A Testis-Specific Long Noncoding RNA, Start, Is a Regulator of Steroidogenesis in Mouse Leydig Cells

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The testis expresses many long noncoding RNAs (lncRNAs), but their functions and overview of lncRNA variety are not well understood. The mouse Prss/Tessp locus contains six serine protease genes and two lncRNAs that have been suggested to play important roles in spermatogenesis. Here, we found a novel testis-specific lncRNA, Start (Steroidogenesis activating IncRNA in testis), in this locus. Start is 1822 nucleotides in length and was found to be localized mostly in the cytosol of germ cells and Leydig cells, although nuclear localization was also observed. Start-knockout (KO) mice generated by the CRISPR/Cas9 system were fertile and showed no morphological abnormality in adults. However, in adult Start-KO testes, RNA-seq and qRT-PCR analyses revealed an increase in the expression of steroidogenic genes such as Star and Hsd3b1, while ELISA analysis revealed that the testosterone levels in serum and testis were significantly low. Interestingly, at 8 days postpartum, both steroidogenic gene expression and testosterone level were decreased in Start-KO mice. Since overexpression of Start in two Leydig-derived cell lines resulted in elevation of the expression of steroidogenic genes including Star and Hsd3b1, Start is likely to be involved in their upregulation. The increase in expression of steroidogenic genes in adult Start-KO testes might be caused by a secondary effect via the androgen receptor autocrine pathway or the hypothalamus-pituitary-gonadal axis. Additionally, we observed a reduced number of Leydig cells at 8 days postpartum. Collectively, our results strongly suggest that Start is a regulator of steroidogenesis in Leydig cells. The current study provides an insight into the overall picture of the function of testis lncRNAs.

Keywords: long noncoding RNA, spermatogenesis, testis, steroidogenesis, Leydig cell, knockout mouse, testosterone

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INTRODUCTION

The testis has two main functions: spermatogenesis and androgen production. Spermatogenesis is regulated within the seminiferous tubules that contain germ cells and Sertoli cells and consists of three steps: mitosis, meiosis and spermiogenesis (1–3). Some of the mitotically proliferating spermatogonia enter meiosis to differentiate into haploid spermatids via primary and secondary spermatocytes, and mature spermatooza are formed by spermiogenesis. During these processes, Sertoli cells nurse germ cells to support sperm development by releasing glial cell line-derived neurotrophic factor signaling molecules, retinoic acid, and growth factors (4–6). Leydig cells, another type of somatic cells, reside in the interstitial regions among the seminiferous tubules and produce androgens such as testosterone (7–9). Testosterone is a major androgen produced in the steroidogenesis pathway in Leydig cells and plays a critical role in spermatogenesis by targeting Sertoli cells (10, 11). Dysfunction of Leydig cells frequently leads to disruption of spermatogenesis due to insufficient intratesticular testosterone (12, 13).

In mammals, androgen production is precisely controlled by the hypothalamus-pituitary-gonadal (HPG) axis (14–16). The hypothalamus secretes gonadotropin-releasing hormone (GnRH) and induces the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary to Leydig and Sertoli cells in the testis. Testosterone is produced and secreted in response to LH in Leydig cells for androgen production in the steroidogenesis pathway in Leydig cells and plays a critical role in spermatogenesis by targeting Sertoli cells (10, 11). Dysfunction of Leydig cells frequently leads to disruption of spermatogenesis due to insufficient intratesticular testosterone (12, 13).

Recent high-throughput studies have revealed that the testis expresses much more long noncoding RNAs (lncRNAs) than those expressed in other tissues (24, 25) and identified differential expression of many lncRNAs in the testis (26, 27). lncRNAs are noncoding RNAs that are comprised of more than 200 nucleotides and they exhibit a wide variety of non-translational functions including gene regulation (28–32). During meiosis, the functions of several lncRNAs have been revealed. For instance, testis lncRNAs have been shown to function in meiotic entry in spermatogonia, activation of immune-related genes in primary spermatocytes, and regulation of postmeiotic genes (33–36). Moreover, some studies have suggested a function of lncRNAs in testicular somatic cells (37–40). However, the phenotype of knockout mice for testicular lncRNAs has been shown by only a few studies. Deletion of Txx, an X-linked lncRNA, resulted in an increase in apoptotic cells due to meiotic arrest at pachynema, although mutant males were fertile (41). Tslrn1, a testis-specific lncRNA, was found to be involved in the formation of spermatooza (42). In the case of 1700121C10Rik, the knockout mice showed no obvious phenotype (43). These findings confirmed some physiological roles of the lncRNA in germ cells but results of in vivo evaluation of the functions of lncRNAs in Leydig cells have not been reported.

We previously reported the identification of two testis-specific lncRNAs at the Prss/Tessp locus on mouse chromosome 9F2-F3 (44, 45). This locus contains six Prss genes that are specifically or predominantly expressed in the testis and encode serine proteases (Figure 1) (46–48). Among the protein-coding genes, it has been suggested that Prss42/Tessp-2 and Prss43/Tessp-3 are essential for the progression of spermatogenesis and that the noncoding regions are involved in transcriptional regulation of the Prss42/Tessp-2 gene (44–46).

Those studies indicate that there are diverse molecular species and functions of lncRNAs in the testis. However, very little evidence for in vivo functions of lncRNAs in spermatogenesis and steroidogenesis has been provided.

In this study, we identified a novel lncRNA, Start (Steroidogenesis activating lncRNA in testis), transcribed at the Prss/Tessp locus. Start was found to be transcribed downstream of the Prss43/Tessp-3 gene (Figure 1) exclusively in the testis, especially in germline and Leydig cells. Using Start-deficient mice and cultured Leydig cell lines, Start was found to be a regulator of spermatogenesis in the testis. This is the first identification of an lncRNA fine-tuning the steroidogenic pathway in the testis.

MATERIALS AND METHODS

Animals

The experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University and by the Animal Care and Use Committee of the National Research Institute for Child Health and Development. C57Bl/6Ncl mice (CLEA Japan Inc., Tokyo, Japan) and knockout (KO) mice (ICR and mixed background of C57Bl/6 and DBA2, Sankyo Labo Service Corporation) were maintained on 14 hr light/10 hr dark cycles at 25°C and given food and water ad libitum. In the analysis of Start-KO mice, we used male mice at 64–83 dpp as 2.5-month-old adult mice.

Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNAs were extracted with ISOGEN and ISOGEN II (Nippon Gene, Tokyo, Japan) for tissues and cultured cells,
respectively, according to the manufacturer’s instructions. After treatment with TurboDNase (Thermo Fisher Scientific, Rockford, IL, USA), the RNAs were reverse-transcribed into cDNAs with the oligo(dT) primer or random hexamer using Superscript III (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. A random hexamer was used for RT-PCR to check tissue distribution, and oligo(dT) was used for other experiments. PCR was performed using ExTaq polymerase (Takara, Otsu, Japan) or KOD Fx Neo (Toyobo, Tokyo, Japan). For strand-specific RT, “Start full length” reverse and “Start full length” forward primers were used to synthesize Start sense and antisense cDNAs, respectively, and a reaction temperature of 55°C was used to avoid non-specific binding of primers. Primer sequences are shown in Table 1. We repeated experiments with three or more biological replicates to confirm the reproducibility and showed a representative result.

**Rapid Amplification of cDNA Ends Analyses**

For 5’RACE, cDNA was generated using a gene-specific primer 1 (GSP1) and total RNAs from mouse male germ cells. After purification of cDNA with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), oligodeoxycytidine was added by terminal deoxynucleotidyl transferase (Takara). The first-round PCR was performed with an abridged anchor primer (AAP) and GSP2. For the second nested amplification, GSP3 and an abridged universal amplification primer (AUAP) were used.

For 3’RACE, cDNA was prepared by reverse transcription with oligo(dT) connected to an adaptor sequence (AP). The first-round PCR was conducted by using primers, AP and GSP4. The second nested amplification was carried out with the same adaptor primer and GSP5.

All of the PCR products were subcloned into the EcoRV site of a pBluescript II KS(+) vector (Stratagene La Jolla, CA, USA) by the TA-cloning method, and 10 subclones for each sample were sequenced. All of the primer sequences are listed in Table 1.

**Quantitative RT-PCR**

cDNAs were synthesized as described above using the oligo(dT) primer. PCR was performed by using SYBR Green PCR Master Mix for protein-coding genes (Thermo Fisher Scientific) and KOD SYBR qPCR Mix for Start (Toyobo). In both cases, the 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used as previously described (49). The relative expression levels were normalized to endogenous Gapdh or Aip mRNA and calculated by the comparative Ct method (50). Primer sequences are shown in Table 1 and Supplemental Table 1.

**Preparation of Germ Cell, Sertoli Cell, and Sertoli/Leydig Cell Fractions**

Native germ cells were isolated from two adult mouse testes, and their purity was checked by marker gene expression as previously described (46, 49). Sertoli cells were obtained from 11-day-old testes for primary culture as previously described (37, 51). The Sertoli/Leydig cell fraction was obtained from 6-month-old testes by using a discontinuous Percoll gradient (20%, 37%, and 53%) as previously described (44).

**Cytosolic and Nuclear Extraction From Germ Cells**

Subcellular fractions were obtained by using NP-40 lysis buffer (10 mM Tris HCl [pH 7.5], 10 mM NaCl, 3 mM MgCl2, 0.5% NP 40) containing 1× proteinase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (44). The nuclear fraction was collected as a pellet after centrifugation, and the supernatant was used as the cytosolic fraction.

**In Situ Hybridization**

The RNA probes to detect Start were prepared as follows. The full length of Start was amplified by RT-PCR with mouse testis cDNA and a primer pair of Start full length (Table 1) using KOD
Fx Neo and was subcloned into the EcoRV site of a pBluescript II KS+ vector. After the sequence had been confirmed, the full length was cut out by EcoRI and HindIII digestion, blunted by T4 DNA polymerase (Takara), and inserted into the blunted PstI site of a pGEM-3Zf(+) vector. The resulting plasmid was linearized with ApaI and NotI digestion for synthesizing sense and antisense RNA probes, respectively. Digoxigenin (DIG)-labeled RNA probes were synthesized using a DIG RNA labeling kit (Roche Molecular Biochemicals) with SP6 RNA polymerase for a sense probe and T7 RNA polymerase for an antisense probe. In situ hybridization with the Tyramide Signal Amplification Plus system (PerkinElmer, Waltham, MA, USA) was performed according to the procedure reported previously (44, 53). Briefly, adult mouse testes were fixed for 3 h at 4°C with 4% paraformaldehyde in phosphate buffered saline (PBS). After washing with PBS, each testis was dissected into two pieces with a razor blade under a dissecting microscope. The two pieces of testis were dehydrated, embedded in paraffin, and cut into 7-μm-thick sections. The sections were hybridized with 2 ng/μl of the DIG-labeled RNA probe overnight at 45°C. After washing, the sections were incubated with an anti-DIG-horseradish peroxidase antibody (1:50 dilution; Cat# 1207733, Roche Molecular Biochemicals) for 30 min at room temperature. The reaction with tyramide-Cy3 (1:50 dilution in 1X Plus Amplification Diluent (PerkinElmer), followed by 1:100 dilution with distilled water) was performed at room temperature for 20 min. To detect nuclei, the sections were incubated with 10 μg/ml Hoechst 33258 at room temperature for 10 min. The samples were mounted with a Fluoro-KEEPER Antifade Reagent (Nacalai Tesque, Kyoto, Japan) and observed under an LSM 5 LIVE confocal microscope (Carl Zeiss, Oberkochen, Germany).

**Generation of Start-KO Mice**

sgRNA sequences were designed by CRISPRdirect (https://crispr.dbcls.jp/) (54). sgRNAs were synthesized and purified by using a CUGA7 gRNA Synthesis Kit (Nippon Gene) according to the manufacturer’s instructions. The selected sequences were Start sgRNA-UP (AGTACTTCCCTAGGTAAATGGTTTTAGAGCTAGAAATAGCA) and Start sgRNA-DOWN (GTAACATCCTGTCATATTGGTTTTAGAGCTAGAAATAGCA), in which proto-spacer adjacent motif (PAM) sequences are underlined. Oligo DNA sequences used for sgRNA synthesis are listed in Table 1.

For injection, oocytes were collected from F1 hybrid (C57BL/6 x DBA/2) BDF1 female mice that were superovulated by standard procedures (55) and fertilized in vitro with sperms from male mice of the same genetic background. CAS9 protein (100 ng/μl; Nippon Gene) and sgRNAs (250 ng/μl each) were microinjected into the cytoplasm of fertilized oocytes. The oocytes were cultured in KSOM medium overnight, and embryos at the two-cell stage were transferred to pseudo-pregnant ICR female mice on the next day.

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**Table 1** | Oligo DNAs used in this study.

| Name | Forward | Reverse |
|------|---------|---------|
| [RT-PCR] |         |         |
| Start 1418bp | CTGGTTTTTGGTATCCCTGA | ATGCGATATCGGAGCAAGCA |
| Gapdh | CATGACACAGTCATGCCCATC | TAGCCCAAGATGCTCCTAGTG |
| [RACE] |         |         |
| GSP1 |         | AAACAAAATCTGGGCTGGAGAG |
| GSP2 |         | ATGCGATATCGGAGCAAGCA |
| [Cloning] |         |         |
| Start |         |         |
| Otp3b1 | ATGAGCCACCTTGCACTGAGG | CATTCAAGAACAGGAGGAG |
| Lhc |         | TCTCCGGGCTTCAAGAATGAC |
| Gapdh | CATGACACAGTCATGCCCATC | TAGCCCAAGATGCTCCTAGTG |
| [qRT-PCR] |         |         |
| Start |         |         |
| Lhca |         | TGAAGCTCGGTAGCTGAGAG |
| Lhb |         | GGGGTCGCTAGTCAAGC |
| Gapdh | CATGACACAGTCATGCCCATC | TAGCCCAAGATGCTCCTAGTG |
| Ap |         | C однако AAGGAGGAAGAGAG |

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For injection, oocytes were collected from F1 hybrid (C57BL/6 x DBA/2) BDF1 female mice that were superovulated by standard procedures (55) and fertilized in vitro with sperms from male mice of the same genetic background. CAS9 protein (100 ng/μl; Nippon Gene) and sgRNAs (250 ng/μl each) were microinjected into the cytoplasm of fertilized oocytes. The oocytes were cultured in KSOM medium overnight, and embryos at the two-cell stage were transferred to pseudo-pregnant ICR female mice on the next day.
Genotyping
Tail tips were collected from mice 3-4 weeks after birth and treated with Proteinase K solution (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K) at 50°C for 24 hr. After extraction with phenol/chloroform/isoamyl alcohol, genomic DNA was precipitated with isopropanol. Purified DNAs were examined by PCR using a pair of primers listed in Table 1.

Morphological Observation of Testes
Wild-type and Start-KO mice were euthanized, and their body weights were measured. Then testes were isolated and their weights were measured. The testes were fixed with Bouin’s solution at room temperature for 2 days, dehydrated, embedded in paraffin, and sectioned at 7 μm in thickness. The sections were stained with hematoxylin and eosin and observed under a light microscope.

Sperm Count
One cauda epididymis was collected from wild-type and Start-KO adult mice, soaked in 0.7 ml of HTF medium (Nippon Medical and Chemical Instruments, Osaka, Japan), and torn into pieces by a sterile 26G needle. After incubation at 37°C with 5% CO2 in air for 2 hr, whole medium was transferred into a new tube and mixed with 0.7 ml of 10% neutral buffered formalin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) for fixation. The number of sperms was counted manually by using a hemocytometer.

RNA-Sequencing Analysis
RNA-seq analysis was performed as previously described (36, 56). In brief, total RNAs were isolated from a wild-type testis and Start-KO testis (line #2) of mice at the age of 2.5 months using ISOGEN according to the manufacturer’s protocol. The RNAs were treated with TurboDNase to completely remove genome DNA, and the quality of the RNA samples was evaluated by electrophoresis and BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA) with RNA6000 nano tip. cDNA synthesis was performed with 500 ng RNA from each sample by using a TruSeq Stranded mRNA Sample preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer’s protocol. The resulting cDNA library was validated using BioAnalyzer with DNA1000 Chip and quantified using NEBNext Library Quant Kit for Illumina (NEB, Ipswich, MA, USA). A total of 101-cycles single-end sequencing was performed using HiSeq1500 (Illumina) in the rapid mode. After conversion to fastq format, reads were mapped to the mouse reference genome, mm10, with Hisat2 software (v2.1.0) (57). The expression level of each gene was calculated as gene-specific Transcript per Million mapped reads (TPM) using Stringtie (v1.3.3b) (57) with the optional – conservative setting enabled. Total reads, mapping rates, and accession numbers are summarized in Table 2.

Testosterone Measurement
A blood sample from each mouse was collected into a microtube and kept on ice for 1-2 hr until it became clotted. The sample was then centrifuged at 1,000g for 10 min at 4°C. The supernatant was collected as serum and quickly frozen by liquid nitrogen. To measure intratesticular testosterone, tunica albuginea of each adult testis was removed, and the tissue was homogenized in 1 ml of ice-cold PBS. The crude sample was centrifuged at 2,500 rpm for 5 min at 4°C, and the supernatant was collected into a new tube. This centrifugation step was performed twice. The sample was mixed with an equal volume of chloroform, vortexed for 1 min, and centrifuged at 15,000 rpm for 5 min at room temperature. After the centrifugation, the organic phase was collected into a new tube whose cap was removed, and the tube was centrifuged until chloroform was evaporated completely. The precipitate was dissolved in 50 μl dimethyl and diluted with 350 μl enzyme-linked immunosorbent assay (ELISA) buffer (Cayman Chemical, Ann Arbor, MI, USA). The serum from adult mice was diluted 10 or 20 times with milli-Q water for ELISA analysis, and that from 8-days postpartum (dpp) mice was used after 3.3 times dilution. The intratesticular sample was diluted 100 times with ELISA buffer. Intratesticular and serum testosterone concentration were measured by using a Cayman Testosterone EIA kit according to the manufacturer’s instructions.

Immunohistochemistry
Paraffin sections of testes were prepared as above at 7 μm in thickness, and de-paraffinized and hydropthilized with xylene, ethanol, and Milli-Q water. The sections were boiled for 45 min with 10 mM sodium citrate-HCl (pH 6.0) and treated with 3% H2O2-containing PBS. After the H2O2 treatment, the blocking was performed by soaking the sections with 1% bovine serum albumin in 10 mM Tris-HCl (pH 7.5), 0.88% NaCl, and 0.1% Tween20 for 1 hr at room temperature. An anti-HSD3B antibody (no dilution; Cat# sc-515120, Santa Cruz Biotechnology, Dallas, TX) was reacted with the section for 1 hr at room temperature. After washing with PBS, the sections were subjected to the 2nd antibody reaction for 1 hr at room temperature with a HRP-Labelled Polymer Anti-Mouse (1:4 dilution, Dako). After washing with PBS, the sections were stained with ImmPACT Vector Red (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions.

LH Measurement
Serum samples were collected as described above. Serum LH concentration was measured by using a Luteinizing Hormone ELISA kit (CUSABIO, Wuhan, China) according to the manufacturer’s instructions.

Plasmid Constructs for Overexpression
The full length of Start was amplified and inserted into a pBluescript II KS+ vector as described above. The Start sequence was obtained by EcoRI and HindIII digestion from this vector, and the fragment was bluntly by T4 DNA polymerase and inserted into the EcoRV site of a pcAG-Hyg vector (Fujifilm Wako Pure Chemicals). Constructs with insertion of the Start sequence in both directions were obtained.
Cell Culture and Transfection

For each of mouse Leydig cell lines, TM3 and MA-10, the hygromycin concentration for selection was preliminarily determined. TM3 cells were cultured in Dulbecco Modified Eagle Medium (DMEM)/F12 containing 2.5% fetal bovine serum, 5% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml L-glutamine (Invitrogen, Carlsbad, CA, USA). After coating a 3.5-cm dish with 0.1% gelatin before use, 2.0 × 10^5 cells were spread in the dish one day before transfection. The next day, 6.0 µl PEI Max (Polyscience, Warrington, PA, USA) was mixed with 200 µl Opti-MEM (Thermo Fisher Scientific) and 2 µg DNA in that order. The mixture was added to the dish after 20-min incubation. Selection was done with the medium containing 250 µg/ml of hygromycin for 8 days.

MA-10 cells were cultured in DMEM/F12 containing 15% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml L-glutamine on a dish coated with 0.1% gelatin. One-tenth of confluent cells in a 6-cm dish was spread in a 3.5-cm dish one day before transfection. The next day, 24.0 µl PEI Max was mixed with 200 µl Opti-MEM and 8 µg DNA in that order. The mixture was added to the dish after 20-min incubation. Selection was done with the medium containing 100 µg/ml of hygromycin for 8 days.

Statistical Analysis

Results are presented as the mean value ± standard deviation (S.D.) of at least three independent experiments. The data were statistically analyzed by Student’s t-test, Welch’s t-test or one-way analysis of variance (ANOVA) followed by the Tukey HSD test. A P value less than 0.05 was considered statistically significant. All statistical calculations were done by R software (Ver. 3.5.0; https://cran.ism.ac.jp/bin/windows/).

RESULTS

Cloning and Characterization of a Testis-Specific IncRNA, Start

Previously, we revealed the presence of two testis-specific IncRNAs at the mouse Prrs/Tessp locus and we suggested significant roles of these noncoding regions. In this study, we searched for other IncRNAs and found by RT-PCR that a 1418-bp sequence was transcribed in an intergenic region between Prrs43/Tessp-3 and Prrs45/TESPL genes in the testis (Figure 2A). We then determined the 5’- and 3’-ends of this transcript by RACE analyses. In 3’RACE, two bands were specifically amplified by the second PCR (marked as “a” and “b” in Figure 2B), and 10 subclones from each were sequenced. It was found that all subclones contained the same sequence for each band. Due to stronger band intensity, a cytosine from band “a” was determined to be a major transcriptional termination site (Figure 2C). In 5’RACE, two bands were detected by the second PCR (marked as “c” and “d” in Figure 2D), and 10 subclones were checked for each band. For band “c”, three different sequences were detected, but the subclones from band “d” contained a single sequence. Because band “d” was more intense than band “c”, a guanine from band “d” was determined to be a major transcriptional start site (Figure 2E). Note that the transcriptional start site was within the primer sequence used for the initial RT-PCR amplifying the 1418-bp product (Figure 2A). Consequently, this transcript was defined to be a single-exon structure of a 1822-nucleotide sequence with a poly (A) tail at its 3’ end. Based on the function reported here, we named this transcript Start.

RT-PCR was performed using 9 tissues to examine the tissue specificity of Start, and a signal was only observed in the testis (Figure 3A). During the first wave of spermatogenesis, qRT-PCR showed that Start expression was significantly elevated from 7 ddp to 14 ddp and remained at high levels thereafter (Figure 3B). We then separated testicular cells into three fractions, germ cells, Leydig/Sertoli cells and Sertoli cells, and performed RT-PCR. As shown in Figure 3C, Start was expressed in both germ cells and Leydig cells but not in Sertoli cells. To investigate the subcellular localization, germ cells were separated into nuclear and cytosolic fractions. RT-PCR showed more dominant localization in the cytosol, but the signal was also found in the nucleus (Figure 3D). These results indicated that Start is a germ cell and Leydig cell-specific IncRNA and is activated during sperm development.

To further determine the localization of Start, we performed in situ hybridization by the highly sensitive tyramide signal amplification method (53). Antisense and sense probes for Start were hybridized with adult testis sections and the signal was detected as red fluorescence. Consistent with results shown in Figure 3C, a strong signal was detected in Leydig cells, mostly in the cytosolic region, by the antisense probe, while few signals were seen in the section hybridized with the sense probe (Figures 4A, B). In the seminiferous tubules, many more red dots were observed with the antisense probe (Figures 4C–E) than with the sense probe (Figure 4F). At seminiferous epithelial stages II-IV and VI-VII (Figures 4C, D), the signals were found in all types of spermatogenetic cells including spermatogonia (SG), primary spermatocytes at the early and mid-pachytene stages (ePS and mPS), and round and elongating spermatids (RS and ES, respectively). Consistent with the results of RT-PCR shown in Figure 3D, more signals were present in the cytosol of these cell types. At stage X, most of the spermatogenetic cells were stained in a cytosol-predominant manner as in earlier stages (Figure 4E), but late pachytene spermatocytes (IPS) appeared to contain more signals in the nuclei than did other types of germ cells. Similar results were obtained with a testis from another mouse. Collectively, the results showed that Start was dominantly present in the cytosol of Leydig cells and all types of spermatogenetic cells, though transcripts were also localized in the nucleus. Noticeably, Leydig cells carried the strongest signals among all cell types, suggesting that Start plays an important role in Leydig cells.

Generation of Start-Knockout Mice and Morphological Phenotype in Adult Testis

To reveal the physiological role of Start, we generated Start-knockout (KO) mice by the CRISPR/Cas9 system using two guide RNAs designed at the 5’- and 3’-flanking sequences of Start (Figure 5A). Injection of the guide RNAs and CAS9 protein into
fertilized eggs resulted in the generation of four founder mice that had a deletion of Start, and two KO lines, lines #1 and #2, were successfully established by mating with wild-type mice. These KO mice contained 2222-bp (#1) and 2208-bp (#2) deletions, which meant that #1 contained 5 and 9 nucleotides longer deletions than #2 at the 5’ and 3’ regions, respectively (Figures 5A, B). RT-PCR confirmed no expression of Start in these two KO lines (Figure 5C).
study, and all experiments were done with three or more mice except for RNA-seq.

Both #1 and #2 lines of Start-KO mice were fertile, and we observed morphology of the adult testis by collecting testes from 2.5-month-old wild-type and Start-KO mice. The testis weight normalized to body weight was not significantly different between wild-type and KO mice (Figure 5D), and observations of testis sections stained with hematoxylin and eosin did not show any morphological difference (Figure 5E). In addition, the number of sperms collected from the cauda epididymides was not changed in KO adult mice (Figure 5F). To see whether sex differentiation was affected, we investigated the sex ratio among 16 littermates and we found no significant difference (17 males and 11 females in wild-type vs 22 males and 13 females in Start-KO, no significant difference by the chi-square test). Therefore, the adult Start-KO testis appeared to be morphologically normal.

Increased Expression of Testicular Steroidogenic Genes and Decreased Testosterone Level in Start-KO Adult Mice

Subsequently, we attempted to detect differentially expressed genes. Total RNA was purified from whole testes of a 2.5-month-old wild-type and Start-KO mice from the same litter and used for RNA-seq analysis. The resultant reads, mapping rate to the mouse cDNA library, and fastq accessions in the NCBI SRA database are summarized in Table 2. Interestingly, the Star gene encoding a protein to trigger steroidogenesis was found to be upregulated by 2.3 fold in the Start-KO testis (Figure 6A). Furthermore, expression levels of other steroidogenic genes including Cyp11a1, Cyp17a1, Hsd3b1, and Hsd17b3 showed 1.3-1.6-fold increases in the Start-KO testis (Figure 6A). qRT-PCR for these genes using four sets of wild-type and KO mice confirmed that Star and Hsd3b1 genes were

FIGURE 3 | Expression of the Start transcript. (A) Tissue specificity of Start. Total RNAs from nine mouse tissues, as indicated, were collected from adult wild-type mice and used for RT-PCR with a random hexamer for reverse transcription. The Gapdh gene was detected as an internal control. The cycle number of each PCR is shown in parenthesis. We obtained similar results with the three biological replicates, and a representative result of the three experiments is shown. (B) Start expression during testis development. Mouse testes at 7, 14, 21, 28, and 56 dpp were collected, and total RNAs were purified and used for qRT-PCR. The Aip gene was used as an internal control to normalize the Start expression level. Data are presented as means ± S.D. from three independent experiments with three biological replicates. Statistical significance was analyzed by Tukey’s HSD test. *P < 0.05, **P < 0.01. (C) RT-PCR with fractionated testicular cells. Total RNAs from germ cells, Leydig/Sertoli cells, and Sertoli cells were collected and used for RT-PCR. The Gapdh gene was utilized as an internal control. The cycle number of each PCR is shown in parenthesis. (D) RT-PCR for subcellular localization of Start. Total RNAs were purified from nuclear and cytosolic fractions of germ cells and used for RT-PCR. Gapdh was used to amplify a region within exon 6 as an internal control. The cycle number is indicated in parenthesis.
significantly upregulated in the Start-KO testis (Figure 6B). We then measured testosterone concentration in serum and testis. Surprisingly, ELISA analysis showed that the testosterone level was significantly reduced in both serum and testis of KO mice (Figures 6C, D). This is inconsistent with the increase of steroidogenic gene expression, but the results suggested that Start participates in regulation of the steroidogenesis pathway in Leydig cells.

To understand why the testosterone level was low despite high expression of steroidogenic genes in adult Start-KO testes, we performed several experiments. First, we considered the possibility that conversion into 5α-dihydrotestosterone might be augmented in KO mice. However, qRT-PCR indicated that expression of the Srd5a1 gene, encoding 5α-reductase, was not changed in Start-KO testes (Supplemental Figure 1). Second, we investigated the effect of Start deficiency on Androgen receptor (Ar) and found by qRT-PCR that Ar expression was not different in wild-type and Start-KO testes (Supplemental Figure 2A). This suggests that the low level of testosterone might affect the androgen-AR autocrine pathway in adult Start-KO testes. Third,

**FIGURE 4** | Start localization determined by in situ hybridization using the tyramide signal amplification system. (A, B) Representative images of Start localization in interstitial Leydig cells stained with the antisense probe (A) and sense probe (B). Staining with the sense probe was performed as a negative control. (C-E) Representative images of seminiferous tubules at seminiferous epithelial stages II-IV (C), VI-VII (D), and X (E) stained with the antisense probe. The squares indicate the cells enlarged below. As specified in (C-E), the squares emphasize a spermatogonium (SG), primary spermatocytes at the early, mid, and late pachytene stages (ePS, mPS, and lPS, respectively), round spermatid (RS), and elongating spermatid (ES). (F) An image of seminiferous tubules at stage II-VI stained with the sense probe as a negative control. In all images, red dots represent Start signals, and nuclei were co-stained with Hoechst 33342 (grey). The experiment was repeated twice with different testes, and similar results were obtained. Scale bars, 50 µm.
we investigated the effect of Start deficiency on the HPG axis. In the pituitary, an ELISA assay and qRT-PCR showed that the LH level and Lhb mRNA expression were not significantly different in wild-type and Start-KO mice (Figures 7A, B) but that Ar expression was dramatically elevated in KO mice (Figure 7C). In the testis, the expression of Lhcgr, a receptor of LH, which is supposed to be specific to Leydig cells (58), was significantly increased in Start-KO mice (Figure 7D). Presumably,
upregulation of *Star* and *Hsd3b1* in adult *Start*-KO testes resulted from a secondary or indirect effect of the *Start* deficiency on the AR autocrine pathway or on the HPG axis (see “Discussion”).

**Effect of *Start* Deficiency at a Younger Age**

To gain more insight into effects of *Start* deficiency on steroidogenesis, we investigated *Start*-KO mice at 8 dpp. We first conducted morphological observation by preparing cross-sections of testes of wild-type and *Start*-KO mice from the same litter and staining them with hematoxylin and eosin. As shown in Figure 8A, no significant differences were observed within the seminiferous tubules, whereas more interstitial regions containing few cells were observed in the *Start*-KO testis than in the wild-type testis (see regions encircled in red). Indeed, the number of interstitial cells in randomly selected areas (0.25 mm²) in the sections was significantly smaller in *Start*-KO testes at 8 dpp than in wild-type testes (Figure 8B). In contrast, no significant change was observed in interstitial cells between KO and wild-type mice at 2.5 months of age (Figure 8B). Since
interstitial cells mainly consist of Leydig cells but also contain small populations of other types of cells (59, 60), we investigated by immunohistochemistry of HSD3B, a marker of Leydig cells (61, 62), whether the altered number of interstitial cells reflected the Leydig cell population. The number of HSD3B-positive cells was significantly decreased in Start-KO testes at 8 dpp, while it was not altered at 2.5 months of age (Figure 8C). This result was supported by two additional data. First, qRT-PCR indicated a significant decrease of Insl3 expression, another marker gene for Leydig cells (63, 64), in Start-KO testes at 8 dpp but not in adults (Supplemental Figure 3A). Second, the expression of Hsd3b6, a marker gene for adult Leydig cells (65), showed no difference between wild-type and Start-KO testes in adults (Supplemental Figure 3B). These results indicated that the number of Leydig cells was decreased in Start-KO mice at 8 dpp.

Next, we investigated the expression of steroidogenic genes in wild-type and Start-KO testes at 8 dpp. Our qRT-PCR indicated that expression levels of Star and Hsd3b1 genes were significantly low in Start-KO testes (Figure 9A), and serum testosterone level was also found to be lower in Start-KO mice than in wild-type mice at 8 dpp (Figure 9B). This is in contrast to the results for Start-KO mice at 2.5 months of age. To clarify the difference from adults, we examined the expression of hormone receptors in the testis at 8 dpp. While qRT-PCR showed that Ar mRNA expression was not different in wild-type and Start-KO mice as in adults (Supplemental Figure 2B), expression of the Lhcgr gene, which was increased in Start-KO adult testes (Figure 7D), was not changed by Start deficiency at 8 dpp (Figure 9C). Collectively, the results showed that steroidogenic genes were downregulated in the testis and that the testosterone level was decreased in Start-KO mice at 8 dpp.

Activation of Steroidogenic Genes by Start in Leydig Cell Lines

To determine how Start regulates steroidogenic genes in Leydig cells, we utilized two mouse Leydig cell lines, TM3 and MA-10 (66, 67), to perform Start overexpression. The antisense sequence of Start was overexpressed as a negative control. Strand-specific
RT-PCR confirmed successful overexpression of *Start* and its antisense transcript in both cell lines (Figures 10A, C). In TM3 cells, *Start* gene expression was significantly increased by *Start* overexpression (Figure 10B), and in MA-10 cells, not only *Start* but also *Cyp17a1*, *Hsd3b1*, and *Hsd17b3* genes were significantly upregulated by *Start* (Figure 10D). We failed to detect testosterone in the culture media of MA-10 cells overexpressing *Start*, probably due to low capability of testosterone production of this cell line as previously reported (67, 68). These results supported the notion that *Start* acts as an activator of steroidogenic genes in Leydig cells, as shown by results for 8-day-old mice (Figure 9A). Based on these results, we concluded that *Start* activates a subset of genes involved in steroidogenesis in Leydig cells.

**DISCUSSION**

The current study showed that a testis-specific lncRNA, *Start*, was involved in activation of steroidogenic genes in the testis. As we mentioned in the introduction section, testicular lncRNAs have been mostly evaluated in germ cells, and no *in vivo* evaluation of their functions in Leydig cells has been reported. Thus, this is the first evidence for *in vivo* biological functions of an lncRNA in Leydig cells.

**Possible Function of *Start* in Testicular Germ Cells**

While we found a role of *Start* in Leydig cells, it is presumed that *Start* also has some function in germ cells, given its localization in meiotic cells. In general, lncRNAs regulate the expression of their target genes at various levels depending on their subcellular localization (69, 70). *Start* was found to be localized in the cytosol of most germ cells, which raises the possibility that *Start* is related to the regulation of RNA stability or translation of meiotic genes, as was previously reported (71, 72). At the late pachytene stage, *Start* was also localized in the nucleus, suggesting a role in transcriptional gene regulation as was observed for other lncRNAs (73–75). Since expression of various meiotic genes is changed during spermatogenesis (76–78), *Start* may be involved in the regulation of such genes in germ cells.

It is interesting that *Start*, *Tesra*, and *lncRNA-HSVIII*, three lncRNAs at the *Prss/Tessp* locus (Figure 1), are all expressed in germ cells and Leydig cells. At the late pachytene stage, when some of the *Prss/Tessp* genes are activated, they are localized in...
the nucleus. This indicates the possibility that Prss/Tessp genes are regulated by these lncRNAs. Indeed, Tesra contributes to transcriptional activation of the Prss42/Tessp-2 gene by collaborating with an enhancer adjacent to lncRNA-HSVIII (45). However, the contribution of lncRNA-HSVIII to expression of the Prss42/Tessp-2 gene is not clear, and the function of Start in germ cells remains to be elucidated. Therefore, analysis considering the functional relationship of the three lncRNAs at the Prss/Tessp locus is necessary to clarify the function of Start in germ cells.

### Involvement of Start in Development of Fetal Leydig Cells

Our data indicated a decrease in the number of interstitial Leydig cells in Start-KO mice at 8 dpp. This was based on the significantly small number of HSD3B-positive cells shown by immunohistochemistry (Figure 8C) and a significantly low level of InsI3 expression shown by qRT-PCR (Supplemental Figure 3A). Both changes were observed in Start-KO testes at 8 dpp but not in adults. No difference in Hsd3b6 gene expression between wild-type and Start-KO testes supports the notion that Start does not affect adult Leydig cells (Supplemental Figure 3B). Therefore, we concluded that the number of fetal Leydig cells was decreased by deletion of Start at 8 dpp. This suggests the involvement of Start in the development of fetal Leydig cells. According to previous studies, fetal and adult Leydig cells are differentiated by distinct regulatory mechanisms (79, 80). However, the mechanism of the differentiation and proliferation of Leydig cells is not fully understood and is even controversial (80). Therefore, Start may play some role in the differentiation or proliferation of fetal Leydig cells or their progenitor cells, but the detailed mechanism needs further research. Elucidation of the molecular mechanism will provide an insight into the function of Start in the development of fetal Leydig cells.

### Function of Start as a Regulator of Steroidogenesis in the Testis

In the present study, we obtained data supporting the involvement of Start in the regulation of steroidogenesis in Leydig cells. First, many Start transcripts were localized in Leydig cells, where steroid hormones are synthesized (Figure 4A). Second, expression of Star and Hsd3b1 genes was changed in Start-KO mice at 8 dpp and in adults (Figures 6B, 9A). Third, serum testosterone level was lower in Start-KO mice than in wild-type mice both in adults and at 8 dpp (Figures 6C, 9B). Fourth, intratesticular testosterone level was lower in adult Start-KO mice than in wild-type mice (Figure 6D). Fifth, overexpression of Start in two Leydig cell lines significantly increased the expression of steroidogenic genes (Figure 10). Although the decreases in the gene expression and testosterone level might be partially due to the reduction in the number of Leydig cells at 8 dpp, these results strongly suggest that Start is a regulator...
of steroidogenesis in the mouse testis by controlling at least Star and Hsd3b1 gene expression in Leydig cells.

Then, how does Star regulate the expression of steroidogenic genes? In Leydig cells, Star was mostly localized in the cytosol, suggesting its function in post-transcriptional regulation such as mRNA stabilization and translation. In the case of regulation of mRNA stability, lncRNAs are known to form double-strand RNAs with their target mRNAs and induce molecular events.
leading to RNA degradation or stabilization (81, 82). In translational regulation, IncRNAs generally interact with miRNAs to block their function, resulting in enhancement of the translation of miRNA-targeted mRNAs (75, 83, 84). Given that Start is involved in the regulation of multiple target genes and that one of the targets, Star, has been reported to be regulated by an miRNA (85), we prefer the possibility of the mechanism involving the miRNA pathway. This may also be one explanation for the reason why Cyp17a1 and Hsd17b3 genes were upregulated by Start overexpression in MA-10 cells but were not changed in Start-KO testes. There might be miRNAs that can regulate Cyp17a1 and Hsd17b3 genes and interact with Start in MA-10 cells, but they might not be physiologically significant in Start-KO testes. Elucidation of the detailed molecular mechanism by which Start controls the expression of steroidogenic genes is currently in progress.

It is notable that Start-KO mice were fertile despite low levels of testosterone in serum and testis. In general, testosterone production plays a key role in the generation of mature sperm, and many studies have shown that low testosterone levels were associated with male infertility in mammals (86, 87). Meiotic arrest observed in Sertoli cell-specific AR-KO mice supports the notion that testosterone is essential for spermatogenesis (88, 89). However, in some cases, normal progression of spermatogenesis was reported even when testosterone levels were greatly reduced (90, 91), and testosterone levels were not necessarily correlated with fertility (92). These results suggest that a high testosterone level is important for complete spermatogenesis but is not an absolute requirement. In Start-KO mice, the reduced level of testosterone might still be sufficient for spermatogenesis.

Low Testosterone Levels and High Expression Levels of Steroidogenic Genes in Adult Start-KO Testes

Interestingly, Star and Hsd3b1 gene expression was upregulated in adult Start-KO testes despite the low level of testosterone (Figure 6). We do not have a clear explanation for this counterintuitive result, but we have several suggestions based on our data. One possibility is that testosterone is more actively metabolized to other molecules. Although we cannot rule out this possibility, enhanced conversion to 5α-dihydrotestosterone was unlikely considering the results showing no significant change in the expression of Srd5a1 gene in Start-KO testes (Supplemental Figure 1). Another possibility is that enhancement of Star and Hsd3b1 expression is caused by autocrine signaling of the androgen-AR trail in Leydig cells. We did not detect any difference in Ar expression between wild-type and Start-KO adult testes (Supplemental Figure 2), but the low level of testosterone might cause a defect in the AR-autocrine pathway. It has been reported that the expression levels of some steroidogenic genes were significantly decreased but that the expression levels of other steroidogenic genes were increased in Leydig cell-specific AR KO testes (93). This indicates that the deficiency of autocrine signaling via AR in Leydig cells can cause a disturbance of steroidogenic gene expression. In adult Start-KO males, the low level of testosterone might cause a deficiency in this autocrine system and lead to abnormal elevation of Star and Hsd3b1 expression.

There is also the possibility that the low level of testosterone affects the HPG axis in Start-KO adults. In the pituitary, a low level of testosterone often causes an increase in LH secretion (94–96), but in Start-KO mice, neither LH secretion nor Lhb mRNA expression was significantly changed (Figures 7A, B). This was probably associated with a dramatic increase in Ar expression in the pituitary of Start-KO mice (Figure 7C), as shown by studies in other tissues (97–99). Notably, Lhgr expression was found to be significantly increased in Start-KO adult testes (Figure 7D), which is consistent with the upregulation of this receptor gene in AR hypomorphic mutants (100). This was only observed in adults and suggests that the LH signaling pathway in Leydig cells might be enhanced under the condition of normal LH secretion, by which LH-responsive genes such as Star and Hsd3b1 might be upregulated in Start-KO adult testes. Given that mouse models overexpressing human chorionic gonadotropin or a constitutively active LH receptor in the testis showed higher levels of testosterone (101–103), the enhancement of the LH pathway might intrinsically cause an increase in testosterone production. Nonetheless, Start deficiency caused a significant decrease in testosterone level, suggesting that Start is a critical regulator of steroidogenesis in the testis.

At 8 dpp, although an effect of pituitary hormones might remain by minipuberty (104, 105), we verified more direct effects of Start deficiency than in adults and observed downregulation of Star and Hsd3b1 genes. Taken together with the increased expression of steroidogenic genes in Start-overexpressing TM3 and MA-10 cells (Figure 10), the function of Start is likely to be enhancement of the expression of steroidogenic genes.

Most previous studies focused on the functions of testis IncRNAs in germ cells (33–36, 41–43, 45, 106) but not on their roles in Leydig cells (40, 107). The results of the present study shed light on the in vivo function of Start, which plays roles in maintenance of the steroid synthesis pathway in the testis. Thus, this report is the first report on a testis-specific IncRNA that finetunes the steroid synthesis pathway in Leydig cells.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The resultant fastq files for RNA-sequencing analysis of a wild-type testis and Start-KO testis were deposited in the NCBI SRA database under the accession numbers SRR12700727 and SRR12700726 (BioProject: PRJNA665274). Nucleotide sequence data for Start are available in the DDBJ/EMBL/GenBank databases under the accession number LC583804.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Use and Care Committee at Hokkaido University and the Animal Care and Use Committee of the National Research Institute for Child Health and Development.
AUTHOR CONTRIBUTIONS

KO and AK designed the study. KO, SM, AS, NT, YS, and MT performed the experiments. KO, SM, AS, NT, YS, TK, HS, and AK analyzed the data. KO, SM, AS, NT, YS, MT, ST, TK, HS, and AK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTAL MATERIAL

The Supplemental Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.665874/full#supplementary-material
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.