. Trends Cell Biol. 2014;24(10):594–602.
23. Gou LT, Dai P, Yang JH, Yue Y, Hu YP, Zhou Y, et al. Pachytyene piRNAs instruct massive mRNA elimination during late spermiogenesis. Cell Res. 2014;24(6):680–700.
24. Dai P, Wang X, Gou LT, Li ZT, Wen Z, Chen ZG, et al. A Translation-Activating Function of MV2piRNA during Mouse Spermiogenesis. Cell. 2019;179(7):1566–81 e16.
25. Lei Q, Pan Q, Li N, Zhou Z, Zhang J, He X, et al. H19 regulates the proliferation of bovine male germ line stem cells via Igf-1 signaling pathway. J Cell Physiol. 2018;234(1):915–26.
26. Dai J, Vologoshin O, Potapova S, Camerini-Otero RD. Meiotic Knockdown and Complementation Reveals Essential Role of RAD51 in Mouse Spermatogenesis. Cell reports. 2017;18(5):1383–94.
27. Yu Q, Qiu Y, Wang X, Tang J, Liu Y, Mei L, et al. Efficient siRNA transfer to knockdown a placenta specific IncRNA using RGD-modified nano-liposome: A new preeclampsia-like mouse model. Int J Pharm. 2018;546(1–2):115–24.
28. Sun T, Cheng YT, Yan LX, Krittawong C, Qian W, Zhang HJ. IncRNA MALAT1 knockdown alleviates myocardial apoptosis in rats with myocardial ischemia-reperfusion through activating PI3K/AKT signaling pathway. Eur J Med Pharmacol. 2019;23(23):10523–31.
29. Crooke ST, Wang S, Vickers TA, Shen W, Liang XH. Cellular uptake and trafficking of antisense oligonucleotides. Nat Biotechnol. 2017;35(5):230–7.
30. Wheeler TM, Leger AJ, Pandey SK, MaclLeod AR, Nakamura M, Cheng SH, et al. Targeting nuclear RNA for in vivo correction of myotonic dystrophy. Nature. 2012;488(7409):111–5.
31. Xiu B, Chi Y, Liu L, Chi W, Zhang Q, Chen J, et al. LINC002273 drives breast cancer metastasis by epigenetically increasing AGR2 transcription. Mol Cancer. 2019;18(1):187.
32. Wu H, Lima WF, Zhang H, Fan A, Sun H, Crooke ST. Determination of the role of the human RNase H1 in the pharmacology of DNA-like antisense drugs. J Biol Chem. 2004;279(17):17181–9.
33. Yu RZ, Kim TW, Hong A, Watanabe TA, Gaus HJ, Geary RS. Cross-species pharmacokinetic comparison from mouse to man of a second-genera-
tion antisense oligonucleotide, ISIS 301012, targeting human apolipoprotein B-100. Drug Metab Dispos. 2007;35(3):460–8.
34. Rigo F, Chuan SJ, Norris DA, Hung G, Lee S, Matson J, et al. Pharmacology of a central nervous system delivered 2′-O-methylxethyl-modified sur-
vival of motor neuron splicing oligonucleotide in mice and nonhuman primates. J Pharmacol Exp Ther. 2014;350(1):46–55.
35. Geary RS, Norris D, Yu R, Bennett CF. Pharmacokinetic, biodistribution and cell uptake of antisense oligonucleotides. Adv Drug Deliv Rev. 2015;87:46–51.
36. Lennox KA, Behlke MA. Cellular localization of long non-coding RNAs affects silencing by RNAi more than by antisense oligonucleotides. Nucleic Acids Res. 2016;44(2):863–77.
37. Lee JS, Mendell JT. Antisense-Mediated Transcript Knockdown Triggers Premature Transcription Termination. Mol Cell. 2020;77(5):1044–54.
38. Latos PA, Pauler FM, Koerner MV, Senenig HB, Hudson QJ, Stocis RR, et al. Aim transcriptional overlap, but not its IncRNA products, induces impaired Igf2r silencing. Science. 2012;338(6113):1469–72.
39. Engreitz JM, Haines JE, Perez EM, Munson G, Chen J, Kane M, et al. Local regulation of gene expression by IncRNA promoters, transcription and splicing. Nature. 2016;539(7629):1863–70.
40. Luo S, Lu HY, Liu L, Yin Y, Chen C, Han X, et al. Divergent IncRNAs Regulate Gene Expression and Lineage Differentiation in Pluripotent Cells. Cell Stem Cell. 2016;18(5):637–52.
its transcription plays a cis-regulatory role in the adult. Cell Rep. 2012;2(1):111–23.

42. Cunningham DB, Segretain D, Arnaud D, Rogner UC, Avner P. The mouse Tsx gene is expressed in Sertoli cells of the adult testis and transiently in premeiotic germ cells during puberty. Dev Biol. 1998;204(2):345–60.

43. Anguera MC, Ma W, Clift D, Namekawa S, Kelleher RJ, Lee JT. Ttx produces a long noncoding RNA and has general functions in the germline, stem cells, and brain. PLoS Genet. 2011;7(9):e1002248.

44. Goetz P, Chandley AC, Speed RM. Morphological and temporal sequence of meiotic prophase development at puberty in the male mouse. J Cell Sci. 1984;65:249–63.

45. Wen K, Yang L, Xiong T, Di C, Ma D, Wu M, et al. Critical roles of long noncoding RNAs in Drosophila spermatogenesis. Genome Res. 2016;26(9):1233–44.

46. Goyal A, Myacheva K, Gross M, Klingenberg M, Duran Arque B, Diederichs S. Challenges of CRISPR/Cas9 applications for long non-coding RNA genes. Nucleic Acids Res. 2017;45(3):e12.

47. Liu SJ, Horibuchi MA, Cho SW, Birk HS, Malataste M, He D, et al. CRISPR-based genome-scale identification of functional long noncoding RNA loci in human cells. Science. 2017;355:6320.

48. Peddle CF, Fry LE, McClements ME, Maclaren RE. CRISPR Interference-Potential Application in Retinal Disease. Int J Mol Sci. 2020;21:7.

49. Kampmann M. CRISPR-based functional genomics for neurological disease. Nat Rev Neurol. 2020;16(9):465–80.

50. Adams O, Gonzalez-Duarte A, O’Riordan WD, Yang CC, Ueda M, Kristen AV, et al. Patisiran, an RNAi Therapeutic, for Hereditary Transthyretin Amyloidosis. N Engl J Med. 2018;379(1):11–21.

51. Rinaldi C, Wood MJA. Antisense oligonucleotides: the next frontier for treatment of neurological disorders. Nat Rev Neurol. 2018;14(1):9–21.

52. Dorsett Y, Tuschl T. siRNAs: applications in functional genomics and potential as therapeutics. Nat Rev Drug Discov. 2004;3(4):318–29.

53. Dowdy SF. Overcoming cellular barriers for RNA therapeutics. Nat Biotechnol. 2017;35(3):222–9.

54. Inguscio S, Verlengia G, Soukupova M, Zucchi S, Simonato M. Gene Therapy Tools for Brain Diseases. Front Pharmacol. 2019;10:724.

55. Wild EJ, Fabrizi SJ. Therapies targeting DNA and RNA in Huntington’s disease. Lancet Neurol. 2017;16(10):837–47.

56. Beaudet AL, Meng L. Gene-targeting pharmaceuticals for single-gene disorders. Hum Mol Genet. 2016;25(R1):R18–26.

57. Thirumalai A, Page ST. Recent Developments in Male Contraception. Drugs. 2019;79(1):11–20.

58. Rolland AD, Evrard B, Darde TA, Le Beguec C, Le Bras Y, Bensalah K, et al. RNA profiling of human testicular cells identifies syntenic IncRNAs associated with spermatogenesis. Hum Reprod. 2019;34(7):1278–90.

59. Joshi M, Rajender S. Long non-coding RNAs (lncRNAs) in spermatogenesis and male infertility. Reprod Biol Endocrinol. 2020;18(1):103.

60. Fu K, Tian S, Tan H, Wang C, Wang H, Wang M, et al. Biological and RNA regulatory function of MOV10 in mammalian germ cells. BMC biology. 2019;17(1):39.

61. Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn. 2012;14(1):22–9.

62. Luo M, Yang F, Leu NA, Landaiche J, Handel MA, Benavente R, et al. MEIOB exhibits single-stranded DNA-binding and exonuclease activities and is essential for meiotic recombination. Nat Commun. 2013;4:2788.